An anti-NH2-terminal antibody localizes NBCn1 to heart endothelia and skeletal and vascular smooth muscle cells

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An anti-NH2-terminal antibody localizes NBCn1 to heart endothelia and skeletal and vascular smooth muscle cells. NBCn1 has been localized to the basolateral membrane of various epithelia, but thus far it has been impossible to detect the protein in these tissues by using anti-COOH-terminal antibodies. Hence an antibody was developed against the NH2-terminus of NBCn1 and was validated by peptide recognition and immunoblotting on positive control tissues and by binding of an ~180-kDa protein in the rat kidney, cerebrum, cerebellum, and duodenum. In addition, an ~180-kDa immunoreactive band appeared using samples from the aorta, heart ventricles and atria, mesenteric arteries, lung, spleen, liver, pancreas, and epididymis. Immunohistochemical analysis confirmed the previously described labeling in the kidney, duodenum, and the choroid plexus. The anti-NH2-terminal antibody localized NBCn1 to the plasma membrane domains of endothelia and smooth muscle cells in small mesenteric and renal arteries, as well as the capillaries of the heart ventricles, spleen, and salivary glands. NBCn1 was also detected in neuromuscular junctions and vasculature in skeletal muscle. Analysis of variable NBCn1 splicing by RT-PCR revealed that an NH2-terminal sequence, the cassette III, seems absent from cardiovascular NBCn1 and that both cassettes I and II are variable in most epithelia, whereas cassette II is absent from epithelial NBCn1. Thus the development of the NH2-terminal antibody allowed the localization of NBCn1 protein to major cardiovascular tissues where NBCn1 mRNA was previously detected. The NBCn1 is a likely candidate for mediating the reported electroneutral Na+-HCO3- cotransport in vascular smooth muscle.

bicarbonate metabolism; acid/base physiology; immunohistochemistry; sodium bicarbonate cotransporter biology

ACID/BASE TRANSPORTERS are critically involved in the maintenance of pH at the cell, tissue, and organ levels as well as in the whole organism. Sodium-coupled bicarbonate transport has been shown to counteract intracellular acidification or otherwise to contribute to intracellular pH homeostasis in a variety of tissues, and in a number of these, the Na+-HCO3- transporter(s) has/has been identified (1, 2, 3, 4, 12, 14, 15, 20). The transporters are also involved in transepithelial transport of HCO3- and thus exert an important function at the tissue and organ level. The most prominent example thus far is the NBCe1, which mediates bicarbonate reabsorption in the renal proximal tubule by extrusion of Na+ and HCO3- across the basolateral membrane (8). This electronegenic cotransporter may be responsible for up to 80% of the renal HCO3- reabsorption and 20% of the proximal tubular Na+ reabsorption. Interestingly, both electronegenic NBC forms are implicated in transepithelial transfer of Na+ and HCO3-, and, at least in the proximal tubule, the electrical properties are defining the direction of transport. Only one Na+-dependent Cl-/HCO3- exchanger, the NCBE, has been localized to epithelia, i.e., the choroid plexus (16). NCBE may be involved in NaHCO3 secretion into the cerebrospinal fluid, but no studies have yet directly addressed this possibility. The Cl−-independent, electroneutral Na+-HCO3- cotransporter, NBCn1, is thought largely to maintain cellular pH homeostasis.

The NBCn1 (or NBC3) has, nevertheless, been suggested to participate in the renal shortcut pathway for NH4+ (13). It was suggested that NBCn1 facilitates influx of NH4+ from the lumen of renal medullary thick ascending limbs by buffering the H+ formed along with NH3 intracellularly after the dissociation of NH4+. NBCn1 is localized to the basolateral plasma membrane in these cells (20) and thus mediates Na+ and HCO3- uptake from the blood side. The NBCn1 has been localized to the basolateral membrane of other renal tubules as type A intercalated cells of collecting ducts and terminal inner medullary collecting ducts (15, 20). In other epithelia, NBCn1 is also a basolateral protein as in the duodenal mucosa, choroid plexus, and salivary glands (9, 14, 16). Recently, NBCn1 was also found in the rat hippocampal neurons (7). Surprisingly, NBC3 knockout mice revealed no other phenotype than blindness and auditory impairment due to the destruction of neurons in these sensory organs (5). However, there are no reported studies where NBCn1-deficient mice have been challenged by, e.g., the induction of acid/base imbalance, and the implication of NBCn1 in a regulatory response to such conditions is possible.

