The Na\(^+\)-dependent HCO\(_3\)^\(^-\) cotransporter NBCn1 (NBC3, slc4a7) was originally cloned from rat aorta (11) and human skeletal muscle (30). When heterologously expressed in Xenopus oocytes, NBCn1 mediated net acid extrusion dependent on Na\(^+\) and HCO\(_3\)^\(^-\) but independent of Cl\(^-\). The transport was electroneutral and was only partly inhibited by DIDS (11).

Subsequently, NBCn1 has been proposed to be important in a number of cell types: mouse duodenal enterocytes (26), rat outer medullary thick ascending limb epithelial cells (24), mouse retinal and inner ear sensory cells (7), rat inner medullary collecting duct epithelial cells (27), rat hippocampal neurons (12), rat choroid plexus epithelial cells (9), rat osteoclasts (8), and mouse vascular smooth muscle cells (6).

In a few of these studies, a direct link between the presence of NBCn1 and its proposed function has been shown by the use of knockout mice (7) or small interfering RNA-mediated knockdown (6). The molecular identification in the remaining studies hinges on RT-PCR analyses and immunohistochemical localization, using antibodies directed against the transporter (14, 15, 18, 21, 31, 32, 37). The use of antibodies depends on immunoreactivity, which can be influenced by several factors, including the conformation of the antigen, phosphorylation, or masking from other structures (14, 23, 25). Furthermore, eventual splice variants without the targeted epitope will not be immunoreactive. The risk of unspecific binding of antibodies is also of concern.

At least three different antibodies have been raised against the electroneutral Na\(^+\)-HCO\(_3\)^\(^-\) cotransporter: two targeting its COOH terminus [anti-NBC3 raised against the terminal 18 amino acids of the human sequence (21) and anti-NBCn1 raised against the terminal 15 amino acids of the rat sequence (37)] and one targeting its NH\(_2\) terminus [raised against the terminal 20 amino acids of the rat sequence (14)].

While previous antibody-based studies represent major steps forward in our understanding of NBCn1 expression, several potential concerns could be raised, as follows: 1) the labeling patterns obtained with the published antibodies display important differences, 2) in certain tissues (e.g., hippocampus and heart) in which electroneutral Na\(^+\)-HCO\(_3\)^\(^-\) cotransport has been reported (12, 22), no immunohistochemical staining of the intact tissue has been shown, and 3) identification of NBCn1 in some tissues, using Western blot analysis without supporting immunohistochemistry (14), makes interpretations difficult because bands obtained with Western blot analysis could originate from NBCn1 expressed in vascular structures or other widely expressed cell types not directly involved in the specific function of the tissue.

Since much of the current knowledge about NBCn1 is based on the immunoreactivity of the transporter, we performed the present study to investigate the expression pattern of NBCn1, using a technique independent of immunoreactivity. We produced mice with a gene trap insertion into the NBCn1 gene (slc4a7) bringing the LacZ gene, coding for bacterial \(\beta\)-galactosidase, under control of the NBCn1 promoter. In these mice, histochemical staining for \(\beta\)-galactosidase activity can be used
as a reporter for the transcriptional activity of the NBCn1 gene (13).

MATERIALS AND METHODS

Development of the transgenic mouse model. 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. Embryonic stem cells containing the pGT2L×f gene trap vector integrated into the intron sequence between exon 3 and exon 4 in one allele of the NBCn1 gene (slc4a7) were obtained from BayGenomics. The vector includes a promoterless LacZ reporter gene, which, after integration, is under the control of the NBCn1 promoter.

Embryonic stem cells were injected into B6D2F2 mouse blastocysts (38). The chimeric males were bred with C57BL/6 females. Agouti offspring (indicating germ-line transmission of the embryonic stem cells) were tested for the presence of the disrupted NBCn1 allele by PCR, using genomic tail DNA. Heterozygous mice were further bred to obtain homozygous mice on a mixed genetic C57BL/6/129S1/Sv background. For genotyping, a common forward primer was used: 5’-GCA GAG ACT GAA CTC AGC TGT T-3’.

Blinding assay (33). The kidney inner medulla was stained with a COOH-terminal anti-NBCn1 antibody. The kidney inner medulla was finely homogenized with a pestle in ice-cold dissection buffer, centrifuged at 4,000 g for 15 min at 4°C and then treated as previously described (14).

Preparation and staining of tissues. Seven homozygous (NBCn1LacZ/LacZ), three heterozygous (NBCn1LacZ/+), and seven wild-type (NBCn1+/+) mice 7–16 wk old were perfusion fixed with 4% (wt/vol) paraformaldehyde in PBS. Samples from organs of interest were dissected free of surrounding tissue and were washed in PBS overnight at 4°C. Next, tissues were immered in staining solution for 30 min at room temperature (21°C), followed by 18–24 h at 4°C. The staining reaction was stopped by transferring tissues to a solution containing 1% (wt/vol) EDTA and 4% paraformaldehyde in PBS. Tissues were photographed with a digital camera (Olympus C7070WZ) mounted on a Zeiss stereo microscope (Stemi 2000).

