Human CLC-KB Gene Promoter Drives the EGFP Expression in the Specific Distal Nephron Segments and Inner Ear

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Abstract. Human CLC-KB has been identified as a kidney-specific member of the CLC chloride channel family, and mutations of the human CLC-KB gene are known to cause Bartter syndrome type III. A precise understanding of the localization of this channel in the human kidney is imperative to our understanding of the pathophysiology, but this has remained unclear due to the high homology between human CLC-KB and CLC-KA, another kidney-specific member of the same family. The high intraspecies homology also rules out an exact correlation of the human isoforms (CLC-KA and CLC-KB) to the mouse and rat isoforms (CLC-K1 and CLC-K2, respectively). This study created transgenic mice harboring the enhanced green fluorescence protein (EGFP) gene driven by an 11-kbp human CLC-KB gene promoter. Three transgenic lines were generated, and all of them showed EGFP fluorescence in the kidney, with an identical pattern of localization to the thick ascending limb of Henle’s loop, distal tubules, connecting tubules, and intercalated cells of the collecting duct. This localization is exactly the same as that of mouse CLC-K2 identified in a previous report (Kobayashi et al. J Am Soc Neph 12: 1327–1334, 2001). EGFP fluorescence was also detected in the inner ear, more specifically in marginal cells of the stria vascularis and dark cells of the vestibular labyrinth, suggesting that human CLC-KB could play an important role in the fluid transport mechanism of the inner ear. The results (1) confirmed that CLC-KB is the true human homologue of rat and mouse CLC-K2 and (2) established that the 11-kbp human CLC-KB gene promoter is sufficient to elicit the precise expression in specific cell types of the kidney and inner ear.

Human CLC-KB has been identified as a kidney-specific member of the CLC chloride channel family, and mutations of the human CLC-KB gene are known to cause Bartter syndrome type III (1), and this channel is thought to play an important role in the transepithelial chloride transport in the kidney. Human CLC-KA, another member of the CLC chloride channel family that is expressed only in the kidney, shares approximately 90% identity with human CLC-KB in nucleic acid and amino acid sequences. Although the high intraspecies identity rules out a correlation of mouse and rat channels with human counterparts, it has long been assumed that human CLC-KB and CLC-KA are the homologues of mouse and rat CLC-K2 and CLC-K1, respectively. The intrarenal localizations of mouse and rat CLC-K1 and CLC-K2 have been exhaustively studied and identified by immunohistochemistry and in situ hybridization (2–5). However, our intrarenal localization of human CLC-KB in earlier RT-PCR studies failed to exactly match that of mouse and rat CLC-K2 (6); we were therefore unable to confirm that CLC-KB was the true human homologue of CLC-K2.

As both of the CLC-K genes are located right next to each other at chromosome 1p36 (Figure 1), it can be safely presumed that they were created by gene duplication from a common ancestor. When we characterized the 1.5-kbp promoter activity of rat CLC-K2, we found that, although the GA-rich element may play an important role in the promoter activities, this promoter alone is not sufficient for the strict regulation of nephron segment–specific expression (7). A longer promoter sequence will be needed to more precisely characterize the expression of CLC-K2 (CLC-KB).

Conditional gene targeting using the Cre/loxP system is a powerful tool for studying gene function in vivo. To introduce this technique to physiologic and pathophysiologic studies on renal channels or transporters, experiments will require transgenic mice in which kidney-specific promoters drive the Cre recombinase expression in a nephron segment–specific pattern. In studies conducted thus far, promoters have been shown to effectively target the reporter gene expression in the glomerulus (8), the proximal tubule (9), and principal cells of the collecting duct (10). Few promoters are, however, demonstrably functional in the thick ascending limb of Henle’s loop or the distal tubule in transgenic mice.

By generating transgenic mice in which the reporter gene expression is driven by an 11-kbp promoter of the human CLC-KB gene, we hoped therefore to prove (1) that the human CLC-KB gene promoter could drive the reporter gene expression in the same....

