Transcription of Cystic Fibrosis Transmembrane Conductance Regulator Requires a CCAAT-like Element for both Basal and cAMP-mediated Regulation*

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The cystic fibrosis transmembrane conductance regulator (CFTR) gene in man is controlled by a tightly regulated and weak promoter. The architecture of the CFTR promoter suggests regulatory characteristics that are consistent with the absence of a TATA-like sequence, including the ability to initiate RNA transcription at numerous positions. Detailed investigation of the most proximal region of the human CFTR gene promoter through deletion and mutational analysis reveals that expression is contingent on the conservation of the inverted CCAAT sequence. Basal expression of CFTR transcription and cAMP-mediated transcriptional regulation require the presence of an imperfect and inverted CCAAT sequence. Basal expression of CFTR transcripts was found to be constitutive and weak promoter. The architecture of the transcription factor/cyclic-AMP response element binding protein families poten-
tially contribute to the tight transcriptional control rendered by the CFTR gene promoter.

The gene responsible for cystic fibrosis encodes the cystic fibrosis transmembrane conductance regulator (CFTR) gene product (1, 2). The expression of CFTR is predominantly confined to specific epithelial cell types and is ordinarily expressed in low levels. The low levels and cell type-specific expression of CFTR appears to be dictated, primarily, by genomic sequences 5' upstream of the translational start of CFTR, which correspond to functional promoter sequences (3, 4). The requirements for active and cell type-specific CFTR transcription rely on a narrow band of nucleotide sequences, proximal to the multiple transcript initiation sites (5). The levels of CFTR transcripts in individual cell types appear to be concomitant with the ability of certain epithelial cell types, which vary substantially in response to 1) levels of cAMP (6), 2) the stimulation of protein kinase A and C activities (7–9), and 3) phorbol esters (10) likely through signaling pathways or mechanisms that converge ultimately on gene transcription (for review, see Ref. 11). The mechanisms that modulate CFTR gene expression through extracellular and intracellular signals may implicate activities analogous to that of both the C/EBP and ATF/CREB families of transcription factors (12).

The C/EBP-related family of nuclear transcription factors represent a class of proteins characterized by the ability to bind the CCAAT nucleotide consensus sequence and confer either transcriptional activation or repression on target genes (13–16). All members in the C/EBP class of transcription factors contain a basic region and a leucine zipper (bZIP) domain, which correspond to both DNA binding (17, 18) and protein dimerization potentials (19). The C/EBP family of nuclear proteins are also members of a larger superfamily of transcription factors also characterized by the bZIP motif that includes the ATF/CREB family of transcription factors (18). The ATF/CREB family has been characterized based on the recognition of several proteins to the CAMP-response element (CRE) nucleotide consensus (20–24). It has been previously demonstrated that individual C/EBP proteins characterized thus far can homodimerize or heterodimerize within the C/EBP family of bZIP domain proteins to initiate a specific transcriptional effect (15,

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The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; CRE, cyclic AMP-response element; C/EBP, CCAAT-enhancer binding protein; ATF/CREB, activating transcription factor/cyclic AMP response element binding protein; 8-Br-cAMP, 8-bromo-cAMP; PCR, polymerase chain reaction; bp, base pairs; DOTAP, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; hGH, human growth hormone; EMSA, electrophoretic mobility shift assay(s).
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24) and could elicit transcriptional stimulation or repression in response to CAMP (25). It is now believed that the diverse transcriptional regulation of genes containing the recognition sites of either C/EBP and ATF/CREB may include the heterodimerization between different members in each of the C/EBP and ATF/CREB families (26). This mechanism may be utilized to respond to complex signals and transcriptional cues through single sequence elements including a response to CAMP, despite the absence of active CRE, AP1, and AP2 consensus nucleotide sequences (27, 28) within the gene.

In this report we focused on the regulation of CFTR gene transcription mediated by the region encompassing only 135 nucleotides 5′ upstream of the translational start site in pancreatic carcinoma, PANC1, cells. Through deletion analysis of the proximal region of the CFTR gene 5′-flanking sequences, we have identified a single cis-acting element. This cis-acting element is significant to the expression of CFTR gene transcription and represents the most proximal inverted and an imperfect CCAAT consensus essential for any detectable transcription. Analysis of the transcript termini provide evidence for the direct association of the conserved inverted CCAAT sequence in promoting and positioning transcript initiation. Thus, the core CCAAT element of the CFTR gene 5′-flanking region may reflect a weak initiator nucleotide consensus necessary for the generation of the multiple transcripts for CFTR region may also implicate regulation mediated by interleukin-6 (29).

