HNF1α is involved in tissue-specific regulation of *CFTR* gene expression

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The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene shows a complex pattern of expression with tissue-specific and temporal regulation. However, the genetic elements and transcription factors that control *CFTR* expression are largely unidentified. The *CFTR* promoter does not confer tissue specificity on gene expression, suggesting that there are regulatory elements outside the upstream region. Analysis of potential regulatory elements defined as DNase 1-hypersensitive sites within introns of the gene revealed multiple predicted binding sites for the HNF1α (hepatocyte nuclear factor 1α) transcription factor. HNF1α, which is expressed in many of the same epithelial cell types as CFTR and shows similar differentiation-dependent changes in gene expression, bound to these sites *in vitro*. Overexpression of heterologous HNF1α augmented *CFTR* transcription *in vivo*. In contrast, antisense inhibition of *HNF1*α transcription decreased the CFTR mRNA levels. *Hnf1*α knockout mice showed lower levels of CFTR mRNA in their small intestine in comparison with wild-type mice. This is the first report of a transcription factor, which confers tissue specificity on the expression of this important disease-associated gene.

Key words: CFTR (cystic fibrosis transmembrane conductance regulator), HNF1α (hepatocyte nuclear factor 1α), intronic regulatory elements.

INTRODUCTION

The *CFTR* (cystic fibrosis transmembrane conductance regulator) gene shows a complex pattern of expression with temporal and spatial control. Elucidation of critical *CFTR* regulatory elements would enable novel approaches to targeted gene therapy and facilitate development of alternative therapeutic strategies aimed at the manipulation of *CFTR* expression levels. The proximal promoter of the *CFTR* gene lacks detectable tissue-specific and temporal control elements. Hence, it is probable that these elements lie in adjacent 5′ or 3′ sequences or elsewhere in the gene. We previously screened 400 kb encompassing *CFTR* and flanking regions for DHS (DNase I-hypersensitive sites), which are often associated with regulatory elements. DHS were identified 5′ and 3′ to the gene [1,2] and within introns 1 [3], 2, 3, 10, 16, 17a, 18, 20 and 21 [3]. Several DHS show appropriate cell-type specificity [3,4]. The nature and precise mechanisms of action of potential regulatory elements located at each of these sites remains to be elucidated. Our current model for spatial and temporal regulation of *CFTR* expression proposes that multiple regulatory elements in chromatin interact *in vivo*. Specific cell types exhibit different sets of DHS within the gene, suggesting that the expression may be regulated by distinct, although overlapping, sets of transcription factors [4]. To test this hypothesis, regions of *CFTR* that exhibit DHS were evaluated for binding to transcription factors.

The *CFTR* promoter lacks a TATA box and contains AP-1 (activator protein-1), Sp1 (specific protein-1), CRE (cAMP-response element), C/EBP (CCAAT-enhancer-binding protein) and glucocorticoid response elements [5–9]. Regulation of *CFTR* expression by cAMP has been described in [8,10]. Also, binding of C/EBP, ATF/CREB (where ATF stands for activating transcription factor and CREB for CRE-binding protein) and CDP (CAATT displacement protein) to an inverted CCAAT-like element (Y box) in the promoter has been implicated in the regulation of *CFTR* expression [9,11]. A polymorphic YY1 (Ying/Yang 1) site has been identified, which alters activity of the CFTR promoter *in vitro* [12]. However, none of these elements confers tissue-specific regulation on *CFTR* expression. We investigated the transcription factor binding to the core elements in the DHS in introns 10, 17a and 20 of the *CFTR* gene. DHS in all three introns were identified in Caco2 colon carcinoma cells. The intron 17a DHS was also seen in other intestinal, pancreatic and airway cell lines and the intron 20 DHS was evident in airway cell lines and primary human genital duct epithelial cells [13]. Evaluation of cross-species homology between sheep, cow, pig and human *CFTR* showed a high degree of conservation between several predicted regulatory elements enabling finer mapping of the functionally important sequences [14,15].

We identified multiple binding sites for HNF1α (hepatocyte nuclear factor 1α) in the DHS core and showed that these sites bind HNF1α *in vitro*. HNF1α is a homeodomain-containing transcription factor that, in addition to being expressed in the liver, has important functions in regulating gene expression elsewhere in the digestive tract, including the pancreas and intestine (reviewed in [16]). *HNF1*α and *CFTR* show similar expression profiles during post-confluence differentiation of Caco2 colon carcinoma cells. Overexpression of HNF1α before confluence in these cells augments CFTR mRNA levels and antisense inhibition of HNF1α mRNA in a pancreatic cell line down-regulates the *CFTR* expression. *Hnf1*α null mice [17] showed decreased levels of CFTR mRNA in their small intestine in comparison with the WT (wild-type) mice. This is the first identification of a transcription factor, which affects tissue-specific control of *CFTR* expression.

EXPERIMENTAL

Cell culture

The following cell lines were analysed: colon carcinoma Caco2 [18], pancreatic adenocarcinomas Capan1 [18] and NP31 [19],

Abbreviations used: C/EBP, CCAAT-enhancer-binding protein; CFTR, cystic fibrosis transmembrane conductance regulator; CREB, cAMP-response-element-binding protein; DHS, DNase I-hypersensitive sites; EMSA, electrophoretic mobility-shift assay; FAM, 6-carboxy-fluorescein; FP, footprint; HNF, hepatocyte nuclear factor; KO, knockout; Oct-1, octamer-binding protein-1; RT, reverse transcriptase; Sp1, specific protein-1; WT, wild-type.

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Table 1 Primers used to synthesize and clone overlapping DNA fragments of DHS10A, DHS10B, DHS10C, DHS17a and DHS20 of the human *CFTR* gene

Name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Size (bp)	Location
10AB-A	GTATAGTAGCTGTATACA	GGGTAAGAAGCTTGGTTG	290	112 281–112 571*
10AB-B	AAGTGCTTAGTAATTATC	TTAATGGTCCTGTGTAAT	286	112 439–112 725*
10AB-C	CCAACCAAGCTTCTTACC	GTTAAGGAGTAATTGCTA	316	112 553–112 869*
10AB-D	GCTCAAATGTACTCAGTA	AGTGCATAACAACATATC	300	112 679–112 979*
10AB-E	CTTTGTATGTGCAAATCA	GATTCTGTGTATACAACA	301	112 815–113 116*
10AB-F	TGTTGTTATGCACTCCTT	GGACAGGGTAATCTATCT	286	112 966–113 252*
10AB-G	AATGTGTTGTATACACAG	TCTCAGCATGGTTTAATC	296	113 095–113 391*
10C-A	GGGACAGGGTTTCACTATG	GCTCTCTGACTCAATTCCTC	294	121 647–121 941*
10C-B	GTTACATTTTATCTCATTGC	GAGTTGAAATCTGAATTATG	319	121 802–122 121*
10C-C	ACAATAAGCCAGACAGACCA	CCTTGTCCCAGATTAGAGGG	299	121 962–122 261*
10C-D	AGCAGTGACTCAGGAGAAAG	ACCACAAGCTACTCTGATTC	299	122 122–122 421*
17a-C	GTGACAGCCTTAGTGTGG	TTCACACTGGTGCCATTC	312	1491–1803†
17a-D	CTCCCAGTGGTAGCCAAG	ATTGGACTCCTGCCTGTG	284	1662–1946†
20-A	TACATTGTTTTGTGCTGA	CCTCCAATATTATACTAA	317	36 348–36 665†
20-B	GGTAGTAACTAATTCATT	CTGGAATACCCTCTCCAC	299	36 498–36 797†
20-C	TTAGTATAATATTGGAGG	CCATTCCTCGTGAAGCCA	248	36 648–36 896†
20-D	GATGCCAGACAGGGAGAT	CTTATATTACTCCAGACT	299	36 798–37 097†

* Locations on ac000111.
† Locations on ac000061.

