Lipid deprivation increases surfactant phosphatidylcholine synthesis via a sterol-sensitive regulatory element within the CTP:phosphocholine cytidylyltransferase promoter

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Lipid-deprived mice increase alveolar surfactant disaturated phosphatidylcholine (DSPtdCho) synthesis compared with mice fed a standard diet by increasing expression of CTP:phosphocholine cytidylyltransferase (CCT), the rate-limiting enzyme for DSPtdCho synthesis. We previously observed that lipid deprivation increases mRNA synthesis for CCT [Ryan, McCoy, Mathur, Field and Mallampalli (2000) J. Lipid Res. **41**, 1268–1277]. To evaluate regulatory mechanisms for this gene, we cloned the proximal ~ 1900 bp of the 5′ flanking sequence of the murine CCT gene, coupled this to a luciferase reporter, and examined transcriptional regulation in a murine alveolar epithelial type II cell line (MLE-12). The core promoter was localized to a region between −169 and +71 bp, which exhibited strong basal activity comparable with the simian virus 40 promoter. The full-length construct, from −1867 to +71, was induced 2–3-fold when cells were cultured in lipoprotein-deficient serum (LPDS), similar to the level of induction of the endogenous CCT gene. By deletional analysis the sterol regulatory element (SRE) was localized within a 240 bp region. LPDS activation of the CCT promoter was abolished by mutation of this SRE, and gel mobility-shift assays demonstrated specific binding of recombinant SRE-binding protein to this element within the CCT promoter. These observations indicate that sterol-regulated expression of CCT is mediated by an SRE within its 5′ flanking region.

Key words: cholesterol, lipoproteins, sterol regulatory element, transcription.

INTRODUCTION

Alveolar type II epithelia are actively engaged in the biosynthesis of phosphatidylcholine (PtdCho), a vital component of cellular membranes and pulmonary surfactant. PtdCho synthesis within alveolar type II cells requires availability of lipid substrates, such as fatty acids, that are carried as triacylglycerols within serum lipoproteins or complexed to albumin [1]. Fatty acids are key components of the major surfactant molecule, disaturated PtdCho (DSPtdCho), but also directly activate CTP:phosphocholine cytidylyltransferase (CCT; EC 2.7.7.15), the rate-limiting enzyme required for PtdCho biosynthesis [2]. Other lipids that appear to be required for adequate surfactant lipid synthesis are neutral lipids, including cholesterol, triacylglycerols and diacylglycerol, and some anionic phospholipids, such as phosphatidylglycerol. Accordingly, studies demonstrate that exogenous loading with these lipids when presented either as lipoprotein, liposomal or vesicular particles markedly stimulates PtdCho synthesis [3–5]. These effects are mediated by post-translational activation of CCT resulting in an overall increase in the mass of PtdCho or surfactant DSPtdCho.

Unlike loading with exogenous lipoproteins, the response mechanisms that govern PtdCho homoeostasis within alveolar epithelial cells after lipid deprivation remain largely unknown. Type II cells appear to have the capability to sustain adequate biosynthetic activity for surfactant PtdCho in response to rapid changes in lipid substrate availability. Related whole-animal studies examining effects of fasting or essential fatty acid-deficient diets [6,7] show reduced surfactant levels and surface activity [8–12]. However, studies also show that despite decreases in lung weight, levels of PtdCho or phospholipid compositions are maintained in the setting of caloric restriction [13–15]. Fasted animals also maintain the volume of lamellar bodies, an intracellular storage form of surfactant in the lungs [10,16], increase choline incorporation into PtdCho [14], and replenish their surfactant pools by 96 h [17]. These latter results suggest that a compensatory mechanism(s) exists for surfactant PtdCho synthesis to offset caloric or lipid restriction.

Indeed, feedback control mechanisms exist at the molecular level for other membrane lipids, such as cholesterol. For example, cholesterol (or lipoprotein) depletion triggers transcription of the 3-hydroxy-3-methylglutaryl-CoA reductase gene required for cholesterol synthesis [18]. This occurs via activation of the transcription factor, sterol regulatory element (SRE)-binding protein (SREBP). Cholesterol deprivation also increases transcription of the fatty acid synthase gene via SREBP activation [19]. The proximal 5′ flanking region of the CCT gene has an SREBP regulatory element [20]. In recent studies, we demonstrated that cholesterol deprivation of alveolar type II epithelial cells increases CCT protein and mRNA expression primarily by an increase in CCT mRNA synthesis [21]. These results led us to hypothesize that sterols transcriptionally regulate the CCT gene.

Abbreviations used: CCT, CTP:phosphocholine cytidylyltransferase; FBS, fetal bovine serum; LDLR, low-density lipoprotein receptor; LPDS, lipoprotein-deficient serum; MLE-12, murine alveolar epithelial type II cell line; PtdCho, phosphatidylcholine; DSPtdCho, disaturated PtdCho; SRE, sterol regulatory element; SREBP, SRE-binding protein.