For histological examination, selected tissues were immersed in staining solution for 14 h at room temperature before the staining reaction was stopped. Subsequently, tissues were cryosectioned to 30-μm thickness or were paraffin embedded and prepared as 8- to 20-μm sections. Microscopy was performed on a Leica DMRE brightfield microscope equipped with a Leica DM300 digital camera.

RT-PCR analyses. The expression of NBCn1 and β-galactosidase mRNA in heterozygous gene trap mice and wild-type mice was investigated by RT-PCR analyses. Kidney corpuscles were microisolated after enzymatic digestion as previously described (15). mRNA was isolated using Dynabeads mRNA Direct microkit (Dynal, Oslo, Norway) and was DNase treated (RQ DNaSel, Promega; Madison, WI). To confirm successful mRNA isolation, RT-PCR reactions for β-actin were performed. Primers and reaction conditions used for detection of NBCn1 have previously been described (6). Similar conditions were used for β-galactosidase with the following primers: forward, 5’-ATG TGC TGC AAG GCG ATT AA-3’; reverse primer for the wild-type allele was 5’-CTT GTA GCT AGA-3’; reverse primer for the interrupted allele was 5’-ATG TGC TGC AAG GCG ATT AA-3’.

A: representative amplification plot from quantitative RT-PCR experiment shows lower level of NBCn1 mRNA in a homozygous gene trap mouse (gray lines) compared with a wild-type mouse (black lines). Broken lines represent GAPDH, and unbroken lines represent NBCn1.

B: normalized NBCn1 mRNA levels in heterozygous gene trap mice and wild-type mice were 0.79 ± 0.05 and 0.99 ± 0.02, respectively. *P < 0.05 vs. NBCn1+/+.

C: summarized Western blot data (n = 3–4) showing lower NBCn1 protein abundance in homozygous gene trap mice compared with wild-type mice. The level of NBCn1 protein is normalized to wild-type levels. Comparisons were made by unpaired Student’s t-tests. *P < 0.05, **P < 0.01 vs. NBCn1+/+.

D: summarized Western blot data (n = 3–4) showing lower NBCn1 protein abundance in homozygous gene trap mice compared with wild-type mice. The level of NBCn1 protein is normalized to wild-type levels. Comparisons were made by unpaired Student’s t-tests. *P < 0.05, **P < 0.01 vs. NBCn1+/+. 

AJP-Cell Physiol • VOL 294 • FEBRUARY 2008 • www.ajpcell.org
Intracellular pH measurements. Segments of the bladder wall were dissected free from adherent mucosa and connective tissue, mounted in a myograph (DMT, Aarhus, Denmark), and kept at 37°C. Tissues were loaded with 5 μmol/l BCECF-AM in 0.02% DMSO for 30 min. Excitation was performed alternately at 440 and 495 nm while the emission light was collected at 530 nm. The emission ratio (495/440) was converted to intracellular pH (pHi) through calibration with the previously described high-K⁺ nigericin method (1). Fluorescence measurements were carried out using a Leica DM IRB inverted microscope with a ×20 objective (numerical aperture 0.5) connected to a Photon Technology International DeltaScan system. Background fluorescence measured before the loading procedure was subtracted from the measured emissions.

Intracellular acidification was produced with the NH₄⁺ prepulse technique. NH₄Cl (20 mmol/l) was washed out into a Na⁺-free solution containing 600 μmol/l amiloride (to block Na⁺-H⁺ exchange). In the continued presence of amiloride, Na⁺ was returned to the bath solution, and the Na⁺-dependent and amiloride-insensitive pH recovery was recorded. For each animal, one NH₄⁺ prepulse was performed in the presence of CO₂/HCO₃⁻ and one in its nominal absence. Net base influx was calculated from the pH recovery rate by multiplication with the buffering capacity. The net Na⁺-dependent HCO₃⁻ influx was determined as the difference between the net Na⁺-dependent base influx in the presence and absence of CO₂/HCO₃⁻.

Buffering capacity. The buffering capacity was calculated from the change in pH upon washout of NH₄Cl as previously described (6). No significant difference between the buffering capacity in homozygous gene trap mice and wild-type mice was found, and these results were therefore pooled. In the nominal absence of CO₂/HCO₃⁻, the buffering capacity was 53 ± 7 mmol/l at an average pH of 6.77, similar to previous results from mouse vascular smooth muscle cells (6). In the presence of CO₂/HCO₃⁻, the buffering capacity was 41 ± 4 mmol/l at an average pH of 6.93. The finding that CO₂/HCO₃⁻ did not appear to contribute to an increased intracellular buffering capacity is consistent with previous reports from a variety of smooth muscle cell sources (1, 3, 16).

