J. Biochem. 120, 1040-1047 (1996)

Isolation and Characterization of *ECT1* Gene Encoding CTP: Phosphoethanolamine Cytidylyltransferase of *Saccharomyces cerevisiae*[1]

Rho Min-Seok,[2] Yuji Kawamata,[3] Hidemitsu Nakamura, Akinori Ohta,[4] and Masamichi Takagi

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113

Received for publication, July 29, 1996

Saccharomyces cerevisiae mutants that were unable to utilize extracellular ethanolamine for phosphatidylethanolamine synthesis were isolated. Two of them carried recessive chromosomal mutations in a same gene and were defective in CTP:phosphoethanolamine cytidylyltransferase (ECT) activity *in vitro* (Ect⁻). In an Ect⁻ mutant that also carried the *cho1* mutation, phosphatidylethanolamine accounted for less than 2% of total phospholipids, suggesting the importance of ECT in phosphatidylethanolamine synthesis. By screening a genomic library on a low copy number vector, three complementary clones of different size were isolated. A 2.8-kb common DNA region carried an open reading frame (ORF) of 969 bp in length, of which a truncated form failed to complement the Ect⁻ mutation. This ORF was identical to the previously isolated *MUQ1* gene of unknown function. Its deduced amino acid sequence had significant similarity to CTP:phosphocholine cytidylyltransferases of yeast and rat. The entire ORF, when combined with the glutathione *S*-transferase gene and expressed in *Escherichia coli*, exhibited ECT activity. These results indicate that the cloned gene encodes a catalytic subunit of ECT of *S. cerevisiae*.

Key words: CDP-ethanolamine, Kennedy pathway, phosphatidylethanolamine, phosphoethanolamine cytidylyltransferase, *Saccharomyces cerevisiae*.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

Phosphatidylethanolamine (PE) in eukaryotic cells is synthesized through two pathways (*1*). One is the decarboxylation of phosphatidylserine (PS) and the other is the utilization of free ethanolamine or phosphoethanolamine *via* the CDP-ethanolamine pathway. The vital role of the former pathway in PE synthesis in mammalian cells was demonstrated by the isolation of a phosphatidylserine decarboxylase cDNA clone as the one that complements the deficiency in PS synthesis of a Chinese hamster ovary cell mutant (*2*). The importance of this pathway in yeast *Saccharomyces cerevisiae* is obvious, because mutants defective in PS synthesis or PS decarboxylation cannot synthesize most cellular PE unless ethanolamine is supplied extracellularly. Recently, it has been found that more than one gene codes for this enzyme (*3, 4*). The latter pathway has not been studied as extensively as the parallel CDP-choline pathway for phosphatidylcholine (PC) synthesis.

The CDP-choline pathway is constituted by three enzymes, choline kinase, CTP:phosphocholine cytidylyltransferase (CCT) and cholinephosphotransferase. The first and second enzymes of mammals were purified and their structures were elucidated by analyzing their cDNA clones (*5, 6*). The CCT of mammals is the target of the cellular regulation. It is active in a membrane-bound form and its conversion from a soluble, inactive form is regulated in several ways (*7*), of which the exact molecular mechanism is not yet clear. The significance of its nuclear localization in terms of the cellular regulation of PC synthesis is also controversial (*8*). The recent finding of a 112-kDa protein which was copurified with this enzyme may shed light on these unsolved problems (*9*). In yeast, all the structural genes for the enzymes of the CDP-choline pathway were isolated and their structures were analyzed. Of these, *CCT* codes for CCT (*10*), and the gene product was also found to be membrane-bound in yeast cells, but not when it was produced in *Escherichia coli* (*11*), suggesting that the yeast CCT works in a similar manner to its mammalian counterpart. Recently, it has been reported that yeast CCT activity was inhibited by PI/PC exchange protein in its PC-bound form (*12*), which may explain how yeast cells regulate cellular PC content.

The CDP-ethanolamine pathway also consists of three enzymes with similar activities to those of choline-utilizing pathway, and the step catalyzed by CTP:phosphoethanolamine cytidylyltransferase (ECT, EC 2.7.7.14) is supposed

[1] This study was supported in part by a Grant-in-Aid for Scientific Research (04660080) from the Ministry of Education, Science, Sports and Culture of Japan and performed using the facilities of the Biotechnology Research Center, The University of Tokyo.
Present addresses: [2] National Institute of Bioscience and Human Technology, 1-1 Higashi, Tsukuba, Ibaraki 305; and [3] Discovery Research Laboratories I, Discovery Research Division, Takeda Chemical Industries Ltd., 10 Wadai, Tsukuba, Ibaraki 300-42.
[4] To whom correspondence should be addressed. Phone: +81-3-3812-2111 (Ext 5188), Fax: +81-3-3812-9246, E-mail: aaohta@hongo.ecc.u-tokyo.ac.jp
Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; ECT, CTP:phosphoethanolamine cytidylyltransferase; ORF, open reading frame; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

to be the point of regulation (*13*). The ECT of rat hepatocytes was purified to homogeneity in a soluble form and its kinetic properties were studied (*14, 15*), but the regulation and genetic control of the enzyme mostly remain unknown. In the yeast *S. cerevisiae*, the presence of the same enzyme activity was reported (*16*), but the properties and regulation of the enzyme again remain to be studied.

To elucidate the genetic control and overall structure of the ECT, we isolated *S. cerevisiae* mutants that are defective in the activity of the enzyme. One such mutant that was also defective in phosphatidylserine synthesis contained a greatly reduced amount of PE, suggesting the importance of ECT in the synthesis of this aminophospholipid. We cloned a gene, *ECT1*, that complements the mutation. From both the structural similarity of the deduced product to CCTs and the ECT activity detected in extracts of *E. coli* cells where *ECT1* was expressed, we concluded that *ECT1* codes for the structural gene of the enzyme ECT.

