Alternatively Used Promoters and Distinct Elements Direct Tissue-Specific Expression of Nephrin

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Abstract. Nephrin, an essential component of the glomerular ultrafilter, the slit diaphragm, has also been found to be expressed in the central nervous system and pancreas. This study examined the regulation of the nephrin gene by analyzing the expression of different length nephrin promoter–lacZ reporter constructs in transgenic mice. An upstream segment between −4 kb and −4 bp was shown to be sufficient for driving expression in all three tissues. Surprisingly, a 5.7-kb construct lacking the transcription initiation site and the immediate upstream region of the gene could drive expression in the central nervous system. This led to the identification of a novel, alternatively used exon 1B located 1871 bp upstream of the ATG codon of the previously known first exon, now termed exon 1A. The existence and functionality of exon 1B was verified in nephrin knockout mice in which exon 1A is deleted. Deletion of exon 1B and its immediate surrounding sequence, introduced in the 4-kb promoter–lacZ reporter construct, abolished the expression of the transgene in pancreas and spinal cord but not in kidney and brain in transgenic mice. Analysis of five promoter–reporter gene constructs showed that regulatory elements driving expression encoded by exon 1A in kidney and brain are localized in the region between −4 kb and 2.1 kb.

Nephrin is a recently identified protein, central component of the slit diaphragm, which has a major role for the filtration of the plasma. Mutations in the human nephrin gene result in absence of the slit diaphragm, massive proteinuria, and a lethal disorder termed congenital nephrotic syndrome of the Finnish type (CNF) (1). A similar perinatally lethal phenotype has been described in nephrin-deficient mice (2).

Nephrin was the first molecule to be localized to the slit diaphragm (3). The structure of this primary size-selective filter in the glomerulus has been poorly understood, but significant progress has been made recently in identifying and characterizing its components (4). Nephrin has been proposed to form a zipper-like filter structure, which prevents molecules of the size of albumin and larger to penetrate the filter (4). Recently other proteins such as ZO-1, synaptopodin, p-cadherin, CD2AP, podocin, and FAT have been shown to localize in the slit diaphragm region (5–10).

In addition to the kidney, nephrin expression has been observed in the central nervous system, developing spinal cord, cerebellum, mesencephalon, and olfactory bulb (2,11). The presence of nephrin in brain and spinal cord generated a lot of questions regarding its extrarenal functions. Speculations about its involvement in the neurologic symptoms, observed in some CNF patients, have been made (11). Another extrarenal site of nephrin expression is the insulin-producing β-cells of the pancreas (2,12).

The complex pattern of tissue and cell lineage–specific expression (11) and the existence of splice variants (13,14) imply complex regulation of the nephrin gene. Studies on the mouse promoter in transgenic mice have shown that a 4.1-kb segment of the upstream region in the mouse gene can drive expression in both kidney and brain (15), whereas no expression was observed in the pancreas of these animals. In the case of the human promoter, a considerably shorter upstream sequence of 1.25 kb was found to drive expression of a reporter gene in podocytes of transgenic mice, but no data were presented on extrarenal expression with that construct (16).

In the present study, we have examined the regulation of the mouse nephrin gene using a variety of promoter constructs in transgenic mice. The purpose of this work was to try to determine the transcription mechanisms involved in the tissue-specific expression of the gene.

Materials and Methods

Generation and Analysis of Transgenic Mice

Constructs were generated using a SpeI/SalI mouse genomic clone containing 6.2 kb from the 5' upstream region of the nephrin gene and part of the coding sequence until exon 2. Four of the constructs

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RNA Isolation, 5' RACE, and RT-PCR

Total RNA was isolated from mouse kidney and cerebellum using the Trizol reagent (Life Technologies). Identification of the transcription start site was performed using a SMART RACE cDNA amplification kit (Clontech) and gene-specific primers 5’ CAGAAGCGACGACGACTCATCCCTTAGCG3’ and 5’ACTGTGCTTCCTGCTGCTCA3’ from exons 2 and 4, respectively. The PCR products were cloned with Topo TA cloning kit (Invitrogen) and sequenced using an ABI 310 automatic sequencer (Applied Biosystems).

