Multiple Promoters in the WNK1 Gene: One Controls Expression of a Kidney-Specific Kinase-Defective Isoform

Celine Delaloy, Jingyu Lu,[†] Anne-Marie Houot, Sandra Disse-Nicodeme, Jean-Marie Gasc, Pierre Corvol, and Xavier Jeunemaitre*

INSERM U36, College de France, 75005 Paris, France

Received 5 May 2003/Returned for modification 17 June 2003/Accepted 23 September 2003

WNK1 is a serine-threonine kinase, the expression of which is affected in pseudohypoaldosteronism type II, a Mendelian form of arterial hypertension. We characterized human WNK1 transcripts to determine the molecular mechanisms governing WNK1 expression. We report the presence of two promoters generating two WNK1 isoforms with a complete kinase domain. Further variations are achieved by the use of two polyadenylation sites and tissue-specific splicing. We also determined the structure of a kidney-specific isoform regulated by a third promoter and starting at a novel exon. This transcript is kinase defective and has a predominant expression in the kidney compared to the other WNK1 isoforms, with, furthermore, a highly restricted expression profile in the distal convoluted tubule. We confirmed that the ubiquitous and kidney-specific promoters are functional in several cells lines and identified core promoters and regulatory elements. In particular, a strong enhancer element upstream from the kidney-specific exon seems specific to renal epithelial cells. Thus, control of human WNK1 gene expression of kinase-active or -deficient isoforms is mediated predominantly through the use of multiple transcription initiation sites and tissue-specific regulatory elements.

A new family of serine-threonine kinases was recently described. The members of this family lack a lysine at a usually invariant position in the active site and are therefore known as With No Lysine (WNK) protein kinases (17, 20). Rat WNK1 was the first member of this family to be characterized; it has a cysteine in place of the conserved lysine residue in subdomain II of the catalytic domain (20). The active lysine is itself located in subdomain I in both rats and humans. In both species, WNK1 is expressed in a wide variety of tissues, and two major transcripts have been identified. One is produced mainly in heart, muscle, and brain, and the other, shorter transcript is produced mainly in kidney (18, 20). The substrates of WNK1 are unknown, but WNK1 is capable of autophosphorylation on serine residues, an activity that is increased in vitro by increasing the salt concentration (20). Like many other protein kinases, WNK1 enzymes contain an autoinhibitory domain outside the catalytic domain, which is capable of abolishing kinase activity in vitro (21). There is also evidence that the autophosphorylation sites detected in the activation loop of WNK1 may control kinase activity (21).

Mutations in the genes encoding WNK1 and WNK4, two of the other four members of the human WNK family (17), are responsible for pseudohypoaldosteronism type II (PHA2), also known as Gordon syndrome, an autosomal dominant form of human arterial hypertension associated with hyperkalaemia and metabolic acidosis with hyperchloraemia (5). The mutations in the WNK4 gene are missense

mutations clustering in highly conserved domains close to those encoding the coiled-coil domains (18). The location and nature of these mutations suggest that they may result in changes in interactions with as-yet-unidentified protein partners. The clinical and biochemical abnormalities observed in PHA2 (1, 4, 5) and the presence of WNK1 and WNK4 in the distal convoluted tubule and cortical collecting duct of the nephron (18) suggest that these kinases may be involved in the signaling pathways controlling ion reabsorption. Indeed, WNK1 is produced in several epithelia that are involved in chloride reabsorption (2). In vitro, the transient production of WNK4 in *Xenopus* oocytes was recently shown to decrease the sodium flux mediated by the thiazide-sensitive Na-Cl cotransporter (NCC) (19, 23), while WNK1 prevents WNK4 inhibition of the NCC (23).

The human WNK1 gene extends over more than 150 kb, contains 28 exons, and generates two transcripts of approximately 10 and 12 kb. The mutations in the WNK1 gene responsible for PHA2 are large deletions in the first intron that result in higher levels of expression of the gene in the leukocytes of affected individuals (18). This suggests that particular sequences in intron 1 play a critical role in controlling the ubiquitous and tissue-specific expression of the WNK1 gene. To date, nothing is known about the molecular structures of the different WNK1 isoforms and their possible roles in human disease. A kidney-specific alternative splicing event between exons 4 and 5 was identified in a genome-wide analysis of expressed sequence tags, suggesting possible disruption of the kinase domain in the resulting isoform (22). The objectives of this study were therefore to characterize the pattern of WNK1 gene expression, to determine the molecular structures of the human gene transcripts, to compare human and mouse sequences and gene expression, and to characterize the promot-

* Corresponding author. Mailing address: INSERM U36, College de France, 11, place Marcelin Berthelot, 75005 Paris, France. Phone: 33 (0)1 44 27 16 55. Fax: 33 (0)1 44 27 16 91. E-mail: xavier.jeunemaitre@college-de-france.fr.

[†] Present address: Department of Medical Genetics, China Medical University, Shenyang, China.

ers of the human WNK1 gene, as a first step towards a molecular understanding of PHA2.

MATERIALS AND METHODS

Cells and cell culture. All cell culture reagents were purchased from Gibco BRL-Life Technologies. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 nutrient mixture, human embryonic kidney (HEK 293) cells were grown in Dulbecco's modified Eagle medium, and Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle medium with Glutamax, supplemented with 1% nonessential amino acids. All cell culture media were supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin.

DNA sequence analysis. Nucleic acid sequencing was carried out with the Prism AmpliTaq FS dichlororhodamine dye terminator kit (Perkin-Elmer Applied Biosystems) and an automated sequencer (ABI Prism 377).