EXPERIMENTAL PROCEDURES

Materials—[1,2-^{14}C]Ethanolamine was purchased from ICN Biomedicals (Costa Mesa, CA). Phospho[1,2-^{14}C]-ethanolamine was synthesized enzymatically and purified according to the published protocol (*17*). Partially purified ethanolamine kinase from rat kidney was supplied by Dr. K. Ishidate, and later prepared from rat liver following the above protocol. The purity of the radioactive phosphoethanolamine was checked by thin layer chromatography on Silica Gel 60 (Merck, No. 5721) with 96% ethanol-2% NH_4OH, (1 : 2, v/v) as a developing solvent. CTP and phosphoethanolamine were purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

DNA enzymes used to manipulate isolated genes were products of Takara Shuzo (Kyoto) and Nippon Gene (Toyama).

Growth Conditions, Strains, and Plasmids—Construction and general properties of *S. cerevisae* strains YB1803 (*MAT*a *trp1 cho1::LEU2*) and C5558-2B (*MATα trp1 ura3*) were described previously (*18*). Strain EY132 (*MATα trp1 ura3 cho1::LEU2*) was constructed by crossing strain YB1812 (*MATα trp1 cho1::LEU2*) with strain 2026 (*MAT*a, *leu2 ura3 can ino1::LEU2*). Strain YP148 with fragmented Chr. VII was used as standard for electrophoretic separation of chromosomes (*19*).

Construction of plasmid YCpGPSS (*TRP1* G418^R P^{GAL7}-*CHO1/PSS*) bearing the *CHO1* gene, expression of which was under the control of the *GAL7* promoter, was described previously (*18*). Plasmid YCp50GPSS was obtained by combining a *Bgl*II-*Sal*I fragment carrying the *GAL7-CHO1* region in the YCpGPSS with plasmid YCp50.

Minimal synthetic medium, SD, for yeast growth was prepared as described previously (*18*). When necessary, 2% D-glucose in SD was replaced with 1% D-glucose plus 2% D-galactose for partial induction of *GAL7* promoter.

Yeast cells were transformed using the alkali cation treatment method (*20*).

E. coli strain MV1190 {(*Δsrl-recA*)306::*Tn*10 *Δlac-pro thi, supE* [F′ *proAB lacI*^{q}*lacZΔ*M15 *traD*]} was used as a general bacterial host for plasmids.

DNA Manipulations—Restriction enzyme digestion and ligation and other general treatment of DNAs were done according to the published methods (*21*).

A 2.8-kb *Xba*I-*Hin*dIII fragment of plasmid pYECT-C that contained *ECT1* was subcloned into the *Hin*cII site of pUC119 in two different directions (pUCHX and pUCXH) and successive deletions were introduced from one end of the cloned DNA for overlapped sequencing analysis. The nucleotide sequences were determined by the dideoxy nucleotide chain termination method (*22*).

The same 2.8-kb *Xba*I-*Hin*dIII fragment was blunt-ended, then transferred into the *Sac*I site of the YEp352 to construct plasmid YEpECT. This plasmid was used to provide the *ECT1* gene to yeast cells in high copy number.

To construct a *GST-ECT1* fusion gene, plasmid pUCHX35 was used, being a deletion derivative of plasmid pUCHX that was isolated for sequencing analysis was used. In plasmid pUCHX35, the 5′ side of *ECT1* was deleted up to the first two codons. The remaining *ECT1*-coding region was excised by use of *Sac*I, for which one site was in the vector near the 5′ end of *ECT1* and the other site was at about 900 bp behind the *ECT1*. After blunt-ending the fragment, it was inserted into an *Sma*I site of plasmid pGEX-2T (Pharmacia-PL Biochemicals, Milwaukee, WI). The resultant plasmid pGST-ECT carried a *GST-ECT1* in-frame fusion.

Northern Blotting Analysis—Total RNA fraction was isolated from exponentially growing yeast cells by the phenol extraction method (*23*) and separated by denatured agarose gel electrophoresis with formaldehyde as a denaturant. RNAs were transferred to Hybond N+ membranes (Amersham, Arlington Heights, IL) and detected by hybridization with a 0.9-kb *Sph*I fragment as a probe.

Preparation of Yeast Cell Extracts and Measurement of ECT Activity—The yeast cells grown to early stationary phase were collected, washed once with distilled water and suspended in ice-chilled 50 mM Tris-HCl (pH 8.0)-1 mM EDTA-0.3 M sucrose-10 mM 2-mercaptoethanol. The cells were disrupted by mixing them with glass beads (0.4 to 0.5 mm in diameter). Unbroken cells and glass beads were removed by brief centrifugation (1,500 × *g*, 5 min) at 4°C. The resultant supernatant was assayed for ECT activity. The reaction mixture contained following components in 100 μl: 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM CTP, 1 mM phospho[2,3-^{14}C]ethanolamine (1,090 cpm/nmol), and 20 μl of enzyme. The reaction was carried out for 15 min at 30°C and stopped by boiling for 2 min. Radioactive CDP-ethanolamine and phosphoethanolamine were separated by thin layer chromatography on a Silica Gel 60 plate (Merck, No. 5721) using 96% ethanol-2% NH_4OH (1 : 2, v/v) as a solvent. Spots corresponding to CDP-ethanolamine were detected with BAS2000 imaging plate (Fuji Film, Tokyo) and scraped into scintillation vials. Radioactivities were measured by liquid scintillation spectrophotometry.

