ONLINE MUTATION REPORT

Transcript analysis of the cystic fibrosis splicing mutation 1525-1G>A shows use of multiple alternative splicing sites and suggests a putative role of exonic splicing enhancers

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The autosomal recessive disease cystic fibrosis (CF, MIM 219700) is caused by a wide spectrum of mutations in the gene encoding for the CF transmembrane conductance regulator (CFTR, MIM 602421) protein, a cAMP regulated chloride (Cl^-) channel located in the apical membrane of secretory epithelial cells.

About 20% of the more than 1000 *CFTR* mutations reported so far are splicing mutations and generally they represent 15% of all point mutations.[1] Although it is generally agreed that mutations at the conserved splice dinucleotide consensus sites GU (donor) and AG (acceptor) disrupt function of the resulting protein product, it is important to know exactly which alternatively spliced mRNA molecules will result in such situations.

Key points

- The cystic fibrosis (CF) 1525-1G>A mutation abolishes the normal intron 9 acceptor, forcing the spliceosome to use alternative sites. The in frame deletion of exon 10 was proposed to occur and various alternative acceptors in intron 9 present high consensus values (CVs).
- Here, we studied two CF patients with the 1525-1G>A/F508del genotype. The patients are first cousins, both female, of Afghan descent, with typical CF: raised sweat chloride levels, pancreatic insufficiency (PI), and pulmonary disease.
- Analysis of *CFTR* mRNA from the colonic epithelium by RT-PCR and automatic sequencing confirmed the presence of a transcript lacking exon 10 and detected the additional presence of two transcripts using acceptors within exon 10 at positions 1610-1611 (an intense band, exon 10-Alt1) and 1678-1679 (a minor product, exon 10-Alt2).
- These two acceptors were not among those with the strongest CVs. A possible explanation for their usage may be the presence of two exonic splicing enhancers (ESEs) in exons 10 and 11.
- CFTR mediated Cl^- secretion was assessed in rectal biopsies mounted in modified Ussing chambers and was found to be absent in both CF patients.
- Altogether, these results illustrate the difficulties of predicting the effects of splicing mutations (frequent in virtually all genetic diseases), the importance of analysing transcripts and of assessing function of translated products in native tissues, because of its impact on the clinical phenotype.

Identification of such products will provide insights into the splicing process and help to validate mathematical models designed to estimate the potential for novel splice site use.[1] As some alternative splicing products may be translated into functional proteins, a more accurate prediction of alternative transcripts may also be relevant to evaluate the impact of such mutations on the disease phenotype. Moreover, it is important to determine experimentally the effect of mutations at the molecular and functional level, since this may lead to the choice of appropriate therapy. For example, characterising the aberrant transcripts resulting from a splicing mutation may be useful, as manipulation of splicing sites is currently being developed and tested, possibly becoming a therapeutic option in the near future.[2 3]

The 1525-1G>A mutation in intron 9 of the *CFTR* gene (see http://www.genet.sickkids.on.ca/cftr/) was previously described in a CF patient of Indo-Iranian origin with severe gastrointestinal and pulmonary disease.[4] This mutation abolishes the normal AG acceptor of intron 9 and thus alternative acceptors have to be used. It was proposed that 1525-1G>A would cause an in frame deletion, by skipping of exon 10, in the corresponding mature transcript.[4]

Here, we analysed CFTR mRNA from the colonic epithelium of two CF patients with 1525-1G>A in compound heterozygosity with F508del and performed functional analysis of CFTR mediated Cl^- secretion in the same tissue.

MATERIALS AND METHODS

Patients, genotypes, and rectal biopsies

Two patients, first cousins, both female and of Afghan descent, were diagnosed with typical CF at the age of 2 (patient 1) and 3 (patient 2) years of age by using the published criteria,[5] including pancreatic insufficiency (PI), chronic lung disease with *Pseudomonas aeruginosa* infections, and raised sweat tests (102 and 107 mmol/l [Cl^-], respectively). F508del was detected by an allele specific PCR based test kit (Elucigene CF 20 kit, Orchid Diagnostics, Abingdon, UK) and 1525-1G> was detected following sequencing of all 27 *CFTR* gene exons on an ABI Prism 373A automatic sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism Big Dye Ready Reaction Terminator Cycle Sequencing kit (Applied Biosystems). Small superficial rectal biopsies were obtained by forceps biopsy performed at the

