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Novel regulatory mechanisms for the *CFTR* gene

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Abstract

The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene, which when mutated causes cystic fibrosis, encompasses nearly 200 kb of genomic DNA at chromosome 7q31.2. It is flanked by two genes *ASZ1* [ankyrin repeat, SAM (sterile α -motif) and basic leucine zipper] and *CTTNBP2* (cortactin-binding protein 2), which have very different expression profiles. *CFTR* is expressed primarily in specialized epithelial cells, whereas *ASZ1* is transcribed exclusively in the testis and ovary, and *CTTNBP2* is highly expressed in the brain, kidney and pancreas, with lower levels of expression in other tissues. Despite its highly regulated pattern of expression, the promoter of the *CFTR* gene apparently lacks the necessary elements to achieve this. We previously suggested that *cis*-acting regulatory elements elsewhere in the locus, both flanking the gene and within introns, were required to co-ordinate regulated, tissue-specific expression of *CFTR*. We identified a number of crucial elements, including enhancer-blocking insulators flanking the locus, intronic tissue-specific enhancers and also characterized some of the interacting proteins. We recently employed a high-resolution method of mapping DHS (DNase I-hypersensitive sites) using tiled microarrays. DHS are often associated with regulatory elements and use of this technique generated cell-specific profiles of potential regulatory sequences in primary cells and cell lines. We characterized a set of *cis*-acting elements within the *CFTR* locus and demonstrated direct physical interaction between them and the *CFTR* promoter, by chromosome conformation capture (3C). These results provide the first insight into the three-dimensional structure of the active *CFTR* gene.

Keywords

ASZ1; chromatin immunoprecipitation (ChIP); chromosome conformation capture (3C); cortactin-binding protein 2 (CTTNBP2); cystic fibrosis transmembrane conductance regulator (CFTR); DNase I-hypersensitive site (DHS)

Introduction

The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene encompasses 189 kb at chromosome 7q31.2 [1]. *CFTR* is flanked on the 5' side by *ASZ1* [ankyrin repeat, SAM (sterile α -motif) and basic leucine zipper] and 3' by *CTTNBP2* (cortactin-binding protein 2). These neighbouring genes have very different expression profiles: *CFTR* is expressed primarily, although not exclusively, in specialized epithelial cells [2–4], whereas *ASZ1* is transcribed solely in the testis and ovary [5], and *CTTNBP2* is highly expressed in the brain, kidney and pancreas, with lower levels of expression in other tissues [6].

CFTR exhibits tightly regulated expression, both temporally during development and spatially in different tissue types [2,7,8]. However, the *CFTR* promoter resembles that of a housekeeping

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gene, in that it is CpG-rich, contains no TATA box, has multiple transcription start sites and has several putative binding sites for the transcription factor Sp1 (specificity protein 1) [9]. Consistent with promoters of this type, the *CFTR* promoter demonstrates no apparent tissue-specificity, suggesting the involvement of distal regulatory elements in control of *CFTR* expression. It is probable that these elements are associated with DHS (DNase I-hypersensitive sites) within the genomic region encompassing the *CFTR* locus [10–15].

Although 20 years have elapsed since the identification of *CFTR*, the regulatory mechanisms for this gene have not been fully elucidated. The reasons for this slow progress include the paucity of appropriate primary cell types to evaluate *CFTR* expression and technical challenges arising in the global analysis of large genes that are regulated by multiple interacting *cis*-acting elements separated by tens to hundreds of kilobases of genomic DNA. The recent wave of new technologies that have been facilitated by the ENCODE project [16] overcame several of these difficulties. They not only provide novel methods to interrogate the tissue-specific regulation of a gene such as *CFTR*, but simultaneously enable data to be generated from much smaller amounts of biological material than are required for older techniques. Thus primary cell cultures with limited *in vitro* replication potential can be used for reliable experimentation.