Solutions. The dissection buffer for preparing Western blots contained (in mmol/l) 300 sucrose, 25 imidazole, 1 EDTA, 0.0085 leupeptin, and 1 phenylmethylsulfonyl fluoride. The PBS contained (in mmol/l) 137 NaCl, 2.5 KCl, 4.3 Na₂HPO₄, and 1 KH₂PO₄. The staining solution contained (in mmol/l) 5 K₄Fe(CN)₆, 5 K₃Fe(CN)₆, 2 MgCl₂, 0.1% (wt/vol) SDS, 0.1% (vol/vol) Tween 20, and 0.1% (wt/vol) 5-bromo-4-chloro-indolyl-13-D-galactoside (X-Gal). For functional experiments, the bicarbonate containing solution consisted of (in mmol/l) 114 NaCl, 10 HEPES, 25 NaHCO₃, 1.20 MgSO₄, 4.70 KCl, 5.50 glucose, 0.026 EDTA, 1.18 KH₂PO₄, and 1.60 CaCl₂. In bicarbonate-free solutions, NaHCO₃ was substituted with an equimolar amount of NaCl. In Na⁺-free solutions, NaCl was substituted with an equimolar amount of N-methyl-D-glucamine titrated with HCl. Bicarbonate containing solutions were bubbled with 5% CO₂ in air, whereas bicarbonate-free solutions were bubbled with air; pH was adjusted to 7.40 at 37°C.

Statistical analyses. Data are expressed as means ± SE. Unpaired two-tailed Student’s t-test was used for comparison of two groups. Two-way ANOVA with a Bonferroni post test was used for compar-
ison of consecutive measurements between animals. P < 0.05 was considered statistically significant; n equals number of mice. Statistical analyses were performed using GraphPad Prism 4.02 software.

RESULTS

The integration of the gene trap vector into the NBCn1 gene resulted in a significant reduction in NBCn1 expression at the mRNA level (~54%) as well as at the protein level (~72%) in mice homozygous for the mutation, as shown in Fig. 1. The remaining expression of NBCn1 was probably due to an imperfect trap as previously reported for the pGT2L×f gene trap vector (17). The mice showed normal viability and a Mendelian distribution with 21.5 ± 4.0% homozygous gene trap mice, 55.1 ± 4.8% heterozygous gene trap mice, and 23.4 ± 4.1% wild-type mice among a total of 107 mice genotyped.

Expression of β-galactosidase in tissues where the NBCn1 promoter is active resulted in widespread yet distinct staining of tissues after addition of X-Gal. The chromogenic reaction product was primarily localized to intra- or perinuclear areas. Generally, heterozygous mice stained in a similar pattern to homozygous mice, although the staining was less intense.

As shown in Fig. 2A, retinas of homozygous gene trap mice were strongly stained. The X-Gal precipitate was primarily localized to photoreceptor cells, while staining of some ganglion cells was apparent (Fig. 2B). In heterozygous mice, an intermediate level of staining was seen (Fig. 2C), whereas no staining was seen in wild-type mice (Fig. 2D).

Arteries and veins were consistently stained throughout the investigated tissues. Mesenteric and cerebral arteries and veins along with kidney arteries and arterioles displayed strong staining (Fig. 3). Vascular smooth muscle cell staining was predominant, but staining of vascular endothelial cells was also evident (Fig. 3, C–E).

In addition to vascular smooth muscle cells, we find a general staining of nonvascular smooth muscle cells. In trachea, urinary bladder, uterus, and throughout the gastrointestinal tract (esophagus, stomach, duodenum, jejunum, ileum, and colon), smooth muscle cells of homozygous gene trap mice were stained, whereas no such staining was seen in wild-type mice (Figs. 4 and 5).

Prominent staining in the kidney cortex was found in afferent arterioles and corpuscles (Fig. 6, A, C, D, E, and G). No other cortical structures were convincingly stained; in particular, no staining was seen in cortical collecting ducts (Fig. 6F). In the outer medulla, staining was seen in thick ascending limb epithelial cells as well as in collecting ducts (Fig. 6, H and I). The inner medulla was strongly stained due to a ubiquitous staining of collecting ducts (Fig. 6, J–M). Finally, staining of
the epithelial lining of the kidney pelvis was observed (Fig. 6, A and L). Select areas of the gastrointestinal mucosa were stained. Duodenal and colonic mucosae stained strongly, whereas the jejunal and ileal mucosae were essentially unstained (Fig. 5). In the duodenal mucosa, the predominant staining was in enterocytes of villi rather than crypts (Fig. 5, C–E).

Several areas of the brain were stained (Fig. 7, A and B). Most prominently, the pyramidal cell layer of the hippocampus regions CA1 through CA4 was heavily stained as was the dentate gyrus (Fig. 7, F and G). Multiple distinct layers of the cerebral cortex were stained (Fig. 7H). In cerebellum, the dentate nucleus stained considerably, and cortical Purkinje cells were prominent (Fig. 7, C–E). Also, choroid plexus epithelial cells were moderately stained (Fig. 7, I and J).

