Molecular Cloning, Chromosomal Localization, Tissue Distribution, and Functional Expression of the Human Pancreatic Sodium Bicarbonate Cotransporter*

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We report the cloning, sequence analysis, tissue distribution, functional expression, and chromosomal localization of the human pancreatic sodium bicarbonate cotransporter protein (pancreatic NBC (pNBC)). The transporter was identified by searching the human expressed sequence tag data base. An I.M.A.G.E. clone W39298 was identified, and a polymerase chain reaction probe was generated to screen a human pancreas cDNA library. pNBC encodes a 1079-residue polypeptide that differs at the N terminus from the recently cloned human sodium bicarbonate cotransporter isolated from kidney (kNBC) (Burnham, C. E., Amlal, H., Wang, Z., Shull, G. E., and Soleimani, M. (1997) J. Biol. Chem. 272, 19111–19114). Northern blot analysis using a probe specific for the N terminus of pNBC revealed an ~7.7-kilobase transcript expressed predominantly in pancreas, with less expression in kidney, brain, liver, prostate, colon, stomach, thyroid, and spinal cord. In contrast, a probe to the unique 5’ region of kNBC detected an ~7.6-kilobase transcript only in the kidney. In situ hybridization studies in pancreas revealed expression in the acini and ductal cells. The gene was mapped to chromosome 4q21 using fluorescent in situ hybridization. Expression of pNBC in Xenopus laevis oocytes induced sodium bicarbonate cotransport. These data demonstrate that pNBC encodes the sodium bicarbonate cotransporter in the mammalian pancreas. pNBC is also expressed at a lower level in several other organs, whereas kNBC is expressed uniquely in kidney.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF011390 and AF020195.

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¶ The abbreviations used are: pH, intracellular pH; NBC, sodium bicarbonate cotransport protein; kNBC, kidney NBC; pNBC, pancreatic NBC; kb, kilobases; bp, base pair(s); DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; PCR, polymerase chain reaction; EST, Expressed Sequence Tag; BCECF, 2',7'-bis(carboxyethyl)-5,5'-carboxyfluorescein; TMA, tetramethylammonium; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; NS, not significant.

Sodium bicarbonate cotransporter mediates the coupled movement of Na+ and HCO3− ions across the plasma membrane of many cells (1). This transport process is involved in bicarbonate secretion/absorption and intracellular pH (pH),1 regulation.

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channel on the apical membrane (5, 36). Influx of H+ equivalents during the process of apical bicarbonate secretion requires the efflux of H− or the influx of bicarbonate in the steady state. Studies of pig, rat, and guinea pig pancreatic ducts have demonstrated the presence of a basolateral Na+/H− antiporter, which serves an important housekeeping role (5, 8, 37, 38). A basolateral vacuolar-type H+/ATPase and Na(HCO3)− cotransporter are thought to play an important role in agonist-mediated bicarbonate secretion (5–8, 39). Ishiguro et al. (7) reported that basolateral Na(HCO3)− cotransport contributed up to 75% of basolateral bicarbonate uptake during stimulation of transepithelial bicarbonate transport by secretin. Furthermore, in isolated pancreatic acini, Na(HCO3)− cotransport has been shown to participate in the regulation of H+ after acid loads (3). On the basis of HCO3− flux measurements and thermodynamic considerations, it was concluded that this transporter contributes to HCO3− efflux under unstimulated conditions (3, 7) with a stoichiometry of 3:1 (3), although direct measurements of the stoichiometry have thus far not been performed. After depolarization of the basolateral membrane by secretin (40), the electrochemical driving forces would favor basolateral bicarbonate influx (7).