Calu3 lung adenocarcinoma [20] and MCF7 mammary gland carcinoma [21]. The Caco2, Calu3 and MCF7 cell lines were grown in Dulbecco's modified Eagle's medium, Capan1 and NP31 in RPMI 1640.

Mouse tissues

Hnf1α knockout mice, segregating a *hnf1α* null allele on a C57BL/6J background, have been described previously [17,22]. Tissues from the proximal region of the small intestine of mice and their WT littermates were kindly provided by Dr J. Ferrer (Hospital Clinic i Universitari, Barcelona, Spain); the strain was generated in the laboratory of Dr F. Gonzalez (National Cancer Institute, Bethesda, MD, U.S.A.). Genotypes were confirmed locally.

Preparation of nuclear extracts

Nuclear extracts were prepared by standard methods [45] and protein concentrations were determined using a Bio-Rad DC Protein assay kit.

DNase I footprinting

To localize putative regulatory elements by *in vitro* DNA footprinting, primers were designed to clone overlapping DNA fragments of approx. 300 bp covering the area of each DHS (Table 1). Fragments were amplified using plaque-forming units-DNA polymerase (Stratagene) and cloned into the *Srf*I site of pPCR-script Amp SK with the PCR-Script™ Amp cloning kit (Stratagene). Inserts were sequence-verified. FP (footprint) probes were excised from the vector, with *Cla*I and *Sac*I or with *Not*I and *Eco*RI to label the sense or antisense strand respectively and gel-purified. The probe (1–3 pmol) was labelled by the incorporation of 25 μCi [α-^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences, Chalfont St. Giles, Bucks., U.K.) with 2 units of Klenow DNA polymerase (Roche, Welwyn Garden City, Hertfordshire, U.K.) for 30 min at room temperature (20 °C). After an additional 5 min incubation with 0.1 mM ddCTP, the reaction was stopped at 70 °C for 5 min. Blunt-end probes were labelled with 25 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Biosciences) using polynucleotide kinase at 37 °C for 30 min. Unincorporated nucleotides were removed with G25 spin-columns (Amersham Biosciences). Labelled probes were incubated with nuclear extracts in binding buffer [20 mM Hepes (pH 7.8)/20 % (v/v) glycerol/100 mM KCl/0.2 mM EDTA/0.2 mM dithiothreitol] for 15 min at room temperature. DNA was digested with 0.2–3 units DNase I (Amersham Biosciences) for 2 min at room temperature, the reaction was stopped and samples were phenol/chloroform extracted and precipitated with ethanol. Samples were analysed on a 5 % (acrylamide/bisacrylamide, 20:1) gel.

Electrophoretic mobility-shift assays (EMSAs)

EMSA probes were designed on the basis of the DNase I footprinting results and are summarized in Table 2. Complementary oligonucleotides with or without a short 5′ overhang, as described, were annealed to generate double-stranded probes. EMSAs were performed by incubation of the probes with nuclear extracts in binding buffer (as described for DNase I footprinting) for 15 min with or without competing oligos or antibodies. Samples were then directly loaded on 0.5× TBE (0.0445 M Tris/0.0445 M borate/1.25 M EDTA) 4 % (acrylamide/bisacrylamide, 79:1) PAGE gels and separated at 25 V/cm at 4 °C. For supershift reactions, nuclear extracts and probes were incubated for 10 min, followed by 20 min incubation with 2 μg of specific antibody (as described below in the Western-blot method).

Semi-quantitatative RT (reverse transcriptase)–PCRs for HNF1α and *CFTR*

Total RNA was isolated from approx. 100 mg of cells using RNA isolator (Sigma-GenoSys, Pampisford, Cambridge, U.K.). RT–PCR was performed as described previously [23–25]. The E1R and E1L primers for *CFTR* amplify a fragment of 768 bp and the 9B and 67A primers for β-glucocerebrosidase amplify a fragment of 572 bp. The human HNF1α-F 5′-TCTACAACTGGTTTGCCAACC-3′ and HNF1α-R 5′-GGCTTCTGTACTCAGCAGGC-3′ primers amplify a product of 311 bp [26], the forward primer is identical with both human and mouse sequences, whereas the reverse primer is not. The mouse-specific HNF1α reverse primer was 5′-GGCCTCTGTGCTCAGCAGGC-3′. β-Glucocerebrosidase was co-amplified as a housekeeping gene control with

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Table 2 Sequences of gel shift probes after labelling

Filled 5′-overhangs are indicated by lower-case letters. Primers that contain mutations (underlined) altering a putative HNF1 transcription factor-binding site are denoted by m.

Name	DNA sequence (5′ to 3′)	Location
10AB-FP1	tgcTTAGTAATTATCAAATATTAATAACAATGACActa	112 442–112 479*
10AB-FP1m†	tgcTTAGTAAT**GCCA**AAATATTAATAACAATGACActa	112 442–112 479*
10AB-FP5	tagATAAAGTAAGTTAATCTTTATTGCACACTTATcta	112 988–113 019*
10AB-FP5m	tagATAAAGTAAGTTAATCTT**GCCA**GCACACTTATcta	112 988–113 019*
10AB-FP4	agagTTAATAAGCTTTATATTAGCAATTACTCctta	112 832–112 867*
10C-FP2	ccagCTTTGTTACTTATTGAGCAACCACTACAAGcaca	122 022–122 066*
10C-FP2m	ccagCTTTGTTACTTAT**GCCA**CAACCACTACAAGcaca	122 022–122 066*
10C-FP3	tctgGGACAAGGGGGAGGAGAGTACTGTCTCTTATcag	122 250–122 287*
10C-FP3m5	tctgGGACAAGGG**TT**AGGAGAGTACTGTCTCTTATcag	122 250–122 287*
17a-FP2	ctgcTTTGTAGATTATTAGCTaaatca	1543–1569‡
17a-FP2m	ctgcTTTGTAGAT**ATGC**AGCTaaatca	1543–1569‡
17a-FP3	aagagGGTAACTCATTAATAAAATAACAaatc	1741–1772‡
17a-FP3m	aagagG**TAGC**CTCATTAATAAAA**GCTA**Aaatc	1741–1772‡
20-FP1	tttcTGAGGATTAAATGAGTTAATAACTAGTACTCctc	36 521–36 558‡
20-FP1m	TttgTGAGGATTAAATGAGTTAATAAC**GCCA**ACTCctc	36 521–36 558‡
20-FP3	tagAAAAAGCTGAGGGGCAAAAGGTCAGAGGAGGCcac	36 841–36 878‡
20-FP4	TtagGAAAAAGAAGTTAATACTTGACAAGTGCCAAcatg	36 892–36 930‡
20-FP4m	ttagGAAAAAGAAGTTAATACT**GCCA**AAGTGCCAAcatg	36 892–36 930‡
HNF1	ATCGGGTTAATGATTAACACGTA	
SP1	ATTCGATCGGGGCGGGGCGAGC	
Scrambled	atgATAATGTTAAAATTCAAATTCTTACAACTAAGcat	

* Primer locations correspond to ac000111.
‡ Primer locations correspond to ac000061.

CFTR or with HNF1α. cDNA synthesis reactions for both test and control with specific 3′-primers were performed in the same tube, then cDNA was split into two equal aliquots and PCR with each specific primer set was performed independently. Each RT–PCR assay was performed at least three times on separate occasions and in all cases the results were consistent. PCR assays were evaluated in exponential phase. RT–PCR products were loaded on agarose gels, normalizing for a β-glucocerebrosidase product, and digital images of ethidium bromide-stained gels were analysed by ImageQuant (Amersham Biosciences). Results are presented as Excel bar charts.

