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Alternative splicing of a previously unidentified CFTR exon introduces an in-frame stop codon 5′ of the R region

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The cystic fibrosis transmembrane conductance regulator (CFTR) has been extensively characterized as the carrier of the basic defect in cystic fibrosis. CFTR is part of a growing family of proteins encoded by a single gene, the variant isoforms of which are generated by alternative splicing or RNA editing. We have analyzed the CFTR mRNA in the region of exons 10–11 in T84 cells and detected an alternatively spliced exon (10b) accounting for about 5% of the CFTR mRNA. The exon 10b found in both the human and mice genomes, introduces an in-frame stop codon. The resulting mRNA is translated into a truncated CFTR protein, identified in T84 cells by immunoprecipitation with the CFTR-specific monoclonal antibody MATG 1061. The insertion of a differentially spliced exon carrying an in-frame stop codon is a novel cellular mechanism for the production of a protein sharing common sequences with another, but having different properties and functions.

Cystic fibrosis; Truncated CFTR; Alternative splicing; T84 cell

1. INTRODUCTION

The gene coding for the cystic fibrosis transmembrane regulator (CFTR) has been identified as the carrier of the basic defect on cystic fibrosis [1–4]. The function of this gene is not yet completely clear although most of the evidence indicates CFTR as a protein involved in the transport of electrolytes, particularly in the efflux of chloride ions across the cell membrane [5–7]. The overall structure of the CFTR (see top drawing in Fig. 1) consists of two hydrophobic transmembrane domains followed by two nucleotide binding sites and a regulatory domain [2,8].

CFTR has been extensively characterized, but little attention has been given to the fact that its sequences are not only found in a unique mRNA but also in some variants: one results from the deletion of exon 9 by pre-mRNA alternative splicing [10]; another, from the insertion of an extra 260 bp between exons 23 and 24, which shortens the intracytoplasmic carboxy-terminus [11]. It has previously been reported that some CFTR cDNAs obtained from T84 cells contained 'aberrant sequences', one of them between exons 10 and 11 [2]. Furthermore PCR analysis of the region spanning exons 10 and 11 frequently showed extra bands which usually have been considered as artifacts [9]. This considerable variation in the structure of mRNA has not yet resulted in the identification of variant CFTR proteins. However, there are several published reports in which there is evidence of their existence: in fact, anti-CFTR polyclonal antibodies can detect several protein bands in Western blots of T84 cell extracts [12]. These bands come from a specific antibody–antigen interaction as they can be competed off by the peptide used to produce the antibody. The CFTR proteins are then part of a more complex system than previously envisaged, resembling the ever growing family of proteins encoded by a single gene the alternative splicing of which generates variant isoforms which may modulate critical functions [13–15]. We describe here a thorough analysis of the region between exons 10 and 11 and identify both in human and mouse an alternatively spliced exon that results in a drastically altered version of CFTR (CFTR 36, Fig. 1, bottom drawing) which can be detected in T84 cells by immunoprecipitation.

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Abbreviation: CFTR, cystic fibrosis transmembrane conductance regulator.

2. MATERIALS AND METHODS

2.1. *Cell culture*

T84 (ATCC CCL248) colon carcinoma cell line was plated and grown to confluency in MEM supplemented with 5% FBS, in the presence of 2 mM glutamine and 50 μg/ml gentamycin (Gibco/BRL).

2.2. *Oligonucleotide primers*

We used the following primers for human (h) and mouse (m) sequences: h15′ (exon 1, 5′ TAGTAGGTCTTTGGCATTAG 3′); h85′

(exon 8, 5′ GAAGTAGTGATGGAGAATGT 3′); h85′bis (exon 8, 5′ GGAATATAACTTAACGACTACA 3′); hC16B (exon 10, 5′ GTTTTCCTGGATTATGCCTGGCAC 3′); hC16D (exon 10, 5′ GTTGGCATGCTTTGATGACGCTTC 3′); h10b5′ (exon 10b, 5′ ACTTGGAACTGGAGATG 3′); h10b3′ (exon 10b, 5′ TGCCTGCTCCTATGACACTGA 3′); h10bmid (exon 10b, 5′ CACTTGCGTATGTAATGATG 3′); h113′ (exon 11, 5′ TTCTTGCTCGTTGACCTCCA 3′); h133′ (exon 13, 5′ GTTAGCCATCAGTTTACAGA 3′); m10b33′ (exon 10b, 5′ AAATCACAAGCATGAGGA 3′); m10b5′int (exon 10b, 5′ TGTTCAACTCCGAGATCT 3′).

