Mutants of Saccharomyces cerevisiae Defective in sn-1,2-Diacylglycerol Cholinephosphotransferase

ISOLATION, CHARACTERIZATION, AND CLONING OF THE CPT1 GENE*

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A colony autoradiographic assay for the sn-1,2-diacylglycerol cholinephosphotransferase activity in Saccharomyces cerevisiae was developed. Twenty-two mutants defective in cholinephosphotransferase activity were isolated. Genetic analysis revealed that all of these mutations were recessive, and three complementation groups were identified. The cholinephosphotransferase activities in membranes prepared from cpt1 mutants were reduced 2–10-fold compared to wild-type activity. The cholinephosphotransferase activities of two cpt1 isolates differed from wild-type activity with respect to their apparent Km for CDP-choline. The residual cholinephosphotransferase activities of cpt1 isolates were more sensitive to inhibition by CMP than the wild-type activity. The CPT1 gene was cloned by genetic complementation of cpt1 using a yeast genomic library. In strains transformed with the CPT1-bearing plasmid, a 5–10-fold overproduction of cholinephosphotransferase activity with wild-type kinetic properties was observed. The CPT1 gene was localized to a 1.2–2.4-kilobase region of DNA by transposon Tn5 mutagenesis and deletion mapping. An insertional mutant of the CPT1 gene was constructed and introduced into the chromosome by integrative transformation. The resulting cpt1 insertional mutant fell into the cpt1 complementation group. The cholinephosphotransferase activity in membranes prepared from the cpt1 insertional mutant was reduced 5-fold and exhibited CMP sensitivity. The sn-1,2-diacylglycerol ethanolaminephosphotransferase activities in membranes from all of the cpt1 isolates including the insertional mutant were normal. The data indicate that the cloned CPT1 gene represents the yeast cholinephosphotransferase structural gene, that the yeast choline- and ethanolaminephosphotransferase activities are encoded by different genes, and that the CPT1 gene is nonessential for growth.

Phosphatidylethanolamine is the predominant membrane phospholipid in eukaryotic cells (1). Two major routes exist for the biosynthesis of phosphatidylethanolamine. In the Kennedy pathway (2), phosphatidylethanolamine is produced from sn-1,2-diacylglycerols by the action of diacylglycerol cholinephosphotransferase (EC 2.7.8.2) which uses CDP-choline as substrate; phosphatidylethanolamine is analogously synthesized by the action of diacylglycerol ethanolaminephosphotransferase which uses CDP-ethanolamine as substrate (1). Alternatively, phosphatidylcholine is formed by sequential methylation of phosphatidylethanolamine (1) which is derived from the Kennedy pathway and, in yeast, from the decarboxylation of phosphatidylserine (3). Thus, phosphatidylcholine synthesis is intimately related to the biosynthesis of other phospholipid classes and neutral acylglycerols, suggesting a requirement for complex regulation.

Obtaining an understanding of the molecular mechanisms regulating phosphatidylcholine synthesis in mammalian systems has been impeded by the difficulties inherent to the study of the integral membrane proteins involved. To overcome these difficulties and to gain fundamental insight into the regulation of phosphatidylcholine synthesis, we have chosen Saccharomyces cerevisiae as a model eukaryotic system in which to develop the molecular tools required for detailed analysis of diacylglycerol cholinephosphotransferase. This system offers the advantages of combined genetic and biochemical approaches. Recent progress in the study of other S. cerevisiae lipid biosynthetic enzymes (reviewed in Ref. 3) and previous work from our laboratory (4) have demonstrated the value of such an approach.

The pathways of phosphatidylcholine synthesis in yeast are similar to those in higher eukaryotes (5). A significant body of information regarding the genetics, biochemistry, and complex regulation of the phosphatidylethanolamine methylation pathway of phosphatidylcholine synthesis has emerged in recent years (reviewed in Ref. 3). However, little is known about the role and regulation of the Kennedy pathway and its integration with the methylation pathway. A detailed knowledge of the structure, function, and regulation of the enzymes of the Kennedy pathway and their genes should prove instrumental in addressing these broader issues.

The present work reports the development of a genetic and biochemical system for the detailed analysis of diacylglycerol cholinephosphotransferase. Mutants defective in cholinephosphotransferase activity (cpt mutants) were isolated by a colony autoradiographic assay. These mutants were genetically characterized. The CPT1 gene has been cloned by genetic complementation. Enzymological and genetic evidence implicating the CPT1 gene as the cholinephosphotransferase structural gene was obtained.