Purification of the GST-ECT1 Fusion Gene Product—Overproduction and purification of *GST-ECT1* fusion gene product were done as described (*24*). Briefly, a fresh saturated culture of *E. coli* strain MV1190 that carried the plasmid pGST-ECT was inoculated into 1,000 ml of LB medium at 10% inoculum size. After 1 h of aeration at 37°C, the fusion gene were induced by addition of 1 mM of isopropyl thio-β-D-galactoside (IPTG) and further incubation for 3 h. Cells were collected by centrifugation (5,000 × *g*, 10 min) at 4°C and washed once with chilled phosphate-

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

buffered saline (PBS). Cells were resuspended in 10 ml of PBS and disrupted by sonifying for a total of 3 min (Branson Sonifier, 20 kHz, 50% pulse output). The lysate was solubilized with Triton X-100 (final 1%) and insoluble materials were removed by centrifugation (10,000 $\times g$, 10 min) at 4°C. The supernatant was loaded on a Glutathione-Sepharose 4B column (Pharmacia-PL Biochemicals) and proteins that specifically bound to the resin were eluted according to the supplier's protocol.

Miscellaneous Analytical Methods—Yeast chromosomes were separated by pulse field agarose gel electrophoresis using a CHEF-DRII apparatus (Bio-Rad, CA). DNAs were transferred to nylon membranes after acid-treatment and alkaline denaturation according to the published protocols (*21*). *ECT1* DNA was detected by hybridization with a 1.2-kb *Hin*dIII-*Cla*I fragment from the plasmid pYECT-C as a probe. Radiolabeled DNAs were prepared by the random primer method (*25*) using a labeling kit (Takara Shuzo, Kyoto) and [α-^{32}P]dCTP (Amersham).

To measure incorporation of [^{14}C]ethanolamine into trichloroacetic acid (TCA)-insoluble materials, yeast cells were grown to $A_{600}=0.2$ in 10 ml of SD medium including 1 mM choline, washed twice with 10 ml of water and resuspended in 10 ml of fresh SD medium supplemented with 200 μM of [^{14}C]ethanolamine (5 mCi/mmol). Aliquots of 1 ml were withdrawn and immediately mixed with 5 ml of chilled 5% TCA containing 5 mM ethanolamine. The whole cell suspension was filtered through GF/C filter (Whatman) and trapped cells were washed 5 times with 5 ml of 5% TCA. Filters were dried to remove TCA and subjected to liquid scintillation counting to measure radioactivity.

Yeast phospholipids were labeled with 10 μCi of [^{32}P]P$_i$ per ml of SD medium for at least 5 generations. They were extracted, separated and quantitated as described previously (*26*).

Protein concentration was determined by the dye-binding method (*27*) using Bio-Rad protein assay kit (Bio-Rad). Bovine serum albumin was used as a standard.

RESULTS

Isolation of Mutants Defective in ECT Activity—An *S. cerevisiae* strain that had lost phosphatidylserine synthase activity due to disruption of the *CHO1/PSS* gene was used as a parental strain. Plasmid YCpGPSS (*TRP1* G418^R P^{GAL7}-*CHO1/PSS*) was included in it to avoid a possible severe growth defect resulting from the mutational inactivation of ECT. The strain, YB1803/YCpGPSS, required ethanolamine or choline for its growth in the presence of D-glucose as a sole carbon source, but not when D-galactose was supplied. About 20,000 mutagenized colonies were first grown on D-galactose-containing agar medium, and by replica-plating, four candidate mutants that grew on SD plates containing 1 mM choline but not on plates containing 1 mM ethanolamine were isolated. Mutants 101, 102, and 107 were defective in ECT activity *in vitro* (Table I). Of these, the mutant 101 had lost mating ability, but mutants 102 and 107 had recessive single nuclear mutations that were responsible for their growth characteristics, and they were not complementary to each other. Mutant 102 was crossed with a strain C5558-2B (*MATα trp1 ura3*), and one *ura3* derivative, strain EU102/YCpGPSS, which contained the original mutation Ect$^-$ and plasmid YCpGPSS, was isolated for further characterization.

TABLE I. **Phosphoethanolamine cytidylyltransferase activities of isolated mutants.**

Strain	ECT activity (nmol/min/mg protein)
101	0.06
102	0.19
107	0.04
YB1803/YCpGPSS	2.9

Properties of the Mutant Strain EU102—We first tested whether the mutant strain was able to grow without plasmid YCpGPSS. The strain EU102/YCpGPSS was incubated in the glucose medium containing 100 μg/ml of L-tryptophan and 1 mM choline for 2 days and a portion of culture was spread over agar medium of the same composition. By replica-plating, colonies that failed to grow on the medium without tryptophan or choline, and also failed to grow on 2% galactose-supplemented medium without choline, were selected. A few percent of colonies, which were small in size, had this phenotype. Thus, the strain EU102 without plasmid YCpGPSS was able to grow in choline-supplemented medium. The strain EU102, however, grew at much slower rate than the strain YB1803. The presence of plasmid YCpGPSS improved the growth of strain EU102 even under the repressed condition for the *GAL7-CHO1* fusion gene on the plasmid. This is probably because of the leaky expression of the fusion gene (*18*), which will result in synthesis of some amount of PS and its decarboxylated product PE.

Figure 1 shows that strain EU102 failed to uptake [^{14}C]ethanolamine into cellular TCA-insoluble materials, whereas wild-type strains X2180-1A and YB1803 incorporated it in a time-dependent manner. Strain EU102 actively uptook labeled choline (data not shown). These results indicate that this mutant is defective in incorporation of extracellular ethanolamine into phospholipids.

