Molecular Medicine 5: 211-223, 1999

Molecular Medicine

Original Articles

In Vivo Analysis of DNase I Hypersensitive Sites in the Human *CFTR* Gene

Danielle S. Moulin,[1] Ania L. Manson,[2] Hugh N. Nuthall,[1] David J. Smith,[1] Clare Huxley,[2] and Ann Harris[1]

[1]Paediatric Molecular Genetics, Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Oxford, U.K.
[2]Section of Molecular Genetics, Division of Biomedical Sciences, Imperial College School of Medicine, London, U.K.

Communicated by D. Weatherall. Accepted March 1, 1999.

Abstract

Background: The cystic fibrosis transmembrane conductance regulator gene (*CFTR*) shows a complex pattern of expression. The regulatory elements conferring tissue-specific and temporal regulation are thought to lie mainly outside the promoter region. Previously, we identified DNase I hypersensitive sites (DHS) that may contain regulatory elements associated with the *CFTR* gene at −79.5 and at −20.5 kb with respect to the ATG and at 10 kb into the first intron.

Materials and Methods: In order to evaluate these regulatory elements in vivo we examined these DHS in a human *CFTR* gene that was introduced on a yeast artificial chromosome (YAC) into transgenic mice. The 310 kb human *CFTR* YAC was shown to restore the phenotype of CF-null mice and so is likely to contain most of the regulatory elements required for tissue-specific expression of *CFTR*.

Results: We found that the YAC does not include the −79.5 kb region. The DHS at −20.5 kb is present in the chromatin of most tissues of the transgenic mice, supporting its non-tissue-specific nature. The DHS in the first intron is present in a more restricted set of tissues in the mice, although its presence does not show complete concordance with CFTR expression. The intron 1 DHS may be important for the higher levels of expression found in human pancreatic ducts and in lung submucosal glands.

Conclusion: These data support the in vivo importance of these regulatory elements.

Introduction

Transcription of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (1) is tightly regulated both spatially and temporally. Its expression is limited primarily to specific epithelial cell types within the respiratory system, the small intestine and colon, pancreas and gall bladder, kidney, genital ducts, and sweat glands (2–6). In the postnatal airway, highest levels of *CFTR* mRNA are detected in the serous portion of submucosal glands with low levels in the surface epithelium (6). In the intestine, *CFTR* mRNA is seen primarily in the crypt cells with gradients of expression decreasing along the crypt-villus axis and from the small intestine to the large intestine (5). CFTR mRNA and protein are found in the ductal epithelium of the pancreas and gall blad-

Address correspondence and reprint requests to: Dr. Ann Harris, Paediatric Molecular Genetics, Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Oxford, OX3 9DS, U.K. Phone: 44-1865-222341; Fax: 44-1865-222626; E-mail: aharris@molbiol.ox.ac.uk

der and possibly also in pancreatic acini (4). Temporal regulation of *CFTR* transcription is seen in the airway where abundant *CFTR* transcription throughout the epithelium of the fetus decreases substantially after birth (7,8). *CFTR* expression in the reproductive tract is also cyclical in some species (9,10).

Relatively little is known of the mechanisms underlying this strict cellular control of *CFTR* expression, and there is no consistent evidence for tissue-specific control elements in the promoter (11–13). We searched for regulatory elements in the *CFTR* genomic DNA outside the immediate promoter region by looking for DNase I hypersensitive sites (DHS) that are often associated with sequence elements involved in the regulation of transcription. We identified three DHS in the *CFTR* gene, two in the 5′ flanking sequence (−79.5 kb and −20.5 kb with respect to the *CFTR* ATG), and one in intron 1 (at 185 + 10 kb, where 185 refers to the last base in exon 1) (14,15). These DHS were identified in human cell lines and cultured primary human genital duct cells that express endogenous *CFTR*. The sites at −79.5 and −20.5 kb showed no correlation with *CFTR* expression in the cell types investigated, although they may still play a role in the complex series of events involved in the regulation of *CFTR* transcription. The 185 + 10 kb DHS showed complete correlation with *CFTR* expression only being evident in cells that express *CFTR* and not in those that lacked *CFTR* expression (15,16). In vitro analysis of binding of proteins to this region by electromobility shift assays (EMSA) and DNase I footprinting revealed that some proteins that are only present in *CFTR*-expressing cells bound to specific elements. When assayed by transient expression in a cell line expressing endogenous *CFTR*, the element at 185 + 10 kb augmented reporter gene expression under the control of the *CFTR* promoter but had no effect in nonexpressing cells (15). In vitro evaluation of regulatory elements in a large complex gene such as *CFTR* can only generate limited data, primarily because chromatin-mediated effects are difficult to reproduce. Our aim here was to use transgenic mice carrying the human *CFTR* gene, on a yeast artificial chromosome (YAC), to evaluate whether regulatory elements at the −79.5, −20.5, and 185 + 10 kb are important for *CFTR* expression in vivo.

A 310 kb YAC containing the intact human *CFTR* gene [37AB12, (17)] has previously been introduced into transgenic mice (18). The transgenic mice were bred with the "Cambridge" CF null mice to produce mice that only express the human *CFTR* transgene (18). These mice had a normal phenotype, in contrast to the CF null mice, suggesting that the 37AB12 YAC contained regulatory elements to direct *CFTR* expression in many of the usual sites. Expression of the human transgene showed cell type–specific expression in these mice equivalent to that of the endogenous mouse gene in the intestine and salivary glands. However, in some tissues where human *CFTR* expression would be expected, it was not observed. In sites where there is a difference in *CFTR* expression patterns between mouse and humans, the transgene expression followed the mouse pattern. The YAC contains 310 kb of genomic DNA, including 200 kb spanning the coding region of the human *CFTR* gene and about 70 kb of upstream flanking sequence. Hence, it is likely to contain at least the −20.5 kb and 185 + 10 kb regions, though probably not the −79.5 kb region.

Here we show that the YAC does not extend as far 5′ as the −79.5 kb region and hence this DHS is not an absolute requirement for *CFTR* expression. We have also evaluated the presence of the DHS at −20.5 kb and 185 + 10 kb in chromatin extracted from the YAC transgenic mouse tissues and found that their appearance often correlates with CFTR expression.

Materials and Methods

Cosmid Clones for YAC Mapping

Probes (Fig. 1) were derived from the cosmid contig described in the original *CFTR* cloning paper (19) and were kindly donated by Dr. Joanna Rommens.

Transgenic Mice

Details of generation of the mice carrying the 37AB12 YAC and crosses with the "Cambridge" null mice have been described previously (18). The mice used in this study were homozygous for the YAC transgene and for the CF knockout. Mouse tissues were collected immediately after death and placed in liquid nitrogen for RNA extraction or processed directly for chromatin extraction. Only data from the T30 strain of transgenic mice are presented here.