Although the functional characteristics of pancreatic Na(HCO3)− cotransport have begun to be investigated, the protein responsible for this function has not been identified. We report here the cloning the human pancreatic Na(HCO3)− cotransporter (pNBC). The predicted pNBC polypeptide is 1079 amino acids in length, whereas the NBC variant expressed in kidney (kNBC) consists of 1035 amino acids. The C-terminal 994 amino acids of pNBC and kNBC are identical. pNBC has a unique N terminus of 85 amino acids that replaces the initial 41 amino acids in kNBC. Expression of the cDNA encoding pNBC in Xenopus oocytes results in sodium-dependent and chloride-independent HCO3− transport, which is inhibited by DIDS.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of pNBC—A 159-bp PCR product (2795–2854 bp in human pNBC) was generated using the human pancreas NBC EST clone W39298 (L.M.A.G.E) clone as a template, random primer-labeled with 32P and used to screen a human pancreas cDNA library (CLONTECH, Palo Alto, CA). A similar approach was utilized by Burnham et al. (30) while the present studies were in progress to obtain an NBC clone from human pancreas. Standard hybridization conditions were employed (42 °C, 50% formamide, 5% standard saline phosphate EDTA (SSPE), 5% Denhardt’s solution, 0.5% SDS, 0.2 mg/ml prehybridization herring sperm DNA). The filters were washed three times with 1× SSC/0.1%SDS (42 °C) and once with 0.1× SSC/0.1%SDS (25 °C) (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate). Positive clones were verified by sequencing. Two overlapping clones (7.1) and (9.2.1) were obtained that contained the entire coding region. To obtain a full-length clone containing the complete open reading frame, these two clones were spliced together using a common Spel restriction site and subcloned into pPCR-Script SK(+) (Stratagene) Riboprobes were synthesized by in vitro transcription and labeled with 35S-CTP. For generation of the antisense riboprobe, the plasmid was linearized with SstI and transcribed by T7 RNA polymerase. For generation of the sense riboprobe, the plasmid was linearized with KpnI and transcribed with T3 RNA polymerase. The RNA transcripts were purified by phenol-chloroform extractions and Sephadex G-50 spin columns (Sigma). The final products were suspended in Tris-EDTA buffer with 0.1 M dithiothreitol. The RNA hydrolysates were then sheared by alkaline hydrolysis at 68 °C for 5 min. After the shearing, the reaction was neutralized by adding 3 mM sodium acetate, pH 5, to make a final acetate concentration of 0.3 M. Slices of mouse pancreas (1 mm) were fixed in 4% formalin, and 5-μm sections were obtained from the tissue blocks. The sections were mounted on glass slides (Fisher). The slides were washed and digested with proteinase K. To reduce nonspecific background staining, the slides were succinylated with succinic anhydride and acetylated with acetic anhydride. The riboprobes were hybridized for 18 h at 45 °C. The slides were then washed for 15 min in 2× SSC at room temperature, followed by a wash (15 min) in 1× SSC/50% formamide at 45 °C, then three washes in 2× SSC/0.1% Triton X-100 at 60 °C for 15 min each, followed by two washes in 0.1× SSC at 60 °C for 15 min each. Human genomic DNA was labeled with 32P and used to screen a human genomic library (Genome Systems, St. Louis, MO). DNA from clone F335 was identified by sequencing and was then labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected with antidigoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole, dihydrochloride for one color experiments. Probe detection for two color experiments was accomplished by incubating the slides in fluorescein isothiocyanate-labeled antidigoxigenin antibodies and Texas red avidin followed by counterstaining with 4,6-diamidino-2-phenylindole, dihydrochloride. Xenopus Oocyte Expression—The plasmid containing the complete...
coding sequence of pNBC was linearized by digestion with KpnI and capped cRNA was prepared with T3 RNA polymerase using a T3 mMessage mMachine kit RNA capping kit (Ambion, Austin, TX) as recommended by the manufacturer. This cRNA was used for the Xenopus oocyte expression studies. An aliquot of the synthesized cRNA was run on a denaturing gel to verify the expected size before oocyte injection. Defolliculated oocytes were injected with 50 nl of sterile water or a solution containing 1 ng/ml capped pNBC cRNA (prepared as described above). They were then bathed in Barth’s medium at 18 °C.