**EXPERIMENTAL PROCEDURES**

Materials—[25P]methyl [1H]choline chloride, [γ-32P]ATP, Aquasol-2, and ENHANCE fluorographic enhancer were obtained from New England Nuclear. Amino acids, antibiotics, choline kinase (grade II), phosphohexokinase choline, CMP, ethyl methanesulfonate, CMP-morpholidot, ethanolamine, and bovine serum albumin (essentially fatty acid-free) were purchased from Sigma. Thiophenyl chlorid, benzylxycarbonyl chloride, 10% palladium on activated charcoal, and N,N-dimethylformamide (gold label) were from Aldrich. sn-1,2-Diacylglycerol was prepared by phospholipase C digestion of L-a-diacylglycerophosphatidylcholine (6) obtained from Avanti Polar Lipids, Inc. Choline chloride was purchased from Eastman Kodak. ATP was

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from Pharmacia P-L Biochemicals. Enzymes used in generating ATP were from Boehringer Mannheim. Materials for growth media were from Difco. Restriction enzymes, T4 DNA ligase, and cloning vector pUC18 were purchased from Bethesda Research Laboratories. All other reagents were the highest quality commercially available.

**Properties of** _Saccharomyces cerevisiae_ strains DBY746 (a his3-1 leu2-3 leu2-112 trp1-289) and AH22 (a leu2-3 leu2-112 his4-S19 can1) were obtained from the Yeast Genetic Stock Center and from Dr. G. Stiles (Massachusetts Institute of Technology), respectively. Yeast were cultured in either YPD (1% yeast extract, 2% peptone, and 2% dextrose) or synthetic minimal medium (2% dextrose) 0.67% yeast nitrogen base without amino acids (with supplemental amino acids provided at 20 μg/ml as required. Where appropriate, canavanine sulfate was added at 80 μg/ml. Agar plates included 2% Difco agar.

**Escherichia coli strain HB101** (7), obtained from Dr. P. Modrich (Duke University Medical Center), was used as a recipient for transformation and in the isolation of plasmid DNA. Strain LE392 (supE44, supF88, λ−) (7) was obtained from our laboratory stock and employed to prepare and titer bacteriophage λ467. Strain R477 (RP477) (leuB6, Su−, X−) (8) was from Dr. C. Ruetz (University of Wisconsin-Madison) and used as a host for transposon Tn5 mutagenesis and for the xenogenetic selection of the yeast LEU2 gene. Bacteria were grown on LB or M9 medium prepared as described by Maniatis et al. (9). Where appropriate, ampicillin was included at 50 μg/ml and kanamycin at 20 μg/ml. Amino acids were provided at 50 μg/ml. Agar plates included 1% agar.

The yeast genetic methods employed were adapted from standard protocols (9). DBY746 was mutagenized to 50% survival with ethyl methanesulfonate (10), plated on YPD agar at 500−1000 colonies/plate, and grown at 30 °C for screening by the colony autoradiographic assay described below.

**Tn5 Mutagenesis**—Bacteriophage λ467 (6291, rec:Tn5, cl857, 0am29, Pam80) (11, 12) was obtained from Dr. R. Webster (Duke University Medical Center) and used as a source of transposon Tn5. It was propagated and titered as described by Miller (13). Plasmid pH1 was mutagenized with Tn5 according to the protocol of deBruin et al. (12). E. coli strain R477 was transformed with pH1 and infected with λ467 at a multiplicity of infection of 3, and transposition events were selected on LB plates containing ampicillin and kanamycin. The colonies from 10,000 independent transpositions were pooled, plasmid DNA was isolated, and E. coli strain HB101 was transformed with the plasmid pool, selecting for ampicillin and kanamycin resistance. The insertions were mapped to low resolution by determining which EcoRI fragment of pH1 contained Tn5. Insertions within the insert region of pH1 were mapped to high resolution by determining the distance from the Xhol sites flanking Tn5 to the EcoRI sites of pH1, with ambiguities being resolved by mapping relative to a second restriction site outside of the EcoRI fragment containing Tn5.

**Plasmids**—A yeast genomic library constructed in the shuttle vector YEp35 (14−16) was provided by Dr. K. Nasmyth (Medical Research Council Laboratories, Cambridge, England). Plasmid pUC18 were purchased from Bethesda Research Laboratories. All other plasmids were from Boehringer Mannheim. Materials for growth media were from 3910 supplemented with yeast nitrogen base, trizma base, and yeast extract. The abbreviations used are: kb, kilobase; MOPS, 4-morpholinepropanesulfonic acid.

**Yeast Mutants in Diacylglycerol Cholinephosphotransferase**—Yeast cultures (200 ml) grown on selective minimal medium with supplemental amino acids to an A600 of 1.0−2.0 were harvested at 1,000 × g for 10 min, washed once with 100 ml of water, and washed once with 25 ml of 20% glycerol, 50 mM MOPS/NaOH (pH 7.5), and 1 mM EDTA (GME buffer). The cell pellet was resuspended in a total volume of 1.0 ml with GME buffer and disrupted by glass beads in a mini-beadbeater vial as previously described (4). The homogenate was transferred to a 1.5-ml Eppendorf tube and centrifuged at 4,000 × g for 10 min to remove yeast spheroplasts. The supernatant was then diluted to 10 ml with GME buffer and centrifuged at 100,000 × g for 1 h. The pellet was resuspended with the aid of a Teflon homogenizer in 0.5 ml of GME buffer and frozen in aliquots at −70 °C. The preparation was maintained at 0−4 °C throughout all steps of the membrane isolation. Membrane protein was estimated by the method of Peterson (20) using bovine serum albumin as standard.

