

Intronic enhancers coordinate epithelial-specific looping of the active *CFTR* locus

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The regulated expression of large human genes can depend on long-range interactions to establish appropriate three-dimensional structures across the locus. The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encompasses 189 kb of genomic DNA, shows a complex pattern of expression with both spatial and temporal regulation. The flanking loci, *ASZ1* and *CTTNBP2*, show very different tissue-specific expression. The mechanisms governing control of *CFTR* expression remain poorly understood, although they are known to involve intronic regulatory elements. Here, we show a complex looped structure of the *CFTR* locus in cells that express the gene, which is absent from cells in which the gene is inactive. By using chromatin conformation capture (3C) with a bait probe at the *CFTR* promoter, we demonstrate close interaction of this region with sequences in the middle of the gene about 100 kb from the promoter and with regions 3' to the locus that are about 200 kb away. We show that these interacting regions correspond to prominent DNase I hypersensitive sites within the locus. Moreover, these sequences act cooperatively in reporter gene constructs and recruit proteins that modify chromatin structure. The model for *CFTR* gene expression that is revealed by our data provides a paradigm for other large genes with multiple regulatory elements lying within both introns and intergenic regions. We anticipate that these observations will enable original approaches to designing regulated transgenes for tissue-specific gene therapy protocols.

cis-acting elements | enhancer:promoter interactions | regulation of expression | cystic fibrosis transmembrane conductance regulator

Understanding the three-dimensional organization of individual loci within the human genome and how this relates to the regulation of gene expression is the focus of intense study. Many new technologies are being developed to locate and classify the functional elements of the human genome (1). These elements may contribute to the transcription of ubiquitously expressed genes and are also likely responsible for regulating genes with expression that is temporally and spatially controlled. *Cis*-acting regulatory elements located within noncoding regions of genomic DNA can influence the organization of chromosomes and the transcriptional activity of genes. These *cis* sequences include distal enhancers that may reside large distances from the gene promoters they control. Variations in these enhancer/promoter interactions and the nuclear localization of the genes they regulate are thought to be contributing factors in the diversity of transcriptional profiles between different cell types. Moreover, they are important in adjusting these profiles throughout cellular differentiation and development (2–7). These long-range associations are facilitated by the looping of chromatin, whereby regulatory elements come together with requisite nuclear factors to function within “transcriptional hubs” where transcriptional activity is coordinated (8).

Here, we present evidence that the cystic fibrosis transmembrane conductance regulator (*CFTR*) locus adopts a looped

conformation to facilitate expression. The *CFTR* gene encompasses 189 kb at human chromosome 7q31.2 and mutations within it cause the common genetic disease cystic fibrosis (CF) (9). *CFTR* encodes a membrane-associated chloride ion channel that is expressed at the highest levels in chloride-secreting epithelial cells of the small intestine, pancreas, and male genital duct, and at lower levels in the respiratory epithelium and certain other sites (10–14). The *CFTR* promoter has been characterized as a “housekeeping-like” promoter and does not possess the regulatory elements responsible for the diverse expression profile of the gene (15–17). Thus, it is likely that elements outside the basal promoter region contribute to its diverse expression profile. We previously used classical methods of chromatin analysis to map potential regulatory elements in a number of cell lines that express the *CFTR* gene and identified several functionally important elements (18–23). In this study we aimed to use recent technologies to comprehensively map potential regulatory elements of the *CFTR* locus and to establish their mechanism of action. Moreover, we used a number of human primary cell types relevant to CF pathology, in addition to evaluating pertinent cell lines.

Using high-resolution tiled microarrays, we detected multiple intronic and extragenic DNase I hypersensitive sites (DHS), regions of open chromatin that are depleted of nucleosomes and are often associated with gene regulatory elements (24). We demonstrate that several of these DHS regions bind both tissue-specific and general transcription factors and also possess cooperative enhancer activity *in vitro*. Moreover, we show that *in vivo*, these enhancers interact directly with the *CFTR* promoter region. The recent advances in methodology to evaluate regulatory elements in the human genome *in vivo*, combined with a biological approach to the expression and function of *CFTR*, have enabled us to perform an in-depth study of the organization of the entire *CFTR* locus. We demonstrate the properties of key regulatory elements for *CFTR* and show a transcriptionally active human gene adopting distinct conformations in different cell types.