Measurement of Oocyte Intracellular pH (pH<i>i</i>)—3–6 days post-injection, optical recordings were made at 22–24 °C. Intracellular pH was measured using the fluorescent probe 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) and a microfluorimeter coupled to the microscope (41). Individual defolliculated oocytes were held in place with pipettes attached to low suction with vegetal pole surface closest to the objective. Before loading with BCECF, the background intensity from each oocyte was digitized at 500 nm and 440 nm (530-nm emission). The oocytes were loaded with 32 µM BCECF-acetoxyethyl ester for 1 h before experimentation in the following solution: NaCl (108 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM), and Hepes (8 mM), pH 7.4. Calibration of intracellular BCECF in the oocytes was performed at the end of each experiment by monitoring the 500/440-nm fluorescence excitation ratio at various values of pH, in the presence of high K-glutamate standards as described previously (42). Three experimental protocols were performed: 1) Na<i></i>-removal/addition. The oocytes were bathed in a test solution containing Na-free solution for 30 min: NaCl (108 mM); KCl (2 mM); CaCl2 (1 mM); MgCl2 (1 mM); NaHCO3 (8 mM) and bubbled with 1.5% CO2, pH 7.4. After a steady state was reached, Na was removed by bathing the oocytes in the following Na-free solution: TMA-CI (100 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM), TMA-HCO3 (8 mM) bubbled with 1.5% CO2, pH 7.4; 2) Na<i></i>-removal/addition with DIDS (0.3 mM). In the DIDS-containing experiments, the oocytes were exposed to 0.3 mM DIDS for 30 min before and throughout the influx period. 3) Influx Measurements—Defolliculated oocytes were injected with pNBC cRNA (50 nl, 1 µg/ml) and allowed to incubate in Barth’s medium for 3–6 days at 18 °C before use. The oocytes were preincubated for 1 h in 1 ml of a Na-free solution containing: TMA-CI (108 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM) and Hepes (8 mM), pH 7.4. The oocytes were then transferred into 1.4 ml of the following Na-free solution: NaCl (100 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM), NaHCO3 (8 mM) bubbled with 1.5% CO2, pH 7.4. After a steady state was reached, Na was removed by bathing the oocytes in the following Na-free, Cl-free solution with EIPA (10 µM): TMA-CI (100 mM); sodium glutamate (100 mM); potassium glutamate (2 mM); calcium glutamate (7 mM); magnesium glutamate (2 mM); Hepes (8 mM), pH 7.4. The oocytes were then exposed to 0.3 mM DIDS for 30 min with EIPA (10 µM); sodium glutamate (100 mM); potassium glutamate (2 mM); calcium glutamate (7 mM); magnesium glutamate (2 mM); NaHCO3 (8 mM), pH 7.4. Bubbled with 1.5% CO2, pH 7.4. After a steady state was reached, Na was removed by bathing the oocytes in the following Na-free, Cl-free solution with EIPA (10 µM); TMA-OH (100 mM); t-glutamic acid lactone (100 mM); potassium glutamate (2 mM); calcium glutamate (7 mM); magnesium glutamate (2 mM); TMA-HCO3 (8 mM) bubbled with 1.5% CO2, pH 7.4.

22Na<i></i> Influx Measurements—Defolliculated oocytes were injected with pNBC cRNA (50 nl, 1 µg/ml) and allowed to incubate in Barth’s medium for 3–6 days at 18 °C before use. The oocytes were preincubated for 1 h in 1 ml of a Na-free solution containing: TMA-CI (108 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM) and Hepes (8 mM), pH 7.4. The oocytes were then transferred into 1.4 ml of the following Na-free solution: NaCl (100 mM), KCl (2 mM), CaCl2 (1 mM), MgCl2 (1 mM), NaHCO3 (8 mM) bubbled with 1.5% CO2, pH 7.4. After a steady state was reached, Na was removed by bathing the oocytes in the following Na-free, Cl-free solution with EIPA (10 µM); TMA-OH (100 mM); t-glutamic acid lactone (100 mM); potassium glutamate (2 mM); calcium glutamate (7 mM); magnesium glutamate (2 mM); NaHCO3 (8 mM) bubbled with 1.5% CO2, pH 7.4.

**RESULTS AND DISCUSSION**

**Isolation of cDNA Clones and Characterization of Multiple Human NBC Transcripts**—The cloning of pNBC was based on the identification of human pancreatic cDNA clone in the GenBank™ data base. To obtain full-length pNBC, we screened a human pancreas Agt10 cDNA library using a probe generated by PCR amplification of the human EST sequence. Two overlapping clones were obtained that were fused at a shared Spel restriction site to generate a full-length clone containing the entire open reading frame. To confirm that the pNBC sequence was derived from a bona fide transcript, reverse transcription-PCR was used to generate a full-length PCR product containing the complete open reading frame. Analysis of the full-length clone revealed a 1079-amino acid open reading frame beginning with the initial methionine as well as 117 bp of 5'-untranslated region. Additional overlapping 3'-untranslated rapid amplification of cDNA ends sequences were almost identical to the sequence recently published by Burnham et al. (30) except for minor changes likely due to polymorphisms. The nucleotide sequence of human pNBC has been submitted to the GenBank™ (accession number AF0711390).