Abbreviations: CF, cystic fibrosis; CCH, carbachol; CFTR, CF conductance transmembrane regulator; CV, consensus value; CVA, CV for alternative acceptor; CVN, CV for the normal acceptor; ESE, exonic splicing enhancers; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; I_{SC}, short circuit current; RT, reverse transcription; PI – pancreatic insufficient; PTC, premature termination (stop) codon; SEM, standard error of the mean

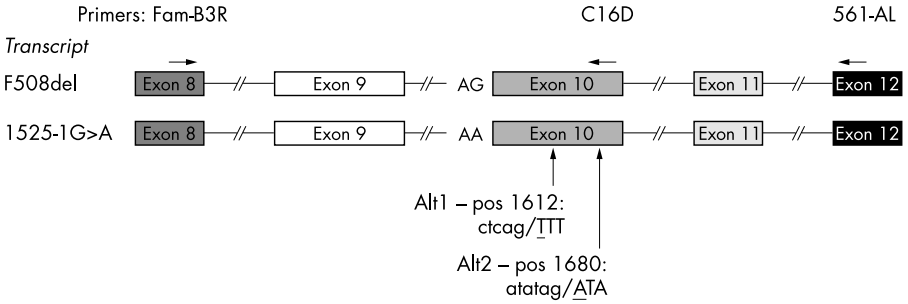

Figure 1 Schematic representation of region in the *CFTR* gene analysed, showing the location of PCR primers and positions of the two exonic alternative splicing sites used by the 1525-1G>A transcripts at positions 1610-1611 (Ex 10-Alt1) and 1678-1679 (Ex 10-Alt2), respectively.

University Children's Hospital Freiburg when the patients were 18 and 16 years of age, respectively, following approval by the hospital ethical committees and the patients' written informed consent. Rectal biopsies from three non-CF subjects were also analysed.

mRNA analysis

One rectal biopsy from each patient was soaked in extraction buffer and total RNA was extracted as before.[6] RT-PCR amplification was carried out in the region of exons 8-10 with primers Fam-B3F and C16D as published before[6] and in the region of exons 8-12 also with Fam-B3F and the following 561-AL: 5'ATACAAATCAGCATCTTTGTATACTG3' (fig 1). RT-PCR products were qualitatively analysed by fragment analysis as described previously[6] and collected manually into different fractions after separation by the DHPLC Transgenomic WAVE® System (Omaha, NE) for reamplification before sequencing. The same primers as above were used for sequencing and products were analysed on the ABI Prism™ 3100 sequencing system (Applied Biosystems).

Consensus values

The complete sequences of intron 9 and exon 9 were submitted to the Spliceview program[7] (free online at: http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html) in order to predict potential cryptic acceptor splice sites. About 48 alternative intronic acceptors were found with scores (CVAs) equal to or higher than the normal acceptor (CVN). For confirmation, CVAs were also calculated as before,[8] according to previously described methods,[9 10] for five of those 48 acceptors lying close to the normal exon-intron junctions, as well as for the normal one (CVN). Branch sites for normal and alternative acceptors were considered as previously,[8] that is, the adenine residue with the highest CV (between -18 and -37 relative to AG).

Ussing chamber experiments

Rectal tissue biopsies were mounted into perfused micro-Ussing chambers and bioelectric measurements were performed as described previously.[11] To determine CFTR mediated Cl^- secretion, tissues were pre-treated with indomethacin (10 µmol/l, basolateral) and amiloride (10 µmol/l, luminal) and allowed to equilibrate for 60 minutes. Then cAMP dependent and cholinergic Cl^- secretion was determined by activation with IBMX/forskolin (100 µmol/l and 1 µmol/l, basolateral) and carbachol (100 µmol/l, basolateral), respectively. For each subject, transepithelial measurements were performed on three rectal biopsies. Data for transepithelial measurements are shown as original recordings or as mean (SEM) (n=number of biopsies).

RESULTS AND DISCUSSION

Detection and characterisation of aberrant splicing products

We qualitatively analysed here transcripts from the colonic epithelium of two patients with typical CF and the 1525-1G>A/F508del genotype.

RT-PCR analysis in the region of exons 8-12 was performed in order to observe whether exon 10 skipping occurs. In this region, besides the correctly spliced product, termed C (that is, F508del transcripts), two major peaks are observed (fig 2A), one, termed A, with about 190 nt less and another, termed B, with about 90 nt less. From the size of these products, we postulate that product A results from skipping of the whole exon 10, that is, splicing between intron 9 donor and intron 10 acceptor, and that the B product most probably corresponds to usage of an internal acceptor site at Q493 (nt 1610-1611) as acceptor (in exon 10). Another three very minor peaks are also observed. Although the results shown in fig 2A suggest that the aberrantly spliced transcripts (peaks A and B) are present in similar amounts to the F508del transcript alone, these results should not be interpreted quantitatively as the PCR reaction was allowed to proceed for 35 cycles (that is, out of the exponential phase) in order to allow detection of minor peaks. Additionally, it is expected that the aberrant mRNAs amplify at higher efficiency,[6] as they have, respectively, 190 nt and 90 nt less than the F508del transcript. However, the experiment was repeated with a lower number of cycles and without correcting for size specific amplification efficiency differences, the sum of the aberrant transcripts accounts for 53% whereas F508del ones account for 47% (data not shown). It is expected that, if such a correction is introduced, these percentages will be roughly similar.