Originally, NBC3 was cloned from human skeletal muscle (17) and the NBCn1 from rat aorta (6). Nevertheless, it has been impossible to detect the protein in these tissues with the use of the available antibodies directed against the COOH-terminus of NBCn1. Furthermore, the presence of NBCn1 mRNA has been shown in the human heart, rat heart, lung, liver, and spleen by Northern blot analysis or RT-PCR (6, 17). Although the extreme COOH terminus seems to be preserved in all splice variants of NBCn1, none of these tissues has displayed NBCn1 immunoreactivity. Hence it was speculated whether the COOH terminus was somehow masked by either of the at least three variable regions or cassettes of NBCn1. In this study, an anti-NH2-terminal NBCn1 antibody is used to localize NBCn1 protein in cardiovascular and other tissues that are known to express the NBCn1 encoding mRNA. Further-
more, the initial analysis of NBCn1 splice variation is reported and related to the antibody recognition profile of anti-NH$_2$-terminal and anti-COOH-terminal antibodies.

**MATERIALS AND METHODS**

**Animals.** The animal protocols were approved by the Institutional Animal Care and Use Committee, in accordance with the licenses for the care and use of experimental animals issued by the Danish Ministry of Justice. Adult male Wistar rats (300–350 g; from Taconic Europe, Eiby, Denmark) had free access to water and pelleted food (Altromin, Lage, Germany) until use. The rats were anesthetized by isoflurane inhalation before being killed or before perfusion fixation and organ removal.

**RT-PCR and sequencing.** Total RNA from fresh tissues was extracted with the use of the RNeasy mini kit (Qiagen, Germantown, MD). After DNase treatment (RNase-Free DNase, Promega, Madison, WI), the RNA was reverse transcribed with the use of 2 U/µl reverse transcriptase (Superscript II; Invitrogen, Taastrup, Denmark) in the presence of either poly(T) primers or specific reverse primers for NBC transcripts (gene-specific reverse transcription). PCR (HotStar Taq Master Mix, Qiagen) with 10–20% cDNA and 1 pmol of each primer was performed for 30 cycles: a hot start at 95°C for 15 min, denaturation at 95°C for 30 s, annealing at 56–60°C (dependent on primer optimum) for 30 s, and elongation at 72°C for 1 min. Negative PCR controls included omission of reverse transcriptase or omission of cDNA. PCR for β-actin was performed to validate each batch of template before use. NBCn1 primers were designed to reveal either 5’ or 3’ variation and are listed in Table 1. PCR products were separated by 2% agarose gel electrophoresis and photographed under ultraviolet illumination. The PCR products in which these were used were previously validated by nucleotide sequencing.

**Antibodies.** A 20-amino acid NH$_2$-terminal peptide (MEADGAGEQMRPPLTRGPFDE with a COOH-terminal cysteine for affinity purification) was conjugated to keyhole limpet hemocyanin and used for rabbit immunization on the basis of the published rat SLC4A7 sequence (GenBank AF080106). Two rabbits were injected with the peptide, and the resulting antisera were affinity purified with the use of the immunizing peptide coupled to an agarose column (Sulfolink; Pierce, Rockford, IL). Previously described antibodies against aquaporin-1 (AQP-1) (19) and NBCn1d (20) were also applied for immunolabeling.