Generation of cell lines carrying stably transfected HNF1α cDNAs and HNF1α antisense ribozyme

The mouse HNFα cDNA was donated by Dr G. Crabtree (Stanford University, Stanford, CA, U.S.A.) and the HNF1α ribozyme [33] by Dr C. Hu (Washington University School of Medicine, St. Louis, MO, U.S.A.). The HNFα cDNA is in a vector (pBJ5) that lacks a mammalian-selectable marker and therefore was co-transfected with 1/20th the amount of pSV2neo [27]. The HNF1α ribozyme is in pcDNA3. DNA was transfected into the Caco2 and MCF7 cell lines with LIPOFECTAMINE™ or Lipofectin (Invitrogen, Paisley, Renfrewshire, Scotland, U.K.) and into the Capan1 cell line with Effectene (Qiagen, Crawley, West Sussex, U.K.) according to the manufacturer's instructions. G418-resistant clones were selected after 3–5 weeks.

Western blots

Proteins in the nuclear extracts were resolved using SDS/PAGE (10% gel) [28]. Proteins were electrophoretically transferred to Hybond-C Super membranes (Amersham Biosciences), and blocked in 5% (w/v) fat-free, dried, skimmed milk (Marvel, St. Albans, Herts., U.K.) in PBS (140 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_3/1.4 mM KH_2PO_3, pH 7.3) for 1 h. Immunodetection with the antibody was performed as described previously [29]. The HNF1α/HNF1β (sc8986), HNF1α (sc6548) Sp1 (sc420), Oct-1 (octamer-binding protein-1; sc232) and C/EBPβ (sc-746) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence reagents (ECL®) and ECL-sensitive film (Amersham Biosciences) were used for the final detection of antibodies.

Real-time quantitative RT–PCR (TaqMan) using the ABI Prism 7700 Sequence Detection System

Primer and probe sequences were designed using the human *CFTR* (GenBank no. M28668) and mouse *cftr* cDNA sequence (GenBank accession no. M60493) and ABI Primer Express 1.0 software. The human CFTR 5′-primer was located in exon 5, TAQEX5F 5′-AGCTGTCAAGCCGTGTTCTAGATA-3′ and the 3′-primer in exon 6a, TAQEX6AR 5′-ATGAGGAGTGCCACTTGCAAA-3′. The human CFTR probe spanned the exon 5/exon 6a boundary, 5′-CACACGAAATGTGCCAATGCAAGTCCTT-3′ and contained fluorophore FAM (6-carboxy-fluorescein) at the 5′-end and quencher TAMRA at the 3′-end.

The mouse cftr 5′-primer, located in exon 16, was m*cftr*F2996 5′-ACGCCCCTATGTCGACCAT-3′ and the 3′-primer in exon 17a was m*cftr*R3146 5′-ACGACTATTATAGCTCCAATCACAATGA-3′; the mouse *cftr* TaqMan probe, spanning the exon 16/exon 17a boundary, was 5′-AGAATCTGTTAAGAATCCCACCTGCTTTCA-3′ and contained fluorophore FAM at the 5′-end and quencher TAMRA at the 3′-end. rRNA primer (18 S) and probes (5′-primer, 5′-CGGCTACCACATCCAAGGAA-3′; 3′-primer, 5′-GCTGGAATTACCGCGGCT-3′; and 5′-FAM, 3′-TAMRA-modified probe 5′-TGCTGGCACCAGACTTGCCCTC-3′) were supplied by MWG-Biotech (Milton Keynes, U.K.). A one-step RT–PCR approach was adopted for quantitative analysis. Independent assays were performed on each experimental sample to determine levels of both CFTR/cftr mRNA and 18 S rRNA. Variations in RNA loading between samples were controlled by normalizing levels of CFTR/cftr mRNA to levels of 18 S rRNA. CFTR/Cftr and 18 S rRNA RT–PCR assays (final volume, 25 μl) were performed using the TaqMan Gold RT–PCR kit (Applied Biosystems, Warrington, Cheshire, U.K.) and contained 1$\times$ buffer A, 5.5 mM $MgCl_2$, 0.3 mM each of dATP, dCTP, dGTP and 0.6 mM dUTP, 0.025 unit/μl AmpliTaq Gold, 0.25 unit/μl MultiScribe RT, 0.2 unit/μl RNase inhibitor, 300 nM of F primer and 900 nM R primer for human CFTR and 300 nM each of 5′- and 3′-primers for mouse cftr and 100 nM probe, and included 50 ng of experimental total RNA template for CFTR. The human rRNA RT–PCR assays used 300 nM F primer, 900 nM R primer, 0.5 ng RNA and 5.5 mM $MgCl_2$. The mouse rRNA assay used 300 nM F and R primers. Experimental total RNA samples were diluted to approx. 10 ng/μl for the CFTR assays and to 0.01 ng/μl for the 18 S rRNA assays. Reactions were performed in MicroAmp Optical 96-well plates covered with MicroAmp Optical caps (Applied Biosystems). The TaqMan RT conditions were 48 °C for 30 min followed by AmpliTaq Gold activation at 95 °C for 10 min and 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min.

Results were analysed using ABI Sequence Detector software version 1.6.3. Relative standard curves for cftr/CFTR and 18 S rRNA levels were generated from seven, sequential, 5-fold dilutions of a common arbitrary sample of mouse intestinal RNA or Caco2 RNA for mouse and human assays respectively. The arbitrary values determined for cftr/CFTR were divided by the appropriate 18 S rRNA value to give an expression ratio. Standards and experimental samples were assayed three times

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Table 3 Location of DNase I FPs

The transcription factors binding to DHS10A FP2 and FP3, DHS10B FP4, DHS10C FP1 and DHS20 FP2 have not yet been identified. CDP, CAATT displacement protein; ISL1, islet-1 transcription factor; GKLF, gut-enriched Krüppel-like factor; ARP1, apolipoprotein A1 regulatory protein-1.

Name	Intron	Sequence (5′-3′)	Location	Factor
10A-FP1	10	TTATCAAATATTAATAACAATGACA	112 452–112 476*	HNF1
10B-FP5	10	AATAAAGTAAGTTAATCTTTATTGCACACTTATTATAA	112 987–113 024*	HNF1
10C-FP2	10	ACTTATTGAGCAACCACT	122 033–122 050*	HNF1
10C-FP3	10	GGACAAGGGGGAGGAGAGTACTGTCTC	122 254–122 280*	Sp1
17a-FP2	17a	CAGTATATTTCTGCTTTGTAGATTATTAGCTAAATCAAGTCACATAAACTTCCTTAATTTAGATAC	1533–1598†	HNF1 (CREB, CDP)‡
17a-FP3	17a	GTAAAAAAAGAGGGTAACTCATTAATAAAATAACAAATCATATCT	1734–1778†	HNF1(× 2) (ISL1, GKLF)‡
20-FP1	20	TGTGAGGATTAAATGAGTTAATAACTAGTA	36 523–36 552†	HNF1
20-FP3	20	AGAAAAAGCTGAGGGGCAAAAGGTCAGAGGAGGC	36 842–36 875†	ARP1
20-FP4	20	GAAAAAGAAGTTAATACTTGACAAGTGCCAAC	36 895–36 927†	HNF1

* Location of DNase I FPs on ac000111.
† Location of DNase I FPs on ac000061.
‡ These TRANSFAC-predicted sites have not been evaluated further.

with each sample analysed in triplicate in each experiment. Reaction controls were performed which omitted RT and/or template RNA. Standard curves were fitted by linear regression and goodness of fit was reported as the coefficient of determination (R^2). Data were analysed by standard statistical software.