Chromatin Extraction

Chromatin was extracted from transgenic mouse tissues, normal mouse ileum, and Calu3 cells as

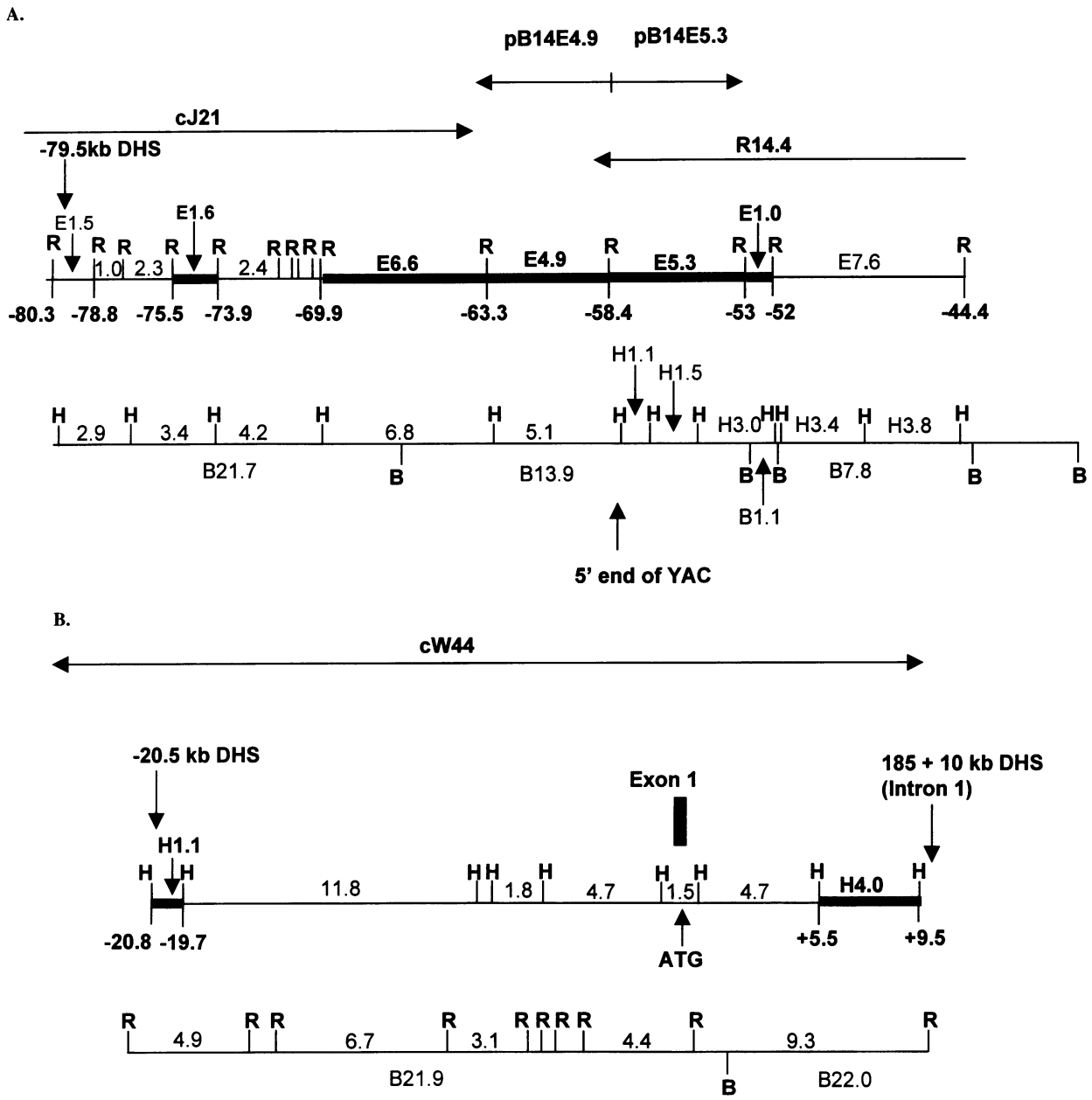

Fig. 1. Map of the region from −80 kb to −40 kb from the start of the *CFTR* gene (A) and of the region spanning the −20.5 and 185 + 10 kb DHS (B). (A) The extent of cosmids cJ21, phage R14.4, and plasmids pB14E4.9 and pB14E5.3 are shown (top). *Eco*RI sites (marked R) and fragments are also shown (middle). Fragments used as probes for mapping the 5′ end of the YAC are indicated by bold lines and type (not all data are shown), and distances 5′ of the ATG translation start site are indicated beneath the line. The −79.5 kb DHS is also indicated. The lower section shows *Hin*dIII sites and fragments above the line (marked H) and *Bam*HI sites and fragments below it (marked B). The probable 5′ end of the YAC is indicated by an arrow below the line. (B) The extent of the cosmid cW44 is shown (top). The middle section shows *Hin*dIII sites (marked H), with *Hin*dIII fragments used as probes indicated by bold lines and labeled in bold type. The locations of the −20.5 kb and 185 + 10 kb DHS, and exon 1 are also shown. The position of the ATG translation start site and distances from it are indicated below the line. The lower section shows *Eco*RI sites (marked R) and sizes of *Eco*RI fragments above the line and *Bam*HI sites (marked B) and fragments below it.

described previously (14,15,20). For most tissues, chromatin was extracted from the whole organ. For the small intestine, the samples were enriched for epithelial cells by washing the contents from the intestine, opening it by cutting along its length, and scraping the surface with a microscope slide.

Table 1. Primers used for PCR

Primer	Primer Sequence (5′ to 3′)	Location[a]	Annealing Temperature (°C)	Extension Time (min)	Product Size (bp)
MOU4-Ava	CCCCCGGGATCCTTCTGGATGATACAGAACC	−79.5 kb	60	2	214
MOU8-Eco	GGAATTCGGATCCTATGACATGAAGACCAACC				
HUG20C	CCCCCGGGATCCATCCTAACTCACAGGCAAC	−20.5 kb	60	2	274
HUG20D	GAATTCGGATCCTTGATAGAGGACGAGATAC				
TSR7-PacI	CCTTAATTAAGGATCCTCATCTTTATCTTCATTGTC	185 + 10 kb	60	2	199
TSR8-SacII	TCCCCGCGGATCCTAACTCATTGTACTGACGAG				
4i-5	TCACATATGGTATGACCCTC	Exon 4	60	3	438
4i-3	TTGTACCAGCTCACTACCTA				

[a]Location denotes map position with respect to *CFTR* ATG.

RNA Extraction

Total RNA was extracted from transgenic mouse tissues, normal mouse ileum, and Calu3 cells by standard methods (21).

Polymerase Chain Reaction (PCR)

Agarose plugs containing 37AB12 YAC DNA were treated with β-agarase to release template DNA for PCR reactions. The primers used to amplify genomic and YAC DNA are shown in Table 1. Southern blot analyses of YACs were carried out by standard methods.

Reverse Transcriptase-PCR (RT-PCR)

All tissues studied were evaluated for transcription of human and mouse *CFTR* mRNA expression by RT-PCR (14). The locations of primers used for RT-PCR are shown in Table 2 and described in detail elsewhere (22,23). The β-actin primers were β3′ATGCCATCCTGCGTCTGGACCTGGC and β5′AGCATTTGCGGTGCGACATGGAGGG, producing a 607 bp fragment from mouse RNA and a 581 bp fragment from human RNA.

