Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 274-281

# CHARACTERIZATION OF DNASE I HYPERSENSITIVE SITES IN THE 120kB 5' TO THE CFTR GENE

Annabel N.Smith, Catherine J.C. Wardle and Ann Harris[1]

Paediatric Molecular Genetics, Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Received April 27, 1995

The chromatin structure of 120kb of genomic DNA 5' to the CFTR gene has been analysed in a number of CFTR expressing and non-expressing cell types, including primary genital duct epithelial cells. Novel DNAse I hypersensitive sites have been observed at -79.5kb and -20.5kb 5' to the ATG translation start codon of the CFTR coding sequence. Neither of these sites appears to show strong correlation with CFTR expression in the cell types investigated, hence they are unlikely to reflect the sites of binding of the major CFTR tissue specific regulator(s). However, they may still play an important part in the complex series of events involved in the regulation of CFTR transcription. 

**INTRODUCTION** The cystic fibrosis transmembrane conductance regulator (CFTR) gene (1,2) which, when mutated results in the disease cystic fibrosis, exhibits a complex mechanism of regulation. The gene shows tight tissue specific regulation, being expressed mainly in specialized epithelial cells (3-5) within the respiratory system (3,6), the intestine, the pancreas and gall bladder (5,7), urinogenital tract and sweat gland (8). In addition to this distinct pattern of tissue specific expression the CFTR gene shows clear temporal regulation. First, analysis of the developmental profile of CFTR expression (9-11) has shown that while CFTR is transcribed in many epithelial cells as early as the 12th week of gestation with a similar tissue distribution to that seen postnatally, within the lung there is a substantial down regulation of CFTR expression at birth (11). Further, within the reproductive tract of at least some species, expression of the CFTR gene seems to be cyclical (12).

Functional analyses of the basal human (13-15) and mouse (16) CFTR promoters have revealed these to be weak elements in driving reporter genes in *in vitro* assay systems. Further, data from different groups analysing the human CFTR promoter in similar assay systems have been inconsistent. A number of DNase I hypersensitive sites that show some degree of correlation with CFTR expression have been observed within

[1] Corresponding Author. Fax: 44-1865-222626.

0006-291X/95 $5.00

the 5' region, between -3,000bp relative to the transcription start site and + 100 bp into intron 1 (13,15). However these sites have only been examined in a few longterm cell lines that either do or do not express CFTR mRNA and protein. To date the genetic elements that confer tissue specific regulation on CFTR expression have not been clearly identified within a 26kb region flanking the promoter (13,15).

In order to identify further sites involved in the tissue specific control of CFTR expression in humans, we have examined 120kb of genomic DNA 5' to the CFTR gene basal promoter. We have identified DNAse I hypersensitive sites within this region by screening with cosmid and phage clones (2). We have analysed a larger number of cell types than were examined in previous studies and have included primary ductal epithelial cells (17) derived from epithelia that express CFTR *in vivo*. We have detected novel hypersensitive sites at -79.5kb and -20.5kb 5' to the ATG start codon of the CFTR coding sequence in addition to sites reported previously in the promoter region (13,15). These two novel sites are unlikely to be the major elements conferring tissue specificity on CFTR expression as neither site appears to show strong correlation with the presence of CFTR mRNA in the cell types investigated, but they may still play an important part in the complex mechanisms that are clearly involved in the regulation of CFTR transcription.

## MATERIALS AND METHODS

**Cell culture.** The cell lines Caco-2 (18), HT-29 (19), Capan-1 (18) and MCF7 (20) were cultured in DMEM;the lymphoblastoid cell line 37566 was cultured in RPMI 1640. Primary human mid-trimester foetal vas deferens and epididymis epithelial cells (17) and SV40 Ori- transformed vas deferens (RVP) and epididymis (REP) cell lines (21) were cultured as originally described.

**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. RNA was isolated by the method of Chirgwin et al (22). RT-PCR was carried out as described previously (23).