**Membrane fractionation and generation of protein samples.** Tissues were removed from anesthetized rats that were then decapitated. The tissue samples were homogenized in dissection buffer [0.3 M sucrose, 25 mM imidazole, 1 mM EDTA (pH 7.2), containing 8.5 mM NH$_4$Cl and 1 mM phenylmethylsulfonyl fluoride] with the use of an Ultra-Turrax T8 homogenizer (IKA Labortechnik), with two 15-s bursts, and centrifuged at 4,000 g for 15 min at 4°C to remove nuclei, whole cells, and large cellular fragments. The pellet was discarded, and the supernatant was transferred to new tubes. For deglycosylation, Medium (Dako) and inspected on a Leica DMRS confocal microscope (Dako, Glostrup, Denmark) for 1 h in PBS-T. Excess antibody was removed by extensive washing, and bound antibody was detected by ECL chemiluminescence kit (Amersham, Little Chalfont, UK). Immunoblotting was also performed after the antibody was preincubated with the immunizing peptide for 24 h at 5°C in PBS-T supplemented with 1% BSA and 2 µM NaN$_3$. After being washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Dako, Glostrup, Denmark) for 1 h in PBS-T. Excess antibody was removed by extensive washing, and bound antibody was detected by ECL chemiluminescence kit (Amersham, Little Chalfont, UK). Immunoblotting was also performed after the antibody was preincubated with the immunizing peptide for 24 h at 5°C in PBS-T supplemented with 1% BSA and 2 µM NaN$_3$ to confirm the recognition of the immunogen by the antibody.

**Immunohistochemistry.** Rat tissues were fixed by perfusion via the abdominal aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). The tissues were dehydrated and embedded in paraffin, and 2-µm sections were cut with the use of a rotary microtome (Leica, Heidelberg, Germany). The sections were dewaxed and rehydrated, and endogenous peroxidase was blocked by 0.5% H$_2$O$_2$ in absolute methanol. The sections were boiled in 10 mM Tris (pH 9) supplemented with 0.5 mM EDTA and then incubated with 50 mM NH$_4$Cl and blocked in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated overnight at 4°C with the primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100.

For brightfield microscopy, the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) in PBS with BSA and Triton X-100. The staining was visualized by 0.05% 3,3′-diaminobenzidine tetrahydrochloride dissolved in PBS with 0.1% H$_2$O$_2$. Mayer’s hematoxylin was used for counterstaining, and the sections were dehydrated in graded alcohol and xylene and mounted in hydrophobic Eukitt mounting medium (O. Kindler, Freiburg, Germany). Microscopy was performed on a Leica DMRE brightfield microscope equipped with a Leica DM300 digital camera.

For fluorescence microscopy, sections were double labeled with primary antibodies against the NH$_2$ terminus of NBCn1, the AQP-1, or by incubation with α-bungarotoxin. The sections were then incubated with a biotinylated anti-AQP-1 with the use of streptavidin FITC (Dako) as the visualizing reagent or α-bungarotoxin (Alexa 488 conjugate, Molecular Probes). After being washed, sections were mounted with a coverslip in Glycergel Antifade Medium (Dako) and inspected on a Leica DMRS confocal microscope.

Table 1. Primer sequences

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<tr>
<th>Primer/Direction</th>
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<tr>
<td>Cassettes I and II&lt;br&gt;Forward</td>
<td>GCTGGATATGACGCCAGCACCTC</td>
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<tr>
<td>Reverse</td>
<td>AACATTGGATGCTGAGCTTCTGC</td>
<td>397 bp with cassette I and without cassette</td>
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<tr>
<td></td>
<td>II; 358 bp without cassettes I and II</td>
<td></td>
</tr>
<tr>
<td>Cassette III&lt;br&gt;Forward</td>
<td>CAAGCTCATGATCGATGGGC</td>
<td>334 bp with cassette III</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACTGCTGAGCCTTTTCAAAGG</td>
<td>223 bp without cassette III</td>
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with an HCX PlApo ×64 (1.32 numerical aperture) objective. The immunofluorescence images were merged with differential interference contrast images to reveal the relationship between the tissue structures and the fluorescence labeling.

