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Transcriptional regulation of *CFTR* gene expression

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TABLE OF CONTENTS

1. ABSTRACT

Cystic Fibrosis results from mutation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The gene was identified in 1989, but more than 20 years later, the regulatory mechanisms controlling its complex expression are still not fully understood. Though the promoter binds transcription factors and drives some aspects of *CFTR* gene expression, it cannot alone account for tissue specific control. This implicates other distal *cis*-acting elements in cell-type-specific regulation of *CFTR* expression. Several of these elements, including intronic enhancers and flanking insulators, were identified within or near the *CFTR* locus. Here we summarize the known regulatory mechanisms controlling *CFTR* transcription, including those acting through elements in the promoter and others elsewhere in the locus. A special focus will be elements that contribute to tissue specific regulation of expression.

2. INTRODUCTION

Cystic Fibrosis, the most common lethal autosomal recessive disorder in Caucasians, results from mutation of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. *CFTR* was identified by positional cloning in 1989 (1),(2),(3). The gene spans 189kb on the long arm of chromosome 7 (7q31.2), and consists of 27 exons, which encode a 1480aa protein. CFTR, a member of the ATP-binding cassette (ABC) family of transporters, functions primarily as an epithelial chloride channel. Expression of *CFTR* in specialized epithelia of the airway, reproductive organs, pancreas and intestinal tract is of primary clinical importance, as aberrant expression in these tissues leads to the most severe phenotypes in CF patients (4),(5),(6). However, the gene is also expressed in a wide range of other cell types, from hypothalamic neurons to lymphocytes, where it may influence processes ranging from the onset of puberty to the immune response and

inflammation (7),(8),(9). In contrast, the genes flanking *CFTR*, ankyrin repeat, SAM and basic leucine zipper domain containing 1 (*ASZ1*) and cortactin-binding protein 2 (*CTTNBP2*), have markedly different expression patterns. *ASZ1* is expressed exclusively in the testis and ovary (10), while *CTTNBP2* is expressed ubiquitously, with high expression in the kidney, pancreas and brain (11). In addition to tight spatial regulation of expression, *CFTR* also exhibits temporal regulation during development, with high levels of *CFTR* expression in the fetal lung (12), and relatively low levels in the adult airway as a whole, where expression is highest in ciliated cells of the superficial epithelium (13),(6).

The regulatory mechanisms controlling the complex expression pattern of *CFTR* are not yet fully understood. The *CFTR* promoter is a 'housekeeping'-type promoter, it is CpG rich, contains no TATA box, and contains a number of putative Sp1 (specificity protein 1) binding sites (14). As expected, the *CFTR* promoter exhibits no tissue-specificity in reporter assays (15). This suggests that distal cis or trans-acting regulatory elements are involved in the cell type-specific regulation of *CFTR* expression.

3. THE PROMOTER

While the promoter is not solely responsible for regulating tissue specific expression of *CFTR*, it contains several important regulatory motifs. In addition to Sp1 sites, several putative AP-1 (activator protein 1) sites are also present, one of which contains a sequence variant that is observed on some mutant CF chromosomes (16). These AP-1 sites are likely functional, as activation of AP-1 with phorbol myristate acetate results in repression of *CFTR* transcription (17),(14). In contrast, *CFTR* promoter activity is enhanced in the presence of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) through a site at nucleotide position -1103 relative to the translational start codon[1] (18). This enhancement is abolished upon treatment with the NF-kB pathway inhibitor pyrrolidine dithiocarbamate (18),(19).

In addition to the mutation in the AP-1 site, three known mutations affect transcription factor binding to the promoter. The first, a T to A substitution at position -102, significantly increases *CFTR* expression by creating a YY1 (yin yang 1) site (20). This mutation is also encompassed by a CArG-like motif, which binds the transcription factor SRF (serum response factor) (21). The second, a G to T change at position -94, results in decreased transcriptional activity by disrupting the binding of the SP1 and USF transcription factors to the promoter (22). The third, a G to A mutation at position -33A, enhances a FOXI1 (forkhead box protein I1) binding site in HVP (SV40 ori-transformed epithelial vas deferens) cells, but not in HeLa (human cervical carcinoma) cells. FOXI1 binding to this site represses *CFTR* expression in HVP cells, a novel function for the factor (23).

Expression of the CFTR promoter is also regulated by cellular cAMP levels through a CRE (cyclic AMP response element) sequence at position -48. This element binds CREB (cAMP response element binding protein) *in vitro*, and can drive cAMP-mediated reporter expression (24). An inverted CCAAT element between positions -132 and -119 is also involved in the cAMP-response. Deletion of this element, which binds C/EBP (CCAAT-enhancer binding protein), ATF1 (activating transcription factor 1), CREB1(cAMP response element binding protein 1) and CDP/cut (CCAATT displacement protein) (25), inhibits CFTR expression (26). This element is required for both basal and cAMP-mediated activation of CFTR expression (26). Binding of CDP/cut, however, reduces CFTR transcription by outcompeting the transcriptional activators hGCN5 and ATF1. Additionally, CDP/cut recruits HDACs (histone deacetylases), which alter the chromatin structure to a transcriptionally inactive state (25).