DNase I Hypersensitivity Assays

Chromatin from transgenic mouse tissues was probed for DNase I hypersensitive regions by standard methods (24). Each batch of chromatin was tested with the RA2.2 probe that detects a constitutive DHS in the human and mouse α-globin gene cluster (25). The DHS at −20.5 kb

Table 2. Comparison of human and mouse RT-PCR primer sequences[a]

Primer	Exon	Size (bp)	Human Sequence	Mouse Sequence
A1R	1		**C**GAGA**G**A**C**CATGCAGA**G**GTC	**G**GAGA**C**A**T**CATGCAGA**A**GTC
6S1L	6	667	GTAACAA**C**TCCCAGA**TT**AGC	GTAACAA**G**TCCCAGA**GA**AGC
C1R	11		GTGGAGGTCA**A**CG**A**GCAAGA	GTGGAGGTCA**G**CG**T**GCAAGA
C2L	13	812	TCTTCA**CTT**AT**TT**CCA**AGCC**	TCTTCA**GT**GA**T**GT**T**CA**GTGT**
F1R	19		**AC**GTGAAGAAA**GA**TGA**C**ATC	**AT**GTGAAGAAA**AG**TGA**T**ATC
F2L	24	877	CA**TGT**C**AA**CA**TTT**AT**GC**T**G**C	CA**GAG**C**CC**CA**GGG**AT**CC**A**CC**

[a]Bases that differ between human and mouse sequences are indicated in bold type.

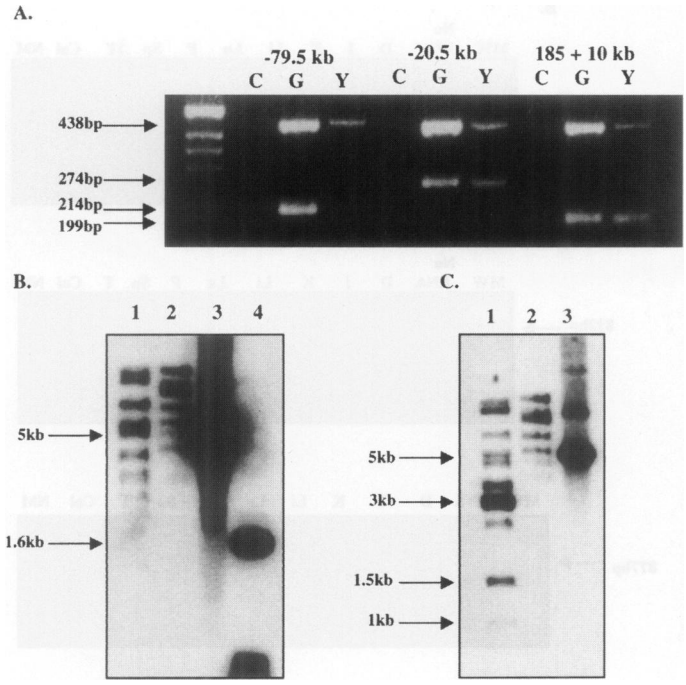

Fig. 2. Mapping of the 5′ end of the CFTR YAC. (A) PCR amplification of 37AB12 YAC DNA and human genomic DNA with primers specific for the −79.5 kb region (214 bp product, primers MOU4-Ava and MOU8-Eco), the −20.5 kb region (274 bp product, primers HUG20C and HUG20D), and the 185 + 10 kb region (199 bp product, primers TSR7-PacI and TSR8-SacII). Each PCR reaction also contains primers for exon 4 (438 bp product, primers 4i-5 and 4i-3). For each set of three reactions, lane C contains the product of the no-DNA control, lane G contains the PCR products obtained using human genomic template DNA, and lane Y contains those using 37AB12 YAC template DNA. (B, C) Southern blots of 37AB12 DNA cleaved with *Hin*dIII and probed with pB14E4.9 (B) and pB14E5.3 (C). In both panels lane 1 contains 37AB12 YAC DNA; lane 2, a control YAC from outside the *CFTR* locus also cleaved with *Hin*dIII; and lane 3, probe DNA. In (B) lane 4 is a 1 kb ladder (Gibco BRL). The multiple bands in the two YAC lanes are probably due to cross-hybridization to repetitive elements in the human DNA.

was detected with the H1.1 probe and that at 185 + 10 kb with the H4.0 probe (see Fig. 1B). The DHS at 185 + 10 kb was previously referred to as the 181 + 10 kb DHS (15).

Results

Mapping of the 5′ End of the CFTR YAC

Long-range mapping had previously shown that the YAC contained about 70 kb of upstream genomic DNA as well as the intact *CFTR* gene (18). PCR assays were carried out to determine whether the DHS at −79.5 kb, −20.5 kb and 185 + 10 kb were in fact present in the YAC (Table 1 shows the primers and expected products). The YAC DNA and genomic DNA were amplified in parallel for each DHS (Fig. 2A). Each PCR reaction also contained a control pair of primers that amplified a 438 bp fragment spanning exon 4 which was known to be present in the intact YAC. The PCR assays for the DHS at −20.5 and 185 + 10 kb gave the expected product with the YAC as well as the genomic DNA, indicating that these sites are present in the YAC. The PCR assay for the DHS at −79.5 kb did not give the expected product from the YAC even though the fragment was generated from the genomic DNA and the control exon 4 assay was efficient. Therefore, the *CFTR* YAC probably does not contain the DHS at −79.5 kb.

Final mapping data for the 5′ end of the YAC were provided by hybridization of various probes to Southern blots of YAC DNA. The pB14E4.9 probe (Fig. 1A) did not hybridize to *Hin*dIII-digested YAC DNA (Fig. 2B), whereas the pB14E5.3 probe (Fig. 1A) hybridized to the expected *Hin*dIII fragments of 1.1, 1.5, and 3 kb (Fig. 2C). (There are additional bands on the gel due to cross-hybridization with repetitive human DNA as shown by the bands also present in the lane with DNA from an unrelated YAC.) Since the YAC library was generated by partial *Eco*R1 digestion of human genomic DNA, the end of the genomic DNA within the YAC would be expected to lie at an *Eco*RI site. Thus the *CFTR* YAC probably extends to the *Eco*RI site at 58.4 kb 5′ to the *CFTR* ATG (Fig. 1A) and the YAC does not include the DHS at −79.5 kb.

