RESULTS

Aberrant splicing along the CFTR gene

Exon 12 showed 10%+2% aberrantly spliced CFTR transcripts, while in exons 3, 4, 14a, 16, 17b and 22 only correctly spliced transcripts were identified (data not shown). The splicing pattern of these exons was not modulated by any of the studied splicing factors (data not shown). We also analyzed 9 constitutively spliced exons (5, 6a, 7, 11, 14b, 17a, 18, 19, 20) that as expected were not modulated by the studied factors.

Quantification of CFTR mRNA

Since we used differential RT-PCR reactions in which the shorter product might preferentially be amplified, the different transcripts were serially mixed in varying, known quantities ranging from 100% correctly to 100% aberrantly spliced transcripts, and subjected to the same PCR conditions. Experiments were repeated 3 times The analysis of exon 9^+ (447bp) and exon 9^- (264bp) transcripts revealed slight differences between the actual and the experimental relative proportions (Fig 1S). Thus, all experimental proportions of exon 9^+ and exon 9^- transcripts were accordingly adjusted. However, no differences were found for correctly (304bp) and aberrantly (388bp) spliced transcripts, in the 84bp region (data not shown).

METHODS

Cell-lines

Epithelial cell lines were established from nasal polyps of CF patients using E6/E7 genes of the human papilloma virus 18 and hTERT (CFP22a) or by E6/E7 only (CFP15a) (Lundberg et al., 2002; Yankaskas et al., 1993). CFP15a, which carries the 3849+10kb C->T splicing mutation and the W1282X nonsense mutation, is associated with IVS8-7T and 12GT repeats. The cells were grown in BEBM (Cambrex). IB3 and T84 cells were grown in DMEM and DMEM-F12, respectively.

Western Blot analysis

Total protein was extracted from cells using standard procedures (Harlow and lane, 1987). Equal amounts of protein were loaded on SDS-PAGE and transferred to nitrocellulose membrane (Sartorius AG). The following antibodies were used: mouse monoclonal against SRp20 (Santa Cruz, 1:100), hnRNP A1 (abcam, 1:1000), SF2/ASF (a gift from Krainer, 1:500), mAb104 (a gift from Stamm, 1:100), rabbit polyclonal against Htra2-β1 (a gift from Stamm, 1:2000), goat polyclonal against α-actin (Santa Cruz, 1:1000). Immunodetection was accomplished by enhanced chemiluminescence (Santa Cruz luminal reagent, Santa Cruz) followed by autoradiography on film (FUJIFILM).

CFTR functional analysis

Cells were loaded overnight with N-(6-methoxyquinolyl) acetoethyl ester (MQAE). The rate of Cl^- efflux was measured in response to exchange of extracellular Cl^- with nitrate (NO_3^-), an anion that passes through the CFTR but unlike Cl^- does not quench the indicator's fluorescence. Activation of the CFTR chloride channel was stimulated by the cAMP agonist, forskolin. Specifically, following overnight incubation with MQAE, cells were washed twice

with Cl^- buffer and further incubated in this buffer for 15 min prior to the experiment. The Cl^- buffer was then washed twice and replaced with NO_3^- buffer (time=0), and the fluorescent level in the cells was immediately read for 1 min, in cycles of 13 seconds. Cells were activated with 1ng forskolin in NO_3^- buffer, and immediately read for additional 3 min also in 13s cycles. The fluorescent measurements were performed using the FLUOstar galaxy fluorescent reader (BMG LabTechnologies). T84 cells were included in each experiment to verify that the system is functional. CFP22a or IB3 cell lines were used to verify that the splicing modulation is specific for splicing mutations. It is important to note that the CFTR channel activity (measured by the Cl^- efflux slope following forskolin administration) can not be compared between different cell lines since it depends strongly on the cell volume, membrane thickness, the number of CFTR and other chloride channels etc.

