Identification of Transcriptional Enhancer Factor-4 as a Transcriptional Modulator of CTP:Phosphocholine Cytidyltransferase α*

CTP:phosphocholine cytidyltransferase (CCT) is the rate-limiting and regulated enzyme in mammalian phosphatidylcholine biosynthesis. There are three isoforms, CCTα, CCTβ1, and CCTβ2. The mouse CCTα gene promoter is regulated by an enhancer element (Eb) located between −103 and −82 base pairs (5'-GGTTTCAGGAATTGCGGAGGTGG-3') upstream from the transcriptional start site (Bakovic, M., Waite, K., Tang, W., Tabas, I., and Vance, D. E. (1999) Biochem. Biophys. Acta 1436, 147–165). To identify the Eb-binding protein(s), we screened a mouse embryo CDNA library by the yeast one-hybrid system and obtained 19 positive clones. Ten CDNA clones were identified as transcriptional enhancer factor-4 (TEF-4). The TEF-binding consensus sequence, 5'-A/T(A/G)(A/G)(A/T)ATG(G/C/T)(G/A)-3', was identified within the Eb binding region. Gel-shift analysis using radiolabeled Eb fragment as a probe showed that cell extracts from yeast expressing hemagglutinin-tagged TEF-4 caused a marked band retardation that could be prevented with an anti-hemagglutinin antibody. When COS-7 cells were transfected with TEF-4, CCTα promoter-luciferase reporter activity and CCTα mRNA levels increased. A TEF-4 deletion mutant containing a DNA-binding domain, mTEA(+), stimulated the CCTα promoter activity, whereas protein lacking the DNA binding domain, mTEA(-), did not. Unexpectedly, when the ATG core of the TEF-4 binding consensus within the Eb region was mutated, promoter activity was enhanced rather than decreased. Thus, TEF-4 might act as a dual transcriptional modulator as follows: as a suppressor via its direct binding to the Eb element and as an activator via its interactions with the basal transcriptional machinery. These results provide the first evidence that TEF-4 is an important regulator of CCTα gene expression.

In higher eukaryotes, CTP:phosphocholine cytidyltransferase (CCT) is a rate-limiting and regulated enzyme in the synthesis of phosphatidylcholine (1–4). The first mammalian CCT was purified from rat liver (5, 6) and was used for isolation of the corresponding cDNA encoding a 367-amino acid (CCTα) protein (7). The predicted CCTα structure consists of a nuclear localization signal, a catalytic domain, a helical lipid-binding domain, and a phosphorylation domain (1–4, 8–10). Recently, cDNAs for two other isoforms, CCTβ1 (10), and its splicing variant CCTβ2 (11), were cloned. All isoforms contain a highly homologous catalytic domain and an amphitropic helical domain that binds lipids (10). CCTα and CCTβ2 also contain a highly phosphorylated domain at their carboxyl terminus (1–4, 9, 11), whereas CCTβ1 lacks this domain. The roles of individual domains in the regulation of CCTα enzyme activity have been extensively studied. CCTα activity is modulated by the binding of specific lipids to the helical domain (1–4, 12, 13) and by phosphorylation at the carboxyl-terminal domain (1–4, 9, 14–17).

In addition to regulation at the protein level, CCTα is regulated at the transcriptional and post-transcriptional levels in various cells and tissues. CCTα mRNA was increased in colony-stimulating factor 1-stimulated macrophages (18), in hepatic tissues after partial hepatectomy (19), and during growth and development (20). However, the question of whether the elevation of CCTα mRNA levels was due to an increase in transcription or due to stabilization of the produced transcript needs to be further investigated (21–23).

Tang et al. (24) isolated the murine CCTα gene (Ctpt) and showed that the exon/intron organization of the gene closely resembles the functional domains of the enzyme. The gene is transcribed from two transcriptional start sites. The 5' promoter region lacks TATA/CAT boxes, but contains GC-rich regions. Bakovic et al. (25) demonstrated that three GC regions are regulatory as follows: a “loose” Sp1 site at −31/−9, a cluster of three overlapping Sp1 sites (−88/−50), and a canonical Sp1-binding site (−148/−128). More recently, the same authors showed that transcription factors Sp1 (26, 27), Sp2 (28), and Sp3 (28) can competitively bind to these regions and that the promoter activity may depend on the relative abundance of those three factors (29, 30).