Northern blot analysis. Poly(A)⁺ RNA was purified from confluent monolayers of HEK 293, MDCK, and CHO cells by using the RNeasy kit and Oligotex (Qiagen). Fifteen micrograms of RNA was electrophoresed on denaturing formaldehyde gel and transferred to a nylon filter (Hybond-N). Blots of RNA from human and mouse tissues, containing 2 µg of poly(A)⁺ RNA per lane, were purchased from BD Clontech. [α -³²P]dCTP-labeled random-primed (RadPrime DNA labeling system; Invitrogen) 0.2- to 0.6-kb PCR fragments of the human or mouse WNK1-coding regions were used as probes, and hybridization was performed according to the manufacturer's instructions with ExpressHyb hybridization solution (BD Clontech). After probing, the filters were washed under standard conditions and placed against X-ray film for 1 to 7 days (Biomax film; Kodak).

RNA isolation and primer extension analysis. Total RNA was isolated from human kidney cells and leukocytes by the procedure of Chomczynski and Sacchi (3). Primer extension was carried out as previously described (10) with a radio-labeled antisense sequence corresponding to the end of exon 2, with yeast tRNA used as a negative control.

5' rapid amplification of cDNA ends (5'RACE) assay. Human heart and kidney Marathon-Ready cDNA amplification kits were purchased from BD Clontech and were used according to the manufacturer's instructions. Reverse primers binding to exon 1, 2, or 6 were used. Nested PCR products were analyzed by agarose gel electrophoresis. Major bands were excised from the gel, purified (QIAquick gel extraction kit; Qiagen), and subcloned into the pGEM-T vector (Promega). A large number of recombinant clones were picked for subsequent sequencing analysis.

Alternative splicing. Total RNAs from human and mouse kidney, heart, and skeletal muscle were reverse transcribed by using Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) (Gibco BRL-Life technologies) with random hexamer primers (Roche). Exon 9, 11, and 12 splice variants were detected with primers flanking exons 8, 10, and 13. The novel splice variant of human exon 4 was detected with primers binding to exons 3 and 4. The amplified fragments were analyzed by agarose gel electrophoresis, and products differing from the expected size were subcloned into the pGEM-T vector (Promega) and sequenced.

Analysis of WNK1 gene expression by RT-PCR. The full-length and kidney-specific WNK1 isoforms from human cDNA templates were detected with forward and reverse primers binding to exons 1 and 2 (full-length isoform), forward and reverse primers binding to exons 4a and 6 (kidney-specific isoform), and forward and reverse primers binding to exons 25 and 26 (both isoforms).

Real-time quantitative RT-PCR. Total RNA (2 µg) from human kidney was converted to cDNA by reverse transcription with the MMLV RT enzyme (Gibco BRL-Life Technologies) in a 20 µl reaction mixture with random hexamers (Roche). Standard curves were generated from serial 1/4 dilutions of cDNA. Real-time quantitative PCR (QRT-PCR) assays were carried out on an iCycler iQ system (Bio-Rad) by using intercalation of SYBR Green (Applied Biosystems) as a fluorescence reporter. Primers were designed to discriminate between catalytic and noncatalytic WNK1 isoforms: a forward primer from exon 2 (Ex2-F, 5'-CGTCTGGAACACCTAAAACGTATCT-3') and a reverse primer from exon 3 (Ex3-R, 5'-CACCAGCTTCTTAGAAGCTTTGATCT-3') were used to amplify catalytic isoforms of WNK1, and primers from exon 4a (Ex4a-F, 5'-TTGTCATCATAAATTCTCATTGCTG-3') and exon 5 (Ex5-R, 5'-AGGAATTGCTACTTTGTCAAACT-3') were used to amplify the kidney-specific isoform. Forward and reverse primers from the 5' sequence of exon 1 (Ex1-F, 5'-TACCACCACTGAGACCG-3'; Ex1-R, 5'-AGCTCCAGTGCAGTGGCATTGGA-3') were used to amplify the full-length WNK1 isoform under P1 control.

Construction of human WNK1 promoter-luciferase reporter plasmids. The luciferase reporter plasmids used in this study were derived from pGL3-Basic (Promega). Promoters P1 and P2 were amplified from BAC AC004765 (gi:

4731046) by PCR with primers containing *NheI* or *KpnI* and *BglII* restriction sites at the 5' end, so that cleavage with these enzymes generated cohesive ends. The renal promoter rP was amplified with primers containing *MluI* and *XhoI* restriction sites at the 5' end. Products were digested and cloned in both sense and antisense orientations into the unique *NheI*, *KpnI* or *MluI*, and *BglII* or *XhoI* sites of pGL3-Basic. Plasmids rP-3447, rP-5269Δ[−4284; −3447], and rP-5269Δ[−5054; −4284] were created by digesting rP-5269 with *MluI*-*BsmBI*, *EcoRI*-*BsmBI*, and *EcoRV*-*EcoRI*, respectively. The [−5054; −4284] fragment was excised from rP-5269 by *EcoRI*-*BsmBI* digestion and was inserted in the sense or antisense orientation into the *MluI* sites of plasmids rP-620 and pGL3-SV40 creating rP-620 and simian virus 40 (SV40) promoter-enhancer vectors. Fragments [−4284; −4111], [−4171; −3896], [−3928; −3710], [−3747; −3447], [−3604; −3447], and [−3604; −3447] upstream from exon 4a were amplified by PCR and cloned in plasmid pGL3-SV40. We used insertless pGL3-Basic as a negative control and pGL3-SV40, containing the SV40 promoter, as a positive control.