RESULTS

Core regions of the DHS in introns 10, 17a and 20 contain multiple predicted binding sites for HNF1

Three DHS in intron 10 at 1716 + 13.2 kb (DHS10A),+ 13.7 kb (DHS10B) and + 23 kb (DHS10C) (1716 is the last base in exon 10), one in intron 17a at 3271 + 0.7 kb (3271 is the last base in exon 17a) and one in intron 20 at 4005 + 3.7 kb (4005 is the last base in exon 20) [13], were evaluated to identify binding sites of putative transcriptional regulators. Overlapping 250–300 bp fragments spanning each DHS were investigated by *in vitro* DNase I footprinting using nuclear extracts from Caco2 cells that express abundant CFTR. Protected regions, indicating protein–DNA interactions, were found within each of the DHS. The locations of the DNase 1 FPs and the predicted transcription factor binding sites that coincide with them, derived from the Transfac 4.0 database [30], are shown in Table 3. Seven of the DNase I FPs correspond to predicted binding sites for the hepatic nuclear transcription factor HNF1. DNase I FPs in introns 10A, 10B and 10C that coincide with HNF1-binding motifs are shown in Figure 1 and those in intron 17a in Figures 3(A) and 3(B).

HNF1α binds the DHS in introns 10, 17a and 20 *in vitro*

Putative HNF1-binding motifs identified by DNAse 1 footprinting were evaluated for binding to HNF1 *in vitro* by performing EMSA experiments with oligonucleotide probes containing the protected regions. Figures 2, 3(C) and 3(D) show evidence that proteins in nuclear extracts from Caco2 cells bind specifically to probes corresponding to FP1 in DHS10A, FP5 in DHS10B, FP2 in DHS10C, FP1 and FP4 in DHS20, and FP2 and FP3 in DHS17a. Addition of Caco2 nuclear extracts produced DNA–protein complexes (lanes 2) with reduced gel mobility compared with the probe alone (lanes 1). Formation of the two complexes (I and II) was specifically inhibited by addition of unlabelled autologous probe (lanes 3) or by a probe containing the HNF1 consensus sequence (lanes 5), but not by a control probe containing a mutation in the HNF1 consensus site [31] (lanes 4). The specificity of HNF1 binding was confirmed using an antibody against HNF1, which resulted in a super-shifted complex (III) (lanes 6) compared with the addition of an unrelated antibody specific for C/EBP (Figure 2, lanes 7) or Oct-1 (Figures 3C and 3D, lanes 7). Hence, HNF1 binds *in vitro* to the predicted motifs in DHS10, DHS17a and DHS20 of the *CFTR* gene.

Correlation between HNF1 and *CFTR* expression *in vivo*

The colon carcinoma cell line Caco2 spontaneously differentiates from crypt- to villous-like enterocytes on reaching confluence [32]. We have previously shown that CFTR mRNA expression is low in Caco2 cells before confluence, but rises substantially after confluence [24]. Others have reported similar confluence and differentiation-related alterations in the expression of HNF1α in Caco2 cells [33]. To determine whether HNF1α protein and CFTR mRNA show co-ordinate expression patterns in the same Caco2 cell population, the expression levels of each gene were evaluated at increasing confluence by Western blot and RT–PCR respectively.

Figure 4(A) shows a Western blot in which HNF1 protein was detected in 20 μg of nuclear extract isolated from Caco2 cells at different levels of confluence, using an antibody that binds to HNF1α and HNF1β. The amounts of both HNF1α (α) and HNF1β (β) increase as the cells approach confluence (0) and plateau approx. 4 days after confluence is reached (4). Figure 4(B) shows CFTR mRNA expression detected by a semi-quantitative RT–PCR assay in which the 768 bp *CFTR*-specific product is compared with the 572 bp β-glucocerebrosidase house-keeping gene product. Expression of β-glucocerebrosidase is independent of Caco2 confluence. Assays were performed by synthesizing cDNA in a single reaction containing specific complementary primers for both CFTR and β-glucocerebrosidase. The cDNA sample was divided for analysis by specific PCRs. Temporal and kinetic aspects of the confluence-dependent increase in expression of CFTR mRNA corresponded closely to the increase observed for HNF1 protein expression.

CFTR expression in other cell lines that contain HNF1

Figure 4(A) also shows the presence of HNF1 in nuclear extracts of several cell lines that have been previously evaluated for *CFTR* expression [1,3,4]. The MCF7 breast carcinoma cell line does

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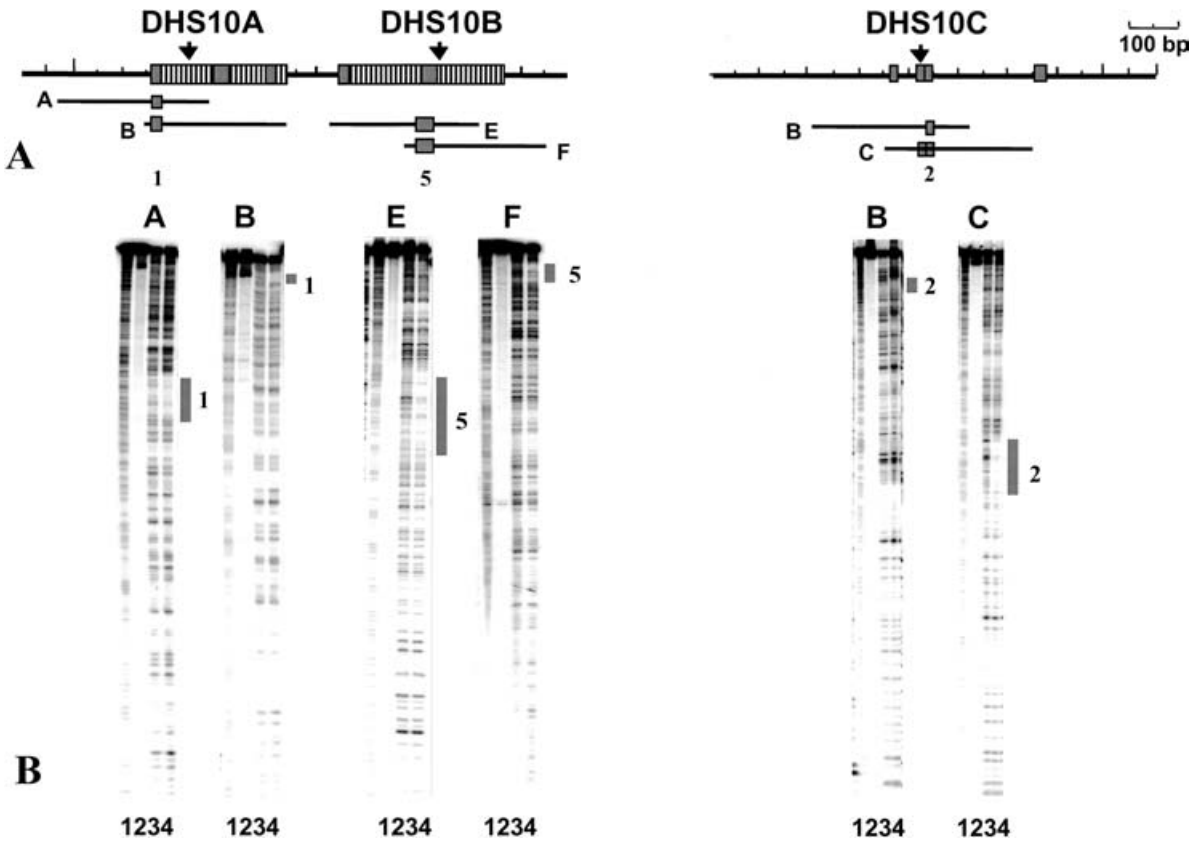

Figure 1 Identification of predicted binding sites for HNF1 in DHS10A, DHS10B and DHS10C by DNase I footprinting

(**A**) Linear maps of the regions of DHS at intron 10A, 10B and 10C with each DHS marked above the line. Below the line are shown the probes used in footprinting experiments. The stippled boxes represent DNase I FPs and the striped bars on the gene denote regions of $>70\%$ homology between the human and sheep *CFTR* sequences. (**B**) DNase I FP reactions for DHS10A and DHS10B with probes A, B, E and F, and DHS10C with probes B and C. FPs corresponding to HNF1-binding motifs are numbered according to (**A**) and are shown as stippled lines. On each gel: lane 1, AG ladder; lane 2, probe alone; lane 3, probe with DNase I; and lane 4, probe with DNase I and 40 μg of nuclear extract from Caco2 cells.

