A Regulatory Element in Intron 1 of the Cystic Fibrosis Transmembrane Conductance Regulator Gene*

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The cystic fibrosis transmembrane conductance regulator (CFTR) gene exhibits a tightly regulated pattern of expression in human epithelial cells. The mechanism of this regulation is complex and is likely to involve a number of genetic elements that effect temporal and spatial expression. We have identified a putative regulatory element within the first intron of the CFTR gene at 181+10kb. The region containing this element was first identified as a DNase I hypersensitive site that was present in cells that express the CFTR gene but absent from cells not transcribing CFTR. In vitro analysis of binding of proteins to this region of DNA sequence by gel mobility shift assays and DNase I footprinting revealed that some proteins that are only present in CFTR-expressing cells bound to specific elements, and other proteins that bound to adjacent elements were present in all epithelial cells irrespective of their CFTR expression status. When assayed in transient expression systems in a cell line expressing CFTR endogenously, this DNA sequence augmented reporter gene expression through activation of the CFTR promoter but had no effect in nonexpressing cells.

The cystic fibrosis transmembrane conductance regulator (CFTR) gene (Rommens et al., 1989; Riordan et al., 1989) shows a tightly regulated pattern of temporal and spatial expression (Crawford et al., 1991; Harris et al., 1991; Denning et al., 1992; Trezise et al., 1993). Very little is known about the genetic elements and transcription factors that regulate CFTR expression. The basal promoter of the CFTR gene has been analyzed in some detail (Chou et al., 1991; Yoshimura et al., 1991, 1991a; Koh et al., 1993), although the data are somewhat inconsistent. The minimal promoter sequence found between −226 and +98 bp with respect to the transcription start site defined by Chou et al. (1991) is sufficient to drive low levels of expression of a reporter gene (Chou et al., 1991; Koh et al., 1993). However, Chou et al. (1991) also identified an element immediately upstream of −277 that repressed reporter gene transcription in several cell lines.

Analysis of the DNA sequence of the basal CFTR promoter reveals a number of elements that may be involved in regulation of transcription. There are several potential binding sites for the AP-1 transcription factor (GGAGTCAG) and three putative binding sites for the transcription factor Sp1 (GGGGCGG). There is evidence for in vitro regulation of CFTR gene expression by phorbol esters (Trapnell et al., 1991). A cAMP-response element (TGACATCA) has been defined within the CFTR promoter at −48 to −41 with respect to the transcription start site defined by Yoshimura et al. (1991a) (McDonald et al., 1995). Two purine/pyrimidine repeat elements have been identified in the 5′-flanking region of the CFTR gene, one of which has been shown to be sensitive to nuclease treatment in supercoiled plasmids, suggesting a non-B DNA structure (Hollingsworth et al., 1994; McDonald et al., 1994).

There is little data on the control of cell specificity of CFTR expression, although there is ample evidence for such regulation. The human CFTR gene is expressed at significant levels mainly in the epithelia lining the pancreas, intestine, bile ducts, male genital ducts, and certain regions of the airway epithelium including the inferior turbinate of the nose, the trachea, and the serous portion of submucosal glands (Crawford et al., 1991; Denning et al., 1992; Engelhardt et al., 1993). There is evidence that expression of the CFTR gene may be hormonally regulated in epithelia within the reproductive system (Trezise et al., 1992; Rochwerger et al., 1994). Some degree of cell type-specific control has been inferred for uncharacterized elements within the immediate 5′-untranslated region. A number of DNase I hypersensitive sites that show some degree of correlation with CFTR expression have been observed between −3,000 bp relative to the transcription start site and +100 bp into intron 1 (Yoshimura et al., 1991b; Koh et al., 1993). However, these sites have only been examined in a few long term cell lines that either do or do not express CFTR mRNA and protein and hence may not adequately reflect cell-specific regulation of expression of the CFTR gene in vivo. Transgenic mouse experiments in which 19 kb of genomic DNA 5′ to the CFTR gene were placed 5′ of a reporter gene failed to achieve expression (Griesenbach et al., 1994).