**Synthesis of Radiolabeled CDP-choline**—[32P]− and [methyl−3H]phosphorylcholine were prepared enzymatically using choline kinase from _S. cerevisiae_ as reported by Vance (21). The reaction mixture consisted of 0.22 mM phosphorylcholine, 1.1 mM choline chloride (5 mCi, 80 Ci/mmole), 10 mM MgCl2, 36 mM Tris-HCl (pH 8.0), 10 mM ATP, and 0.1 unit of choline kinase in a total volume of 280 μl. The [32P]-labeled derivative was similarly prepared except that the reaction mixture contained 0.36 mM [3-32P]ATP (1 mCi, 1 Ci/ mmole) and 1.1 mM choline chloride. The specific activity was reduced by addition of 15 μmol of unlabeled phosphorylcholine; the pH was adjusted to 9 with concentrated ammonium hydroxide; and the material was converted to its zwiterionic salt form by adsorbing it to a 5-ml column of Dowex 1-X2 formate washing with 5 volumes of water, and eluting with 5 volumes of 50 mM formic acid. The eluent was concentrated and reconstituted in 1 ml of formic acid, and its concentration was determined by phosphate analysis according to the method of Ames and Dubin (22). To convert radiolabeled phosphorylcholine to CDP-choline, a modification of the direct condensation with cytidine 5′-monophosphate using the Vilsmeier-Haack reagent (23) was employed. To 10 μmol of phosphorylcholine dried thoroughly in vacuo was added 40 μl of freshly prepared 1.0 M thionyl chloride (40 μmol) in anhydrous _N,N_-dimethylformamide. After 5 min at 25 °C, 3.4 mg (10 μmol) of CMP was added. The reaction mixture was vortexed until clear and held at 25 °C for 1 h after which 200 μl of water was added. The quenched reaction mixture was held for 1 h at 26 °C, and then the pH was adjusted to 9 with concentrated ammonium hydroxide. The material was submitted to a 5-m column of Dowex 1-X2 formate and washed with 5 volumes of water to remove the side product dicholine pyrophosphate and an unidentified product. The material was then eluted with a 0–50 mM gradient of formic acid in a total volume of 100 ml of the order of elution was: unreacted phosphorylcholine, CDP-choline, followed by unreacted cytidine monophosphate. The fractions containing CDP-choline were pooled and lyophilized, and dissolved in water. The concentration was determined by phosphate analysis. The fraction was taken to dryness, and its radiochemical purity was >99% as assessed by paper chromatography on Whatman No. 3M paper using solvent system 1 (23) as the developing solvent system.

**Synthesis of Radiolabeled CDP-ethanolamine**—Ethanolamine [32P]− phosphosphate was prepared from 25 mCi of [32P]−, by including yeast choline kinase (0.1 unit) and ethanolamine (added as 100 mM ethanolamine hydrochloride (pH 8) to a final concentration of 2 mM) in a standard
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enzytic $^{32}$P]ATP-generating system (24). After incubation at 37 °C for 1 h, 50 μmol of unlabeled ethanolamine phosphate was added, and the product was purified by ion exchange chromatography as described above for choline phosphate. After lyophilization, the ethanolamine$[^{32}$P]phosphate was dissolved in 250 μl of 1 M NaOH and reacted with 15 μl of benzylxycarbonyl chloride (100 μmol) for 30 min at 0 °C (25) to convert the free amine to the benzyl carbamate. The protected ethanolamine phosphate was then applied to a 5-ml Bio-Rad AG 50W-X4 H+ column, and the fully protonated product was collected in five successive 1-ml water washes. After lyophilization, the residue was dissolved in 250 μl of anhydrous N,N-dimethylformamide and reacted with 21 μg (30 μmol) of CMP-morpholide for 12 h, at 0 °C (26) to form CDP-ethanolamine benzyl carbamate. The product was deprotected by catalytic transfer hydrogenation in the presence of 0.5 M ammonium formate and 25 mg of 10% palladium on carbon for 5 min with 0.4% Coomassie Blue in ethanol and destained with 10% trichloroacetic acid, the paper was washed with 6×25-ml portions of ice-cold 2% trichloroacetic acid to quench the reaction and precipitate the phosphatidylethanolamine. Both assays were linear with time and protein in the range employed.