**Structure of pNBC**—The overall structure of pNBC is similar to kNBC (30) and other members of the anion exchange gene family (43). Specifically, pNBC has 12 predicted transmembrane regions and hydrophilic intracellular N- and C-terminal regions. As shown in Fig. 1, the sequences of pNBC and kNBC
are identical at the Ser residue at position 42 of kNBC and position 86 of the pancreatic sequence. Unlike the kidney sequence, the N terminus of pNBC before the region common to both polypeptides contains blocks of charged amino acids. Further distinctive features of the N terminus of pNBC are 1) the consensus phosphorylation site for protein kinase A beginning at Ser 190, 2) the consensus phosphorylation sites for protein kinase C beginning at Ser 38 and Ser 65, and 3) the casein kinase II phosphorylation site beginning at Ser 68. In contrast, amino acids 1–41 of kNBC lack consensus phosphorylation sites.

**Tissue Expression of pNBC and kNBC**—The expression of pNBC and kNBC was examined in various human tissues by Northern blot analysis (Fig. 2). Specific probes were prepared that contained the unique N-terminal region of each isoform. A probe to the common 3′-coding region of pNBC and kNBC recognized transcripts in RNA samples from the pancreas, with less expression in kidney, brain, liver, prostate, colon, stomach, thyroid, and spinal chord. Burnham et al. (30), using a probe to nucleotides 2737 to 2973 of kNBC (2837 to 3073 in pNBC), detected transcripts in kidney, pancreas and brain. Importantly, the results of the present study demonstrate that the 5′-coding region of pNBC differs from the 5′ terminus of the human NBC sequence reported by Burnham et al. (30). The C-terminal 994 amino acids of pNBC and kNBC are identical. pNBC has a unique N terminus of 85 amino acids that replaces the first 41 amino acids in kNBC. This observation is consistent with the failure of a probe derived from the 5′ terminus of kNBC to detect the ~7.7-kb pNBC transcript in the Northern blot experiments. As shown in Fig. 2, the expression of the ~7.6-kb kNBC transcript was restricted exclusively to kidney. The kNBC probe failed to detect a transcript in any tissue other than kidney (despite longer exposure times, and lower stringency), suggesting that the kNBC N terminus is unique. The mechanism responsible for the tissue-specific expression of kNBC and pNBC is unknown but may involve activation of a downstream promoter and/or differential splicing of the primary transcripts in kidney.

In separate experiments, a Northern blot from a variety of human tissues was screened with a probe derived from the unique 5′ portion of the pancreas cDNA to confirm that the sequence is represented in the ~7.7-kb mRNA transcript. As shown in Fig. 2, this probe detected a ~7.7-kb mRNA abundant in pancreas and present at lower levels in kidney, brain, liver, prostate, colon, stomach, thyroid, and spinal chord. The size of the transcripts in most tissues was identical to that of the pancreatic NBC transcript identified with a probe common to the 3′-coding region of pNBC and kNBC. However, in thyroid, a transcript of higher mobility was detected by both the specific pNBC probe and the C-terminal probe common to kNBC and pNBC. This transcript may represent a variant of pNBC.

**In Situ Hybridization of pNBC**—To determine in greater detail the distribution of pNBC within the pancreas, we used in situ hybridization with a pNBC 35S-labeled riboprobe. Mouse pancreas was used because of tissue availability. Mouse pNBC was cloned using a PCR-based strategy. The sequence of mouse pNBC was found to be 93% identical to the human sequence and has been deposited in GenBankTM (accession number AF020195). Microautoradiography analysis of frozen pancreas sections hybridized with the pNBC riboprobe showed strong expression in the pancreatic ducts and pancreatic acini. A signal was not detected in the islets.

**Chromosomal Localization of Human NBC**—The initial experiment resulted in the specific labeling of the long arm of a group B chromosome, which was believed to be chromosome 4 on the basis of size, morphology, and banding pattern. A second experiment was conducted in which a biotin-labeled probe that is specific for the centromere of chromosome 4 (D4Z1) was cohybridized with clone F335. This experiment resulted in specific labeling of the centromere of chromosome 4. Measurements of 10 specifically labeled chromosomes 4 demonstrated that clone F335 maps to a position that is 19% that of the distance from the centromere to the telomere of chromosome arm 4q, an area that corresponds to band 4q21. A total of 80 metaphase cells were examined, with 71 exhibiting specific labeling (Fig. 4).

