Membrane lipid biosynthesis in *Chlamydomonas reinhardtii*: expression and characterization of CTP:phosphoethanolamine cytidyltransferase

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CTP:phosphoethanolamine cytidyltransferase (ECT) is considered to be the regulatory enzyme in the CDP-ethanolamine pathway of phosphatidylethanolamine (PE) biosynthesis. The ECT cDNA of *Chlamydomonas reinhardtii* encodes a protein of 443 amino acid residues, which is longer than the same protein in yeast, rat or human. The translated product of cloned cDNA was expressed as a fusion protein in *Escherichia coli*, and was shown to have ECT activity. The deduced amino acid sequence has 41 % identity with that of human or rat, and 30 % with yeast. The ECT protein has a repetitive internal sequence in its N- and C-terminal halves and a signature peptide sequence, RTXGVSTT, typical of the cytidyltransferase family. The first 70 amino acid residues do not match the N-terminal part of the cytidyltransferases from other organisms, and we hypothesize

that it is a subcellular targeting signal to mitochondria. ECT and organelle marker enzyme assays showed that the total activity of ECT correlates well with that of fumarase, a marker enzyme for mitochondria. Northern blots showed an increase in mRNA abundance during reflagellation, indicating a possibility of transcriptional regulation. A notable change in the enzyme activity in *C. reinhardtii* cells was observed during the cell cycle, increasing during the dark and then decreasing during the light period, while the mRNA level did not alter, providing evidence for post-translational regulation.

Key words: *Chlamydomonas reinhardtii*, CTP:phosphoethanolamine cytidyltransferase, gene expression, membrane, lipid, phosphatidylethanolamine.

INTRODUCTION

Phosphatidylethanolamine (PE) is a major membrane component in both prokaryotic and eukaryotic cells. In mammalian cells, PE can be biosynthesized by three different pathways: the CDP-ethanolamine pathway, decarboxylation of phosphatidylserine (PS) and exchange of ethanolamine with the base moiety of pre-existing phospholipids [1]. In plant cells, PE may be produced by either the CDP-ethanolamine pathway or by decarboxylation of PS [2]. Among these pathways, the CDP-ethanolamine pathway is believed to play a central role in the synthesis of PE in many cases [3]. This pathway is catalysed by three enzymes: ethanolamine kinase (EC 2.7.1.82), CTP:phosphoethanolamine cytidyltransferase (ECT) (EC 2.7.7.14) and ethanolaminephosphotransferase (EC 2.7.8.1). ECT catalyses the synthesis of CDP-ethanolamine from CTP and phosphoethanolamine, and is considered to be a regulatory enzyme in the CDP-ethanolamine pathway, primarily based on analogy with the CDP-choline pathway. However, little evidence has been reported for the regulation of ECT or CDP-ethanolamine pathway in PE biosynthesis in eukaryotic cells.

The ECT gene or its cDNA has previously been cloned from yeast (*ECT1*) [4], human (cDNA) [5] and rat (cDNA) [6]. The human ECT protein was found to have 36 % similarity to yeast protein, while rat ECT was 89 % similar to that of human. Both human ECT and rat ECT are longer than the yeast ECT protein in both the N- and the C-terminal regions. There is a large repetitive sequence in the N- and C-terminal halves of human, rat and yeast ECT. Both parts of the repetitive sequence contain a HXGH (His-

Xaa-Gly-His) motif, which is also present in the most conserved region of the N-terminus of members of the cytidyltransferase superfamily [7] and class-I aminoacyl-tRNA synthetases [8]. Since this motif is thought to be associated with the active sites of these proteins, two catalytic domains have been suggested for ECT [9]. In addition, the peptide sequence RTXGVSTT, which was considered a signature sequence for the cytidyltransferase family, is found in the ECT of all three organisms.

No ECT cDNA or structural gene has been cloned from plants or from *Chlamydomonas reinhardtii*.

