Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2008;22:93-100

Accepted: June 03, 2008

Small Nuclear RNAs U11 and U12 Modulate Expression of TNR-CFTR mRNA in Mammalian Kidneys

Jackson Souza-Menezes[1], Deepali N. Tukaye[2], Horacio Javier Novaira[1], William B. Guggino[2] and Marcelo M. Morales[1]

[1]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, [2]Department of Physiology, School of Medicine, The Johns Hopkins University, Baltimore

Key Words
CFTR • TNR-CFTR • Kidney • Small Nuclear RNA • Splicing and mRNA

Abstract
TNR-CFTR, discovered as a splice variant of CFTR (Cystic Fibrosis Transmembrane conductance Regulator), is distributed in different tissues such as human and rat kidney, trachea, lungs etc and is a functional chloride channel. In Kidneys, our findings show TNR-CFTR to have an unique distribution pattern with low levels of expression in renal cortex and high levels of expression in renal medulla. As shown by us previously, TNR-CFTR mRNA lacks 145 bp corresponding to segments of exons 13 and 14. This deletion causes a frame shift mutation leading to reading of a premature termination codon in exon 14. Premature termination of translation produces a functional half molecule of CFTR; TNR-CFTR. Our analysis of TNR mRNA has shown that the putative alternatively spliced intron has in its 5' and 3' conserved element CT and AC, respectively, that can be recognized by snRNAs U11 and U12. With these findings, we hypothesize that TNR-CFTR mRNA alternative splicing is probably mediate by splicing pathways utilizing U11 and U12 snRNAs. In this study, we have determined sequences of snRNAs U11 and U12 derived from rat kidney, which show significant homology to human U11 and U12 snRNAs. We show that there is significantly lower expression of U11 and U12 snRNAs in renal cortex compared to renal medulla in both humans and rats. This renal pattern of distribution of U11 and U12 snRNAs in both humans and rats closely follows distribution pattern of renal TNR-CFTR. Further, we have shown that blocking U11 and/or U12 mRNAs, by using antisense probes transfected in Immortalized Rat Proximal Tubule Cell line (IRPTC), decreases TNR-CFTR mRNA expression but not wild-type CFTR mRNA expression. Our results suggest that expression of U11 and/or U12 snRNAs is important for non-conventional alternative splicing process that gives rise to mRNA transcript coding for TNR-CFTR.

Introduction

We previously [1] have shown that CFTR pre-mRNA in renal medulla can form a splice variant called TNR-CFTR. At a genomic level, TNR-CFTR mRNA lacks 145 bp corresponding to segments of exons 13 and 14, which encode last part (7%) of the regulatory (R)

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/08/0224-0093$24.50/0

Accessible online at:
www.karger.com/cpb

Marcelo Marcos Morales
Instituto de Biofísica Carlos Chagas Filho-UFRJ, CCS- Bloco G, 21949-900
Rio de Janeiro, RJ, (Brasil)
Tel. +55 21 2562-6572, Fax: + 55 21 2280-8193
E-Mail mmorales@biof.ufrj.br

domain of CFTR molecule. This deletion causes a shift in reading frame leading to reading of a premature termination codon in exon 14. Premature termination of translation produces a functional half molecule of CFTR. The splice machinery involved in this process has not been deciphered. However, our analysis of mRNA sequence of TNR-CFTR has shown that the putative alternatively spliced intron has in its 5' and 3' conserved element CT and AC respectively which are similar to well known nonconventional intron splice site sequences 5'AT/3'AC [2].

Splicing is an essential intranuclear process occurring in eukaryotic cells allowing for removal of noncoding sequences and generation of a composite mRNA of coding sequences from pre-mRNA. Splicing is known to occur in two forms, classical splicing and alternative splicing. Pre-mRNAs that undergo classical splicing generate a single variety of mRNA and hence a single protein. Alternative splicing however allows for splicing process to generate more than one combination of coding sequences and hence a variety of mRNAs/protein variants. Alternative splicing therefore plays a central role in regulation of cellular gene expression and homeostasis [2, 3].

Splicing requires assembly of splicesosome, a nuclear ribonucleoprotein complex that is composed of over 200 different proteins and small nuclear RNAs (snRNAs) forming functional units called small nuclear ribonucleoproteins (snRNPs) that form a dynamic and elaborate network of RNA-RNA, RNA-protein and protein-protein interactions[3]. The snRNAs U1, U2, U4, U5 and U6 are part of classic splicing machinery and are involved in consensus sequence recognition at intron-exon boundaries which is an important step for splicing [3]. On the other hand U11 and U12, which are two low-abundance snRNAs, are involved in identification of non classical splice sequences 5'AT/3'AC and hence participate in minor pathway splicing [4].