RESULTS AND DISCUSSION

Filter Paper Assay and Mutant Selection—A colony autoradiographic assay (4, 28, 29) was devised to facilitate the rapid screening of a large number of mutagenized cells for defects in cholinephosphotransferase. The assay was based on the incorporation of radiolabeled CDP-choline into phosphatidylethanolamine in permeabilized yeast colonies on filter papers using endogenous diacylglycerol as the phosphorylcholine acceptor. The radioactive products of the in situ reaction were characterized by extraction of the papers followed by thin layer chromatography (see “Experimental Procedures”). Greater than 97% of the radioactivity remaining after washing was chloroform-extractable, and greater than 99% of this co-migrated with authentic phosphatidylethanolamine during thin layer chromatography. The dependences of the assay on time and substrate concentration were also investigated. As shown in Fig. 1A, incorporation into phosphatidylethanolamine was linear up to 90 min, demonstrating that endogenous diacylglycerol was not limiting in the assay. The CDP-choline concentration required for 50% maximal activity (Fig. 1B) was similar to the apparent $K_M$ for CDP-choline measured in membrane preparations (60 μM, see Table II). Thus, delivery of the radioactive substrate to the enzyme was efficient. A CDP-choline concentration of 100 μM was chosen for mutant

![Fig. 1. Time course and substrate dependence of the cholinephosphotransferase colony autoradiographic assay.](image-url)
screening in order to maximize incorporation while retaining sensitivity to mutants having an altered $K_d$ for CDP-choline. Taken together, these experiments establish a colony autoradiographic assay for yeast cholinephosphotransferase.

The colony autoradiographic assay was used to screen 15,000 colonies of DBY746 mutagenized to 50% survival with ethyl methanesulfonic acid. By comparing the autoradiographic patterns to the Coomassie Blue-stained filters, 22 isolates were identified which had reproducibly decreased in situ cholinephosphotransferase activities. Fig. 2 shows the identification of one of these mutants, HJ110, as an example of the screening method. The extent of reduction of CDP-choline incorporation ranged from slight to nearly complete (see mutants shown in Fig. 3). Since the relative differences observed between various mutant strains were highly reproducible in separate experiments, a qualitative scale reflecting the colony autoradiographic phenotype was devised (see Table 1).

**Complementation Analysis**—Genetic complementation analysis of the mutants was undertaken as a first step in their characterization. The colony autoradiographic assay phenotype proved suitable for this purpose (see Fig. 3). The cholinephosphotransferase activities of diploids formed in crosses of each mutant to the wild-type strain AH22 were normal; thus, all of the $cpt$ mutations were recessive. The mutant allele present in HJ110 was designated $cpt1$, and a strain bearing this allele and which was suitable for genetic crosses to the remaining mutants, HJ111 ($a$ leu2-3 leu2-112 his4-519 can1 $cpt1$), was constructed by standard genetic methods.

Seventeen of 21 of the remaining mutants failed to provide phenotypic complementation when crossed to HJ111 and were thus designated $cpt1$. Four mutants fully complemented the $cpt1$ mutation of HJ111. The mutant allele of one of these, HJ214, was designated $cpt2$, and an additional mater strain bearing it, HJ213 ($a$ leu2-3 leu2-112 can1 his4-519 $cpt1$), was constructed. The complementation data for the remaining mutants as well as HJ110 against $cpt2$ is shown in Fig. 3. All of the remaining mutants complemented the $cpt2$ allele of HJ213. The mutant allele of HJ128 was designated $cpt3$. Since the remaining mutants, HJ120 and HJ244, were phenotypically weak, further complementation grouping was not pursued. Random spores of HJ111 × HJ214 and HJ111 × HJ128 exhibited wild-type cholinephosphotransferase activity by filter paper assay at a frequency sufficient to preclude intragenic complementation within the $cpt1$ locus (data not shown).

**Table 1**

| Strain       | Genetic designation | Filter paper phenotype | Specific activity (nmol/min/mg) |
|--------------|---------------------|-----------------------|--------------------------------|
| DBY746/YEp13 | Wild-Type           | 5+                    | 2.1                            |
| HJ110/YEp13  | $cpt1$              | 1+                    | 0.2                            |
| HJ112/YEp13  | $cpt1$              | 2+                    | 0.3                            |
| HJ135/YEp13  | $cpt1$              | 2+                    | 0.3                            |
| HJ137/YEp13  | $cpt1$              | 2+                    | 0.4                            |
| HJ135/YEp13  | $cpt3$              | 3+                    | 1.1                            |
| HJ125/YEp13  | $cpt1$              | 1+                    | 1.2                            |
| HJ213/YEp13  | $cpt2$              | 2+                    | 1.7                            |
| HJ128/YEp13  | $cpt3$              | 2+                    | 1.9                            |
| DBY746/pRH1  |                     |                       | 10.0                           |
| HJ110/pRH1   |                     |                       | 8.4                            |

Thus, the mutants selected fell into three complementation groups.