To increase resolution and distinguish these smaller peaks, another PCR reaction in the region of exons 8-10 was performed with the same forward Fam-B3F primer and the C16D reverse primer[6] (fig 1). In this region, five peaks (designated D to H) are clearly observed (fig 2B).

All the fragments obtained were identified by sequencing (fig 3), following separation by DHPLC (see Methods and fig 2C), confirming that product A results from skipping of exon 10, product B results from the usage of an internal acceptor site at Q493, which we term Ex10-Alt1 (positions 1610-1611, fig 3), D (121 nt) corresponds to skipping of exon 9 plus use of AG at Q493, and F (236 nt) corresponds to use of alternative AG dinucleotide at R516 (which we term Ex10-Alt2), that is, positions 1678-1679 in exon 10 (fig 1).

We also confirmed by sequencing (data not shown) that fragment E (205 nt) corresponds to F508del transcript without exon 9, fragment G (304 nt) is an alternative transcript using Alt1 (same as B above, see also fig 1), and product H (388 nt) is normally spliced F508del transcript. Skipping of exon 9 is a generally described phenomenon and levels of transcripts lacking exon 9 inversely correlate with the length of the polypyrimidine tract in intron 8[12] and positively with the number of (TG) repeats in the same region.[13]

A summary of the RT-PCR products obtained using 8-12 primers (A) or 8-10 primers (B) and their identification, following sequencing, is given in fig 4.

Contrary to skipping of other *CFTR* exons, transcripts lacking exon 10 (or the other aberrant transcripts detected here) were never observed in non-CF controls (A Ramalho, unpublished results).