To identify the regulatory elements responsible for CCTα transcription, Bakovic et al. (25) prepared various promoter deletion constructs of Ctpt linked to the luciferase reporter. Subsequent functional assays revealed the presence of regions

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‡ The abbreviations used are: CCT, CTP:phosphocholine cytidyl

transferase; 3-AT, 3-aminotriazole; Ctpt, CTP:phosphocholine cytidyltransferase α gene; G3PDH, glycero-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction; TEF, transcriptional enhancer factor; GSTm, glutathione S-transferase; CMV, cytomegalovirus.
responsible for basal expression (~52+/−38) as well as positive and negative regulatory elements (~201+/−90). Furthermore, gel-shift assays indicated several unidentified proteins capable of binding to the region −103/+82, (GTTCGAGGAAATGCGGAGGTGGGAG) and the neighboring region −130+/−103.

In this study, we utilized the yeast one-hybrid system (31, 32) to clone cDNA encoding Eb-binding proteins. One of them was identified as a member of the transcription enhancer factor (TEF) family of proteins, TEF-4 (33, 34). TEF-4 is closely related to TEF-1 that was previously isolated as a regulatory protein of the SV40 enhancer (35). We report that TEF-4 regulates expression of the CCTg gene in COS-7 and 3T3-L1 cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**MATCHMAKER Yeast One-Hybrid System for screening DNA-binding proteins, the Saccharomyces cerevisiae strain YM4271, and the 11-day mouse embryo MATCHMAKER cDNA library constructed in the pACT2 vector were purchased from CLONTECH (Palo Alto, CA). The promoter-less luciferase vector, pGCL3-basic, carrying Photinus pyralis luciferase, the control pRL-CMV vector, carrying Renilla reniformis luciferase, and the Dual-luciferase Reporter Assay System were obtained from Promega (Madison, WI). FuGENE™ transfection reagent, used for transfecting Dulbecco’s modified Eagle medium (DMEM) and Neuro-2a cell’s, was purchased from Roche Molecular Biochemicals, Sigma, and Life Technologies, Inc., respectively. COS-7 and 3T3-L1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). 3-Aminotriazole (3-AT) was from Sigma.

**Construction of Plasmids and Yeast Strains Containing the Eb Sequence—**The triple repeat of Eb (5′-TGGTTTTCAGGAATGCGGAGGTTTGCG-3′) flanked by an EcoRI site at the 5′-end and by StuI and XbaI sites at the 3′-end (5′-GAATTCCGTGGGAATCTCGGTTATTCATTCGAGAAGTGTTGGGGTTTTCTAGGAAATGCGGAGGTGTTTCCAGGATTCGTTTTCAGGAATGCGGAGGTGGGTTTTCAGCGTCTGCAGTCTAGA-3′), and its complementary sequence, were synthesized by the DNA core facility at the University of Alberta. Two strands (50 pmol each) were annealed in 100 μl of 25 mM Tris-HCl, pH 8.0, 0.5 mM MgCl2, and 25 mM EDTA by heating to 95 °C for 10 min followed by slow cooling to room temperature. The annealed sequence (3Eb) was digested and inserted between the EcoRI and XbaI sites of the yeast phIS and pLaZ vectors (CLONTECH) to prepare phIS-Eb and pLaZ-Eb plasmids, respectively. The correct orientation of 3Eb was ascertained by digestion with StuI and sequencing. pHis-Eb and pLaZ-Eb were linearized with AflII and NcoI, respectively, and the linearized products were transferred into YM4271 yeast strain. HIV or SD-/ Ura selective medium, and stable integrants for phIS-Eb and pLaZ-Eb were isolated and named YHIS-Eb and YLaZ-Eb, respectively. The yeast strain YHIS-Eb was transformed with the pACT2 mouse embryo cDNA library and incubated in SD-/His−/Leu− medium at 30 °C overnight. The cell lysate was used for the dual-luciferase assay according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency by using the ratio of the activities obtained with the CCTa promoter deletion constructs (see above) and 0.001 μg of phRL-CMV Renilla vector as a transfection control, and 0.5 μg of either pcTEF-4, pCtMTEA−, pmCMV− or pmCMV+ or pcDNA control. Transfection was initiated by dropwise addition of DNA suspensions to the cell culture. Forty-eight hours later, the cells were harvested, lysed in 200 μl of Passive lysis buffer (Promega), and 10 μl of the cell lysate was used for the dual-luciferase assay according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency by using the ratio of the activities obtained with the CCTa promoter deletion constructs (see above) and the pRL-CMV construct carrying the cytomegalovirus promoter-luciferase fusion for yeast as described (36).