Analysis of Transgene Expression

RT-PCR was used to detect *CFTR* mRNA from transgenic mouse duodenum, ileum, kidney, liver, lung, pancreas, spleen, and testis and genital ducts combined (Fig. 3). In addition, the RT-PCR was carried out on RNA from normal mouse ileum and from Calu3 cells (Calu3 is a human airway adenocarcinoma cell line that expresses endogenous *CFTR*). The positive Calu3 reactions verified the efficiency of the primers to amplify human *CFTR* cDNA (lanes labeled Cal in

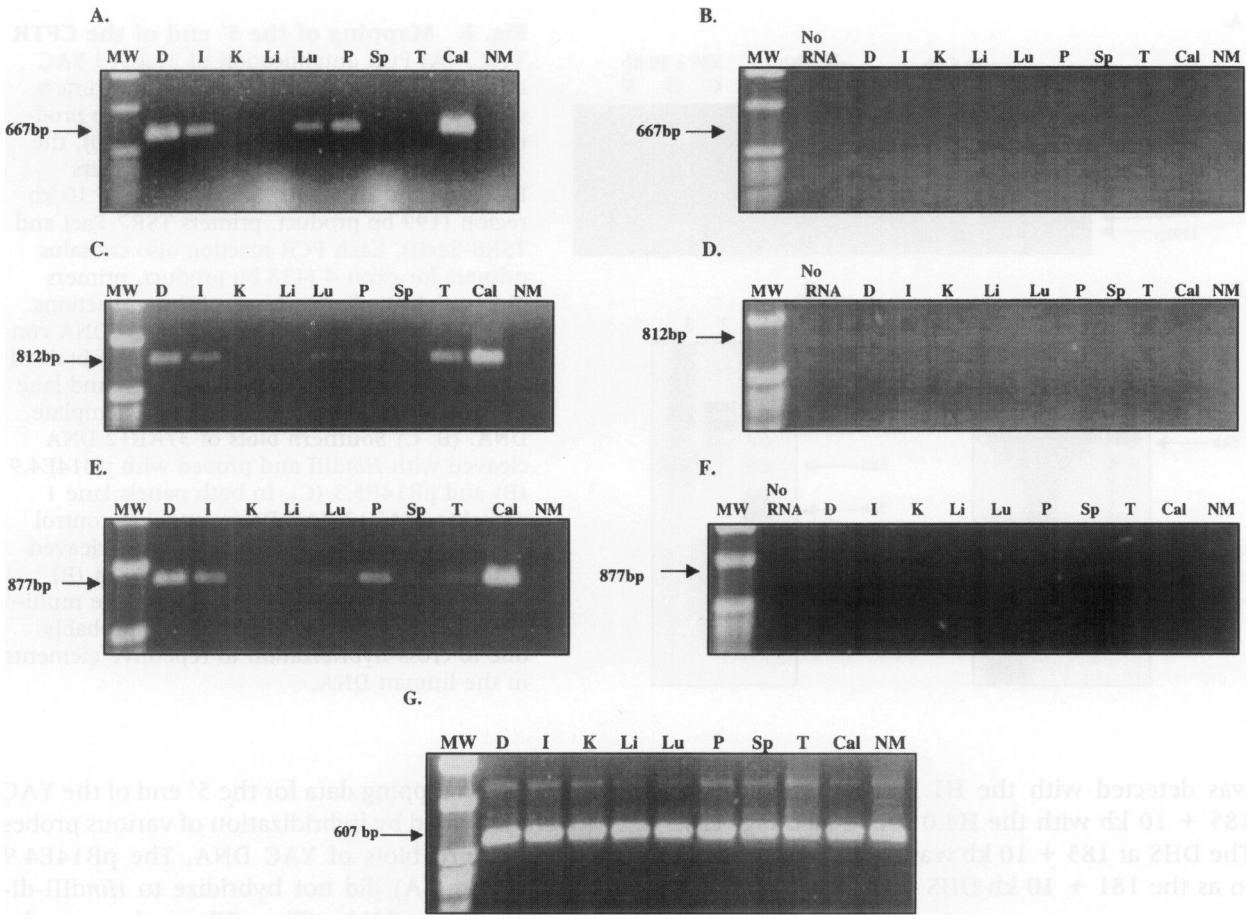

Fig. 3. RT-PCR products generated using human *CFTR*–specific primers. (A, C, E) RT-PCR products generated from template RNA extracted from transgenic mouse duodenum (D), ileum (I), kidney (K), liver (Li), lung (Lu), pancreas (P), spleen (Sp), testis and genital ducts (T), Calu3 cells (Cal), and normal mouse ileum (NM). (B, D, F) A no-RNA control and no–reverse transcriptase controls for each tissue. Panels A and B show RT-PCR products generated using the primers A1R and 6S1L; C and D show products using the primers C1R and C2L; E and F show products using primers F1R and F2L. RT-PCR products were electrophoresed in a 1% agarose gel. Molecular weight (MW) is the 1 kb DNA ladder (Gibco BRL). (G) The 607 bp RT-PCR products generated from the same transgenic mouse RNA samples using the β-actin primers β-3′ and β-5′.

Fig. 3A, C, and E). The normal mouse ileum negative control reactions showed that the primers were specific for human *CFTR* mRNA (lanes labeled NM in Fig. 3A, C, and E). All RT-PCR reactions were accompanied by no-RNA and no–reverse transcriptase controls (Fig. 3B, D, and F). Mouse β-actin primers were used to check that RNA in each sample could be amplified by RT-PCR (Fig. 3G).

Three sets of human *CFTR*-specific primers were used for RT-PCR, AIR/6SIL, C1R/C2L, and F1R/F2L (Table 2). The primers were derived from the human *CFTR* cDNA sequence but have strong homology to the equivalent mouse sequence (see Table 2). However, none of them generated a product from normal mouse ileum RNA. All products obtained using the human-specific primers on transgenic mouse RNA were therefore amplified from the human transgene. This was further validated by complete digestion of the RT-PCR products using enzymes with recognition sites only in the human and not the relevant mouse fragment.

The primers A1R and 6S1L, located in exons 1 and 6, respectively, amplified a product of the expected size in samples from transgenic duodenum, ileum, lung, and pancreas whereas no product was seen in samples from kidney, liver, spleen, and testis and genital ducts (Fig. 3A). The primers C1R and C2L, located in exons 11 and 13, respectively, amplified a product of the expected size in samples from transgenic duode-

Table 3. Summary of RT-PCR and DHS results[a]

	185 + 10 kb DHS	−20.5 kb DHS	RT-PCR mRNA	In Situ Hybridization (18)	Adult Human Expression[c]	Adult Mouse Expression[c]
Small intestine	+	+	+	+++	++++	++++
Colon	NA	+	NA	+	++	++
Stomach	NA	+	NA	−	+	+
Kidney	+	+	−	NA	++	++
Lung	−	(+)	+	−	+	+
Liver	+	+	−	NA	+	+
Pancreas	−	(+)	+	−	+++	+
Testis	−	−	+[b]	NA	+	++
Genital ducts	−	−	+[b]	−	++	++
Spleen	NA	−	−	NA	NA	NA

[a] +, presence and −, absence of DHS or *CFTR* mRNA, respectively. NA, not analysed.

[b] Combined testis and genital duct RNA were used for RT-PCR analysis (these data are for primer sets C1/C1L and F1R/F2L, not A1R/6S1L).

[c] From previously published data.

num, ileum, lung, pancreas, and testis and genital ducts whereas no product was seen in samples from kidney, liver, or spleen (Fig. 3C). The primers F1R and F2L, located in exons 19 and 24, respectively, amplified a product of the expected size in samples from transgenic duodenum, ileum, lung, pancreas, and testis and genital ducts whereas no product was seen in samples from kidney, liver, or spleen (Fig. 3E).