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Materials and Methods

Generation of Transgenic Mice

The transgene used herein (hKBP-EGFP) consists of an 11-kbp human CLC-KB gene promoter fused to the coding region of the enhanced green fluorescence protein (EGFP) gene. In the first step of the construction, the Not I site of the pEGFP-1 (Clontech, Palo Alto, CA) was switched to the PvuI site, the BamHI-AflII fragment of this pEGFP-1 was subcloned into the SmaI site of the pBluescript II (Stratagene, La Jolla, CA), and the KpnI site of this plasmid was changed to the Not I site (pBluescript II-KN-EGFP). Next, a 12.5-kbp human genomic DNA was amplified by the LA-PCR method (TakaRa, Tokyo, Japan) with primers specific for the 3′ end of the CLC-KA gene and the 5′ end of the CLC-KB gene (primer hCLCA8, TCAGTCCCTCTCGTACATC, and primer hCLCB7, CTCGACATCCTCTTCGTGACATC, and primer hCLCB7, CTCGACATCCTCTTCGTGACATC, and primer hCLCB7, CTCGACATCCTCTTCGTGACATC, and primer hCLCB7, CTCGACATCCTCTTCGTGACATC). This DNA fragment was subcloned into the pGEM-T Easy Vector System (Promega, Madison, WI) by the TA-cloning technique. Using this plasmid as a template, the 11-kbp human CLC-KB gene promoter (hKBP) spanning from the end of the CLC-KA gene to just before the translation start site of the CLC-KB gene was amplified by LA-PCR and inserted into the HindIII-EcoRI site of the above mentioned pBluescript II-KN-EGFP. (Both of the primers used in this PCR reaction contained restriction enzyme sequences at their 5′ ends: hKBP pr 5, CCAAGCTTGGCAGTCCTCTCTCGTACATC; hKBP pr 6, CGGAATTCGGGCCCCTCTGGGAGAAGCGGATT.) All cloning junctions were confirmed by sequence analysis. The transgene segment of the final plasmid was excised by digestion with NotI, isolated from the remainder of the plasmid backbone with the Prep-A-Gene DNA Purification System (Bio-Rad, Hercules, CA), and further purified by the QIAGEN-tip 20 plasmid kit (Qiagen, Valencia, CA). The transgene was microinjected into one-cell fertilized mouse embryos of C57BL/6 mice using standard procedures. Founder transgenic mice were determined by PCR and Southern blot analyses, and offspring were genotyped by PCR.

Southern Blot Analyses

Genomic DNA was purified from tail biopsies and subjected to Southern blot analyses or PCR. For Southern blotting, 20 μg of genomic DNA was digested with EcoRI and probed with EGFP cDNA. There is a unique EcoRI site in the transgene; a 12-kbp band was therefore diagnostic of its presence.

Polymerase Chain Reaction

The primers used to screen DNA from tail biopsies for the presence of EGFP were located within the EGFP structural sequence. The upper primer was GAGCTAAAGGGCCACACAGTT, the lower primer was CCCTTCAGCTCGATGCGGT, and the resulting amplification product was 320 bp.

Immunoblot Analyses

Tissues of transgenic mice and wild-type mice were homogenized in RIPA buffer (1 × phosphate-buffered saline [PBS], 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]), and protein contents were determined by the Bradford method (Bio-Rad). Twenty micrograms of proteins were applied to SDS-PAGE and blotted onto a nitrocellulose membrane. After blocking, the membrane was incubated at room temperature for 1 h with a monoclonal antibody against the EGFP protein at a dilution of 1:1000 (JL-8, Clonetech). After several washings, a secondary antibody (anti-mouse IgG HRP-conjugated; DAKO, Glostrup, Denmark) at a dilution of 1:100 was applied to the membrane and incubated at room temperature for 1 h. The membrane was then washed several more times and examined by the ECL Western Blotting Detection System (Amersham Pharmacia Biotech, Buckinghamshire, England).

Detection of EGFP Fluorescence

Animals were anesthetized with diethyl ether. Kidneys were removed after in vitro perfusion with 4% paraformaldehyde in a phosphate buffer and immersed overnight in the same fixative at 4°C. The fixed tissue was cryoprotected by immersion in 20% sucrose in PBS at 4°C and then shock-frozen in liquid nitrogen. Frozen 10-μm sections were cut with a cryostat, thaw-settled on APS-coated slides, mounted with an aqueous mounting medium (Mount-Quick “Aqueous”; Daido Sangyo, Tokyo, Japan), and examined under a confocal laser microscope (Carl Zeiss Japan, Tokyo, Japan).

To prepare for EGFP fluorescence detection in the inner ear, transgenic mice were transcardinally perfused with 10% neutral buffered formalin, and the inner ears were removed, opened, and postfixed in the same fixative for 2 h at RT. The tissue was decalcified in 5% EDTA (pH 7.3) at 4°C for 1 wk, embedded in paraffin, sliced into 8-μm-thick sections, and examined under a confocal laser microscope. Sections of the inner ear corresponding to the EGFP fluorescence microscopy were also stained with hematoxylin and eosin for histologic orientation.