**Generation of probes.** Cosmids, phage and plasmid clones encompassing 120kb of genomic DNA 5' to the CFTR gene were kindly provided by Drs Johanna Rommens and Lap Chee Tsui (2). They included the following: cW44 and cJ21 are cosmid clones; R17, W3, TE17b and TE19bB are phage clones and R14.4 E1.0 and E8.0 and B14 E5.0 and E5.4 are pBluescript subclones of the phage clones R14.4 and B14 respectively (see figure 1). The BBMT2 probe, containing a 340bp BssHII-BglI fragment of the CFTR gene from -242 to + 98 relative to the transcription start site defined by Rommens et al (1), in the vector pMT.IC3 was donated by Dr TC Suen. Individual BamHI, EcoRI or HindIII fragments were excised from the cosmid, phage or plasmid clones, gel purified, genecleaned and used as probes.

**Methylation assays.** 30 $\mu$g of genomic DNA was cleaved first with EcoRI, DNA samples were then ethanol precipitated, resuspended in NaCl-free buffer and samples divided into three 10 $\mu$g aliquots. One aliquot was further digested with 40U MspI to reveal the fragment sizes generated by MspI sites in the area and another aliquot was digested with 40U of HpaII to distinguish between methylated and non-methylated sites. Digested DNAs were resolved by electrophoresis in 0.8% agarose gels and transferred to Hybond N membranes by standard procedures. Membranes were hybridized with pre-reassociated $^{32}$P labelled probes by standard methods.

**DNAse I hypersensitivity assays.**

Chromatin from a panel of cell types was probed for DNase I hypersensitive regions by standard methods (24). Nuclei were treated in parallel aliquots to digestion with a dilution series of DNAse 1 (0 units; 15 units; 30 units; 60 units; 120 units DNAse I [FPLC*pure*, Pharmacia]). DNase I treated DNA samples were then cleaved with the relevant restriction enzymes, BamHI, EcoRI or HindIII, resolved on 0.8% agarose gels and blotted onto Hybond N membranes. Probing of membranes was carried out by standard methods.

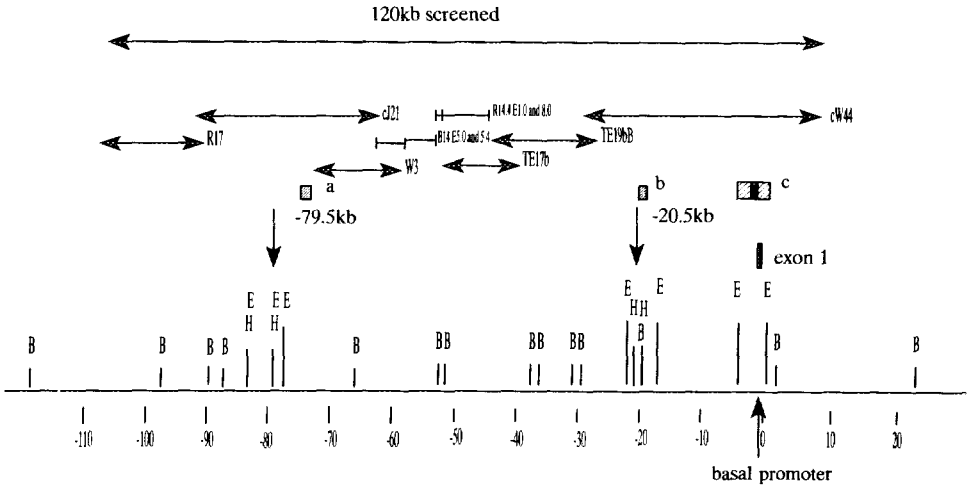


FIGURE 1.
LONG RANGE MAP OF THE 120KB 5' TO THE CFTR GENE.
The restriction map for the enzyme BamHI (B) is shown above the solid line. In addition, certain relevant sites for the enzymes EcoRI (E) and HindIII (H) are shown. The scale is in kilobases, where zero denotes the position of the ATG start codon of the CFTR coding sequence.The cosmid, phage and pBluescript clones used as a source of probes in this study are indicated by the horizontal arrows and bars. cW44 and cJ21 are cosmid clones and R17, W3, TE17b and TE19bB are phage clones. R14.4 E1.0 and E8.0 and B14 E5.0 and E5.4 are pBluescript subclones of the phage clones R14.4 and B14. The hatched boxes represent (a) cJ21 E1.6, (b) cW44 H1.1 and (c) cW44 E4.3 probes referred to in Figures 3, 4 and 5, respectively. The BBMT2 probe is shown as a solid line within the cW44 E4.3 box. The vertical arrows at -79.5kb and -20.5kb mark the location of the novel DNAse I hypersensitive sites identified in this study.