U11 and U12 are mostly nuclear proteins and exhibit some conserved features like presence of a trimethylguanosine cap structure on their 5' end and Sm-antigen binding site [5]. It has been described in Hela cells that U11 and U12 snRNPs interact together to form two-snRNPs complex [6] , which is suggested to be important for in vitro [6] and in vivo [2] splicing of a minor class of pre-mRNA introns that posses AT and AC at their 5' and 3' conserved element respectively [4]. These 5'AT-3'AC classes of introns are quite distinct from vast majority of introns that posses GT and AG at their 5' and 3' ends forming classical intron-exon boundaries [2, 4].

Presence of conserved elements CT and AC at 5' and 3' ends, respectively, of the putative alternatively spliced intron for generation of TNR-CFTR that can be recognized by U11 and U12, led us to hypothesize that alternative splicing for generation of TNR-CFTR recruited complexes requiring U11 and U12 instead of classic U2 dependent splicing machinery. Here, we have investigated involvement of U11 and U12 snRNAs in splicing of CFTR pre-mRNA to generate TNR-CFTR mRNA. We have studied patterns of U11 and U12 distribution in renal medulla vs. cortex and shown that their distribution patterns follow distribution of TNR-CFTR expression in these kidney regions in humans and rats (rat renal medulla expresses TNR-CFTR homologous to human TNR-CFTR as shown by us previously [1]). We have also demonstrated sequence homology between human and rat U11 and U12 snRNAs and comparable expression patterns in the two species. Finally, we have shown that reduction in expression of U11 and/or U12 in IRPTC (Immortalized Rat Proximal Tubule Cells), by antisense RNA silencing, leads to a corresponding reduction in TNR-CFTR mRNA expression without affecting CFTR mRNA expression. These findings suggest that alternative splicing occurring in kidney to generate TNR-CFTR mRNA requires U11 and U12 mediated alternative splicing pathway.

Material and Methods

Human and rat renal sampling

Human renal total RNA was obtained from kidney tissues used in other experiments by our group [1] and it was stored at -70°C since extraction. It was from three normal adult human kidneys (16, 40, and 48 years old) procured by the International Institute for Advancement of Medicine (Philadelphia, PA) and by the Human Tissue Network (Philadelphia, PA). The tissue had been shown not to have undergone warm ischemia and perfusion defects. The organs were prepared but not used for transplant procedure due to technical problems or failure in cross matching. Serology was negative and no prior history of renal or hypertensive disorders were documented for individuals from whom tissues were obtained. Kidneys were obtained after perfusion with ice-cold neutral buffered salt solution and they were kept on ice for 1-12 h. After washing in ice-cold phosphate-buffered saline (PBS), pH 7.2, kidneys were sliced longitudinally and renal medulla separated from renal cortex. The procedures are in accordance with ethical standards formulated in the Helsinki Declaration of 1975 (revised in 1983).

For rat kidneys, organs were obtained after perfusion with ice-cold neutral buffered salt solution, separated into cortex and medulla (as described above) and subjected to total RNA extraction. Animal protocols are in accordance with National

Research Council's guide for care and use of laboratory animals.

Transient transfections

Rat proximal tubule cells (IRPTC) were grown to confluence and on day before transfection, cells were detached by exposure to 0.05% trypsin in Ca^{2+} and Mg^{2+} free solution and reseeded in six-well plates to be 90% confluent by next day. Transient transfection into IRPTC was achieved using a Rous Sarcoma Virus (RSV) promoter-driven mammalian expression vector (pRSV-anti-senseU11 and pRSV-antisenseU12) (Invitrogen, San Diego, CA). Rat U11 or U12 was ligated in this plasmid in an anti-sense orientation in order to hybridize and inhibit transiently RNA for U11 and/or U12. Cells were transiently transfected with 2-3 μg of the construct plasmid vector containing U11 and/or U12 antisense sequence with 6 μl Lipofectamine 2000 reagent (Invitrogen) diluted in 250 μl serum and antibiotic-free DMEM which was added to cells in a final volume of 500 μl per well. After this, cells were incubated at 37°C for 3h with medium mentioned above. Later, this medium was removed and 2 ml supplemented DMEM were added per well and cells were incubated at 37°C for 48 h. In parallel we performed control experiments with transfection of pAvS6-1acZ reporter gene vector.

Reverse Transcription and Polymerase Chain Reaction

Total RNA from renal cortex, medulla and rat proximal tubule cell line (IRPTC) was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [7]. The pairs of primers used in rat samples were design based on human sequences. Two pairs of oligonucleotide primers were synthesized and they correspond to first 21 nucleotides (nt) and last complementary 19 nt of human U11 cDNA sequence (5' aaa aag ggc ttc tgt cgt gag 3' and 5' aaa ggg cgc cgg gac caac 3'). The two primers for rat U12 were made as follows: one from first 22 nt and other from last complementary 18 nt of human U12 cDNA sequence (5' tgc ctt aaa ctt atg agt aagg 3' and 5' cgg gca gat cgc aac tcc 3'), respectively. The primers for U2 amplification was designed as follow: one corresponding to first 21 nt and another corresponding to last 20 nt of human U2 cDNA sequence (5' atc gct tct cgg cct ttt ggc 3' and 5' tgg tgc acc gtt cct gga gg 3'). Primers used for CFTR and TNR-CFTR PCR reactions in IRPTC cells were 5' cag gca gaa gaa gac agt 3' and 5' cag ctt gtt gaa ggt tga 3', which span a DNA segment of 615 bp. As an internal control rat GAPDH primers, were used (5' gtc ttc acc acc atg gag 3' and 3' cat gac aac ttt ggc atc 5' that span a segment of 211 bp).