Because the expression control elements of the CFTR gene had not been well defined, we screened a larger region of genomic DNA than had been analyzed previously in an attempt to identify these elements. The chromatin structure of 120 kb of genomic DNA 5′ to the CFTR gene was analyzed in a number...
of CFTR expressing and nonexpressing cell types, including primary human genital duct epithelial cells in addition to long term cell lines. We identified DNase I hypersensitive sites within this region by screening with probes isolated from cosmid and phage clones (Rommens et al., 1989). Novel DNase I hypersensitive sites were observed at −79.5 and −20.5 kb 5' to the ATG translation start codon of the CFTR coding sequence (Smith et al., 1995). Neither of these sites showed strong correlation with CFTR expression in the cell types investigated. Although they may play an important role in the complex series of events involved in the regulation of CFTR transcription, these data do not support the existence of cell-specific control elements at these sites.

Another DNase I hypersensitive site was observed within intron 1 of the CFTR gene. Detection of this site correlated well, quantitatively and qualitatively, with the levels of expression of the CFTR gene in both long term cell lines and primary genital duct epithelial cells. Nuclear extracts from cells that transcribe the CFTR gene contain specific proteins that bind to DNA in the region of this hypersensitive site. Further, analysis of the putative regulatory element through transient assays of reporter gene constructs showed a positive effect on the activity of the CFTR promoter in cells that express the CFTR gene endogenously.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The following cell lines were analyzed: primary human mid-trimester fetal vas deferens and epididymis epithelial cells (Harris and Coleman, 1989) and SV40 Ori–transformed vas deferens (RVP) and epididymis (REP) cell lines (Coleman and Harris, 1991); Caco2 (Fogh et al., 1977), HT29 (Huet et al., 1987), Capan1 (Fogh et al., 1977), HPAF (Kim et al., 1989); MCF7 (Soule et al., 1973), and a lymphoblastoid cell line 37566. The 16hBE14o–SV40 Ori–transformed bronchial epithelial cell line (Keznes et al., 1994) was kindly donated by Dr. D. Gruenert.

**Reverse Transcription-PCR**—All cell types studied were tested for CFTR mRNA expression by reverse transcription-PCR (RT-PCR) at the time of isolation of nuclei for chromatin analysis (Smith et al., 1995).

**Generation of Probes**—The cW44 cosmid was kindly provided by Drs. Johanna Rommens and Lap-Chee Tsui (Rommens et al., 1989). The XBS.5 (5 kb Xhol/BamHI), H4.0 (4 kb HindIII), and EBL1 (1.7 kb EcoRI/BamHI) subfragments of this cosmid are shown in Fig. 1.

**DNase I Hypersensitivity Assays**—Chromatin from a panel of cell types was probed for DNase I hypersensitive regions by standard methods (Higgs et al., 1990).

Amplification and Cloning of Fragments of Intron 1—The location of oligonucleotides used for PCR amplification of fragments of intron 1 T5R3/4, T5R5/6, and T5R7/8 are shown in Fig. 1. IAIR, CGGATCCGAGAAATAAGCAAGCATTGACGTTTTTCTTG; TSR3, TCCCCGGCGATCCAAGGAGAGATCGAGAACAC; TSR5, CTTCATATGAGTCATCCATTGTTGAAAACCTACTGAC; TSR6, TCCCCGCGGATCCCAAGGAGATCGAGAACAC; TSR7, CTTTAATTAGGACTCCACTTATCTTATCTTCTTCTC; TSR8, TCCCCGGCGGATCCACTAATTGGTACTGAGCAG.

Primers for PCR amplification of intron 1 subfragments were designed to have BamHI and either PstI (forward primers) or SacI (reverse primers) restriction sites at their 5’ end. Fragments 3/4, 5/6, and 7/8 and the B50.7 (750 bp) fragment amplified by primers IAIR and TSR8 spanning the site in intron 1 were cloned into pCR1 (in Vitrogen). All subcloned fragments were sequenced to exclude PCR artifacts.

**Gel Mobility Shift Assays and DNase I Footprinting**—DNA fragments were labeled by fill-in reactions with Klenow DNA polymerase and α-32p]dATP, dCTP, and/or dTTP. Oligonucleotides were end labeled with T4 polynucleotide kinase and [α-32p]dATP. Preparation of nuclear extracts and gel mobility shift assays were carried out as described previously (Hollingsworth et al., 1994). DNase I footprinting reactions used standard methods (Philipson et al., 1990). Oligonucleotide sequences for competition experiments are ASMF1R to ASMF6R: ASMF1F, GTACCTTTGGAGATCACMRG TGGCTCAAAGTAC; ASMF2F, GTACCTTTGAAGATGCACTAAGTG; ASMF3R, TCTCGAATGATCGAGC; ASMF4R, TCTCGAATGATGAGC; ASMF5R, TCTCGAATGATGAGC; ASMF6R, TCTCGAATGATGAGC.