## RESULTS

EXPRESSION OF CFTR mRNA. Expression of CFTR was measured by reverse transcription-PCR (RT-PCR) in all the cell cultures used in this study at the time nuclei were isolated for hypersensitivity assays. Clearly, though this is not an accurate quantitative estimation of levels of CFTR expression, in the presence of an internal control for a house-keeping gene such as glucocerebrosidase it provides a useful gross measure of CFTR mRNA. Figure 2 shows the results of RT-PCR assays on all the cells analysed for DNAse I hypersensitivity. The upper band corresponds to a 769bp fragment from the CFTR cDNA, the lower band is a 572bp fragment from the glucocerebrosidase cDNA used as an internal control. These data are summarized in table 1.

DNASE I HYPERSENSITIVE SITES. All BamHI fragments of genomic DNA included in the region between the BamHI site 5' to the end of the phage clone R17 and the first BamHI site 3' of exon 1 were screened for DNase I hypersensitive sites, using appropriate BamHI, EcoRI or HindIII fragments of the cosmids, phage and plasmids encompassing this region as probes (figure 1). In order to assess the efficacy of DNAse treatment every batch of DNAse I-treated nuclei was assayed with the RA2.2 probe that is known to detect a constitutive DNAse I hypersensitive site within the $\alpha$-globin gene cluster in all cell types (25). This extensive

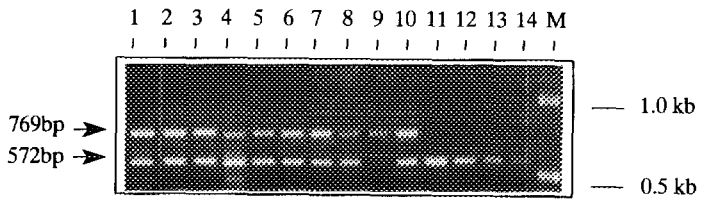


FIGURE 2.
EXPRESSION OF CFTR.
Expression of CFTR in the cell types used in this study as detected by reverse transcription-PCR. The upper band corresponds to a 769bp fragment from the CFTR cDNA. The lower band is a 572bp fragment from the glucocerebrosidase cDNA used as an internal control. Lane 1, HT-29; Lane 2, Caco-2; Lane 3, Capan-1; Lane 4, primary vas deferens epithelial cells A; Lanes 5 and 6, primary vas deferens epithelial cells B and C, respectively; Lanes 7-10, primary epididymis epithelial cells D,E,B and C,respectively; (A-E are derived from 5 different foetuses); Lane 11, immortalized vas deferens epithelial cells, RVP (6); Lane 12, immortalized epididymis epithelial cells, REP (6); Lane 13, MCF7; Lane 14, lymphoblastoid cell line; M, 1kb ladder. The no RNA and no reverse transcriptase negative controls are not shown.

search for DNAse I hypersensitive sites in the 120kb 5' to the transcription start site revealed two such regions of chromatin in addition to sites in the region of the CFTR basal promoter that probably correspond to some of those reported previously (13,15).

**1. A DNASE I HYPERSENSITIVE SITE AT -79.5KB 5' TO CFTR.** The 1.6kb EcoRI sub-fragment of cosmid cJ21 (cJ21 E1.6) shown in figure 1, revealed a DNAse I hypersensitive site in all the cell types examined. In figure 3 the probe cJ21 E1.6 is seen to hybridize to a BamHI restriction fragment of approximately 20kb. A sub-fragment of 12kb can be seen in Lanes 2-5 (15 units; 30 units; 60 units; 120 units of DNAse I respectively) in all the epididymis, Caco-2 and MCF7 cell types. This indicates the presence of a DNAse I hypersensitive site in this region, located approximately -79.5kb 5' to the ATG translation start codon of the CFTR gene (shown by the arrow in figure 1). The site does not appear to be cell type specific as it is seen in all the cell types analysed irrespective of their status with respect to CFTR expression (see Table 1).