Results

Detection of DNase I Hypersensitive Sites Across the *CFTR* Locus. We used DNase-chip to identify DHS in a number of cell types

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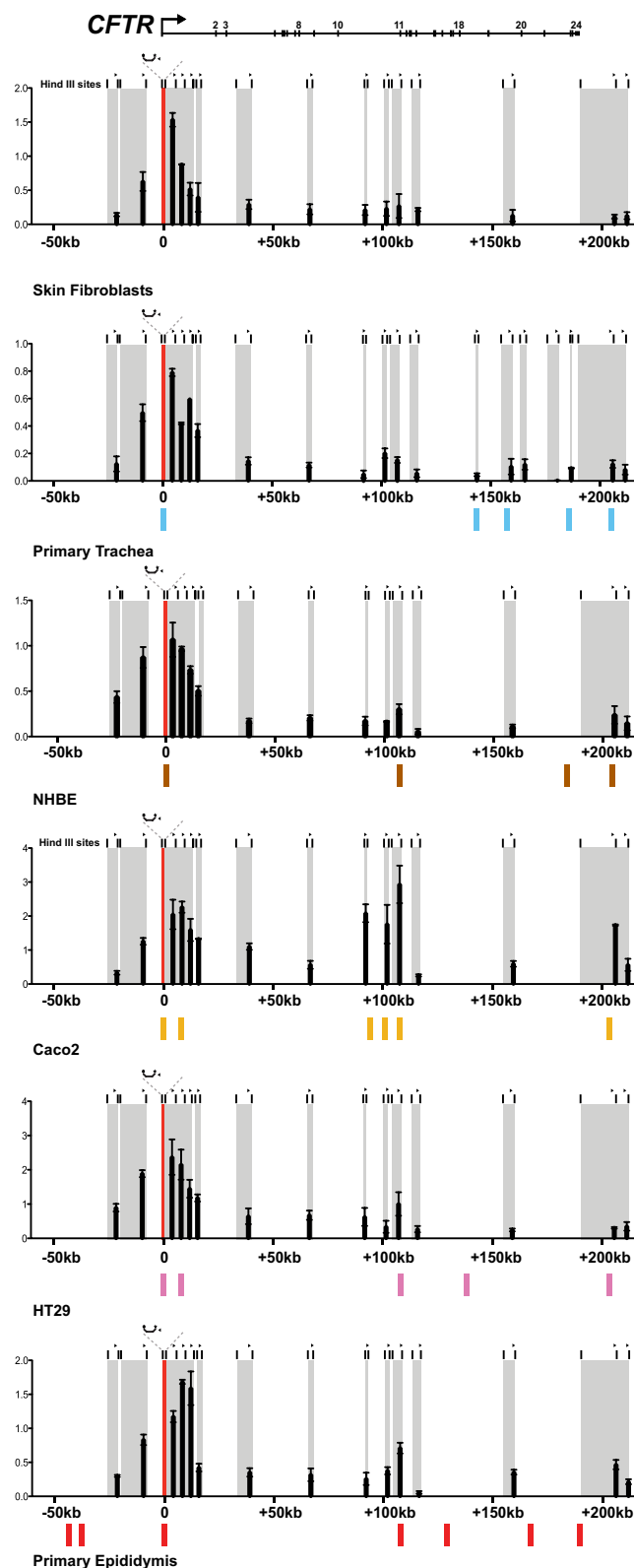


Fig. 2. Long-range interactions between the *CFTR* promoter and specific DHS measured with q3C. The organization of the *CFTR* locus is displayed above the graphs. Each assayed HindIII fragment is represented by a gray bar, with the restriction sites and primer locations along the top of each graph. The red region represents the bait region of the *CFTR* promoter, which includes a primer and Taqman probe adjacent to the 5' HindIII site; this HindIII fragment spans the identified *CFTR* transcriptional start sites. The x axis in each graph represents the position relative to the translational start site; the y axis

directly whether elements within these regions were physically associated with the *CFTR* promoter region.