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To evaluate this hypothesis, various portions of the proximal 5′ flanking sequence of the murine CCT gene were coupled to luciferase coding sequences and expression of this reporter gene was examined in a murine alveolar epithelial type II cell line (MLE-12). We observed that a relatively small region of the CCT genomic sequence (240 bp) contains the core promoter as well as the *cis*-elements necessary to confer sterol regulation.

MATERIALS AND METHODS

Materials

Lipoprotein-deficient serum (LPDS; $d > 1.21$ g/ml) was isolated by ultracentrifugation [22]. Immunoblotting membranes were obtained from Millipore (Bedford, MA, U.S.A.). The ECL® Plus Western blotting detection system was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). A rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acid residues 164–176 of CCT [23] was generated by Covance Research Products (Richmond, CA, U.S.A.). The rodent CCTα cDNA was a gift from Dr Suzanne Jackowski (Department of Biochemistry, St Jude Children's Research Hospital, Memphis, TN, U.S.A.) [24, but see 24a]. The random primed labelling kit used for cDNA labelling was Redprime II™ (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). Radioisotopes, including [γ-^{32}P]ATP, were obtained from New England Nuclear Chemicals (Boston, MA, U.S.A.). MLE-12 cells were kindly provided by Dr Jeffrey Whitsett (Division of Pulmonary Biology, Childrens Medical Center, Childrens Hospital, Cincinnati, OH, U.S.A.) [25]. The immortalized rat fetal type II cell line ('IFT2') was generated as previously described [26]. The H441 and A549 cell lines were obtained from A.T.C.C. Hite's medium was obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA, U.S.A.). C57BL/6J mice were purchased from Jackson Laboratories (Bar-Harbor, ME, U.S.A.).

Animal studies

C57BL/6J mice (100–150 g) were fed *ad libitum* with rat chow (normal diet) for 24 h, or fasted and refed a low-fat/high-carbohydrate diet for 24 h (70 % Carbohydrate Diet, catalogue no. TD 98090; Harlan Teklad, Madison, WI, U.S.A.). After dietary manipulation, mice were killed using pentobarbital (50 mg/kg; intraperitoneal injection). The anterior cervical area was shaved, prepped with betadine ointment, and the trachea cannulated with 0.040-inch diameter plastic tubing. The lungs were lavaged, and a crude alveolar surfactant pellet was isolated as previously described [27]. Whole lung slices from mice were prepared as previously described [28–30]. A mixture of M199 medium and 0.67 % noble agar (final concentration) heated to 37 °C was instilled into the lungs. The mice were subsequently placed on ice to solidify the agar. The lungs were removed, and manually cut into 1 mm slices. Each slice was placed on to a sterile filter paper resting on a stainless steel mesh in a 6-well culture plate, and 4 ml of cold medium [500 ml of M199, 50000 units of penicillin G, 50000 μg of streptomycin sodium, 1250 μg of amphotericin B and 50 ml of fetal bovine serum (FBS)] was added to each well [28]. Following an initial incubation period, the plates were pulsed with 1.25 μCi of [*Me*-^{3}H]choline. Following pulsing, lung slices were rinsed with 4 ml of PBS, placed into polystyrene tubes, and homogenized in 1 ml of PBS. The animal procedures were approved by the University of Iowa Animal Care and Use Committee.

DSPtdCho analysis

Lipids were extracted from 50 μg of protein using the method of Bligh and Dyer [31]. The lipids were dried under nitrogen gas, applied in 50 μl of chloroform/methanol (2:1, v/v) to silica LK5D plates, and developed in chloroform/methanol/light petroleum/acetic acid/boric acid (40:20:30:10:1.8, v/v/v/v/w) [32]. After each plate was dried in a fume hood, the sample lanes and phospholipid standard lanes were briefly exposed to iodine vapours. Samples that co-migrated with the PtdCho standard were scraped from the silica gel, reacted with osmium tetroxide and run in the second dimension and quantitatively assayed for phosphorus content or used for scintillation counting [33].

Enzyme assays

The activity of CCT was determined by measuring the rate of incorporation of [*Me*-^{14}C]phosphocholine into CDP-choline using a charcoal extraction method [3].

Immunoblot analysis

For immunoblot analysis, equal amounts of protein from lung homogenates were used. After dilution in sample buffer, samples were electrophoresed through an SDS/10 % (w/v) polyacrylamide gel and transferred on to a nitrocellulose membrane. CCTα, the predominant murine isoform, was detected by using the ECL® Plus Western blotting detection system and a rabbit polyclonal antibody specific for CCT (1:2000 dilution) [34].