These results are summarized in Table 3 and show that the human *CFTR* transgene is expressed in duodenum, ileum, lung, and pancreas but not in kidney, liver, and spleen. Expression in the testis and genital ducts was observed with the primers C1R/C2L and F1R/F2L but not with primers A1R/6S1L.

Detection of the DNase I Hypersensitive Site at −20.5 kb

The same tissues that had been analyzed by RT-PCR for *CFTR* RNA expression were also analyzed by DNase I digestion of chromatin to detect DHS. The efficiency of the DNase I digestion was confirmed by hybridizing filters carrying *Bam*HI-cleaved DNA from each tissue with the RA2.2 probe (25) which recognizes a constitutive DHS in the mouse α-globin gene cluster.

The DHS at −20.5 kb was detected with the cW44H1.1 probe (Fig. 1B) as shown previously with chromatin extracted from human primary cells and cell lines (14). This probe hybridizes to an *Eco*RI restriction fragment of 4.9 kb in intact chromatin and detects a subfragment of 3.5 kb, and occasionally another subfragment of 3 kb, in chromatin treated with DNase I (14). Chromatin from transgenic mouse tissues was examined for the presence of the −20.5 kb DHS (Fig. 4). The intact 4.9 kb *Eco*RI restriction fragment was seen in all tissues. A 3.5 kb subfragment appeared in chromatin from kidney (Fig. 4A), colon (Fig. 4D), small intestine (Fig. 4E), and stomach (Fig. 4F). Two subfragments, of about 3.5 kb and 2.8 kb, were seen in liver (Fig. 4B). The 2.8 kb fragment could indicate the presence of another DHS in this area. A faint 3.5 kb subfragment was seen in lung (Fig. 4C) and pancreas (Fig. 4G), although its intensity did not fully correlate with the degree of DNase I digestion of the chromatin. No DHS was seen in chromatin from testis (Fig. 4H) or genital ducts (Fig. 4I) or spleen (not shown).

Detection of the DNase I Hypersensitive Site at 185 + 10 kb

The DHS in intron 1 at 185 + 10 kb was detected with the cW44 H4.0 probe (Fig. 1B). This probe detects a 22 kb *Bam*HI restriction fragment in intact chromatin. It detects an intense 8 kb sub-

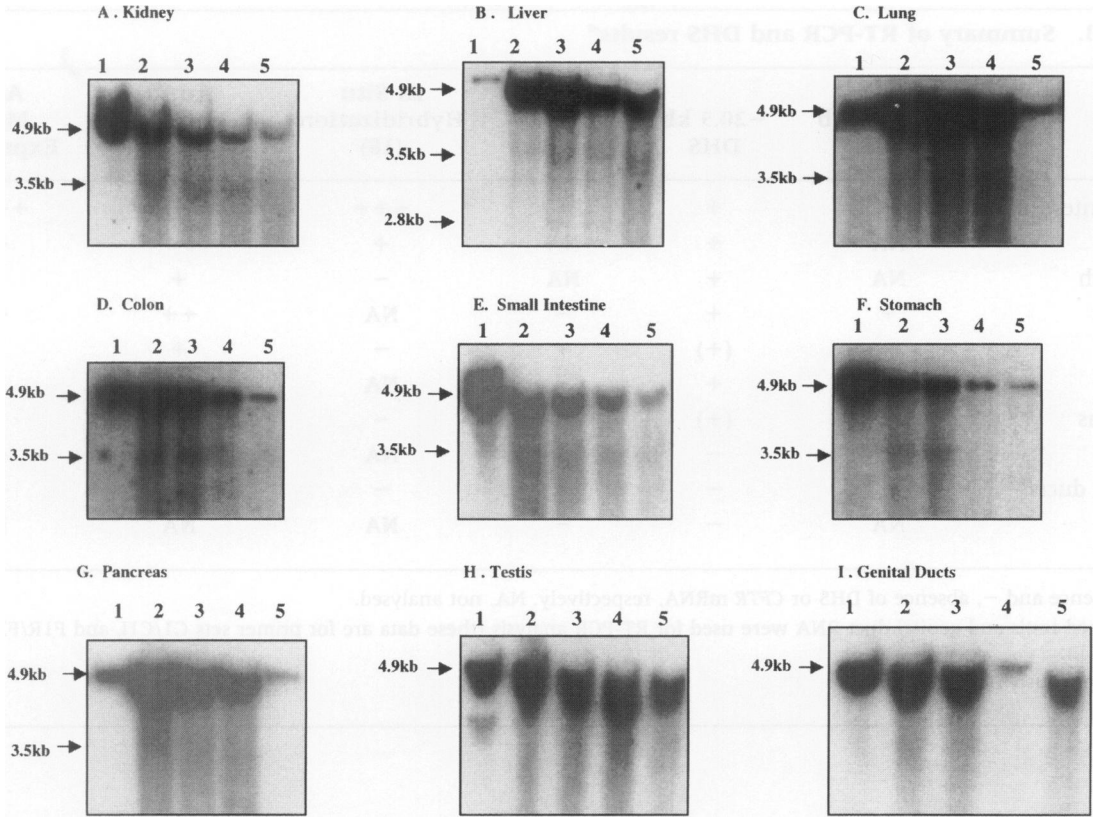

Fig. 4. In vivo evaluation of the −20.5 Kb DHS. (A–I) Chromatin extracted from transgenic mouse tissues, digested with DNase I, cleaved with *Eco*RI, separated on 1% agarose gels, Southern blotted, and probed with cW44 H1.1 to detect the DHS at −20.5 kb. For each tissue, lane 1 shows DNA prepared from nuclei that had not been treated with DNase I and lanes 2–5 show DNA treated with increasing amounts of DNase I: lane 2, 15 units; lane 3, 30 units; lane 4, 60 units; lane 5, 120 units. Molecular weights were estimated with respect to a 1 kb DNA ladder (Gibco BRL).

fragment and fainter 10 kb and 12 kb subfragments in chromatin from *CFTR*-expressing human primary cells and cell lines that have been treated with DNase I (15). Chromatin from kidney, liver, lung, small intestine, pancreas, and testis was examined for the presence of the 185 + 10 kb DHS (Fig. 5). Two *Bam*HI fragments of 22 kb and 13 kb were seen in all tissues. The extra 13 kb *Bam*HI fragment could arise from a partial copy of the YAC that had integrated into the genome of the T30 mice in addition to the intact YAC. This was supported by Southern blotting data showing that the transgenic mice had more copies of the 5′ than the 3′ end of the *CFTR* gene, even though there were definitely intact copies of the YAC present (18). A subfragment of 8 kb was seen in kidney (Fig. 5A), liver (Fig. 5B), and small intestine (Fig. 5D). The presence of this 8 kb fragment in intestinal chromatin that had not been treated with DNase I (Fig. 5D, lane 1) was due to the presence of endogenous DNase in this tissue sample; it was not seen in other intestinal chromatin samples (not shown). An additional subfragment of 10 kb was seen in kidney and small intestine. Small intestine also exhibited another subfragment of 4.8 kb. These extra subfragments suggest that there are additional DHS in intron 1 at 185 + 12 kb (indicated by the 10 kb subfragment) and 185 + 6.8 kb (indicated by the 4.8 kb subfragment). No DHS were observed in lung (Fig. 5C), pancreas (Fig. 5E), or testis (Fig. 5F). A summary of the DHS results is presented in Table 3.