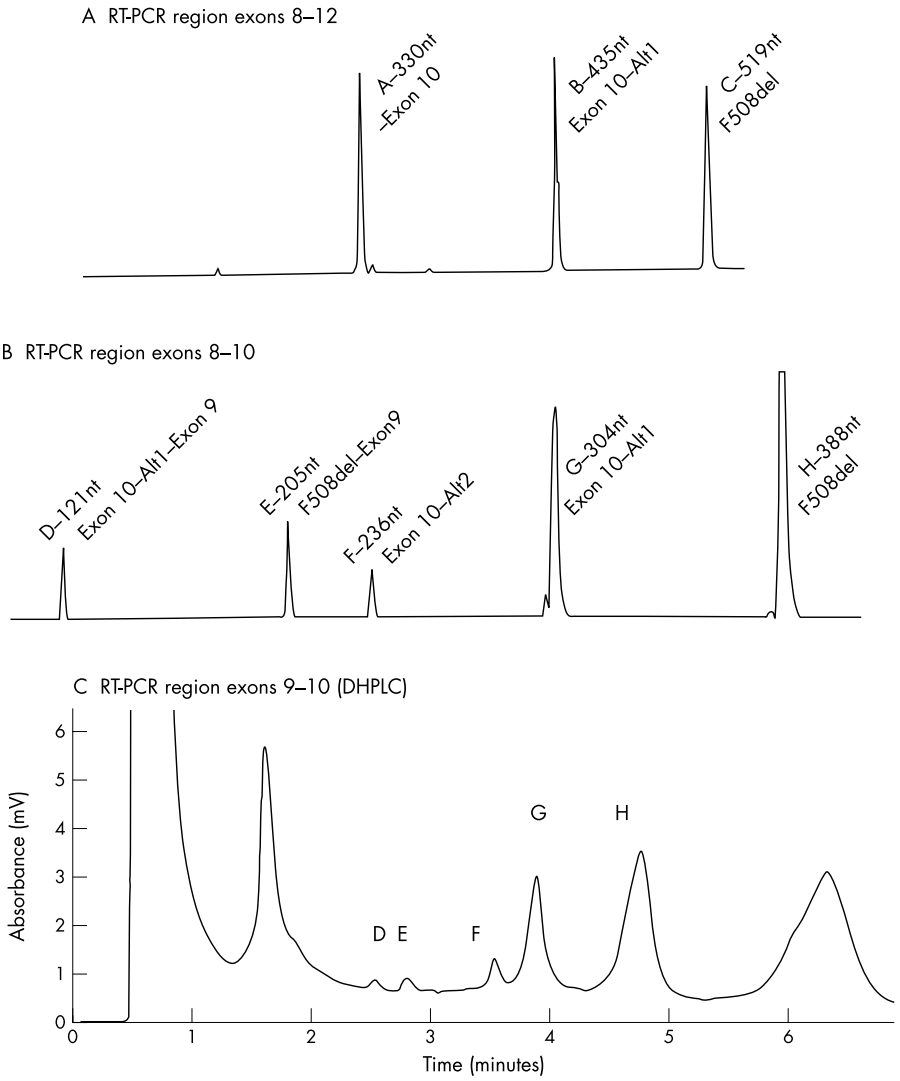

Figure 2 Analysis on a capillary electrophoresis ABI PRISM automatic sequencer of RT-PCR products obtained with Fam-B3F (exon 8, forward) and (A) C16D (exon 10, reverse) or (B) 561-AL (exon 12, reverse) primers. (C) Same as in (B), but instead of separation by the automatic sequencer, different products were separated by DHPLC (see Methods) and collected for further analysis (sequencing). It should be noted that both PCR reactions in (A) and (B) were allowed to proceed for 35 cycles (that is, out of the exponential phase of the reaction) in order to allow detection of minor peaks. Therefore, results presented should not be interpreted quantitatively.

Prediction of splice site usage versus aberrant splicing products detected

Recently, several computational tools were developed to detect the occurrence of potential splice sites in long raw eukaryotic sequences, like those generated by large genomic sequencing programs.[14] Although, like previous ones,[9 10] these methods also provide CV estimates based on nucleotide frequencies at proven splice sites, they do not require previous functional knowledge on the sequences.

Using one of these tools[7] (see Methods), the whole sequence of intron 9 and exon 10 was scanned for potential cryptic acceptors and the score for the normal splice acceptor of intron 10 (CVN) was determined to be 81. From the total number of potential acceptor sites detected by this method, 48 within intron 9 plus another within exon 10 (Ex10-Alt1) were found with a CV equal to or higher than this CVN. For confirmation, CVs according to Shapiro and Senapathy[9 10] were also determined for five of these cryptic acceptors with CVs equal to or higher than 81, which lie closer to the normal exon-intron junctions (and thus have a higher probability of being used) and for the exonic alternative splice sites (Ex10-Alt1 and Ex10-Alt2), the former identified by Spliceview and the second shown here to be used as well, although resulting in a minor product (see above). Table 1 shows that CVAs estimated for these cryptic acceptors by both methods generally correlate quite well. However, CVs for two of them (Int9-Alt4 and Int9-Alt5) were found to be lower than CVN, only when determined according to classical methods.[9 10] Using these methods, CVAs for exonic acceptors Alt1 and Alt2 (this one undetected in Spliceview) were also found to be significantly lower (71.15 and 61.18, respectively) than CVN (72.02, table 1).

CV estimates for branch sites did not change the ranking of alternative acceptors according to their respective strength (table 1). Indeed, generally strong acceptors are coupled to strong branch sites and the same applies for weak acceptors. It was thus surprising that analysis of transcripts did not show the presence of any mRNA resulting from use of any of the strong intronic alternative acceptors identified by the computational method used here. Instead, mRNAs resulting from use of two exonic splice sites were detected, one predicted, but weak (Ex10-Alt1) and another not predicted (Ex10-Alt2), although the latter resulted in a minor product. The reason why the strong intronic alternative sites are not used in vivo is not clear.