Northern-blot analysis of CCT

Total cellular RNA was isolated from cells and murine whole lung using TRI Reagent™ (Sigma, St Louis, MO, U.S.A.) according to the manufacturer's instructions. Total RNA (30 μg) containing ethidium bromide was separated electrophoretically using a 1 % (w/v) agarose gel containing 2.2 M formaldehyde with a circulating running buffer consisting of 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde, and transferred on to a nitrocellulose membrane. The probe for CCTα consisted of a 1.1 kb *Hin*dIII–*Bam*HI fragment of the rodent CCTα cDNA [24, but see 24a] radiolabelled by the random primer method and hybridized to nitrocellulose membranes at 42 °C using standard hybridization protocols [24, but see 24a]. The blot was washed four times for 1 min at 22 °C, then for 10 min at 42 °C as described previously [34], and subjected to autoradiography.

PCR and cloning of the murine CCT promoter

The mouse CCT gene contains two transcriptional start sites 35 nt apart and is devoid of TATA or CAAT boxes, but rich in GC regions typical of housekeeping genes [20]. Using published sequence information for mouse CCT [20] we amplified an approx. 2000 nt fragment of the proximal 5′ region of the gene by PCR using C57BL/6J mouse genomic DNA (100 ng) as a template. Primers (0.4 μM) 5′-TTGTGTGTTTTCACCCCT-TATG-3′ (left) and 5′-TCAACTCCTCCAGGCTCCGGT-3′ (right) were incubated in a reaction mixture using PCR Supermix (Life Technologies) containing 1.5 mM $MgCl_2$, 200 μM dNTP and *Taq* DNA polymerase (1 unit/50 μl of reaction solution). PCR conditions included an intial cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 68 °C for 2 min, plus a final extension at 68 °C for 10 min. Amplification resulted in a 1938 bp product that was then cloned into pCR4-TOPO (Invitrogen). The product was digested with *Not*I and *Spe*I, gel purified, sequenced by the University of Iowa DNA

Core Facility and its identity with the published sequence confirmed. We then directionally subcloned this fragment into pGL3basic (Promega, Madison, WI, U.S.A.) upstream of the firefly luciferase coding region. To examine constitutive and sterol-regulated expression of the CCT gene, various deletion constructs were generated by restriction digestion or by separate PCR cloning. All cloned fragments include the proximal 5′ flanking region, two adjacent transcription start sites and up to 71 nt of the first exon corresponding to the 5′ untranslated region of the mouse CCT transcript [20]. These constructs were also coupled to luciferase, and then used for transient transfection studies and compared with expression of the low-density lipoprotein receptor (LDLR) promoter–luciferase construct [35–37].

The putative SRE within the 5′ flanking region (−156/−147 bp relative to the first transcriptional start site) of the CCT gene, GTCACCCCAC, was mutated to GT**AAA**CCCAC using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.) and primers 5′-CCGCTCAGTAAACCCACGCGCCCGG-3′ (left) and 5′-CCGGGCGCGTGGGTTTACTGAGCCG-3′ (right).

Cell culture and transient transfection

Cells were maintained in Hite's medium containing 10% (v/v) FBS at 37 °C in a 5% (v/v) CO_2 atmosphere. After reaching confluence, the cells were harvested using 0.25% trypsin/0.1% EDTA and plated on to 12-well tissue culture dishes, allowed to reach 60–80% confluence, and then transfected for 90 min. Transfections were conducted in 0.5% Hite's medium using Fugene 6™ reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.) with 0.75 μg of luciferase plasmid and 0.25 μg of cytomegalovirus-β-galactosidase, as a control for transfection efficiency. Immediately after transfections, cells were transferred to medium containing 10% (v/v) FBS or 10% (v/v) LPDS supplemented with lovastatin (2 μM) and cyclodextrin (100 μg/ml) for 18 h. Cell lysates were prepared for analysis of luciferase and β-galactosidase activities and the results were expressed as luciferase/β-galactosidase light units as previously described [38].

SRE DNA binding activity

Oligonucleotides that correspond to the putative native SRE or a mutated SRE were synthesized and annealed together:

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SRE:          gatcAGTCACCCCACG
                  TCAGTGGGGTGCctag
Mutated SRE: gatcAGTAAACCCACG
                  TCATTTGGGTGCctag
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Purified SREBP (200 ng/μl) purified from *Escherichia coli* was diluted 1:10 (v/v) in a buffer solution that contained 25 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl and 10% (v/v) glycerol just prior to use in the DNA binding assay [37,39]. Double-stranded SRE oligonucleotide was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and incubated with purified SREBP for 20 min at 4 °C in a reaction mixture that contained 5% (w/v) Ficoll, 12.5 mM Hepes, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM KCl, 10 ng/μl poly(dI-dC), 0.025% non-fat dried milk powder and 5% (v/v) glycerol. A 50–250-fold excess of unlabelled oligonucleotides was used for competition experiments. Samples were then analysed on a non-denaturing polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA) run at 150 V. The gel was subsequently dried and subjected to autoradiography as previously described [40].