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diluted in 100 µl of 20 mM Hepes buffer, pH 7.6. This mixture was incubated for 15 min at room temperature. This DOTAP/DNA mixture was then transferred to 1.5 ml of medium. This medium was used to replace the existing medium in the cell cultures and gently rocked, briefly, for even distribution of the DOTAP/DNA mixture. Transfected cells were incubated for 6 h at 37°C in a 5% CO₂ atmosphere. At this time, transfected cells were replaced with fresh medium and cultured for an additional 48 h before assaying the cultures and culture medium for chloramphenicol acetyltransferase activity (32) and human growth hormone (hGH). The levels of human growth hormone secreted into the medium from transfected cells were assayed by hGH enzyme-linked immunosorbent assay (Boehringer Mannheim) according to the manu- facturer’s instructions. The values reported are normalized to the level of chloramphenicol acetyltransferase expression directed by the Rous sarcoma virus long terminal repeat sequences in each experiment. Positive and negative controls were included in each experiment conducted here using plasmid constructs containing the cytomegalovirus promoter fused to the human growth hormone gene and the plasmid pØGH, respectively. Levels of hGH were evaluated relative to the expression of pCMV/hGH.

Stable co-transfections of pancreatic carcinoma cells were carried out using DOTAP, identically, as described above with the constructs, pCF-197/wt/hGH or pCF-197/hGH or the control plasmid pØGH and the selectable marker, pMC1neoA (Stratagene, Inc.) at a molar ratio of 15:1 (reporter gene/selectable marker gene) in PANC1 cells. These co-transfections were carried out in the same manner as above with the addition that cells were plated on 60-mm plates at a lower density of approximately 5 × 10⁶ cells/plate. Following transfection, the culture medium containing the DOTAP/DNA mixture was replaced by the selection medium containing 400 µg/ml Geneticin® (G418) (Life Technologies, Inc.). Stable cell populations were established after 14 days in selectable medium. Genomic DNAs were isolated from individual cell populations, and the integration of the transgenes along with the copy number were then confirmed by Southern blot hybridization analysis using probes spanning the bacterial sequences of the transgenes. Monoclonal populations of stably transfected cells pCF-197/wt/hGH, pCFCF-197, and pØGH were then expanded in 60-mm plates in the presence of selectable medium, and the plasmid pØGH and hGH transgene construct were used for the preparation of cytoplasmic RNA (33).

Ribonuclease Protection Assays—The CFTR/hGH antisenese RNA probe was constructed by generating PCR fragments from the pCF-197/wt/hGH, pCF-197/hGH, and pØGH plasmid constructs and cloning them into the pCR II plasmid (In- virogen Corp.). Individual clones were obtained to represent each of the transgene constructs integrated as stably transfected cell lines. Plasmids were sequenced and used to generate specific (α-3²P]JUTP-labeled 5′sp RNA polymerase transcripts, spanning between position –197 of the CFTR 5′ genomic region to 137 nucleotides downstream of the Basal promoter of the hGH gene. In the control, lacking CFTR genomic sequences, a PCR fragment of 425 bp was generated spanning between the pUC12 backbone of the pØGH vector and the hGH gene (30). The resulting 3²P-labeled RNA transcript incorporates 137 nucleotides of the human growth hormone gene from the vector backbone and 285 and 282 bp of 5′ flanking sequence of the CFTR gene, corresponding to the wild-type and mutant constructs, respectively. Both the 492- and 489-nucleotide (α-3²P]JUTP-labeled 5′sp RNA polymerase transcript was examined by polyacrylamide gel electrophoresis, and the RNA transcript was excised from the gel and purified from the polyacrylamide gel. Cytoplasmic RNA was isolated from stably transfected cell lines both CFTR-197/wt/hGH, pCF-197/hGH, and pØGH plasmid constructs. Cytoplasmic RNAs (50 µg) were denatured and allowed to anneal to the antisense RNA probe (–5 × 10⁶ cpm) for 16 h at 52°C. The RNA mixture was digested with ribonuclease A (40 µg/ml) and T1 (2 µg/ml) for 30 min at 30°C. Digestion of RNA was terminated with the addition of sodium dodecyl sulfate and protease K. RNA was extracted with phenol/chloroform mixture and precipitated. The precipitated fragments of cytoplasmic RNAs were resolved on a 4% polyacrylamide gel.

Preparation of Nuclear Extracts, Electrophoretic Mobility Shift Assays, and DNase Protection Assays—Nuclear extracts from human PANC1 cells were prepared essentially as described previously (34) with modifications to the preparation of nuclear extracts described previously (35). The extracts were frozen under liquid N₂ and stored at −80°C until further use. The protein concentrations of the extracts were determined by UV absorbance. The double-stranded oligonucleo- tide corresponding to sequences of the CFTR inverted CCAAT element 5′-tgagctgtgttggggaacaaggt3′ was synthesized and labeled at the 5′ end by incubating with [γ-3²P]ATP and T4 polynucleotide kinase. The binding reactions were carried out at 4°C for 30 min with 0.1 ng of the 3²P-labeled oligonucleotide, 0.5 µg of nonspecific competitor DNA (poly(dl-dC)) and either 2 or 10 µg of PANC1 nuclear extract in the following buffer conditions: 20 mM Hepes, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 5% (v/v) glycerol. For competition experiments, unlabeled competitor oligonucleotides corresponding to nucleotide sequences of the wild type CFTR inverted CCAAT element, the mutated inverted CCAAT element 5′-tgagctgttggggaacaaggt3′, and C/EBP consensus 5′-tgcagattgcgaatctgca3′ (Santa Cruz Biotechnology, Inc.), the rat albumin promoter (DEI site) 5′-tgcagattgcgaatctgca-3′ (15), and the human c-fos C/EBP target site 5′-tgcagattgcgaatctgca-3′ (36) were added in molar excess to the binding reactions prior to the introduction of oligonucleotides. A control oligonucleotide (5′-gaggctgttggggaatcgtcag3′) of the human hemopexin gene A site (37) was used to determine the effectiveness of polyclonal antibodies specifically directed against C/EBFs. DNA-protein complexes were resolved by electrophoresis on a 5% polyacrylamide/6% bisacrylamide (80:1) in Tris acetate/EDTA buffer at 10 V/cm at 4°C. Antibody supershift assays were performed similarly, that either polyclonal antibody (1 µg) to C/EBPα (Santa Cruz Biotechnology, Inc.) or preimmune serum was added with bovine serum albumin (5 µg) directly to the DNA/nuclear extract mixture and allowed to incubate at 4°C for an additional 30 min. DNA-protein and antibody complexes were resolved on a 5% polyacrylamide gel.