**Preparation of Cell and Nuclear Extracts—**TEF-4-positive yeast, Y41b, was cultured in 15 ml of SD-/His−/Leu medium containing 45 mM 3-AT. After 48 h, 12 ml of the late log-phase culture was transferred into 100 ml of the same medium and cultured for an additional 36 h. The cells were collected by centrifugation at 600 × g for 10 min, resuspended in 1 ml of homogenization buffer (0.1 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM phenylmethlysulfonyl fluoride, 0.01 mM β-mercaptoethanol, 20% glycerol). After vortexing with glass beads to break the cell walls, the cells were subjected to three cycles of freezing and thawing and centrifuged at 10,000 × g for 5 min to remove the cell debris (38). The yeast transformed with pY41b were cultured in SD/Leu medium and treated in a similar manner to obtain cell extracts. Nuclear extracts from mammalian cells were prepared according to Andrews and Faller (39) with minor modification (25).

**Preparation and Purification of Glutathione S-Transferase TEF-4 Fusion Protein (GST-TEF-4)—**Full-length TEF-4 cDNA was digested from pY41b with EcoRI and XhoI and ligated into the pGEX-KG (Promega) vector to obtain the GST-TEF-4 expression plasmid. GST-TEF-4 was transformed into BL21(DE3) and induced with 0.5 mM isopropyl-1-thiogalactopyranoside. The bacteria were subsequently transferred to 10 volumes of growth media and grown for 1 h. Isopropyl-1-thiogalactopyranoside was then added to the culture to a final concentration of 0.5 mM, and the incubation was continued for 3 h. Cells were collected by centrifugation, resuspended in 0.5 mM of lysis buffer (25 mM Hepes-NaOH, pH 7.5, 20 mM KCl, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 0.02% Triton X-100, and sonicated for 2 min. After sonication, the lysate was centrifuged at 4000 × g for 10 min. The supernatant was further incubated at 4 °C for 1 h. After centrifugation at 40,000 × g for 1 h, the supernatant was used for the GST-TEF-4 purification.
X-100, sonicated, and centrifuged at 10,000 × g for 15 min. To obtain purified proteins, the supernatant was applied to a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). GST-TEF-4 and GST proteins were eluted according to the manufacturer’s instructions.

Electromobility Gel-shift and Supershift Assays—The double-stranded Eb oligonucleotide was annealed (70 °C, 10 min) in 100 μl of 25 mM Tris- HCl, pH 8.0, 0.5 mM MgCl₂, and 25 mM NaCl and then cooled to room temperature. An aliquot (10 pmol) of the double-stranded oligonucleotide was 5’-end labeled with [³²P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase and purified on a Sephacyr S-200 column (Amersham Pharmacia Biotech). A DNA protein-binding reaction was performed for 30 min at room temperature in 40 μl of 1× binding buffer (40 mM Tris- HCl, pH 7.8, 4 mM MgCl₂, 2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 200 μg/ml bovine serum albumin, 20% glycerol, and 0.2% Nonidet P-40) containing 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech), 1 μl of the radiolabeled probe (50,000–80,000 cpm), and one of the following: yeast cell extracts, mammalian nuclear extracts, purified GST-TEF-4 or GST proteins. In some cases, unlabelled double-stranded Eb (100-fold molar excess), anti-hemagglutinin antibody (Roche Molecular Biochemicals), or anti-GST antibody (Amersham Pharmacia Biotech) was included in the incubation mixture. The reaction was stopped by addition of 4 μl of 6× DNA loading buffer. The labeled probe was separated from DNA protein complexes by electrophoresis on 6% nondenaturing polyacrylamide gels in 0.5× Tris borate/EDTA buffer (44.5 mM Tris- HCl, pH 8.3, 44.5 mM boric acid, and 1 mM EDTA) at 4 °C until the xylene cyanol dye reached 5 cm from the bottom of the gel. Autoradiography was carried out by exposure of the gel to Kodak X-omat XAR2 film with an intensifying screen at −70 °C for 16–48 h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—All values are expressed as means ± S.D. Group means were compared by Student’s t test or the Cochran-Cox test after analysis of variance to determine the significance of difference between the individual means. Statistical significance was assumed at p < 0.05.