The *cho1* null mutants, which lacked active PS synthase and hence were impaired in PE formation through PS decarboxylation, contained significant amounts of PE in

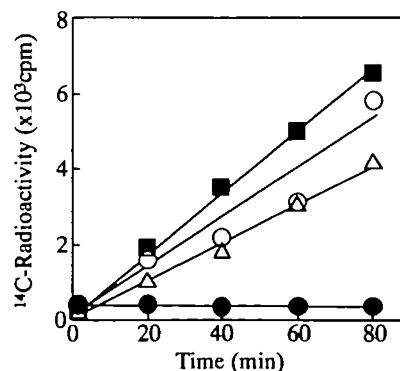


Fig. 1. **Incorporation of [^{14}C]ethanolamine into TCA-insoluble materials.** Each strain was grown to $A_{600}=0.2$ and uptake of [^{14}C]ethanolamine into cellular TCA-insoluble materials was measured as described in "EXPERIMENTAL PROCEDURES." Results are expressed as ^{14}C-radioactivity (cpm) per ml of culture. Symbols are: open circle, X2180-1A (wild type); open triangle, YB1803 (*cho1::LEU2*); closed circle, EU102 (Ect$^-$ *cho1::LEU2*); closed square, EU102 with plasmids YCpGPSS and YEpECT.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

choline-supplemented media (*26, 28*). To know the effect of the mutation on PE synthesis in strain EU102, phospholipid composition of the strain was analyzed by [^{32}P]P_i-homogeneous labeling in a choline-supplemented medium (Table II). Strain EU102 contained only 1.9% PE in total phospholipids, far less than that (20.3%) found in the *cho1* strain YB1803. This result indicates that PE formation of the choline-supported *cho1* null mutant was blocked by the mutation of EU102, that is, the mutated gene in the strain EU102 should be also responsible for PE formation from endogenous ethanolamine. It is likely that the choline-supported *cho1* null mutant synthesizes most PE by way of CDP-ethanolamine.

Isolation of ECT1 Gene—To isolate a complementary gene for the Ect$^-$ mutation in strain EU102, an *S. cerevisiae* genomic DNA library which had been constructed on the vector YCp50 (*29*) was screened using the strain EU102/YCPGPSS as a host. Ura$^+$ transformants were first selected on a minimal medium containing 1% D-glucose and 2% D-galactose, and then they were replica-plated to SD medium containing 1 mM ethanolamine. After incubation for 12 h at 30°C, the replicas were replica-plated to the same fresh agar medium and transformants that grew on it were selected. From such transformants, three plasmids of different size, pECT-A, pECT-B, and pECT-C, were recovered (Fig. 2A). Figure 3 shows growth of the transformant bearing the plasmid pECT-C or vector YCp50. Judging from their restriction maps, they had a common region of about 3 kb. This was excised from the smallest plasmid, pECT-C, as an *Sal*I fragment and subcloned on YCp50. From the resultant plasmid pECT-CSS, a series of deletion plasmids was constructed (Fig. 2B). As indicated, the complementary region was present within a 2.8-kb *Hin*dIII-*Xba*I fragment and both sides of the internal *Xho*I site were necessary. This *Hin*dIII-*Xba*I fragment also restored [^{14}C]ethanolamine incorporation of the mutant EU102 (Fig. 1, closed square). It was localized on the larger fragment of the fragmented chromosome VII in the strain YP148 by hybridization analysis after pulse field gel electrophoresis (data not shown). Thus we named the isolated gene with complementary activity on the *Hin*dIII-*Xba*I fragment *ECT1* and determined its nucleotide sequence.

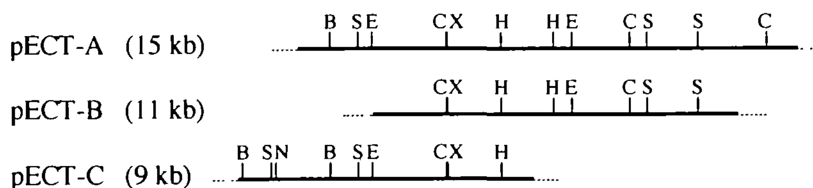
Characteristics of the Structure of ECT1—In the nucleotide sequence of the 2,829-bp *Hin*dIII-*Xba*I fragment (only relevant sequences are shown in Fig. 4), we found a 969-bp open reading frame (ORF) from the nucleotide position of 514 to 1482 from the *Hin*dIII end (1 to 969 in Fig. 4), which likely belongs to *ECT1* because this ORF covers a unique *Xho*I site at the nucleotide 1230 (underlined at 718 in Fig. 4). A nucleotide sequence from the position 1 to 367 was probably a truncated ORF without a 3′ end. There was a small ORF of 252 bp at the 3′ flanking region of the 969-bp ORF, from nucleotide 1658 to 1909

TABLE II. **Phospholipid composition of the mutant strain EU102.**[a]

Strain	Enzyme activity		Distribution of ^{32}P-radioactivity (%)						
	ECT	PSS	PC	PI+PS	PMME	PE	PG	PA	CL
EU102	−	−	62.9	28.8	nd	1.9	0.9	2.0	3.4
YB1803	+	−	58.0	14.0	<0.1	20.3	0.4	1.4	5.3
X2180-1A	+	+	37.9	30.9	1.6	22.6	0.2	3.3	3.4

[a] Cells were grown in SD medium with 1 mM choline at 30°C. Abbreviations are: PSS, phosphatidylserine synthase; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PMME, phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; nd, not detectable.

A



B

Plasmid	Growth on SDE	ECT activity (nmol/min/mg)
pECT-CSS	+	2.90
pECT-CSX	−	0.20
pECT-CXS	−	0.18
pECT-CXbH	+	2.20
pECT-CRB	+	ND*

SBgE XbSa CX H Bg S

1 kb

* Not determined

Fig. 2. **Three clones that complemented Ect$^-$ mutation and localization of the complementary gene on plasmid pECT-C.** Panel A: Restriction maps of three overlapping clones. Approximate sizes of the cloned DNAs are indicated in parenthesis. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; S, *Sal*I; X, *Xho*I. Panel B: An *Sal*I fragment of pECT-C was subcloned on vector YCp50 and indicated derivatives were introduced into the strain EU102 (Ect$^-$ *cho1::LEU2*) carrying plasmid YCpGPSS. The resultant transformants were tested for growth on SD media and analyzed for ECT activity.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

(not shown in Fig. 4). Its transcriptional direction was opposite to that of the 969-bp ORF. A search for similar sequences in a DNA database revealed that the nucleotide sequence of the putative *ECT1* gene was identical to that of *MUQ1*, which was found downstream of *PRP18* encoding a component of U5 small nuclear ribonucleoprotein (*30*). The nucleotide sequences flanking the putative *ECT1* ORF, that is, the upstream truncated ORF and the downstream small ORF, were identical to those previously published, *PRP18* and *STF2* (*31*), respectively. There was no other possible protein coding region on the sequenced *Hin*dIII-*Xba*I fragment, and the 969-bp ORF thus seemed to be that of *ECT1*.