RESULTS

Deletion of a Functional CFTR Gene Promoter Demonstrates Both Basal Transcription and cAMP-mediated Regulation—Characterization of specific cis-acting elements involved in directing transcription have been complicated by the low levels of CFTR gene transcription demonstrated in most epithe- lial cell types (5). Therefore, we decided to test the promoter function of nucleotide sequences 5′ upstream of the CFTR gene by performing transient transfections in PANC1 cells. It is known that expression of a reporter in Panc1 cells, which is a 5′ deletion mutant of the CFTR promoter, was not induced by 8-Br-cAMP. The promoter sequence extending upstream of the open reading frame of the CFTR gene was cloned into the pØGH backbone, and the DNA sequences from the 5′ deletion mutant of the CFTR promoter were then labeled with [3²P]JUTP. These DNA sequences were excised in different molar ratios and used to transfect Panc1 cells. When many other cell lines tested, PANC1 had been shown to support relatively high levels of reporter gene activity when directed by the CFTR promoter in a transfection assay (5). In this study, we utilized the hGH gene as a reporter, taking advantage of its sensitivity to transcriptional regulation and stability of its transcripts (30). A series of deletions of the CFTR gene promoter were constructed by the polymerase chain reaction using end points at selected sites up to 912 nucleotides upstream of the open reading frame. The CFTR gene promoter sequence was cloned into the pØGH plasmid vector, transfected into Panc1 cells, and assayed for hGH expression. Fig. 1 shows that the major elements of transcriptional control of CFTR gene promoter function in the pancreatic cell line are confined to sequences approximately 135 nucleotides from the translational start site. In control cultures (hatched bars) not induced by 8-Br-cAMP, the promoter sequence extending 912 nucleotides from the open reading frame produced significant levels of hGH expression, approximately 5-fold greater than control cells transfected with reporter gene constructs lacking all CFTR gene sequences (pØGH). Subsequent deletion from the 5′ end up to 135 nucleotides from the translational start of CFTR diminished basal transcription activity only by 20%; however, deletion of a further 19 bp (pCF-116/hGH) re-
Evidence of a tagenesis—8-Br-cAMP. That of the pØGH control construct, abolishing the effect of this plasmid exhibited markedly reduced levels of basal expression of the hGH gene, as shown in the schematic diagram. Plasmid constructs were transiently transfected into the cell line PANC1, which was either left untreated or treated with 8-Br-cAMP, and hGH reporter gene expression levels were determined as described under "Materials and Methods." The expression of hGH levels were evaluated and normalized to the level of constitutive expression of chloramphenicol acetyltransferase generated by the internal control plasmid pRSVcat. Values represented in the graph are shown as the mean percent plus the standard error of hGH expression relative to the level of constitutive expression generated by the plasmid pCMV/hGH, indicated at the top of the graph. Numbers refer to the number of nucleotides relative to the translational start site consensus for CFTR when fused to the hGH reporter gene in each of the plasmid constructs represented. Mutation to the wild-type inverted CCAAT sequence (5'-ggaattdggagaataaatt-3') is denoted as the nucleotide sequence ggaattggaatatt. The plasmid hGH construct pØGH is the parental vector, lacking 5'-flanking sequences of the CFTR gene, included in this experiment. The # refers to the number of data points generated for each of the constructs shown.

...duced transcriptional activity to a level 50% lower than that of the negative control, pØGH, which lacked all CFTR nucleotide sequences. This reduction of promoter activity in pCF-116/hGH to a point below the clearly detectable level of hGH expression pØGH likely indicates that cryptic promoter sites exist within the pØGH plasmid backbone or the hGH gene, and that the activity of these sites is suppressed by sequences within the proximal 116 bp of the CFTR promoter. Consistent with this interpretation is the finding that successive deletion of sequences downstream of –116 restored hGH expression to the background levels expressed by pØGH (data not shown); however, we did not perform experiments to substantiate the presence of DNA element corresponding to transcriptional repression.