In *C. reinhardtii*, three major phospholipids, PE, phosphatidylglycerol (PG) and phosphatidylinositol (PI), are present, while two other phospholipids found in most other organisms, PS and phosphatidylcholine (PC), are absent [10]. PE is a major extraplasmid lipid in this organism, and appears to be synthesized by the CDP-ethanolamine pathway of PE biosynthesis. The occurrence of PE and the absence of PC provide a unique opportunity to study the regulation of PE synthesis in a model organism without overlapping substrate utilization, and may provide valuable information on the importance of this lipid with respect to understanding its function in membranes, and the regulation of its biosynthesis in lower plants and algae. In the present paper, we report the cloning of ECT cDNA from *C. reinhardtii* and the biochemical characterization of the heterologously and homologically expressed gene products. We found that the *C. reinhardtii* ECT is longer than the same protein in yeast and mammals, and it is likely to be a mitochondrial protein that may be regulated at the transcriptional level during reflagellation and at the post-translational level during the cell cycle.

Abbreviations used: CCT, CTP:phosphocholine cytidyltransferase; ECT, CTP:phosphoethanolamine cytidyltransferase; EST, expressed sequence tag; IPTG, isopropyl β -D-thiogalactoside; MBP, maltose-binding protein; ORF, open reading frame; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TAP, Tris/acetate/phosphate; UTR, untranslated region.

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The nucleotide sequence data for *Chlamydomonas reinhardtii* ECT reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AAO60076.

EXPERIMENTAL

Materials

Restriction endonucleases and other DNA-modifying enzymes were from New England BioLabs (Beverly, MA, U.S.A.). Primers were made by Integrated DNA Technologies (Coralville, IN, U.S.A.). CTP, phosphoethanolamine and CDP-ethanolamine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

cDNA library preparation

The cDNA library used was the Core cDNA library cloned into Lambda Zap II and purchased from the *Chlamydomonas* Genomics Project (Duke University, Chapel Hill, NC, U.S.A.). The cDNA in Lambda Zap II phages was transfected into *Escherichia coli* by using a ZAP-cDNA Synthesis Kit (Stratagene, Valencia, CA, U.S.A.), as described by the manufacturer. *E. coli* colonies were pooled for plasmid preparations that were used as templates in PCR-based cDNA cloning of ECT.

cDNA cloning

Rat and human ECT protein sequences (accession numbers NP_446020 and NP_002852) were used to search the *C. reinhardtii* EST (expressed sequence tag) database (http://www.biology.duke.edu/chlamy_genome) to find highly homologous EST clones. These EST clones were assembled into two separate contigs that corresponded to the N- and C-terminal sequences of ECT in human, rat and yeast. The two contigs contain putative start and stop codons, based on the translated sequence analysis. The primers that successfully produced the correct cDNA by PCR included forward primers 5'-TGAACCGCGGTAACCAAGCT-3' and 5'-ATGGTCTTACTCGATTCGGTC-3', and reverse primers 5'-GCATCAGTAACCTCACACG-3' and 5'-CAACTCCTGC-ACGTACTGCTT-3'.

DNA sequencing and sequence analysis

The PCR products spanning the complete ECT cDNA coding region were subjected to agarose gel electrophoresis and extracted from the gel using a QIAquick gel extraction kit (Qiagen, Valencia, CA, U.S.A.). The purified DNA fragments were sequenced on both strands using Applied Biosystems BigDye terminators (Foster City, CA, U.S.A.). The contig alignment of the complete coding sequence of ECT cDNA and the ESTs for the ECT gene in the *C. reinhardtii* EST database was performed for 3'-UTR (untranslated region) and 5'-UTR sequences. The complete coding region was also sequenced on both strands of the PCR product, and afterwards, it was cloned into an *E. coli* expression vector pMAL-c2X (New England BioLabs).

Nucleotide and deduced amino acid sequences were compared with sequences in the databases at the National Center for Biotechnology Information (NCBI) by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequence analysis and alignments were obtained by using the multiple sequence alignment programs from the European Bioinformatics Institute EMBL-EBI server (<http://www.ebi.ac.uk/clustalw/>). The Prosite database from EBI server (<http://ca.expasy.org/prosite/>) was used to find putative consensus motifs for specific domains in ECT.