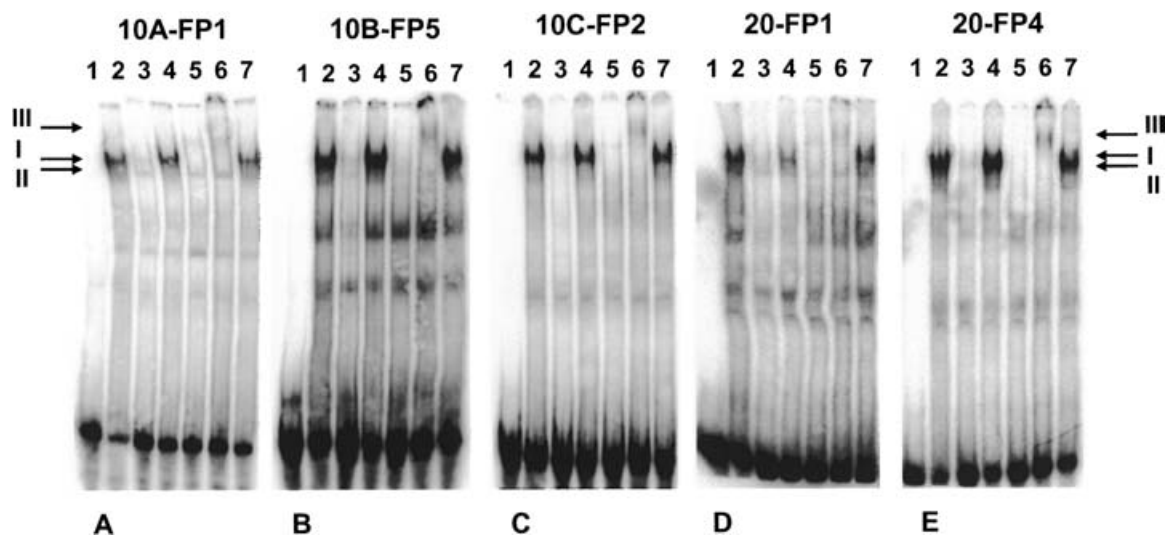

Figure 2 EMSAs demonstrate binding of HNF1 to DNase I FPs in DHS10A, DHS10B, DHS10C and DHS20

Sequences of the probes used are shown in Table 2: (**A**) DHS10A FP1, (**B**) DHS10B FP5, (**C**) DHS10C FP2, (**D**) DHS20 FP1 and (**E**) DHS20 FP4. On each gel: lane 1, probe alone; lane 2, probe with 15 μg of Caco2 nuclear extract; lane 3, probe with 15 μg of Caco2 nuclear extract and competition with 20-fold excess of cold probe; lane 4, probe with 15 μg of Caco2 nuclear extract and competition with 20-fold excess of an oligonucleotide homologous with the probe but with a mutation destroying the predicted HNF1-binding motif; lane 5, probe with 15 μg of Caco2 nuclear extract and competition with 20-fold excess of HNF1 consensus sequence oligonucleotide; lane 6, supershift reaction with 2 μg of an antibody specific for HNF1; and lane 7, supershift reaction with 2 μg of an irrelevant antibody to C/EBP. Arrows I and II show the major specific complexes formed by interaction of the probes with proteins in the Caco2 nuclear extracts and III is the supershifted complex.

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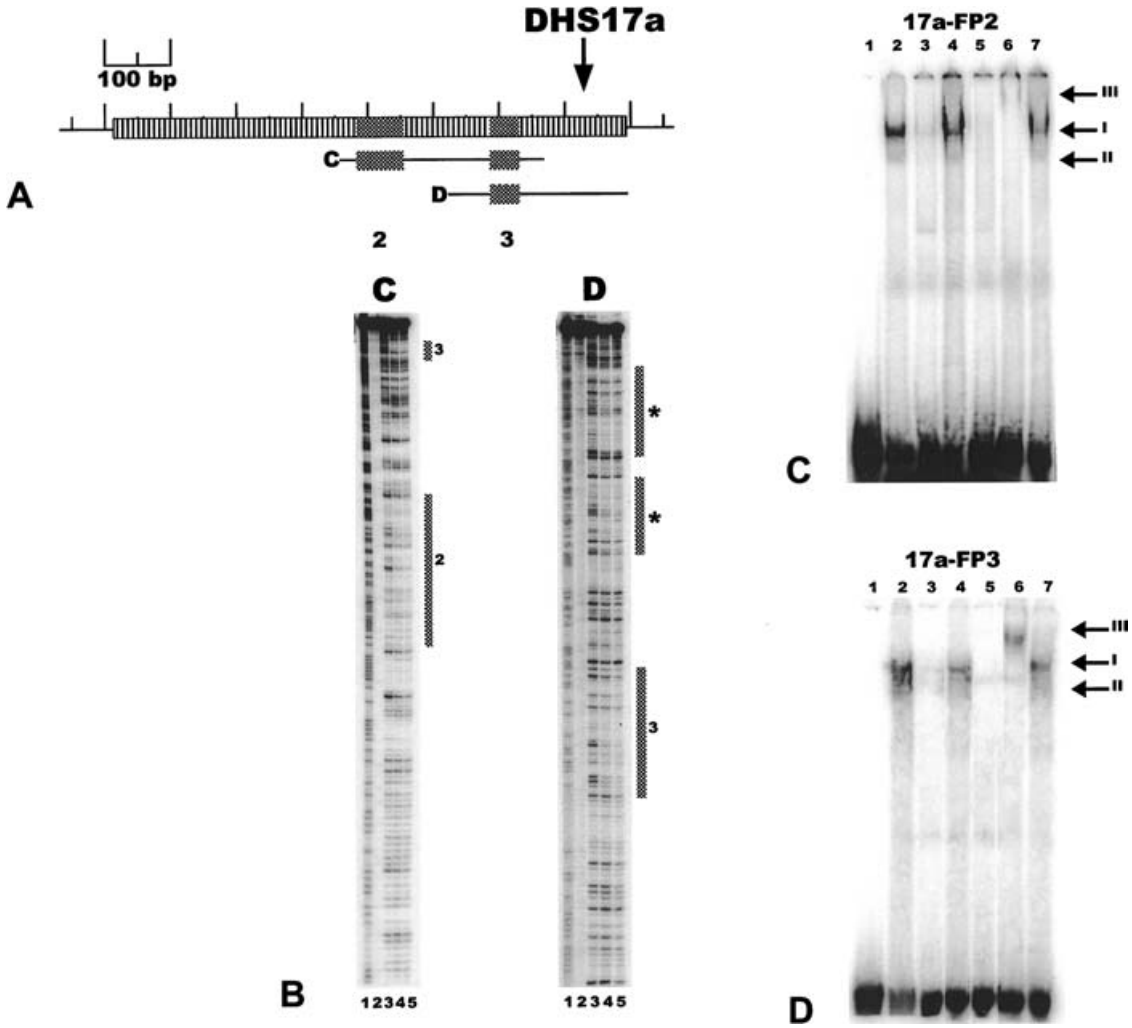