The noncoding region upstream of the *ECT1* ORF was only 146 bp (Fig. 4). To ascertain whether this ORF is transcribed, we performed Northern blot analysis on RNA of the strain EU102/YCpGPSS carrying the plasmid pECT-CXbH by using an internal 647-bp *Sph*I-*Xho*I fragment as a probe. A 1.2-kb mRNA product was detected (data not shown) and we concluded that this ORF was actively transcribed. There are three small ORFs in the intermediate region (start and terminal codons are indicated by open boxes in Fig. 4), the functions of which are unknown. The transcriptional element of many *myo*-inositol-regulated genes of phospholipid biosynthetic enzymes, of which the consensus sequence is CATRTGAA, was not found in the flanking 5′ upstream region.

The overall hydropathic profile of the *ECT1* product suggests that it is not a type of integrated membrane protein with hydrophobic membrane-spanning segments. There are two weakly hydrophobic regions at the carboxy terminus, from Val-250 to Ala-268 and from Gly-282 to Gly-290. Helical structures which are typical of many membrane-spanning polypeptides, however, were not predicted for either region.

ECT1 Codes for ECT—We searched protein database for proteins similar to the product of *ECT1*. Three proteins with similar functions, yeast and rat CCTs (*6, 11*) and CTP: *sn*-glycerol-3-phosphate cytidylyltransferase of *Bacillus subtilis* (*32*) were extracted (Fig. 5). Within the deduced amino acid sequence from Pro-7 to Leu-160 of the *ECT1* product, 52 and 55 amino acids are identical to those from Pro-103 to Leu-257 of *S. cerevisiae* CCT and from Pro-76 to Leu-230 of rat CCT, respectively. These regions of yeast and rat CCTs were very similar to each other, that is, 99 amino acids were identical. The same areas also had similarity to CTP:*sn*-glycerol-3-phosphate cytidylyltransferase of *B. subtilis*, and three well-conserved regions were detected (Fig. 5A). In addition to this alignment, another part of *ECT1* product, from Val-200 to Ala-277, was

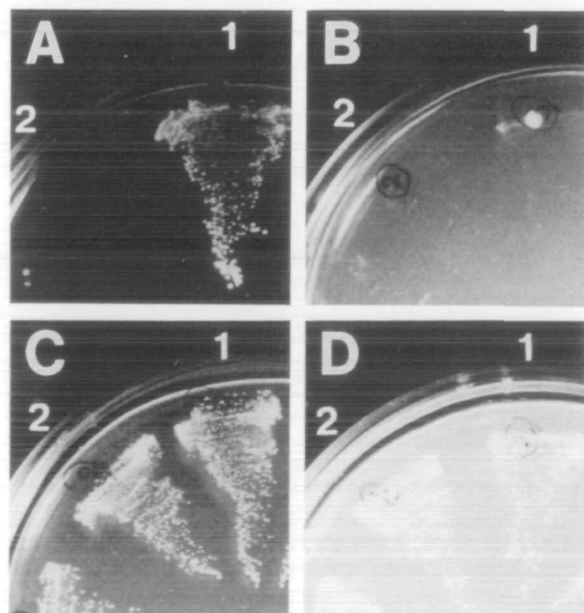


Fig. 3. **Growth of the mutant on SD medium supplemented with ethanolamine and its restoration by plasmid pECT-C.** Strain EU102 (Ect⁻ *cho1::LEU2*) harboring plasmid YCpGPSS and with plasmid pECT-C (1) or vector YCp50 (2) was grown on various synthetic agar media at 30°C. Panel A, SD with 1 mM ethanolamine; Panel B, SD without supplement; Panel C, SD with 1 mM choline; Panel D, SDG without supplement.