RESULTS

Cloning of the Putative Regulatory Proteins That Bind to the Eb Element of the CCTα Promoter—A yeast integrant (YLacZ-Eb) carrying three repeats of Eb in the front of the his3 coding region was constructed and used to screen for the Eb-binding proteins and promoter activation by the one-hybrid expression system. The Yhis-Eb yeast strain was transformed with the pACT2 mouse embryo cDNA library encoding fusions of the yeast gal4 activation domain, a cDNA of an unknown protein, and a hemagglutinin tag at its 5′-end. The yeast transformation efficiency was 1 × 10⁴ colony-forming units/μg as determined on SD−/Leu selective medium. After screening 1.5 × 10⁶ colonies, 19 positive clones were obtained that were able to grow on the SD−/−Leu selective medium containing 1 μg ml⁻¹ 3-AT. All the colonies obtained from those yeast transformants were 10/19 encoded transcriptional enhancer factor-4 (33, 34); 1/19 encoded ribosomal protein L22 (40); 1/19 encoded ribosomal protein S4 (41); 1/19 encoded phosphatidylinositol phospholipase Cα (42); 1/19 encoded a mouse ortholog of the human FRAF-related protein (43); 1/19 encoded DNA polymerase I (44); and 4/19 encoded unknown cDNAs. Thus, the most frequently isolated clones were those encoding transcriptional enhancer factor TEF-4. Since we found that the TEF-binding consensus (35), 5′- (ACT/A)(AG/A)(ATG (CT/T/G)-3′ was present in the Eb promoter region, we decided to focus on TEF-4 and further analyze its role in CCTα gene transcription. We selected the clones pY41b and pY51b, isolated from the yeast Y41b and Y51b, that encoded the full-length or almost full-length TEF-4 (pY41b from the position −24 and pY51b from the position +94 relative to the translation start codon).

To confirm that TEF-4 clones can drive transcription of other yeast genes carrying the Eb sequence in their promoters, the yeast integrant YLacZ-Eb carrying a 3-fold repeat of Eb follow by the β-galactosidase lacZ gene was transformed with pY41b, pY51b plasmids, or pYcont (pACT2 vector), cultured on SD−/Leu selective medium, and tested for the expression of β-galactosidase. Formed colonies were examined for β-galactosidase by the colony-lift filter assay. The colonies of YLacZ-Eb transformed with pY41b and pY51b expressed β-galactosidase and turned blue in the presence of the β-galactosidase substrate. However, the colonies of YLacZ-Eb transformed with either pGAD53m encoding a fusion of p53 with the GAL4 activation domain (which does not bind to the Eb region) or the pYcont empty vector, did not yield blue colonies, as expected. We next used YS3BLUE yeast strain that carried the p53-binding sequence in front of lacZ as a positive control. YS3BLUE transformed with pGAD53m plasmid expressed β-galactosidase and the colonies turned blue. When the YS3BLUE yeast was transformed with plasmids pY41b and pY51b encoding TEF-4, which does not bind to the p53 sites, with the empty vector pYcont, no blue color was obtained. The untransformed YLacZ-Eb or YS3BLUE yeast produced a similar negative response. Together, these results indicate that pY41b and pY51b encodes TEF-4 that binds to the Eb region of heterologous yeast promoters and drives transcription of the his3 and lacZ genes. The results also confirm the validity of the TEF-4 clones and their expression.