One microgram of total RNA from renal cortex, medulla and IRPTC was reverse-transcribed with SuperScriptTM (Gibco BRL) at 37°C for 60 min for complementary DNA (cDNA) synthesis. The reaction was terminated by extraction with Phenol chloroform isoamilic alcohol and precipitation with ethanol. Three different cDNA sources were used: a) primed with oligonucleotide dT (Stratagene), b) primed with anti-sense U11 primer, and c) primed with anti-sense U12 primer. RNA, extracted from IRPTC cells, was reversed transcribed using only oligonucleotide dT. Negative control corresponded to a 1 μg aliquot of total RNA used for cDNA synthesis in absence of reverse transcriptase enzyme, and was called RT(-). Synthesized cDNA were used for subsequent PCR containing 2.5 U of Pfu DNA polymerase (Stratagene), 0.2 μM of primers, 0.2 μM of each dNTP, 20 mM Tris-Cl (pH 8.8), 10 mM KCl, 10 mM $(NH4)_2SO4$, 2.0 mM $MgSO_4$, 1% Triton® X-100, and 1 mg/ml nuclease-free bovine serum albumin (BSA) in following conditions: an initial denaturation at 94°C for 5 min; followed by 40 cycles, each consisting of annealing at 58°C for 1 min (for U11), 53°C for 1 min (for U12) and 62°C for 1 min (for U2); elongation at 68°C for 1 min and denaturation at 94°C for 1 min. Final elongation was at 68°C for 7 min.

For semi-quantitative analyses of CFTR and TNR-CFTR, cDNA were used in presence of: 2.5 units of Taq DNA polymerase (Gibco BRL, N.Y, USA), 0.2 μM of each primer (CFTR and GAPDH), 0.2 μM of each nucleotide and commercial buffer (Gibco BRL, N.Y, USA) containing 1.5 mM MgCl2. PCR was performed under following conditions: initial denaturation at 94°C for 4 min, followed by 36 cycles characterized by: denaturation (94° C 1 min), annealing (58° C 1 min) and extension (72° C, 1 min). The reaction was concluded with an extension for 10 minutes at 72°C. The semi-quantitative method of RT-PCR used for studies of expression of CFTR and TNR-CFTR mRNA in IRPTC was validated in preliminary experiments. Firstly, optimal PCR conditions that yielded a single band on agarose gel electrophoresis were determined for CFTR, TNR-CFTR and GAPDH genes in the same reaction tube. Secondly, to determine whether the method was semi-quantitative, serial quantities of total RNA (62·5, 125, 250, 500, 1000 and 2000 ng) extracted from IRPTC were used for RT-PCR amplification for CFTR, TNR-CFTR and GAPDH genes in the same reaction tube. Thirdly, experiments were performed to determine optimal number of PCR cycles that yielded PCR products in linear phase of amplification of all genes in the same reaction. Finally, to ensure that reactions were consistent, PCR reactions were performed at least twice. Only one of these reactions was arbitrarily selected for inclusion in final densitometry analysis For semi-quantitative PCR comparing normal cells and transfected cells, optimal total amount of RNA was 1000 ng and optimal number of PCR cycles was 36. All reactions included a negative control RT (-). Identity of amplification product was confirmed by determination of molecular size on agarose gel electrophoresis with a 100 bp DNA molecular marker (Gibco BRL). Results are presented as mean ± S.E.M.

CFTR, TNR-CFTR and GAPDH bands from samples were analyzed by densitometry (computer software Scion Image Alpha 4.0.3.2, Scion Corporation, USA). Expression was normalized by dividing CFTR and TNR-CFTR values by corresponding internal control values of GAPDH amplified in the same reaction tube.

Sequencing of snRNAs U2, U11 and U12 in rat kidney

PCR products of 134 base pairs (bp), 150 bp, and 189 bp corresponding to rat renal snRNAs U11, U12, and U2, respectively, were obtained and ligated to plasmid pCR-Scripit SK(+) (Stratagene). These products were then used to transform *Epicurian coli* XL1-blue supercompetent cells (Stratagene). Following Sequenase version 2.0 kit (US Biochemicals) and using M13 forward and reverse primers, nucleotide sequences of PCR products were determined.