Statistical analysis

The data are expressed as means ± S.E.M. Statistical analysis was performed using Student's t test [41].

RESULTS

Effect of lipid deprivation on DSPtdCho

Long-term restriction of essential fatty acids reduces surfactant levels [6,7], whereas acute effects of lipid deprivation on surfactant metabolism have not been investigated. We hypothesized that because of compensatory mechanisms existing within the PtdCho biosynthetic pathway, acute lipid deprivation would have no effect or lead to a transient increase in alveolar surfactant content in mice. To evaluate this hypothesis, mice were fed normal chow or fasted and refed a low-fat diet enriched with carbohydrate for 24 h as described previously [42]. After dietary manipulation, animals were killed, lungs lavaged and tissue homogenates isolated. Compared with mice fed normal rat chow, the levels of DSPtdCho, the major surface-active lipid within surfactant, increased by approx. 80% in mice administered a modified low-fat/high-carbohydrate diet (Figure 1A; $P < 0.001$). These results show that lipid-deprived mice acutely increase steady-state levels of surfactant lipid, perhaps by altering PtdCho synthesis.

To investigate effects of lipid deprivation on PtdCho synthesis, additional studies were performed using lung slices prepared from mice given standard or modified diets as described above. Murine lung slices were pulsed with [*Me*-^{3}H]choline and incorporation of the label into DSPtdCho, the major phospholipid of alveolar surfactant, was then determined. The incorporation of [*Me*-^{3}H]choline into DSPtdCho increased by 83% in lung preparations isolated from mice fed a low-fat/high-carbohydrate diet compared with the standard diet (Figure 1B). Similar studies showed that [*Me*-^{3}H]choline incorporation into PtdCho also

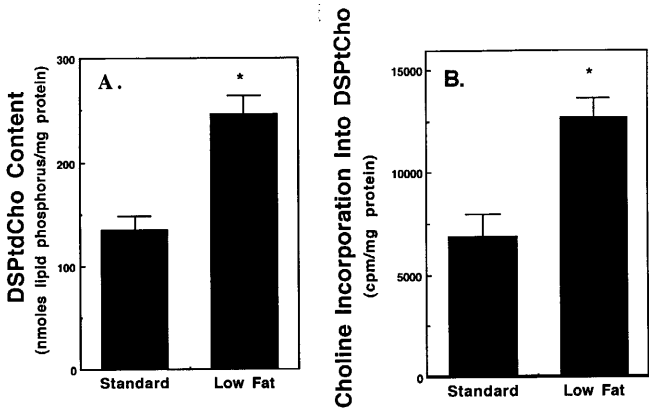


Figure 1 Effect of lipid deprivation on DSPtdCho biosynthesis in mice

(**A**) C57BL/6J mice were fed *ad libitum* with rat chow (standard diet) for 24 h, or fasted and refed a low-fat/high-carbohydrate diet for 24 h. After dietary manipulation, mice were killed and lavaged. Lavage DSPtdCho content was assayed using a phosphorus assay on the DSPtdCho fraction following TLC. The data are expressed as nmols of phospholipid phosphorus/mg of protein. (**B**) Lung slices from mice that were administered standard and modified diets, as described above, were pulsed with [*Me*-^{3}H]choline and incorporation of the radiolabel into DSPtdCho was then assayed. The data are expressed as c.p.m./mg of protein. The data in each panel are expressed as means ± S.E.M. from three independent experiments. *$P < 0.01$ compared with control.

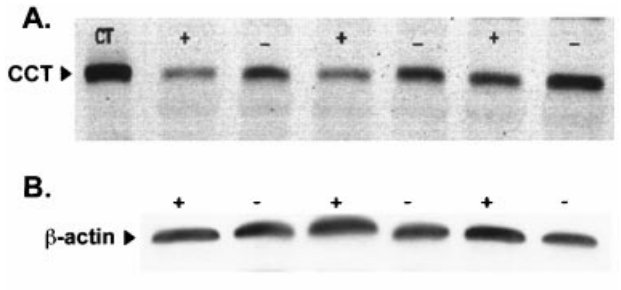


Figure 2 Effect of lipid deprivation on expression of CCT protein in mice

(**A**) Mice were fed *ad libitum* with rat chow (standard diet) for 24 h (+), or fasted and refed a low-fat/high-carbohydrate diet (−) as in Figure 1. After dietary manipulation, mice were killed and the lungs were isolated. Whole-lung homogenates (100 μg of protein) were separated by SDS/PAGE [10% (w/v) polyacrylamide], transferred on to nitrocellulose, and probed with an anti-CCT rabbit polyclonal antibody. (**B**) Immunoreactive β-actin content was determined for comparison using similar methods. Each band with either a + or − sign above it represents the amount of CCT protein in the lungs from one mouse.