The large vessels of the heart were stained, as was heart atria but not ventricles (Fig. 8, A and B); this was reflected by staining of atrial cardiomyocytes, whereas ventricular cardiomyocytes were unstained (Fig. 8, C and D). No staining of skeletal muscle was seen (Fig. 8E).

We performed RT-PCR analyses to investigate the expression of NBCn1 and β-galactosidase mRNA in selected tissues. In heterozygous gene trap mice, we confirmed the expression of β-galactosidase and NBCn1 in duodenal mucosa and smooth muscle, colonic mucosa and smooth muscle, bladder wall, kidney cortex, and kidney corpuscles (Fig. 9). A similar expression pattern for NBCn1 was found in wild-type mice, whereas no expression of β-galactosidase was seen in these mice (Fig. 9).

To further confirm that NBCn1 is expressed in the stained tissues and investigate whether the staining correlates to functional importance of the transporter, we examined the pHi regulatory function of bladder smooth muscle cells in situ. Under resting conditions (Fig. 10, A and B), smooth muscle cells from wild-type mice displayed an intracellular steady-state pH of 7.35 ± 0.02. In homozygous gene trap mice, this value was reduced to 7.14 ± 0.03 (P < 0.01). When bladder smooth muscle cells from wild-type mice were investigated under bicarbonate-free conditions, a major reduction in steady-state pHi (ΔpHi = -0.28 ± 0.08; n = 4; P < 0.05) was observed, similar to previous results from mouse vascular smooth muscle cells (6). In the nominal absence of CO2/HCO3–, no significant difference in steady-state pHi was observed between wild-type and homozygous gene trap mice (Fig. 10B). Taken together, these results are consistent with NBCn1 mediating net bicarbonate uptake in bladder smooth muscle cells under resting conditions.
Fig. 5. β-Galactosidase staining of the gastrointestinal tract. A: whole-mount preparation of duodenum from homozygous gene trap mouse. The duodenal wall (arrow) as well as the duodenal mucosa displayed prominent staining. Note the strong staining of the duodenal villi (marked by dotted lines) compared with the subjacent band of tissue (asterisks). B: no staining was observed in the duodenum from wild-type mice. C: section (8-μm thickness) of duodenal villus from homozygous gene trap mouse showing staining of enterocytes (arrow). D: section (8-μm thickness) of duodenal crypts and the smooth muscle cell layer from homozygous gene trap mouse. Strong staining of the longitudinal and circumferential smooth muscle layer was found. No compelling staining was seen in the duodenal crypts. Insert: high magnification of duodenal crypt epithelium with goblet cell (GC). E: no staining of duodenal villi (top), crypts, or smooth muscle cells (bottom) was found in wild-type mice. F: whole-mount preparation of jejunum from homozygous gene trap mouse; the smooth muscle cell layer (arrows) was stained, whereas no staining of the mucosa was seen. The dotted lines mark villi. G: whole-mount preparation of ileum from homozygous gene trap mouse; the smooth muscle cell layer (arrows) was stained, whereas no staining of the mucosa was observed. The dotted lines mark villi. H: in wild-type mice, no staining of jejunum (top) or ileum (bottom) was seen. I: whole-mount preparation of colon from homozygous gene trap mouse; the colonic wall (arrows) and mucosa (Mu) were strongly stained. J: no staining of colonic tissue from wild-type mice was detected. K: section (20-μm section) of colonic crypt from homozygous gene trap mouse. Staining of the colonic epithelium (arrow) is apparent. L: sections (20-μm thickness) of the colonic smooth muscle layer. Staining was seen in smooth muscle cells from homozygous gene trap mice (top) but not in wild-type mice (bottom). M: section (20-μm thickness) of colonic crypt from wild-type mouse. No staining was observed.

AJP-Cell Physiol • VOL 294 • FEBRUARY 2008 • www.ajpcell.org
Additionally, we investigated the ability of bladder smooth muscle cells to recover from an intracellular acid load. After an NH$_4^+$ pulse in the presence of CO$_2$/HCO$_3^-$, the pH recovery rate was decreased in homozygous gene trap mice compared with wild-type mice (Fig. 10A). In bicarbonate-free solution, no significant difference was observed between the base influx seen in wild-type and homozygous gene trap mice [J = 0.85 ± 0.02 mmol·l$^{-1}·$min$^{-1}$ (n = 4) and J = 0.82 ± 0.11 mmol·l$^{-1}·$min$^{-1}$ (n = 5), respectively; $P = 0.43$]. When the HCO$_3^-$-independent recovery is taken into account, the amidolide-insensitive Na$^+$- and HCO$_3^-$-dependent base influx was ~65% reduced in homozygous gene trap mice compared with wild-type mice (Fig. 10C).