2.3. *RNA extraction, cDNA synthesis and PCR amplification*

Total RNA was extracted from 1×10^8 T84 cells using the guanidinium thiocyanate lysis method [16]. cDNA was synthesized using a specific primer (h133′), mapping into exon 13 of the CFTR sequence. At the end of the reaction, the enzyme was heat-inactivated for 10 min at 95°C and 1/10 of the transcription reaction was subsequently used for PCR amplification, in the presence of 1 μM of each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$ and 2.5 U *Taq* polymerase (Perkin-Elmer-Cetus), in a Perkin-Elmer thermal cycler 480. The amplification was obtained after 40 cycles consisting of 60 s at 93°C, 60 s at 56°C and 60 s at 72°C. The final product was analysed by electophoresis in 1% agarose gel. The fragments produced by PCR were repaired by DNA polymerase-Klenow treatment, phosphorylated and purified by low-melting agarose electrophoresis. The purified fragment was cloned into the *Sma*I site of pUC18, and its sequence determined according to standard protocols [16].

2.4. *Genomic clones*

Two lambda clones (λXT1 and λXT2) isolated from a mouse genomic library, containing exon 10 and the majority of the intron between exons 10 and 11, were a kind gift from Dr. P. Scambler [17]. DNA was extracted, digested and fractionated according to standard protocols [16]. Southern blot was performed using the cloned human exon 10b as a probe.

2.5. *Expression of CFTR 36 in T84 cells*

The following monoclonal antibodies were utilized. Mouse anti-human CFTR (IgG_{2a}) raised against amino acids 503–515 of CFTR (MATG 1061) which recognizes the first half of the nucleotide binding domain 1 [18], was a generous gift from Dr. A. Pavirani (Transgene, Strasbourg, France). Mouse anti CD4 antigen OKT4, (IgG_{2b}) was from ATCC and was used as irrelevant antibody. CFTR was labeled and immunoprecipitated according to standard methods [19]. Briefly, T84 cells grown in T25 flasks were incubated overnight with [^{35}S]methionine (100 μCi/ml) in methionine-free medium, washed with phosphate-buffered saline and lysed. Lysed cells were divided in two aliquots and incubated either with MATG 1061 or OKT4 antibody as ascites fluid at 1:100 final dilution. *Staphylococcus aureus* (Calbiochem) was added for immunoprecipitation and washed with RIPA buffer. Pellets were resuspended in sample buffer, incubated for 10 min at 37°C, subjected to SDS-PAGE, fluorography and autoradiography.

3. RESULTS AND DISCUSSION

3.1. *Identification of the exon 10b in human and mouse CFTR genes*

CFTR mRNA extracted from T84 cells, was used as template for cDNA synthesis. The cDNA region spanning exons 8–13 was then subjected to PCR using suitable primers; the resulting amplified fragments were analyzed in agarose gel. Fig. 2A shows the agarose gel electrophoresis analysis of the PCR products. It can be seen that using T84 cell-derived cDNA and primers 85 bis and 113, two bands are obtained; they were both positive to hybridization with the oligonucleotide hC16B present in exon 10. Subsequent cloning and sequencing showed that the bottom band was 517 bp long and has the published CFTR sequence (exons 8–11) [2] while the upper band was 636 bp long and contained an alternatively spliced exon (named 10b) between exons 10 and 11. While this manuscript was in preparation similar data was reported by Will et al. [23]. The exon 10b sequence introduces an in-frame stop codon, so that the mRNA processed this way would be translated in a CFTR protein, truncated at the level of the first nucleotide binding fold (see Fig. 1, bottom drawing).

To make sure that exon 10b belongs to a CFTR mRNA in which the frame is not modified by other possible alternative splicings, we amplified, cloned and sequenced the region from the initial ATG in exon 1 to the end of exon 10b, using primers h15′ and h10b3′ for the amplification. The clones produced have the normal