**Table 1**
**EXPRESSION OF CFTR AND PRESENCE OF DNASE 1 HYPERSENSITIVE SITES**

| Cell Type | Level of CFTR Expression | -79.5kb site | -20.5kb site |
|---|---|---|---|
| HT-29 | +++ | ++ | ++ |
| Caco-2 | +++ | + | ++ |
| Capan-1 | ++ | + | + |
| primary vas deferens epithelial cells (vas) A,B* | ++ | ++(A) | +ʷ(B) |
| primary epididymis epithelial cells (epid) E* | ++ | ++(E) | +(E) |
| *immortalized vas deferens epithelial cells* (RVP) | +/- | + | + |
| immortalized epididymis epithelial cells (REP) | +/- | ++ | ++ |
| MCF7 | - | ++ | ++ |
| lymphoblastoid cell line (37566) | - | + | +ʷ |

The relative level of CFTR expression in the cell types used, based on the RT-PCR results and the prominence of DNAse I hypersensitive sites at -79.5kb and -20.5kb.

* A,B,E denote cultures of primary foetal genital duct cells arising from 3 different foetuses. ʷdenotes subfragment generated by DNAse 1 hypersensitive site feint.

Methylation assays for the region of genomic DNA flanking this site were uninformative due to absence of any MspI restriction sites.

**2. A DNASE I HYPERSENSITIVE SITE AT -20.5KB 5' TO CFTR.** The 1.1kb HindIII sub-fragment of cosmid cW44 (cW44 H1.1) shown in figure 1 detects a DNAse I hypersensitive site in all of the cell types analysed. In figure 4, the probe cW44 H1.1 is seen to hybridize to an EcoRI restriction fragment of approximately 4.8kb . A sub-fragment of approximately 3.5kb can be seen in Lanes 2-5 (15 units; 30 units; 60 units; 120 units of DNAse I respectively) in the REP, epididymis and MCF7 cell types. This indicates the presence of a DNAse I hypersensitive site in this region, located approximately -20.5kb 5' to the ATG translation start codon of the CFTR gene (shown by the arrow in Figure 1). The site does not appear to be cell type specific as it is seen in all the cell types analysed irrespective of their status with respect to CFTR expression (see Table 1). Though there is substantial variation in the prominence of this site in different cell types, this does not correlate with levels of CFTR expression. A second EcoRI sub-fragment of approximately 3.0kb is also detected by the cW44 H1.1 probe, as illustrated in figure 4 in Lanes 1-5. This may indicate the presence of a second DNAse I hypersensitive site or cross-hybridization of the probe to another sequence. Methylation assays for the region of genomic DNA flanking the -20.5kb site generated complex results. Preliminary data (not shown) suggest that this site is unmethylated in all cell types analysed.

**3. DNASE I HYPERSENSITIVE SITES IN THE IMMEDIATE 5'REGION.** Three major DNAse I hypersensitive sites were detected by the BBMT2 probe on BamH1 digests of DNAse I treated DNA, with an additional 2 sites being detected in some cell types (data not shown). This probe hybridizes to a BamHI fragment of approximately 22kb and sub-fragments at approximately 6.3kb, 3.2kb and 2.5kb. The 6.3kb fragment is often