**RESULTS**

Expression of CFTR mRNA—Expression of CFTR was measured by reverse transcription-PCR in all the cell cultures used in this study at the time nuclei were isolated for hypersensitivity assays (Smith et al., 1995). This assay does not provide a highly accurate quantitative estimation of levels of CFTR mRNA expression; however, it provides a useful qualitative method of verifying production of CFTR mRNA by the cells under investigation when performed with an internal control for a house-keeping gene such as glucocerebrosidase. These data are summarized in Table I.

A Novel DNase I Hypersensitive Site in Intron 1—A diagram showing the relative positions of the XBS.5 (5 kb Xhol/BamHI, where the BamHI site is located at the cosmid end), H4.0 (4 kb HindIII), and EBL1 (1.7 kb EcoRI/BamHI) subfragments of the cW44 cosmid that were used as probes are shown in Fig. 1A. The efficacy of DNase I treatment of every preparation of DNA treated nuclei was evaluated with the RA2.2 probe that is known to detect a constitutive DNase I hypersensitive region in the promoter of the human papilloma virus type 111 (HPV111) genome.
is barely detectable in the transformed genital duct-derived cell lines REP and RVP, which have greatly down-regulated CFTR expression following transformation. This site is not evident in the 16HBE14o— bronchial epithelial cell line that expresses high levels of CFTR following SV40 Ori— transformation (not shown).

Sequence Analysis of the 181 + 10kb Hypersensitive Site—The Xb5.0 fragment of the cv44 cosmid (from the more distal Xho site 3' to the end of exon 1 to the BamHI site at the end of the cosmid, see Fig. 1) spanned the hypersensitive site. This fragment was subcloned into the BamHI and SalI/Xho sites of pUC119. Because the DNase I hypersensitive site was known to be in close proximity to the HindIII and EcoRI sites at 1.7 and 1.9 kb from the 3' end of the Xb5.0 fragment (see Fig. 1A), these sites were utilized for subcloning the EcoRI and HindIII fragments. The inserts were sequenced. A partial restriction map of 850 bp flanking this region is shown in Fig. 1B.

Gel Mobility Shift Assays—Overlapping fragments of approximately 250 bp (3/4, 5/6, and 7/8) were generated from within the 850-bp fragment of intron 1 by PCR. No specific gel mobility shifts were observed with fragments 3/4 or 5/6. However, several proteins bound to fragment 7/8 as illustrated in Fig. 3. Two gel mobility shift bands were generated by nuclear extracts from all cell lines tested, irrespective of whether they transcribe CFTR (bands C1 and C2). At least one other protein complex was seen in long term cell lines transcribing CFTR, Caco2, HT29 (Fig. 3, band a1), Capan1, REP, and RVP. These protein-DNA complexes were specifically competed by excess cold fragment 7/8 but not by the 5/6 fragment (Fig. 3). Primary epidemiology and primary vas deferens nuclear extracts also caused gel mobility shifts of fragment 7/8 and the formation of a complex of at least three components (a2, b1, and b2, Fig. 4A). The lymphoblastoid cell line 37566 showed a gel mobility shift to form a complex lying between a2 and b1 (Fig. 4A).

Further mapping of the location of the DNA-protein complexes detected by gel mobility shift analysis was achieved by competition with subfragments of the 7/8 element (see Fig. 4C). In each case the labeled probe was the entire 7/8 fragment, and all complexes were abolished by the presence of excess unlabeled 7/8 fragment (Fig. 4A). The 38-bp Alu fragment did not compete with any of the protein-DNA complexes (not shown). All protein-DNA complexes were eliminated by an excess of 112-bp ScrI fragment (Fig. 4B) and 74-bp Alu/ScrI fragments (not shown), suggesting that all proteins causing gel shifts were binding between the ScrI and Alu sites. In competition experiments (not shown) the 93-bp ScrI fragment showed weak competition with the a1, a2, b1, and b2 complexes, suggesting the relevant protein complex might also involve sites close to the 3' end of this fragment. The addition of excess unlabeled 130-bp Sau3a fragment blocked the formation of complexes seen at bands a1, a2, b1, and b2 (Fig. 4A). Adding an excess of the 75-bp Sau3a fragment eliminated the complexes seen at bands c1 and c2 (Fig. 4B). Hence, the complexes a1, a2, b1, and b2 detected in CFTR-expressing cells probably include proteins that bind to DNA sequences lying 5' to the Sau3a site (Fig. 4C), and the complexes seen in all cell lines, c1 and c2, involve DNA-protein interactions primarily 3' to the Sau3a site.