To understand further the nature of the $cpt$ mutants, the cholinephosphotransferase activities were characterized. For this purpose, the mutant strains were transformed with the shuttle vector YEp13 so that the results could be directly compared to strains bearing hybrid plasmids which complement the $cpt1$ mutation (see "Cloning of the CPT1 Gene"). Membranes of parent and mutant strains were prepared and assayed for cholinephosphotransferase activity at a CDP-choline concentration of 250 μM. This concentration was chosen since its position on the in vitro CDP-choline dependence curve approximated that used in the colony autoradiographic assay. The results are shown in Table 1. Only $cpt1$ mutants were defective in cholinephosphotransferase activity.
under these conditions. The qualitative scale reflecting the extent of the filter paper phenotype correlated well with the cholinephosphotransferase activities assessed in membranes. Mutants exhibiting a strong phenotypic defect had 5-7-fold reductions in \( \text{in vitro} \) cholinephosphotransferase activities, whereas more moderately affected \( \text{cpt1} \) mutants were associated with intermediate activities. In contrast, the \( \text{cpt2} \) and \( \text{cpt3} \) isolates exhibited near normal cholinephosphotransferase activities under these conditions despite their strong defects in the colony autoradiographic assay. This initial \( \text{in vitro} \) screening suggested that the \( \text{cpt1} \) complementation group most likely represented the desired mutants in the cholinephosphotransferase structural gene. Therefore, the \( \text{cpt2} \) and \( \text{cpt3} \) isolates were not pursued further. The finding of additional complementation groups strongly affected in the cholinephosphotransferase autoradiographic assay may have important implications in future studies of regulation.

**Enzymological Analysis**—Additional characterization of the cholinephosphotransferase activity of several \( \text{cpt1} \) mutants was performed. Kinetic constants determined from Lineweaver-Burk plots of the CDP-choline dependences are shown in Table II. The 8-fold reduction in \( V_{\text{max}} \) and normal \( K_M \) for CDP-choline shown for \( \text{HJ135} \) was typical of other phenotypically strong \( \text{cpt1} \) mutants (data not shown). In contrast, \( \text{HJ125} \) showed a normal \( V_{\text{max}} \) but a 4.5-fold increase in \( K_M \) for CDP-choline (Fig. 4). Similarly, \( \text{HJ115} \) exhibited a 2-fold higher \( K_M \) value for CDP-choline than wild-type and a slightly decreased \( V_{\text{max}} \). These alterations in the kinetic properties of cholinephosphotransferase in \( \text{cpt1} \) mutants strongly suggest that the \( \text{cpt1} \) locus represents the cholinephosphotransferase structural gene. As discussed under “Experimental Procedures,” the cholinephosphotransferase assay employed was only partially dependent on exogenous diacylglycerol. Kinetic interpretation of diacylglycerol dependencies was therefore difficult. However, the yeast cholinephosphotransferase could be assayed in Triton X-100-mixed micelles where a complete dependence on exogenous diacylglycerol was observed. The apparent \( K_M \) for \( \text{sn-1,2-dioleoylglycerol} \) determined for each of the strains in Table II using this assay was similar to wild-type (data not shown).

The yeast ethanolaminephosphotransferase activity has been reported to be more strongly inhibited by CMP than is the cholinephosphotransferase activity (31). Therefore, the effect of CMP on the residual cholinephosphotransferase activity of \( \text{cpt1} \) mutants was measured to assess the possibility that the residual activity reflects a cross-specificity of ethanolaminephosphotransferase for CDP-choline. As shown in Fig. 5, the residual cholinephosphotransferase activity of \( \text{HJ110} \) was inhibited 75% in the presence of 1 mM CMP, whereas that of \( \text{DBY746} \) was only inhibited 30%. Curves nearly identical to the inhibition profile of \( \text{HJ110} \) were obtained for the other strong \( \text{cpt1} \) mutants (\( \text{HJ112}, \text{HJ135}, \) and \( \text{HJ137} \)) (data not shown). This property could reflect an alteration of the activity of cholinephosphotransferase as a consequence of mutation within its structural gene. Since the cholinephosphotransferase activities of four independent \( \text{cpt1} \) mutants were found to possess this property, this explanation was considered unlikely. Alternatively, the residual cholinephosphotransferase activity could reflect the activity of a

![Fig. 4. Double-reciprocal plot of the CDP-choline dependence of the cholinephosphotransferase activities of strains \( \text{HJ125}/\text{YEp13} \) and \( \text{DBY746}/\text{YEp13} \). Membranes from each strain were assayed for cholinephosphotransferase activity as described under “Experimental Procedures” as a function of CDP-choline concentration. Linear regression analysis of the data was used to construct the lines as shown and to determine the kinetic constants reported in Table II.](image)

![Fig. 5. Inhibition of \( \text{HJ110}/\text{YEp13}, \text{DBY746}/\text{YEp13}, \) and \( \text{HJ110}/\text{pRH1} \) by CMP. Membranes from each strain were assayed for cholinephosphotransferase activity as described under “Experimental Procedures” with the addition of the indicated concentrations of CMP. The activities are expressed as percent remaining relative to no addition.](image)
second enzyme which exhibits the observed inhibition by CMP, Ethanolaminephosphotransferase or a previously unidentified cholinephosphotransferase is a likely candidate (see "Concluding Discussion").