Transient transfections and reporter gene assays. We used 1 µg of luciferase reporter plasmids per well to transfect cultured cells in 12-well dishes, using cationic liposomes (Lipofectamine 2000) (Invitrogen) for MDCK cells and FuGEN 6 transfection reagent (Roche) for HEK and CHO cells according to the manufacturer's instructions. To control for transfection efficiency, cells were cotransfected with 200 ng of PCH110 (12). Forty-eight hours after transfection, cells were assayed for luciferase and β-galactosidase activities in an ML3000 luminometer. Luciferase activity measured with the Promega luciferase assay system was normalized to β-galactosidase activity measured with a chemiluminescence reporter assay system (β-galactosidase reporter gene assay; Roche).

In situ hybridization. Paraffin-embedded sections of mouse kidney and probes were prepared as previously described (14). Segments of the mouse WNK1 gene orthologous to exon 1, exons 2 to 4, and exon 4a of human WNK1 were amplified from mouse kidney cDNA. Amplified products 600 bp in size were cloned in pGEM-T, and their identity was checked by DNA sequencing.

Nucleotide sequence accession number. The nucleotide sequence of exon 4a of the human WNK1 gene has been deposited in the GenBank database under accession number AY231477.

RESULTS

Mapping of multiple transcription start sites for two proximal promoters. We carried out primer extension with RNA from human leukocytes, which produce the long isoform of WNK1, and the kidney, which produces the shorter isoform, to identify the transcription start sites for the WNK1 transcripts. Two main bands were obtained with an antisense primer binding to exon 2: a short one (308 bp) from the kidney and a longer one (1,200 bp) from leukocytes (Fig. 1A). The specificity of the assay was confirmed by the lack of an extension product when yeast tRNA was used as the template.

The main 1,200-bp extension fragment from leukocyte RNA indicated a major transcription start site about 200 to 300 bp upstream from the start codon in exon 1. We then performed 5'RACE-PCR to identify more precisely the 5' end of the WNK1 transcripts. With a primer binding to exon 1 and mRNA extracted from human heart, in which the long transcript predominates, we obtained a 650-bp band, which we cloned and sequenced (Fig. 1B, left panel). The transcription start sites for the proximal promoter (P1) were found to map to nucleotide positions −179 and −219 with respect to the first ATG codon in exon 1 (Fig. 1C).

We looked for possible sequence similarity between the WNK1 proximal promoter P1 and the 5' flanking region of the mouse WNK1 gene. These two sequences displayed 69% overall identity over a stretch of 3.3 kb (Fig. 2A). We identified a 153-bp region of extended homology (88% identity) immediately surrounding the major transcription start site that mapped to a position 219 bp upstream from the translation start site. Figure 2B shows the comparison between the P1

FIG. 1. Determination of multiple proximal transcription initiation sites for the human WNK1 gene. (A) Primer extension analysis of total RNAs from human kidney and leukocytes, using a primer complementary to sequences in exon 2. A control reaction was performed with yeast tRNA. Molecular size markers are given on the left. Two major products of 1,200 and 308 bp are indicated by arrows; faint bands around 440, 410, and 390 bp in kidney are indicated by arrowheads. (B) 5'RACE-PCR on heart cDNA with a primer complementary to sequences in the exon (EX) 1 and on kidney cDNA with a primer complementary to sequences in exon 2. Agarose gel electrophoresis of PCR products is shown, with positions of molecular size standards indicated on the left and right. (C) Schematic representation of the multiple transcription initiation sites (bent arrows). Positions are given relative to the first translational start site in exon 1 (ATG +1).

proximal promoters of the mouse and human WNK1 genes. This region lacks a TATA box but contains putative binding sites for Sp1, Oct-1, and HES-1, all of which are activator elements that are able to initiate gene transcription. It also contains a repressor element, CUP, which acts by connecting a CUP/Sp1 element with a downstream C/EBP binding site (16), and a putative C/EBP α binding site. This promoter region is very rich in G and C residues, with CpG islands characteristic of housekeeping genes, consistent with the ubiquitous production of this full-length isoform.

The major 308-bp extension fragment detected in the kidney was also observed in leukocytes, although it generated a much weaker signal in these cells (Fig. 1A). Three other faint bands at 440, 410, and 390 bp were also observed. Positive exon 1 sequence amplification from these extracted bands showed that they corresponded to exon 2 preceded by the end of the exon 1 sequence. This indicates that the 5' end of the major mRNA is encoded by the first exon of the gene, 618 bp downstream from the first ATG and 22 bp upstream from another in-frame start codon. Weak bands corresponding to PCR products 450 to 500 bp in size were generated by 5'RACE-PCR from human kidney cDNA, using a primer specific for exon 2 (Fig. 1B, right panel). The sequencing of these products revealed multiple transcription start sites in exon 1, located at four main sites, i.e., bp 623 (located within 6 bp of the site

identified by primer extension), 533, 520, and 481 (corresponding to the other minor extension fragments) downstream from the first ATG codon (Fig. 1C). These results demonstrate the presence of a second promoter region, P2, within the coding sequence of exon 1. It should be noted that the transcripts generated under the control of this second promoter contain the entire kinase-coding domain, with the first amino acid of the kinase domain encoded by the fourth codon of this truncated transcript.