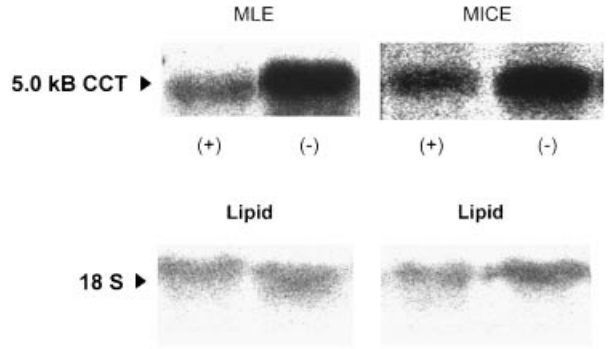


Figure 3 Effect of lipid deprivation on expression of steady-state CCT mRNA

(**A**) MLE-12 cells were cultured in 10% (v/v) FBS (+ lipid) or 10% (v/v) LPDS (− lipid) for 72 h. Cells were then harvested, total RNA extracted and processed for Northern blotting for detection of a 5 kb CT mRNA transcript. (**B**) Mice were fed *ad libitum* with rat chow (standard diet) for 24 h (+ lipid), or fasted and refed a low fat/high-carbohydrate diet (− lipid) as in Figure 1. After dietary manipulation, mice were killed and total cellular RNA was isolated and used for Northern-blot analysis. The data are representative of one of three independent experiments. Levels of 18 S mRNA were measured to show relative loading of gels.

increased by approx. 2-fold in lung slices from animals given the modified diet compared with the control diet (results not shown). These results indicate that dietary lipid restriction increases alveolar surfactant lipid, in part, by stimulating its biosynthesis.

Effect of lipid deprivation on CCT expression

The molecular mechanisms underlying changes in surfactant synthesis after dietary restriction remain poorly understood. We previously demonstrated that lipid deprivation increases CCT activity and immunoreactive protein content in primary rat alveolar type II cells and in MLE-12 cells [21]. We hypothesized that lipid restriction increases PtdCho synthesis *in vivo* via increased expression and activity of CCT. To test effects of lipid deprivation *in vivo*, mice were administered an identical dietary protocol to that outlined in Figure 1. Consistent with findings in cell culture, a low-fat/high-carbohydrate diet increased levels of

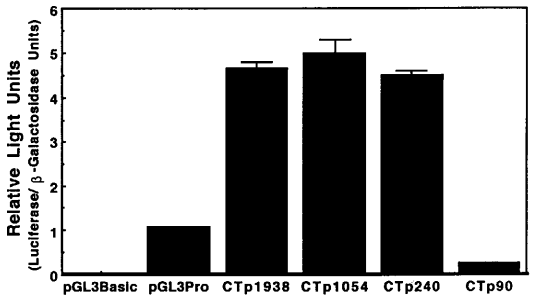


Figure 4 Deletional analysis of the CCT promoter in MLE-12 cells

Transfections were conducted in 0.5% Hite's medium with Fugene 6^{TM} reagent and 0.75 μg of test plasmid for 90 min using one of six different luciferase reporter vectors: 1, pGL3basic, a negative control that contains no promoter; 2, pGL3Pro, a positive control that contains the simian virus 40 promoter; and 3–6, CTp1938 (−1867/+71), CTp1054 (−983/+71), CTp240 (−169/+71) and CTp90 (−19/+71), the experimental vectors containing fragments of the CCT promoter cloned into pGL3basic. Luciferase and β-galactosidase activities in cellular extracts were determined by luminometer readings. Data are presented as means ± S.E.M. ($n = 7$).

CCT protein in lungs without altering levels of β-actin protein (Figure 2). CCT activity increased 57% in these studies. To determine if the increase in immunoreactive CCT was due to an increase in its synthesis, we assayed steady-state CCT mRNA levels *in vitro* and *in vivo*. As we have previously shown, MLE-12 cells cultured in lipoprotein-deficient medium for 72 h were observed to have increased levels of the 5 kb CCT transcript [21] (Figure 3A), effects attributed to increased CCT mRNA synthesis [21]. When CCT mRNA was analysed in mice we detected relatively low levels of CCT mRNA from animals fed a standard diet. However, after exposure to a low-fat/high-carbohydrate diet, levels of steady-state CCT mRNA increased approx. 2-fold (Figure 3B). These results indicate that effects of lipid deprivation on DSPtdCho synthesis *in vivo* are associated with enhanced expression of a regulatory enzyme at the pre-translational level. Furthermore, MLE-12 appears to be a suitable model to study molecular mechanisms for lipid regulation of CCT expression as changes similar to the intact animal model were seen in these cells.