In skin fibroblasts, no significant interactions were detected between the promoter and any other region of the locus. These cells show a characteristic pattern of interaction frequency that gradually decreases for fragments at increasing distances from the bait region, signifying a decrease in random ligation events. In contrast, in the intestinal cell types (Caco2 and HT29), the primary epididymis cells, and the NHBE cells, we detected interactions between the promoter and HindIII fragments close to and spanning the intron 11 DHS and regions 3' to the gene. These associations were strongest in Caco2 cells in which the three DHS in intron 10 also demonstrated a high interaction frequency with the promoter. The DHS in intron 10 may be directly interacting with the promoter or exhibiting a bystander effect because of the stronger association of the intron 11 DHS with the promoter, as these DHS map within about 17 kb of each other. The interactions were moderate in HT29, primary epididymis, and NHBE cells. Several of these interactions were weakly evident in the primary tracheal epithelial cells, which display a different pattern of DHS. Although other intronic DHS exist in these cells, none were found to interact with the promoter region.

An Element Within *CFTR* Intron 11 Is a Strong Transcriptional Enhancer.

Our DNase-chip analysis revealed a strong DHS within *CFTR* intron 11 at 1811 + 0.8 kb in several cell types. Because we previously demonstrated enhancer function associated with several intronic DHS in the *CFTR* gene (19, 23, 26, 27), we next tested whether the intron 11 DHS exhibited similar properties. The entire region flanking this site was cloned, in forward and reverse orientations, into the enhancer site of a luciferase reporter vector (23) in which 787 bp of the *CFTR* minimal promoter drives luciferase expression. These constructs were evaluated for firefly luciferase expression following transient transfection into Caco2 cells (Fig. 3A). Positive controls included the intron 1 DHS region enhancer in both orientations and negative controls were provided by the intron 10a,b DHS that we demonstrated previously to have no enhancer activity (23). The fragment encompassing the DHS in intron 11 enhanced *CFTR* promoter activity almost 20-fold in the forward and reverse orientation. The intron 1 enhancer (27) acts as a modest enhancer with about fivefold effect on the *CFTR* promoter, while the +15.6 kb DHS region does not show enhancer activity.

We next assayed whether these intronic elements act cooperatively to influence the *CFTR* promoter. To achieve this, constructs were built in which combinations of two or three DHS regions were cloned into the enhancer site of the vector in linear order equivalent to their locations with the *CFTR* gene. The intron 10a,b DHS region had no effect on luciferase activity when combined with the other enhancer elements. However, in all other cases the combination of two or three DHS elements in the same construct had a cooperative effect on the *CFTR* promoter (Fig. 3B). The combination of the intron 1 DHS element with the intron 11 DHS enhancer doubled the *CFTR* promoter activity in comparison to the intron 11 element alone. Similarly, the combination of the intron 1 DHS element with the +15.6 kb DHS element had a cooperative effect on luciferase expression, as did joining the intron 11 DHS element with the +15.6 kb DHS fragment, although alone the latter element does

represents the interaction frequency relative to the interaction frequency between two HindIII fragments within the ubiquitously expressed *ERCC3* gene. Below each graph the major DHS of each cell type (see Fig. 1) are represented by colored bars. Data for each cell type are from a single representative 3C experiment (each experiment was performed at least twice). Error bars represent SEM of at least two PCR reactions for each fragment.