In addition to supporting basal transcriptional activity, the same CFTR promoter constructs also demonstrate cAMP-stimulated expression of the hGH gene (Fig. 1, black bars). Essentially the same level of activity in the presence of 8-Br-cAMP, representing 3–4-fold stimulation over untreated controls, was seen in cells transfected with promoter constructs extending 135 or more bp upstream of the translational start site. Deletion of sequences upstream –116 bp reduced activity to below that of the pØGH control construct, abolishing the effect of 8-Br-cAMP.

... Functional Inverted CCAAT Element Is Identified by Mutagenesis—Evidence of a cis-acting element residing between a 19-bp sequence between –135 and –116, was demonstrated by the site-directed mutagenesis of this nucleotide sequence. This 19-bp sequence of interest contains a suspected inverted CCAAT element, like those previously implicated in directing constitutive and inducible RNA polymerase II-mediated transcription of several genes (39), either in tandem with or in the absence of a TATAA element (40). We altered the inverted CCAAT sequence 5'-ggaatgagaataaatt-3' present in pCF-197 wild-type construct to a 5'-ggaagaaatt-3' by site-directed mutagenesis to yield the construct pCF-197/hGH. Cells transfected with this plasmid exhibited markedly reduced levels of basal expression of hGH but also abolished the induction of hGH expression by 8-Br-cAMP (Fig. 1). Taken separately, this result supports the contention that conservation of the inverted CCAAT element may be an essential requirement for the basal expression of CFTR gene transcription.

In addition to mediating basal expression of CFTR gene transcription, the inverted CCAAT element was strictly required for cAMP-mediated induction of transcriptional activity. Moreover, the absence of any demonstrable cAMP-mediated activation of CFTR gene transcription via existing sequences homologous to both CRE and AP1 suggest that this inverted CCAAT element is, alone, sufficient for cAMP responsiveness within the CFTR gene (data not shown). Recent data have indicated that the induction of CFTR gene expression was mediated by cAMP likely acting through putative cis elements of the CFTR gene promoter (6). The best studied transcriptional response by cAMP was identified through the analysis of the conventional CRE nucleotide consensus (20). Despite the presence of both CRE and AP1 consensus sites upstream in the CFTR gene 5'-flanking region (3,4), these sequences appeared to lack substantial cis-acting potential within the context of the CFTR gene (5,41). To map the nucleotide sequences responsible for the observed induction of CFTR by cAMP, deletion constructs (Fig. 1) were examined for their ability to support cAMP-mediated induction of CFTR gene transcription. Constructs pCF-912/hGH, pCF-492/hGH, pCF-262/hGH, pCF-197/hGH, and pCF-135/hGH each averaged a stimulation of 3-fold, upon induction with the CAMP analog, 8-Br-cAMP (Fig. 1). This activation was detected following a 12-h treatment of cells with 8-Br-cAMP. A 19-bp deletion downstream of the –135 nucleotide position abolished cAMP induction as indicated by construct pCF-116/hGH (Fig. 1). Mutation to the inverted CCAAT sequence demonstrates the strict requirement of the conserved CCAAT sequence to mediate the induction by 8-Br-cAMP on hGH expression. Construct pCF-197/hGH contains a three-nucleotide deletion in the inverted CCAAT sequence to C–T. The loss in cAMP-mediated regulation directly corresponds to the deletion of three nucleotides in the inverted CCAAT element. In addition, the absence of any CRE consensus indicates the level of CAMP-inducible transcription of CFTR is mediated by the inverted CCAAT element, alone. The CAMP-mediated induction of hGH expression directed by the CFTR gene promoter from construct pCF-197/hGH requires the conservation of the inverted CCAAT element consensus in the CFTR gene promoter. This result is consistent with other examples of CAMP induction mediated by the CCAAT element, including the recent examination of the G-protein1,2 subunit gene promoter (42). Thus, the results indicate here that the potential role of the inverted CCAAT element in the CFTR gene is to mediate both the basal transcription of CFTR and induction by cAMP.

Inverted CCAAT Element of the CFTR Promoter Directs CAMP-mediated Transcription in a Heterologous Gene—The inverted CCAAT element from the 5'-flanking sequences of the CFTR gene demonstrates both transcriptional cis activation and CAMP-mediated transactivation. To test heterologous CAMP-mediated transcription, the thymidine kinase promoter of the herpes simplex virus was fused to the human CFTR gene 5'-flanking sequence. Both wild-type and mutant CFTR templates (pCF-197wt/hGH and pCF-197ΔhGH) were utilized for PCR amplification of DNA to produce a 158- and 155-bp fragment of the CFTR promoter, respectively. The human CFTR gene sequences were then fused upstream of the thymidine kinase promoter in constructs pCF-eGlu1tk/hGH, pCF-eGlu2tk/hGH, and pCF-eGlu3tk/hGH. These constructs were then tested for the ability to confer transcriptional activation...
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The inverted CCAAT sequence directs basal and cAMP-mediated transcription in both orientations. A mutation, identical to that directed in construct pCF-197Δ/hGH, was placed within the context of the thymidine kinase promoter to produce the plasmid p[CF-ccaat][tk/hGH]. Mutation to the inverted CCAAT consensus by deleting three nucleotides demonstrates the requirement of the CCAAT consensus for trans-activation through the thymidine kinase promoter. Deletion to the core CCAAT sequence eliminates any stimulatory activity as reflected by the negative control parental plasmid backbone, ptk/hGH. The induction by 8-Br-cAMP of human growth hormone expression in PANC1 cells transfected with p[CF-197]/tk/hGH and p[CF-ccaat][tk/hGH] indicates a greater than 3- and 4-fold increase, respectively. Comparatively, HeLa S3 cells reflect a much more modest change of approximately 2-fold. Plasmid p[CF-ccaat][tk/hGH] failed to generate any increase in the expression of human growth hormone in either cell line, indicating the role of the inverted CCAAT sequence in either orientation was sufficient to confer the induction by cAMP.