Analyses of cDNA and Amino Acid Sequences

Homology searches were performed using FASTA (Genetics Computer Group) and BLAST (GenBank). For signal peptide predictions, SPScan program (Genetics Computer Group) and SIGFIND (19) database were used. Mouse/human homology searches and analysis for putative transcription factor recognition sites were performed using mVISTA and rVISTA scan, respectively (20–22).

Immunohistochemical Analyses

Newborn, nephrin knockout (n = 4), and wild-type and heterozygous littersmates (n = 12) were perfused transcardially under ether anesthesia with 1 ml of Tyrode Ca²⁺ -free solution at 37°C followed by perfusion with 2 ml of an ice-cold mixture of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (pH 6.9) at 37°C. After perfusion, the bodies were immersed in the same fixative for 60 min and for 60 more min after opening the brain surface. The

Figure 1. 5' upstream region of mouse nephrin and constructs used for expression studies in transgenic mice. (Top) Schematic illustration of the 5' end and upstream region of the gene. Boxes represent alternatively used first exons 1A and 1B as well as exon 2. Locations of restrictions sites used for cloning are indicated. (Middle) Four lacZ reporter gene constructs, all having the same 3'-end of the nephrin gene starting at # -4 bp, as counted from the translation initiation codon of the renal cDNA. (Bottom) Nephrin promoter constructs with its 3'-end starting at position # -546 upstream of the translation initiation site. (Side) The expression pattern of the five constructs in transgenic mice is depicted to the right of the construct schemes; k, kidney; b, brain; s, spinal cord; p, pancreas. The number of independent mouse lines generated with each construct is shown in parentheses on the right.
brains were washed in 10% sucrose in 0.1 M phosphate buffer (pH 7.4) overnight and then snap-frozen using CO₂. Fourteen-micrometer thick coronal brain sections were cut on a cryostat (Microm, Heidelberg, Germany) and thaw-mounted on chrome alum-gelatin–coated glass slides. Immunohistochemical analyses were performed using the tyramine signal amplification (TSA) (23). A polyclonal rabbit anti-mouse antiserum (1:200) raised against the intracellular part of nephrin (2) was applied to the sections overnight at 4°C, followed by incubation with horseradish-peroxidase-conjugated swine anti-rabbit IgG (1:100; Dako A/S, Copenhagen, Denmark) and components of the TSA-Plus Fluorescein System (DuPont, New England Nuclear, Boston, MA). Sections were mounted in a mixture of glycerol and 0.1 M phosphate-buffered saline (3:1), pH 7.4, containing 0.1% 4-phenylenediamine (Sigma Chemicals, St. Lois, MO), and examined by Bio-Rad Radiance Plus (Bio-Rad, Hemel Hemstead, UK) confocal laser scanning microscope installed on a Nikon (Nikon, Tokyo, Japan) Eclipse E600 fluorescence microscope. Digital images resulting from the confocal scanning microscopy were optimized for image resolution. The specificity of the antibodies was tested by preabsorption tests with an excess (10⁻⁶ M) of nephrin protein.

Results

To localize cis-regulatory elements responsible for the tissue-specific expression of nephrin, reporter gene constructs containing most of the 5' UTR and different length segments of the upstream region were expressed in transgenic mice. One construct devoid of 546 bp containing the 5' UTR and the immediate upstream region was made as negative control. Two to four mouse founder lines were generated with each of the constructs. A diagram of the constructs and the reporter gene expression pattern achieved with each of them is shown on Figure 1.