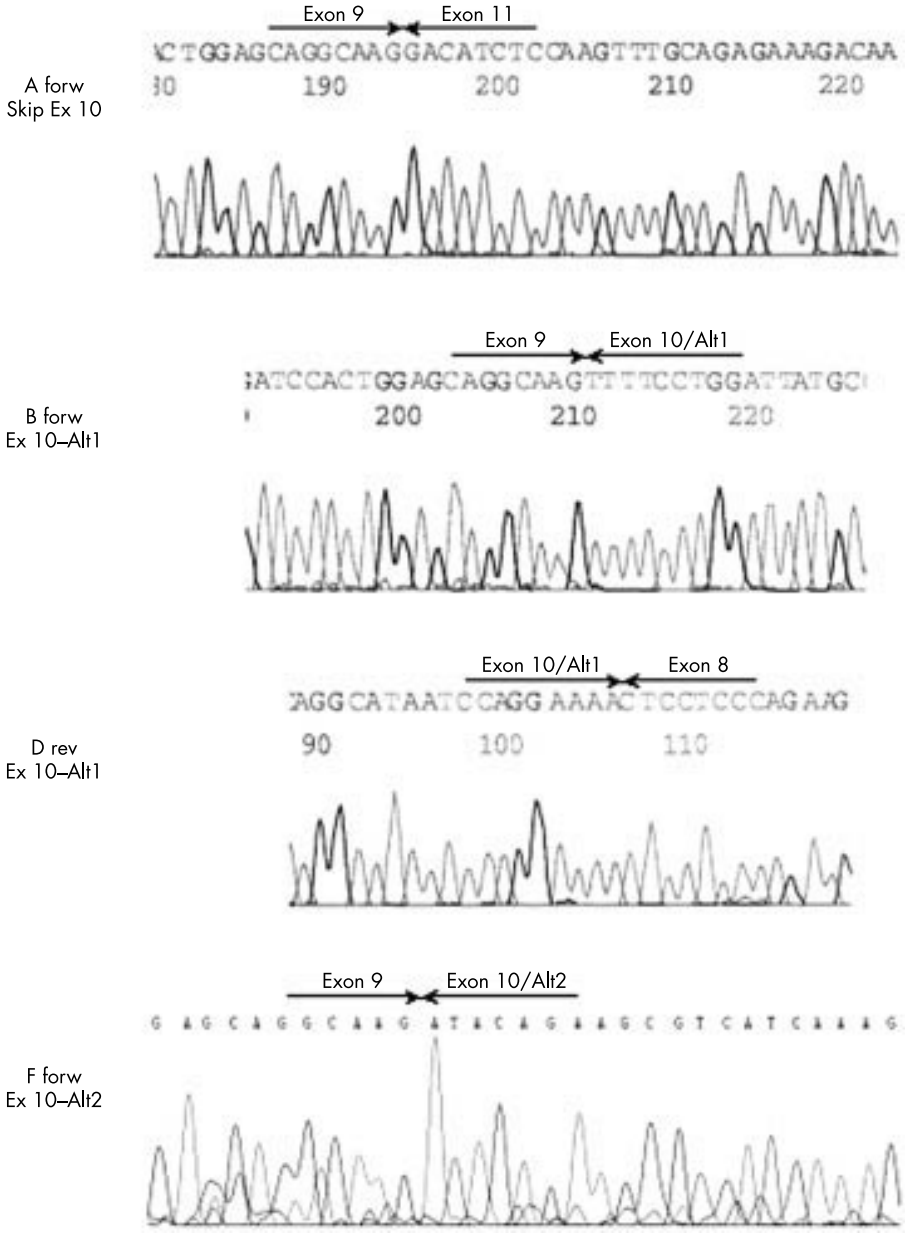

Figure 3 Automatic sequencing of RT-PCR products A, B, D, and F (as defined in fig 2). A forw, sequence of transcript showing junction of exon 9 and exon 11 (exon 10 skipping); B forw, sequence of transcript showing junction of exon 9 and position 1612 in exon 10 (Ex10-Alt1); D rev, reverse sequencing of the transcript showing junction of exon 8 and position 1612 in exon 10 (Ex10-Alt1), same acceptor used as in B but transcript lacking exon 9 as well; F forw, sequence of transcript showing junction between exon 9 and position 1680 in exon 10 (Ex10-Alt2).

Table 1 also shows translation properties of the predicted alternative transcripts. Only Int9-Alt2 and Ex10-Alt1 are in frame with (the rest of) exon 10. All the other four predicted alternative intronic, as well as Ex10-Alt2, result in out of frame transcripts. However, from all these predicted alternative transcripts, using intronic or exonic sites, the only one that does not bear a premature stop codon (PTC) is Ex10-Alt1.

It is also possible that potential splice sites resulting in introns of relatively small size (in the range of 60-300 nt, for example, Int 9-Alt1-4) are not used because of spliceosome steric hindrance. Indeed, it was suggested that splicing may be susceptible to intron size constraints,[15] since human genome analysis shows that only about 15% of genes have introns <100 bp.[16] Confirming these data, the smallest intron in the *CFTR* gene (intron 22) is ~600 nt. On the other hand, the huge size of the resulting exons (of about 10 kb) could also constitute a constraint to usage of other acceptors (for example, Int 9-Alt1-4). The presence of these, or of another large alternatively spliced transcript, cannot be ruled out in the current situation, as the method used here would miss such a large fragment.