The Inverted CCAAT Region Mediates Transcription from Multiple Start Sites—Previous studies of transcript initiation along the CFTR gene previously revealed several start sites (1, 3, 5), although their precise locations remain to be established. To determine if gene transcription from multiple initiation sites is associated with the inverted CCAAT sequence of the CFTR gene, we used a ribonuclease protection assay to identify the 5′ termini of the transcripts. PANC1 cells were stably co-transfected with individual hGH reporter transgene constructs pCF-197wt/hGH or pCF-197Δ/hGH or the control plasmid pØGH in combination with a selectable gene. Clonal populations of stably transfected cells carrying the hGH transgenes were established and confirmed by genomic DNA hybridization analysis (data not shown), and cytoplasmic RNA was isolated. Individual antisense 32P-labeled RNA probes, were created corresponding to the wild-type and mutant CFTR transgene constructs, pCF-197wt/hGH and pCF-197Δ/hGH, respectively. These probes, spanned the sequences between the CFTR gene promoter and the human growth hormone gene. As shown in Fig. 3, a protection assay of the wild-type CFTR transgene, isolated from two independent cell clones pCF-197wt/hGHpac-A and pCF-197wt/hGHpac-E, yielded several distinct protected fragments within the CFTR 5′ genomic region within a 153-bp sequence upstream of the open reading frame for CFTR. In contrast, mutant transgenes from the two independent clones, pCF-197Δ/hGHpac-G and pCF-197Δ/hGHpac-K, lacking the wild-type inverted CCAAT consensus, failed to produce transcripts from any of those end points. Control assays using native PANC1 cell cytoplasmic RNA (not shown) and PANC1 cells stably transfected with the pØGH parental vector showed no protected transcripts (Fig. 3) as expected. In Fig. 3, qualitative comparisons were made between the pattern of transcript termini rendered by the wild-type and mutant inverted CCAAT element CFTR gene constructs within the region encompassing the 5′-flanking nucleotide sequences of the CFTR gene. The result from this experiment indicates that despite the variable sites of transcript initiation, transcripts may be directed through a weak nucleotide consensus, which is associated with the inverted CCAAT element. The results here may imply a role of the inverted CCAAT element of the CFTR gene in stabilizing a basal transcription mechanism through the cis-acting element.

DNA-binding Proteins Interacting with the Inverted CCAAT Element Region of the CFTR Gene Promoter—To determine if CFTR gene regulation, mediated by the inverted CCAAT element, is due to specific DNA-protein interactions, electrophoretic mobility shift assays (EMSA) were performed. Fig. 4A
shows that nuclear extracts prepared from PANC1 cells bind the oligonucleotide, corresponding to the inverted CCAAT sequence of the CFTR gene promoter. A single major band was seen, as well as two weaker migrating complexes. Formation of the predominant complex and one of the minor complexes was specifically depleted by increasing concentrations of unlabeled oligonucleotide. To identify DNA-binding proteins, competition with unlabeled oligonucleotides corresponding to the cognate sequence of the C/EBP protein consensus was used to confirm the specificity of DNA binding to the 32P-labeled cognate sequence of the inverted CCAAT element of the CFTR gene. In Fig. 4B, oligonucleotides corresponding to several known C/EBP targets were used in a competition EMSA. 10-fold amount of unlabeled oligonucleotide were included in the reactions prior to the addition of the 32P-labeled CFTR inverted CCAAT element oligonucleotide. Oligonucleotide targets for C/EBP binding from the albumin gene, c-fos gene and the dyad symmetric consensus sequence for C/EBP binding (43) (16) (18), respectively, competed for binding. This result occurred despite the relatively modest excess of unlabeled oligonucleotide (10-fold). We suggest that the same or related proteins that bind to the C/EBP elements of these genes could target the CFTR gene promoter. A saturating excess (100-fold) of the oligonucleotide corresponding to a deletion mutant, without the inverted CCAAT element failed to compete for binding to the wild-type inverted CCAAT element of the CFTR gene when compared with the controls shown. Interestingly, nuclear extracts prepared from PANC1 cells treated with cAMP failed to demonstrate induction or banding patterns that differed from untreated PANC1 cells (not shown).