Immunohistochemical Analyses

Frozen sections prepared in the manner described above were used for immunohistochemistry. The primary antibodies included (1) anti-AQP1 antibody (Chemicon International Inc. Temecula, CA), (2) anti-AQP2 antibody (13), (3) anti-NKCC2 (bumetamide-sensitive Na+K+/2Cl− co-transporter) antibody (11), (4) anti-TSC (thiazide-sensitive Na+/Cl− co-transporter) antibody (12), and (5) anti-NCX (Na+/Ca2+ exchanger) antibody (Affinity Bioreagents, Inc. Golden, CO), all of which had been generated in rabbits except the anti-NCX antibody, which was a mouse IgM monoclonal antibody. The anti-
NKCC2 antibody and anti-TSC antibody are a generous gift from Dr. Steven C. Hebert. The secondary antibody for the anti-AQP1 antibody, anti-AQP2 antibody, anti-NKCC2 antibody, and anti-TSC antibody was an anti-rabbit IgG Cy3-conjugated antibody (Sigma, St. Louis, MO), and the secondary antibody for the anti-NCX antibody was an anti-mouse IgM rhodamine-conjugated antibody (ICN Pharmaceuticals, Inc. Aurora, OH). After blocking, frozen sections were incubated at room temperature for 1 h with the anti-AQP1, anti-AQP2, anti-NKCC2, and anti-TSC antibodies at a dilution of 1:100, and at room temperature for 2 h with the anti-NCX antibody at a dilution of 1:50. Sections were then washed several times and incubated at room temperature for 1 h with the secondary antibodies at a dilution of 1:200. After more washing, the slides were mounted and examined under a confocal laser microscope.

Results

As illustrated in Figure 1, an 11-kbp human CLC-KB gene promoter including intron 1 was fused to the EGFP cDNA before the SV40 polyA signal sequence. After selecting 16 mice by PCR analysis out of 76 live-born offspring, we used Southern blot analyses to confirm that 10 of the 16 selected mice conveyed the transgene (Figure 2). Finally, only three of the ten founder transgenic mice were successfully bred to establish transgenic lines and show the EGFP expression (6–4, 4–4, and 5–4 in Figure 2). The patterns of EGFP expression in these three mice were almost identical.

As shown in Figure 3A, the immunoblots that used an antibody against the EGFP protein clearly demonstrated that EGFP was vigorously expressed in the transgenic mouse kidney, whereas no specific signal was found in the wild type. In immunoblots of various tissues of the transgenic mouse, it was interesting to note that, in addition to the band for the kidney, a weak but clear band was seen in the lane for the brain and a very weak band was seen in the lane for the heart (Figure 3B).

The EGFP is a GFP chromophore variant that produces a bright green light when illuminated by blue light. It does not require any cofactors, substrates, or additional gene products from Aequorea Victoria, and it remains functional even in fixed frozen sections (Clontech). Fluorescence microscopy of the transgenic mouse kidney provided high-contrast images of the EGFP fluorescence with minimum background (Figure 4, A through C). As shown in Figures 4A through 4C, the EGFP fluorescence was present in the cortex and outer medulla but absent from the inner medulla. A more detailed inspection revealed that the fluorescence was localized in the cytoplasm of the tubule cells along the distal nephron segments. Blood vessels and interstitial cells were not positive for the EGFP fluorescence.

To determine the exact intrarenal distribution of the EGFP fluorescence, we stained the transgenic mouse kidney with the antibodies against several channels and transporters. Figure 5A shows that EGFP-expressing tubules were not stained by AQP1, which suggests that EGFP was not present in the proximal tubules or the thin descending limb of Henle’s loop. In contrast, Figures 5B through 5D demonstrate that EGFP-positive tubules were also positive for NKCC2, TSC, and NCX, which indicates that EGFP was co-expressed with NKCC2 in the thick ascending limb of Henle’s loop, TSC in the distal tubule, and NCX in the connecting tubules. Figure 5E further shows that the EGFP fluorescence was absent from the AQP2-positive principal cells of the collecting duct but present in the intercalated cells. As a whole, these results demonstrate

![Figure 2. Southern blot analyses of the founder transgenic mice. Genomic DNA was digested with EcoRI and probed with the EGFP cDNA. The 12-kbp diagnostic band is indicated with an arrow, and the final three founder transgenic lines are designated with their temporary names (6–4, 4–4, and 5–4).](image)

![Figure 3. Immunoblot analyses of transgenic and wild-type mice. (A) Whole kidney proteins from representative transgenic and wild-type mice were fractionated by SDS-PAGE, transferred onto a membrane, and probed for EGFP by immunoblotting using the monoclonal antibody generated against the EGFP protein. The 27-kD EGFP band is indicated with an arrow. Open arrows indicate nonspecific bands. T, transgenic; WT, wild type. (B) Tissue homogenates from various tissues of transgenic mice were probed for EGFP by immunoblotting using the monoclonal antibody generated against the EGFP protein. The 27-kD EGFP band is indicated with an arrow. Open arrows indicate nonspecific bands. K, kidney; L, liver; Sp, spleen; H, heart; P, lung; St, stomach; SI, small intestine; LI, large intestine; B, brain.](image)
that EGFP was expressed in exactly the same distal nephron segments in which mouse CLC-K2 is expressed, namely, the thick ascending limb of Henle’s loop, the distal tubule, the connecting tubule, and intercalated cells of the collecting duct.