Construction of MBP (maltose-binding protein)-fusion protein and expression in *E. coli*

Two primers were designed with an adapter sequence of *EcoRI* or *HindIII* to amplify the complete coding region of cDNA. The PCR product of 1.3 kb from these primers was digested with *EcoRI*

and *HindIII* and then cloned into the *EcoRI/HindIII* site of an *E. coli* expression vector pMAL-c2X (New England BioLabs). The construct, designated as pMAL-ECT, was sequenced to confirm the correct translation frame. Competent *E. coli* DH5 α cells were transformed with pMAL-ECT. The fusion protein was produced by the transformed *E. coli* grown at 37 °C in Luria-Bertani medium containing 50 μ g/ml ampicillin, and induced at an A_{600} of 0.7 with 0.2 mM IPTG (isopropyl β -D-thiogalactoside). After induction at 30 °C for 12 h, the cells were harvested and the fusion protein was purified with a pMAL protein fusion and purification system as described by the supplier (New England BioLabs). Cell extracts of the IPTG-treated and untreated cells, plus purified fusion protein, were used for protein analyses. SDS/PAGE was performed using 10 % polyacrylamide mini-slab gels. Proteins were stained with Coomassie Brilliant Blue. Protein concentrations were determined by the dye-binding method using Bio-Rad protein assay kits (Bio-Rad, Hercules, CA, U.S.A.) with BSA as a standard [11].

ECT activity assay

ECT enzyme activity was measured as described previously [12] in a 50 μ l final volume containing 20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT (dithiothreitol), 2 mM CTP, 2 mM phosphoethanolamine and 20 μ l of cell extract (30 μ g of *E. coli* protein) or 10 μ l of purified protein (15 μ g). The mixture was incubated at 30 °C for 30 min or as otherwise specified. The reaction was stopped by boiling for 5 min. The products were separated by TLC on a Silica Gel 60 plate (Merck, Darmstadt, Germany) with 100 % ethanol/0.5 % NaCl/25 % NH₄OH (50:50:5, by vol.) as the developing solvent. After development, the plates were air-dried and then sprayed with ninhydrin. Spots corresponding to CDP-ethanolamine and phosphoethanolamine were detected by their bright purple colour as compared with standards run on the same plates.

Cell fractionation and enzyme assays

The cell-wall-deficient mutant strain of *C. reinhardtii* CC406 cw15 (mt⁻) was grown in Tris-minimal phosphate medium [13] at 22 °C, with bubbling of 5 % CO₂ in air under continuous light. The cells were harvested in log phase by centrifugation at 1000 g at 4 °C for 10 min, washed once with cold 20 mM Hepes/KOH (pH 7.5) at 4 °C, and concentrated with a final centrifugation. The cellular fractionation was performed by sequential centrifugation of homogenates as described previously [10]. All centrifugations were performed at 4 °C, and the samples were kept on ice in all steps of the procedure. The cellular fractions were identified by the use of the marker enzymes Rubisco for chloroplasts [14], fumarase for mitochondria [15] and diacylglycerol:CDP-ethanolamine ethanolaminephosphotransferase for endoplasmic reticulum [16]. The ECT assay was conducted as described above, except that 100 μ g of microsomal protein and 30 μ M [¹⁴C]phosphoethanolamine (54 mCi/mmol) were used in the reaction. The radiolabelled CDP-ethanolamine produced in the enzyme assays was quantified using liquid-scintillation spectrometry after being re-collected from the TLC plates. The fractionation was conducted independently three times with similar results and the data presented in Table 2 were from a single representative experiment.

Southern and Northern blot analyses

Genomic DNA from *C. reinhardtii* wild-type 137C was isolated as described [17]. Genomic DNA (5 μ g) was digested with restriction endonucleases (New England BioLabs), resolved on an 0.8 % agarose gel, transferred on to a Nytran membrane

cECT	1	MVLLDSVKQNPLLWTCAGVVGLGVSLLSWKAAKAAELYVPIQYQYRPGTISGWLANALA	
hECT	1	-----MIRNGRGAAGG	
rECT	1	-----MIRNGHGAGGA	
yECT	0	-----	
cECT	61	NHSKSRKRPRPVRYLDGCFDMMHYGHANALRQAKAVG---DELVVGLINDAEIMRCKG	
hECT	12	AEQPGPGRRARVRVWCDGCYDMVHYGHSNQLRQARAMG---DYLIIVGVHTDEEIAKHKG	
rECT	12	AGLKGPGGQRTVRVWCDGCYDMVHYGHSNQLRQARAMG---DYLIIVGVHTDEEIAKHKG	
yECT	1	----MTVNLDPDKVWIDGCFDFTHHGHAGAILQARRTVSKENGKLFQGVHTDEEIQHNKG	