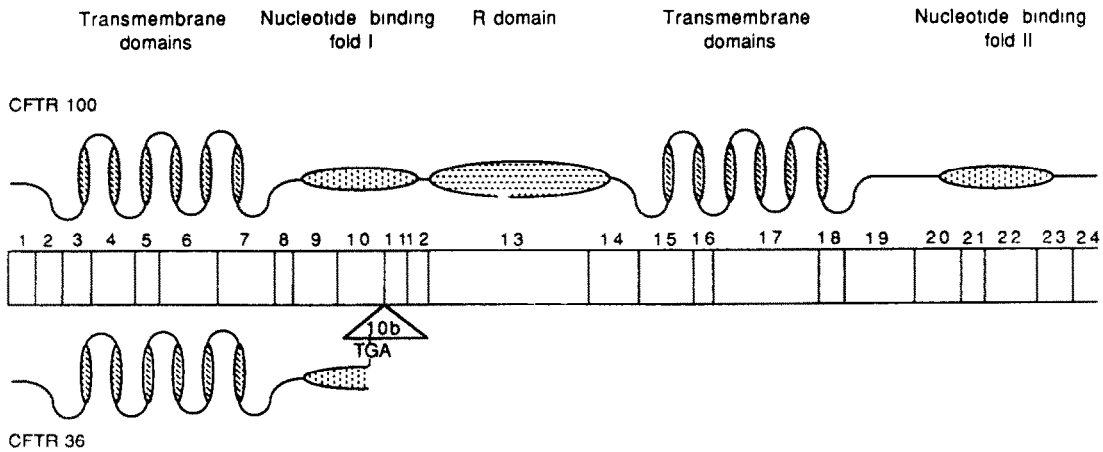

Fig. 1. Schematic representation of the CFTR structure. CFTR 100 protein is shown in the top drawing. It consists of two transmembrane domains, two nucleotide-binding folds and a regulatory domain R. CFTR 36 protein is shown in the bottom drawing. It retains only one transmembrane domain and part of the first nucleotide binding fold. Between the two structures, the exons represented in the CFTR mRNA are shown on a semi-quantitative scale.

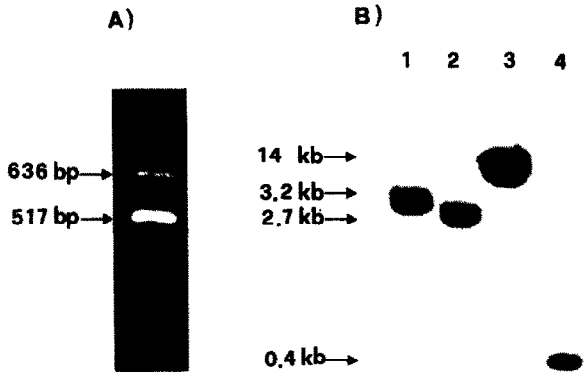

Fig. 2. (A) Gel electrophoresis of CFTR PCR fragments. PCR products obtained using primers h113′ and h85′bis: the concomitant presence of 10b+ and 10b− forms in CFTR cDNA, reverse transcribed from T84 cells is evident. (B) Autoradiograph of a Southern blot of λXT1 digests. Lanes 1, 2, 3 and 4 show respectively the *Eco*RI, *Hind* III, *Sal*I and *Hin*fI digests of λXT1. The digests were fractionated in 1.2% agarose gels for 30 min, transferred to nitrocellulose membranes and hybridized with a clone carrying the human 10b exon ^{32}P-labelled. The running conditions of the gel produced a compression of the higher molecular weight bands.

CFTR sequence from exon 1 to 10 followed by the 10b exon sequence (see Fig. 3). The nucleotide sequence of exon 10b was identical both when it was amplified from human T84 and other sources such as leukocyte mRNA and from human genomic DNA directly. This rules out the introduction of the stop codon by RNA editing mechanisms as observed in the apolipoprotein B system [20]. To confirm the presence of exon 10b within the CFTR gene we have analyzed mouse CFTR genomic clones derived from a region surrounding exon 10 [17]. The new exon 10b was shown to be present also in the mouse genome, mapping 3′ from exon 10. Fig. 3 shows the result of a Southern blot performed on λXT1: DNA in lanes one to four was digested respectively with *Eco*RI, *Hin*dIII, *Sal*I and *Hin*fI and the probe used was the cloned human 10b exon sequence. The results show that exon 10b is entirely contained in a 0.4 kb *Hin*fI fragment. Sequence analysis showed that mouse exon 10b was 74% homologous to the human and presented an in-frame stop codon that would produce a truncated mouse CFTR, with 25 extra amino acids (see Fig. 3). The presence of the in-frame stop codon both in mouse and human exon10b and the 74% nucleotide sequence homology (Fig. 3) lend support to the status of coding sequence of exon 10b.