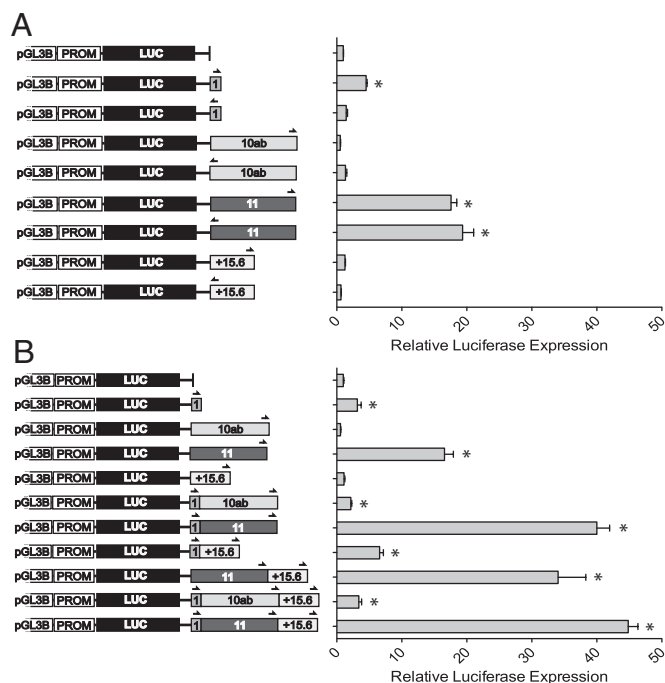


Fig. 3. Intronic enhancers act cooperatively to augment the *CFTR* basal promoter. (A) Caco2 cells were transfected with pGL3B luciferase-reporter constructs containing the 787-bp *CFTR* basal promoter and fragments of the DHS regions found in introns 1, 10a,b, 11, or +15.6 cloned into enhancer site of the vector in either forward or reverse orientations. (B) Caco2 cells were transfected with the single enhancer luciferase reporter constructs (as shown in A), in addition to constructs containing multiple combinations of two or three DHS regions cloned into the enhancer site. For both (A) and (B) data are shown relative to the *CFTR* basal promoter-alone vector; error bars represent SEM ($n = 6$). *, $P < 0.01$ in comparison to the *CFTR* promoter-only vector, by using unpaired t tests.

not act as an enhancer. Maximal *CFTR* promoter activity was seen when all three elements, the enhancers in introns 1 and 11 and the +15.6 kb element, were combined in the same construct. In contrast, when the middle fragment in the enhancer site was the intron 10a,b DHS, this additional enhancement was not evident and luciferase expression was similar to the constructs containing only the intron 1 DHS element. These data demonstrate that intronic enhancers within the *CFTR* locus act cooperatively to augment promoter activity in intestinal epithelial cells.

In Vivo Association of HNF1 and p300 with *CFTR* Regulatory Elements.

The mechanism of interaction between these intronic enhancer sequences and the *CFTR* promoter is of significant interest and is likely to involve multiple protein complexes, including tissue-specific transcription factors and general factors, including chromatin remodeling enzymes and proteins associated with the nuclear scaffold (27, 28, 30, 31). We previously showed that hepatocyte nuclear factor 1 (HNF1) binds directly to the core element within the intron 1 DHS and is necessary for enhancer activity (27). To provide further evidence for the close interaction between the intronic enhancer elements, we performed ChIP experiments with formaldehyde-crosslinked Caco2 chromatin and an HNF1-specific antibody to determine enrichment of enhancer regions (Fig. 4). We observed strongest enrichment at the intron 1 DHS enhancer and modest but significant enrichment at each of the additional intronic DHS observed in Caco2 cells, which suggests either direct or indirect association with HNF1. These data were confirmed by ChIP-chip analysis. We also performed ChIP experiments with an antibody for the