cECT	117	PPVMNEEERHTLVEAVKWVDEILTGVPYDLNPEFVNEFLTCKHRIDYIIHGDDPCLLPDGS	
hECT	68	PPVFTQEERYKMVQAIKWVDEVVPAAPYVT---TLETLDKYNCDFCVHGNDITLTVDGR	
rECT	68	PPVFTQEERYKMVQAIKWVDEVVPAAPYVT---TLETLDKHNCDFCVHGNDITLTVDGR	
yECT	57	TPVMNSSERYEHTRSNRWCSEVVEAAPYVTD---NWMKDYQCQYVVGDDITIDANGE	
cECT	177	DAYAHAKKLGRFKMVKRTTEGVSTTDIVGRMLTCSRNVHFINADEPHPLAKSFSMGTFREE	
hECT	124	DTYEEVKQAGRYRECKRTQGVSTTDLVGRMLLVTKAHHSSQEMSSEYREYADSF-----	
rECT	124	DTYEEVKQAGRYRECKRTQGVSTTDLVGRMLLVTKAHHSSQEMSSEYREYADSF-----	
yECT	113	DCYKLVKEMGRFKVVKRTYGVSTTEIHRILTKKSLPPTHDPYPTTQELS-----	
cECT	237	SN-----AEASTSDASTRTLSKFLPTSRRLVQFSNGRVAPEGARIVYIDGAFDCF	
hECT	179	-----KCPGGRNPWTGVSQFLQTSQKIIQFASGKEPQPGETVIYVAGAFDLF	
rECT	184	TPAGDTLSSEVSSQCPGQSPWTGVSQFLQTSQKIIQFASGKEPQPGETVIYVAGAFDLF	
yECT	164	-----FYSVAQDAVSKHCYVQFQRLDNLVNGGYKFDAEDCVYVDGDFDLF	
cECT	288	HPGHVKILQAAK--AQGDFLLVGLHTDEDVQARRGPHLPIMNLHERSLSVLSCKYVDEVV	
hECT	226	HIGHVDFLEKVHRLAERPYYIAGLHFDQEVNHYKGKNYPIMNLHERLSVLACRYVSEVV	
rECT	244	HIGHVDFLEKVHRLAERPYYIAGLHFDQEVNRYKGKNYPIMNLHERLSVLACRYVSEVV	
yECT	210	HMGDIDQLRKLMKDLHPDKKLIVGITTSYSS-----TIMTMKERVILSVLSCKYVDAVI	

cECT	346	IGSPCVITEDLMTTFNISVVVRGSMSETSMGLPVEEERYEVPRRMGLFTELPSPSDVSAR	
hECT	286	IGAPYAVTAELLSHFKVDLVCHG--KTEIIPDRDGSDFYQEPKRRGIFRQIDSGSNLTDD	
rECT	305	IGAPYSVTAELLNHFKVDLVCHG--KTEIIPDRDGSDFYEEPKRRGIFCQIDSGSDLTDD	
yECT	264	IDADATSMQYN-----CEKYHIGTAVLTAAGKFSEYLTKE	
cECT	406	NIHRIIVDKRAAFEARNAKKVKGEAYYTGAQYVQEL-----	443
hECT	344	LIVQRIITNRLEYEARNQKKEAKELAFLEAARQAAQPLGERDGDF	389
rECT	362	LIVQRIIKNRLEYEARNQKKEAKELAFLEALRQQAQPRGETD---	404
yECT	300	LIVKRVESQREVYIARNQKKGMSI-----	323

Figure 1 Alignment of amino acid sequences of *C. reinhardtii* ECT and other ECTs in human, rat and yeast

The amino acid sequences of *C. reinhardtii* ECT (cECT; GenBank® accession number AA060076), human ECT (hECT; NP_002852), rat ECT (rECT; NP_446020) and yeast ECT (yECT; BAA09310) are compared. The conserved HXGH motifs are indicated with asterisks, and the RTXGVSTT signature in cytidyltransferase family is underlined. Residue numbers for amino acids are shown on the left. Gaps in alignments are indicated by dashes. Residues that are identical in three or more sequences are shaded in grey.