A high level of sequence similarity between the putative human and mouse P2 promoter regions (83% identity) showed that such alternate transcription start sites may also exist in the mouse gene. Although no TATA box was identified, these G+C-rich sequences are highly compatible with promoter activity. They contain numerous consensus Sp1 binding sites and several putative binding sites for other transcription factors (Fig. 2C), including the CCAAT binding factor. They also contain consensus recognition sequences for transcriptional repressors such as WT1-KTS and CUP and for transcriptional activators known to be produced in the brain, heart, and testis (NF-ATp) or that may be involved in regulating kidney development (AP-2alphaA).

The two proximal promoters are active in vitro. We analyzed the regulatory mechanisms controlling the production of the two WNK1 isoforms under control of the P1 and P2 promoters

FIG. 2. Comparison of human and mouse WNK1 proximal promoters. Sequences were aligned with the BLAST 2 program. Sequences of the human (h) WNK1 promoters are shown in the top lines of each panel, and sequences of the mouse (m) WNK1 promoters are shown in the bottom lines of each panel. Nucleotide positions are given relative to the first ATG codon of exon 1. (A) Schematic representation of similarities in the proximal 3.5-kb flanking region of the first exon and exon 1 sequences of the WNK1 genes of humans and mice. Percent nucleic acid identities are indicated. (B) Region of the proximal P1 promoter region with the highest level of similarity is shown. (C) Similarities in the flanking translation start sites of the P2 promoters of the human and mouse genes. I, sequence identity; -, gaps. Horizontal lines indicate consensus transcription factor binding sites identified with the TESS program; bent arrows indicate the transcription initiation sites mapped by 5' RACE-PCR. The translation start site for P2 transcripts is in boldface.

by characterizing the 5' flanking region. This was achieved by cloning various fragments upstream from the luciferase reporter gene. Transient luciferase assays were carried out with the renal HEK 293 and MDCK cell lines and the nonrenal CHO cell line. Cells were also transfected with pGL3-Basic containing sequences cloned in the antisense orientation as negative controls. As shown in Fig. 3A, HEK, MDCK, and CHO cells express the two kinase domain-containing WNK1 isoforms. The same amount of RNA subjected to Northern blot analysis was used for the three cell lines. A greater expression of the full-length WNK1 transcripts was observed in HEK cells than in MDCK and CHO cells. Furthermore, quantitative RT-PCR experiments with HEK cells showed that the amount of P2 transcript was two-fold greater than the amount of P1 transcript in HEK cells (Fig. 3B).

The 1,200-bp sequence upstream from the first ATG in exon 1 (P1-1200) and the 613-bp sequence located between the two ATGs (ATG +1 and ATG +640) in exon 1 (P2+626) gave high levels of luciferase activity in all three cell lines when inserted in the native (Fig. 4A and B) but not in the reverse

(data not shown) orientation. When the 19-bp sequence containing the transcription start site identified by 5' RACE-PCR was deleted from the basal P2+626 promoter, the resulting P2 Δ 19 construct was found to have barely detectable promoter activity. The level of transcriptional activity of the fused sequences of the P1-1200 and P2+626 constructs (P2+P1-1200 construct) was lower than that of P1-1200, suggesting that transcriptional interference occurred due to promoter competition. Although P2+626 was less active in CHO cells than in renal cell lines, none of the fragments upstream from each of the 5' untranslated regions gave convincing lineage-restricted expression of the reporter gene.

The P1–2500 construct exhibited 20% of the activity of the P1–1200 promoter, suggesting that the sequence between nucleotide positions –2500 and –1200 represses transcription. We investigated the promoter specificity of this silencer effect by inserting the [–2500; –1200] sequence upstream from the P2, P2+P1, and PSV40 promoter constructs (Fig. 4C). This sequence was found to repress transcription from the SV40 promoter but not from the P2 promoter. Indeed, it appeared to

FIG. 3. Characterization of the human WNK1 renal promoter (rP). (A) Northern blot analysis of WNK1 expression in cultured cells. Fifteen micrograms of poly(A)⁺ RNA from MDCK cells, HEK cells, and CHO cells was hybridized with human WNK1 exon 1 (Ex 1) probe (specific for the full-length transcript) (P1), human exon 5-6 probe (specific for both kinase domain-containing and -defective isoforms), or human probe specific for transcripts ending at the second polyadenylation signal (polyA probe). Hybridization with the exon 1 probe revealed two bands, except for MDCK cells. These two bands result from the use of two polyadenylation sites, as shown when the blot was hybridized with the probe specific for the second poly(A) signal: only the larger band is observed. A shorter band appeared for the MDCK cells when exon 5-6 was probed, corresponding to the renal kinase-defective isoform. (B) Endogenous expression of WNK1 isoforms in cultured HEK 293 cells. QRT-PCR was used to quantify WNK1 transcripts. The full-length isoform (under P1 control) was amplified with primers binding to the 5' sequence of exon 1, both kinase domain-containing isoforms (under P1 and P2 transcriptional control) were amplified with primers binding to exons 2 and 3, and the kidney-specific kinase-defective isoform was amplified with primers binding to exons 4a and 5. Relative expression of the different WNK1 isoforms is shown. (C) Functional analysis of the proximal 5' flanking region of exon 4a. Reporter constructs containing the indicated lengths of the 5' rP relative to the transcription initiation site (+1) are indicated on the left. Luciferase activity normalized with respect to that for pGL3-Basic is shown, after the transfection of CHO, HEK 293, or MDCK cells. Histograms represent means, and bars indicate the minima and maxima for at least three experiments. (D) Comparison of human (h.) and mouse (m.) minimal promoter sequences. Sequences were aligned with BLAST 2. I, sequence identity; -, gaps. Lines indicate consensus transcription factor binding sites identified with the TESS program, and the bent arrow indicates the transcription initiation site mapped by 5'RACE-PCR.

activate transcription from the P2 promoter. In HEK and MDCK cells, the effect of this sequence on transcription from the P2+P1-1200 promoter was approximately equivalent to the mean of its repressor effect on P1 and its activator effect on P2, while in CHO cells, in which P2+P1-1200 is barely de-

tectable, the effect is equivalent to its activator effect on P2 promoter.