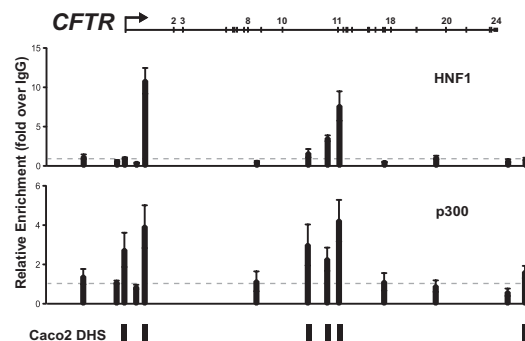


Fig. 4. HNF1 and p300 associate with multiple DHS of the *CFTR* locus in Caco2 cells. Real-time PCR analysis of Caco2 chromatin immunoprecipitated with indicated antibodies at various locations along the locus. Each value shown is relative to enrichment measured with isotype-matched IgG control (dotted line). Data for each antibody are from a single representative ChIP experiment (each experiment performed at least twice). All data points were calculated as percentage of input material and then normalized to background 18s rRNA levels; error bars represent SEM of at least two PCR reactions for each fragment. Black bars below graphs represent locations of Caco2 DHS (see Fig. 1).

histone acetyltransferase p300, a chromatin-associated enzyme found at transcriptionally active elements (32). p300 was significantly enriched only at each Caco2 intronic DHS, the +15.6 kb DHS, and the *CFTR* promoter. Thus, in Caco2 cells the direct interaction between the intronic DHS and *CFTR* promoter involves a protein scaffold that includes the tissue-specific transcription factor HNF1 and the general transcriptional activator p300 to coordinate the active *CFTR* locus.

Discussion

Elucidating the regulatory mechanisms for the *CFTR* gene has been a significant challenge, in part because of the large size of the gene, the lack of tissue-specific control elements in the promoter, and the paucity of relevant cell types for analysis. Here we demonstrate that the active *CFTR* locus exists in a complex conformation involving the direct interaction of several intronic enhancers with the promoter. These interactions are most evident in epithelial cells of intestinal lineage where *CFTR* is most highly expressed; however, they are also apparent in epithelial cells derived from the respiratory system, which generally express *CFTR* at lower levels. The very low levels of *CFTR* expression observed in airway cell types (see Fig. 1B) may be achieved primarily via promoter-mediated mechanisms or by inhibition of promoter-intronic enhancer contacts. In contrast, intestinal and genital duct cells appear to require the contribution of the intronic enhancers to maintain high *CFTR* expression. These promoter-enhancer interactions do not occur in skin fibroblasts, which lack *cis* regulatory elements detectable by DHS mapping in the *CFTR* locus. Other human nonepithelial cell types have been assayed for DHS using DNase-chip, including GM06990 (B lymphoblastoid), primary H9 (undifferentiated embryonic stem cells), CD4⁺ T cells, and K562 (erythroleukemia) (33) (data available at <http://genome.ucsc.edu/ENCODE>). None of these cell types exhibit the DHS within *CFTR* introns 1, 11, or at +15.6 kb that we have detected in the various epithelial cell types used in this study.

The existence of an epithelial-specific looped conformation for *CFTR* is consistent with observations in other mammalian genes and gene loci that achieve tightly regulated transcriptional activation via direct interactions of regulatory elements, including the T-helper 2 (T_H2) cytokine locus, the α - and β -globin loci, and others (2–6). A unique aspect of the *CFTR* locus is that key interacting elements lie within introns, in addition to those distal to the gene. In this context, our model for the active *CFTR* locus

predicts that the interaction of multiple enhancers and both tissue-specific and general transcription factors confers the complex regulation of *CFTR* expression. This is consistent with our previous data, in which removal of the intron 1 DHS region from a human *CFTR* YAC resulted in a 60% decrease in *CFTR* expression levels in a human intestinal cell line and in the intestine (but not the lungs) of transgenic mice carrying the YAC (27). We would predict that removal of multiple enhancer sites and the promoter would be required to completely extinguish *CFTR* expression in any epithelial cell type. We demonstrate here that one of the tissue-specific transcription factors interacting with the enhancers in intestinal cells is HNF1, which we have previously shown to be involved in *CFTR* expression both in vitro and in vivo through interactions at multiple intronic sites (27, 28). HNF1 has been implicated in a similar mechanism of transcriptional regulation in the human *ADH* gene, where it binds a distal enhancer and augments gene expression (34). We also show that one of the general transcription factors involved in the *CFTR* transcriptional hub is the widely expressed factor p300. This protein has histone acetyl transferase activity and is known to be a marker of transcriptionally active elements, including promoters and enhancers (32, 35). In a recent study of p300-enriched sites using tiled microarrays, Heintzman et al (36) also determined several regions close to the intron 11 enhancer to be associated with p300 binding in several *CFTR*-expressing cell types, corroborating our data from Caco2 cells. The presence of p300 in the *CFTR* transcriptional unit is consistent with our previous work, demonstrating increased histone acetylation at certain regulatory elements within the locus (30).