General Localization of Tissue-Specific Enhancers in the Mouse Nephrin Gene

Two constructs containing 6242 (nephA) and 4013 bp (nephB) of the upstream region and the 5' UTR (with the exception of bases −1 to −3), were strongly expressed in podocytes (Figure 2A) and central nervous system (data not shown). Weak lacZ staining was also observed in pancreas (Figure 2B). Essentially the same expression pattern has been described for the endogenous nephrin gene (2). A third construct (nephC), containing 2148 bp of the upstream region and the 5' UTR, did not yield any expression, indicating that the region between −2148 and −4013 contains cell lineage–specific enhancer elements. When a 700-bp segment between bases −1249 and −1908 was deleted from construct nephB (nephB−Δ700), expression was observed only in podocytes (not shown) and brain (Figure 2C), but not in the spinal cord (Figure 2C) and pancreas (not shown).

Identification of a Novel Exon–Exon 1B

Two mouse founder lines were made with the negative control construct nephA−Δ500. This construct lacked the 5' UTR, including the transcription initiation site and part of the immediate promoter region; therefore, no expression was expected. Surprisingly, the transgenic embryos showed strong lacZ staining in the central nervous system (Figure 2D) similar to that shown for the native nephrin promoter (2). No staining was observed in kidney or pancreas of mice containing this construct (not shown).

The highly tissue-specific expression from the negative control construct, nephA−Δ500, led us to believe that there might be an alternative transcription start site, and a novel exon(s) located upstream from the main transcription initiation site. To clarify this, we determined the 5' end of both kidney and cerebellar mRNA by 5' RACE. In the case of kidney mRNA, we found that the transcription start site was at position −381 bp, as counted from the translation initiation codon (ATG) in the cDNA (data not shown). The 5' RACE of mRNA from cerebellum yielded different results. In this case, the analysis revealed an alternative 5' end at position −1871 and, respectively, of a novel exon 1490 bp upstream from the transcription initiation site shown for kidney mRNA. The new exon (designated exon 1B) was spliced together with exons 2 through 29, with the sequence of exon 1 (from now on exon 1A) being absent from the cDNA (Figure 3). The sequence of exon 1B is shown in Figure 4. It is 271 bp in size and has a typical GT-donor site at its 3'-end. For both kidney and cerebellar nephrin isoforms, the first nucleotides from the cDNA are in agreement with the requirements for transcriptional initiation in mammalian cells (24). The ATG codon of the alternative exon was found to fit the Kozak consensus sequence (RNNatgG where R is purine) (25).

The alternatively used exons 1A and 1B give rise to different
sequences in the amino-terminus of the nephrin protein (Figure 4). As previously published (11), exon 1A encodes a typical signal peptide sequence. In contrast, analysis of the amino-terminus encoded by exon 1B, performed with SPScan (Genetics Computer Group) and SIGFIND (19), did not reveal a signal peptide cleavage site.

To examine if the novel nephrin isoform is possibly specific for rodents, we searched for the exon 1B sequence in the upstream region of the human nephrin gene. A BLAST search carried out with the public database yielded the presence of such a tentative exon about 3.7 kb upstream of the ATG translation initiation codon in exon 1A (GenBank accession no. AC002133). Similar results were achieved with BlastN search in Celera database (GA_x54KRE9Q4RC). The possible N-terminal sequence of this putative human brain–specific isoform is shown on Figure 4. Thus, the human nephrin gene is also likely to generate the two nephrin isoforms described here for the mouse, but so far there are no reports on the presence of nephrin in human brain.

Evidence for Central Nervous System Specificity of the Nephrin Isoform Encoded by Exon 1B

The identification of this new splice isoform of nephrin prompted us to reconsider the situation with the nephrin knock-out mice previously created in our laboratory. As described elsewhere, the nephrin knockout was generated by disruption of exon 1A (2). Neither nephrin mRNA nor nephrin protein were observed in the kidneys of the −/− mice. However, on the basis of the results obtained in the present study, one could expect that the knockout animals still express nephrin in the brain through the use of exon 1B. To explore this, we performed immunohistochemical staining of brain sections from newborn −/− mice using antiserum raised against the intracellular part of the mouse nephrin (2). At the light microscopy level, this did indeed yield quite similar distribution of immunoreactivity in the brains of the knockout mice as observed in their wild-type or heterozygous littermates (Figure 5). In the cerebral cortex, a moderate, diffuse signal, most prominent in the cortical lamina I of the cingulate and retrosplenial cortical areas was observed (Figure 5, A through C). Weak staining was seen in the deeper cortical layers, apparently in processes running perpendicularly to the surface. Immunostaining was also observed in the choroid plexus, particularly in the apical parts of the epithelial cells (Figure 5D). The strongest staining was found in the cells located in the pia and arachnoid mater.