**Immunogold electron microscopy.** Tissue blocks prepared from the perfusion-fixed rat hearts [4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4)] were cryoprotected with 2.3 M sucrose and rapidly frozen in liquid nitrogen. The samples were freeze-substituted by sequential equilibration over 3 days in methanol containing 0.5% uranyl acetate at temperatures raised gradually from −80° to −70°C, then rinsed in pure methanol for 24 h while the temperature was increased from −70° to −45°C, and infiltrated with Lowicryl HM20 and methanol 1:1, 2:1, and, finally, pure Lowicryl HM20 before ultraviolet polymerization for 2 days at −45°C and 2 days at 0°C. Immunolabeling was performed on ultrathin Lowicryl HM20 sections. Sections were pretreated with a saturated solution of NaOH in absolute ethanol (2−3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris (pH 7.4) containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C with the NH₂-terminal NBCn1 antibody diluted in 0.05 M Tris (pH 7.4) containing 0.1% Triton X-100 with 0.2% milk. After being rinsed, sections were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.EM10; BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate at temperatures raised gradually from 80° to 70°/H11011 to 64° to 50° C and 2 days at 0°C. Immunogold electron microscopy using immunoelectron microscopy was performed on known positive tissues with the use of either the affinity-purified antibody or the preabsorbed antibody after overnight incubation with the immunizing peptide (right). Lanes represent renal cortex and outer stripe of outer medulla (Cox), renal inner stripe of outer medulla (ISOM), and renal inner medulla (IM), sample buffer (SB, heart, cerebrum (Chr), cerebellum (Chbl), and duodenum (Duod)).

**RESULTS**

**Validation of the anti-NH₂-terminal NBCn1 antibody.** The newly developed antibody was evaluated by means of 1) recognition of the immunizing peptide by the antibody; 2) preabsorption of the antibody with the immunizing peptide before immunoblotting and immunohistochemistry; and 3) finding again the previously described localization. Figure 1A shows that the anti-NH₂-terminal NBCn1 antiserum recognized the immunizing peptide when this was subjected to gel electrophoresis and immunoblotting. Immunoblotting was performed on known positive tissues with the use of either the affinity-purified antibody or the preabsorbed antibody after overnight incubation with the immunizing peptide (Fig. 1B). The expected ~180-kDa band is observed in protein samples from the renal cortex/outer medulla, ISOM, and IM, as well as from the heart, cerebrum, cerebellum, and duodenum (Fig. 1B, left). The negative immunodetection after the anti-NBCn1 antibody was preabsorbed is shown in Fig. 1B, right. The two immunoblots were run in parallel. Figure 1C shows the reduction in apparent molecular size of the detected protein resulting from deglycosylation by PNGase treatment. The anti-NH₂-terminal antibody detects the proteins in all three kidney samples (Fig. 1C, left) that are deglycosylated to a similar degree, ~40 kDa, as the proteins recognized by the previously characterized anti-COOH-terminal NBCn1 antiserum (Fig. 1C, right). The results indicate that the anti-NH₂-terminal antibody recognizes the same N-glycosylated protein as the anti-COOH-terminal NBCn1 antiserum.

The previously described labeling of NBCn1 in e.g., the duodenum, kidney, and choroid plexus was confirmed with the use of the anti-NH₂-terminal NBCn1 antibody as illustrated in Fig. 2. In the kidney, labeling is observed in the basolateral plasma membrane domain of the thick ascending limbs as well as in type A intercalated cells of collecting ducts, as illustrated in Fig. 2A. No labeling appeared in the type B intercalated cells. As shown in Fig. 2B, the staining is also found basolaterally in the terminal inner medullary collecting ducts. In duodenal mucosa, the labeling is most prominent in the villus cells and restricted to the basolateral plasma membrane domain, as it was in the epithelial cells of the choroid plexus (Fig. 2, C and D, respectively). The insets in Fig. 2, A–D, illustrate the disappearance of staining when the antibody was preabsorbed by the immunizing peptide.