We applied several of these recently developed protocols in combination with classical methods to elucidate *CFTR* regulatory elements. Moreover, we examined *CFTR* expression in primary human airway epithelial cells and genital duct cells to elucidate the critical regulatory elements in normal cells in addition to the cancer cell lines that have been examined to date. These experiments generated structural and functional evidence for a *CFTR* transcriptional hub in which intronic enhancer elements are brought into close proximity to the *CFTR* promoter to activate transcription.

Identification of DHS in the *CFTR* gene

We used a high-resolution tiled microarray-based assay, DNase-chip [17], to map DHS across the *CFTR* locus in a number of cell types. To perform DNase-chip, chromatin-bound DNA is digested with a small amount of DNase, so that DHS are preferentially digested. DNase-digested ends are made blunt and ligated to biotinylated linkers. DNA is then sheared and DHS ends are enriched on a streptavidin column. A second linker is added before the DNA is amplified, labelled with dyes, and hybridized to tiled microarrays. Using this technique, we identified a number of cell-type-specific DHS within and flanking the *CFTR* locus (Figure 1A). Moreover, since the microarrays used in this analysis were the Nimblegen ENCODE arrays, we simultaneously generated data on other genes within the 30 Mb covered by the ENCODE pilot project [16]. These include a number of other genes involved in differentiated functions of the airway epithelium. More recently, the development of genome-wide approaches to evaluate regulatory elements, such as DNase-chip, DNase-seq and FAIRE [17–20], has enabled simultaneous analysis across the whole genome. The data generated by these experiments have tremendous power to dissect transcriptional regulatory pathways in specific cell lineages, such as primary airway epithelial cells.

Figure 1(A) shows the identification of DHS by DNase-chip within approx. 1.5 Mb spanning the *CFTR* locus and including the *Met* proto-oncogene, *CAPZA2* [capping protein (actin filament) muscle Z-line α 2], *ST7* (suppressor of tumorigenicity 7 isoform B), *WNT2* [Wingless-type MMTV (murine-mammary-tumour virus) integration site 2], *ASZI*, *CFTR* and *CTTNB2*. A number of DHS are observed, some of which are apparently common to many cell types, such as the DHS associated with the promoter of the *CAPZA2* gene (Figure 1A, arrow a) and others are cell-type-specific, an example being the DHS at the *CFTR* promoter in 16HBE14o- cells [21] (Figure 1A, arrow b), the only cell type shown that expresses *CFTR*.

Ubiquitous DHS are also evident, for example, arrow c in Figure 1(A) marks a ubiquitous site in the last intron of the *CTTNB2* gene.

Within the *CFTR* gene itself, many of the DHS identified by DNase-chip correspond to ones that we saw previously by classical methods of DHS mapping, but a significant number are novel and some are only evident in primary airway cells that we have now evaluated for the first time (C.J. Ott, N.P. Blackledge, J.E. Kerschner, G.E. Crawford, C.U. Cotton and A. Harris, unpublished work). Figure 1(A) shows the DHS profile in skin fibroblasts that do not express *CFTR*, Beas2B cells [22] that express very low levels of the transcript and 16HBE14o- that express high levels of *CFTR* mRNA. The airway lines show very few DHS within the *CFTR* locus, in contrast with intestinal and genital duct cells ([23]; C.J. Ott, N.P. Blackledge, J.E. Kerschner, G.E. Crawford, C.U. Cotton and A. Harris, unpublished work). Of interest is a prominent DHS in intron 10 of the gene (Figure 1A, arrow d) that warrants further investigation.

Moving from the DHS to the regulatory element

Not all DHS contain *cis*-acting regulatory elements; some may be associated with structural elements that function in chromatin organization. However, for each DHS, we pursue a number of experimental methods to investigate whether they are associated with important regulatory elements. These are well illustrated by our analysis of a DHS in the first intron of *CFTR* at 185 +10 kb (where 185 is the last base in *CFTR* exon 1) and of DHS flanking the locus at -20.9, +6.8 and +15.6 kb. For all DHS, an initial bioinformatics search for cross-species homologies can often be informative and reinforce predictions for functional importance. However, since there is some divergence in the patterns of regulation of expression of the *CFTR* gene in different species, particularly rodents and human, extensive cross-species conservation is not a pre-requisite for further investigation of a DHS.