If Int9-Alt5 were used by the spliceosome, however, an intron close in size to the normal intron 9 (~10 kb) would result. Similarly, the resulting exon from use of this site would only possess an additional ~160 nt, amounting to a total of ~490 nt, that is, an usual exon length. On the other hand, although Int9-Alt5 is predicted to be weaker (CVA=70.18) than the normal acceptor (CVN=72.12), this can hardly be the only argument for its exclusion by the spliceosome, because the much weaker Ex10-Alt2 (CVA=61.18) is used. Enhanced degradation of messengers bearing PTCs is a generally described mechanism, termed nonsense mediated decay, or NMD,[17] which was also described for CFTR transcripts.[18] It is

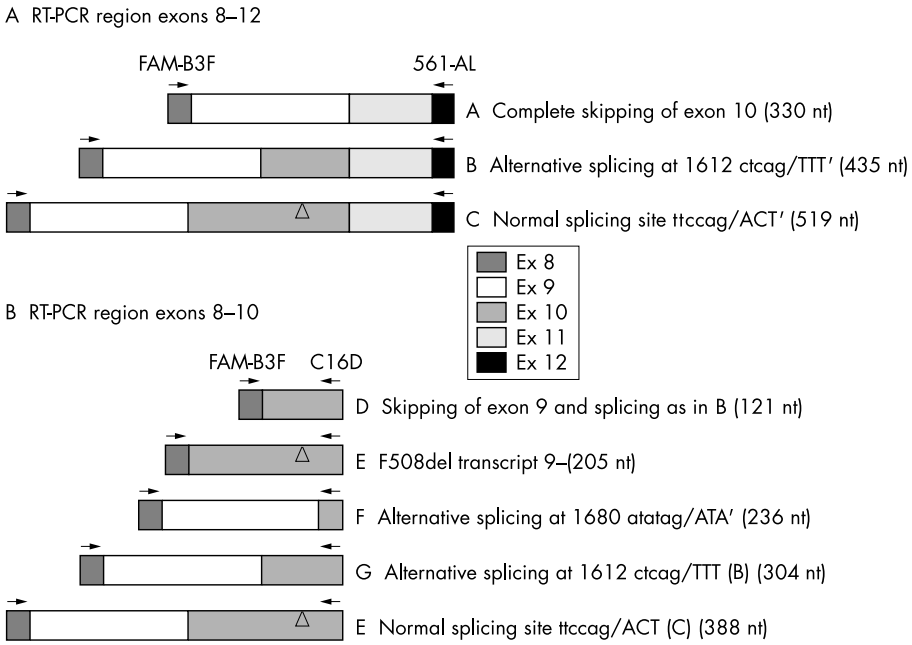

Figure 4 Schematic representation of the multiple products resulting from alternative splicing owing to the presence of the 1525-1G>A mutation. (A) Products resulting from reaction with set of primers with Fam-B3F and 561-AL. (B) products resulting from reaction with set of primers Fam-B3F and C16D. Products are designated A-H, as in fig 2. The symbol Δ refers to transcripts bearing the F508del mutation.

thus possible that the alternative transcript resulting from usage of the Int9-Alt5 site is formed, but is degraded because of NMD.

More difficult to explain is use of the weak Ex10-Alt2 alternative acceptor which also bears a PTC. It has been described that the "weakness" of splice sites can be compensated for by the presence of 5′ or 3′ exonic splicing enhancers, or ESEs.[19] These are *cis* acting splicing enhancers that stimulate spliceosome assembly at nearby sites through binding of SR proteins.[20] Although ESEs have been difficult to identify, a recently developed computational method predicted hexanucleotide motifs which were experimentally confirmed to enhance splicing activity in vivo.[21] We have looked for such motifs within *CFTR* sequences of both exons 10 and 11 and found two, namely GAAGAA (ex 10, 1589-1594) and GAATCA (ex 11, 1763-1768).

If this sequence in exon 11 acts as an ESE, it could "force" the spliceosome to assemble at the normal intron 10 acceptor (which has a relatively low CVN of 72.02), but also when intron 9 acceptor is mutated, explaining the exon 9-exon 11 aberrant splicing observed here. If the sequence in exon 10 acts as an ESE, it could explain why the two weak exonic alternative acceptors, Ex10-Alt1 and 2, are used instead of Int9-Alt5 or any of the other strong intronic alternative acceptors (table 1). Indeed, one of the exonic alternative acceptors (Ex10-Alt1) lies only 15 nucleotides away from this putative ESE in exon 10 and the second (Ex10-Alt2) about 85. The nearest cryptic alternative intronic acceptor (Int9-Alt5) lies about 250 nucleotides away from this putative ESE.