**Tissue expression profile of NBCn1 by immunoblotting.** Immunoblotting with the use of the anti-NH₂-terminal antibody with protein samples from various tissues revealed an ~180-kDa band, as shown in Fig. 3A. A sharp band was seen in the aorta, both ventricles, and atrium of the heart, the mesenteric artery, kidney cortex, cerebrum and cerebellum, lung, trachea, pancreas, liver, parotid gland, and in the epididymis. The 180-kDa band appeared broader and more blurry in the spleen, IM and ISOM of the kidney, duodenum, jejunum, ileum, and colon. In addition to the 180-kDa band, an additional sharper band was observed of ~140 kDa in certain tissues: the spleen, kidney cortex, lung, trachea, gastric fundus and pylorus, duodenum, jejunum, and ileum; a weak band was observed in epididymis. Figure 3B shows that only relatively

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**Fig. 1. Immunoblot validation of anti-NH₂-terminal NBCn1 antibody.** A: peptide recognition by anti-NH₂-terminal NBCn1 antibody. After SDS-PAGE, immunizing peptide was electrotransferred onto nitrocellulose and incubated with anti-NH₂-terminal NBCn1 antiserum and visualized by chemiluminescence with use of horseradish peroxidase-conjugated secondary antibody. B: preabsorption test of anti-NH₂-terminal NBCn1 antibody. Selected ranges of known positive tissues were subjected to immunoblotting with affinity-purified anti-NH₂-terminal NBCn1 (Nt) NBCn1 antibody (left) or same antibody after overnight incubation with immunizing peptide (right). Lanes represent renal cortex and outer stripe of outer medulla (Cox), renal inner stripe of outer medulla (ISOM), and renal inner medulla (IM), sample buffer (SB, heart, cerebrum (Chr), cerebellum (Chbl), and duodenum (Duod)). C: effect of deglycosylation on protein migration. Samples from rat kidney were treated enzymatically with PNGase F (dg) and immunoblotted with use of anti-NH₂-terminal NBCn1 (left) or samples from rat kidney. Anti-COOH-terminal (Ct) NBCn1 antiserum was applied on ISOM samples (right).
few tissues were NBCn1 positive when the anti-COOH-terminal antibody was applied. Only spleen, IM, and ISOM of the kidney, duodenum, jejunum, colon, liver, and epididymis contained an immunoreactive 180-kDa band. Samples from the cerebrum, cerebellum, lung, trachea, and the parotid gland may also be positive. As shown in Fig. 3C, the anti-NH2-terminal NBCn1 antibody recognized a sharp 180-kDa protein in samples from skeletal muscle and a broad band in the control ISOM.

Immunohistochemical detection of NBCn1 in cardiovascular tissues. Immunolabeling was revealed in a range of vascular tissues with the use of the anti-NH2-terminal NBCn1 antibody, as shown in Fig. 4. In the aorta, NBCn1 immunoreactivity was detected in endothelial cells and vasa vasorum (Fig. 4A). The

![Image](http://ajpheart.physiology.org/)

**Fig. 2.** Immunohistochemical localization of NBCn1 in known positive tissues. Anti-NH2-terminal NBCn1 antibody was applied to semithin sections of rat kidney, duodenum, and brain for immunohistochemical verification of antibody sensitivity. A: immunolabeling in transition between ISOM and IM. Dashed line marks transition and arrows mark basolateral plasma membrane domain of medullary thick ascending limbs (mTAL) of Henle’s loop and a subset of collecting duct cells in ISOM (OMCD) and initial inner medulla (IMCD). B: immunostaining of terminal inner renal medulla. Arrows mark collecting duct cells (IMCD3). C: duodenal mucosa subjected to anti-NBCn1 immunolabeling. Arrows show basolateral plasma membrane domain of villus enterocytes. Lp, lamina propria. D: immunolabeling in rat brain. Basolateral plasma membrane domain of choroid plexus epithelium is marked by arrows. Bl, blood side of epithelium; Ar, arteriole. Scale bars = 50 μm. Insets show result of preabsorbing the antibody with immunizing peptide.