These results demonstrate that the cloned 1,200-bp fragment of P1 contains an orientation-specific promoter that is highly active in HEK 293, MDCK, and CHO cells. The cloned

FIG. 4. Activity of WNK1 promoters P1 and P2 in cultured cells. (A) Schematic representation of the structural organization of the two proximal WNK1 promoters; 5' flanking sequences (horizontal lines), the first exon (grey box), main transcription start sites (bent arrows), and the ATG codon corresponding to the translation start site are indicated. Reporter constructs used for luciferase assays are identified, with the length of the promoter of each construct, inserted upstream from the luciferase gene, given relative to the first ATG codon in exon 1. (B) Normalized luciferase activity for constructs containing P1, P2, or both promoters in transfected HEK 293, MDCK, and CHO cells. The relative luciferase activity of pGL3-Basic is considered to be 1. The SV40 promoter served as a positive control. Data are means from at least three experiments. (C) Promoter specificity of the transcriptional effect mediated by the [-2500; -1200] region, tested in three cell lines. The chimeric constructs used are presented schematically on the left; [-2500; -1200] was cloned upstream from the human WNK1 or SV40 promoter. The normalized luciferase activities for these constructs were divided by the activity for the promoter construct without the [-2500; -1200] sequence, resulting in the rate of transcriptional activation mediated by [-2500; -1200].

613-bp [+13; +626] P2 fragment contains an orientation-specific promoter that is less active in CHO cells than in renal cells, and essential elements are located between nucleotide positions +607 and +626. Furthermore, positive and negative regulatory elements located from bp -1200 to 2500 upstream from the first ATG modulate the *in vitro* activity of the proximal promoters.

The two major ubiquitously produced transcripts differ in their polyadenylation sites. Multitissue Northern blots probed with a C-terminal cDNA (exons 13 to 18) revealed the presence of two major transcripts (Fig. 5B). The shorter, 9-kb

isoform was found mainly in the kidney, whereas the longer, 10.5-kb isoform was present mainly in skeletal muscle, heart, and brain. Another probe corresponding to the sequence between the two predicted consensus polyadenylation sites, located 823 and 2636 bp, respectively, downstream from the translation stop codon, was used with the same blot (Fig. 5A). This probe detected the 10.5-kb isoform in all tissues and the short isoform specifically in the kidney (Fig. 5C). Thus, alternative polyadenylation results in the production of two mRNAs, differing in length by 1.8 kb, but is not responsible for the structure of the short renal transcript.

FIG. 5. Northern blot analysis of human WNK1 transcripts. (A) Schematic representation of the 3' end of the human WNK1 (hWNK1) gene, showing the locations of two predicted consensus polyadenylation sites (AATAAA) with positions given relative to the stop codon in exon 28. The probe used to discriminate transcripts according to their polyadenylation sites is indicated by a line. UTR, untranslated region. (B) Northern blot of RNAs from multiple human tissues hybridized with a probe complementary to exon 13-18 sequences. RNA size standards are indicated. (C) The same blot hybridized with a probe binding between the two predicted polyadenylation sites.

Other alternatively spliced forms. In order to further characterize WNK1 transcripts, we carried out a systematic search for alternative splicing events by RT-PCR with mRNA extracted from human heart and skeletal muscle, in which the full-length transcript predominates, and from human kidney, in which the short specific transcript is produced. Five different human transcripts were detected, resulting from alternative splicing of exons 9, 11, and 12 either separately or in combination (Fig. 6A). Exon 9 was present in about half of the transcripts (Fig. 6B, upper left panel), whereas the major product amplified from exons 11 to 13 was a 250-bp molecule corresponding to a transcript in which exons 11 and 12 were removed by splicing (Fig. 6B, lower left panel). A 530-bp product, corresponding to mRNA from which only exon 11 had been removed by splicing, and a 990-bp product, corresponding to mRNA containing all of the exons, were barely detectable in the three tissues tested. No kidney-specific splicing was observed. Exons 9 (84 bp), 11 (459 bp), and 12 (279 bp) are in frame and encode residues 714 to 741, 792 to 944, and 945 to 1037, respectively, of the protein. These residues are located downstream from the kinase domain (which ends at amino acid 494), between the two coiled-coil domains (residues 553 to 589 and 1796 to 1821, respectively). They have no known function but are well conserved among species (>82% identity between human, mouse, and rat sequences). Mouse exons 11 and 12 were most frequently removed by alternative splicing, whereas exon 9 was not (Fig. 6B, right panels).

A rare alternative splicing event was discovered within intron 3. A minor larger band, observed in kidney and heart (Fig. 6C), corresponds to a transcript generated from an alternative acceptor splice site located 83 nucleotides upstream from the normal acceptor splice site of exon 4. The resulting 83-bp insertion contains an in-frame stop codon resulting in the production of a putative protein ending at catalytic kinase subdomain VII.