DNase I Footprinting of the 7/8 Fragment—Fig. 5 shows DNase I footpring of the 7/8 fragment following binding of nuclear extracts from the MCF7 and HPAF cells lines that do not transcribe CFTR, the Caco2 carcinoma cell line, and two independent primary epididymsm cell cultures that transcribe the CFTR gene. A footprint between bases 726 and 746 (AT-GTATCTTTAGCAGTAATTTAC) (see Fig. 1C) was seen in all cell lines. The precise appearance of this footprint was slightly
different when nuclear extracts from primary cells were used, as illustrated by the appearance of a hypersensitive site close to the Sau3a site at 730. Nuclear extracts from the primary epithelial cells gave two additional footprints at bases 712–726 (GTCACTTATTAACAAT) and 671–683 (GTACTTTGGAATC), separated by another hypersensitive site close to the Scrfl site. The footprint at bases 712–727 is weakly evident with Caco2 nuclear extracts and on occasion is seen with MCF7 nuclear extracts.

The DNase I footprint data suggest a complex pattern of DNA-protein interactions within this region of the 7/8 fragment. The results obtained with nuclear extracts from the primary cells suggest that the complex of protein(s) may be altering chromatin structure as revealed by the presence of DNase I hypersensitive sites. It is probable, given the complexity of the DNase I footprint and gel mobility shift data, that a number of proteins are interacting with this region of genomic DNA.

Oligonucleotide Analyses—Several oligonucleotides were chosen on the basis of the DNase I footprints (shown in Fig. 5) to further elucidate the precise location of the DNA-protein interactions described in the previous paragraph. Gel mobility shift assays were performed in which oligonucleotides ASTM1F/R, ASTM2F/R, ASTM3F/R, ASTM4F/R, ASTM5F/R, and ASTM6F/R (Fig. 1C) were used to compete for the gel mobility shifts produced when labeled fragment 7/8 was incubated with nuclear extracts from Caco2, primary epididymis, and MCF7.

Competition with a 100-fold molar excess of oligonucleotides ASTM2F/R resulted in a reduction in the amounts of band a1 on gel mobility shift reactions in Caco2 (Fig. 6A). This suggested that the 20 base pairs of ASTM2F/R encompassed at least one site of DNA-protein interaction for the a1 complex. However, when oligonucleotide ASTM2F/R was labeled and used in gel mobility shift assays (not shown), it was inefficient (in comparison with the whole 7/8 fragment) at generating a DNA-protein complex with nuclear extracts from Caco2 cells, suggesting that nucleotides lying outside this 20-bp sequence (presumably 5′ to it, on the basis of the 7/8 restriction fragment competition experiments above) may be important for the formation of complex a1. Further evidence for this was provided by gel mobility shift experiments with the ASTM5F/R oligonucleotide, which generated the a1 complex more efficiently than ASTM2F/R (not shown). ASTM2F/R was also seen to inhibit formation of the a2/b1/b2 complexes seen with primary epididymis cell nuclear extracts (Fig. 6C). Here again, the competition was incomplete, suggesting that other elements outside this sequence (presumably 5′ to it) were important in the generation of these complexes. The ASTM5F/R oligonucleotide was also effective in competition of the a1/a2/b1/b2 complexes (competition of a1 is shown in Fig. 6B) with little effect on the noncell-specific c1 and c2 complexes.

Competition with oligonucleotides ASTM4F/R showed no in-
Fig. 4. Gel mobility shift profiles of the 205-bp 7/8 fragment with nuclear extracts from CFTR+ and CFTR− cell lines and competition with the subfragments of the 7/8 DNA fragment as shown. A and B, lanes 1, no nuclear extract; lanes 2, MCF7 (CFTR−); lanes 3, 37566 (CFTR−); lanes 4, primary epididymis (CFTR+); lanes 5, Caco2 (CFTR+). Complexes a1, a2, b1, b2, c1, and c2 are marked. C, restriction map of the 7/8 fragment.