Figure 3 Identification of HNF1-binding sites in DHS17a by DNase I footprinting and EMSAs

(**A**) Linear map of the region of intron 17a that encompasses the DHS (marked above the line), the striped bar on the gene denotes a region of > 70 % homology between the human and sheep *CFTR* sequences. Below the line are shown the probes used in footprinting experiments. The stippled boxes represent DNase I FPs. (**B**) DNase I FP reactions for DHS17a, probes C and D, FPs corresponding to HNF1-binding motifs are numbered according to (**A**) and are shown as stippled lines. Stippled boxes marked with * denote other FPs. On each gel: lane 1, AG ladder; lane 2, probe alone; lane 3, probe with DNase I; and lanes 4, 5 probe with DNase I and 30 and 60 μg of nuclear extract respectively from Caco2 cells. (**C**, **D**) EMSAs of probes shown in Table 2. On each gel: lane 1, probe alone; lane 2, probe with 15 μg of Caco2 nuclear extract; lane 3, probe with 15 μg of Caco2 nuclear extract and competition with 100-fold excess of cold probe; lane 4, probe with 15 μg of Caco2 nuclear extract and competition with 100-fold excess of an oligonucleotide homologous with the probe but with a mutation destroying the predicted HNF1α-binding motif; lane 5, probe with 15 μg of Caco2 nuclear extract and competition with 100-fold excess of HNF1α consensus sequence oligonucleotide; lane 6, supershift reaction with 2 μg of an antibody specific for HNF1; and lane 7 supershift reaction with 2 μg of an irrelevant antibody to Oct-1. Arrowed complexes I and II are the major specific complexes formed by interaction of the probes with proteins in the Caco2 nuclear extracts and III is the supershifted complex.

not express *CFTR* and lacks HNF1 protein. Calu3 is a lung adenocarcinoma cell line, which expressed abundant CFTR and is seen here to express HNF1α and HNF1β. NP31 and Capan1 are pancreatic adenocarcinoma cell lines that express low levels of CFTR in comparison with those detected in Caco2 post-confluence. HNF1α and HNF1β are just detectable in the Capan1 cell line but not in NP31, hence the expression of *CFTR* is not solely dependent on HNF1 expression in all cell types.

Overexpression of HNF1α augments *CFTR* expression levels *in vivo*

To investigate whether overexpression of HNF1 altered *CFTR* expression levels *in vivo*, Caco2 cells were stably transfected with a mouse hnf1α cDNA. Since we have shown that both HNF1α and CFTR mRNA levels are low before confluence in this cell line (Figure 4), we investigated the effect of *HNF1*α expression from the transgene before confluence. The transgene promoter is not subject to the same regulation as the endogenous *HNF1*α gene, so it was predicted that higher levels of *HNF1*α expression before confluence would augment *CFTR* expression levels. Figure 5 shows expression of HNF1α in a Caco2 clone, CaH11, which expresses high levels of the mouse hnf1α in contrast with the control vector-transfected CaP2 clone. The primers used for the RT–PCR were 100 % homologous with mouse hnf1α and detected the mouse transcript with higher efficiency when compared with the endogenous human HNF1α. The mouse hnf1α mRNA was distinguished from the endogenous human *HNF1*α transcript by restriction enzyme digestion (*Pst*I cleaves the human cDNA only and *Stu*I is specific for the mouse gene; results not shown). The bar charts in Figure 5 of ImageQuant-based quantification of

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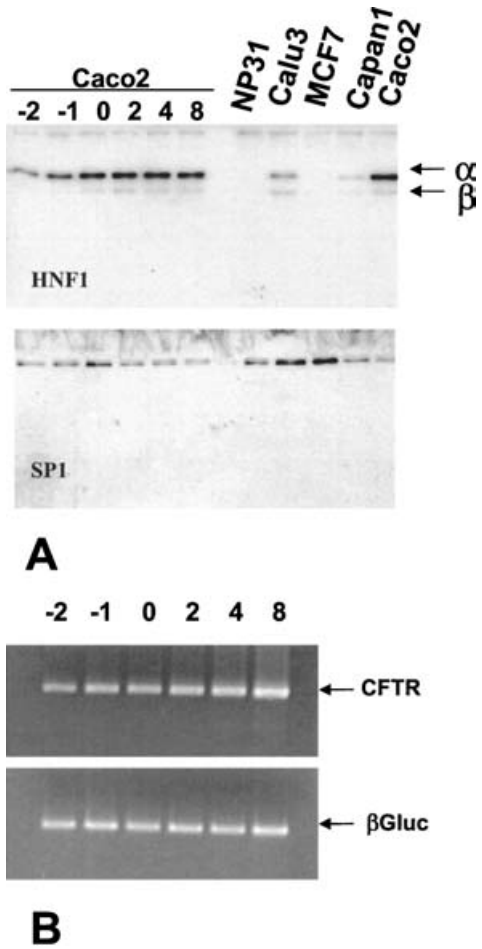

Figure 4 Levels of CFTR mRNA and HNF1 protein show a parallel increase post-confluence in Caco2 cells

CFTR and HNF1 expression evaluated in RNA or nuclear extracts made in parallel from cultures of Caco2 cells at the following levels of confluence: — 2 days, 50 % confluence; — 1 day, 80 % confluence; 0, confluence; and 2, 4 and 8 days, post-confluence. (**A**) Western blot of Caco2 nuclear extracts (20 μg): the upper panel is probed with an antibody specific for HNF1 and the lower panel with an irrelevant antibody to Sp1. Also shown are 20 μg of nuclear extracts from NP31, Calu3, MCF7 and Capan1 cell lines. (**B**) Semi-quantitative RT–PCR for CFTR in Caco2 RNA. The 768 bp CFTR-specific product in the upper panel is compared with the 572 bp β-glucocerebrosidase housekeeping gene product in the lower panel.

CFTR and HNF1 normalized to β-glucocerebrosidase show that increased levels of HNF1α mRNA expression are observed when the CaH11 clone is 50 % and particularly 70 % confluent (b) in comparison with the control clone CaP2. Further, the increase in *HNF1α* expression correlates with augmented expression of *CFTR* (a) in comparison with the control clone CaP2. A 70 % increase in HNF1α levels at 50 % confluence and an 83 % increase at 70 % confluence are associated with 78 and 183 % respective increases in *CFTR* expression at the same points of confluence. An additional Caco2 clone carrying the mouse *hnf1α* gene (CaH10) showed equivalent up-regulation of *CFTR* expression (results not shown). These experiments were performed in triplicate, with RNA extracted from two independent cell culture confluence series, and were reproducible. Overexpression of the mouse hnf1α cDNA at confluence no longer influenced *CFTR* expression in CaH11 cells (results not shown). This is not surprising, if the endogenous HNF1α expression in confluent Caco2 cells is sufficient for maximal activation of *CFTR* expression.