To compare DNA binding affinities between cAMP-induced and basal levels of transcription, directed by the proximal 5′ end of the human CFTR gene, nuclear extracts prepared from PANC1 cells treated with 8-Br-CAMP and untreated cells were used for DNase I protection assays. The footprint visualized in Fig. 5 is indicative of protection of the CFTR gene promoter, is positioned between −146 and −90 upstream of the open reading frame of CFTR, and presents a large region protected from deoxyribonuclease hydrolysis (Fig. 5). Interestingly, this footprint is primarily a characteristic of the extract derived from the untreated control and not from nuclear extracts isolated from cells treated with 8-Br-CAMP. The footprint demonstrated in nuclear extracts from untreated PANC1 cells generates extensive binding, overlapping the inverted CCAAT element of the human CFTR gene. Comparatively, DNA binding affinity is slightly detectable in PANC1 cells stimulated with 8-Br-CAMP (Fig. 5) in accord with the stimulation of reporter gene transcription by cAMP as shown in Figs. 1 and 2. This result is consistent with a DNA-protein complex constitutively bound to CCAAT box sequences previously shown to contribute to the weak and basal levels of human gp91-phox gene transcription through the displacement of more potent transcriptional activators (44). This model accounts for the relative absence of change in the detection of multiple DNA-protein complexes by EMSA from various cell types despite dramatic changes in the level of gene transcription mediated by the CCAAT box (44).
CCAAT Nucleotide Binding Complex—Experiments to identify protein species as part of the DNA-protein complex(es) associated with the inverted CCAAT element of the CFTR gene promoter were performed by an antibody supershift analysis using EMSA. The experiment conducted shown as Fig. 6A used the 32P-labeled oligonucleotide, corresponding to the wild-type sequence of the inverted CCAAT element of the CFTR gene in the presence of nuclear extracts isolated from PANC1 cells. An antibody directed against the related C/EBP protein, C/EBP\(\delta\), was used to identify C/EBP\(\delta\) within the inverted CCAAT-bound complex. The addition of C/EBP\(\delta\)-specific antiserum (1:100 dilution), but not the preimmune serum (lane 6), supershifted a single detectable band shown in the lanes 5 and 7. Using a 5-fold amount of specific competitor, oligonucleotide sequences corresponding to the C/EBP \(\delta\) consensus of 5'-tgcagattgcagcaatctgca-3' readily competed for binding to the inverted CCAAT element of the CFTR gene. As shown in Fig. 6A, lane 7, nuclear extracts prepared from PANC1 cells following the treatment with cAMP demonstrated no apparent increase or change in the amount of C/EBP\(\delta\) in the complex bound to the inverted CCAAT element. Efforts to demonstrate supershifted complexes with antibodies specific for C/EBP\(\alpha\), C/EBP\(\beta\), or CRP1 do not indicate the presence of these proteins in the DNA-protein complex (not shown), although this does not preclude the involvement of other C/EBP family members with the inverted CCAAT element of the CFTR gene promoter. As a comparative control, the antibody directed against C/EBP\(\delta\) was...
tested with PANC1 nuclear extract bound to the cognate oligonucleotide. A serum control (shown in the last lane) used as a polyclonal antibody against the RelB protein (Santa Cruz Biotechnology, Inc.). Arrows indicate position of complexes formed in the absence of antibody.

ATF1 and CREB1 Appear in Complexes Bound to the Inverted CCAAT Element In an effort to understand the regulation of CFTR transcription mediated by cAMP, investigation of the CREB and the ATF families of transcription factors would provide evidence for a role of these factors in response to cAMP-mediated transcription. An EMSA antibody supershift was performed using nuclear extracts prepared from PANC1 cells. Polyclonal antibodies against CREB1, CREB2, ATF1, ATF2, and ATF3 were used to detect the presence of these proteins in protein complexes bound by the CFTR gene inverted CCAAT element. As shown in Fig. 7 bandshifts were detected as the major DNA-protein complex in each of the lanes containing nuclear extract, but only antibodies to CREB1 and ATF1 could supershift the complex in their corresponding lanes. This would indicate the presence of CREB1 and ATF1 in DNA-protein complexes formed within the CFTR gene inverted CCAAT element.