**Fig. 3.** Comparison of protein detection by anti-NH2-terminal and anti-COOH-terminal NBCn1 antibodies. A: immunoblot performed with use of anti-NH2-terminal NBCn1 antibody on various rat tissue samples. Mesenteric a, mesenteric arteries; cortex, renal cortex and outer stripe of outer renal medulla; Submandib gl, submandibular gland; Subling gl, sublingual gland. B: immunoblot performed in parallel with immunoblot in A, with application of the previously described anti-COOH-terminal NBCn1 antiserum. C: immunoblot performed with use of anti-NH2-terminal NBCn1 antibody on protein samples from rat ISOM and rat skeletal muscle (Sk Musc, from entire quadriceps femoris muscle).
smooth muscle cells also seem to stain, but it is uncertain whether this is unspecific staining because the labeling is cytosolic. In mesenteric arteries, labeling is seen in the endothelium as in the aorta. However, in the mesenteric arteries, the smooth muscle cells of the tunica media stain near cell borders as demonstrated by the gridlike appearance of the labeling in the longitudinally cut vessel (Fig. 4B). Also, in intrarenal arteries, NBCn1 labeling is detectable in the endothelium and in the smooth muscle cells in what seems to correspond to cell boundaries, as illustrated in Fig. 4C. This vessel is cut transversally, and hence the staining of the myocyte layer appears striped rather than gridlike. Figure 4D shows that liver staining is restricted to the endothelium of the small hepatic arteries and the branches of the portal vein and that no labeling appears in the intralobular bile duct. Also, no labeling is seen in the parenchyma or in the central vein. It should be noted that endothelial NBCn1 immunoreactivity does not seem to be a general feature, and in many tissues, endothelia apparently do not stain. This is exemplified by the labeling pattern in kidney, duodenum, and choroid plexus.

In the heart, labeling is seen in the capillaries in the myocardium but not in the cardiomyocytes. In both the atria and ventricles, the labeling is confined to the endothelia, as illustrated in Fig. 5, A and B, respectively. Double labeling immunofluorescence microscopy of the rat heart atrium confirmed that NBCn1 labeling colocalized with the water channel AQP-1 in the endothelia (Fig. 5C). The NBCn1 staining is shown in red and AQP-1 in green, whereas yellow color indicates colocalization. The immunogold electron micrograph shown in Fig. 5D shows that NBCn1 is expressed in both the luminal and the basal plasma membrane of the endothelial cells in the rat heart ventricle. No labeling appeared in the myocytes, except for rare gold particles situated in a few mitochondria.

Expression of NBCn1 in skeletal muscle. In skeletal muscle, NBCn1 labeling was found in the vasculature and in structures resembling the neuromuscular junctions (Fig. 5E). Double labeling skeletal muscle with NBCn1 and fluorescence tagged α-bungarotoxin verified the expression of the bicarbonate transporter in the junctions, as illustrated in Fig. 5F. Interestingly, there seems to be little microsection localization of the NBCn1 and α-bungarotoxin; that is, the red and green fluorescence were both found within the neuromuscular junction but the labeled spots rarely overlapped. This may indicate that NBCn1 is expressed on the motor neuron terminals or that it is situated in sarcosomal areas devoid of the nicotinic acetylcholine receptor.