Cloning of the CPT1 Gene—A yeast genomic library constructed in the vector YEpl3 was transformed into HJ110, and the resulting transformants were screened for complementation of cpt by the colony autoradiographic assay. Approximately 18,000 transformants were screened, and six plasmids were selected which reproducibly conferred increased cholinephosphotransferase activity relative to HJ110/YEp13. Plasmid DNA was isolated from these strains and transformed into E. coli strain HB101 from which quantities of plasmid sufficient for physical analysis were purified. Restriction endonuclease mapping revealed that four of the plasmids contained a common 5.0-kb region of insert DNA. A representative of this group, pRH1, was chosen for further characterization. The insert DNA of pRH1 is shown in Fig. 6. The ability of pRH1 to complement the cpl defect in a plasmid-transmissible fashion was shown by colony autoradiography. Co-segregation of phenotypic correction with the LEU2 gene carried on the YEp13 portion of pRH1 was demonstrated by growing HJ110/pRH1 for 10 generations on YPD, plating single colonies onto YPD plates, replica printing to synthetic minimal plates lacking leucine, and performing cholinephosphotransferase colony autoradiography on the master print. In this experiment, 80% of the colonies had simultaneously become auxotrophic for leucine and defective in the filter paper assay; there was an exact co-segregation of the two phenotypes.

Enzymological characterization of strains transformed with pRH1 was undertaken. As shown in Table I, the presence of pRH1 in HJ110 fully restored the cholinephosphotransferase activity and led to an approximately 30- and 5-fold overproduction of activity in mutant and wild-type backgrounds, respectively. This degree of overproduction is typical of other membrane-bound enzymes whose structural genes have been cloned into YEp13 (33, 34) and is within the range expected based on the copy number of YEp13 (35, 36). The overproduced cholinephosphotransferase activity expressed in strains transformed with pRH1 was wild-type with respect to substrate dependences, thermal inactivation (data not shown), and CMP inhibition profile. As seen in Table II, pRH1 completely corrected the Km defect present in HJ125. The overexpressed cholinephosphotransferase activity in HJ110/pRH1 was wild-type with respect to inhibition by CMP (Fig. 5). The overproduction of cholinephosphotransferase activity directed by the cloned CPT1 gene which is indistinguishable from wild-type activity in several genetic backgrounds is strong evidence that the cloned gene represents the cholinephosphotransferase structural gene.

Sublocalization of the CPT1 Gene—In order to localize the coding region of the presumptive CPT1 gene within the pRH1 insert DNA, pRH1 was mutagenized with transposon Tn5 as described under "Experimental Procedures." Twenty Tn5 insertions were found within the insert region of pRH1. The locations of these were precisely determined and are shown in Fig. 6 (triangles).

Each of the 20 pRH1::Tn5 insertions was transformed into HJ110, and their functional ability in complementing the cholinephosphotransferase activity defect was evaluated by the colony autoradiographic assay. Four contiguous Tn5 insertions (Fig. 6, filled triangles) failed to provide phenotypic complementation, setting a lower limit to the size of the CPT gene of 0.8 kb. The Tn5 insertions which were fully active and thus carried no nonessential DNA set the upper limit of size at 2.4 kb. Additional localization data were obtained by deletion mapping. A complete HindIII digest of pRH1 was recircularized to construct pRH103; this construction deleted the region from the leftmost HindIII site within the insert to the Bsr322 HindIII site 0.35 kb into the YEp13 sequences to the right of the insert border. Upon transformation into HJ110, pRH103 failed to complement the cholinephosphotransferase filter paper assay defect, indicating that the CPT gene extended at least up to the leftmost HindIII site from the inactive Tn5 insertions (Fig. 6). Thus, the minimal size of the CPT gene was determined to be 1.2 kb.

Construction of a cpl Insertional Mutant—To establish that the CPT1 gene cloned is the CPT1 gene, a one-step gene disruption experiment (37) was performed. For this purpose, an integrative plasmid containing the CPT gene disrupted by a yeast selectable marker was required. Since pRH1 contained no restriction sites within the known CPT coding region convenient for the placement of a yeast selectable marker, the disrupted copy of the CPT1 gene in pRH1::Tn5-9 (Fig. 6, asterisk) was subcloned into pUC18 so that the restriction sites within Tn5 could be used to insert the LEU2 fragment of YEp13 (see "Experimental Procedures"). The structure of the plasmid constructed, pRH105, is shown in Fig. 7.