Characterization of the kidney-specific transcript. As the production of the short kidney-specific transcript could not be accounted for by the use of different polyadenylation sites or specific alternative splicing, we systematically checked for the presence of each exon by Northern blotting. We found that the human renal transcript lacked the first four exons of the gene (the Northern blot hybridized with a probe specific for exons 1 to 3 [not shown]) but contained exon 5 (Fig. 7A). Thus, the major renal isoform is truncated from residue 1 to 437, encoding all of kinase subdomains I to IX and part of subdomain X. A similar pattern was observed in the mouse, suggesting that an alternative promoter is present in intron 4 in both species.

We carried out 5'RACE-PCR on human kidney cDNA with an exon 6-specific primer to identify the transcription start site of the human renal isoform. Alignment of the resulting 700-bp fragment (Fig. 7B) with a BAC sequence covering human WNK1 (AC004765) revealed the presence of a novel 341-bp exon (exon 4a) located between exons 4 and 5. A single transcription start site was identified in all ($n = 6$) sequenced clones, 251 bp upstream from an in-frame ATG codon located in exon 4a. Part of this exon has been observed in a genome-wide screen of alternative splicing by automated analysis of expressed sequence tags in the human genome (22). No splicing between exons 4 and 4a was detected by RT-PCR (not shown). We investigated the tissue specificity of the truncated transcript by probing human and mouse Northern blots with a human or mouse exon 4a-specific probe. A kidney-specific signal was obtained with this probe (Fig. 7E). This tissue specificity was confirmed by RT-PCR with both human and mouse tissues, with primers corresponding to the 5' (exons 1 and 2) or 3' (exons 25 and 26) part of the gene or primers amplifying exons 4a to 6 (Fig. 7F). This new exon is highly conserved between human, mouse, and rat, with 90% identity over the 90 bp of coding sequence (Fig. 7D). It encodes a cysteine-rich region between residues 19 and 27 (Fig. 7D).

Functional characterization of the renal promoter rP. We investigated whether the proximal 5' flanking region of exon 4a contains a functional promoter and evaluated its cell specificity by carrying out reporter gene assays with renal MDCK and HEK 293 cells and with nonrenal CHO cells. Northern blot analysis showed that the full-length WNK1 transcripts are expressed in the three cell lines but that only the renal WNK1 isoform is expressed in MDCK cells (Fig. 3A and B). A 3.4-kb fragment corresponding to the sequence immediately upstream from exon 4a was cloned upstream from the luciferase reporter gene (Fig. 3C). Deletion analysis showed that truncation of the promoter from nucleotide -3447 to -70 did not significantly modify its low activity. In contrast, when the promoter region was lengthened from -3447 to -5269, the resulting plasmid rP-5269 had a very high level of transcriptional activity selectively in MDCK cells (41 ± 4 versus $1.9 \pm$

FIG. 6. Alternative splicing in human and mouse WNK1 genes. (A) Schematic representation of WNK1 splicing events identified by RT-PCR in various tissues. The genomic segment spanning WNK1 is represented by a horizontal line, exons are represented by numbered grey boxes, and the splicing events are shown by broken lines. Exons 9, 11, and 12 are independently spliced. (B) Agarose gel electrophoresis of RT-PCR products from human, kidney, heart, and skeletal muscle RNAs (left panels) and mouse kidney, heart, and skeletal muscle RNAs (right panels). Amplified exons (Ex), from which primers were chosen, are indicated between the gels. Amplified fragments are indicated by arrows, with sizes given in base pairs. (C) Identification of a rare exon 4 alternative splicing event. Left panel, RT-PCR on human kidney, heart, and skeletal muscle RNAs with primers binding to exons 3 and 4. Right panel, schematic representation of the splicing between exons 3 and 4. The donor splice site (gt) and acceptor splice site (ag) are indicated. The grey box indicates the 83-bp fragment inserted following the rare splicing event (Ins83).

0.3 for rP-3447) (Fig. 3C), suggesting a strong cell-specific enhancer.

Thus, elements essential for basal transcriptional activity of the renal isoform of the human WNK1 gene are located between positions -70 and +14, and a strong positive regulatory region is located between -3447 and -5269.

Comparison with the mouse proximal 5' flanking region of exon 4a revealed very little overall sequence identity. Nevertheless, the proximal 81-bp [-70; +10] promoter is evolutionally conserved between mouse and human (77% identity) and contains consensus binding sites for PU-1, GR, and C/EBP α

(Fig. 3D). Furthermore, extensive similarity was found at position -2200 (92% identity over 53 bp) and between nucleotides -5044 and -4644 (86% identity over a 400-bp sequence). However, deletion of the 5.2-kb promoter from nucleotide -5054 to -4284 had no effect on transcriptional activity, while deletion from nucleotide -4284 to -3447 significantly decreased activity in MDCK cells (Fig. 3C). Thus, the renal enhancer is located between nucleotides -4284 and -3447.