A *cis*-acting element in the first intron of the *CFTR* gene acts as an HNF1 (hepatocyte nuclear factor 1)-dependent enhancer of the *CFTR* promoter

Using DNase-chip, we detected a cell-type-specific DHS within the first intron of *CFTR* (Figure 1B, arrow) that corresponds to a regulatory element that we identified in our earlier work [11,24] and has been confirmed by others [25]. This element (known as 7/8 based on primer sets used to amplify the region [11]) was shown to positively regulate *CFTR* promoter activity specifically in intestinal cells both *in vitro* and *in vivo*: removal of the element from a YAC (yeast artificial chromosome) containing human *CFTR* reduced expression levels of the human gene by approx. 60% in trans-genic mice carrying the YAC, but only within the epithelium of the small intestine [24]. More recently, we showed that this element functions as a classical, tissue-specific enhancer in transient transfection experiments and can also independently recruit general factors necessary for transcription initiation [23]. To determine the nuclear factor(s) interacting with the regulatory sequence, we performed an *in vitro* DNase I footprinting analysis, which revealed a significant protected sequence encompassing a conserved HNF1-binding site. Expression of HNF1 α correlates with *CFTR* expression and this transcription factor binds *in vitro* to another cluster of intronic DHS in *CFTR* [26]. *In vivo*, HNF1 α contributes to the maintenance of normal mouse *Cfr* expression levels in the small intestine [26]. More recently, we showed that HNF1 binds to the intron 1 enhancer both *in vitro*, by EMSA (electrophoretic mobility-shift assay), and *in vivo*, by ChIP (chromatin immunoprecipitation) [23]. We have now demonstrated that a number of other intronic DHS within the *CFTR* locus are associated with enhancer elements that co-operate to activate *CFTR* expression (C.J. Ott, N.P. Blackledge, J.E. Kerschner, G.E. Crawford, C.U. Cotton and A. Harris, unpublished work).

The *CFTR* locus is flanked by enhancer-blocking insulators

The *ASZ1* and *CTTNBP2* genes, which flank *CFTR*, are located within approx. 50 kb of the gene and due to their highly divergent patterns of expression we investigated whether there were functional elements preventing transcriptional interference between these loci. By conventional DHS mapping, we initially identified two enhancer-blocking insulators 5' and 3' to the *CFTR* gene that had distinct properties. First, a DHS located at – 20.9 kb with respect to the translation start site was associated with a classical CTCF (CCCTC-binding factor)-dependent insulator element [27]. CTCF, a ubiquitously expressed, zinc finger DNA-binding protein [28,29], often establishes independently regulated domains of gene activity. A second element, located 3' to *CFTR*, within a DHS at +15.6 kb (with respect to the translational end point) also demonstrated enhancer-blocking activity but this was independent of CTCF binding. The +15.6 kb DHS was marked by a peak of euchromatin-specific histone modifications, unlike the – 20.9 kb DHS [27], supporting the hypothesis that these elements function by different mechanisms.

In addition to the prominent site at +15.6 kb other DHS were evident 3' to the coding region of the gene, in particular, a complex cluster of sites at +5.4, +6.8, +7.0 and +7.4 kb from the *CFTR* translation end point [13]. The DHS at +5.4 and +7.0 kb were observed in a variety of cell types irrespective of *CFTR* expression. However, the DHS at +6.8 and +7.4 kb were only found in a restricted number of *CFTR*-expressing cell types, including primary epididymis cells [30], suggesting that they may contain tissue-specific regulatory elements that participate in controlling *CFTR* expression [13]. We subsequently demonstrated by ChIP that the +6.8 kb DHS is associated with a tissue-specific CTCF recognition site that binds CTCF *in vivo* (Figure 2) [31]. Both Caco2 cells and primary epididymis cells express *CFTR* but the +6.8 kb DHS is evident only in primary epididymis cells. Enrichment of the +6.8 kb DHS after ChIP with an antibody specific for CTCF is evident in primary epididymis cells, but not Caco2. The +6.8 kb DHS core also displays enhancer-blocking activity comparable with that of other known insulator elements, including the one at the *CFTR* – 20.9 kb DHS [27,31].