**DISCUSSION**

We investigated the expression profile for NBCn1, using a method visualizing the activity of the NBCn1 promoter. This method is of particular value because it is independent of immunoreactivity. Previously, three different antibodies have been used to investigate the expression of NBCn1, but important differences in the expression patterns obtained are apparent (Table 1). With the present β-galactosidase-based method, we provide supporting evidence for NBCn1 expression in a number of tissues in which electroneutral Na$^+$-HCO$_3^-$ cotransport has previously been reported and provide novel evidence for the expression of NBCn1 in a number of tissues in which NBCn1 has not previously been identified. It should be noted that while the present study was performed on mice, the previously published studies have been performed on various species. Discrepancies may thus be partially explained by species differences.

One concern using the β-galactosidase method is the potential risk for unspecific staining. With the described protocol, however, virtually no staining was seen in wild-type mice. The considerable number of animals investigated and a high degree of reproducibility allow us to draw firm conclusions. The inclusion of heterozygous mice, which throughout the investigated tissues displayed an intermediate staining, is a strong argument for specificity of the observed staining.

The distinct staining of the retina, seen in the present study, supports the previously reported immunolocalization and is consistent with the gradual retinal degeneration and development of blindness in NBCn1 knockout mice (7). With the use of the COOH-terminal anti-NBC3 antibody, NBCn1 was previously identified in the synaptic termini of photoreceptor cells (7). Similarly, the β-galactosidase staining was mainly restricted to photoreceptor cells, although staining of some ganglion cells was observed.

The prominent staining of vascular smooth muscle cells and vascular endothelial cells is in line with the labeling obtained using the NH$_2$-terminal antibody (14), whereas such labeling has not been seen with either of the COOH-terminal antibodies (21, 32, 37). RT-PCR analyses have also localized NBCn1 to arteries (6, 11, 26) where Na$^+$-dependent HCO$_3^-$ transport is known to be important (1, 6). With the use of small interfering RNA-mediated knockdown, NBCn1 has been shown to be responsible for the Na$^+$-dependent HCO$_3^-$ transport in mouse vascular smooth muscle cells (6). Here, it accounts for at least 50% of the net acid-extruding capacity and is likely to be involved in contractile changes seen upon removal of CO$_2$/HCO$_3^-$ (6). Some evidence exists for Na$^+$-dependent HCO$_3^-$ transport in vascular endothelial cells, although this work has so far been restricted to cultured cells (34, 35). Further studies into the importance of NBCn1 in endothelial cells of intact arteries are needed.

The functional importance of NBCn1 in nonvascular smooth muscle cells has not until now been directly investigated, and NBCn1 has not earlier been localized to this tissue. In the present study, the NBCn1 promoter is found to be active in a wide array of smooth muscle cells from trachea, urinary bladder, uterus, and the gastrointestinal wall, making it a likely candidate for the previously reported electroneutral Na$^+$-dependent HCO$_3^-$ transport present in nonvascular smooth muscle cells (2). By RT-PCR analyses, we confirm the expression of NBCn1 in smooth muscle cells from the bladder and the gastrointestinal wall. Furthermore, we show that disruption of the NBCn1 gene and the consequent reduction in NBCn1 expression result in a marked reduction in the amidolide-insensitive Na$^+$-dependent HCO$_3^-$ transport present in bladder smooth muscle cells. Also, in resting smooth muscle cells from homozygous gene trap mice, the steady-state pH$_i$ in the presence of CO$_2$/HCO$_3^-$ was significantly reduced. In the nominal absence of CO$_2$/HCO$_3^-$, no difference in net acid extrusion or steady-state pH$_i$ was seen when compared with wild-type mice. These results are in agreement with previous results from vascular smooth muscle cells (6) and strongly suggest a central role for NBCn1 in smooth muscle pH$_i$ regulation. The results further suggest a good correlation between β-galactosidase staining, NBCn1 and β-galactosidase mRNA expression, and functional importance of the transporter, illustrated in a tissue where NBCn1 has not previously been immunohistochemically identified.

Two very different expression patterns for NBCn1 have previously been reported in the kidney. 1) The COOH-terminal anti-NBC3 antibody labels rabbit and rat collecting ducts from cortex, outer medulla, and initial inner medulla exclusively. In rabbits, labeling is apical on type A intercalated cells (32), whereas in rats, apical staining of type A cells as well as basolateral staining of type B cells is seen (21). Recently, a study using the COOH-terminal anti-NBC3 antibody on human kidney preparations reported labeling of thick ascending limb epithelial cells (15). 2) The COOH- and NH$_2$-terminal anti-NBCn1 antibodies label thick ascending limb epithelial cells in addition to outer and inner medullary but not cortical collecting ducts. In rats, the NH$_2$-terminal antibody labels basolateral plasma membrane domains of type A but not type B intercalated cells (14). The results from the present study support the expression pattern reported using anti-NBCn1 antibodies, although the applied method does not allow for localization of the transporter to cellular subtypes or subcellular regions. Using β-galactosidase staining, prominent promoter activity was observed in renal vascular structures, including arcuate arteries, afferent arterioles, and corpuscles. The novel finding that NBCn1 is actively transcribed in kidney corpuscles was confirmed by RT-PCR analyses. The functional importance of NBCn1 in the kidney still remains unclear. It has been suggested that NBCn1 may contribute to the countercurrent multiplication system responsible for medullary ammonium build-up and secretion (24). This has not been experimentally verified, although NBCn1 has previously been shown to be upregulated.
C598 ANTIBODY-INDEPENDENT LOCALIZATION OF NBCn1 IN MICE