The [-4284; -3447] fragment was then inserted upstream from the proximal rP-620 promoter driving the luciferase reporter gene. This distal fragment conferred a ~12-fold higher

FIG. 7. Characterization of the kidney-specific transcript. (A) Northern blot analysis of human (left panels) and mouse (right panels) WNK1 transcripts. Multitissue Northern blots were hybridized with a human WNK1 cDNA probe specific for exon 4 (EX4) (upper panels) or exon 5 (lower panels). (B) 5'RACE-PCR on kidney cDNA, using a primer complementary to sequences in exon 6. Agarose gel electrophoresis of PCR products is shown with molecular size standards indicated on the left and the size of the major band indicated on the right. (C) Comparison of the genomic structures of the human (h) (upper panel) and mouse (m) (lower panel) WNK1 genes. The genomic segment spanning WNK1 is represented by a horizontal line, and exons are indicated by numbered vertical lines. The novel exon 4a is indicated in boldface with the novel renal promoter (rP) (bent arrow). The genomic structure of the mouse gene was deduced from the WGS supercontig NW 000264 (gi: 20832062) and NW 036612 (gi: 20737037), partially sequenced. (D) Comparison of human (top line) and mouse (bottom line) exon 4a coding sequences. Sequences were aligned with BLAST 2 program. I, sequence identity. In-frame ATG codons are underlined. Deduced protein sequences are indicated, with conserved residues in grey. Double lines indicate cysteine-rich regions. (E) Same blots as in panel A; human (upper panel) and mouse (lower panel) multitissue Northern blots were hybridized with an exon 4a-specific probe. (F) Analysis of WNK1 transcripts by RT-PCR on human and mouse kidney, heart, and skeletal muscle RNA templates. The long isoform was amplified with primers specific for human exons 1 and 2 (top panels), whereas the short renal isoform was amplified with primers specific for human exons 4a to 6 (middle panels) and both isoforms were amplified with primers specific for human exons 25 and 26 (bottom panels).

TABLE 1. Cell specificity of the renal enhancer^a

Promoter-luciferase construct	Relative luciferase activity ^b in:	
	MDCK cells	CHO cells
rP-620	1.00 (0.98, 1.04)	1.00 (0.94, 1.08)
E _S -rP-620	11.12 (9.56, 12.62)	1.23 (1.00, 1.36)
E _A -rP-620	12.90 (9.25, 15.79)	1.47 (1.28, 1.72)
SV40	1.00 (0.75, 1.23)	1.00 (0.85, 1.18)
E _S -SV40	23.32 (20.44, 25.63)	1.30 (1.10, 1.43)
E _A -SV40	18.30 (16.75, 19.61)	1.51 (1.13, 1.87)

^a Reporter plasmids were transfected into renal MDCK cells and nonrenal CHO cells. E_S and E_A, [-4284; -3447] in sense or antisense orientation, respectively.

^b The results are the mean (minimum, maximum) from at least three experiments.

activity to the proximal rP promoter specifically in MDCK cells, independent of its orientation (Table 1). A similar effect was observed when it was cloned upstream from the SV40 promoter, with a ~20-fold increase in reporter gene activity in renal cells and a nonsignificant increase in CHO cells (Table 1). Therefore, the enhancer activity seems to be specific to renal epithelial cells and independent of the promoter.

Mapping of the renal enhancer. To determine more precisely the location of the enhancer, serial deletion fragments of the [-4284; -3447] enhancer region were cloned upstream from the SV40 promoter and tested for their enhancer activity in MDCK cells. The more distal fragment [-3604; -3447] had full enhancer activity when it was inserted in the sense or antisense orientation, with about 15-fold-higher transcriptional activity (Table 2).

We looked for sequence similarity between this 157-bp human enhancer region of the renal promoter and the 5' flanking region of the mouse WNK1 exon 4a. We identified two 51- and 47-bp sequences of extended homology (78 and 81% identity, respectively, at positions -8601 and -1655 upstream from the mouse ATG of exon 4a) and several other conserved sequences of about 20 bp in length.

In addition, comparison between the enhancer fragment and promoter regions of kidney-specific expressed genes also revealed interesting sequence similarities. In particular, 79% similarity was observed between the [-3589; -3447] rP WNK1 sequence and the human [-1102; -929] sequence promoter of the NCC gene. Similarity of 83% between the [-3589; -3447]

rP WNK1 sequence and the [-1409; -1231] 5' flanking region of the human kallikrein gene was observed (nucleotide positions are indicated with respect to the ATG codon for the NCC and kallikrein genes). These similarities are highly suggestive of a common enhancer upstream from these three genes that have a similar expression profile restricted to the distal convoluted tubule.

Profiles of production of catalytic and noncatalytic isoforms in the kidney. We assessed the relative abundances of isoforms containing and not containing the entire kinase domain in the kidney by performing QRT-PCR on human kidney mRNA, using primers amplifying either exons 2 and 3 or exons 4a and 5. Experiments performed in triplicate showed that 10.5 times more short kinase-defective transcript than kinase domain-containing transcript is produced (Fig. 8A). Furthermore, the full-length transcript (under the P1 promoter) accounts for 82% of the kinase domain-containing transcripts (under the P1 and P2 promoters). In similar experiments performed with human heart and muscle specimens, only the kinase domain-containing transcripts were produced (data not shown). These results are consistent with those obtained with Northern blots.

A striking difference in tissue distribution was shown in the mouse adult kidney by in situ hybridization. Both the exon 1-specific probe (which detects the full-length P1 isoform) as well as the exon 2-4-specific probe (which detects the P1 and P2 kinase domain-containing isoforms) gave uniform, low-level hybridization in all cells (Fig. 8B, panels a and b). In contrast, the exon 4a-specific probe (which detects the kinase-defective isoform) gave high levels of hybridization exclusively in the kidney cortex (Fig. 8B, panel c) and was present predominantly in the distal convoluted tubule (Fig. 8B, panels d and e). A similar pattern was observed in rat kidney (not shown).