Table 1 Summary of potential splice site acceptors in the region of intron 9/exon 9, respective CVs, and translation properties of corresponding mRNAs

Acceptor site	Sequence (AG position)	CV splice view	CV[10]	CV branch*	In frame	Premature stop (position)
Int 9-Alt1	TTTTTCTAG TTTG (1524 + 63/64)	89	78.51	74.79 (-20 nt)	No	Yes (+39 nt)
Int 9-Alt2	AGTTTGTAG TGCT (1524 + 70/71)	85	73.48	74.79 (-27 nt)	Yes	Yes (+39 nt)
Int 9-Alt3	TATTTTCAG TGC (1524 + 199/200)	84	75.41	72.65 (-37 nt)	No	Yes (+72 nt)
Int 9-Alt4	CTTAACCAG AACA (1524 + 299/300)	82	70.09	67.15 (-28 nt)	No	Yes (+36 nt)
Int 9-Alt5	TGTGCATAG CAGA (1525-164/5)	81	70.18	70.97 (-27 nt)	No	Yes (+9 nt)
Int 9-norm	TATTTCCAG ACTT (1525-1/2)	81	72.12	59.81 (-37 nt)	Yes	No
Ex 10-Alt1	TGTTCTCAG TTTT (1610-1611)	83	71.15	66.94 (-19 nt)	Yes	No
Ex 10-Alt2	TGAATATAG ATAC (1678-1679)	†	61.18	63.02 (-29 nt)	No	Yes (+213 nt)
Int 10-norm	TGGTAATAG GACA (1714-1/2)	83	72.02	65.60 (-20 nt)	Yes	No

*Branch site considered was the one with highest CV. Position of branch alanine is indicated between brackets and is relative to the new exon border (see Methods).
†Not recognised as potential splice acceptor (CV below 72).