The major advances in our understanding of regulatory mechanisms for the *CFTR* gene that arise from this work were enabled by the application of relatively recent technologies, including DNase-chip and 3C. A comparison of the power of these techniques with classic methods is warranted, particularly with respect to DHS mapping. We previously used a Southern blot-based method to screen for DHS across 400 kb spanning the *CFTR* locus in a number of cell lines, and identified a number of important regulatory elements within the locus (18–23). While our experiments using DNase-chip were able to reproducibly and reliably detect DHS of the locus that functionally associate with the *CFTR* promoter, we were unable to detect several DHS that we observed in earlier studies. These include the site at –20.9 kb from the *CFTR* translational start site (18, 21) that was evident in many cell types and in human YAC-transgenic mice (25), as well as a cluster of four sites +6.8 to +7.4 kb distal to the translational stop site of *CFTR* (20). The –20.9 kb and +6.8 kb sites both possess enhancer-blocking insulator activity and associate with CCCTC-binding factor (CTCF) (30, 31). The lack of detection of the –20.9 kb and +6.8 kb DHS in the current studies is likely because of their locations within or adjacent to repetitive regions, which are excluded from the tiled arrays; alternatively, the classic method of DHS mapping may be more sensitive than DNase-chip. Cell lines may also have evolved in culture and this process was accompanied by changes in the chromatin structure. Many groups have noted the loss of *CFTR* expression with prolonged culture of epithelial cell lines, which would be consistent with gradual changes in chromatin structure and modification. This phenomenon reinforces our approach of using primary human cell cultures in the definition of regulatory elements for *CFTR*.

The potential involvement in *CFTR* transcription of DHS flanking the *CFTR* gene warrants further comment, particularly because several sites bind CTCF and may contribute to the chromatin structure of the active locus. In primary genital duct cells that exhibit the +6.8 kb DHS, corresponding to a functional CTCF-binding site (31), this region may mark the end of the active domain. In contrast, other cells (such as Caco2) that lack the +6.8 kb DHS and show very low occupancy of CTCF in this

region may recruit the +15.6 kb DHS region and the end of the looped domain could then be provided by a more distal CTCF binding site at (+48.9 kb:116950000). The precise definition of elements at the 3' end of the locus that interact with the *CFTR* promoter cannot be provided by the 3C data presented here, as one HindIII fragment encompasses both the 6.8 kb region and the +15.6 kb DHS. In further experiments using 3C libraries generated with EcoRI (Fig. S1), which cleaves between +6.8 and +15.6 (at +11.2 kb) and also 3' to the +15.6 kb DHS (at +17.2 kb and 20.1 kb), all these fragments interact with the *CFTR* promoter bait, demonstrating their close proximity in the active locus and suggesting that they could potentially contribute to a transcriptional hub. Consistent with these data are the observed binding of Rad21 (a subunit of the cohesin complex known to interact with CTCF on chromatin) at the –20.9 kb, +6.8 kb, and +48.9 kb sites (30, 31) (Fig. S2).