SnRNAs renal expression analysis by RNase Protection Assay (RPA)

For RNase protection assay of both U11 and U12 transcripts from same samples of rat and human kidney cortex and medulla, four different nucleotide sequences were used as template for antisense RNA synthesis. For human U11 and U12, a 134 nt probe (full-length) and a 101 nt probe (corresponding to nt 36 to 137) were used respectively. Probes for rat U11 and U12 were prepared from corresponding cDNA sequences ligated into vector plasmid pCR-Script SK (+). A probe of 189 nt corresponding to full-length rat U2 was used. All probes were prepared by linearization of 1 μg of template DNA using restriction enzymes ECO RI for human U11 and U12, SstI for rat U11 and U12, and Hind III for rat U2. As an internal control pTRI-Actin-HUMAN (250 nt) or pTRI B-Actin-125-RAT (125 nt) (Ambion) were used. RNA polymerases SP6, T7, and T3 were used to transcribe *in vitro* human U11 and U12 templates, rat U11 and U12 templates, and rat U2 template, respectively, to generate radio labeled antisense RNA probes using protocol described in Maxiscripit kit (Ambion) in presence of [α^{32}P]UTP (800 Ci/mmol, Amersham Corp.). For β-actin control templates T3 RNA polymerase was used. All probes, including human and rat β-actin controls, were evaluated with 10 μg of yeast tRNA in presence and absence of RNase A and T_1 following Ambion RPA II™ kit protocol. Radiolabeled antisense probes ($1x10^6$ cpm/sample) were mixed with 10 μg of total tissue RNA. Samples were hybridized at 45° C for 18 hours. Then a combination of RNase A and T_1 (RPA II kit, Ambion) was used to treat samples at 37° C for 30 min. Protected fragments were then analyzed in 8 M urea 5% polyacrylamide gels. Gels were transferred to chromatography papers and exposed to X-ray films with an intensifying screen at -70° C. The density of corresponding bands was analyzed by computer software (ImageQuant-Molecular Dynamics).

Statistical analyses

Results are presented as mean ± SEM. Student T-test is used to compare expression of U11, U12, and U2 between cortex and medulla. One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test are used to compare mRNA expression of CFTR and TNR-CFTR in cell culture experiments. Differences are considered significant when $p < 0.05$.

Results

TNR-CFTR has tissue and species specific expression [1]. We have shown that TNR-CFTR is not only expressed in human Kidney, trachea and lungs but also in rat kidney. Mice do not express TNR-CFTR and sequence analysis of mouse CFTR pre-mRNA demonstrates that it lacks conserved minor pathway splicing sequences, 5'CT/3'AC and polypyramidine tract, identified in the putative alternatively spliced intron of CFTR pre-mRNA in humans and rats. We have also

Fig. 1. Comparison between human and rat cDNA sequences of U11 and U12. A - Shows the results of cDNA sequence comparison between human and rat U11. Three nucleotide mismatches were revealed at positions 17, 31, and 109 (bold letters). B - Illustrates the results obtained by comparison of human and rat cDNA sequences of U12. Eight nucleotide mismatches were observed at positions 23, 50, 62, 94, 97, 98, 105, and 131 (bold letters). Moreover, an insertion of a Thymine at position 101 (bold and underlined) just before an Adenine in rat sequence was seen.

shown that pattern of TNR-CFTR expression in rats closely follows that in humans. This makes rat kidney a good model system for study of TNR-CFTR processing.

Detection and sequencing of snRNAs U11 and U12 from rat kidney

In this study, we first established presence of U11 and U12 in rats and then compared their sequences to human U11 and U12 sequences for degree of sequence homology. To detect presence of snRNAs U11 and U12 in rat kidney, RNA isolated from rat renal cortex and medulla was reversed transcribed and amplified by PCR using primers corresponding to initial and final portions of both human cDNA sequences of U11 and U12. 134 bp and 150 bp RT-PCR products were obtained from these reactions corresponding to expected full-length size of U11 and U12, respectively, in both cortex and medulla of rat kidney. Both 134-bp and 150-bp RT-PCR products were then sub-cloned and sequenced to identify their nucleotide sequences. Eight different PCR products derived from eight rats were sequenced to arrive at sequence homology between human and rat U11 and U12.

Comparison of U11 cDNA sequence of human and rat (Figure 1A) showed high degree homology. Differences were identified corresponding to positions