3.2. *CFTR 36 expression in T84 cells*

The confirmation that a CFTR mRNA containing exon 10b was biologically active was obtained by the detection of the corresponding protein by specific immunoprecipitation. T84 cells were metabolically labelled and the products of the subsequent immunoprecipitation with the CFTR specific antibody MATG 1061 are shown in Fig. 4, lane 1. Two major bands indicated as CFTR 100 and CFTR 36 (named this way by analogy with the ApoB100/ApoB 48 system [20]) are clearly visible. These bands are absent when the irrelevant antibody OKT4 is used (see Fig. 4, lane 2). CFTR 36 had an apparent MW of 61 ± 1 kDa (n = 3), which

A)

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gcatgacctgtggacggtcccatttgcgaaacccagacattcatgactctgtttgcactctgactgctgaagttgatctgttt

ctcagtgtgctgcatcaagGCTTGGAATCAGAGATGGGGATGGGATATCCTCTTCCTCATGCTTGTGATTTTGTTCAACTCCG
                    :::::::   :::::: : :  : :::: :::::::::: ::::: :::::::::::::: :
                  ACTTGGAACTGGAGATGAGAACAGAATATTATCTTCCTCATTTTTGTGTTTTTGTTCAACTCTA

AGATCTTCAAAGTCCCCTGTGTGTCGTGATGCT---CAGTGTCACAGGAGAATGTGAGTgtggaagggcaagcaatgcacaac
:  ::: ::::: : : :: :: : :: ::: :   :::::::: ::::: : :  ::
ATGTCTGCAAAG-CACTTGCGTAT-GTAATGATGCTCAGTGTCATAGGAGCAGG-CAG-
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B)

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                10|10b
SYDEYRYKSVVKACQLQQAWNQRWGWDILFLMLVILFNSEIFKVPCVSQPA
:::: :  :: :::::   :: :
SYDETRTRSVIKACQLEETWNWRQPA
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Fig. 3. (A) Comparison between mouse (upper line) and human (bottom line) exon 10b sequences (capital letters). Small letters correspond to the mouse intronic regions. The mouse junctions are deduced by homology with the human sequence. The dots indicate the homologies between the two sequences. In-frame stop codons are underlined. (B) Comparison between the last predicted amino acids of CFTR 36 protein in mouse (top line) and man (bottom line). The exon 10–10b boundary derived from the human cDNA sequence is represented by a vertical line, the stops are underlined.

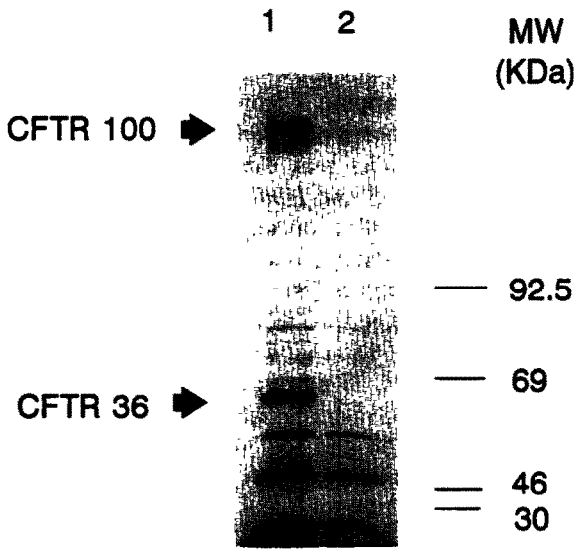

Fig. 4. Gel electrophoresis of the products obtained by immunoprecipitation of T84 cell proteins after labelling with [^{35}S]methionine. Lane 1 shows the immunoprecipitation products obtained using the CFTR specific MATG 1061 antibody. Lane 2 shows the result obtained with the irrelevant antibody OKT4.

is in strict agreement with the predicted mass of 62 kDa deduced from the cDNA sequence. The CFTR36/CFTR100 ratio is higher than expected from their respective mRNAs abundance (see Fig. 2A, lane 1). This may reflect a different immunoprecipitation efficiency for both proteins, or a different stability of the respective mRNAs.

At the moment we can only speculate about the possible function of the truncated protein CFTR 36. Its most interesting structural feature will be the absence of the R domain (see Fig. 1, bottom panel), the critical regulatory sequence containing most but not all of the CFTR 100 protein kinase A substrate sites. The interaction between the transmembrane domains to form the ion channel may be intermolecular instead of being intramolecular as proposed for CFTR 100 [21]. CFTR 36 and/or other CFTR related proteins generated from the CFTR pre mRNA by alternative splicing could be a logical and plausible explanation for some of the inconsistencies reported between the CFTR 100 function, a low conductance chloride channel with a linear current–voltage relationship, and the actual chloride transport deficiencies observed in CF patients whose regulation is defective [22]. CFTR alternative splicing variants such as CFTR 36 should also be taken into account in the functional implications of mutations preceeding exon 11 and in any design for gene therapy. A gene replacement involving only the major cDNA sequence may not provide all the CFTR functions.

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