Evaluation of TEF-4 Binding to the Eb Promoter Element—To confirm the binding of TEF-4 to the Eb region, we performed gel-shift analysis using the radiolabeled Eb oligonucleotide as a probe. The labeled Eb fragment was incubated with cell extracts isolated from the yeast expressing TEF-4 (Y41b) or control (Ycoint), and DNA-protein complexes were separated by electrophoresis and visualized by autoradiography (Fig. 1A). When Y41b extract was used, two slower migrating bands in addition to the faster moving probe were observed (Fig. 1A, lane 2). The intensities of the two slower bands were markedly decreased after competition with a large excess of unlabeled probe (Fig. 1A, lane 4), suggesting that both originated from a protein that binds to Eb. However, the lower band was also visible in the experiments with control yeast extracts (Fig. 2A, lane 3) indicating that only the upper band was specific for TEF-4/DNA binding. This was further confirmed by a competition experiment with an antibody specific for the hemagglutinin tag, which was part of the TEF-4 fusion protein. When various amounts of the anti-hemagglutinin antibody were added to the reaction mixture, the intensity of the upper band decreased, whereas the intensity of the lower band was not affected (Fig. 1B). These results clearly support the notion that the upper band was specific for the interaction of the TEF-4 fusion protein with Eb.

Antibodies specific for TEF-4 are not available. To confirm that the binding to Eb is specific for TEF-4 protein regions and not for the hemagglutinin tag, we cloned and purified a fusion protein, glutathione S-transferase-TEF-4 (GST-TEF-4). We also made pure GST and used it as a negative control in parallel gel-shift assays. As shown in Fig. 1C, migration of the Eb band was retarded only in the presence of purified GST-
TEF-4 (lane 2) and not in the presence of GST (lane 3). The addition of an anti-GST antibody (lane 4) or cold Eb competitor (lane 6) prevented formation of the retarded bands. Purified GST-TEF-4 also caused a band retardation of the SV40 enhancer element GT-IIC (lane 8) that is known to be regulated by TEF proteins. Furthermore, GST-TEF-4 did not bind to the CCTα basal promoter fragment −90/+38 that does not contain a TEF-4 consensus sequence (data not shown). Taken together, we conclude that TEF-4 is responsible for the formation of the protein complexes within the Eb region of the CCTα promoter.

DNA Binding Properties of TEF-4 after Its Expression in Mammalian Cells—To test whether or not TEF-4 could also bind to the Eb promoter element after expression in mammalian cells, we prepared a mammalian TEF-4 expression plasmid, pcTEF-4, and transfected COS-7 cells with pcTEF-4 or control vector pcDNA. We isolated cell nuclear extracts and performed gel-shift analysis using Eb and mutated Eb (Ebm in which the TEF-4 ATG-binding core was mutated to GCT) as probes. Nuclear extracts from COS cells transfected with pcTEF-4 produced two slowly migrating bands in addition to the rapidly migrating Eb band (Fig. 2A, lane 3). The intensities of the two retarded bands markedly decreased after competition with an excess of cold Eb probe (lanes 4 and 5). The upper band was also visible in the experiments with control nuclear extracts (lane 2), indicating that some endogenous nuclear proteins or the protein complex of TEFs with some other factors also bind to Eb. The migration of the lower band, however, coincided with the migration of the purified GST-TEF-4, suggesting that this band originated from the binding of TEF-4 overexpressed in COS-7 cells. When the mutated element Ebm was used instead, no protein binding was detected with nuclear extracts from TEF-4-expressing cells (Fig. 2A, lane 8) nor did the purified GST-TEF-4 fusion protein bind to the mutated probe (Fig. 2B, lane 3). These results suggested that both overexpressed TEF-4 and an endogenous nuclear protein from COS-7 cells can bind to the Eb promoter element by recognizing the same ATG core sequence within the TEF binding consensus.

Activation of Chimeric CCTα Promoter-Luciferase Reporters

**Fig. 1.** Gel-shift and supershift analysis for the TEF-4/hemagglutinin tag or purified TEF-4/GST fusion protein binding with the Eb promoter element. A, the end-labeled Eb probe was incubated with 65 μg of the extract from yeast carrying pY41b (lanes 2 and 4) or pYcont (lane 3). A 100-fold molar excess of unlabeled probe was used for competition with the labeled Eb probe (lane 4). B, the end-labeled Eb probe was incubated with 65 μg of the extract from yeast carrying pY41b. Cell extracts were incubated without (lane 1) or with 2 (lane 2), 4 (lane 3), or 8 μg (lane 4) of anti-hemagglutinin antibody. C, the end-labeled Eb (lanes 1–7) or GT-IIC probe (lanes 8 and 9) were incubated with 1 μg of purified GST-TEF-4 (lanes 2, 4, 6, and 8) or purified GST (lanes 3, 5, 7, and 9). In lanes 4 and 5, the purified proteins were incubated with 5 μg of anti-GST antibody. In lanes 6 and 7, a 100-fold molar excess of unlabeled Eb probe was added. Bovine serum albumin was used so that the protein content in each lane was equal. The arrows indicate the positions of specific DNA-protein complexes. Each experiment was repeated twice with similar results.