The disrupted CPT1 gene of pRH105 was liberated by digestion with BamHI and PstI and transformed into DBY746, selecting for leucine prototrophy to detect integrative events. Five out of 10 transformants had acquired a strong defect in cholinephosphotransferase activity when screened by the colony autoradiographic assay. The extent of the defects was similar to that of HJ110 (see Fig. 8). One of these, designated HJ001, was selected for further study. Both the leu" and cpl" phenotypes of HJ001 were mitotically stable on nonselective medium, indicating that the disrupted CPT1 gene was stably integrated into the chromosome.

Characterization of the cpl Insertional Mutant—Complementation analysis for HJ001 is shown in Fig. 8. The cpl insertional mutant complemented the cpl2 and cpl3 (data not shown) mutations but failed to complement the cpl1 mutation.

Since no four-spore asci were generated upon sporulation of HJ001 x AX22, random spores were analyzed for cholinephosphotransferase activity and growth on leucine. In this
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Fig. 7. Integrative plasmid constructed for gene disruption of chromosomal CPT1. As described under “Experimental Procedures,” a disrupted copy of the CPT gene was mobilized on the 11.3-kb EcoRV/KpnI fragment of pRH1::Tn5-3 (Fig. 6, *) and subcloned into pUC18. The 2.2-kb Xhol/Sall LEU2 fragment of YEp13 was then inserted utilizing the XhoI sites within Tn5. The resulting integrative plasmid, pRH105, is shown above along with the origin of its various regions. To disrupt the chromosomal CPT1 gene, 2 μg of pRH105 was cleaved with BamH I and Pst I and transformed into DBY746. Integrative events were detected by selecting for leucine prototrophy, and cholinephosphotransferase colony autoradiography was used to identify a cptl insertional mutant.

Fig. 8. Complementation analysis of the cptl insertional mutant. The colony autoradiographic assay shows the activity of the cptl insertional mutant (HJ001) and crosses of HJ001 to cpt mater strains. The cpt insertional mutation is recessive to the wild-type gene (× AH22), fully complements cpt2 (× HJ213) and cpt3 (data not shown), and fails to complement cptl (× HJ111). The activities of HJ213, HJ111, and DBY746 were included as controls.

Table III

| Strain  | Genetic designation | Cholinephosphotransferase activity | Ethanolaminephosphotransferase activity |
|---------|---------------------|-----------------------------------|----------------------------------------|
|         |                     | nmol/min/mg protein               | mmol/min/mg protein                    |
| HJ011   | Wild-type           | 2.3                               | 1.8                                    |
| HJ001   | cptl                | 0.5                               | 1.6                                    |

Fig. 9. CMP sensitivity of the residual cholinephosphotransferase activity of the cptl insertional mutant. Membranes prepared from the cptl insertional mutant (HJ001) and a stable integrant of pRH105 having normal cholinephosphotransferase activity (HJ011) were assayed as described under “Experimental Procedures” with the addition of the indicated concentration of CMP. The activities are expressed as percent remaining relative to no addition. ▲, HJ001; △, HJ011.

experiment, 65/160 (41%) segregants were found to be defective in cholinephosphotransferase activity and prototrophic for leucine, and 95/160 (59%) were found to have wild-type cholinephosphotransferase activity and were auxotrophic for leucine. This segregation pattern was indicative of nuclear inheritance of a single gene and demonstrated tight linkage of the LEU2 gene to the cptl locus. These results indicate that the disrupted cptl construct integrated at the cptl locus by homologous recombination and established that the cloned CPT gene is the CPT1 gene. Enzymological analysis revealed that the cholinephosphotransferase activity of the insertional mutant was reduced 5-fold relative to a control strain which was transformed to leucine prototrophy by pRH105 but had not acquired a cptl mutation (Table III). The residual cholinephosphotransferase activity exhibited a wild-type K_M for CDP-choline (data not shown) and, unlike the wild-type activity, was strongly inhibited by CMP (Fig. 9). Since the integrated cptl gene in HJ001 was intentionally disrupted near the middle of the known functional region of the gene, it is assumed that the CPT1 gene product would be nonfunctional. The data indicate that the CMP-sensitive residual activity does not represent an altered activity of the CPT1 gene product (see “Concluding Discussion”). Thus, the ability of a haploid strain containing the cptl insertional mutation to grow shows that the CPT1 gene is nonessential for growth.

