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CFTR mRNA and its truncated splice variant (TRN-CFTR) are differentially expressed during collecting duct ontogeny

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Abstract The collecting duct epithelium originates from the embryonic ureter by branching morphogenesis. Ontogeny-dependent changes of CFTR mRNA expression were assessed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in primary monolayer cultures of rat ureteric buds (UB) and cortical collecting ducts, microdissected at different embryonic and postnatal developmental stages. The amount of wild-type CFTR-specific PCR product in UB declined to 20% of the initial value between embryonic gestational day E15 and postnatal day P1. After birth the CFTR product increased transiently between P1 and P7 by a factor of 10 and decreased towards day P14. PCR products specific for TRN-CFTR, a truncated splice variant, however, were low in early embryonic cells, increased markedly between day E17 and P2, and reached a plateau postnatally. Therefore, mRNA encoding TRN-CFTR does not appear to have a specific embryonic-morphogenetic function. By contrast, such function is suggested for wild-type CFTR mRNA as its abundance was high in early embryonic nephrogenesis, as well as during a postnatal period shortly before branching morphogenesis is completed.

Key words: Embryonic kidney; Nephrogenesis; Cystic fibrosis transmembrane conductance regulator; Ureteric bud

1. Introduction

The collecting duct epithelium of the metanephric kidney develops by budding and branching of the embryonic ureter. This branching morphogenesis, in homology to lung and other parenchymal organs, forms a tree of fluid-filled tubular epithelia [1]. The ureteric buds (UB) of the outermost branches close off this system most distally. With the end of morphogenesis UBs are connected to the mesenchyme-derived nephron and, with initiation of glomerular filtration, differentiate into the NaCl-reabsorbing cortical collecting duct (CCD) epithelium [2]. In rat principal cells this differentiation is characterized by acquisition of Na^+- and K^+-selective channels in the apical plasma membrane and by downregulation of embryonic Cl^- channels [3]. In addition, mRNA encoding the ubiquitous volume-regulatory Cl^- channel ClC-2 is highly abundant in rat UB cells, but downregulated in CCD [4].

It is of interest that ClC-2 mRNA in rat developing lung epithelium is expressed in an identical temporal pattern. Downregulation of ClC-2 parallels the switch of fetal respiratory epithelium from fluid secretion to reabsorption upon birth [5]. A second Cl^- channel, the secretory CFTR (cystic fibrosis transmembrane conductance regulator [6,7]) channel, is differentially expressed during (human, rabbit, and rat) lung [8–10] and (rat and human) kidney development [11,12]. In human, recessive mutations in the CFTR gene cause cystic fibrosis (CF), the most common lethal disease among Caucasians, and the lung is one of the severely affected epithelial organs in CF patients.

The gene product of CFTR, in addition to its Cl^--conductive properties, regulates other apical membrane ion channels [13]. Specifically, CFTR when switched on by PKA-dependent phosphorylation activates outwardly rectifying Cl^- channels (ORCC) in lung [14] and inactivates epithelial Na^+ channels in lung [15] and kidney [16] and in this way most probably promotes secretion and inhibits reabsorption.

Lung and kidney are both normally developed in CF patients [17] implying that Cl^- secretion by the fetal respiratory epithelium utilizes Cl^- channels other than CFTR (e.g. ClC-2). Nevertheless, the regulatory function of CFTR and its reported developmental expression may hint at its possible role in controlling fluid within tubular lumina during morphogenesis. The present study, therefore, investigated the spatial and temporal pattern of CFTR expression in the collecting duct, a developing NaCl-reabsorbing epithelium. CFTR mRNA was determined by in situ hybridization in embryonic rat kidney. In addition, CFTR mRNA was evaluated quantitatively by reverse transcriptase-polymerase chain reaction (RT-PCR) in primary monolayer cultures of UBs and CCDs microdissected at different developmental stages (embryonic day E15 to postnatal day P14). The data demonstrate: (i) CFTR mRNA is expressed by embryonic UB cells in situ and in primary culture. (ii) CFTR mRNA is downregulated in embryonic UB towards birth, transiently upregulated in postnatal UB (P3–6) and early CCD cells, but downregulated at day P14. (iii) A truncated splice variant of CFTR (TRN-CFTR [18]), in contrast, is upregulated around birth.