(Schleicher & Schuell, Keene, NH, U.S.A.), and hybridized with a radiolabelled probe derived from the 0.4 kb of PCR fragment of the 3' coding region of cDNA. The DNA transfer, hybridization and washes were as described in [18]. For Northern blots, cells of the 137C strain were grown on Tris/acetate/phosphate (TAP) medium with a 12 h/12 h light–dark cycle until the cell density reached approx. 10^7 cells/ml. The cells were deflagellated by pH shock [19]. The flagella were removed and the cells were re-suspended in fresh TAP for reflagellation. Cell samples were taken at different times during reflagellation for total RNA isolation. Total RNA was isolated as described in [20]. Total RNA (30 µg) was electrophoresed on a 1% agarose gel containing formaldehyde, transferred on to a Nytran membrane, and hybridized to the same probe used in a Southern blot as described [18].

Enzyme activity during the cell cycle

Cells of *C. reinhardtii* 137C were grown in Tris-minimal phosphate medium [13] at 22 °C, with bubbling of 5% CO₂ in air with a 12 h/12 h light–dark cycle. After 5 days of cell growth under synchronization, samples of cells were taken at various times

during a cell cycle. The cells of each sample were pelleted at 1000 g for 10 min at 4 °C, followed by freezing in liquid nitrogen. The total RNA and protein were isolated as described [20,21]. The Northern blot of total RNA and ECT enzyme assays were performed as described above.

RESULTS

The nucleotide and deduced amino acid sequences of *C. reinhardtii* ECT

The cDNA was 2019 bp long, including a 192 bp 5'-UTR, a 1329 bp ORF (open reading frame) and a 498 bp 3'-UTR. The ORF encodes a polypeptide of 443 amino acids. Like human, rat and yeast ECTs, *C. reinhardtii* ECT has a large internal repetitive sequence. Both of the repeat sequences have HXGH motifs (at positions 84–87 and 288–291). In addition, the *C. reinhardtii* ECT has other conserved residues that are considered important for catalytic activity in the cytidyltransferase family, such as the sequence RTXGVSTT (residues 93–100) and an aspartate residue located two amino acid residues before the HXGH motif in the N-terminal half of the protein (Figure 1).

Figure 2 Hydropathy and α -helix amphiphilicity profiles of the predicted protein sequence of *C. reinhardtii* ECT

(A) Hydropathy was analysed by the method of Kyte and Doolittle [22]. Positive values represent hydrophobicity. (B) The sequence was analysed for amphipathic α -helices using the algorithm of Chou and Fasman [23]. The predicted membrane-spanning domain is indicated as TM.

Table 1 Identity (%) and similarity (%; in parentheses) between CTP:phosphoethanolamine cytidyltransferases from *C. reinhardtii*, human, rat and yeast

	<i>Chlamydomonas</i>	Human	Rat	Yeast
<i>Chlamydomonas</i>	—	41	41	30
Human	(62)	—	88	34
Rat	(62)	(91)	—	32
Yeast	(45)	(49)	(47)	—
<i>C. reinhardtii</i> *	—	50	50	40
N- and C-terminal halves†	33	30	32	25

* Identity of *C. reinhardtii* CTP:phosphoethanolamine cytidyltransferase without the N-terminal 75 residues to the same proteins in other organisms.

† Identity between N- and C-terminal halves within each ECT protein in different organisms.

A hydrophobicity profile indicates that the overall sequence of *C. reinhardtii* ECT is hydrophilic, but contains one clear hydrophobic region in the N-terminus capable of forming a transmembrane domain (residues 15–40) (Figure 2). Analysis of the secondary structure predicted a region (residues 20–50) that could

form an amphipathic α -helix. This corresponds well with the hydropathy profile.

Sequence similarity to ECT of other organisms

The predicted *C. reinhardtii* ECT protein is 41% identical with human and rat ECTs, and 30% with yeast (Table 1). *C. reinhardtii* ECT is longer than those of yeast (323 residues), human (389 residues) and rat (404 residues), which appears to result from an extended N-terminal region. The N-terminus has 75 amino acid residues that do not match the N-terminus of any other ECT (Figure 1). When these 75 residues are not included in the comparisons, identity of the *C. reinhardtii* ECT protein increases to 50% when compared with human or rat ECT, and 40% with yeast. The first 75 amino acid residues appear to include a subcellular targeting sequence, as predicted by several prediction programs for subcellular localization.