The three-dimensional structure of the active *CFTR* locus

A number of techniques have been developed over the last several years to interrogate the three-dimensional structure of genes within the nucleus [32–36]. These methods, which usually capture the chromatin structure *in vivo* by formaldehyde cross-linking of proteins and associated DNA, enable the mapping of regions of a locus that are in close physical proximity, despite their linear separation on the chromosome. These close interactions are often associated with transcriptionally active genes and presumably facilitate the co-operation of *cis*-acting regulatory elements and gene promoters. They may also delineate the boundaries of an active locus and compartmentalize it away from neighbouring genes with different cell-type-specific expression patterns.

Since we identified enhancer-blocking insulators flanking the *CFTR* locus that bind CTCF, and this protein is thought to be involved in regulating nuclear organization, we next evaluated the three-dimensional structure of the *CFTR* locus in a number of cell types that express *CFTR* or in which the gene is silent. This also enabled us to determine whether the intron 1 enhancer element, which is located 10 kb distal to the *CFTR* promoter, was brought into its close proximity by a looping mechanism to augment gene expression. We employed the technique of chromosome conformation capture (3C), which enables the identification of direct interactions between different parts of a locus in three dimensions [32]. In 3C experiments, DNA–protein interactions in intact nuclei are fixed by formaldehyde. The cross-linked chromatin is then digested with a restriction enzyme followed by ligation. If there is a protein-mediated interaction between a remote regulatory element, for example in an intron, and the

gene promoter, new chimaeric DNA molecules are formed that contain both elements. These chimaeric fragments can be determined by PCR with carefully designed primers. A fixed Taqman probe and reverse primer were designed within a HindIII fragment at the *CFTR* promoter, and multiple forward primers were generated within distal regions across the *CFTR* locus (Figure 3A). These forward primers were located within HindIII fragments encompassing the – 20.9 kb, +6.8 kb and +15.6 kb DHS, and within specific intronic HindIII fragments. The assay fragments were positioned approx. 25–50 kb apart, such that they would give a good overall representation of the structure of the *CFTR* locus (Figure 3A). Real-time PCRs using the ‘fixed’ reverse probe/primer and each of the ‘variable’ forward primers enabled quantification of ligation events (subsequently referred to as ‘interaction frequency’) between the *CFTR* promoter and specific distal regions within each 3C sample. 3C was performed using chromatin prepared from primary epididymis cells and skin fibroblasts. The results shown in Figures 3(B) and 3(C) demonstrate that the three-dimensional structure of the *CFTR* locus shows a dramatic difference between *CFTR*-expressing cells (epididymis) and non-expressing cells (fibroblasts). In epididymis cells, the 3′-flanking region (encompassing the +6.8 kb DHS) is closely associated with the promoter region, as shown by its enrichment after 3C using a probe in the *CFTR* promoter. We have also demonstrated strong interactions between the promoter, the intron 1 DHS and other intronic DHS in other cell types [23] (C.J. Ott, N.P. Blackledge, J.E. Kerschner, G.E. Crawford, C.U. Cotton and A. Harris, unpublished work). In contrast, there are no significant interactions between the *CFTR* promoter and distal parts of the gene in skin fibroblasts.

We predict that looping of *CFTR* (Figure 3D), possibly induced by CTCF, enables key intronic *cis*-acting regulatory elements and others flanking the gene to move into close proximity to the *CFTR* promoter, so activating cell-type-specific expression.