2. Materials and methods

2.1. In situ hybridization

Hybridization with non-radioactive digoxigenin-labeled probes and their detection was performed according to [19]. Embryonic kidneys (day E17) from Wistar rats (Charles River, Braunschweig, Germany) were fixed in Karnovsky's solution (1:1 diluted in H_2O) and embedded in paraffin. Paraffin sections (5 μm) were mounted on siliconized slides. The sections were hydrated in decreasing concentrations of ethanol then pretreated with 1 M NaSCN at 80°C and with 0.15% proteinase K in 100 mM Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl)/150 mM NaCl, pH 7.5 at 37°C for 2.5 min, postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at 4°C,

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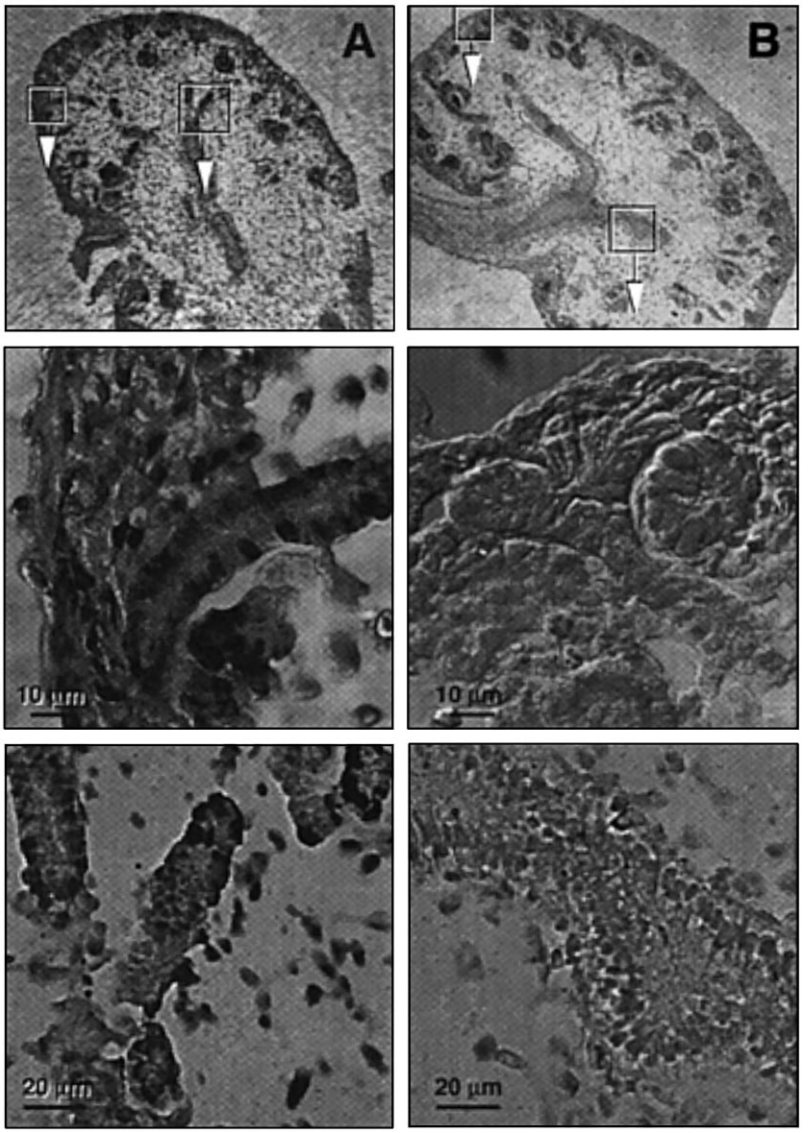

Fig. 1. In situ hybridization using digoxigenin-labeled CFTR-specific DNA probes. Light micrographs (DIC optics) of paraffin-embedded sections from embryonic (day E17) rat kidney showing overviews (top) and, in more detail, UB in the outermost cortex (middle) and ureteric trunks in the medulla (bottom). Total binding (A) is compared with non-specific background binding (B). The CFTR probe did not discriminate between wild-type and TRN-CFTR mRNA.

washed in 100 mM Tris-HCl/150 mM NaCl, pH 7.5, and dehydrated in increasing concentrations of ethanol.