While the similarity between *C. reinhardtii*, human, rat and yeast ECTs is present across the entire sequence of the proteins, there are greater similarities when the N- and C-terminal regions (residues 71–207 and 259–426) are compared than in the middle (residues 208–278). When the N- and C-terminal halves within the same ECT protein were aligned for each organism, an identity of 33% was found in *C. reinhardtii*, 30% in human, 32% in rat and 25% in yeast (Table 1).

Location of the ECT in cells

The cellular location of the ECT enzyme was determined by testing activities in various cellular fractions and comparing the results with marker enzyme distributions (Table 2). The results showed that the total and specific activities of ECT were well correlated with those of fumarase, which is a marker enzyme of the mitochondria, but not with those of Rubisco and EPT, which serve as marker enzymes for chloroplasts and endoplasmic reticulum respectively. Endoplasmic reticulum and mitochondria have been shown to be the primary candidates for sites of ECT activity in animals and higher plants [24–26]. This result is consistent with our early prediction using the deduced amino acid sequence of the ECT cDNA, which shows that the ECT protein is targeted to mitochondria after translation.

Expression of ECT in *E. coli* cells and ECT activity

An in-frame fusion of the ECT gene to MAL, which codes for MBP, was expressed in *E. coli* under the control of a promoter inducible by IPTG. The expected overproduction of the fusion protein upon induction was observed (Figure 3A). Figure 3(B) shows the production of CDP-ethanolamine from phosphoethanolamine and CTP in a reaction catalysed by both the cellular

Table 2 Percentage of the total activity (%), and specific activity of marker and ECT enzymes in various cellular fractions of *C. reinhardtii*

EPT, ethanolaminephosphotransferase; supt, supernatant. Units for specific activity: fumarase, mmol/mg per h; Rubisco, nmol/mg per h; EPT, pmol/mg per h; ECT, pmol/mg per h. Units for total activity: fumarase, mmol/h; Rubisco, nmol/h; EPT, pmol/h; ECT, pmol/h. Unit of chlorophyll: mg/ml. The fractionation was conducted independently three times with similar results, and the data are from a single representative experiment.

Fraction	Chlorophyll	Fumarase		Rubisco		EPT		ECT	
		%	Specific	%	Specific	%	Specific	%	Specific
760 g pellet	96.24	45.13	39.00	73.05	12.60	12.27	76.20	56.08	35.88
3000 g pellet	2.04	1.64	18.60	4.29	10.20	9.00	219.00	3.38	28.50
10 000 g pellet	1.01	0.56	6.60	3.39	8.40	38.15	933.60	3.11	27.00
100 000 g pellet	0.71	3.11	21.00	11.84	16.20	40.58	565.80	2.27	11.22
100 000 g supernatant	0.00	49.56	21.60	7.43	0.60	0.00	0.00	35.16	11.40

Figure 3 Expression of MBP-ECT fusion gene in *E. coli* and its ECT activity

(A) Cell extracts from induced or uninduced bacterial cultures and purified fusion protein were subjected to SDS/PAGE. Lane 1, protein markers [molecular mass (MM) in kDa]; lane 2, cell extract from uninduced cells; lane 3, cell extract from induced cells; lane 4, purified chimaeric protein. (B) ECT enzymic assays were performed using the same cell extracts and purified fusion protein as in (A). In the enzyme assay, the reaction mixture included 30 μ g of protein from *E. coli* cell extract or 15 μ g of purified protein. The reaction products were separated by TLC as described in the Experimental section. Lane 1, substrate standards; lane 2, reaction with uninduced cell extract; lane 3, reaction with induced cell extract; lane 4, reaction with purified protein. P-Ethn, phosphoethanolamine; CDP-Ethn, CDP-ethanolamine. The activity assay of the expressed ECT in *E. coli* was repeated independently more than three times, and the result shown is typical of all the replicate experiments that gave the same results.

extract and purified fusion protein from the overexpressed cells. No CDP-ethanolamine was produced by an extract from cells that carry only the vector pMAL-c2X (results not shown). In addition, we tested the enzyme activity of the fusion protein under different conditions (Figure 4) and found the activity to be dependent on the presence of CTP, phosphoethanolamine and Mg^{2+} . These results clearly indicate that the cDNA described in the present study codes for the structural gene of *C. reinhardtii* ECT.