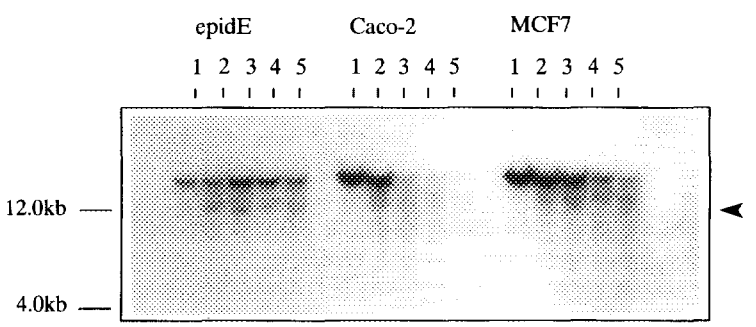


FIGURE 3.
DETECTION OF A DNASE I HYPERSENSITIVE SITE AT -79.5KB 5' TO CFTR.
A Southern blot of genomic DNA extracted from epididymis E, Caco-2 and MCF7 nuclei treated with DNAse I, digested with BamHI and probed with the cJ21 E1.6 fragment shown in Figure 1. Lane 1 shows DNA prepared from nuclei which had not been treated with DNAse I. Lanes 2-5 show DNA prepared from nuclei treated with increasing amounts of DNAse I: Lane 2, 15 units; Lane 3, 30 units; Lane 4, 60 units; Lane 5, 120 units DNAse I (FPLC*pure*, Pharmacia). The numbers on the left represent the migration of DNA size markers (1kb ladder, GIBCO BRL) in kb. The probe cJ21 E1.6 hybridizes to an approximately 20kb BamHI restriction fragment. A sub-fragment of approximately 12kb can be seen in Lanes 2-5 in all of the cell types studied, marked by the arrowhead on the right. This indicates the presence of a DNAse I hypersensitive site in this region, located approximately -79.5kb 5' to the ATG translation start codon of the CFTR gene (shown by the arrow in Figure 1).

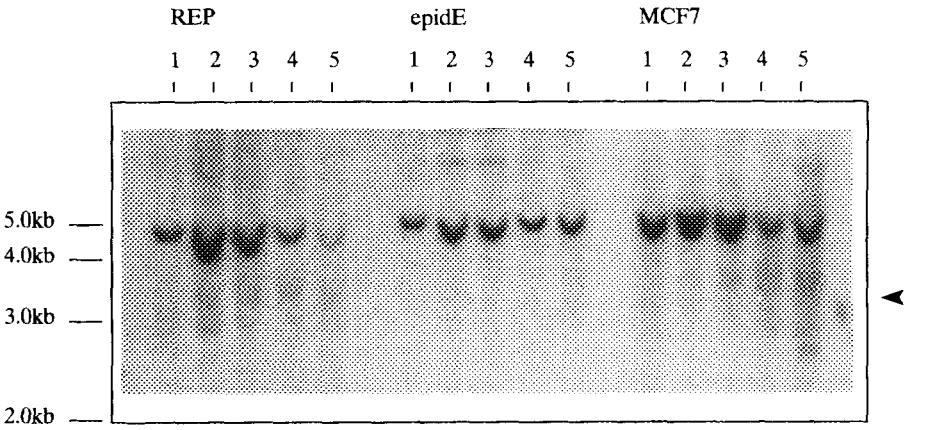


FIGURE 4.
DETECTION OF A DNASE I HYPERSENSITIVE SITE AT -20.5KB 5' TO CFTR.
A Southern blot of genomic DNA extracted from REP, epididymis E and MCF7 nuclei treated with DNAse I, digested with EcoRI and probed with the cW44 H1.1 fragment shown in Figure 1. Lane 1 shows DNA prepared from nuclei which had not been treated with DNAse I. Lanes 2-5 show DNA prepared from nuclei treated with increasing amounts of DNAse I: Lane 2, 15 units; Lane 3, 30 units; Lane 4, 60 units; Lane 5, 120 units DNAse I. The numbers on the left represent the migration of DNA size markers in kb. The probe cW44 H1.1 hybridizes to an approximately 4.8kb EcoRI restriction fragment. A sub-fragment of approximately 3.5kb can be seen in Lanes 2-5 in all of the cell types studied, marked by the arrowhead on the right. This indicates the presence of a DNAse I hypersensitive site in this region, located approximately -20.5kb 5' to the ATG start codon of the CFTR gene (shown by the arrow in Figure 1). A second sub-fragment of approximately 3.0kb can also be seen in Lanes 1-5. This may indicate the presence of a second DNAse I hypersensitive site or cross-hybridization of the probe to another sequence.