Deletional and mutational analyses

To examine whether induction of CCT mRNA observed after lipid deprivation has a transcriptional basis, the 5′ flanking region of the CCT gene was cloned, various portions of the CCT promoter were coupled to luciferase, and constitutive and sterol-regulated transcriptional activity were assessed in MLE-12 cells. Results in Figure 4 show that three out of four constructs showed robust activity that was considerably greater than activity displayed by the strong simian virus 40 promoter. The smallest active construct, which contains 169 bp of the 5′ flanking region (CTp240), exhibited high levels of expression and probably contains the core promoter. We then tested these constructs in MLE-12 cells cultured in the presence of FBS or LPDS (Figure 5). Our results show that constructs CTp1938 (−1867/+71), CTp1054 (−983/+71) and CTp240 (−169/+71) confer sterol-regulation to the luciferase gene as we observed a 2–3-fold increase in luciferase activity in these constructs when cultured with LPDS rather than FBS. This activation of the CCT promoter was comparable, though to a lesser degree, with the human LDLR promoter, which exhibited a 5-fold induction of reporter activity in the presence of sterol-depleted medium compared with

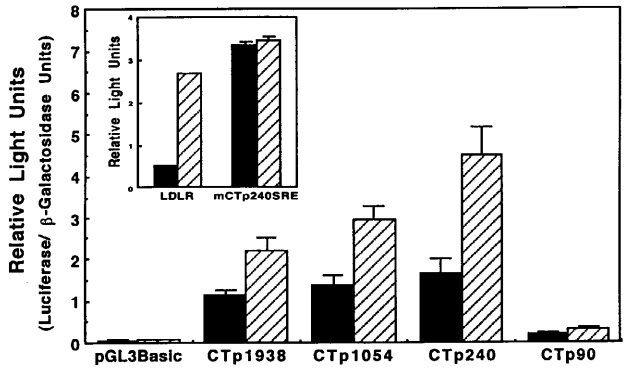


Figure 5 LPDS activates the CT promoter in MLE-12 cells

Transfections were conducted as described in Figure 4 using pGL3basic, CTp1938, CTp1054, CTp240 and CTp90. After transfections, cells were exposed to medium containing 10% (v/v) FBS (black bars) or 10% (v/v) LPDS (hatched bars) for 18 h. For comparison, we tested effects of LPDS on the activation of a positive control plasmid encoding the human LDLR coupled to luciferase (inset). We also tested the effects of LPDS on a CCT promoter–luciferase construct harbouring mutations within the candidate SRE (mCTp240SRE; inset). Luciferase and β-galactosidase activities in cellular extracts were determined in a luminometer. Data are presented as means $\pm$ S.E.M. (main panel, $n = 6$; inset, $n = 3$).

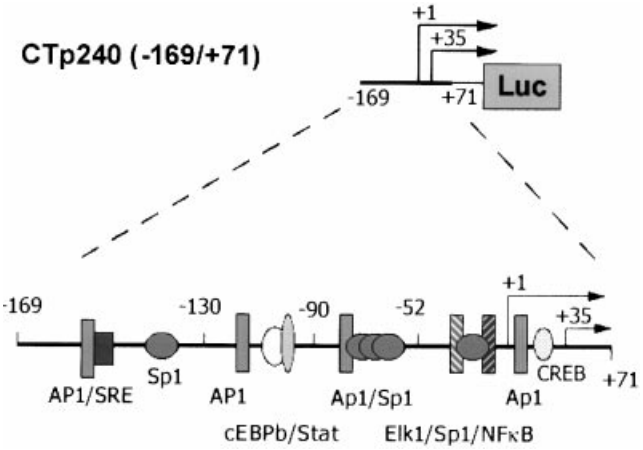


Figure 6 Consensus regulatory elements identified within the CCT promoter

Schematic diagram showing regulatory elements found within a 240 bp ($-169/+71$) segment of the mouse CCT promoter. Consensus elements for nuclear factors were identified using the TFSEARCH program.

FBS (Figure 5, inset). Furthermore, sterol regulation was not observed in the CTp90 ($-19/+71$) construct (Figure 5).

Sequence analysis of the proximal 5′ flanking region using the TFSEARCH program at http://www.cbrc.jp/research/db/TFSEARCH.html revealed the presence of a putative consensus SRE (GTCACCCCAC) between -156 to -147 bp (Figure 6). Mutation of two nucleotides within this SRE abolished the sterol-regulated activation of luciferase activity (Figure 5, inset), confirming that this SRE was necessary for sterol regulation of the CCT promoter. Mutation of the SRE, however, did not reduce the constitutive activity of the promoter sequence, suggesting that the SRE is distinct from core promoter elements. Together, these results indicate that the 240 bp 5′ flanking sequence contains the basal promoter as well as the sterol-regulated *cis*-acting element(s) of the murine CCT gene. To