Ethanolaminephosphotransferase Activities—To investigate the relationship between the yeast choline- and ethanolaminephosphotransferases, membranes possessing defective and overproduced cholinephosphotransferase activities were assayed for ethanolaminephosphotransferase activity. Tables
III and IV show that the large reductions in cholinephosphotransferase activity in cpl mutants were associated with only slightly reduced ethanolaminephosphotransferase activities. Ethanolaminephosphotransferase activity decreased 5–15% as compared to the decline in cholinephosphotransferase activity. The converse was also observed. When the cholinephosphotransferase activity was elevated due to the presence of the CPT1 gene of pRH1, the ethanolaminephosphotransferase activity increased to an extent approximately 8% of the increase in cholinephosphotransferase activity. Similar results were obtained in all mutant strains bearing pRH1 that were examined. The isolation of mutants defective in cholinephosphotransferase but not ethanolaminephosphotransferase establishes genetically that the two enzymatic activities are encoded by different genes. The attenuated response of the ethanolaminephosphotransferase activities to variation in cholinephosphotransferase activities could be interpreted to reflect a specificity of the cholinephosphotransferase for CDP-ethanolamine; thus, cholinephosphotransferase appears to utilize CDP-ethanolamine as substrate at a rate approximately 10-fold less than CDP-choline.

Concluding Discussion—Enzymological characterization of the cpl mutants isolated in this work supports the conclusion that the cpl locus represents the structural gene for yeast cholinephosphotransferase. The cpl complementation group encompassed independent isolates which were defective in cholinephosphotransferase activity to varying extents, indicating one whose defect was completely attributable to an altered $K_m$ for CDP-choline. The overproduction of cholinephosphotransferase activity having wild-type properties in several genetic backgrounds bearing the cloned CPT1 gene further supports this view. Since we have demonstrated a genetic link between the cloned CPT1 gene and the cpl complementation group, we further conclude that the cloned gene is the cholinephosphotransferase structural gene.

The residual cholinephosphotransferase activity present in strong cpl mutants and the cpl insertional mutant clearly differed from the major wild-type activity in its CMP sensitivity; this strongly suggests the presence of a second cholinephosphotransferase activity in yeast. However, no conclusion regarding the identity of the enzyme(s) responsible for the residual activity is justified. As discussed earlier, the presence of the CMP-sensitive activity in all strong cpl mutants and the cpl insertional mutant argues against it reflecting an altered activity of the CPT1 gene product. The second activity may be due to a weak activity of ethanolaminephosphotransferase using CDP-choline as substrate. In view of the evidence presented here that cholinephosphotransferase exhibits weak activity using CDP-ethanolamine, it would not be surprising if a similar cross-specificity is manifested by ethanolaminephosphotransferase which could quantitatively account for the residual cholinephosphotransferase. Ethanolaminephosphotransferase was found to be CMP-sensitive as previously reported (31). However, the residual cholinephosphotransferase of the cpl insertional mutant was somewhat high when compared to that observed in other cpl mutants and was not associated with a proportionately elevated ethanolaminephosphotransferase activity. In this regard, it is noteworthy that the residual activity of the insertional mutant was also surprisingly high given its dramatically reduced activity in the colony autoradiographic assay. Several factors may underlie these observations. Whereas alterations in the predominant cholinephosphotransferase activity were reflected in the colony autoradiographic assay, physical barriers such as subcellular compartmentalization may preclude the assay from detecting the minor residual activity. Moreover, physiological conditions and growth properties may influence the level of the second activity or its expression, explaining differences between activities observed in the colony autoradiographic assay and membranes. Finally, the residual activity or its expression could be regulated by the CPT1 gene or its gene product, accounting for differences in its level in various cpl mutants, especially the insertional mutant. Thus, we conclude that the residual cholinephosphotransferase activity is due to a cross-specificity of ethanolaminephosphotransferase and/or the presence of a second cholinephosphotransferase. This possibility may bear on our observation that the CPT1 gene is nonessential for cell growth. Our current efforts to obtain mutants in ethanolaminephosphotransferase will assist in the resolution of this problem.

Our data support and extend previously reported enzymological evidence that the yeast cholinephosphotransferase and ethanolaminephosphotransferase activities are catalyzed by separate enzymes (31). Our demonstration of the genetic distinguishability of the two activities constitutes conclusive evidence for distinct enzymes.

The genetic foundation and molecular tools assembled in this work should greatly facilitate further studies of the structure, function, and regulation of yeast cholinephosphotransferase. We anticipate that future studies of the physiologic significance of cpl mutants and detailed analysis of the CPT1 gene and gene product will yield fundamental insight into the regulation of phosphatidylcholine biosynthesis.

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### Table IV

| Strain | Genotype | CPT activity | Ethanolamine activity |
|--------|----------|--------------|-----------------------|
| HJ12/YEp13 | cpl1 | 0.3 | 1.2 |
| HJ37/YEp13 | cpl1 | 0.4 | 1.4 |
| HJ135/YEp13 | cpl1 | 0.3 | 1.4 |
| DBY746/YEp13 | Wild-type | 2.1 | 1.5 |
| DBY746/pRH1 | | 10.0 | 2.1 |
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