**Fig. 2.** Gel-shift analysis using TEF-4 expressed in COS-7 cells or purified GST-TEF-4 with Eb or mutated Eb (Ebm) probe. A, the end-labeled Eb probe (lanes 1–5) or Ebm probe (lanes 6–8) was incubated with 20 μg of nuclear extract from COS-7 cells transfected with vector control (lanes 2, 4, and 7) or pcTEF-4 (lanes 3, 5, and 8). A 100-fold molar excess of unlabeled probe was used for competition with labeled Eb (lanes 4 and 5). B, the end-labeled Eb probe (lane 1) or Ebm probe (lanes 2 and 3) was incubated with 1 μg of purified GST (lane 2) or purified GST-TEF-4 (lanes 1 and 3). The arrows indicate the positions of specific DNA-protein complexes. Each experiment was repeated twice with similar results.
by TEF-4—To examine whether or not TEF-4 can mediate transcription of the CCTα gene, we prepared various CCTα promoter deletion constructs linked to the luciferase reporter and transfected either COS-7 or 3T3-L1 cells with pcTEF-4 or pcDNA control. The expression of luciferase activity was determined by dual-luciferase assays and normalized for transfection efficiency after cotransfection with pcDNA (0.5 μg) and pRL-CMV (0.001 μg) were transfected into COS-7 cells with pcDNA (0.5 μg) (lane 1, white bar) or pcTEF-4 (0.5 μg) (black bar), or mutated TEF-4, pcmTEA(-) (lane 3, hatched bar) or pcmTEA(+) (lane 4, striped bar). * and ** represent p < 0.05 and p < 0.01, respectively, compared with pcDNA or pcTEF-4 treatment. Values are the means ± S.D. from three independent dishes. Each experiment was repeated three times with similar results.

To substantiate the notion that TEF-4 functionally interacts with the EB region in mammalian cells, we prepared luciferase reporter constructs containing point mutations in the EB region (ATG to GCT) of the LUC.C8 (−201/+38) and LUC.C7 (−1268/+38), named LUC.C8m and LUC.C7m, respectively. We also prepared a luciferase reporter containing point mutations in the EB region (ATG to AGC) of the LUC.C7 (−201/+38), named LUC.C7m2, to which TEF-5 was reported not to bind (36). When COS-7 cells were transfected with these mutated EB constructs the luciferase activity increased above that of the wild-type constructs. Furthermore, TEF-4 expression enhanced the luciferase activity of the mutated constructs relative to that of the wild-type constructs (Fig. 3A). These results suggest that TEF-4 functionally binds to the EB region of CCTα promoter where it acts as a transcriptional repressor. Taken together, deletion and mutation analyses show that TEF-4 can act as both a positive and a negative modulator of the CCTα promoter activity; the two functions combined predominantly produce a stimulatory effect.

To establish which domain(s) of TEF-4 is responsible for its stimulatory activity and binding to the CCTα promoter, we expressed TEF-4 deletion mutants pcmTEA(+) and pcmTEA(−) in COS-7 cells and measured luciferase activity from the LUC.D3 core promoter construct (stimulatory effect) and LUC.C7 (stimulatory and inhibitory effects) construct. The TEF-4 deletion mutant containing only the so-called TEA-DNA binding domain, pcmTEA(+), retained its stimulatory effect on luciferase expression from both LUC.D3 and LUC.C7 promoter-reporter constructs (Fig. 3B, lane 4). A second TEF-4 dele-
ment was repeated three times with similar results. Values are means ± S.D. from three independent dishes. Each experiment was repeated three times with similar results.