Some previously undetected DHS were seen in the *CFTR* gene, both in cell lines that had been evaluated previously by classic methods and in primary tracheal epithelial cells that were investigated here. The most interesting new DHS lies in intron 11. This enhancer lies almost exactly at the midpoint of the locus, and its association with the promoter results in the formation of two ≈100 kb chromosome loops. We are currently determining the molecular basis of the enhancer activity in the intron 11 DHS element and additional transcription factors that directly bind to this region. We previously demonstrated that HNF1 binds directly to the tissue-specific enhancer in the intron 1 DHS (27), and this factor is also a strong candidate for involvement at the intron 11 DHS element, as the ≈1.5 kb region contains a number of predicted binding sites for HNF1 and is enriched by ChIP with an antibody specific for this factor. Possibly in certain cell types, HNF1 recruits and coordinates the other nuclear factors necessary for *CFTR* transcription. In the current series of experiments we concentrated on the analysis of elements associated with DHS that show enhancer activity and that interact directly with the *CFTR* promoter. However, a number of other DHS were identified in each of the cell types that do not encompass such elements. We previously showed that some of these sites had modest enhancer activity (23), although other sites appear to be associated with cell type-specific *CFTR* expression by alternative mechanisms, as yet unidentified. It is also possible that some of these DHS may contain regulatory elements for other genes, particularly because many of the sites do not show a direct correlation with *CFTR* expression. The genes that flank *CFTR*, *ASZ1* on the 5' side, and *CTTBP2* on the 3' side, have different expression profiles than *CFTR*. Moreover, while the present study focused on regulatory elements detected within the *CFTR* locus itself, it is possible that other *cis* or *trans* elements outside this region also coordinate regulation of the gene.

The diversity in DNase-chip data generated from the airway-derived cell types is of interest and may suggest different regulatory mechanisms in these cells. The NHBE cells, which are a mixture of bronchial and tracheal epithelial cells, provide evidence for similar *CFTR* regulatory mechanisms to those of intestinal epithelial cells. In contrast, the pHTE cultures of tracheal cells alone exhibit a different set of DHS and, moreover, show only slight evidence for intralocus looping. We are currently investigating each of these lung cell types further to dissect their *CFTR* regulatory pathways.

We anticipate that understanding the complex mechanisms by which large, disease-associated genes maintain their transcriptional activity in vivo will provide previously unexplored avenues for therapy development. Endogenous enhancers, such as those identified here, may be incorporated into next-generation gene therapy vectors to provide stable expression, at appropriate levels, in the relevant cell types. Moreover, identification of the specific transcription factors acting upon genes such as *CFTR* will begin to reveal the cellular signaling

pathways involved in regulating transcript levels, which might also be targeted for therapeutic benefit.

Materials and Methods

Cell Culture. The human colon carcinoma cell lines Caco2 and HT29, primary skin fibroblasts (ATCC# GM08333), were grown by standard methods. Primary human fetal male epididymis cells and primary tracheal epithelial cells were cultured as previously described (10, 37). NHBE cells were a mixture of primary human bronchial and tracheal epithelial cells (Lonza, CC-2541).

DNase-Chip. DNase-chip was performed as previously described (24), with modifications (27), and experiment-analyzed with ACME statistical software (38). Data are publicly available at <http://www.ncbi.nlm.nih.gov/geo/GSE18735>.

qRT-PCR. *CFTR* expression was assayed as described previously using a Taqman primer/probe set spanning *CFTR* exons 5 and 6 (TAQEX5/6) (28).

Quantitative Chromosome Conformation Capture (q3C). 3C was performed as described previously (29), with minor modifications (31).

Transient Promoter/Enhancer Reporter Assays. Sequences encompassing the DHS in introns 10a,b, 11, and at +15.6, were amplified by PCR using *Pfu* DNA polymerase (Stratagene). Primers are shown in Table S2. Reporter assays were performed by standard methods using a reporter gene construct driven by the 787 bp *CFTR* minimal promoter (19, 23).

Chromatin Immunoprecipitation. ChIP was performed as described previously (27). Immunoprecipitations were performed with antibodies specific for HNF1 (Santa Cruz, sc-8986), p300 (Santa Cruz, sc-585X), RAD21 Abcam (ab992-50), or IgG (Santa Cruz, sc-2027).

Primer Sequences. All primer sequences and locations used for RT-PCR, plasmid cloning, 3C, and ChIP are listed in the Table S2.

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