Previous reports taught us that at least one of the CLC-K chloride channels was present in the inner ear, more specifically, in marginal cells of the stria vascularis and dark cells of the vestibular labyrinth (14,15). Our EGFP fluorescence image of the inner ear of the transgenic mouse demonstrated a localization identical to that shown in the previous reports, indicating that CLC-KB was expressed in the inner ear (Figure 6, A through D).

**Discussion**

We have demonstrated that an 11-kbp of the human CLC-KB gene promoter can faithfully target the reporter gene expression in the distal nephron segments of transgenic mice in a manner identical to mouse CLC-K2.
Before we started making our transgenic mice, we needed to decide on a suitable promoter sequence length that would optimize our chances of obtaining satisfactory results. A 1.5-kbp upstream sequence is already known to be insufficient for the precise expression (6), and comparison of the human genomic sequences of CLC-KB and CLC-KA genes tells us that approximately 2 kbp of 5' flanking sequences are well preserved in these two genes. From these data, we decided that a promoter region longer than 2 kbp should be included in the transgene. As the first intron is known to frequently contain enhancers, we used the 11-kbp promoter sequence of the human CLC-KB gene, a sequence that includes the first intron. Our transgenic mice clearly demonstrated that this 11-kbp promoter sequence reproduced the tissue-specificity of the human CLC-KB gene expression, thereby suggesting that essential cis-elements are included within this promoter region.

Due to the extremely high homology among the CLC-K channels, there has been controversy about the correlation of mouse and rat CLC-K2 and CLC-K1 to their human counterparts. In our transgenic mice, the human CLC-KB promoter directed the EGFP expression to exactly the same distal nephron segments as mouse CLC-K2. Although the actual intrarenal localization of CLC-KB in the human kidney is still unknown, we tentatively conclude that CLC-KB is the true human homologue of CLC-K2. Mutations of the human CLC-KB gene have been shown to cause Bartter syndrome type III (1), and knowledge of the intrarenal localization of this gene product should shed light on the pathophysiology.

Aside from the kidney, the inner ear is the only other part of the body where potential CLC-KB (CLC-K2) expression has been noted. Targeting our investigation to the inner ear, we detected the same pattern of EGFP fluorescence in the inner ear that had been demonstrated in the previous studies (14,15). Our results strongly suggested that CLC-KB is expressed in the inner ear, but it is unclear whether the same can be said for CLC-KA. On the other hand, our immunoblot studies showed that our transgenic mice expressed a significant amount of the EGFP protein in the brain and a small amount in the heart. Although our fluorescence microscopy studies verified EGFP expression in the brain and its localization to the medullary reticular formation (data not shown), we were unfortunately unable to detect the endogenous CLC-K2 expression in the

![Figure 5](image-url) Figure 5. Nephron segment-specific expression of EGFP in the transgenic mouse kidney. Immunohistochemical analyses of the transgenic mouse kidney were performed with antibodies against AQP1, bumetamide-sensitive Na⁺/K⁺/2Cl⁻ co-transporter (NKCC2), thiazide-sensitive Na⁺/Cl⁻ co-transporter (TSC), Na⁺/Ca²⁺ exchanger (NCX), or AQP2. (A) AQP1; magnification, × 400. (B) NKCC2; magnification, ×400. (C) TSC; magnification, ×400. (D) NCX; magnification, ×400. (E) AQP2; magnification, ×400. In all pictures, green fluorescence indicates EGFP and red fluorescence indicates the channels or transporters employed.
bodies of these neurons by immunohistochemical analyses. Although our fluorescence microscopy failed to detect any EGFP expression in the heart (data not shown), there must be a very low expression of CLC-KB in the heart, because the immunoblot analysis is a more sensitive method for detecting protein expression than fluorescence microscopy. The physiologic significance of CLC-KB in brain and heart remains to be elucidated in future studies.

In summary, we generated transgenic mice with reporter gene expression under the control of the 11-kbp human CLC-KB gene promoter and showed that this promoter provided a nephron segment–specific pattern of expression identical to that of mouse and rat CLC-K2. Using this promoter and the Cre/loxP technology, it is now possible to generate transgenic mice in which specific genes are overexpressed or deleted in the distal nephron segments.

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References

Figure 6. EGFP fluorescence in the inner ear. EGFP fluorescence appeared in only restricted cell-types in the inner ear. (A and B) stria vascularis; magnification, ×200. Arrows indicate the marginal cells. (C and D) vestibular labyrinth; magnification, ×200. Arrows indicate the dark cells, and arrowheads indicate the crista ampullaris. Panels B and D are H&E-stained.