CFTR RNA level analysis

The level of CFTR RNA in the CF-derived cell lines (IB3, CFP22a and CFP15a) was analyzed using LightCycler real-time PCR (software version 3.5, with a FastStart DNA Master SYBR Green I kit, Roche Diagnostics). The CFTR levels, amplified by the forward primer – 5'GAGGGTAAAATTAAGCACAGT 3' and the reverse primer – 5'TGCTCGTTGACCTCCA 3', were normalized to those transcribed from a control gene, the ribosomal protein S9 gene (RPS9). This gene was amplified by the forward primer – 5'AGACCCTTCGAGAAATCTCGTCTCG 3' and the reverse primer – 5'TGGGTCCTTCTCATCAAGCGTCAGC 3'. For each pair of primers, a standard curve was performed and annealing temperatures as well as elongation times were optimized to exclude PCR artifacts**.** Experiments were repeated at least 3 times

REFERENCES

Harlow, E., and Lane, D. (1987). "Antibodies: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Lundberg, A.S. et al. (2002) Immortalization and transformation of primary human airway epithelial cells by gene transfer. *Oncogene*, **21**, 4577-4586.

Yankaskas, J.R. et al. (1993) Papilloma virus immortalized tracheal epithelial cells retain a well-differentiated phenotype. *Am. J. Physiol.*, **264**, C1219-1230.

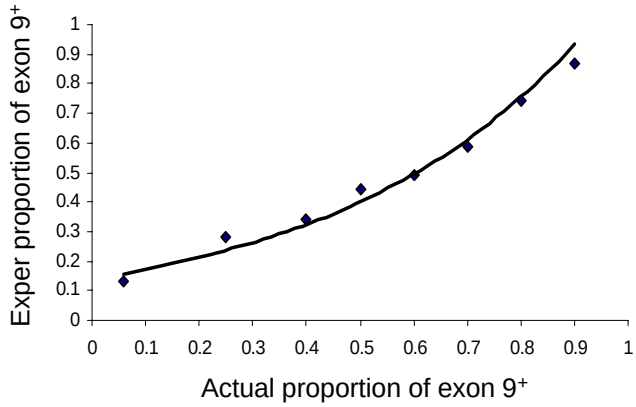
FIGURE LEGENDS

Fig. 1S. Determination of proportion of exon 9^+ CFTR transcripts. Actual versus experimental proportions are presented. The regression line is indicated (R^2= 0.967).

Table I: Primers used for detecting aberrantly spliced CFTR exons in CFP15a cells

Primer	Sequence	Location (exon)	Annealing temperature(°C)
2R	5’ TGAGGAAAGGATACAGACAGCG 3’	2	58
5x3	5’ CTAGAACACGGCTTGACAGC 3’	5	
R117	5’ ACCCGGATAACAAGGAGGAA 3’	4	55
6bx3	5’ CCAGCAGTATGCCTTAACAG 3’	6b	
6bx5	5’ CAGAGAGCTGGGAAGATCAGTG 3’	6b	58
8-9R	5’ AAATAATTCCCCAAATCCCTCCTCC 3’	8+9	
8Rx5	5’ AAGCAAGAATATAAGACATTGG 3’	8	55
F10Rx3*	5’ TTGGCATGCTTTGATGACGC 3’	10	
X10	5’ GATTATGGGAGAACTGGAGC 3’	10	58
13Ai3s	5’ TGGTCGAAAGAATCACATCC 3’	13	
13d	5’ CCAGTGGTAGACCTCTGAAG 3’	13	55
15x3	5’ CCAGTGGTAGACCTCTGAAG 3’	15	
15x5	5’ GTAGCCGACACTTTGCTTG 3’	15	58
17bx3	5’ GTAAATTCAGAGCTTTGTGG 3’	17b	
17ax5	5' CCAAACCTCACAGCAACTCA 3'	17a	60
19x3	5' CGAGAGTTGGCCATTCTTGT 3'	19	
18x5	5’ GTAAACTCCAGCATAGATGTGG 3’	18	60
Fx20*	5’ GCCTCTTGGGAAGAACTGGAT 3’	20	
18x5	5’ GTAAACTCCAGCATAGATGTGG 3’	18	62
ex21R	5’ CCACTGTTCATAGGGATCCAA 3’	21	
x21B	5’ TTGGATCCTATGAACAG 3’	21	50
x23A	5’ TTACTGTGCAATCAGCAA 3’	23	

*Fluorescently labeled with 6-FAM.


Exper proportion of exon 9+
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Actual proportion of exon 9+