CFTR-specific DNA probes were amplified and digoxigenized by PCR (PCR labeling mix, Boehringer, Mannheim, Germany) from a ureteric bud (E17) cDNA (cDNA preparation and PCR conditions: see below). Probes were precipitated by Na-acetate/ethanol, digested with *Alu*I into fragments of 218 bp, 207 bp, 113 bp, 37 bp, and 32 bp. Fragments >100 bp were purified by QIAquick Spin Columns (QIAquick PCR Purification Kit; Qiagen, Hilden, Germany). As a negative control non-labeled PCR product was prepared identically to that of the digoxigenized probe; control hybridizations were performed with a mix (final concentrations) of 25 ng/µl of non-labeled probe and 1 ng/µl of digoxigenized probe.

Tissue sections and the probe were denatured at 80°C and hybridized for 24 h at 37°C in a buffer (53% deionized formamide, 10% dextran sulfate, 10% Denhardt's solution and $2\times$SSC, pH 7.0) containing the digoxigenin-labeled probe at a final concentration of 1 ng/µl. After hybridization, the sections were immersed in $2\times$SSC at 65°C, $0.1\times$SSC at 45°C for 5 min, washed in 100 mM Tris-HCl/150 NaCl, pH 7.5, incubated in blocking buffer (Boehringer, Mannheim), and finally incubated in a dilution of an anti-digoxigenin antibody (1:500 in Tris-HCl) conjugated with alkaline phosphatase for 30 min at room temperature. Alkaline phosphatase, in the presence of 4-nitroblue tetrazolium chloride (NBT) plus 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), produces a precipitate of dark purple color. Color developed within 2 h and was monitored using a light microscope equipped with DIC optics.

2.2. Primary monolayer cultures

UB and CCD were obtained by microdissection (in Ca^{2+}- and Mg^{2+}-free phosphate-buffered solution at 4°C) of the most distal dichotomous tubular branches of the outer renal cortex of Wistar rats at different embryonic and postnatal stages (embryonic day E15 to postnatal day P14). These V-shaped branches were explanted on a 3-fold dried layer of newborn rat tail collagen, and attached to the matrix. UB and CCD cells formed confluent monolayers within 1–3 days of culture in nephron culture medium [20] supplemented with 10% fetal calf serum (FCS) and bovine pituitary extract (50 µg/ml; Sigma Chemicals, Deisenhofen, Germany). Culture medium was replaced 24 h before an experiment (after 2–4 days of culture) by medium containing 1 µM aldosterone (Sigma) instead of FCS.

2.3. Preparation of cDNA

Total RNA (1–2 µg) from 6–12 monolayers (about 5 mm in diam-

eter) was isolated according to [21] using Tri Reagent (Biozol, Eching, Germany) and pooled. The whole isolated RNA was reverse transcribed with the ReadyToGo T-Primed First-Strand Kit (Pharmacia, Freiburg, Germany). cDNA was precipitated, washed, diluted in 40 μl of 10 mM Tris-HCl/1 mM EDTA pH 8.0, and stored at −80°C.

2.4. Polymerase chain reaction (PCR)

The concentrations of cDNA preparations from different developmental stages were adjusted to the same level using β-actin as a standard. The cDNA specific for the Cl^- channel CFTR (GenBank accession number M89906) and β-actin (GenBank accession number X00351) were amplified using sequence-specific oligonucleotide primers giving rise to products of 607 bp and 351 bp, respectively (CFTR sense: 5′-CGC AGG TTC TCA GTG GAC GAT GCC-3′ (position 556–579), CFTR antisense: 5′-CCT CAA CCA GAA AAA CCA GCA CGC A-3′ (position 1138–1162), β-actin sense: 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′ (position 384–413), β-actin antisense: 5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′ (position 705–734); primers were synthesized by Life Technologies, Eggenstein, Germany). The PCR reaction mixture contained 2 μl of H_2O-diluted cDNA (1:25 for CFTR and 1:100, 1:1000, and 1:10 000 for β-actin, respectively), 20.6 μl H_2O, 0.2 μl dNTPs (25 mM; Promega, Ingelheim, Germany), 0.25 μl sense primer, 0.25 μl antisense primer (both 10 μM), 0.125 μl AmpliTaq Gold polymerase (5 U/μl; Perkin Elmer, Weiterstadt, Germany) and 2.5 μl 10-fold reaction buffer (15 mM $MgCl_2$). Samples were incubated in a MJ-Research thermal cycler (DNA Engine PTC 200, Biozym, Oldendorf, Germany), initially for 10 min at 95°C. PCR amplification was performed by 35 (CFTR) or 30 cycles (β-actin) of 45 s at 94°C, 1 min at 62°C (CFTR) or 56°C (β-actin) and 1 min at 72°C and a final extension at 72°C for 7 min. The amount of β-actin PCR product (for quantification see below) was directly proportional to the template concentration of the cDNA dilutions (1:100, 1:1000, and 1:10 000), indicating the non-saturating behavior of the PCR reaction. In the CFTR PCR, partial heat inactivation of the polymerase was compensated by prolonging the extension period after every cycle by 1 s. 12 μl of each PCR reaction was separated by electrophoresis in a non-denaturing 5% polyacrylamide gel. Identity of the PCR products was confirmed by restriction analysis (not shown) and sequencing after cloning in the pCR-Script Amp SK(+) vector (Stratagene, Amsterdam, The Netherlands).