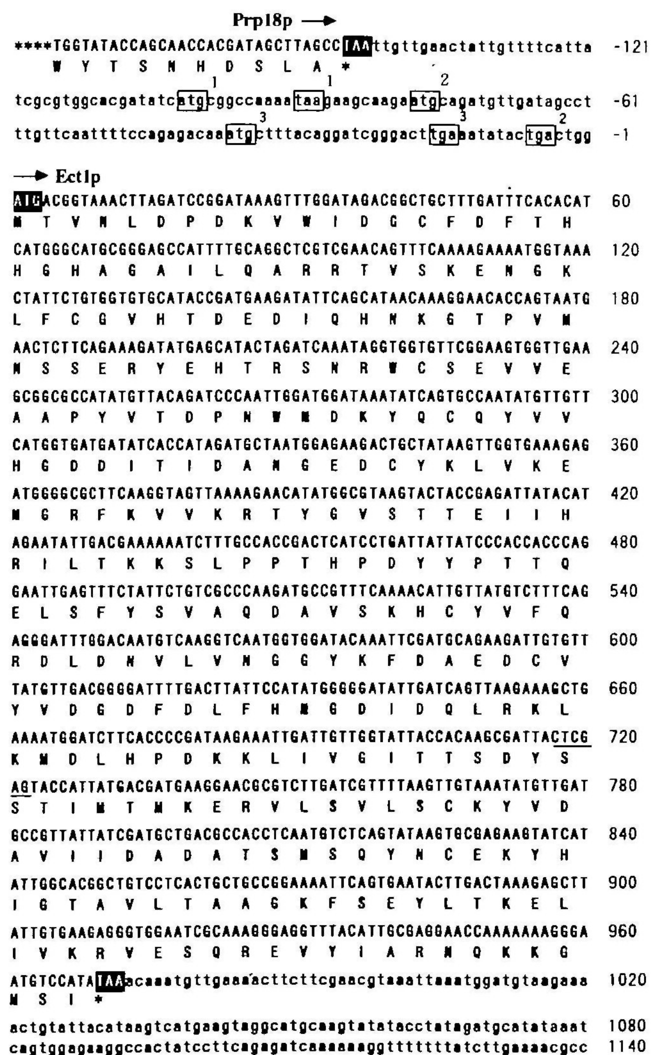


Fig. 4. **Nucleotide and deduced amino acid sequences of *ECT1* and its adjacent area.** Only the relevant region of the analyzed sequence is illustrated. Closed boxes are starting and terminating codons of *ECT1* and *PRP18*. Numbered open boxes are those of short open reading frames in the 5′ flanking region of *ECT1*. *Xho*I site is underlined. The accession number of the entire 2,829-bp sequence to the DDBJ/EMBL is D50644.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

Fig. 5. **Similarity to other cytidylyltransferases.** Amino acids that are identical in three or more sequences are boxed. Sequences are phosphoethanolamine cytidylyltransferase of *S. cerevisiae* (YECT), phosphocholine cytidylyltransferases of *S. cerevisiae* (YCCT) and rat (RCCT), and *sn*-glycerol-3-phosphate cytidylyltransferase of *B. subtilis* (TAGD).

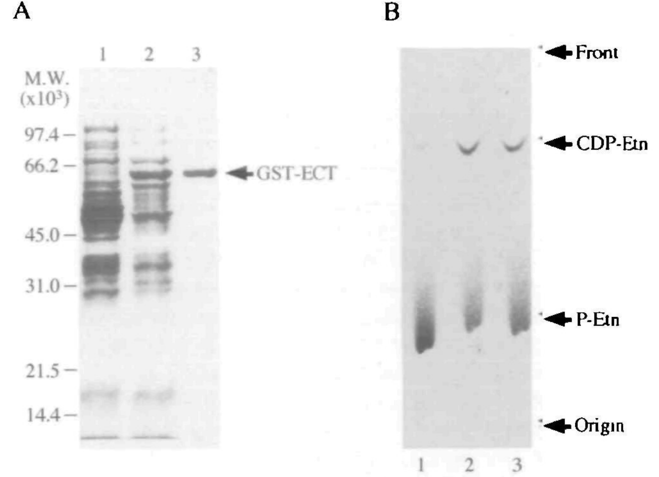


Fig. 6. **Expression of *GST-ECT1* fusion gene in *E. coli* and its ECT activity.** In panel A, cells from induced or uninduced culture were disrupted as described in "EXPERIMENTAL PROCEDURES." Cell lysates were centrifuged at $10,000\times g$ for 5 min and the supernatant was subjected to SDS-PAGE analysis (panel A) and ECT assay (panel B). Lanes are: 1, not induced; 2, induced; 3, purified fusion protein. In the ECT assay, reaction mixture included 20 μg of protein of *E. coli* extract or 10 μg of purified protein. Reaction products were separated by TLC as described. Radioactivity of the products by the induced and uninduced cell extracts were 3,080 cpm/min/mg protein and 530 cpm/min/mg protein, respectively. Abbreviations are: P-Etn, phosphoethanolamine; CDP-Etn, CDP-ethanolamine.

similar to the above conserved region, but in this case, the third well-conserved region was not included (Fig. 5B). Since there is no reason to consider that the yeast ECT has a completely different reaction mechanism from CCT, and since *ECT1* complemented the defective mutation in ECT activity, it is likely that *ECT1* is a structural gene for ECT.

To confirm the ECT activity of the *ECT1* gene product, we prepared an in-frame fusion of *ECT1* to *GST* which codes for glutathione *S*-transferase and expressed it in *E. coli* under the inducible promoter by use of IPTG. Figure 6A shows the expected overproduction of the fusion protein upon induction, and Fig. 6B shows the production of CDP-[^{14}C]ethanolamine from [^{14}C]ethanolamine by the cellular extract and purified fusion protein from the overproduced cells. The induced cell extract gave about 6-fold more radioactive CDP-ethanolamine than the uninduced cell extract. No radioactive CDP-ethanolamine was produced by an extract from cells carrying only the vector (data not shown). These results clearly indicate that the *ECT1* gene product has ECT activity.

DISCUSSION

It is known that the aminoalcohol kinases and phosphotransferases in the CDP-ethanolamine and CDP-choline pathways of yeast are functionally redundant (*33*). The successful isolation of mutants defective in ECT activity, as mutants that are unable to grow on ethanolamine-supplemented medium under conditions limiting PE synthesis from PS, suggests that the enzyme involved in the CDP-choline synthesis, CCT, have little ECT activity. On the other hand, *cct* mutants were only defective in PC synthesis from choline (*16, 34*), which means that ECT has very low, if any, CCT activity. As a whole, the enzymes involved in CDP-aminoalcohol synthesis in yeast do not seem to be redundant in their activities and could be good targets of distinctive regulation of PE and PC syntheses.

The predicted structure of *ECT1* gene product had two regions with significant similarity to those of other three known cytidylyltransferases, yeast and rat CCTs and *Bacillus sn*-glycerol-3-phosphate cytidylyltransferase. The N-terminal conserved region, which is highly conserved between yeast and rat CCTs, was supposed to be a catalytic site. Another conserved region is apparently a repeat of the first half of the N-terminal conserved region. This repetition is unique to ECT, and not being observed in the other three cytidylyltransferases, and its functional implication is not clear. It might be a degenerated form of the dimeric structure that has been reported for the rat CCT, and the second repeat may have regulatory role for the ECT activity.