Fig. 5. DNase I footprint of the 7/8 element. Protected sequences are shown on the right. Lanes 1 and 14, AG ladder; lane 2, no DNase I; lanes 3 and 8, no nuclear extract; lane 4, 20 μg of nuclear extract from MCF7 (CFTR−); lane 5, 40 μg of nuclear extract from MCF7 (CFTR−); lane 6, 20 μg of nuclear extract from HPAP (CFTR−); lane 7, 40 μg of nuclear extract from HPAF (CFTR−); lane 9, 20 μg of nuclear extract from Caco2 (CFTR+); lane 10, 40 μg of nuclear extract from Caco2 (CFTR+); lane 11, 20 μg of nuclear extract from primary epididymis cell culture i (CFTR+); lane 12, 40 μg of nuclear extract from primary epididymis cell culture i (CFTR+); lane 13, 40 μg of nuclear extract from primary epididymis cell culture ii (CFTR+).

Inhibition of the a1 complex (Fig. 6B), hence it is likely that the a1/a2/b1/b2 complexes are interacting with DNA 5′ to the end of oligo 4. This was confirmed by gel mobility shift experiments (not shown) using the MseI fragment lying between 720 and 748 (see Fig. 6D) as a probe, which only generated the c1 and c2 complexes. Competition with oligonucleotides ASTM4F/R showed inhibition (though incomplete) of formation of the c1 and c2 complexes seen in all cell types analyzed (Fig. 6B). Further, the ASTM4F/R oligonucleotide alone was effective at forming the c1 and c2 complexes when used as a probe in gel mobility shift assays (not shown). However, oligonucleotide ASTM3F/R, which overlaps the 3′ 19 bp of ASTM4F/R, was only able to cause slight inhibition of the c1 and c2 complex in Caco2 at 500-fold excess (Fig. 6A), suggesting that the important DNA-protein interactions required for generating the c1 and c2 complexes are close to the Sau3a site.

Neither oligonucleotides ASTM1F/R nor ASTM6F/R showed competition with any of the DNA-protein complexes detected in the primary epididymis cell nuclear extracts or those from any of the other cell lines (not shown). Hence the precise nature of the DNase I footprint observed in the region of ASTM1F/R (GTACTTTGGAATC) with the epididymis cell nuclear extracts (Fig. 5) remains obscure.

In summary (Fig. 6D) the DNA-protein interactions that generate the gel mobility shifts c1 and c2 seen with nuclear extracts from all the cell types that we have analyzed occur between the MseI site at 720 and the MseI site at 748 with the key sites in the complex lying closer to the 5′ half of this fragment. The DNA-protein interactions that generate the gel mobility shifts a1, seen in Caco2 and HT29 and a2, b1, and b2 seen in primary epididymis nuclear extracts (none of these complexes being seen in the cell lines Panc-1, HPAF, and MCF7 that do not transcribe CFTR) are 5′ to the MseI site at 720 but 3′ to the end of oligonucleotide ASTM6F/R at 699. The efficiency of the ASTMS5F/R oligonucleotide in generating the a1 complex in Caco2 nuclear extracts confirms this localization. The ability of the ASTMS2F/R oligonucleotide to form the a1 complex, even if inefficiently, suggests that the base pairs involved in this interaction do not extend greatly to the 5′ end of this oligonucleotide at 711.

Transient Expression Assays—Constructs containing the 787-bp CFTR basal promoter fragment (designated 245) driving luciferase expression with the 5′/6, 7/8, or B50.7 (IA1R-TSR8) fragments doped into the enhancer site of the PGL2B vector (3′ to the CFTR promoter) were co-transfected with pdlCMVcat (Ma et al., 1992) into the cells lines Caco2, 16HBE14o−, and MCF7, and both CAT and luciferase activities were assayed. The results of the transfection experiments are shown in Fig. 7. Each experiment included transfections that were carried out in quintuplicate in 60-mm culture dishes. Luciferase values were corrected for transfection efficiency based on CAT activities for each transfected dish. In each experiment, controls were transfections with the pGL2B vector alone. Luciferase activities were expressed as a fraction of that
obtained with the pGL2B+245 CFTR promoter element construct.