2.5. Quantitative evaluation of the RT-PCR reaction

Gel bands were stained with fluorescence DNA dye (VistraGreen; Amersham, Braunschweig, Germany) and scanned in a Storm 840 Fluorophosphor Imager (Molecular Dynamics, Krefeld, Germany). The fluorescence intensity of the scanned DNA bands was quantified by ImageQuant software (Molecular Dynamics). Four independent cDNA preparation series were performed. In each series 5–7 developmental stages were investigated. To compare the data from different series, the absolute β-actin-corrected CFTR values were normalized to the largest value of each series.

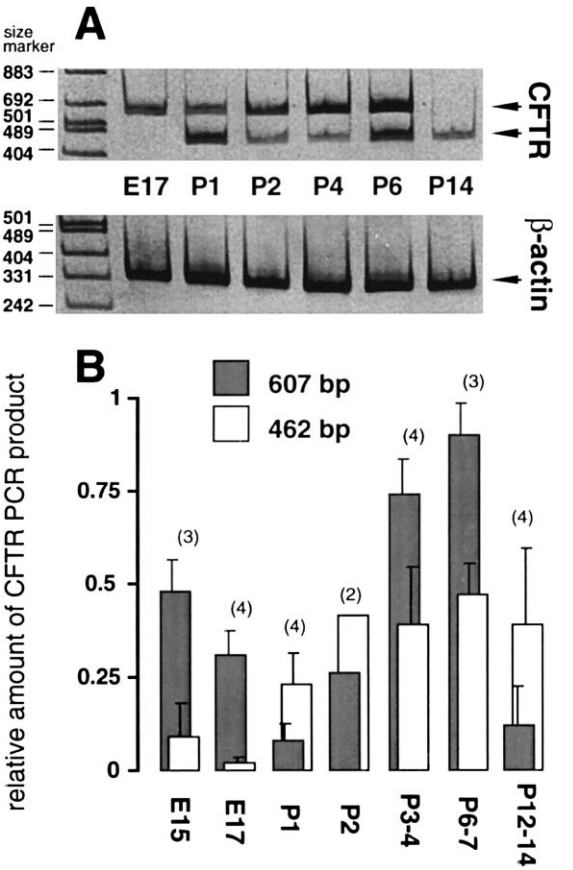

Fig. 2. Quantitative RT-PCR. A: 5% acrylamide gels showing (in VistraGreen fluorescence stain) CFTR- (upper gel) and β-actin-specific PCR products (lower gel) of cDNAs. Complementary DNAs were prepared from rat UB and CCD, microdissected at different developmental stages (embryonic day E15 to postnatal day P14) and grown in primary monolayer culture. B: Amount of PCR products specific for CFTR (as shown in A), normalized by the β-actin PCR product and plotted against developmental age. Both CFTR-specific products (bands of 607 and 462 bp) from four different cDNA preparation series are depicted (means ± S.E.M., n = number of cDNAs).

3. Results and discussion

This study has attempted to evaluate quantitatively the time-dependent expression of CFTR mRNA in a developing NaCl-reabsorbing epithelium. To this end, primary monolayer cultures of UB and CCD derived from gestational day E15 to postnatal day P14 were analyzed by RT-PCR. These cultured cells have been shown previously [3] to express a principal cell-like morphology independent of the developmental stage the cells originated from. Despite their identical morphology, cultured UB and CCD cells differ markedly in their physiology [3]: CCD cultures have been characterized by patch clamp experiments as principal cells by the presence of apical amiloride-sensitive Na^+ and secretory K^+ channels. UB cultures, in contrast, express different types of Cl^- conductances in a developmental stage-dependent way. Importantly, UB cultures derived from gestational day E17 exhibit whole cell currents identical to those of cells at the tip of freshly dissected E17 UB (unpublished own observation). This strongly suggests that UB and CCD primary monolayer cultures mirror in vitro the in vivo developmental stage of differentiation.