RT-PCR analysis of NBCn1 splice variation. RT-PCR was performed on selected tissues to confirm the presence of NBCn1 RNA in the different tissues and to investigate whether there was a connection between the presence of the cassettes I, II, and III and the ability of the anti-NH2-terminal and anti-COOH-terminal NBCn1 antibody to recognize the protein. Figure 6A shows the products after RT-PCR with the use of a primer pair to amplify the cassettes I and II of NBCn1. The higher-molecular-weight band indicates the presence of cassette I, and a lower band indicates its absence. Both the presence and absence of cassette I were found in the ISOM and IM of the kidney, in cerebrum, cerebellum, pyrulus, duodenum, ileum, colon, the submandibular, sublingual, and parotid glands, trachea, spleen, and epididymis. No bands were detected in the liver and heart under these reaction conditions, nor was the cassette II detected in any tissues because the reaction theoretically should yield a product size of 766 bp. Figure 6B shows similar analysis of the presence of cassette III. Although inclusion of cassette III seemed to dominate in most tissues, mRNA excluding cassette III was found in the ISOM and IM.
of the kidney, cerebellum, duodenum, ileum, liver, trachea, spleen, and epididymis. In the rat heart, the product indicating the absence of cassette III was dominating. Thus the expression of both cassettes of NBCn1 seems to vary at the mRNA level within a variety of epithelial tissues.

DISCUSSION

The anti-NH2-terminal NBCn1 antibody revealed a labeling pattern in the kidney, duodenum, and choroid plexus identical to what was found previously with the use of the anti-COOH-terminal antibody (14–16, 20). Nevertheless, some controversy remains regarding the renal localization of rat NBCn1, because the anti-human NBC3 antibody does not stain rat medullary thick ascending limbs but rather stains type A intercalated cells apically and type B in the basolateral membrane domain (11, 18). Because we cannot exclude that the anti-NH2-terminal and anti-COOH-terminal antibodies fail to recognize a type-B cell-specific variant of NBCn1, this issue is still not fully resolved. The findings obtained with the anti-NH2-terminal and anti-COOH-terminal antibodies are, however, supported by both NBCn1 mRNA expression and functional NBCn1 expression in the mTAL. It remains to be clarified whether or not intercalated cells type B actually express NBCn1/NBC3 mRNA. Interestingly, all three antibodies display identical labeling patterns in all nonrenal epithelia studied thus far (unpublished observations).

Immunoblotting with the use of the anti-NH2-terminal NBCn1 antibody shows three apparently distinct bands: a fuzzy 180-kDa band, a sharp 180-kDa band, and an ~140-kDa band. It seems as though the fuzzy band is found in tissues...
where immunohistochemistry reveals epithelial staining and the sharp band is found in tissues where the endothelium is primarily labeled. Preparations of isolated renal medullary thick ascending limbs or duodenal enterocytes yield only the 180-kDa broad band (not shown). The variations in band appearance in the different tissues and sometimes within a single tissue may possibly be accounted for by differences in glycosylation and phosphorylation of the protein or by variable splicing.

One reason the anti-NH$_2$-terminal antibody recognizes NBCn1 in more tissues by immunoblotting may be that there is a structural hindrance for recognition by the anti-COOH-terminal or anti-hNBC3 antibodies. It has been speculated that the COOH-terminus is bound to cytoskeletal or other proteins in a way that masks the epitope for the anti-COOH-terminal and anti-hNBC3 antibodies. This is unlikely because the proteins are detached and denatured during immunoblotting. Thus a simpler explanation would be that the anti-NH$_2$-terminal antibody reveals NBCn1 immunoreactivity with a better signal-to-noise relationship than the previous antibodies.

Three variable regions have been described in the NBCn1. The cassette I consists of 14 amino acids in the long intracellular NH$_2$-terminus and was formerly called the A-cassette; cassette II is the following 123 amino acids; and cassette III, formerly the B-cassette, consists of 36 amino acids in the COOH-terminus (7). It appears that both cassettes I and III are variable in epithelial NBCn1, whereas cassette II seems absent.