Discussion

The data presented here provide the first in vivo analysis of potential regulatory elements located at −20.5 kb with respect to the ATG and at 10 kb into the first intron of the human *CFTR* gene. By using a YAC carrying the intact human *CFTR*

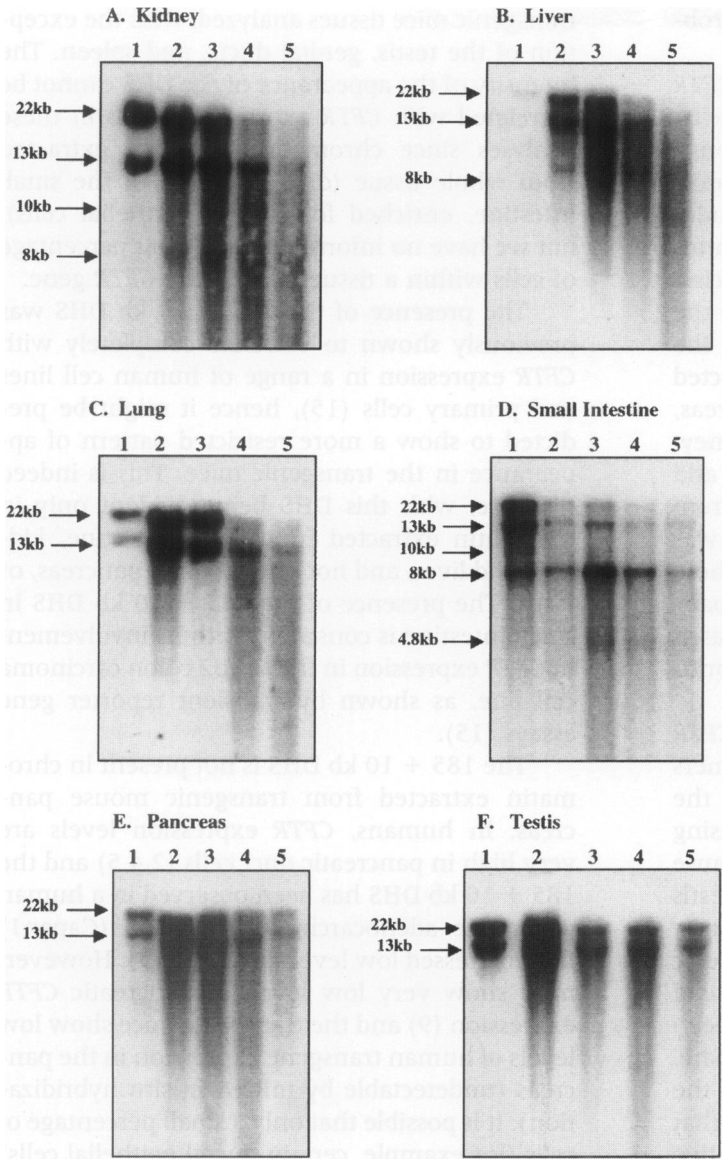

Fig. 5. In vivo evaluation of the 185 + 10 kb DHS. (A–F) Chromatin extracted from transgenic mouse tissues, digested with DNase I, cleaved with *Bam*HI, electrophoresed on a 1% agarose gel, Southern blotted, and probed with cW44 H4.0 to detect the DHS in intron 1 of the *CFTR* gene, at 185 + 10 kb. For each tissue, lane 1 shows DNA prepared from nuclei that had not been treated with DNase I and lanes 2–5 show DNA treated with increasing amounts of DNase I: lane 2, 15 units; lane 3, 30 units; lane 4, 60 units; lane 5, 120 units. Molecular weights were estimated with respect to a 1 kb DNA ladder (Gibco BRL).

gene, introduced into CF knockout mice, we were able to evaluate the involvement of these regulatory elements in *CFTR* gene expression. If regulatory elements at these sites are involved in driving *CFTR* gene expression, then the DHS would be expected to appear in chromatin extracted from tissues of these transgenic mice that express the *CFTR* gene. The absence of the DHS would indicate that they are not needed for expression in these tissues. However, the DHS might still appear even if they are not important in expression. The fact that two independent transgenes with the YAC carrying the intact human *CFTR* gene (T30 and T57) were both able to complement the null mice and gave roughly the same expression in all tissues examined (18) argues against position effects of neighboring chromatin playing a significant role in *CFTR* expression and the DHS in these mice.

The DHS that was previously detected at −79.5 kb was not present on the YAC. As the YAC expresses well in the small intestine and salivary glands of transgenic mice, this indicates that the −79.5 kb DHS is not important for expression in these tissues. However, it could be important for expression in such tissues as the kidney, and Brunner's glands (18), where expression of the gene from the YAC is not found. The two regions at −20.5 kb and 185 + 10 kb in the YAC were both able to form DHS in some tissues in the transgenic mice. This indicates that the mouse transcriptional machinery is able to

interact with these sites and that they are probably important for *CFTR* gene expression.

An initial investigation of human *CFTR* transgene expression in the CF knockout mice by mRNA in situ hybridization (18) showed abundant expression in the crypts of the small intestine with a decreasing gradient from the duodenum to the colon, as well as in salivary glands within the ductal epithelium and mucous acini. No *CFTR* transgene expression was seen in the pancreas, lung, or Brunner's glands. In the present study human *CFTR* mRNA was detected by RT-PCR in the small intestine, lung, pancreas, testis and genital ducts but not in the kidney, liver, or spleen. The results for the lung and pancreas are of interest as it was not clear from the in situ hybridization data whether there was any expression of the human transgene in these tissues. The positive results for the lung and pancreas by RT-PCR could be due to the increased sensitivity of RT-PCR over mRNA in situ hybridization (18).

We consistently detected human *CFTR* mRNA in testis and genital ducts using primers for exons 11 to 13 and exons 19 to 24 of the transgene, but no product was obtained using primers in exons 1 and 6. This could be because mRNA transcribed from the transgene in testis and genital ducts lacks the sequence for primer binding in exon 1, exon 6, or both. Testis-specific alternative splicing of *CFTR* mRNA and the use of testis-specific transcription start sites have been reported in mice and humans (26–29). To date, only events that insert novel sequences into the *CFTR* mRNA have been reported, and none that result in deletion of exons. However, alternative splicing causing exon loss from the human *CFTR* transcript has been reported in nasal epithelial cells, among other cell types (22). Such testis-specific alternative splicing of the human *CFTR* gene might be relevant to the male infertility associated with CF in humans, although CF mice are fertile. However, the defect is generally thought to be within the genital duct epithelium rather than the testis itself.