**DISCUSSION**

The expression of CFTR is controlled by different regulatory mechanisms (6, 7, 9, 45-48), suggesting that an array of signals could be required to maintain the activity of the CFTR cAMP-mediated Cl⁻ channel. Intracellular signaling by cAMP is a major component in the modulation of CFTR activity (49). With regard to the processes that modulate the function of CFTR through cyclic AMP-mediated activation of Cl⁻ channels, a complementary model has included the stimulatory effects mediated by cAMP on CFTR gene transcription (6), therefore providing further provocation of epithelial cells to direct the expression of sufficient levels of CFTR through signaling pathways mediated by cAMP. In this report, the CFTR gene 5'-flanking region serves as a model promoter to examine the role of nucleotide sequences proximal to both transcriptional and translational start sites in the complex regulation of CFTR gene transcription. The expression of CFTR gene transcription is consistent with the characteristically weak promoter that utilizes multiple initiation sites for transcription (3, 4). Through deletion and mutagenesis of the 5'-flanking sequences of the CFTR gene, we demonstrate that the basal transcription of CFTR in PANC1 is conserved by using sequences extending only 135 nucleotides upstream of the open reading frame for translation. Experiments were performed and indicate the role of an inverted CCAAT sequence in directing both basal and cAMP-inducible regulation of CFTR. The presence of the cis-element containing both an inverted and imperfect repeat of the canonical CCAAT sequence of ATTTGGGAAGCAAAT within the deleted 19-bp element indicates a possible relationship between the inverted CCAAT element and transcriptional regulatory function. Analysis of the inverted CCAAT element, proximal to many transcript initiation directed by the CFTR gene (3-5), demonstrates that conservation of the inverted CCAAT consensus is essential for maintaining basal levels of reporter gene activity in a transient expression assay. Based on this characterization of the CFTR gene promoter, mutation of nucleotides within the inverted CCAAT sequence virtually abolished the expression of reporter gene activity. Although these studies cannot account for cell type-specific differences in the expression of CFTR gene transcription, this provides some fundamental understanding of the nucleotide requirements for directing CFTR gene transcription.

Despite the absence of nucleotide consensus for CRE, AP1, or AP2 activity within the context our promoter constructs, we have evaluated reporter gene levels of transfected PANC1 cells stimulated with cAMP. The cis-acting element, which apparently directs basal transcription, can also account for the induction of CFTR gene transcription by cAMP in these studies. These unexpected results from our transfection studies performed in this report indicate that cAMP-mediated regulation resides within the nucleotide sequences also directing basal levels of transcription. To address the logical flaw in interpreting the cAMP-mediated regulation conferred by the same nucleotide sequences directing basal expression of CFTR gene transcription, we had constructed heterologous gene promoter constructs using the CFTR gene 5'-flanking element fused to the herpes simplex virus thymidine kinase promoter. Our results suggest that the same sequence elements promoting basal CFTR gene transcription confer cAMP-mediated regulation through the context of a heterologous gene. Although models that demonstrate the ability to direct cAMP-mediated transcription in the absence of conventional cAMP-responsive regulatory elements are relatively few, some recent investigations implicate the activity of the CCAAT element in directing both basal and cAMP-mediated transcription (50). This was also previously demonstrated with the human G-protein sub gene promoter (42). It has been more recently described that the regulation of the human tryptophan hydroxylase gene transcription requires an inverted CCAAT motif for maintaining basal expression and cAMP-mediated transcription (51). The absence of a CRE, AP1, or AP2 consensus sequence within the region of the CFTR promoter that we have examined here reflects some similarities to these unconventional genetic models for cAMP-mediated regulation. The kinetics of cAMP-mediated transcription in these models (42, 51) bears some similarities to the cAMP induction of CFTR gene expression (6) in relationship to the time required for stimulation to occur (>=6 h). This is in contrast to stimulation mediated by cAMP through a conventional cis-acting CRE, such as that previously demonstrated with the somatostatin gene (52), requiring only minutes for transcriptional stimulation. This would suggest that transcription mediated by cAMP can occur through sev-
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Several different mechanisms, accounting for differences in the kinetics of gene transcription mediated by cAMP. The delayed response to intracellular increases of cAMP to the activation of gene transcription appears to fall into a category of genes possibly requiring de novo protein synthesis (51). This is in contrast to the rapid transcriptional response to intracellular levels of cAMP mediated by the CRE not requiring new protein synthesis (53). We indicate cAMP-responsive transcription within the CFTR gene promoter is likely due to the inverted CCAAT sequence. This result is indicated from co-transfection studies despite the absence of more conventional sequences responsive to cAMP such as the CRE (52), AP1 (54), and AP2 (55) sites within the nucleotide elements studied in this report. Interestingly, the inverted CCAAT element of the CFTR gene was able to stimulate both basal levels as well as cAMP-mediated transcription within the context of a heterologous gene promoter. Although the placement of such cis-acting sequence elements of the CFTR gene within the context of a heterologous gene promoter may demonstrate a misleading effect with regard to actual CFTR promoter function, here we demonstrate the ability of the inverted CCAAT element to only confer cAMP-responsive transcriptional control.