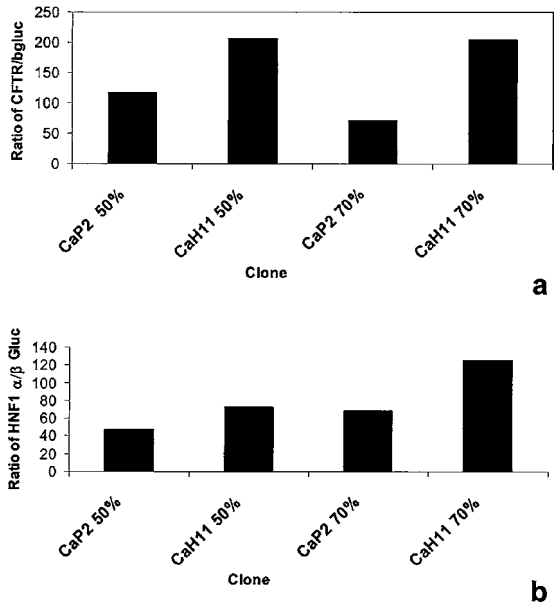

Figure 5 HNF1α overexpression augments levels of CFTR mRNA in Caco2 cells

Bar charts showing the ratios of *CFTR* (**a**) and *HNF1α* (**b**) expression compared with the β-glucocerebrosidase control. *HNF1α* and *CFTR* expression were evaluated by semi-quantitative RT–PCR in Caco2 cells transfected with mouse HNF1α (CaH11) or the pBJ5 vector (CaP2) at 50 and 70 % confluence. The primer pair for HNF1α uses the 3′-primer homologous with the mouse gene.

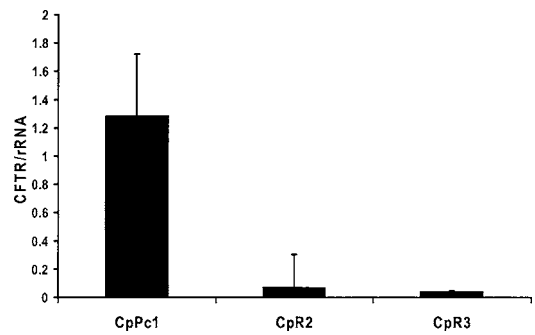

Figure 6 HNF1α antisense ribozyme decreases levels of CFTR mRNA in Capan1 cells

CFTR expression measured by quantitative RT–PCR using an ABI Prism 7700 sequence detection system in Capan1 clones that contain the pcDNA3 vector (CpPc1) or express the HNF1α antisense ribozyme (CpR2 and CpR3). Mean values of triplicate assays are shown and data are derived from Ct (threshold cycle) values produced for a CFTR assay normalized to 18 S rRNA.

Antisense-mediated inhibition of HNF1α decreases CFTR expression levels *in vivo*

To determine whether a decrease in HNF1α levels influences CFTR mRNA expression *in vivo*, constructs containing an HNF1α antisense ribozyme [33] were introduced into Capan1 cells. Two Capan1 clones that expressed the ribozyme (CpR2 and CpR3) and showed a decrease in HNF1α as evaluated by a semi-quantitative RT–PCR (results not shown) were analysed further. *CFTR* expression was compared in these lines and a pcDNA3 vector-only transfected line (CpPc1). Figure 6 shows quantitative TaqMan RT–PCR assays for CFTR mRNA normalized to rRNA. The expression of the HNF1α antisense ribozyme in clones CpR2

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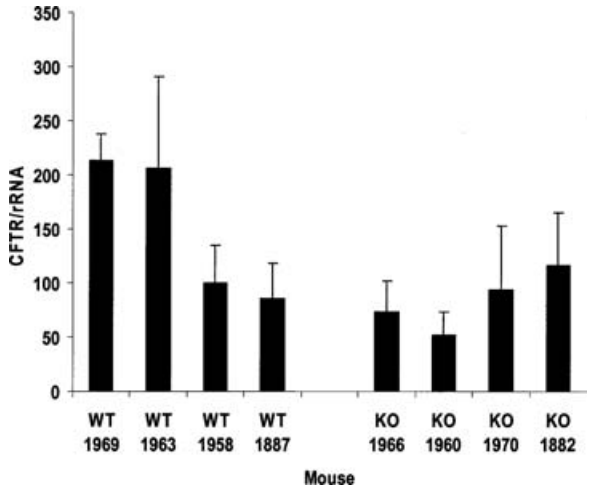

Figure 7 *Hnf1α* null mice show lower levels of *cftr* expression than WT mice

Cftr expression measured by quantitative RT–PCR using an ABI Prism 7700 sequence detection system. Proximal small-intestine tissue was from four WT mice (1969, 1963, 1958 and 1887) and four KO mice (1966, 1960, 1970 and 1882). Mean values of triplicate or sextuplet assays are expressed as a percentage of CFTR mRNA in small intestine of WT mouse 1958. Data are derived from Ct (threshold cycle) values for a CFTR assay normalized to 18 S rRNA.

and CpR3 is accompanied by a significant decrease ($P < 0.001$) in CFTR mRNA expression as compared with the vector-transfected control CpPc1. These experiments were performed in triplicate on two different cultures of the Capan clones and shown to be reproducible. In a further series of experiments, the HNF1α antisense ribozyme was stably expressed in Caco2 cells, but was shown to inhibit *HNF1α* and *CFTR* expression only at an early stage of culture before confluence (results not shown). Possibly the high levels of HNF1α, expressed in Caco2 cells in comparison with Capan1 (see Figure 4), render the antisense ribozyme relatively ineffective by confluence and the levels of HNF1α remain more than sufficient to drive maximal expression of CFTR mRNA.

Expression of *Cftr* in the small intestine of *Hnf1α* KO (knockout) mice

Expression of *Cftr* in RNA, extracted from small intestines of *Hnf1α* null mice and their WT littermates, was evaluated by quantitative RT–PCR. Figure 7 shows the ratio of *Cftr* to rRNA in four WT mice (1969, 1963, 1958 and 1887) and four KO mice (1966, 1960, 1970 and 1882). Although there is clearly an individual variation in *Cftr* expression levels between mice of the same genotype, and the ratio of *Cftr* to rRNA overlaps in the WT and KO groups, *Cftr* expression levels are generally higher in the WT when compared with the *Hnf1α* null mice. Statistical evaluation of the ratios of CFTR/rRNA for the WT and KO groups of mice using an unpaired *t* test, assuming equal variance, gave a value of $P = 0.037$ which identifies a significant difference in *Cftr* levels expression in *Hnf1α* null mice and WT mice.

DISCUSSION

Very little is known about transcription factors that regulate expression of the *CFTR* gene, with the exception of non-tissue-specific factors interacting with its promoter. Our search for potential regulatory elements that lie outside the *CFTR* promoter identified several intragenic DHS that might contain regulatory elements. Guided by regions of strong similarity between the sheep, cow, pig and human *CFTR* intronic sequences, we analysed DNA–protein interactions by *in vitro* DNase I footprinting. The identification of multiple predicted binding motifs for the HNF1 transcription factor within the core of the DHS allowed us to perform further *in vitro* and *in vivo* experiments to establish whether HNF1 is indeed involved in the regulation of expression of the *CFTR* gene. We provide data to support the hypothesis that HNF1α is one of the transcription factors that contributes to *CFTR* regulation. We propose that HNF1α binds to a number of regulatory elements located at DHS within *CFTR* and simultaneously interacts with multiple other transcription factors to influence *CFTR* gene expression.