A

B

C

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AJP-Cell Physiol • VOL 294 • FEBRUARY 2008 • www.ajpcell.org

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Fig. 6. β-Galactosidase staining of the kidney. A: midsection of kidney from homozygous gene trap mouse. Low-magnification photograph showing punctate cortical staining, homogenous medullary staining, and pronounced staining of the kidney pelvis. B: strong staining of arcuate artery from homozygous gene trap mouse. C: section (0.2-cm thickness) of kidney cortex from homozygous gene trap mouse; staining was seen in a number of brain stem nuclei, in the cerebellar cortex, and in the dentate nucleus (DN). D: section (8-μm thickness) of cerebral cortex from homozygous gene trap mouse. Strong staining was localized to Purkinje cells. E: no staining was detected in wild-type mice. F: section (30-μm thickness) of outer medulla from homozygous gene trap mouse; strong staining of thick ascending limb (TAL) and collecting duct epithelial cells was seen. G: no staining was detected in wild-type mice. H: midsection of kidney from wild-type mouse showing no staining. I: section (0.5-cm thickness) of kidney cortex from homozygous gene trap mouse showing staining in a number of brain regions. J: section (30-μm thickness) of outer medulla from wild-type mouse; no staining was detected in the outer medulla of wild-type mice. K: higher magnification of inner medulla from homozygous gene trap mouse displaying strong staining of collecting ducts. L: section (30-μm thickness) of choroid plexus showing staining of the epithelial lining (arrow) of the kidney pelvis from homozygous gene trap mouse. M: no staining was seen in the inner medulla of wild-type mice.

Fig. 7. β-Galactosidase staining of the brain. A: sagittal section (0.5-cm thickness) of brain from homozygous gene trap mouse showing staining in a number of brain regions. B: no brain staining was observed in wild-type mice. C: section (0.5-cm thickness) of cerebellum and brain stem (BS) from homozygous gene trap mouse. Staining was seen in a number of brain stem nuclei, in the cerebellar cortex, and in the dentate nucleus (DN). D: section (8-μm thickness) of hippocampus from homozygous gene trap mouse. Strong staining was seen in CA1 through CA4 and the dentate gyrus. E: no staining was detected in wild-type mice. F: section (8-μm thickness) of cerebellum. The cortical staining was localized to Purkinje cells. G: no staining was detected in wild-type mice. H: section (8-μm thickness) of hippocampal staining. I: section (8-μm thickness) of cerebral cortex from homozygous gene trap mouse displaying staining in multiple layers. J: section (8-μm thickness) displaying moderate staining of choroid plexus epithelial cells (arrow) from homozygous gene trap mouse. K: no staining was seen in wild-type choroid plexus epithelial cells (arrow).
Fig. 8. β-Galactosidase staining of the heart and skeletal muscle. A: whole-mount preparation of heart from homozygous gene trap mouse. Staining was seen in heart atria but not heart ventricles. Also, the large vessels were stained. The border between the atria and the ventricles is marked by the dotted line. B: whole-mount preparation of heart from wild-type mouse. No staining of the heart and associated vessels was observed. C: section (8-μm thickness) of heart atrium from homozygous gene trap mouse. Atrial cardiomyocytes were stained. D: section (8-μm thickness) of heart ventricle from homozygous gene trap mouse. No staining was detected in ventricular cardiomyocytes. E: whole-mount preparation of anterior tibial muscle. No staining of skeletal muscle was seen in homozygous gene trap mice.

Fig. 9. Agarose gels showing RT-PCR results for β-galactosidase (top; β-Gal) and NBCn1 (bottom). For each primer set, a band of the appropriate size has been sequenced to validate the assay. RT-PCR experiments for β-actin were performed to confirm successful isolation of mRNA. + and −, RT+ and RT−, respectively.