Figure 4. Nucleotide sequence of mouse exon 1B. The exon sequence is shown with capital letters. The box depicts the transcription initiation site as determined by the cDNA analyses. The putative ATG translation initiation codon is underlined, and the gt donor splice site is shown in small underlined letters. The putative cDNA-derived mouse protein sequence is shown under the nucleotide sequence. The alternative amino acid sequence shows the putative N-terminus of the human analogue based on homology between the mouse exon 1B and the human genomic sequence (GenBank accession no. AC002133).
A moderate signal was observed in the hypothalamus: in the fornix and the presumable axons of the magnocellular neurons terminating in the posterior pituitary (Figure 5G). A preadsorption test of the antiserum with nephrin protein entirely abolished the nephrin immunoreactivity (Figure 5H). Immunostaining of knockout kidneys was used as a control and showed no signal for nephrin (data not shown).

**Identification of Homologous Regions in the Promoter Sequences of Mouse and Human**

A sequence alignment of the nephB construct against 7.9 kb from the upstream and 5' UTR sequence of the human nephrin gene. Only regions with homology higher than 50% are shown on the plot, with sequences reaching above 75% homology being in dark color. The five regions with high degree of homology are designated with roman numbers I to V. (Middle) Schematic presentation of the nephB construct containing exon 1B and the 5' UTR of exon 1A. The region deleted in construct nephB-Δ700 is shown with dotted line. The arrows represent the ATG codons. (Bottom) schematic presentation of the 7.9-kb upstream region of the human nephrin, which was used for the homology search. Exon 1 is presented with a box. Again, the arrow shows the position of the ATG codon. The regions I through V, found to have more than 75% homology with the mouse sequence, are presented with darker boxes.

(Figure 5, E and F). A moderate signal was observed in the hypothalamus: in the fornix and the presumable axons of the magnocellular neurons terminating in the posterior pituitary.
Discussion

The present work provides new information on the regulation and expression of the mouse nephrin gene based on studies in transgenic mice. Thus, a novel alternatively used first exon, 1B, was identified upstream of exon 1. The mRNA initiated from exon 1B was shown to be expressed in the central nervous system. This novel product had a similar expression pattern in the brain as the product of exon 1A. The biologic role of the two alternative nephrin isoforms in the brain is still obscure. Additionally, the expression pattern of different promoter-reporter gene constructs indicated that the regulatory elements of the mouse nephrin gene driving expression encoded by exon 1A in kidney and brain are localized in the region between −4 kb and −2.1 kb.

Our results with nephB-Δ700 construct showing strong kidney and brain expression indicate that an important podocyte-specific enhancer element is located in the region between −4 kb and −2.1 kb. The strong sequence homology between the mouse and the human genes in this region revealed in the VISTA-plot supports this notion (Figure 6). This is in agreement with the previous work of Moeller et al. (15) and Eremina et al. (16), who localized such elements to upstream gene regions smaller than −5.4 kb and −4.1 kb, respectively. However, in a more recent article, Moeller et al. (26) showed evidence for weak kidney activity in a construct containing 1.25 kb from the upstream region of the mouse nephrin gene. This contradicts the present study, as a construct containing 2.15 kb did not reveal expression of the reporter gene in podocytes or any other tissues normally expressing nephrin in four independent mouse lines. These results could mean that the 1.25-kb upstream region only has a weak kidney enhancer that is inhibited by suppressor elements in the region between −2.15 kb and −1.25 kb, while other significant podocyte-specific regulatory elements reside in the region −4 kb to −2.1 kb. This hypothesis could explain the peculiar results we obtained with a 1214-bp promoter construct (unpublished data). We observed podocyte staining in one of two founder lines generated with that construct. However, the mice from both lines also had clearly ectopic, extrarenal lacZ staining in tissues such as brain, cartilage, and bone. This may be due to an integration site-dependent influence over the weak podocyte-specific elements contained in this fragment.