These results suggest that the DNA binding domain of TEF-4 is critical and sufficient for its function as a transcriptional modulator of the CCTα promoter which, as demonstrated in Fig. 3A, was predominantly through its effect on basal transcription. On the other hand, the full-length GST-TEF-4 fusion protein containing the TEA domain did not bind to the core promoter region (−90/+38) since this region does not have a consensus binding site for TEF-4 (data not shown). Thus, it appears that TEF-4 does not stimulate the basal activity through direct binding to the core promoter. Instead, we postulate that TEF-4 stimulates the expression of some other transcription factor(s) necessary for the basal CCTα gene transcription and/or directly interacts through its TEA domain with a nuclear protein(s) that is critical for CCTα transcription.

To confirm that the multiple effects of TEF-4 on the CCTα promoter activity are not restricted to COS cells, we performed similar functional assays in 3T3-L1 cells. The results are shown in Fig. 3C. The exogenous TEF-4 expressed in 3T3-L1 cells enhanced the basal activity of the deletion promoter series and of the TEF-4 consensus site mutant LUC8Sm as was the case in COS-7 cells.

TEF-4 Expression Increases CCTα mRNA Abundance—COS-7 cells were transfected with pcDNA or its control vector pcDNA to determine whether or not TEF-4 could also increase the amount of CCTα mRNA by acting on the natural promoter as it did on the promoter-luciferase reporter chimeras. As shown in Fig. 4, TEF-4 increased the CCTα mRNA level about 1.4-fold compared with that in control cells. These results confirm that TEF-4 can modulate the abundance of CCTα transcripts in cultured cells.

DISCUSSION
The Physiological Role of TEF-4 Is Unknown—In the transcription enhancer factor family, TEF-1 was initially purified from HeLa cells, based on its binding affinity for the SV40 enhancer element GT-IIC (45), and then subsequently cloned and characterized (35). TEF-1 consists of 426 amino acids forming four separate structural domains as follows: a DNA-binding domain (TEA/ATTS domain) at its amino terminus, proline-rich and serine-threonine-tyrosine-rich domains in the middle region, and a zinc finger motif at its carboxyl terminus. The TEA region directly interacts with consensus DNA elements, and the proline-rich and zinc finger domains modulate TEF-1 binding to these elements (46). TEF-1 also contains potential phosphorylation sites for calmodulin-, cAMP-, and cGMP-dependent protein kinases (47) that could also modulate its function as a transcription factor.

TEF-4, initially named as the “embryonic TEA domain-containing factor” or ETF, was identified in neural precursor cells by subtraction screening with adult mouse brain mRNA (33). Based on its level and pattern of expression, TEF-4 has been implicated in the developmental regulation of neural (33), kidney (34), and other tissues (34), but its actual physiological role remains unknown. TEF-4 contains 66% sequence identity to TEF-1, mostly in the TEA region, hence other domains may be important for TEF-4-specific activity (33). Two other members of the family, TEF-3 (34) and TEF-5 (36), were identified by PCR amplification using degenerate primers corresponding to the highly conserved TEA domain. TEF factors activate promoters via binding to the consensus sequence (5′-A/T)(A/G)(A/G)(A/TATG)(G/A)-3′ containing a conserved ATG core. Although it was known that nuclear factors could activate viral promoters by binding to GT-IIC and Sp1/SphII enhancer elements (48), until recently the actual targets for TEFs in the mammalian genome were largely unknown. TEF-1 was reported to stimulate transcription of α-myosin heavy chain in myocytes (49) and TEF-5 to regulate the cell-specific expression of chorionic somatomammotropin-B in placenta (36). There were no reports of promoters that could be targeted for regulation by TEF-4. Therefore, this is the first report demonstrating that a mammalian gene, CCTα, can be regulated by TEF-4 through modulation of its basal transcription and through binding to the TEF consensus site within the distal enhancer region.