Embryonic UB cells (rat E17) expressed CFTR mRNA in vivo as demonstrated in the present study by in situ hybridization. Specific hybridization signals with the CFTR cDNA probe were observed in tubule cells (Fig. 1; compare total binding (A) with non-specific background binding (B)). Single cells at the outermost distal tip of the outgrowing UB and from the ureteric trunk were positive for CFTR mRNA suggesting CFTR expression along the entire developing collecting duct. This pattern has been shown also by immunohistochemistry for the CFTR protein in fetal human [11] and rat kidney [12].

Consistent with the in situ data, CFTR mRNA was expressed in vitro by primary monolayer cultures grown from microdissected UB and CCD. Two CFTR-specific PCR products were amplified with cDNAs prepared from these monolayers. In addition to the expected 607 bp PCR product, a 462 bp fragment was found. Restriction analysis with the enzymes

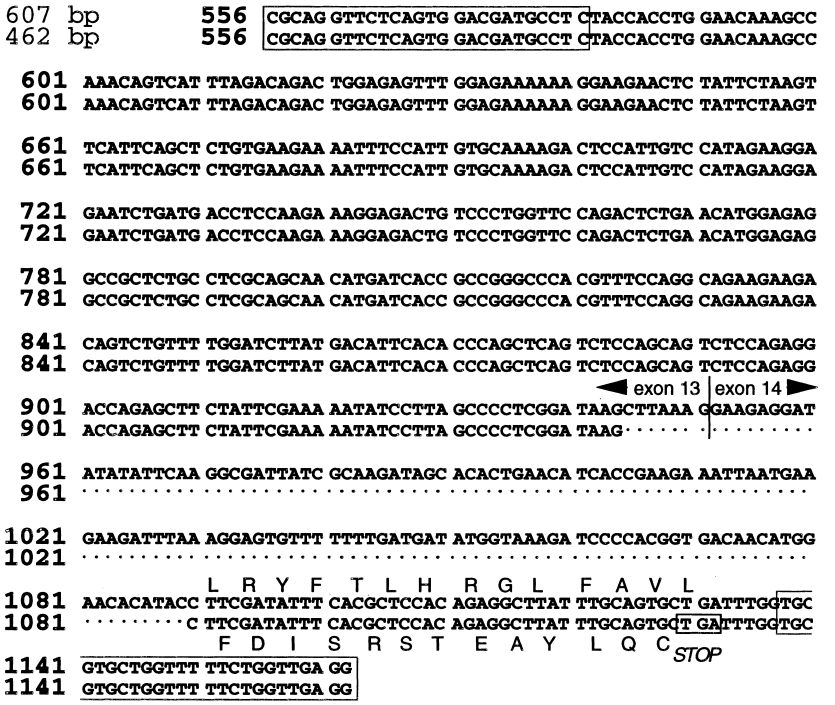

Fig. 3. DNA sequences of the 607 bp and 462 bp CFTR-specific PCR products. The 607 bp fragment encodes parts of exons 13 and 14. The exon junction is indicated by a vertical line. In the 462 bp fragment, 145 bp (bp 945–1089) are deleted resulting in a frame shift and introduction of a stop codon (box). In this region the deduced amino acids encoded by the cDNA and its splice variant are indicated by their single letter abbreviations. The nucleotide positions shown on the left side correspond to the published rat cDNA sequence (GenBank accession number M89906). Positions of forward and reverse PCR primers are indicated by boxes.

*Hin*dIII and *Apa*I and DNA sequencing confirmed that both PCR products were derived from the CFTR gene.

The CFTR protein consists of six transmembrane α-helices (TMD I), a first cytoplasmic nucleotide binding domain (NBD I), followed by a cytoplasmic regulatory (R) domain and six further transmembrane α-helices (TMD II), and it is terminated by the cytoplasmic NBD II. The cDNA fragment amplified by the CFTR primers included parts of exons 13 and 14 which encode a portion of the regulatory domain of the CFTR protein.