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Abbreviations

ASZ1	ankyrin repeat, SAM, (sterile α -motif) and basic leucine zipper
3C	chromosome conformation capture
CAPZA2	capping protein (actin filament) muscle Z-line α 2
CFTR	cystic fibrosis transmembrane conductance regulator
ChIP	chromatin immunoprecipitation
CTCF	CCCTC-binding factor
CTTNBP2	cortactin-binding protein 2
DHS	DNase I-hypersensitive site(s)
HNF1	hepatocyte nuclear factor 1
WNT2	Wingless-type MMTV (murine-mammary-tumour virus) integration site 2
YAC	yeast artificial chromosome

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Figure 1. Identification of DHS within part of ENCODE region 1

(A) Averaged DNase-chip hybridization data from three (skin fibroblasts), two (16HBE14o-) or a single (Beas2B) experiment were analysed with ACME statistical software [37]. The y-axis for each DHS track represents $-\log_{10}(P\text{-value})$ between 0 and 16 as determined by ACME. Below the DHS tracks are shown the genes mapping to this region: all are on the forward strand except *WNT2*, *ASZ1* and *CTTNBP2*. A description of arrows a–d is given in the text. (B) Magnification of the DNase-chip data from the *CFTR* promoter region in skin fibroblasts, 16HBE14o- and Caco2 cells. Map location 0 represents the beginning of *CFTR* exon 1. Significant peaks representing DHS are apparent in the 5' promoter region of the *CFTR* + cells, whereas Caco2 cells display another significant DHS within the first intron (data taken from [23]).

Figure 2. *In vivo* binding of CTCF at the +6.8 kb DHS region

Immunoprecipitations were performed with a CTCF-specific antibody and chromatin from (A) Caco2 cells and (B) primary epididymis cells. No antibody control samples were also prepared, in which chromatin was incubated with Protein A–agarose beads alone (results not shown). Samples were subjected to Taqman quantitative PCR analysis using probes specific for intron 17a and regions of interest 3' to *CFTR*. CTCF-specific enrichment of each of these regions is shown relative to levels at intron 17a (which contains no predicted CTCF-binding sites). The vertical broken line represents the location of the *CFTR* translation end point, and the *x*-axis on the right of this is drawn to scale (i.e. each data point accurately reflects the relative positions of Taqman amplicons). Immunoprecipitations were repeated at least twice. PCRs

were performed in triplicate and C_t (threshold cycle value) values were averaged. Results are means $\pm$ S.E.M. Data taken from [31].

Figure 3. 3C analysis of the *CFTR* locus

(A) Scale figure of the *CFTR* gene with exons marked by vertical bars and the translation start site represented by a bent arrow. Small vertical lines above the gene denote HindIII sites and half arrowheads show the locations of 3C primers. Due to space constraints, primers are not drawn to scale, and the fixed *CFTR* promoter HindIII fragment is expanded, showing the location of Taqman probe (joined ball and star) and the reverse primer. The -20.9 kb DHS and +6.8 kb DHS locations are also indicated. (B) 3C data from primary human skin fibroblast cells. (C) 3C data from primary human epididymis cells. The x-axis for each chart is drawn to scale, with units representing base pairs relative to the *CFTR* translation start point (0). Both charts are aligned with the *CFTR* gene figure above. Vertical dashed lines represent HindIII

sites. The interaction frequency between a fixed HindIII fragment at the *CFTR* promoter (shaded grey) and HindIII fragments at various regions across the *CFTR* gene was measured by Taqman quantitative PCR. The interaction frequency at each point is expressed relative to the interaction between two HindIII fragments within the ubiquitously expressed *ERCC3* (excision repair cross-complementing group 3) gene. Results shown represent the means $\pm$ S.E.M. for two independent experiments. Each real-time PCR was performed three times. Data are from [31]. **(D)** A looping model for the active *CFTR* gene [31]. In *CFTR*-expressing cell types, such as primary epididymis cells, elements in the *CFTR* 3' flanking region are in close proximity to the *CFTR* promoter. This 3' flanking region includes the tissue-specific +6.8 kb DHS, shown here to bind CTCF, as well as other previously described DHS [13,27]. Protein factors bound at each of these sites interact with the promoter-bound transcription machinery, thus forming an active chromatin hub (ACH) and helping regulate expression of the *CFTR* gene. Besides the DHS from the 3' flanking region, intronic DHS such as the intestine-specific intron 1 element [11,23] and others may also contribute to the *CFTR* ACH in a tissue-specific manner.