Fig. 10. Bladder smooth muscle cells in situ from homozygous gene trap mice have a decreased capacity to regulate intracellular pH (pHi). A: average trace (n = 4–5) showing the decreased Na−-dependent pHi recovery rate and the decreased steady-state pHi observed in bladder segments from homozygous gene trap mice compared with wild-type mice in the presence of CO2/HCO3−. B: average steady-state pHi, measured in the presence (+HCO3−) and absence (−HCO3−) of CO2/HCO3−. Steady-state pHi was significantly lower in homozygous gene trap mice in the presence of CO2/HCO3− but not in its nominal absence. Comparisons were made with a two-way ANOVA with a Bonferroni post test. C: the net amiloride-insensitive Na−- and HCO3−-dependent base influx (in mmol·l−1·min−1) was significantly smaller in bladder segments from homozygous gene trap mice compared with wild-type mice. Comparison was made with unpaired Student’s t-test. **P < 0.01 vs. NBCn1+++. NS, no significant difference.
Table 1. The expression pattern of NBCn1 obtained with the present β-galactosidase-based method compared with previously applied methods

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>β-Galactosidase (PS)</th>
<th>NH4-Terminal Antibody (14)</th>
<th>COOH-Terminal Antibody (Anti-NBCn1)</th>
<th>COOH-Terminal Antibody (Anti-NBCn3)</th>
<th>RT-PCR</th>
<th>Northern Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina</td>
<td>+</td>
<td>+</td>
<td>(37) (21, 32)</td>
<td>+ (6, 11, 26)*</td>
<td></td>
<td></td>
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<tr>
<td>Cardiovascular tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vascular smooth muscle cells</td>
<td>+</td>
<td>+</td>
<td>− (37)</td>
<td>− (21, 32)</td>
<td>+ (11, 30)</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Vascular endothelial cells</td>
<td>+</td>
<td>+</td>
<td>− (37)</td>
<td>− (21, 32)</td>
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<td></td>
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<tr>
<td>Heart</td>
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<tr>
<td>Ventricles</td>
<td>−</td>
<td>−</td>
<td>− (14)</td>
<td>+ (14)</td>
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<tr>
<td>Cardiomyocytes</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Capillary endothelia</td>
<td>+</td>
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<tr>
<td>Atria</td>
<td>−</td>
<td>−</td>
<td>− (14)</td>
<td>+ (14)</td>
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<td></td>
</tr>
<tr>
<td>Cardiomyocytes</td>
<td>+</td>
<td>−</td>
<td></td>
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<tr>
<td>Capillary endothelia</td>
<td>+</td>
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<tr>
<td>Nonvascular smooth muscle cells</td>
<td></td>
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</tr>
<tr>
<td>Trachea</td>
<td>+</td>
<td>+/−†</td>
<td>(+)−(14)†</td>
<td>+ (14)</td>
<td>− (30)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal wall</td>
<td>+</td>
<td>−</td>
<td>− (26)</td>
<td>+ (PS)</td>
<td>− (30)</td>
<td></td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>(11)/−(30)</td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td></td>
<td>− (37)</td>
<td></td>
<td>+ (PS)</td>
<td></td>
</tr>
<tr>
<td>Corpuscles</td>
<td>+</td>
<td>−</td>
<td>− (37)</td>
<td>− (21, 32)</td>
<td>+ (PS)</td>
<td></td>
</tr>
<tr>
<td>Collecting ducts</td>
<td>−</td>
<td>−</td>
<td></td>
<td>+ (21, 32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer medulla</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+ (14, 27)</td>
<td></td>
</tr>
<tr>
<td>Thick ascending limb</td>
<td>+</td>
<td>+ (14, 15)</td>
<td>+ (24, 27, 37)</td>
<td>+ (15)/−(21, 32)</td>
<td>+ (24)</td>
<td></td>
</tr>
<tr>
<td>Collecting ducts</td>
<td>+</td>
<td>+</td>
<td>+ (24, 27)</td>
<td>+ (21, 32)</td>
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<tr>
<td>Inner medulla</td>
<td>+</td>
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<td>+ (14, 27)</td>
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</tr>
<tr>
<td>Collecting ducts</td>
<td>+</td>
<td>+</td>
<td>+ (27, 37)</td>
<td>+ (21)†</td>
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<tr>
<td>Pelvis epithelial cells</td>
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<tr>
<td>Gastrointestinal mucosa</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td>− (30)</td>
<td></td>
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<tr>
<td>Esophagus</td>
<td>−</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>−</td>
<td></td>
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</tr>
<tr>
<td>Duodenum</td>
<td>+</td>
<td>+ (14, 15)</td>
<td>+ (15, 26)</td>
<td>+ (15)</td>
<td>+ (14, 26, PS)</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>−</td>
<td>+</td>
<td>+ (14)†</td>
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</tr>
<tr>
<td>Ileum</td>
<td>−</td>
<td>+</td>
<td>+ (14)†</td>
<td>+ (14)</td>
<td></td>
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</tr>
<tr>
<td>Colon</td>
<td>+</td>
<td>+ (14)†</td>
<td>+ (14)†</td>
<td>+ (14, PS)</td>
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<tr>
<td>Brain</td>
<td>+</td>
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<td></td>
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<tr>
<td>Cerebrum</td>
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<tr>
<td>Hippocampus</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortex</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purkinje cells</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>Dentate nucleus</td>
<td>+</td>
<td></td>
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<tr>
<td>Choroid plexus</td>
<td>+</td>
<td>+</td>
<td>+ (28, 29)</td>
<td>+ (28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>−</td>
<td>−</td>
<td>− (14)</td>
<td></td>
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<tr>
<td>Neuromuscular junctions</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+ (30)/−(11)</td>
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</table>

The summarized results originate from studies using different animal species, and discrepancies may thus be partially explained by species differences. † Identification of NBCn1; (+), inconclusive identification; −, studies in which NBCn1 could not be identified. * Obtained from whole vessel homogenates. ‡ Only observed with Western blotting of whole tissue homogenates; not seen with immunohistochemistry. § Initial inner medullary collecting ducts. Numbers in parentheses refer to the reference list. PS, present study.