To address the relevance of the inverted CCAAT sequence in directing the transcriptional machinery to the CFTR gene, stably transfected cells were obtained using two independent CFTR promoter transgene constructs. RNA isolated from the transgenic cells, containing wild-type and mutant inverted CCAAT sequence fused to reporter sequences, were analyzed by ribonuclease protection mapping. Results from the ribonuclease protection assay indicate the direct association of the conserved inverted CCAAT sequence with the positioning and pattern of the transcription start sites, using antisense probes specific for each of the constructs tested shown in Fig. 3, thus providing evidence for the involvement of an inverted CCAAT nucleotide consensus in directing basal transcription. Although many examples have alluded to the role of CCAAT sequences in directing RNA polymerase II-dependent transcription (40), there are no apparent studies that demonstrate a direct role for a CCAAT consensus nucleotide sequence as a component of the basal transcription apparatus. One explanation for the lack of evidence is due to the absence of example promoters and cell types that could represent a model environment demonstrating both basal and inducible transcription involving a CCAAT nucleotide consensus, exclusively. Another explanation may involve multiple and complex levels of regulation directed at specific CCAAT elements, making the evaluation between basal and inducible regulation difficult to interpret. Due to the relatively weak promoter function of the CFTR gene, this may well represent a novel feature of transcriptional regulation characteristic of the CFTR gene. In addition, specific 32p-labeled RNA antisense probes were created to span specifically between the CFTR promoter and the human growth hormone gene. Therefore, the qualitative evaluation of the protected fragments was restricted to transcripts generated only from the CFTR gene promoter fragment from the window of nucleotides depicted in Fig. 3. A study by Koh et al. (5) indicated that the presence of an additional putative exon upstream of the CFTR promoter, referred to as exon 1a, may utilize alternative or cryptic starts in transcription. Although this may represent an additional level of regulation for CFTR gene expression, we have no indication of this yet in the cell type we have studied in this report (data not shown). Through the context of the promoter sequences examined, we indicate the possible significance of the inverted CCAAT sequence in basal regulation of CFTR gene transcription. This would indicate that the conservation of the inverted CCAAT sequence is associated with the transcriptional start site selection and represents a sequence requirement for basal transcription of the CFTR gene.

Studies to compare the affinity of DNA binding overlapping the inverted CCAAT sequence between cAMP-mediated and basal transcription indicate extensive protection of DNase hydrolysis in untreated cells when compared with cAMP-stimulated cells. Contrary to our own expectations, extensive protection of the nucleotide sequences overlapping the inverted CCAAT element of the human CFTR gene was more characteristic of basal expression and not of cAMP-stimulated expression in the PANC1 cell type. Although EMSA experiments performed here do not yet account for some form of displacement of transcriptional activation through binding to the inverted CCAAT element, one explanation may be due to the sensitivity of the DNase protection assay to detect subtle changes in DNA binding affinities. Arguably, the constitutive binding of a repressor to the CCAAT element, which may act to displace transcriptional activation as shown previously (44), could function in tandem with other nuclear protein(s) to allow some access of more potent trans-activators to the CCAAT sequence. Thus, this mechanism may allow more or less activation of CFTR gene transcription through the competition between positive and negative effectors of CFTR gene transcription. The relief of repressor activity by other factors may represent a plausible mechanism for activation of CFTR gene transcription, but this hypothesis has not yet been experimentally tested.

Gel shift and competition analysis of the CCAAT element indicate that in fact CCAAT binding protein(s) are responsible as least in part for the interaction with the CFTR gene-inverted CCAAT nucleotide consensus. Interestingly, competition with nucleotide consensus elements corresponding to both the albumin and c-fos gene CCAAT sequences (56) (36), respectively, readily competed for the CFTR gene-inverted CCAAT element, suggesting that specific CCAAT enhancer binding proteins may target the CFTR gene promoter. A further attempt to characterize such interaction by specific gene products was performed by antibody supershift analysis to immunologically characterize the C/EBP species from PANC1 cell nuclear protein bound to the inverted CCAAT element of the CFTR gene. Results of experiments shown in Fig. 6 indicate the presence of C/EBPβ in nuclear complexes bound to the CFTR-inverted CCAAT sequence. Due to the involvement of C/EBPβ (NF-IL6p) in a response to cytokine-mediated inflammation by interleukin-6 (57, 37), we cautiously speculate the involvement of interleukin-6 in a signaling pathway in regulating CFTR gene transcription.

Immunological detection of CREB1 and ATF1 associated with protein complexes bound to the inverted CCAAT element of the CFTR gene promoter (Fig. 7) may indicate another level of regulation associated with cAMP-responsive transcription factors despite the absence of a CRE nucleotide consensus within the sequences examined in this report. Although the absence of a CRE certainly may not preclude CREB or ATF proteins from targeting promoters devoid of such cis-acting elements, a recent example by Vallejo et al. (58) demonstrates that regulation of the somatostatin gene requires protein-protein interaction between both C/EBP and ATF/CREB factors to elicit a response by cAMP through the somatostatin gene CRE. Inversely, C/EBP proteins have been shown to bind, specifically, to the phosphoenolpyruvate carboxykinase gene CRE with high affinity to promote cAMP-mediated transcriptional activation (59). With regard to the CFTR gene, this paradox may be accounted for by a reversed mechanism, suggesting the inverted CCAAT element as the cis-acting target directing multiple protein interactions between members of the
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C/EBP and ATF/CREB families of transcription factors. In addition, studies by Roessler et al. (60) demonstrate C/EBP as an effector of cAMP-mediated transcription through the combined interactions with liver-specific transcription factors to modulate the CAMP regulation of phospholipase C activity. The models described here may represent complex features analogous to CFTR gene regulation. Therefore, through the identification of trans-acting factors responsible for directing CFTR gene transcription, we can begin to elucidate the mechanisms regulating CFTR gene expression that may help us better understand the pathology associated with cystic fibrosis.

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