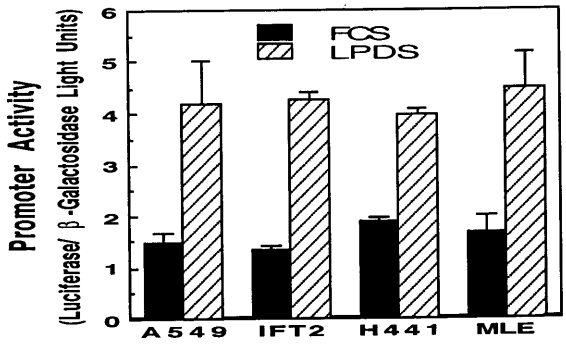


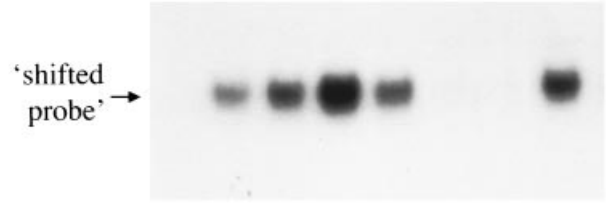
Figure 7 LPDS activation of the CCT promoter is not species specific

Transfection conditions were identical to those described in Figure 5. Expression of the CTp240 experimental vector in the MLE-12 cells was compared with human cell lines (A549 and H441), and an immortalized rat fetal type II cell line (IFT2). After transfections, cells were exposed to medium containing FBS (black bars) or LPDS (hatched bars) and values were normalized for transfection efficiency as in Figure 4. Data are presented as means $\pm$ S.E.M. The data are from of three separate studies.

confirm these findings, the 240 bp construct was also tested in A549 (human), H441 (human) and immortalized fetal type II (rat) cell lines (Figure 7). Similar to MLE-12 cells, these cell lines exhibited a 2–4-fold induction in CCT transcriptional activity in response to sterol deprivation. Overall, these latter studies indicate that transcription factors that control sterol-regulation of the CCT gene are present in rodent and human lung epithelia and can bind the human SRE without species specificity.

SRE DNA binding activity

We then tested the putative SRE (-156 to -147 bp) to see if it could specifically bind purified recombinant SREBP in a mobility-shift DNA binding assay. Labelled oligonucleotides corresponding to the native SRE sequence were able to bind to purified SREBP (Figure 8). In addition, this binding could be efficiently competed with an excess of unlabelled oligonucleotide corresponding to the SRE, but not by an excess of an SRE



SREBP (μl)	-	1	2.5	5	1	1	1	1
Cold SRE	-	-	-	-	-	50x	250x	-
Cold mSRE	-	-	-	-	-	-	-	250x

Figure 8 Gel mobility-shift analysis demonstrates SREBP binding to the consensus SRE within the CCT promoter

End-labelled SRE oligonucleotide was incubated with purified recombinant SREBP. As the mass of SREBP is increased an inreasing amount of probe is retarded. Bound protein is efficiently competed with 50 × or 250 × unlabelled (Cold) SRE oligonucleotide but not by a mutated SRE (mSRE). The data are representative of one of three independent experiments.

sequence where 2 bp had been mutated to abolish binding. Our data confirm that an SRE sequence contained within the CCT promoter binds specifically to SREBP to retard its mobility (Figure 8).

DISCUSSION

Few studies have investigated the physiological events related to surfactant PtdCho metabolism after lipid or caloric deprivation. There is even less information on the biochemical or molecular processes that might serve as an adaptive mechanism after alveolar epithelia are deprived of critical lipid substrates utilized in PtdCho biosynthesis. The present paper is the first study demonstrating that lipid (or sterol) deprivation leads to an increase in DSPtdCho synthesis coupled with enhanced expression of the rate-limiting enzyme involved in surfactant PtdCho synthesis. Our molecular studies, for the first time, also link the SREBPs to the regulation of the CCT promoter, thus providing a novel feedback control mechanism relating PtdCho biosynthesis to sterol deprivation. Our *in vitro* studies using MLE-12 cells show that a relatively small portion of the 5′ flanking region, containing this enhancer sequence, confers constitutive activity and responds to sterol-depleted medium.

In contrast with other studies showing a decrease in surfactant content, a protocol of fasting and refeeding with a low-fat/high-carbohydrate diet resulted in a nearly 2-fold increase in surfactant content in alveolar lavage (Figure 1). These results differ significantly from others where feeding rats to induce essential fatty acid deficiency resulted in a decrease in alveolar surfactant content and altered fatty acid composition of PtdCho [6–8]. Notable differences between our work and those of others include differences in species, the dietary regimens used, and, perhaps more importantly, the duration of dietary manipulation. In prior studies, regimens were administered over several weeks resulting in modified surfactant composition and impaired biophysical activity, whereas we assessed short-term effects of lipid deprivation. We doubt that species differences could account for the study results because our preliminary data show similar effects for surfactant synthesis with rats using the same dietary protocol (results not shown). Based on the current results, and existing literature, it is likely that a rapid feedback response mechanism is activated almost immediately after lipid availability is restricted to the alveolar epithelium.