The overall amino acid sequence of yeast ECT is hydrophilic. Neither helical membrane-spanning domains nor amphipathic helical structures are predicted. The latter structure has been reported in rat CCT C-terminal region as a possible membrane-binding domain (*6*) and is also probably present in yeast CCT (residues 260 to 280) immediately after the highly conserved region. There are short stretches of hydrophobic amino acids after the 229th residue, but their implication awaits future analysis. Rat CCT is active in a membrane-bound form, although its transition mechanism from the soluble state is not completely elucidated. In the case of yeast CCT, most of its activity was recovered in the membrane fraction, despite its overall hydrophilic amino acid sequence (*11*). Rat ECT, which was mostly recovered in the soluble fraction, was localized in proximity to rough endoplasmic reticulum (ER)

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

as well as in cytoplasmic space by immunoelectron microscopy (*35*). These analyses suggest that CCTs and also ECT reside on or near ER, where choline- and ethanolamine-phosphotransferase reactions are expected to occur. Such a location will help the rapid flux of phospholipid precursors. Yeast ECT may also be associated with ER membrane, since the GST-Ect1 fusion protein that was produced in *E. coli* was mostly recovered in the membrane fraction in our preliminary experiment. Finally, there is a C-terminal sequence QKKGMSI, which resembles the C-terminal QKKKQSAN of yeast CCT. These sequences are not exactly the same as the ER retention motif in higher eukaryotes, KKXX or KXKXX (*36*), but might work for association of these enzymes with yeast ER.

The close chromosomal localization of *ECT1* to the *PRP18* gene raises the question of the transcription of *ECT1*. Horowitz and Abelson reported that the major 2.05-kb transcripts of *PRP18* covered the *ECT1* locus, but *ECT1* has its own transcript that started from 55 bp behind the 3′ end of the preceding *PRP18* gene (*30*). Their results mean that most of the promoter elements of *ECT1* are located within the region where its transcription might be susceptible to interference by the transcription of *PRP18*. Although overlapping of transcriptional units has not been reported so often in yeast, some ORFs on chromosome III are located very close to or overlapping adjacent ORFs (*37*), and hence this might be a rather common organization of yeast chromosome.

Although this study has not proved that the original Ect^- mutation in the mutant EU102 is located within the *ECT1* locus, this is highly probable, because the mutation was complemented by a structural gene coding for ECT on a low copy number vector and all isolated clones carried the same *ECT1* gene. The availability of an *ect1 cho1* double mutant should provide an excellent opportunity for analyzing the physiological role of PE synthesis in eukaryotic cells. PE synthesis may contribute to yeast cellular activity in two ways. One depends on its structural aspect. Its head group ethanolamine has a unique feature among phospholipids of eukaryotic cells: it is smaller than others and tends to form hydrogen bonds with phosphate oxygen of adjacent phospholipid molecules. PE under aqueous conditions, therefore, exhibits a higher phase-transition temperature than PC with the same acyl residues and often forms a hexagonal II structure (*38*). Thus PE should have unique structural and functional roles in eukaryotic membranes. The other way involves its metabolism. PE is a precursor of phosphoethanolamine in glycosylphosphatidylinositol-anchored proteins (*39*), which may be important for organization of yeast cell wall. We found in our preliminary experiment that the strain EU102 failed to grow on choline-supplemented medium at 37°C, which may be indicative of a vital function of this peculiar phospholipid in yeast. Construction of a null *ect1* mutant and analysis of its properties are under way in our laboratory.

We thank Dr. Isao Shibuya for generous support and encouragement at the initial stage of this study. We also thank Dr. Kouzou Ishidate for the supply of rat ethanolamine kinase and Yu Fukuda for advice and discussions.