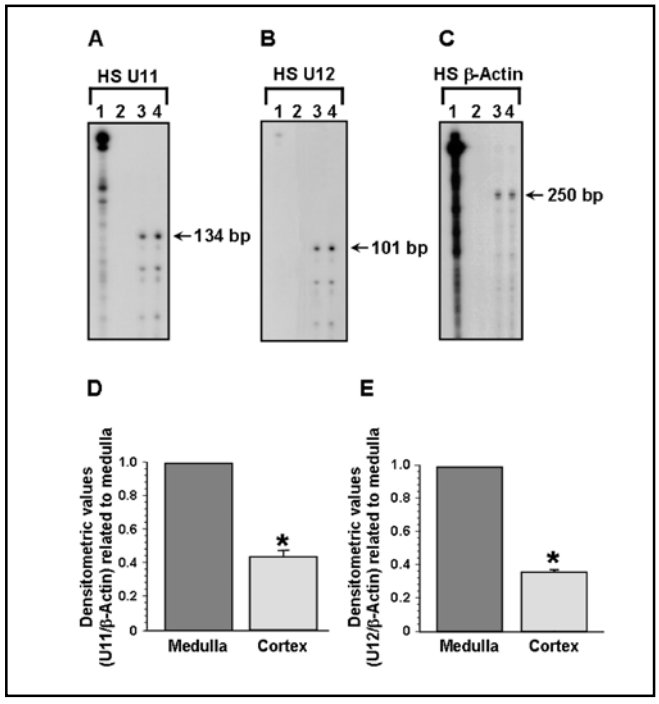

Fig. 2. Distribution of snRNA U11 and U12 in human renal cortex and medulla determined using RPA. A: Protected bands corresponding to snRNA U11 (134 bp). B: Protected bands corresponding to snRNA U12 (101 bp). C: Protected bands corresponding to β-actin (250 bp). D: The graphic representation of densitometric values (U11/ β-actin) normalized to medulla. The density of bands in cortex is about 44 ± 4% of medulla. E: The graphic representation of densitometric values U12/β-actin normalized to medulla. The density of bands in cortex is about 36 ± 2% of medulla. All values are represented as means ± SEM. * $p < 0.05$, n = 4.

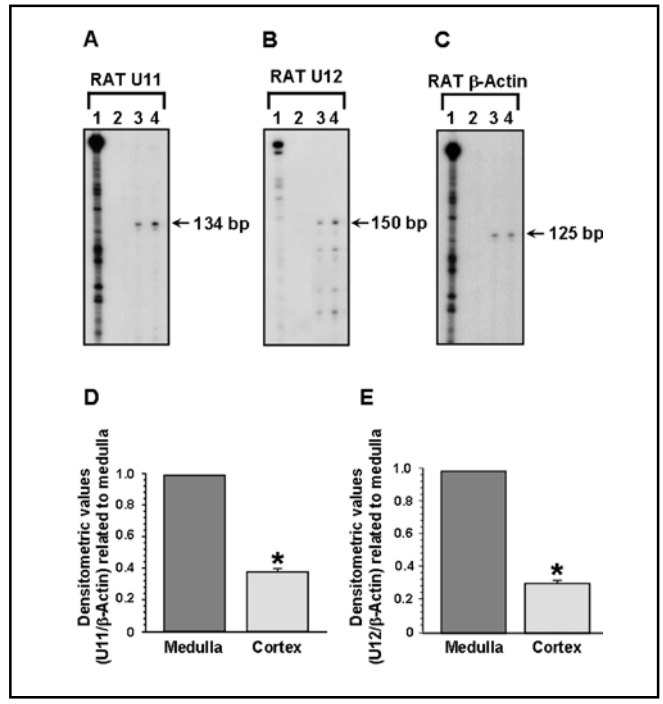

Fig. 3. Distribution of snRNA U11 and U12 in rat renal cortex and medulla determined using RPA. A: Protected bands corresponding to snRNA U11 (134 bp). B: Protected bands corresponding to snRNA U12 (150 bp). C: Protected bands corresponding to β-actin (125 bp). D: The graphic representation of densitometric values (U11/ β-actin) normalized to medulla. The density of bands in cortex is about 38 ± 2% of medulla. E: The graphic representation of densitometric values U12/β-actin normalized to medulla. The density of band in cortex is about 33 ± 1% of medulla. All values are represented as means ± SEM. * $p < 0.05$, n = 4.

17, 31 and 109 where G, T and T were substituted by A, C and C, respectively in rat U11. Human and rat cDNA sequences of U12 were also found to be very homologous (Figure 1B). Here, identified differences corresponded to positions 23, 50, 62, 94, 97, 98, 105, and 131 where A, A, A, C, C, C, T, and G were substituted by C, G, T, T, T, A, C, and A, respectively in rat U12. We also observed an insertion of a T (thymine) at position 101 before an A (adenine).

SnRNAs U2, U11 and U12 expression in human and rat kidneys

Having established presence of U11 and U12 in rat kidney and their homology to human U11 and U12, we next looked at distribution pattern of U11 and U12 in both human and rat kidney tissue for similarities in expression.

RPA technique was used to analyze snRNAs U11 and U12 expression in rat and human renal cortex and medulla. Hybridization of total RNA with ^{32}P-labeled antisense RNA probes showed that human (Figure 2) and rat (Figure 3) U11 and U12 are expressed in renal cortex and medulla. It was observed that U11 is expressed at lower levels in renal cortex compared to medulla of both human and rat (44% and 38% of renal medulla values, respectively, n = 4, $p < 0.05$). U12 was also found to have lower expression in renal cortex compared to medulla of both human and rat (36% and 33% of renal medulla values, respectively, n = 4, $p < 0.05$). It was observed by RPA that level of expression for rat U2 was not significantly different between renal cortex and medulla (n = 4) (Figure 4). Human and rat β-actin internal controls were used to demonstrate that differences in U11 and U12 expressions were not due to intrinsic differences within the assay. Signal intensity of both controls did not vary significantly between cortex and medulla in human and rat kidneys.