Expression of *CFTR* appears to be dependent on cellular oxygenation, since it exhibits transcriptional repression during hypoxia. This regulation is HIF-1 dependent, and occurs through a HRE (hypoxia response element) located at position -850 (27). Additional hypoxic regulation may occur through HIF-1 mediated regulation of *NF-kB* and *CREB* (28). *CFTR* transcription is also repressed during the UPR (unfolded protein response). This repression is reported to be mediated by the binding of the UPR-activated transcription factor ATF6 at the *CFTR* promoter during endoplasmic reticulum stress, a condition that activates the UPR (29). Moreover, NF-E2-related factor 2, a key inducer of antioxidant defenses, may negatively regulate *CFTR* expression (30).

DNA methylation plays an important role in the promoter function of many genes. Actively transcribed genes tend to have hypomethylated promoters, while inactive promoters generally exhibit hypermethylation. In particular, the hypermethylation of CpG islands in promoters can repress gene expression by recruiting repressor complex factors thus inhibiting transcription factor binding. *CFTR* has a CpG-rich promoter, suggesting that methylation may be involved in regulating transcription of the gene. One study reported an increase in methylation at MAZ (myc-associated zinc finger protein) and Sp1 binding sites in the *CFTR* promoter following ER stress. Moreover, hypermethylation of specific sites in the *CFTR* minimal promoter was observed in some cell lines expressing *CFTR* in the absence of ER stress (29). However, comprehensive methylation analysis by another research group showed that the *CFTR* minimal promoter was almost completely unmethylated in all cell types, independent of *CFTR* expression (31). The only exception was the *CFTR* negative human breast adenocarcinoma cell line MCF7, in which the promoter is almost completely methylated. This is consistent with earlier data that showed methylation of the *CFTR* promoter in some long-term cell lines (15).

4. IDENTIFYING REGULATORY ELEMENTS

While the promoter is important for basal expression of *CFTR*, it does not explain the complex tissue

specific regulation of *CFTR* expression. This points to distal cis or trans-acting regulatory elements as the critical regulators of this process. These distal elements are most likely to be found within the genomic region encompassing the *CFTR* locus, and may be associated with DHS (DNase-I Hypersensitive Sites). Active chromatin generally exhibits increased sensitivity to DNase I digestion relative to inactive chromatin, while regions bound by structural or regulatory proteins often exhibit dramatically increased sensitivity to the nuclease (32). In order to identify DHS which may contain elements involved in the regulation of *CFTR* expression the 400kb of genomic DNA centered on the *CFTR* locus was analyzed using classical DHS mapping techniques (33),(34),(35),(36),(37),(38). More recently, DNase-ChIP, which involves hybridizing DNase-I digested DNA to a tiled microarray, was used to generate high-resolution DHS maps in several cell types (39),(40). Together, these techniques led to the identification of several functional elements involved in regulation of *CFTR* transcription, including enhancers and insulators.

5. ENHANCERS

Several elements were characterized in the *CFTR* locus that enhance transcription driven by the *CFTR* minimal promoter. Sequences located in intron 1, intron 11, intron 20, and intron 21 are all able to enhance luciferase reporter expression driven by the *CFTR* minimal promoter in the human colon carcinoma cell line Caco2 (33),(41),(40),(42). The intron 1 element, which binds the HNF1 (hepatocyte nuclear factor 1) transcription factor *in vitro* and *in vivo* as shown by ChIP (41), also acts as an intestinal-specific enhancer in the genomic context of the *CFTR* locus. Deletion of the core of the intron 1 element from a YAC (yeast artificial chromosome) carrying the human *CFTR* locus results in a 60% reduction of YAC-derived *CFTR* in both Caco2 cells and the small intestine of YAC-transgenic mice (43). HNF1, which also binds to the intron 10a, intron 10b, intron 10c, intron 17a and intron 20 DHS *in vitro* (44), is upregulated in Caco2 cells upon post-confluence differentiation, corresponding with an increase in *CFTR* transcription, and can enhance *CFTR* expression in Caco2 cells when overexpressed prior to confluence (44).

Enhancers likely makes a critical contribution to tissue-specific regulation of *CFTR* transcription. The activity of the intron 1 enhancer is specific to the intestine and genital duct while the intron 11 element shows a similar cell-type restriction. Moreover, it is probable that additional elements may enhance expression in other cell types. A complex set of cell type-specific intronic enhancers may in fact be common for large loci. The murine *Gli3* locus, which is similar in size to *CFTR*, harbors a variety of conserved intronic enhancers. These enhancers, alone and in combination, drive expression in a range of tissues, including the limbs, face and a wide range of nervous tissues (45).