The 607 bp PCR product represented the fragment expected from the published rat cDNA sequence. Its amount differed markedly depending on the developmental stage in vivo from which the cultures had been derived (Fig. 2A). The β-actin-normalized amount of the 607 bp fragment was higher in embryonic (day E15 and E17) and postnatal (P3–4 and P6–7) cultures when compared to those of perinatal (P1 and P2) and late postnatal (P12–14) stages (Fig. 2B). These results suggest a decreasing expression during embryonic and a transiently high expression during postnatal development. In particular, the CFTR mRNA abundance in UB cells declined by a factor of 5 between day E17 and day P1 and increased by a factor of 10 between day P1 and day P6–7. A similar decrease has been found for the CFTR protein during embryonic development in whole human fetal kidney (between the 13th and 23rd week of gestation [11]). Remarkably, CFTR mRNA in the distal epithelium of human fetal lung has been shown by in situ hybridization to decrease in an analogous manner between the first and third trimester [22]. Taken together, both organs downregulate distal epithelium (bud) CFTR mRNA during embryonic development but, in contrast to UB epithelium, the transient increase in CFTR mRNA after birth has not been observed in human lung distal epithelium [22].

In the 462 bp fragment, 145 nucleotides (position 945–1089, GenBank accession number M89906) were deleted by alternative splicing using sequences (5′-AAG**ct** … … **ac**CTT-3′) which are not conventional splice donor and acceptor consensus sequences. The deletion affected portions of exons 13 and 14. Due to the shift in reading frame, 13 new amino acids and a premature stop codon were introduced in exon 14 (Fig. 3). This splice variant has been termed TRN-CFTR [18]. The 145 bp deleted in TRN-CFTR mRNA encode the last 7% of the R-domain. The TRN-CFTR protein, therefore, lacks TMD II and NBF II.

The amount of the TRN-CFTR-specific 462 bp PCR product was low in the embryonic cultures when compared to the perinatal and postnatal cultures (Fig. 2B). This indicates a 10-fold increase in mRNA abundance between the late embryonic and the peri- and postnatal period. In contrast to the 607 bp fragment, the amount of the 462 bp PCR product was not reduced in postnatal CCD (P12–14) cultures (Fig. 2B).

The protein encoded by TRN-CFTR mRNA probably corresponds to a 75 kDa protein which has been identified by Western blot in the developing human kidney [11]. In these whole kidney preparations, the 75 kDa CFTR protein was upregulated by a factor of 3–4 between week 16 and 23 of gestation and it remained highly expressed postnatally. This temporal pattern resembles very closely that observed in the rat UB cultures of the present study, taking into account the different time course of kidney development in both species: morphogenesis in rat is completed at day P6–7 postnatally and in human before the 32nd gestational week. In adult rat

and human kidney TRN-CFTR mRNA has been observed in inner and outer medulla, but not in cortex [18] suggesting that TRN-CFTR mRNA expression is downregulated during postnatal maturation of the CCD.

TRN-CFTR mRNA encodes a functional protein, with respect to cAMP-activated Cl^- conductance and regulation of ORCC Cl^- channels, when expressed in oocytes and in a mammalian CFTR-defective cell line [18]. However, both the protein expressed by TRN-CFTR mRNA in vitro [23] and the 75 kDa protein in vivo [11] are rarely inserted in the plasma membrane. This implies that a putative regulation of reabsorptive Na^+ channels by TRN-CFTR is not likely in nephrogenesis.

In contrast to TRN-CFTR, wild-type CFTR channels have been demonstrated in the apical membrane of UB and CCD of fetal and postnatal human [11] and in CCD of postnatal rat kidney [24]. Although rat CCD principal cells are not a route of transcellular Cl^- secretion, i.e. CFTR does not generate a relevant cAMP-activated apical Cl^- conductance [25], CFTR in these cells might regulate reabsorptive Na^+ channels as has been demonstrated in mouse M1 principal cells [16]. Rat UB cells in primary monolayer culture have been shown to express mRNA encoding the α-subunit of the epithelial sodium channel (unpublished own data) but they do not exhibit amiloride-sensitive Na^+ currents [3]. It may be suggested, therefore, that CFTR, which is highly expressed in the UB cultures between postnatal day P3 and day P7 (Fig. 2B), inhibits preexisting Na^+ channels up to the time when the UB is connected to its filtrating glomerulus and reabsorptive mechanisms in the epithelium are switched on.

In conclusion, the outgrowing and branching embryonic collecting duct expresses both the CFTR mRNA and the TRN-CFTR splice variant. The abundance of each of these mRNAs in the terminal UB epithelium changes differentially with the developmental stage of morphogenesis.

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