Another conclusion that can be drawn when comparing the results obtained with constructs nephB and nephB-Δ700 is that important regulatory elements, required for expression in pancreas and spinal cord, reside in the region between −1249 and −1908. Previous reports on studies in transgenic mice with lacZ expression constructs similar to nephB or longer did not report on expression in the pancreas. This can probably be explained by the extremely weak and difficult to observe expression, rather than by difference in the sequence of the constructs used in these studies.

The observation that construct nephA-Δ500 lacking the minimum promoter and the 5′ untranslated region was expressed strongly in brain and spinal cord was intriguing, and it led us to identify the novel alternative exon 1B. The existence of this isoform was nicely demonstrated in our nephrin knockout mice, where exon 1A was deleted and exon 1B was clearly functional. We thus observed nephrin in the brain by immunostaining, although the kidneys remained completely negative for both mRNA and the nephrin protein (2). RT-PCR also showed that exon 1A is expressed in both kidney and brain, whereas exon 1B is brain-specific (data not shown).

Interestingly, it was shown here for the first time that nephrin is expressed in brain choroid plexus. This was observed for both nephrin −/− and wild-type mice (Figure 5, E and G). Similar results were obtained for rat brains (data not shown). Choroid plexus is involved in the blood filtration in the blood/brain barrier, and nephrin has been shown to be essential for the filtration in kidney; therefore, one could speculate about a possible role of nephrin for this process in brain.

The biologic role and exact location of the two nephrin isoforms in the brain remain to be clarified. Although exon 1A codes for a typical signal peptide, analysis of the sequence encoded by exon 1B did not reveal a possible signal peptide nature. Searches in the Celera and public databases showed no homology of the alternative nephrin isoform to previously characterized sequences. Light microscopy immunohistochemical analyses of both exon 1A and exon 1B isoforms in the brain have not revealed any clear differences in location. Immunoelectron microscopic studies are being performed to shed more light on the location of the two isoforms in the brain.

Whatever the function of the two isoforms in the brain, it is clear that the renal isoform of nephrin is essential for life. Whether or not the exon 1B isoform has some important function in the brain remains to be found. One future study attempting to solve this question should include deletion of both exons 1A and 1B.

An extensive body of data has been accumulated on transcription factors and signaling pathways involved in the development of kidney podocytes, and the involvement of some of them in nephrin expression has been studied. For example, Podl and LMX1B, known to play role for podocyte differentiation, have been shown to have no effect on nephrin expression (16,28). Another transcription factor with a major role in kidney development and podocyte differentiation is WT1. Although no induction of nephrin expression was observed in rat embryonic kidney cells transfected with inducible WT1 (29), a more recent study shows that absence or even reduction of WT1 expression causes decrease of the nephrin expression or completely abolishes it (30). These results show that WT1 may have important role for regulation of the nephrin expression but requires activation of other transcription factors. Although the current knowledge about actual transacting factors controlling nephrin gene expression is limited, it is of great interest that modulation of the renin/angiotensin system and protein kinase C have been shown to affect regulation of the nephrin gene in kidney cells (31,32).

Thorough studies on the regulatory mechanisms of the nephrin gene may have clinical importance and may help to elucidate the role of nephrin for podocyte differentiation and its function in central nervous system. Our data show that the nephrin gene expression is controlled by tissue-specific elements in the region −4 kb to −2.1 kb and alternative splicing...
in the 5’ end of the gene. The present work gives a basis for future detailed studies on the transcription factors involved in the regulation of the nephrin gene.

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