6. INSULATORS

The *CFTR* locus is flanked by enhancer blocking insulators, which repress functional interaction between enhancers and promoters in a position-dependent manner, and form boundaries between discrete regulatory domains. These insulators serve to transcriptionally isolate *CFTR* from the neighboring genes, which exhibit very different expression patterns, despite being only 50kb away on either side. Insulators were characterized in DHS located -20.9kb 5' of the translational start codon, and at +6.8kb and +15.6kb downstream of the translational stop codon. All three elements function as enhancer blocking insulators in cellular assays (46),(47). The enhancer blocking activity of the -20.9kb and +6.8kb insulators is dependent on CTCF binding (46),(47). CTCF is a ubiquitously expressed zinc-finger DNA binding protein known to form barriers between independent transcriptional units (48),(49). The +15.6kb enhancer does not bind CTCF, but is marked by a peak of euchromatin-specific histone modification and binds *in vitro* to several factors known to interact with the *CFTR* promoter, specifically CREB/ATF, AP-1 and C/EBP (46),(34). This suggests that the +15.6kb element functions by a different mechanism that the -20.9kb or +6.8kb insulators. The mechanism may involve hormone receptor binding, as the segment of the +15.6kb element responsible for enhancer blocking binds an RXRα-VDR (retinoid X receptor α-vitamin D receptor) heterodimer complex *in vitro* (46). Nuclear receptor proteins, including RXRα and VDR, perform many of the same functions as, and functionally cooperate with, CTCF (50),(51),(52),(53). While the -20.9kb and +15.6kb DHS are seen in both *CFTR* expressing and non-expressing cell types, the +6.8kb DHS is restricted to primary genital duct cells and adult lung tissue. Moreover, CTCF is enriched *in vivo* at the +6.8kb DHS in primary epididymis, while it is not in Caco2 cells, which lack the DHS (47),(54),(34). This suggests that the +6.8kb DHS may play an important role in regulating male genital duct-specific expression of *CFTR*.

7. THREE-DIMENSIONAL STRUCTURE OF THE LOCUS

As CTCF is known to play a role in organizing higher order chromatin structure, and physical proximity with the promoter is a possible mechanism by which intronic enhancers may function, the three-dimensional organization of the *CFTR* locus was investigated using chromosome conformation capture (3C) (55)**.** Several regions 5' and 3' to the locus and within it were shown to interact with the *CFTR* promoter in some *CFTR* expressing cells. These interactions are not observed in cells where the gene is transcriptionally silent (40),(56). Fragments containing either the intron 1 or intron 11 elements interact with the *CFTR* promoter in intestinal cell lines, consistent with their roles as enhancers in these cell types (40),(41). Similarly, fragments containing the intron 11 DHS and the enhancer blocking insulator at +6.8kb interact strongly with the *CFTR* promoter in primary epididymis cells (47). The promoter also interacts with the insulator element at -20.9kb in the CFTR expressing HeLa S3, HT29 (human colon carcinoma) and Caco2 cell lines (56), but this interaction is only observed in 3C using EcoRI digestion, not HindIII digestion (40). This may be due to the larger

size of the EcoRI fragment encompassing the -20.9kb DHS, compared to the HindIII fragment in the same area. Long range interactions also occur between the *CFTR* promoter and more distal elements, including the –79.5kb DHS (37) in HeLa S3, HT29 and Caco2 (56) and several DHS downstream of the *CFTR* locus in HT29 (40). These data suggest a model in which the active boundary elements, together with tissue-specific enhancers are looped in close association with the promoter in cells where *CFTR* is actively transcribed, while the looping is absent in cells where *CFTR* is transcriptionally silent.

Interestingly, *CFTR* locus looping is also largely absent from cells which express low levels of the transcript, such as airway epithelia (40). This suggests that the regulatory mechanisms controlling *CFTR* transcription may be fundamentally different in cells that express the gene at low or high levels. While it is possible that there are distal elements such as silencers interacting with the *CFTR* promoter in the airway it is also possible that promoter-mediated mechanisms are primarily responsible for transcriptional regulation in these cells.

8. CONCLUSIONS AND PERSPECTIVES

Major advances in our understanding of the mechanisms that regulate tissue specific regulation of *CFTR* gene expression occurred over the past few years. However, there is still much to be learned. Novel functional elements were characterized in the promoter, including a hypoxia response element. Similarly, the importance of intronic enhancers and insulator elements that flank the locus has been elucidated. These elements, which functionally interact with the promoter in a cell type-specific manner, control, at least in part, the spatial regulation of *CFTR* expression, particularly in intestinal epithelial cells. Further work is required to uncover the mechanisms controlling *CFTR* expression in other differentiated cell types.

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Footnotes: [1] All discussed promoter variants are numbered relative to the translational start codon.

Key Words: cystic fibrosis transmembrance conductance regulator (CFTR), gene regulation, cis-acting elements, chromatin looping, insulators

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