The −20.5 kb DHS has been previously seen in a wide variety of human epithelial cell lines and primary cells irrespective of their *CFTR* expression status (14), hence we predict that it might contain a regulatory element involved in basal *CFTR* expression. As such, it would be expected to appear in a wide variety of tissues in the YAC transgenic mice. Our results confirm this prediction, as the −20.5 kb DHS was seen to some extent in chromatin extracted from all the transgenic mice tissues analyzed, with the exception of the testis, genital ducts, and spleen. The intensity of the appearance of the DHS cannot be correlated with *CFTR* expression levels in these analyses since chromatin has been extracted from whole tissue (or in the case of the small intestine, enriched for surface epithelial cells), but we have no information on what percentage of cells within a tissue express the *CFTR* gene.

The presence of the 185 + 10 kb DHS was previously shown to correlate completely with *CFTR* expression in a range of human cell lines and primary cells (15), hence it might be predicted to show a more restricted pattern of appearance in the transgenic mice. This is indeed the case, with this DHS being evident only in chromatin extracted from small intestine, kidney, and liver, and not seen in lung, pancreas, or testis. The presence of the 185 + 10 kb DHS in small intestine is consistent with its involvement in *CFTR* expression in the Caco2 colon carcinoma cell line, as shown by transient reporter gene assays (15).

The 185 + 10 kb DHS is not present in chromatin extracted from transgenic mouse pancreas. In humans, *CFTR* expression levels are very high in pancreatic duct cells (2,4,5) and the 185 + 10 kb DHS has been observed in a human pancreatic adenocarcinoma cell line (Capan1) that expressed low levels of *CFTR* (15). However, mice show very low levels of pancreatic *CFTR* expression (9) and the transgenic mice show low levels of human transgene expression in the pancreas (undetectable by mRNA in situ hybridization). It is possible that only a small percentage of cells (for example, certain ductal epithelial cells) in the transgenic mouse pancreas express the human *CFTR* gene at a high level and that they contribute too small a fraction to the total tissue chromatin for the DHS to be detected. Alternatively, the −20.5 kb DHS, which is present in pancreatic chromatin from the transgenic mice, might be involved in the low-level expression detected in the transgenic mouse pancreas by RT-PCR, while the 185 + 10 kb site might contain a regulatory element involved in the high levels of *CFTR* gene expression normally seen in human pancreatic duct cells. The lack of the 185 + 10 kb site in the mouse chromatin (probably due to the lack of appropriate transcription factors) would then explain the lack of high-level expression in the mouse pancreas.

Similarly, the 185 + 10 kb DHS is not present in chromatin extracted from transgenic mouse lung. The *CFTR* gene is normally ex-

pressed at low levels in the surface epithelium of the adult human lung, with higher levels in the serous portion of submucosal glands (6). Mice express low levels of *CFTR* mRNA in the surface epithelium and have very few submucosal glands restricted to the tracheal submucosa (9). Low levels of human *CFTR* mRNA were detected by RT-PCR in lungs from the transgenic mice. The presence of the −20.5 kb DHS, but not the 185 + 10 kb DHS, in chromatin extracted from the transgenic lung tissue might be accounted for by the −20.5 kb DHS being responsible for the low level of expression in the epithelial cells, while the 185 + 10 kb DHS is important for higher expression levels in the human submucosal glands.

It is not clear why the 185 + 10 kb DHS is seen in chromatin extracted from liver, since neither mouse nor human *CFTR* genes are expressed at significant levels in liver and no human transgene *CFTR* mRNA is detected in liver RNA. *CFTR* expression is seen at high levels in gall bladder epithelium but gall bladders were removed from the transgenic mouse tissues prior to extraction of mRNA and chromatin. Similarly, the 185 + 10 kb DHS was present in chromatin extracted from transgenic mouse kidney but no human *CFTR* mRNA was detected in the transgenic mouse kidney by RT-PCR. However, endogenous *CFTR* mRNA is expressed in the normal human and mouse kidneys. These data suggest that, although the 185 + 10 kb DHS appears, the human *CFTR* transgene is unable to respond to some of the mouse transcription factors that regulate *CFTR* expression in the kidney.

The results from the testis and genital ducts, in which the human YAC *CFTR* transgene was expressed but neither the −20.5 kb or 185 + 10 kb DHS were evident, suggest that neither of these regions is important in testis or genital duct expression. Human testis exhibits low and diffuse levels of *CFTR* expression, which does not appear to be associated with any specific cell type (30). In contrast, *CFTR* is expressed at higher levels in the epithelium of the seminiferous tubules of mouse testis at all stages of spermatogenesis (9). *CFTR* is also known to show specific temporal regulation of expression in the male and female reproductive systems in rodents (10). Control of expression in these tissues may be operating through different regulatory elements.

In conclusion, the data provide evidence for the in vivo importance of the DHS at −20.5 kb as this site is present in chromatin from most of the transgenic mouse tissues. The 185 + 10 kb DHS has a more restricted distribution and is not found in lung or pancreatic tissues of the transgenic mice. The lack of formation of this DHS might be responsible for the very low levels of *CFTR* expression in the mouse lung and pancreas, which would indicate that the 185 + 10 kb DHS is important for the higher levels of expression in these human tissues. Finally, neither DHS is present in chromatin from testis or genital ducts, and expression in these tissues may be operating through other regulatory elements that have not been identified but that are present on the YAC.

However, the results also illustrate the problems of using the mouse as a model for investigation of the subtleties of human *CFTR* tissue-specific regulation. The *CFTR* gene shows a divergent pattern of expression in rodents (9) and humans (2–6). The data presented here show that the murine transcriptional machinery can interact with the −20.5 and 185 + 10 kb DHS and give adequate levels of *CFTR* mRNA expression in many tissues. However, lack of transgene expression in certain other sites, such as the kidney and Brunner's glands, might result from the incompatibilities between the mouse and human transcriptional control mechanisms for *CFTR*. Analysis of the promoter regions of the human, mouse, and rat *CFTR* genes has shown them to be highly divergent (31,32). Although some putative regulatory elements are loosely conserved, most of them are species-specific. A more extensive cross-species comparison of the *CFTR* promoter region (33) from eight mammalian species including human, monkey, cow, rabbit, mouse, and rat suggests that the cow and rabbit might be more suitable models in which to analyze human *CFTR* gene regulation in vivo. Recent advances in cloning technology (34–36) will enable this type of analysis to be carried out in ovine models, which are known to show a pattern of *CFTR* gene expression and regulation that is very similar to that of the human gene (37,38).

Acknowledgments

We are grateful to Johanna Rommens for cosmids and plasmids pB14E.9 and pB14E5.3 and to Erick Denamur for helpful discussions. This work was supported by the Cystic Fibrosis Trust, the Wellcome Trust, and AFLM. D.S.M. was a recipient of a Wellcome Trust prize studentship, H.N.N. of a Cystic Fibrosis Trust studentship.