TEF-4 Has Both Stimulatory and Repressive Actions on the CCTα Promoter—Our initial studies demonstrated that unknown nuclear factors bound to the −103–82, or Eb, region of the mouse CCTα promoter and modulated transcription (25). In the present study, we isolated an Eb-binding nuclear factor, TEF-4, by functional cloning using the yeast one-hybrid system. The sequence alignments suggested that the primary structure of the TEA domain of TEF-4 is indistinguishable from that of TEF-1 (Ref. 34 and data not shown). Indeed, we showed (Fig. 1C) that purified GST-TEF-4 binds to the GT-IIC motif present in the SV40 enhancer as previously shown for TEF-1 (35). Consequently, we might expect that other TEF members, TEF-1, TEF-3, and TEF-5, would also bind to the Eb sequence. However, only TEF-4 cDNA was functionally cloned in the present study suggesting that if the cDNAs for other TEFs were present in the library, the TEA domain was not the sole determinant of TEF-4 DNA binding affinity and function (46). The gel-shift and supershift experiments with yeast and mammalian proteins and purified GST-TEF-4 fusion protein clearly demonstrated that the Eb region within the CCTα promoter represents a true binding site for TEF-4. However, we did not test other members of the TEF family for their binding and regulatory properties. Thus, we do not exclude the possibility that other TEF members may also be relevant for the regulation of expression of CCTα.

Previous reports showed that overexpressed TEF-1 represses the expression of the GT-IIC-containing reporter genes in HeLa cells due to the presence of a limited amount of a putative
coactivator (35, 50). Transfection of TEF-4 failed to induce any obvious effect on the luciferase gene expression from a tetramerized GT-IIC oligonucleotide ligated upstream of the thymidine kinase promoter-reporter (33). However, we report in this paper for the first time that TEF-4 is a functional transcription factor that is able to enhance the activity of the CCTα promoter-luciferase reporters and increase the level of CCTα mRNA in transfected cells. Previous studies have shown that the level of CCTα mRNA is increased upon stimulation of cells by colony-stimulating factor-1 (18), after partial hepatectomy (19), or during tissue growth and differentiation (20). Our data suggest that TEF-4, and perhaps other members of the family, might play a positive regulatory role in transcription of the CCTα gene under these stimulatory conditions.

The present results demonstrate that overexpression of TEF-4 significantly enhances the CCTα promoter activity but that the activation was moderate compared with the vector control. We demonstrated that TEF-4 might play multiple roles in the regulation of the CCTα promoter. We established that TEF-4 stimulates basal transcription since overexpressed TEF-4 increases the activity of the basal promoter construct −52/+38 that lacks the TEF-4 consensus binding site. By preparing mutated TEF-4 proteins, we demonstrated that the TEA-DNA binding domain is important and sufficient for its stimulatory activity. These results suggest that either TEF-4 stimulates transcription of a basic transcription factor(s) or that TEF-4 directly interacts via its TEA domain with pre-existing protein(s) or cofactor(s) required for basal transcription. Recently, it was found that the p160 family of nuclear receptor coactivators, SRC1, TIF2, and RAC3, act as bona fide coactivators of TEF-4 and other members of the TEF family of transcription factors (51). These coactivators are potential targets for the action of TEF-4 on the CCTα promoter.

Unexpectedly, when the TEF-4 binding region Eb within the CCTα promoter was mutated, the overexpressed TEF-4 did not decrease but further enhanced the luciferase activity (Fig 3A). These data suggest that after binding to the Eb region TEF-4 acts as a transcriptional repressor and might explain why transactivation by TEF-4 was attenuated in longer promoter constructs and why the effect of TEF-4 on CCTα transcription was relatively modest. Repression of transcription by TEF proteins is known to occur. The cause for TEF-1 repression has been suggested to be due to titration by the overexpressed TEF-1 of another limiting transcriptional coactivator (35). It is well established that the E-box motif for TEF-1 and the M-CAT motif for Max are both required for expression of the cardiac α-myosin heavy chain gene in rat myocytes (52). Low concentrations of either TEF-1 or Max alone modestly activate expression of the gene and repress the gene at higher concentrations. However, when TEF-1 and Max were cotransfected, a synergistic transactivation of the α-myosin heavy chain gene occurred, which was explained not only by TEF-1 and Max binding to their respective DNA motifs but also by protein-protein interactions between them. In this respect it is noteworthy that TEF-1 repressed the human chorionic somatomammotropin B promoter in BeWo choriocarcinoma cells (53) via direct protein-protein interactions with TATA-binding proteins. However, it was established that another member of the family, TEF-5, specifically binds to several functional motifs in the human chorionic somatomammotropin-B gene enhancer and thus stimulates its activity (36).

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Identification of Transcriptional Enhancer Factor-4 as a Transcriptional Modulator of CTP:Phosphocholine Cytidyltransferase α

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