REFERENCES

1. Paltauf, F., Kohlwein, S.D., and Henry, S.A. (1992) Yeast mutant with thermolabile CDP-choline synthesis: Isolation and characterization of a cholinephosphate cytidylyltransferase mutant in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression* (Jones, E.W., Pringle, J.R., and Broach, J.R., eds.) pp. 415-500, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Kuge, O., Nishijima, M., and Akamatsu, Y. (1991) A cloned gene encoding phosphatidylserine decarboxylase complements the phosphatidylserine biosynthetic defect of a chinese hamster ovary cell mutant. *J. Biol. Chem.* **266**, 6370-6376
3. Clancey, C.J., Chang, S.-C., and Dowhan, W. (1993) Cloning of a gene (PSD1) encoding phosphatidylserine decarboxylase from *Saccharomyces cerevisiae* by complementation of an *Escherichia coli* mutant. *J. Biol. Chem.* **268**, 24580-24590
4. Trotter, P.J., Pedretti, J., Yates, R., and Voelker, D.R. (1995) Phosphatidylserine decarboxylase 2 of *Saccharomyces cerevisiae*: Cloning and mapping of the gene, heterologous expression, and creation of the null allele. *J. Biol. Chem.* **270**, 6071-6080
5. Weinhold, P.A., Rounsifer, M.E., and Feldman, D.A. (1986) The purification and characterization of CTP:phosphorylcholine cytidylyltransferase from rat liver. *J. Biol. Chem.* **261**, 5104-5110
6. Kalmer, G.B., Kay, R.J., Lachance, A., Aebersold, R., and Cornell, R.B. (1990) Cloning and expression of rat liver CTP: phosphocholine cytidylyltransferase: An amphipathic protein that controls phosphatidylcholine synthesis. *Proc. Natl. Acad. Sci. USA* **87**, 6029-6033
7. Kent, C. (1990) Regulation of phosphatidylcholine biosynthesis. *Prog. Lipid Res.* **29**, 87-105
8. Wang, Y., Sweitzer, T.D., Weinhold, P.A., and Kent, C. (1993) Nuclear localization of soluble CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **268**, 5899-5904
9. Feldman, D.A. and Weinhold, P.A. (1993) Identification of a protein complex between choline-phosphate cytidylyltransferase and a 112-kDa protein in rat liver. *J. Biol. Chem.* **268**, 3127-3135
10. Tsukagoshi, Y., Nikawa, J., and Yamashita, S. (1987) Molecular cloning and characterization of the gene encoding cholinephosphate cytidylyltransferase in *Saccharomyces cerevisiae. Eur. J. Biochem.* **169**, 477-486
11. Tsukagoshi, Y., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) Expression in *Escherichia coli* of the *Saccharomyces cerevisiae CCT* gene encoding cholinephosphate cytidylyltransferase. *J. Bacteriol.* **173**, 2134-2136
12. Skinner, H.B., McGee, T.P., McMaster, C.R., Bell, R.M., and Bankaitis, V.A. (1995) The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. *Proc. Natl. Acad. Sci. USA* **92**, 112-116
13. Tijburg, L.B.M., Geelen, M.J.H., and van Golde, L.M.G. (1989) Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biochim. Biophys. Acta* **1004**, 1-19
14. Sundler, R. (1975) Ethanolaminephosphate cytidylyltransferase. Purification and characterization of the enzyme from rat liver. *J. Biol. Chem.* **250**, 8585-8590
15. Vermeulen, P.S., Tijburg, L.B.M., Geelen, M.J.H., and van Golde, L.M.G. (1993) Immunological characterization, lipid dependence, and subcellular localization of CTP:phosphoethanolamine cytidylyltransferase purified from rat liver: Comparison with CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **268**, 7458-7464
16. Nikawa, J., Yonemura, K., and Yamashita, S. (1983) Yeast mutant with thermolabile CDP-choline synthesis: Isolation and characterization of a cholinephosphate cytidylyltransferase mutant. *Eur. J. Biochem.* **131**, 223-229
17. Tijburg, L.B.M., Vermeulen, P.S., and van Golde, L.M.G. (1992) Ethanolamine-phosphate cytidylyltransferase. *Methods Enzymol.* **209**, 258-263
18. Hamamatsu, S., Shibuya, I., Takagi, M., and Ohta, A. (1994) Loss of phosphatidylserine synthesis results in aberrant solute sequestration and vacuolar morphology in *Sacchromyces cerevisiae. FEBS Lett.* **348**, 33-36
19. Gerring, S.L., Connelly, C., and Hieter, P. (1991) Positional

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

mapping of genes by chromosome blotting and chromosome fragmentation in *Methods in Enzymology* (Guthrie, C. and Fink, G.R., eds.) Vol. 194, pp. 57-77, Academic Press, New York
20. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163-168
21. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
23. Schmitt, M.E., Brown, T.A., and Trumpower, L. (1990) A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **18**, 3091-3092
24. Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* **67**, 31-40
25. Feinberg, A. and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13
26. Hikiji, T., Miura, K., Kiyono, K., Shibuya, I., and Ohta, A. (1988) Disruption of the *CHO1* gene encoding phosphatidylserine synthase in *Saccharomyces cerevisiae*. *J. Biochem.* **104**, 894-900
27. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-252
28. Atkinson, K.D. (1984) *Saccharomyces cerevisiae* recessive suppressor that circumvents phosphatidylserine deficiency. *Genetics* **108**, 533-543
29. Rose, M.D., Novick, P., Thomas, J.H., Botstein, D., and Fink, G.R. (1987) A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**, 237-243
30. Horowitz, D.S. and Abelson, J. (1993) A U5 small nuclear ribonucleoprotein particle protein involved only in the second step of pre-mRNA splicing in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13**, 2959-2970
31. Yoshida, Y., Sato, T., Hashimoto, T., Ichikawa, N., Nakai, S., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) Isolation of a gene for a regulatory 15-kDa subunit of mitochondrial F1F0-ATPase and construction of mutant yeast lacking the protein. *Eur. J. Biochem.* **192**, 49-53
32. Mauel, C., Young, M., and Karamata, D. (1991) Genes concerned with synthesis of poly(glycerol phosphate), the essential teichoic acid in *Bacillus subtilis* strain 168, are organized in two divergent transcription units. *J. Gen. Microbiol.* **137**, 929-941
33. McGee, T.P., Skinner, H.B., and Bankaitis, V.A. (1994) Functional redundancy of CDP-ethanolamine and CDP-choline pathway enzymes in phospholipid biosynthesis: Ethanolamine-dependent effects on steady state membrane phospholipid composition in *Saccharomyces cerevisiae*. *J. Bacteriol.* **176**, 6861-6868
34. McGee, T.P., Skinner, H.B., Whitters, E.A., Henry, S.A., and Bankaitis, V.A. (1994) A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. *J. Cell Biol.* **124**, 273-287
35. van Hellemond, J.J., Slot, J.W., Geelen, M.J.H., van Golde, L.M.G., and Vermeulen, P.S. (1994) Ultrastructural localization of CTP: phosphoethanolamine cytidylyltransferase in rat liver. *J. Biol. Chem.* **269**, 15415-15418
36. Gaynor, E.C., te Heesen, S., Graham, T.R., Aebi, M., and Emr, S.D. (1994) Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. *J. Cell Biol.* **127**, 653-665
37. Slonimski, P.P. and Brouillet, S. (1993) A data base of chromosome III of *Saccharomyces cerevisiae*. *Yeast* **9**, 941-1029
38. Hauser, H., Pascher, I., Pearson, R.H., and Sundell, S. (1981) Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta* **650**, 21-51
39. Menon, A.K. and Stevens, V.L. (1992) Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. *J. Biol. Chem.* **267**, 15277-15280

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012