References

1. Riordan JR, Rommens JM, Kerem, B-S, et al. (1989) Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA. *Science* **245:** 1066–1073.
2. Crawford IC, Maloney PC, Zeitlin PL, et al. (1991) Immunocytochemical localization of the cystic fibrosis gene product CFTR. *Proc. Natl. Acad. Sci. U.S.A.* **88:** 9262–9266.
3. Denning GM, Ostedgaard LS, Cheng SH, Smith AE, Welsh MJ. (1992) Localization of the cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia. *J. Clin. Invest.* **89:** 339–349.
4. Hyde K, Reid CJ, Tebbutt SJ, Weide L, Hollingsworth MA, Harris A. (1997) The cystic fibrosis transmembrane conductance regulator as a marker of human pancreatic duct development and differentiation. *Gastroenterology* **113:** 914–919.
5. Strong TV, Boehm K, Collins FS. (1994) Localization of cystic fibrosis transmembrane conductance regulator mRNA in the human gastrointestinal tract by in situ hybridization. *J. Clin. Invest.* **93:** 347–354.
6. Engelhart JF, Yankaskas JR, Ernst SA, et al. (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nat. Genet.* **2:** 240–247.
7. Harris A, Chalkley G, Goodman S, Coleman L. (1991) Expression of the cystic fibrosis gene in human development. *Development* **113:** 305–310.
8. Trezise AEO, Chambers JA, Wardle CJ, Gould S, Harris A. (1993) Expression of the cystic fibrosis gene in human foetal tissues. *Hum. Mol. Genet.* **2:** 213–218.
9. Trezise AE, Buchwald M. (1991) In vivo cell-specific expression of the cystic fibrosis transmembrane conductance regulator. *Nature* **353:** 434–437.
10. Trezise AE, Linder CC, Grieger D, et al. (1993) CFTR expression is regulated during both the cycle of the seminiferous epithelium and the oestrous cycle of rodents. *Nat. Genet.* **3:** 157–164.
11. Chou J-L, Rozmahel R, Tsui L-C. (1991) Characterization of the promoter region of the cystic fibrosis transmembrane conductance regulator gene. *J. Biol. Chem.* **266:** 24471–24476.
12. Koh J, Sferra TJ, Collins FS. (1993) Characterization of the cystic fibrosis transmembrane conductance regulator promoter region. *J. Biol. Chem.* **268:** 15912–15921.
13. Yoshimura K, Nakamura N, Trapnell BC, et al. (1991) The cystic fibrosis gene has a "housekeeping"-type promoter and is expressed at low levels in cells of epithelial origin. *J. Biol. Chem.* **266:** 9140–9144.
14. Smith AN, Wardle CJC, Harris A. (1995) Characterization of DNAse 1 hypersensitive sites in the 120 kb 5′ to the *CFTR* gene. *Biochem. Biophys. Res. Commun.* **211:** 274–281.
15. Smith AN, Barth ML, McDowell TL, et al. (1996) A regulatory element in intron 1 of the cystic fibrosis transmembrane conductance regulator gene. *J. Biol. Chem.* **271:** 9947–9954.
16. McDowell TL, Smith DJ, Huthall HN, Moulin DS, Harris A. (Unpublished) Role of a regulatory element in intron 1 of the CFTR gene in airway epithelium.
17. Anand R, Ogilvie DJ, Butler R, et al. (1991) A yeast artificial chromosome contig encompassing the cystic fibrosis locus. *Genomics* **9:** 124–130.
18. Manson AL, Trezise AEO, MacVinish LJ, et al. (1997) Complementation of CF null mice with a human CFTR transgene. *EMBO J.* **16:** 4238–4249.
19. Rommens JM, Iannuzzi MC, Kerem B-S, et al. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245:** 1059–1065.
20. Becker P, Renkawitz R, Schutz G. (1984) Tissue-specific DNAse I hypersensitive sites in the 5′-flanking sequences of tryptophan oxygenase and the tyrosine aminotransferase genes. *EMBO J.* **3:** 2015–2020.
21. Chirgwin JM, Przybyla AE, Macdonald RJ, Rutter WJ. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18:** 5294–5299.
22. Hull J, Shackleton S, Harris A. (1994) Analysis of mutations and alternative splicing patterns in CFTR mRNA derived from nasal epithelial cells. *Hum. Mol. Gen.* **3:** 1141–1146.
23. Chalkley GC, Harris A. (1991) Detection of mutations and polymorphisms in the CF gene by analysis lymphocyte mRNA. *J. Med. Genet.* **28:** 777–780.
24. Higgs DR, Wood WG, Jarman AP, et al. (1990) A major positive regulatory region located far upstream of the human alpha-globin gene locus. *Genes Dev.* **4:** 1588–1601.
25. Vyas P, Vickers MA, Simmons DL, Ayyub H, Craddock CF, Higgs DR. (1992) *Cis*-acting sequences regulating expression of the human alpha-globin cluster lie within constitutively open chromatin. *Cell* **69:** 781–793.
26. Trezise AEO, Buchwald M, Higgins CF. (1993) Testis-specific, alternative splicing of rodent CFTR mRNA. *Hum. Mol. Genet.* **2:** 801–810.
27. Delaney SJ, Rich DP, Thomson SA, et al. (1993) Cystic fibrosis transmembrane conductance regulator splice variants are not conserved and fail to produce chloride channels. *Nat. Genet.* **4:** 426–431.
28. Delaney SJ, Koopman P, Lovelock PK, Wainwright BJ. (1994) Alternative splicing of the first nucleotide binding fold of CFTR in mouse testes is associated with specific stages of spermatogenesis. *Genomics* **20:** 517–518.
29. White NL, Higgins CF, Trezise AE. (1998) Tissue-

specific in vivo transcription start sites of the human and murine cystic fibrosis genes. *Hum. Mol. Genet.* **7:** 363–369.

30. Tizzano EF, Silver MM, Chitayat D, Benichou JC, Buchwald M. (1994) Differential cellular expression of cystic fibrosis transmembrane regulator in human reproductive tissues. Clues for the infertility in patients with cystic fibrosis. *Am. J. Pathol.* **144:** 906–914.
31. Denamur E, Chehab FF. (1994) Analysis of the mouse and rat CFTR promoter regions. *Hum. Mol. Genet.* **3:** 1089–1094.
32. Vuillaumier S, Dixmeras I, Messai H, et al. (1997) Cross-species characterization of the promoter region of the cystic fibrosis transmembrane conductance regulator gene reveals multiple levels of regulation. *Biochem. J.* **327:** 651–662.
33. Vuillaumier S, Kaltenboeck B, Lecointre G, Lehn P, Denamur E. (1997) Phylogenetic analysis of cystic fibrosis transmembrane conductance regulator gene in mammalian species argues for the development of a rabbit model for cystic fibrosis. *Mol. Biol. Evol.* **14:** 372–380.
34. Campbell KH, McWhir J, Ritchie WA, Wilmut I. (1996) Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380:** 64–66.
35. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* **385:** 810–813.
36. Cibelli JB, Stice SL, Golueke PJ, et al. (1998) Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280:** 1256–1258.
37. Tebbutt SJ, Wardle CJC, Hill DF, Harris A. (1995) Molecular analysis of the ovine CFTR gene. *Proc. Natl. Acad. Sci. U.S.A.* **92:** 2293–2297.
38. Harris A. (1997) Towards an ovine model of cystic fibrosis. *Hum. Mol. Genet.* **13:** 2191–2193.