To investigate these mechanisms, we focused on the PtdCho biosynthetic pathway, because two enzymes in this pathway, cholinephosphotransferase and CCT, are lipid regulated. Interestingly, both enzymes are post-translationally activated by fatty acids, and thus lipid-deprivation would be expected to lead to enzyme inhibition with an overall decrease in surfactant PtdCho content explaining prior data [6–8,43]. Indeed, this was observed with cholinephosphotransferase; however, CCT activity was unexpectedly stimulated after lipid deprivation as a consequence of increased mRNA synthesis [21]. In preliminary studies, we observe that lipid deprivation stimulates CCT activity in rat alveolar type II epithelial cells but not alveolar macrophages and fibroblasts (R. K. Mallampalli and A. J. Ryan, unpublished work). Thus such effects in the lung may be restricted primarily to cells involved in surfactant synthesis. Tijburg et al. [44] also showed that fasting and refeeding with a high-sucrose/fat-free diet decreased liver CCT activity and increased the pool size of the CCT substrate, cholinephosphate. These data suggest that liver CCT may also be affected by diet, but that it is regulated differently. Related studies by Batenburg and Whitsett [45] demonstrated that liver enzymes involved in *de novo* fatty acid synthesis are activated by fasting and refeeding but that lung fatty acid lipogenic enzymes are not [45]. CCT expression was not determined in this study [45]. Together, one can speculate that the liver may utilize the *de novo* fatty acid pathway to adapt to lipid deficiency, whereas the lung uses the CDP-choline pathway as a feedback control mechanism to maintain PtdCho homoeostasis. Although the current results provide evidence that lipid deprivation stimulates lung PtdCho synthesis by increasing CCT expression, compensatory effects at other regulatory steps, such as enhanced surfactant secretion or reduced surfactant uptake, might also have contributed to our results on alveolar PtdCho pool size.

Using MLE-12 cells and whole-lung isolates a consistent, and perhaps biologically relevant, observation was that lipid deprivation stimulated expression of CCT mRNA. These changes were of lesser magnitude compared with changes seen for transcripts of other sterol-regulated lipogenic enzymes [42], but coincided with increased surfactant levels *in vivo*. Furthermore, like other sterol-regulated genes, control was at the level of transcription rather than at the level of mRNA stability [19,21,46–48].

To investigate transcriptional mechanisms, transfectional analysis was performed which revealed a constitutively active promoter with high levels of expression localized within a relatively small portion of the 5′ flanking sequence of CCT ($-169/+71$) consistent with observations in hepatoma cells and embryonic fibroblasts [20]. Recent studies by Sugimoto et al. [49] reveal that this region has an enhancer element ($-103/-82$) containing a transcriptional enhancer factor-binding consensus sequence where transcriptional enhancer factor-4 acts as a dual transcriptional modulator. Basal expression appears to reside in a sequence between -52 and -19 (Figure 4 and [20]). The core promoter ($-169/+71$), though small, also appears to contain the SREs, as a 2–4-fold induction was seen in different cell types across species. Interestingly, the genes of several lipogenic proteins, such as the human LDLR gene [46], the rat fatty acid synthase promoter [19], the human squalene synthase gene [47], and the human ATP citrate-lyase gene [48] are tightly regulated by enhancers in close proximity to the core promoter. For example, the promoters of the LDLR and the squalene synthase genes have SREs located approx. 125 bp upstream [46] and approx. 200 bp upstream of their respective cognate transcription start sites [47]. The observed increase in luciferase activity for the CCT gene in response to lipid deprivation corresponds to the increase seen in endogenous CCT mRNA, suggesting that all of the change in CCT mRNA can be accounted for by *cis*-elements in this proximal 5′ flanking DNA sequence. Targeted mutation of the candidate SRE within this core element revealed that this element was necessary for the sterol-regulated transcriptional activation of the CCT gene. Gel mobility-shift assays demonstrated specific binding of SREBP to this consensus sequence. Thus SREBP *trans*-activates the CCT gene, similiar to other related lipogenic genes involved in fatty acid, triacylglycerol and cholesterol biosynthesis. Whether other *cis*-elements in addition to the SRE site are involved in sterol-mediated activation of the CCT gene requires further investigation. In this regard, studies suggest that Sp1-binding transcription factors serve as co-activators with SREBP in regulation of the LDLR, acetyl-CoA carboxylase, and fatty acid synthase promoters [50–52].

Together, our results provide novel information demonstrating co-ordinate regulation of cholesterol and PtdCho metabolism at the molecular level. The results indicate that the 240 bp fragment ($-169/+71$) contains the basal promoter as well as the sterol-regulated *cis*-acting element(s) of the murine CCT gene. The SRE was localized between -156 and -147 bp in the 5′ flanking sequence of the CCT gene. Future studies directed at uncovering

additional regulatory elements involved in up-regulating CCT gene expression under physiological conditions may provide new strategies for modulating surfactant phospholipid synthesis.

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