Genomic copy and expression in *C. reinhardtii*

Southern blot analysis indicated that the *ECT* gene occurs as a single copy in the genome of wild-type strain 137C of *C. reinhardtii* (Figure 5A). Northern blot analysis indicated that the abundance of mRNA from the *ECT* gene increased within 30 min of cell growth during reflagellation after deflagellation by pH shock (Figure 5B). The expression reached its highest level after 90 min of the reflagellation period, and then began to decrease. The mRNA abundance after 30 min of reflagellation was approx. 3-fold higher than in cells before deflagellation, whereas after 90 min it was less than 2-fold.

Enzyme activity and expression of the *ECT* gene during the cell cycle

No significant change was detected in the expression of the *ECT* gene by measuring RNA from the cells during the cell cycle (Figure 6, lower panel). However, a notable change in ECT enzymic activity was observed in the cells during this same period (Figure 6, upper panel). The ECT activity increased

Figure 4 ECT activity of the MBP-ECT fusion protein expressed in *E. coli* under different conditions

The reaction mixture contained 15 μ g of purified protein and was incubated at 30 °C for 30 min as described in the Experimental section. The reaction products were separated by TLC. Lane 1, no enzyme added; lane 2, with enzyme; lane 3, no Mg^{2+} added; lane 4, no CTP added; lane 5, no CDP-ethanolamine added. The data shown are from a single experiment that was repeated three times with the same results.

Figure 5 Nucleic acid analysis of the *C. reinhardtii* *ECT* gene and regulation of its transcription

(A) Southern hybridization of genomic *ECT* gene. Genomic DNA was digested by *EcoRI* (lane 1), *EcoRV* (lane 2), *HindIII* (lane 3), *XmnI* (lane 4), *PvuII* (lane 5) and *PstI* (lane 6), separated on a 0.8% agarose gel, transferred on to a Nytran membrane, and hybridized to a radiolabelled 0.4 kb probe from PCR of 3' coding region of *ECT* cDNA. (B) Northern hybridization of total RNA from *C. reinhardtii* cells during reflagellation. Total cellular RNA was prepared from cells before deflagellation (pre), and during the reflagellation after pH shock at 10, 30, 60, 90 and 120 min, subjected to electrophoresis on a 1% formaldehyde/agarose gel, transferred on to a Nytran membrane and hybridized to the same probe as in the Southern blot.

during the dark period, reaching the highest level just before the light was on, and then decreased during the light stage, reaching the lowest level just before the start of the next dark period.

Figure 6 ECT activity and expression of *ECT* gene during the cell cycle

Upper panel: the ECT activity during the cell cycle. The reaction mixture contained 200 μ g of total protein and was incubated at 30 °C for 30 min as described. The reaction products were separated by TLC and the radioactivity of CDP-ethanolamine was measured by liquid-scintillation spectrometry of the product spot on the gel. Lower panel: Northern blot of total RNA from cells during the cell cycle. Total cellular RNA was prepared from cells during the cell cycle at 2, 6, 10, 14, 18 and 22 h, subjected to electrophoresis on a 1% formaldehyde/agarose gel, transferred on to a Nytran membrane and hybridized to the same probe as in the Southern blot. Values for the enzyme activity are the means $\pm$ S.D. of three replicates of an experiment.