**Functional Expression of pNBC in Xenopus Oocytes**—We examined the functional properties of pNBC using measurements of pH, 22Na, and 36Cl− uptake after injecting the corresponding polyadenylated cRNA into Xenopus oocytes. Polyadenylated cRNA prepared as described above was injected into oocytes and allowed to express for 72 h. Augmented 22Na uptake was observed in oocytes injected with pNBC cRNA (Fig. 5). The uptake was 16-fold greater than control oocytes, p < 0.001. Uptake was significantly inhibited in the presence of DIDS (0.3 mM). Cl− uptake was not significantly affected by pNBC cRNA injection: 0.16 ± 0.05 nmoI/h/oocyte in controls (n = 8) and
Further studies were done in which pH$_i$ transients were measured in control oocytes and those injected with pNBC cRNA (Fig. 6). Resting pH$_i$ was similar in both groups of oocytes, 7.1. After extracellular Na$^+$ removal, in control oocytes, pH$_i$ increased by 0.008 ± 0.001 pH/min (n = 7). In contrast, in the cRNA-injected oocytes, pH$_i$ decreased at a rate of -0.008 ± 0.001 pH/min (n = 5), p < 0.001. Na$^+$ removal caused a similar decrease in pH$_i$ in cRNA-injected oocytes in the absence of chloride with 10 μM EIPA; -0.010 ± 0.001 pH/min (n = 3), p = NS. DIDS (0.3 mM) significantly decreased the Na$^+$-induced pH$_i$ transient in cRNA-injected oocytes to -0.0025 ± 0.0003 pH/min (n = 3), p < 0.05.

The Physiological Role of pNBC-mediated Na(HCO$_3$)$_n$ Cotransport—The highest level of pNBC expression was found in the pancreas, with lower levels of expression in kidney, brain, liver, prostate, colon, stomach, thyroid, and spinal chord. The results of the present study are compatible with previous functional studies that have demonstrated Na(HCO$_3$)$_n$ cotransport in pancreatic ductal cells and acini (3, 5–8, 39). It has been hypothesized that basolateral Na(HCO$_3$)$_n$ cotransport in pancreatic ductal cells plays a modulatory role in ductal fluid secretion (5, 7). Under resting conditions, the basolateral cotransporter would mediate cellular bicarbonate efflux when the basolateral membrane potential is -2 -70 mV (40). After stimulation of bicarbonate secretion by secretin, the basolateral membrane voltage of rat duct cells depolarizes to -2 -40 to -20 mV (40). Under these conditions, the cotransporter would mediate bicarbonate influx (7). After stimulation by secretin, basolateral bicarbonate uptake by guinea pig pancreatic ductal cells is mediated in part by the basolateral Na(HCO$_3$)$_n$ cotransporter (5–8, 39), although a basolateral H$^+$-ATPase may also play a role (5, 6, 8). Two important physiological roles for bicarbonate secretion by pancreatic centroacinar cells and ductal cells have been proposed (44): 1) the solubilization of secreted proteins and vesicular retrieval of secreted proteins from...
the acinar lumen and 2) neutralization of the acidic chyme delivered into the upper intestine from the stomach. In the absence of secretogogues, cellular bicarbonate efflux via the basolateral Na(HCO$_3$)$_2$ cotransporter coupled to apical Na$^+$H$^+$ exchange may mediate transepithelial H$^+$ secretion in the main and common pancreatic ducts (5).

The results of the present study indicate that the pNBC is also expressed at lower levels in kidney, brain, liver, prostate, colon, stomach, thyroid, and spinal chord. The N terminus of pNBC has a unique consensus phosphorylation site for protein kinase A beginning at Lys$^{46}$, consensus phosphorylation sites for protein kinase C beginning at Ser$^{58}$ and Ser$^{65}$, and a casein kinase II phosphorylation site beginning at Ser$^{68}$, which kNBC lacks. Of interest, cAMP stimulates transepithelial bicarbonate secretion and basolateral Na(HCO$_3$)$_2$ cotransporter activity by protein kinase A.

The stoichiometry of the transported species appears to be tissue-dependent. The lack of detectable transcripts in heart though the stoichiometry of the transported species appears to be tissue-dependent. The lack of detectable transcripts in heart and lung with any of the three probes used in this study suggests the possibility that cardiac and lung Na(HCO$_3$)$_2$ cotransport is mediated by an alternative protein(s). The finding that prostate and thyroid have transcripts that are labeled by the pNBC probe is of interest, given that these tissues have not been previously investigated for the presence of functional Na(HCO$_3$)$_2$ cotransport. Furthermore the higher mobility of the thyroid transcript suggests that this tissue expresses a variant of pNBC not present in other organs.

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