As seen from above results, renal distribution pattern

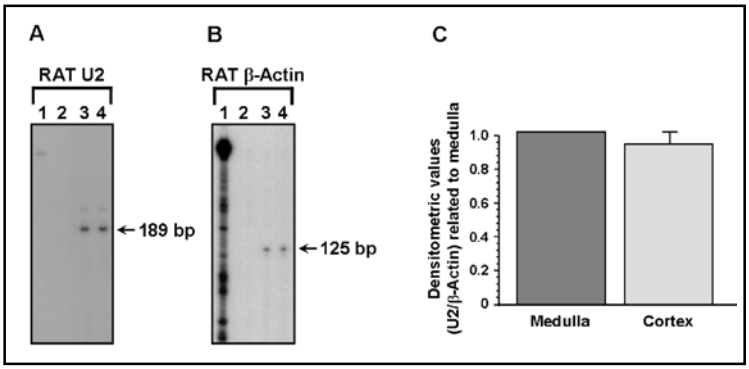

Fig. 4. Distribution of snRNA U2 in rat renal cortex and medulla determined using RPA. A: Protected bands corresponding to U2 (189 bp). B: Protected bands corresponding to β-actin (125 bp). C: The graphic representation of densitometric values U2/β-actin normalized to medulla. All values are represented as means ± SEM. n = 4.

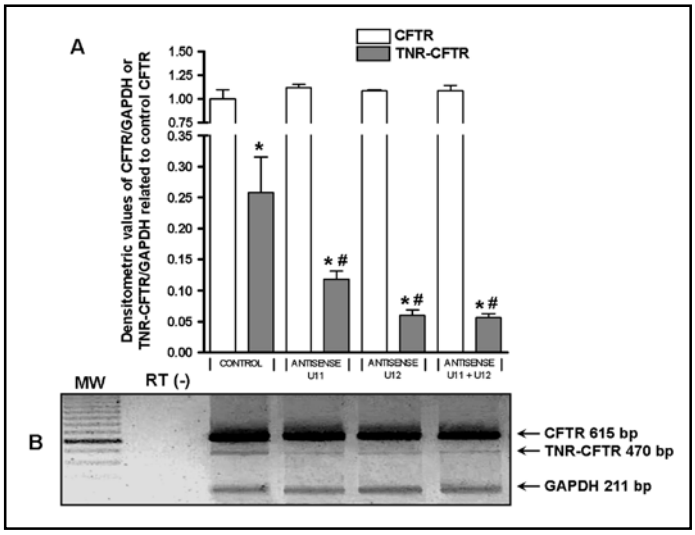

Fig. 5. Expression of CFTR and TNR-CFTR mRNAs in rat proximal tubule cell line determined using antisense RNA. Mock transfected cells (control) and cells transfected with anti-sense probe for U11, for U12 or with both probes are indicated, respectively, as: antisense U11, antisense U12 and antisense U11+U12. A: Data represented as mean ±SE for densitometric values of TNR-CFTR/GAPDH (gray bars) related to CFTR/GAPDH (open bars) obtained in RT-PCR experiments. B: Representative electrophoresis agarose gel of PCR products obtained in the same reaction tube. All values are represented as means ± SEM. * $p < 0.05$ when compared to CFTR control. # $p < 0.05$ when compared to TNR-CFTR control, n = 4. MW = molecular weight, RT (-) = reaction free of reverse transcriptase.

of U11 and U12 are similar in humans and rats with higher expression in renal medulla than renal cortex. This pattern of distribution is identical to pattern of distribution for TNR-CFTR in human and rat renal tissues. These results suggest that TNR-CFTR splicing and U11/U12 expression in rat renal tissue probably parallels behavior of these molecules in human renal tissue. These results further validate the fact that rat renal tissue is a good model for understanding mechanisms involved in expression of TNR-CFTR.

Modulation of TNR-CFTR mRNA by snRNAs U11 and U12

We detected expression of both CFTR and TNR-CFTR mRNAs by RT-PCR in Immortalized Rat Proximal Tubule Cells (IRPTC). Under normal conditions in IRPTC, we found TNR-CFTR mRNA expression to be lower compared to CFTR (26% of CFTR expression, n = 4, $p < 0.05$) (Figure 5). This finding is similar to that seen in human renal tissue as previously demonstrated by us [1]. We then looked at effect of silencing U11 and/or U12 snRNAs on expression of TNR-CFTR in IRPTC. Expression of TNR-CFTR in IRPTC decreased in cells transfected with antisense probes against U11, U12 or U11+U12 compared to TNR-CFTR in control cells (58%, 33% and 30% of control values, respectively, n = 4, $p < 0.05$) (Figure 5). Wild-type CFTR mRNA expression was not affected by antisense probes U11 and/or U12 transfection in the same cell line. Note that silencing of U11 and U12 snRNAs together fails to produce a synergistic effect.

These results suggest that U11 and U12 are candidates involved in alternative splicing pathway of CFTR pre-mRNA to generate TNR-CFTR mRNA and play a significant role in modulating TNR-CFTR mRNA expression.