seen in chromatin prior to DNAse 1 treatment in all cell types suggesting it may be prone to endogenous nuclease activity. These hypersensitive sites are likely to correspond to those reported by others (13,15), however different reports on various cell lines are inconsistent. Though the sites that we have detected do show some correlation with CFTR expression, being most obvious in the HT-29 cell line which drives very high levels of expression of CFTR, primary vas deferens and epididymis epithelial cells that transcribe moderate levels of CFTR may only show these sites weakly. Further the SV40 transformed epididymis cell line REP, that has greatly down regulated CFTR expression, shows these DNAse I hypersensitive sites as strongly as does the HT-29 cell line, suggesting the transformed nature of these cells may be having some impact on the results. These DNAse I hypersensitive sites in the promoter region were not seen in the MCF7 mammary carcinoma cell line or in the 37566 lymphoblastoid cell line, neither of which express CFTR. Methylation analysis of the promoter region using the 4.3kb EcoRI fragment of the cW44 cosmid suggests that this region is not methylated in any of the cell types analysed here, regardless of the levels of expression of CFTR, with the exception of the MCF7 cell line which shows almost complete methylation (Figure 5). However another line that does not express CFTR, the 37566 lymphoblastoid cell line appears to be largely unmethylated in this region (not shown).

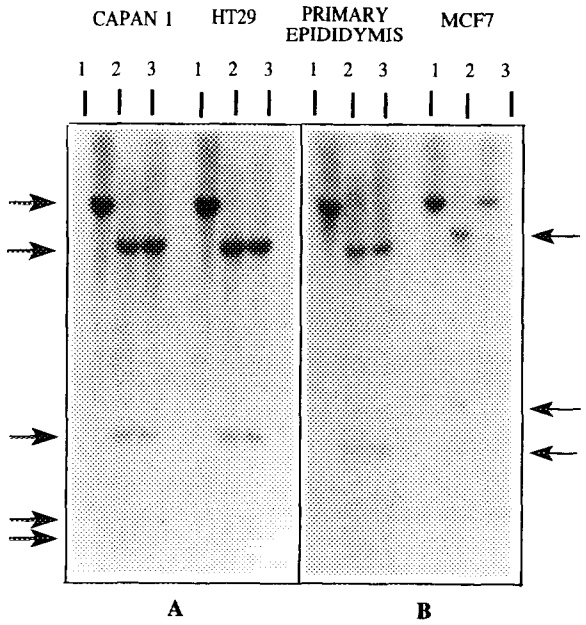


FIGURE 5.
METHYLATION OF THE CFTR BASAL PROMOTER REGION.
Southern blots of genomic DNA, in panel A from Capan-1 and HT-29, and in panel B from primary epididymis G25 and MCF7 probed with the cW44 E4.3 fragment. For each cell type the three lanes are as follows: 1) cleaved with EcoRI; 2) cleaved with EcoRI and MspI; 3) cleaved with EcoRI and HpaII. Arrows denote the 4.3kb EcoRI fragment hybridizing to the cW44 E4.3 probe and Msp1/HpaII cleavage products of 3.3kb (MCF7 only), 3kb, 990bp (MCF7 only), 720bp, 330bp and 270bp.

**DISCUSSION** Of particular importance in the context of potential targeted gene therapy for CF is the isolation of element(s) that confer tissue specificity on CFTR gene expression. Previous analyses of chromatin structure (and methylation status) of the CFTR gene promoter region have identified a number of DNAse 1 hypersensitive sites in a small number cell lines which show some correlation with CFTR expression in those lines. We have examined a much larger area of genomic DNA 5' to the CFTR gene basal promoter and 2.2kb 3' to the major transcription start site. In addition to investigating long term carcinoma cell lines that express CFTR at various levels we have analysed CFTR expressing primary vas deferens and epididymal epithelial cells. Novel DNAse I hypersensitive sites have been observed at -79.5kb and -20.5kb 5' to the ATG translation start codon of CFTR. Neither of these sites appears to show strong correlation with the expression of CFTR in the cell lines analysed, hence they are unlikely to reflect the sites of binding of the major CFTR tissue specific regulator(s). However regulation of expression of the CFTR gene is clearly a complex series of events. The potential regulatory elements that we have defined at -79.5kb and -20.5kb 5' to the ATG translation start codon of the CFTR gene and those reported here and described by others within the basal promoter region are insufficient to construct an accurate model of regulation, however they

may all be involved in some way. Further investigation is required to elucidate the mechanisms of control of CFTR gene expression and it remains likely that CFTR tissue specific control elements may lie elsewhere in the CFTR gene locus.