The results of the luciferase assays showed that none of the cloned fragments from intron 1 had an effect on the CFTR promoter when transfected into MCF7 cells. Similar results were obtained in the 16HBE14o cell line that while transcribing CFTR at a high level as a result of SV40 Ori transformation, does not show the DNase I hypersensitive site at 181 kb that is seen in the other cell types that express CFTR endogenously. However the 7/8 fragment had a positive effect on CFTR promoter activity in the Caco2 cell line. Although the 5/6 fragment had essentially no effect, 1.1 (S.E. = 0.176) promoter alone, the 7/8 fragment augmented luciferase activity by a mean of 2.2-fold (S.E. = 0.311) with respect to the CFTR promoter alone. The BS0.7 fragment that encompasses the 750-bp fragment of the putative regulatory element (see Fig. 1B, 1AIR-TSR8) caused a mean amplification of luciferase activity of 3.4-fold (S.E. = 0.350). An analysis of variance was performed on log transformed data (to correct for non-normality) with experiment number added to the model as a block to control for temporal variation. After correcting the significance levels for multiple comparisons, the 7/8 and the BS0.7 elements were each seen to have significantly greater activity than the 245 CFTR promoter element alone (p < 0.01 in both cases). However, the 5/6 element did not differ in activity from the promoter alone (p > 0.3).

**DISCUSSION**

The identification and isolation of element(s) that control expression of the CFTR gene are of particular importance in the context of potential targeted gene therapy for CF. Previous analyses of chromatin structure (and methylation status) of the CFTR gene promoter region have identified a number of DNase I hypersensitive sites in a small number of cell lines that show some correlation with CFTR expression in those lines (Koh et al., 1993; Yoshimura et al., 1991a); however, to date the picture is incomplete. We have identified DNase I hypersensitive sites in a small number of cell lines that show some correlation with CFTR expression in those lines (Koh et al., 1993; Yoshimura et al., 1991a); however, to date the picture is incomplete. We have identified DNase I hypersensitive sites in a small number of cell lines that show some correlation with CFTR expression in those lines (Koh et al., 1993; Yoshimura et al., 1991a); however, to date the picture is incomplete. We have identified DNase I hypersensitive sites in a small number of cell lines that show some correlation with CFTR expression in those lines (Koh et al., 1993; Yoshimura et al., 1991a); however, to date the picture is incomplete. We have identified DNase I hypersensitive sites in a small number of cell lines that show some correlation with CFTR expression in those lines (Koh et al., 1993; Yoshimura et al., 1991a); however, to date the picture is incomplete.
Through a combination of DNase I footprint analysis and gel mobility shift assays using subfragments of the region (7/8) containing this element and oligonucleotides, we have determined that the regulatory element is located within a segment of about 40 bp. The 40-bp sequence contains two distinct sites of DNA-protein interactions as illustrated in Fig. 6D. The 5′ side of the Msel site at 720 bp contains the sequence AATCCTAAACTCTGTCACCTAA. A minimum of 9 bases (in bold) at the end of this sequence are crucial for the binding of the proteins found specifically in the nuclear extracts of the CFTR-expressing Caco2 and primary epididymal cells. It is probable that additional base pairs may be involved in the interaction. On the 3′ side of the same Msel site is the sequence TAACAAGTGATCTTGGCAATTTCTATT. A minimum of 13 base pairs (in bold) are likely to be involved in DNA binding of the proteins that generate the c1 and c2 complexes detected in CFTR expressing and nonexpressing cells.

Analysis of the DNA sequence shown in Fig. 1C reveals the presence of consensus binding motifs for several known transcription factors within the 7/8 region. These include PUT2 ATGTACTT (Siddiqui and Brandriss, 1988) and inf.1 AAGTGA (Fujita et al., 1987). The PUT2 protein functions in concert with the proline utilization pathway of Saccharomyces cerevisiae. The regulatory element lies upstream of the TATA box of this gene and is essential in proline induction of the PUT2 gene. The PUT2 homology within the 7/8 region lies within the sequence 671–683 that shows protection on DNase I footprints solely with the primary epididymal cell nuclear extracts (see Fig. 5). The relevance of this putative element to regulation of the CFTR gene expression is not inherently obvious.

A region of homology with inf.1 lies at the 5′ end of the ASTM2R oligonucleotide on the reverse strand and is coincident with the base pairs that appear to be responsible for binding of the a1 complex observed in Caco2 nuclear extracts. The inf.1 element is a 6-bp unit of a repeated sequence that mediates virus-induced transcription of interferon β, although any relevance to CFTR expression is obscure.