During NH4+ loading and K+ depletion (19, 20) when transepithelial NH4+ transport is increased.

Transport of acid-base equivalents across the gastrointestinal epithelium may be important for local as well as systemic pH-regulation. Bicarbonate transport is believed to be involved in the protection of the gastrointestinal mucosa against acid-induced injury (4). NBCn1 has earlier been shown to be expressed in duodenal enterocytes (14, 15, 26), and the present study supports this view. Functionally, DIDS-insensitive Na⁺-dependent bicarbonate transport has been described in duodenum and may be important for intracellular pH-regulation and transepithelial bicarbonate secretion (26). Notably, we provide novel evidence for NBCn1 promoter activity in the colonic mucosa where bicarbonate secretion is known to take place, although little is known about the processes involved (5). Up until now, efforts have been centered on transporters on the apical membrane where chloride-dependent transporters and transporters dependent on short-chain fatty acids are the primary candidates (5). Our results suggest, however, that NBCn1 is expressed in the colonic mucosa where it could contribute to intracellular pH-regulation as well as transepithelial transport of sodium and bicarbonate.

The involvement of Na⁺- and HCO3⁻-dependent membrane transporters in the regulation of intracellular pH in cells of the nervous system has long been recognized (10). So far, the major focus has been on electrogenic Na⁺-HCO3⁻ cotransporters (33, 39), although NBCn1 has been suggested to be important in rat hippocampal neurons (12). NBCn1 has been identi-
fied in cerebral as well as cerebellar samples by Western blot analysis or RT-PCR (11, 12, 14, 28), but the precise localization is poorly understood. In the present study, we provide evidence for NBCn1 promoter activity in several regions of the intact brain, including hippocampus, multiple layers of the cerebral cortex, cerebellar Purkinje cells, and the dentate nucleus. Furthermore, our results support recent findings that NBCn1 is expressed in the choroid plexus (9, 28, 29) where it may contribute to the production of cerebrospinal fluid.

In the heart, strong evidence exists for the importance of Na\(^+\)-dependent HCO\(_3\)\(^-\) transport (41), which has been suggested to be involved in cardiac pathology (36, 40). Although the transport in some studies has been reported to be electrogenic, other work has indicated electroneutral transport (41). Despite consistent efforts, the relative contribution from electrogenic and electroneutral transporters to intracellular pH-regulation has not been conclusively determined, and it has been suggested that species and/or cell-type differences in the expression of Na\(^+\)-dependent HCO\(_3\)\(^-\) transporters may exist (41). With the use of immunohistochemistry, NBCn1 has so far not been localized to cardiomyocytes, although expression in heart capillary endothelia has been reported (14). In the present study, however, we find that the NBCn1 promoter is active in atrial but not ventricular cardiomyocytes. This difference may contribute new insights into the importance of electroneutral Na\(^+\)-HCO\(_3\)\(^-\) cotransport for cardiomyocyte function.

The human ortholog of NBCn1 was originally cloned from human skeletal muscle and was shown to localize to this tissue using Northern blot analysis (30). In subsequent studies, however, NBCn1 has not been identified in skeletal muscle by the use of Northern blot analysis (11) nor with the COOH-terminal anti-NBCn1 antibody (14). Recently, NBCn1 has been shown to localize to neuromuscular junctions using the NH\(_2\)-terminal use of Northern blot analysis (11) nor with the COOH-terminal

In conclusion, we find that the NBCn1 promoter is active in a wide array of tissues. In support of previous findings, we show that the NBCn1 promoter is active in kidney thick ascending limb and medullary collecting duct epithelial cells, the epithelial lining of the kidney pelvis, vascular smooth muscle cells and endothelial cells from a broad range of blood vessels, duodenal enterocytes, choroid plexus epithelial cells, hippocampus, and retina. Furthermore, the present study identifies a number of new tissues in which NBCn1 may be important despite lack of previous immunohistochemical evidence, including atrial cardiomyocytes, nonvascular smooth muscle cells, kidney cupules, colonic mucosa, multiple layers of the cerebral cortex, cerebellar Purkinje cells, and the dentate nucleus.

In bladder smooth muscle cells in situ, we show that NBCn1 is responsible for Na\(^+\)-dependent HCO\(_3\)\(^-\) transport active at rest and after intracellular acid loading.

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REFERENCES


ANTIBODY-INDEPENDENT LOCALIZATION OF NBCn1 IN MICE