Discussion

TNR-CFTR was discovered by our group as a splice variant of CFTR with a tissue specific expression in kidneys, trachea and lungs in humans and rats [1]. It also has a species specific expression seen in human and rat but not in mice. TNR-CFTR comprises of the first half of full length CFTR which includes first Transmembrane

domain, first Nucleotide binding domain, and Regulatory domain (93%) (MSD1, NBD1 and R) and thus is a truncated version of full length CFTR [1]. Our functional characterization studies have established TNR-CFTR as a cAMP dependent PKA (protein kinase A) activated Cl^- channel similar to full length WT CFTR [1, 8, 9].

TNR-CFTR mRNA is deficient in 145bp which get deleted as part of the putative alternatively spliced intron which includes portions of exons 13 and 14 of full length CFTR in addition to the intervening intron between these two exons that is primarily spliced to generate full length CFTR mRNA. Furthermore, sequence analysis of TNR-CFTR shows that the putative alternatively spliced intron possesses sequences that are an inverse compliment of conventional splice sites including the polypyrimidine tract. Based on these observations it is safe to conclude that TNR-CFTR generation occurs via alternative splicing of CFTR pre-mRNA and that the non conventional splice sites identified (5' and 3' conserved element CT and AC, respectively) could probably play a role in alternate splicing of full length CFTR pre-mRNA. In this study we have tried to decipher the pathway involved in alternate splicing of TNR-CFTR.

It is known that in minor class introns 5' AT and 3' AC conserved elements are splice site sequences recognized by minor pathway splicosome recruiting U5, U11 and U12 [2, 4]. Having identified similar (5' CT and 3' AC) sequences in the putative alternatively spliced intron to generate TNR-CFTR form CFTR pre-mRNA, we devised to determine if minor pathway snRNAs U11 and/or U12 are involved in modulating alternative splicing of TNR-CFTR. We first looked at expression of U11 and U12 snRNAs in human and rat renal tissue. As evident from Figures 2 and 3 with use of RPA we see that U11 and U12 snRNAs are expressed in human and rat renal medulla and cortex. Densitometric analysis of total amounts of U11 and U12 snRNAs in renal cortex and renal medulla shows that the two snRNAs have a higher level of expression in renal medulla vs. renal cortex. This differential distribution pattern closely follows distribution pattern of TNR-CFTR in corresponding regions of kidney in humans and rats [1]. However RPA of U2 snRNA (Figure. 4), an important component of major splicing pathway machinery, shows that it does not differ in expression amounts between renal medulla vs. cortex. These results support our hypothesis that alternative splicing of TNR-CFTR is modulated more likely by U11 and U12 snRNAs mediated minor pathway than U2 mediated classical pathway [2]. In Figure 5, antisense experiments in IRPTC reasonably show that decreasing levels of U11 and U12 independently or together decreased generation of TNR-CFTR mRNA to a significant extent whereas there is no observable effect on expression of full length CFTR mRNA expression. Also, we observe that silencing of U11 and U12 simultaneously fails to show synergy in decreasing TNR-CFTR mRNA generation indicating that these two modulate TNR-CFTR mRNA generation in the same pathway. With these observations we can safely conclude that TNR-CFTR splicing is mediated via a minor pathway recruiting U11 and U12 snRNAs. Also, since U11 and U12 silencing fails to decrease expression levels of full length CFTR mRNA indicating that splicing pathways involving U11/U12 snRNAs are probably not involved in its generation. This is further supported by the fact that classical splice site sequences are found associated with the intron spliced for generation of full length CFTR mRNA based on sequence analysis. It is to be noted that even though antisense experimental data is gathered from IRPTC (Figure. 5), sequence similarities in rat and human TNR-CFTR [1] and U11/U12 snRNAs (Figure.1) as well as identical renal region specific distribution of these molecules (Figure.2, Figure.3, [1]) provide for reasonable confidence in extrapolation of this data onto humans. Thus, we reasonably surmise that alternative splicing of TNR-CFTR in humans is possibly modulated by U11 and U12 snRNAs.

We acknowledge that these experiments only provide evidence for involvement of U11 and U12 in generation of TNR-CFTR mRNA and hence modulation of its splicing. Sequence analysis has shown the presence of 5'CT / 3'AC conserved elements and polypyramidine tract at the predicted intron exon junctions for the putative alternatively spliced intron of TNR-CFTR. These are very similar to the intron splice sites (5'AT/3'AC) previously shown to be recognized by minor splicesosome pathway recruiting U5, U11 and U12 snRNAs [4]. Also, mice CFTR lacking conserved 5'CT/3'AC elements and polypyramidine tract does not undergo alternative splicing to generate TNR-CFTR as shown by RT-PCR of mice CFTR exons 13 and 14 [data not shown]. With this information we strongly speculate that nonconventional splice sites identified by sequence analysis (5'CT/3'AC) could be most likely target sites for recruiting U11 and U12 snRNAs.