DISCUSSION

In the present study, we report the cloning and characterization of cDNA coding for ECT from *C. reinhardtii*. *C. reinhardtii* ECT does not resemble other cytidyltransferases that have been sequenced. In particular, the N-terminal region of the ECT from *C. reinhardtii* is longer than the ECT of the other three species. This region contains a small, α -helical, hydrophobic section long enough to span the membrane, and this appears to serve as a sub-cellular targeting sequence to mitochondria. It has been shown in castor bean (*Ricinus communis* L.) endosperm that the majority of ECT occurs mainly in the mitochondria, where it is associated with the outer membrane [25,26]. In the present study, we found that the total activity of ECT was the highest in the fraction that had the highest total activity of fumarase, but did not correlate with fractions containing chloroplasts and endoplasmic reticulum. Therefore the ECT is primarily, if not exclusively, a mitochondrial protein in *C. reinhardtii*, as it is in castor bean [25]. In contrast, the enzyme was associated primarily with rough endoplasmic reticulum or the cytosol in mammalian cells [24,27]. Thus the subcellular localization of ECT enzyme appears to be different between mammals and photosynthetic organisms. This indicates that the regulatory mechanism may differ between plants and mammals. It has been proposed for mammalian cells that ECT might reversibly interact with membranes of the endoplasmic reticulum, which could bring the enzyme into close proximity to the third enzyme in the CDP-ethanolamine pathway, ethanolaminephosphotransferase, an established integral microsomal protein that catalyses the final step in the CDP-ethanolamine pathway [28]. A mitochondrial location in photosynthetic organisms seems to contradict this mechanism of ECT regulation. The significance of these differences remains obscure at this time; in-

terestingly, ethanolaminephosphotransferase appears to be microsomal in *C. reinhardtii* [29].

Based on the well-conserved regions in the N-termini of human, rat and other members of the cytidyltransferase superfamily, including rat and yeast CCT (CTP:phosphocholine cytidyltransferase) proteins and GCT (glycerol-phosphate cytidyltransferase) in *Bacillus subtilis*, it has been proposed that the N-terminus contains the catalytic domain of these cytidyltransferases [7]. Since both repetitive sequences in ECT contain the HXGH motif that is present in the most conserved region of the N-terminus of the members in the cytidyltransferase family and class I aminoacyl-tRNA synthetases [8], and this motif is considered to be in the active site of these enzymes [9,30], *C. reinhardtii* ECT might contain two catalytic domains like the ECTs of human, rat and yeast. In addition, there is a RTXGVSTT motif in the ECT sequence (residues 282–296), which is considered to be a signature sequence for the cytidyltransferase family [9]. Therefore *C. reinhardtii* ECT appears to be a real member of this family.

There is very limited evidence for regulation of ECT activity or PE biosynthesis by the CDP-ethanolamine pathway at the level of gene expression. One investigation showed that the mRNA level of the rat *ECT* gene varied during development of rat liver [6]. In the present study, we found that ECT might be regulated at the level of transcription during the reflagellation of *C. reinhardtii* cells, where an increase in mRNA abundance was detected after 30 min of reflagellation. Whether or not this increase in mRNA level is correlated with an enhanced ECT activity is unknown, since an increase in ECT activity may not necessarily result from an enhanced *ECT* mRNA level. Such a disjunction between the mRNA level and ECT activity was found in our results from the measurements of the ECT activity and mRNA abundance during the cell cycle. A clear change in ECT activity was observed during the cell cycle, while no notable variation in ECT mRNA was detected, indicating a post-translational regulation mechanism for ECT in the *C. reinhardtii* cells. The increase in ECT activity appears to be correlated with the cell-division process, as preparation for cell division, which occurs immediately before the light period, may require enhanced *de novo* PE synthesis. Several reports have described the regulation of a *CCT* gene at the pre-translational level in mammalian cells [31–33]. However, in these studies, the increase in CCT mRNA levels resulted from stabilization of pre-existing mRNA instead of an increase in gene transcription. Although in one study, an increase in mRNA of a *CCT α* gene from mouse embryo fibroblast cells was observed during the S phase of the cell cycle, it was not correlated with increased CCT activity and PC biosynthesis in the G₁ phase, but instead was in preparation for mitosis [34]. Positive associations between hepatic cell division and a high expression of *CCT* genes during the perinatal period have been reported [35,36]. Further studies on the correlation between ECT mRNA level and ECT activity will help to elucidate the mechanism of transcriptional or translational regulation of ECT in *C. reinhardtii*.

This research was supported partially by National Science Foundation grants IBN-0212093 to J. V. M. and MCB-9603626 to T. S. M.

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Received 16 February 2004/12 May 2004; accepted 18 May 2004

Published as BJ Immediate Publication 18 May 2004, DOI 10.1042/BJ20040254