Regulatory Proteins—Data from the gel mobility shift and DNase I footprint experiments using the 7/8 fragment suggest that there may be a complex of proteins binding within this region. There are clearly one or more proteins that give rise to the gel mobility shifts at positions c1 and c2. In addition to these proteins, other proteins give rise to the unique gel mobility shifts that are associated with CFTR-expressing cells: a1, seen in the colon carcinoma cell lines, and those seen in the primary male genital duct epithelial cells, a2, b1, and b2. The nature and identity of these proteins is currently unclear. The results of oligonucleotide competition experiments suggest that at least some of the proteins bind directly to DNA.

The pattern of expression of these proteins in different cell types is of interest. All cell types we have analyzed contain proteins that generate the c1 and c2 complexes that bind to oligonucleotide ASTM4F/R. On the basis of gel mobility shifts, these proteins would appear to be the same in all cells. However, the proteins that bind to the region of the ASTM2F/R and ASTM5F/R oligonucleotides in different cell types are likely not to be all the same. Nuclear extracts from intestinal carcinoma cell lines Caco2 and HT29 and the pancreatic adenocarcinoma cell line Capan1 all produce the same prominent gel mobility shift designated a1. The abundance of this complex is greater in the intestinal carcinomas, and this may reflect the CFTR expression levels in these cells (see Table I). Nuclear extracts from primary epididymis cells show at least three gel mobility shift bands (a2, b1, and b2) with mobilities differing from the single predominant complex seen in Caco2 nuclear extracts.

Oligonucleotide competition experiments have shown that the proteins that cause the a1/a2/b1/b2 gel shifts are all interacting with the sequence of ASTM2F/R and ASTM5F/R. However, the DNaseI footprinting data show a greater region of protection of the DNA backbone by nuclear extracts from the primary cells. We have not determined whether the other proteins are in fact binding directly to the DNA or to other proteins in the DNA-protein complex. We are further characterizing the nature of these proteins.

The data from the gel mobility shift assays (taken together with the data showing that the DNase I hypersensitive site at 181–10kb is only seen in cell types that express the CFTR gene) lead us to propose the following model for DNA-protein interactions in this region of the gene. Some protein factors that bind to this region are present in nuclear extracts from most cell types, regardless of their status with respect to CFTR expression, and their presence alone does not create a DNase I hypersensitive site. Additional proteins that bind to the DNA in this region and cause conformational changes in the chromatin structure to expose a DNase I hypersensitive site are expressed in many cell types that endogenously transcribe the CFTR gene. There is evidence for such a mechanism existing in regulatory elements associated with other genes (J. enuwijn et al., 1993). At this stage the nature of any interactions between the individual proteins themselves and between each of them and the DNA backbone remains to be elucidated.

Transient Assays—In vitro transient assays of the activity of the regulatory element (the BS0.7 fragment spanning the 181–10kb site) have shown that it augments the level of the CFTR promoter-mediated expression of the luciferase reporter gene in the Caco2 cell line by a mean of 3.4-fold in 30 independent transfections (six experiments with five dishes of cells for each transfection). Although this enhancement of expression might seem relatively modest, this is not unexpected given the weak activity of the CFTR promoter in driving reporter gene expression in transient assays (Chou et al., 1991; Yoshimura et al., 1991b; Koh et al., 1993). Moreover, the observed enhancement of expression is of the same order of magnitude as has been observed for other regulatory elements in the CFTR gene promoter (McDonald et al., 1995). It is interesting that the 7/8 fragment shows a common gel mobility shift in Caco2 and HT29 but a different shift profile with primary genital duct epithelial cells. Both Caco2 and HT29 are colon carcinoma cell lines that transcribe high levels of CFTR in culture. Although these cell lines are not normal cells, it is likely that they arise from colonic epithelial cells that do express significant amounts of CFTR in vivo. This is in contrast to the 16HBE14o− cell line that is derived from bronchial epithelium, which transcribes very low levels of CFTR in vivo. Thus, high levels of CFTR expression in this line are likely to be a consequence of the SV40 Ori− transformation process. It remains possible that the element that we have identified may be of importance in the expression of CFTR only in specific epithelia such as the intestinal epithelium and male genital duct epithelium.

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