Table 2. Summary of NBCn1 expression profiles

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<thead>
<tr>
<th>Tissue</th>
<th>PCR Cassette</th>
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<th>Immunohistochemistry</th>
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<td></td>
<td>I</td>
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<td>Cerebrum</td>
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<td>Cerebellum</td>
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<td>Epididymis</td>
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<td>Skeletal muscle</td>
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Plus signs denote the presence and minus signs the absence of NBCh1 mRNA, protein, or immunostaining, as indicated. ISOM, inner stripe of outer medulla; IM, inner medulla.
from epithelial NBCn1. Interestingly, the cassette III is absent from the heart, showing a substantial lower band. This feature seems to coincide with the lack of recognition by the anti-COOH-terminal NBCn1 antibodies that may only recognize the “epithelial NBCn1.” It remains to be clarified how many of these transcripts are converted into biologically significant amounts of NBCn1 protein and whether the apparent difference in epithelial and nonepithelial NBCn1 can be confirmed at the protein level.

Table 2 provides a summary of the expression profile for NBCn1 found in a range of tissues by RT-PCR, immunoblotting, and immunohistochemistry. Overall, there is good correlation among the results obtained with the three techniques. The expression of NBCn1 in the cardiovascular structures was found by all three methods, as was the staining in the brain and epididymis. PCR analysis of NBCn1 expression was not performed for the aorta and other arteries in the present study but was previously shown by the cloning from an aorta library (6).

The novel staining of the aorta is interesting because NBCn1 was not detected in this tissue by using the previously applied antibodies. The labeling of endothelia and vasa vasorum appears credible, but the staining of smooth myocytes is less compelling. The staining in these cells seems to be cytosolic, which is a highly unlikely position for a protein that does not undergo acute regulated vesicular trafficking. Moreover, NBCn1 is indeed observed in close proximity to the plasma membrane domain of small-artery myocytes in both the kidney and the mesentery. This is interesting because all prior attempts to detect NBCn1 protein in vascular smooth muscle have failed. Na+-dependent HCO₃⁻ transport has been described in vascular smooth muscle from mesenteric arteries (1). This transport process has been shown to be DIDS sensitive and electroneutral. Interestingly, the vascular NBCn1 is apparently inhibited by this compound, whereas the epithelial form seems relatively DIDS insensitive. Evidence for endothelial Na⁺-dependent HCO₃⁻ transport is sparse, but such transport has been observed in primary cultures of brain endothelia (C. Taylor, M. Barrand, and S. Hladky, Department of Pharmacology, Cambridge University, UK, personal communication). It remains to be clarified whether this transport is indeed mediated by NBCn1.

The human NBC3 was cloned from a skeletal muscle library (17), but thus far antibodies have been unable to detect the protein in this tissue as was the case for the rat aorta NBCn1. The immunostaining as well as immunoblotting presented here confirm the presence of NBCn1 in skeletal muscle. It is of interest that the staining not only appears in the capillaries as confirmed by immunoblotting, but immunohistochemical analysis shows absence of labeling in the submandibular and sublingual glands. The reason for this inconsistency might again rely on relatively low NBCn1 abundance in the glands. NBCn1 staining was primarily found in vascular and ductal structures, which comprise a small fraction of the tissue, and these structures would not be well represented in the loaded proteins in immunoblotting. Nevertheless, previous studies have detected NBCn1 in the rat parotid and submandibular glands (9). In that study, the presence of NBCn1 RNA in these tissues was confirmed by immunoblotting, but immunohistochemical analysis only showed weak labeling of submandibular gland and no labeling of parotid gland.

In conclusion, an anti-NH₂-terminal NBCn1 antibody localized the electroneutral Na⁺-HCO₃⁻ cotransporter for the first time to cardiovascular tissue and to skeletal muscle. NBCn1 was expressed in the endothelia of large arteries, in small arteries, in capillaries of many epithelial tissues, in heart atria and ventricles, and in skeletal muscle. NBCn1 was also found in vascular smooth muscle of small arteries and in the neuromuscular junction of skeletal muscle. The findings call for further investigations into the functional role of NBCn1 in endothelia and muscle.

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