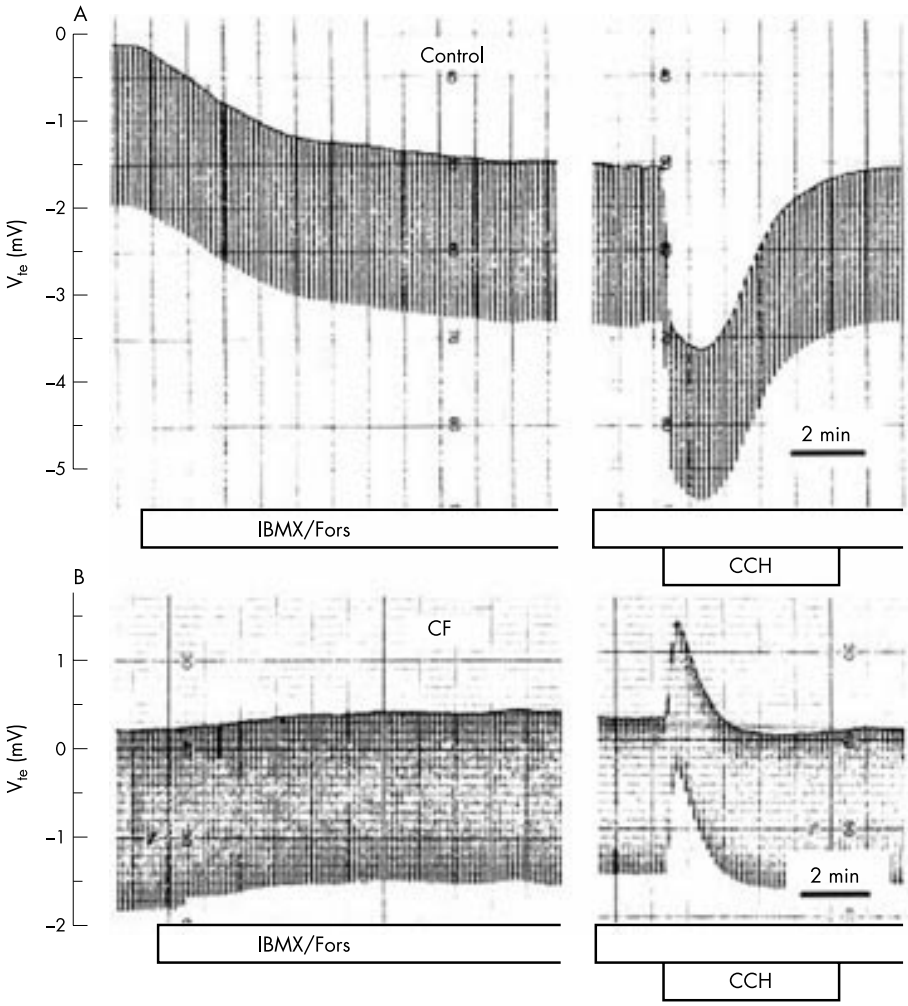

Figure 5 Original recordings of the effects of cAMP dependent activation with IBMX and forskolin (IBMX/Fors, 100 and 1 µmol/l, both sides), and cholinergic activation with carbachol (CCH, 100 µmol/l, basolateral) on V_{te} and R_{te} in (A) non-CF controls and (B) CF (1525-1G>A/F508del) rectal tissues. All experiments were performed in the presence of amiloride and indomethacin. R_{te} was determined from the V_{te} downward deflections obtained by pulsed current injection. Time gaps between recordings were 10 minutes.

Defective intestinal Cl^- secretion in 1525-1G>A/F508del patients

To determine whether any of these alternative CFTR transcripts were translated into functional protein, we performed functional analyses on rectal biopsies of both 1525-1G>A/F508del patients. Previous studies have shown that cAMP dependent and cholinergic Cl^- secretion in human and murine colonic epithelium requires functional CFTR.[22] CFTR is expressed in the luminal membrane of intestinal epithelial cells and is activated by cAMP dependent stimulation. Previous studies indicated that CFTR mediated Cl^- secretion can be further increased by coactivation with cholinergic stimuli, which trigger intracellular Ca^{2+} release and thus increase the activity of Ca^{2+} dependent basolateral K^+ channels, which in turn increase the driving force for Cl^- secretion across the luminal membrane.[11 22]

To test for cAMP dependent Cl^- secretion, rectal biopsies were stimulated with IBMX and forskolin (100 µmol/l and 1 µmol/l, basolateral). As expected from previous studies,[11] cAMP dependent activation of non-CF tissues induced a sustained lumen negative Cl^- secretory response (ΔI_{sc} = -59.6 ± 8.6 µA/cm²; n=9). Coactivation with carbachol (100 µmol/l, basolateral) induced a further increase in Cl^- secretion with a peak response of –81.5 ± 12.6 µA/cm² (n=9) (fig 5A). In contrast, IBMX and forskolin failed to induce Cl^- secretion in rectal biopsies from the two 1525-1G>A/F508del patients (patient 1: ΔI_{sc} = +6.6 ± 2.7 µA/cm² (n=3); patient 2: ΔI_{sc} = +2.2 ± 1.1 µA/cm² (n=3)). Furthermore, coactivation with carbachol induced an inverse (lumen positive) K^+ secretory response (patient 1: ΔI_{sc} = +33.8 ± 9.1 µA/cm² (n=3); patient 2: ΔI_{sc} = +15.3 ± 5.5 µA/cm² (n=3)) but no Cl^- secretory response (fig 5B). Failure to induce Cl^- secretion by both cAMP dependent and cholinergic activation indicates that CFTR function is defective in native tissues from 1525-1G>A/F508del patients.

It cannot be excluded that the minor aberrant transcripts detected here produce functional CFTR in low amounts. However, from the high sensitivity of these functional measurements (that is, the large magnitude of Cl^- secretory responses in non-CF tissues) we predict that such residual Cl- channel function had to be very small (<1%) and would probably not be relevant for in vivo physiology. Indeed, exon 10 codes for a large proportion of the first nucleotide binding domain (NBD1) of CFTR, essential to open the Cl^- channel and initiate its activity by binding and hydrolysing ATP.[23] It is expected that the absence of part (or the whole) of exon 10 causes substantial constraints in protein structure and that such mutants present major defects in channel opening, similar to what happens to the F508del mutation, also located in NBD1.[24]

It therefore seems that none of the alternatively spliced transcripts detected here is translated into functional protein. Thus, production of functional protein as a mechanism influencing use of splice sites does not seem to provide a plausible

explanation for the aberrant splicing described here caused by 1525-1G>A.

CONCLUSION

Altogether, our results show how a splicing mutation can lead to unexpected use of alternative splicing sites that may be very difficult to predict as they seem to result from the interplay between negative and positive elements in the primary transcript that are intrinsically difficult to recognise. Among such factors are: "strength" of the splice site (determined as a CV); length of the resulting intron (unfavourable, if too small); length of the resulting exon (unfavourable, if too long); presence of a PTC in the resulting transcript (causing NMD); and possibly also vicinity to ESEs and temporal order of intron removal. An improvement/deterioration of any one (or more) of these factors determines usage/exclusion of an acceptor.

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