**Acknowledgments** We are grateful to Drs. Johanna Rommens, Lap-Chee Tsui and T.C. Suen for providing cosmid, phage and plasmid clones, to Drs. Douglas Higgs and Charles Craddock, Tony Hollingsworth and Erick Denamur for helpful discussions, to Hugh Nuthall and to Prof. Richard Moxon for support. Funded by the *Cystic Fibrosis Research Trust UK and the Association Francaise de Lutte Contre la Mucoviscidose (AFLM).*

## REFERENCES

1. Riordan, JR., Rommens, JM., Kerem, B-S., Alon, N., Rozmahel, R., Grzelczak, Z., Lok, S., Plavsic, N., Chou, J-L., Drumm, ML., Iannuzzi, MC., Collins, FS., Tsui, L-C. (1989) Science **245**:1066-1073
2. Rommens, JM., Iannuzzi, MC., Kerem, B-S., Drumm, MJ., Melmer, G., Dean, M., Rozmahel, R., Cole, J., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan JR., Tsui, L-C., Collins, FS.(1989) Science **245**:1059-1065
3. Crawford, IC., Maloney, PC., Zeitlin, PL., Guggino, WB., Hyde, SC., Turley, H., Gatter, KC., Harris, A., Higgins CF. (1991) *Proc. Natl. Acad. Sci. USA* **88**:9262-9266
4. Denning, GM., Ostedgaard, LS., Cheng, SH., Smith, AE., Welsh, MJ. (1992) J.Clin.Invest. **89**:339-349
5. Marino, CR., Matovcik, LM., Gorelick, FS., Cohn, JA. (1991) J.Clin.Invest. **88**:712-716
6. Engelhardt, JF., Yankaskas, JR., Ernst, SA., Yang, Y., Marino, CR., Boucher, RC., Cohn, JA., Wilson, JM. (1993) *Nature Genet.* **2**:240-247
7. Fitz, JG., Basavappa, S., McGill, J., Cohn JA. (1993) J. Clin Invest. **91**: 319-328
8. Kartner, N., Augustinas, O., Jensen, TJ., Naismith, AL., Riordan, JR. (1992). *Nature Genet.* **1**:321-328
9. Harris, A., Chalkley, G., Goodman, S., Coleman, L. (1991) Development **113**:305-310
10. Foulkes, AG., and Harris, A. (1993) Pancreas **8**:3-6
11. Trezise, AEO., Chambers, JA., Wardle, CJ., Gould, S., Harris, A. (1993) Hum. Mol. Genet. **2**:213-218
12. Trezise, AEO., Buchwald, M. (1991) Nature **353**:434-437
13. Yoshimura, K., Nakamura, H., Trapnell, BC., Dalemans, W., Pavirani, A., Lecocq, J-P., Crystal RG.(1991) J. Biol. Chem. **266**:9140-44
14. Chou, J-L., Rozmahel, R., Tsui, L-C. (1991) J. Biol. Chem. **266**:24471-24476
15. Koh, J., Sferra, J., Collins, FS. (1993) J. Biol. Chem. **21**:15912-15921
16. Denamur, E., Chehab, FF. (1994) Hum. Mol. Genet. **3**:1089-1094
17. Harris, A., Coleman, L. (1989) J. Cell. Sci. **92**:687-690
18. Fogh, J., Wright, WC., Loveless, JD. (1977) J. Natl. Canc. Inst. **58**:209-214
19. Huet, C., Sahuquillo-Merino, C., Coudrier,E., and Louvard, D. (1987) J. Cell Biol. **105**:345-357
20. Soule, HD., Vazquez, J., Long, A., Albert, S., Brennan, M. (1973) J. Natl. Cancer Inst. **51**:1409-1416
21. Coleman, L., Harris, A. (1991) J. Cell. Sci. **98**:85-89
22. Chirgwin, JM., Przybyla, AE., Macdonald, RJ., and Rutter, W. J. (1979) Biochemistry **18**:5294-5299
23. Chambers, JA., and Harris, A. (1993) J. Cell Sci. **105**:417-422
24. Higgs, DR., Wood, WG., Jarman, AP., Sharpe, J., Lida, J., Pretorius, I-M., Ayyub, H. (1990) Genes and Devel. **4**:1588-1601
25. Vyas, P., Vickers, MA., Simmons, DL., Ayyub, H., Craddock, CF., Higgs, DR. (1992) Cell **69**:781-793