It is yet to be seen what physiological significance TNR-CFTR bears in tissues that it is expressed in. In kidneys, differential expression pattern between renal medulla and cortex provides an interesting observation to speculate role of TNR-CFTR in the physiologically diverse

functions carried out by nephron/ nephron segments in renal cortex vs. medulla. The cortical segments are predominantly involved in solute and water reabsorbtion [10] while the medullary segments are predominantly involved in generation of medullary osmolarity gradient for concentrating urine [11]. A high level expression of TNR-CFTR in renal medulla opens doors to speculation about its involvement in generation of medullary osmolarity gradient and related medullary physiology. Also, in Cystic Fibrosis, an autosomal recessive disease resulting from a mutation in CFTR (ΔF508 in NBD1 in 70% cases), renal involvement is neither significant nor life threatening in majority cases despite abundant expression of CFTR in kidneys [12-15]. It would be interesting to see how ΔF508 mutation affects TNR-CFTR and if abundant expression of TNR-CFTR in kidneys vs. lung [1] has a protective effect on renal function. Studies in our lab are currently underway along these lines to understand physiological relevance of TNR-CFTR expression and differential distribution in kidneys. Following a similar line of reasoning and knowing that alternative splicing occurs in kidney [16], it will not be far fetched to consider that differential expression of U11 and U12 snRNAs has a role in modulating gene expression differentially in the two kidney regions in lieu of diverse physiological processes carried out by them.

To summarize, in this study we have identified for the first time candidates involved in alternative splicing of full length CFTR pre-mRNA to generate TNR-CFTR mRNA. We have provided strong evidence that U11 and U12 snRNAs are involved in modulation of TNR-CFTR mRNA expression in rats (Figure. 5) with reasonable grounds to assume that similar modulation occurs in humans. Also, as these snRNAs are known to recognize nonconventional splice site sequences (5'AT/3'AC), we strongly consider the putative non conventional splice sites (5'CT/3'AC) identified in the alternatively spliced intron as probable sites targeted by U11and U12 in splicing CFTR pre-mRNA to TNR-CFTR mRNA.

Acknowledgements

This study was supported by the National Institute of Health DK37253, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Programa de Apoio aos Núcleos de Excelência (PRONEX), Third World Academy of Sciences (TWAS), Instituto do Milênio, Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and The Brazilian Ministry of Health.

References

1 Morales MM, Carroll TP, Morita T, Schwiebert EM, Devuyst O, Wilson PD, Lopes AG, Stanton BA, H.C.Dietz, Cutting GR, Guggino WB: Both the wild type and a functional isoform of CFTR are expressed in kidney. Am J Physiol 1996;270:F1038-F1048.

2 Hall SL, Padgett RA: Requirement of U12 snRNA for in Vivo Splicing of a Minor Class of Eukaryotic Nuclear Pre-mRNA Introns Science 1996;271:1716-1718.

3 Valadkhan S: snRNAs as the catalysts of pre-mRNA splicing. Curr Opin Chem Biol 2005;9(6):603-8.

4 Tarn WY, Steitz JA: A novel splicesosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. Cell 1996;84(5):801-11.

5 Montzka KA, Steitz JA: Additional low-abundance human small nuclear ribonucleoproteins: U11, U12, etc. Proc Natl Acad Sci U S A 1988;85(23):8885-9.

6 Wassarman KM, Steitz JA: The low-abundance U11 and U12 small nuclear ribonucleoproteins (snRNPs) interact to form a two-snRNP complex. Mol Cell Biol 1992;12(3):1276-85.

7 Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162(1):156-9.

8 Schwiebert EM, Egan ME, Hwang TH, Fulmer SB, Allen SS, Cutting GR, Guggino WB: CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. Cell 1995;81(7):1063-73.

9 Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC: CFTR as a cAMP-dependent regulator of sodium channels. Science 1995;269:847-50.

10 Anzai N, Jutabha P, Kanai Y, Endou H: Integrated physiology of proximal tubular organic anion transport. Curr Opin Nephrol Hypertens 2005;14(5):472-9.

11 Fenton RA, Knepper MA: Mouse models and the urinary concentrating mechanism in the new millennium. Physiol Rev 2007;87(4):1083-112.

12 Morales MM, Capella MA, Lopes AG: Structure and function of the cystic fibrosis transmembrane conductance regulator. Braz J Med Biol Res 1999;32:1021.

13 Morales MM, Falkenstein D, Lopes AG: The cystic fibrosis transmembrane regulator (CFTR) in the kidney. An Acad Bras Cienc 2000;72:399.

14 Al-Shawwa BA, Rao AR: Cystic fibrosis and renal disease: a case report. J Med Case Reports 20071:24.

15 Katz SM, Krueger LJ, Falkner B: Microscopic nephrocalcinosis in cystic fibrosis. N Engl J Med 1988;319:263-266.

16 Arystarkhova E, Sweadner KJ: Splice variants of the gamma subunit (FXYD2) and their significance in regulation of the Na, K-ATPase in kidney. J Bioenerg Biomembr 2005;37(6):381-6.