HNF1 is a homeodomain transcription factor, which, in addition to being expressed in liver, is involved in regulating gene expression in pancreas, kidney, stomach and small intestine [16]. As for CFTR [34], the levels of HNF1 expression in the small intestine are highest in the crypt epithelial cells and gradually decrease to lower levels in the villus tips [35]. The importance of HNF1α in differentiation of the pancreas has been elucidated recently [36] and CFTR expression is detected in the developing pancreas very early in human gestation. How could HNF1 binding at DHS affect *CFTR* transcription? In addition to interacting with binding sites located in the promoters of liver specific or other genes such as α-1 antitrypsin [33] and phenylalanine hydroxylase [37], important intronic HNF1-binding sites have been characterized in several genes. The aldolase B gene is transcribed at high levels in liver, kidney and intestinal tissue, due to the co-operation of a weak liver-specific promoter and an activator in the first intron that contains crucial HNF1 sites [38]. Fibroblast growth factor receptor 4 overexpression in pancreatic cancer is mediated by HNF1α binding to an enhancer sequence within a DHS in the first intron of the *FGFR4* gene [39]. A single 13 bp HNF1 target site is not usually sufficient to direct HNF1-dependent transcription, rather co-operation within a complex network of transcription factors is required. This is consistent with our observation of multiple potentially interacting HNF1 sites in the *CFTR* intronic DHS, which could activate *CFTR* transcription or at least augment expression to the relatively high levels found in intestinal and pancreatic duct epithelial cells *in vivo*. The mechanism by which these sites could interact with the *CFTR* promoter is of interest and HNF1 is known to have a number of germane properties. HNF1 interacts with multiple co-activator proteins, some of which have been implicated in *CFTR* expression, such as CREB-binding protein (CBP/p300, where p300 is a transcriptional activator protein required to drive p53 expression) and CBP-associated factor (P/CAF) [40]. HNF1 has also been shown to recruit histone acetyltransferase activity, direct nucleosomal hyperacetylation [36] and be involved in chromatin remodelling through promoter demethylation [37]. Hence, as has been suggested for other genes [40], the combined action of HNF1 [37] and multiple co-activators could activate transcription of the *CFTR* gene by coupling nucleosome modification and recruitment of the general transcription machinery. It is probable that HNF1α is only one of multiple transcription factors that are involved in the tissue-specific regulation of *CFTR* gene expression and that the mechanism of interactions is complex. The fact that increases in *CFTR* expression achieved by augmenting HNF1α levels in Caco2 are not directly proportional (Figure 5) suggests a complex interaction between HNF1α and the *CFTR* gene.

Our current model of regulation of expression of the *CFTR* gene suggests that multiple regulatory elements interact in chromatin *in vivo*, probably by different mechanisms in individual cell types that exhibit specific sets of DHS within the gene. Expression in different organs and cells is probably regulated by distinct, although overlapping, sets of transcription factors. HNF1-binding motifs

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we have detected to date are located at DHS in introns 10, 17a and 20 of the *CFTR* gene, all of which are evident in Caco2 intestinal carcinoma cells [13]. The intron 17a DHS is also evident in HT29 intestinal epithelial cells, Capan1 pancreatic adenocarcinoma cells and Calu3 airway epithelial cells. The DHS in intron 20 is also detected in chromatin from primary male genital duct epithelial cells (vas deferens and epididymis) [13] and weakly in Capan1 and Calu3 cells. However, the intron 10 DHS is not evident in the Calu3 airway carcinoma cell line that expresses high levels of CFTR mRNA and contains HNF1α protein. HNF1α regulation of *CFTR* expression in this cell line might be through other regulatory elements in addition to those in introns 17a and 20. Presumably, different cell-type-specific transcription factors may be involved in the complex with HNF1α in each cell type.

Heterologous expression of HNF1α alone is insufficient to switch on *CFTR* gene expression in a cell line that does not normally express it. An HNF1α expression plasmid was introduced into MCF7 cells that do not normally express the *CFTR* gene, but MCF7 clones expressing the HNF1α did not exhibit significant up-regulation of CFTR mRNA (results not shown). Presumably, the ineffectiveness of HNF1α to act alone was due to lack of the necessary additional cofactors or modification of the *CFTR* gene chromatin in this cell line inhibiting gene expression by other mechanisms. Further evidence for the recruitment of additional relevant transcription factors to interact with HNF1α at the DHS in intron 10 comes from our evaluation of the effect of activators of *CFTR* transcription on DHS regions [4]. Forskolin, an inducer of intracellular cAMP, which is known to augment *CFTR* transcription [41] was shown to enhance reproducibly the 1716 + 13.2 kb DHS in intron 10 (DHS10A) in Caco2 cells. In addition to HNF1 motifs in this region, clusters of predicted binding sites for CREB and CREB-related proteins have been identified in adjacent sequences. These proteins could provide the additional cofactors to interact with HNF1α and activate *CFTR* transcription *in vivo*.

Mutations in HNF1α are associated with MODY3 (maturity-onset diabetes of the young 3) in man [42]; however, the phenotype of several strains of HNF1α KO mice [17,43] are intriguing in the context of the hypothesis that HNF1α may be involved in regulation of *CFTR* expression. One strain of HNF1α KO mouse, in addition to exhibiting Laron-type dwarfism and non-insulin-dependent diabetes, has vestigial male genital tracts [17]. A diagnostic feature of cystic fibrosis is male infertility that arises due to absence or incomplete vas deferens. Unlike another strain of HNF1α null mouse [43], which showed significant failure to survive after weaning, these mice are suitable for postnatal analysis of *Cftr* expression. We have shown by quantitative RT–PCR that although there is individual variation in *Cftr* expression levels in the small intestine of WT and HNF1α-null mice (KO), with some overlap between the two genotypes, in general WT mice express significantly more *Cftr* when compared with the KO mice ($P = 0.037$). The mechanism of HNF1α involvement in mouse *Cftr* expression remains to be elucidated, as the mouse gene has not been subjected to extensive DHS mapping. However, a comparative genomic approach has enabled us to search for HNF1-binding sites predicted by TRANSFAC in the regions of the mouse *cftr* introns that show similarity with the human *CFTR* intronic DHS regions. There is no mouse:human homology for the HNF1 site at DHS10AB-FP1 (Table 3) but the mouse gene has a predicted HNF1-binding site adjacent to AF162137:215801–215817. The HNF1 site at DHS10AB-FP5 is homologous in mouse and human *Cftr*/*CFTR* but those in DHS10C are not conserved. In DHS17a, although the two HNF1 sites that have been identified in the human *CFTR* gene are not conserved in mouse *Cftr* there are two predicted HNF1-binding sites within the homologous region of intron 17a at AF162137:249273–249289 and 249883–249899. The HNF1 sites in DHS20 show no homology in the mouse gene but again other sites are seen in adjacent regions at AF162137:277337–277353 and 277750–277766. We have previously shown, using a yeast artificial chromosome containing the human *CFTR* gene in transgenic mice [44], that regulation of CFTR/cftr expression in the human and mouse small intestine is in part achieved by similar mechanisms. Thus it is possible that HNF1 contributes to regulation of mouse *cftr* expression by similar mechanisms to those controlling human *CFTR* gene expression, at least in the small intestine.

In summary, we provide evidence for the transcription factor HNF1α playing an important role in *CFTR* expression in human intestinal epithelial cells and pancreatic duct cells. These cells express comparatively high levels of CFTR mRNA and it is possible that HNF1α is recruited to augment basal *CFTR* expression levels only in cells with abundant CFTR. Certain other cell types, e.g. within the lung epithelium, express much lower levels of CFTR and may recruit other factors to interact with the basal transcription machinery. A small ($< 10\%$) increase in expression of functional CFTR would alleviate the disease phenotype. Hence, these results provide a basis for designing gene therapy vectors that show appropriate regulated expressions of CFTR, and for alternative therapeutic strategies that up-regulate HNF1α to increase expression in endogenous *CFTR* transcripts.

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