DISCUSSION

The recent discovery of mutations in the WNK1 and WNK4 genes that are responsible for a particular form of human hypertension (PHA2) has generated considerable interest in this new family of serine-threonine kinases and in the molecular mechanisms leading to high blood pressure (13). The case of the ubiquitously expressed WNK1 gene is particularly intriguing. In two kindreds affected by PHA2, two overlapping large deletions in the first intron of the WNK1 gene were identified. These deletions have no effect on the gene product itself but instead modify WNK1 gene expression, as observed in the leukocytes of affected subjects (18). In this study, we show that WNK1 gene expression is under the control of three alternative promoters, generating several WNK1 isoforms with a tissue-specific distribution and resulting in the production of isoforms with and without the entire kinase domain. Further variations are achieved by use of alternative splicing and alternative polyadenylation sites. A newly discovered exon is under the influence of an alternative promoter and a kidney-specific enhancer. The corresponding kidney-specific transcript has no serine-threonine kinase activity, shedding light on an unsuspected function of this protein.

Several proximal transcription start sites and alternative splicing events were identified for the human WNK1 gene. Exon 1 is surrounded by a large CpG island that spans 1.5 kb, and comparison of the sequence of this region with the human

TABLE 2. Mapping of the renal enhancer^a

Luciferase construct	Luciferase activity ^b in MDCK cells.
SV40.....	1.00 (0.71, 1.34)
E _S [-4284; -4111]-SV40.....	1.55 (1.42, 1.66)
E _S [-4171; -3896]-SV40.....	1.92 (1.81, 2.01)
E _S [-3928; -3710]-SV40.....	1.09 (1.05, 1.13)
E _S [-3747; -3447]-SV40.....	18.03 (16.98, 19.66)
E _S [-3604; -3447]-SV40.....	16.53 (15.88, 17.37)
E _A [-3604; -3447]-SV40.....	14.07 (12.3, 15.7)

^a Normalized luciferase activity of different rP enhancer-SV40 promoter-luciferase constructs transfected in MDCK cells. E_S and E_A, enhancer in sense or antisense orientation, respectively.

^b The results are the mean (minimum, maximum) from at least three experiments.

FIG. 8. Expression pattern of the WNK1 gene in kidney. (A) Quantification of WNK1 transcripts in human kidney by QRT-PCR. The full-length transcript was amplified with primers binding to exon 1 (Ex1), the kinase domain-containing isoforms were amplified with primers binding to exons 2 and 3, and the kidney-specific kinase-defective isoform was amplified with primers binding to exons 4a and 5. Human kidney cDNA was serially diluted 1/4, and 3 μ l of each dilution was used in the PCR assay. Two PCR assays were performed for each dilution. The threshold cycle was measured and plotted against the log of the dilution. (B) In situ hybridization of WNK1 mRNA on a kidney section from an adult mouse. Hybridization with an antisense riboprobe corresponding to the full-length WNK1 isoform (a) and to the kinase domain-containing isoforms (b) shows weak uniform labeling, whereas the probe for the renal isoform (c) shows an intense hybridization signal restricted to the cortex; at high magnification, the labeling can be localized to the DCT (d and e). Asterisks, glomerulus; PCT, proximal convoluted tubule. Magnifications, $\times 3.5$ (a, b, and c), $\times 100$ (d), and $\times 250$ (e).

expressed sequence tag database revealed several potential transcription initiation sites (17). Primer extension and 5'RACE-PCR experiments enabled us to identify two transcription start sites in the 5' flanking region, 219 and 179 bp

upstream from the first translation start codon. We also identified other transcription start sites within exon 1, 623 to 481 bp downstream from the first ATG codon, resulting in a WNK1 isoform with only part of the sequence encoded by exon 1, the

first 639 nucleotides of the coding sequence having been eliminated. However, the predicted protein contains the entire kinase domain, with an ATG (codon 214) located four codons before the codon encoding the first amino acid of subdomain I of the kinase domain. There is no clear evidence that these two putative proteins have different functional roles, although a potential coiled-coil domain is predicted between residues 189 and 220. In that regard, it is interesting that various WNK1 subcellular localizations were recently observed among different epithelia (2).

The two proximal transcription start sites were confirmed to be functional by the identification of two proximal promoters that were functionally active *in vitro*. A very high level of transcriptional activity was observed with the P1 [1200; -1] fragment in various cell lines, with only 20% of this activity obtained with the P1 [2500; -1] construct. Whether the presence of a repressor element within positions -2500 to -1200 can account for quantitative differences in expression in tissues such as heart, skeletal muscle, brain, and various epithelia remains to be tested. Primer extension experiments showed that the level of P2 promoter activity is higher in human kidney than in leukocytes. However, it should be noted that these experiments are very sensitive to structural conformations of mRNA and do not allow a relative estimation of each isoform. Indeed, they suggested that P2 was more dominant than P1 in kidney, whereas the QRT-PCR found that the P2-driven transcript accounts for only 18% of the longer kinase domain-containing transcripts.

Further variation in human WNK1 gene transcripts is achieved by the use of two polyadenylation sites and alternative splicing. Northern blot experiments with rat and human cells have demonstrated the presence of two main mRNA species of unknown molecular structure (18, 20). Both isoforms seem to be present in tissues in which WNK1 is produced, with the shorter of the two transcripts being produced mainly in the kidney. Our study demonstrates that the two ubiquitous isoforms, approximately 9 and 10.5 kb in length, are generated by the use of two different polyadenylation sites separated by 1.8 kb at the 3' end of the human WNK1 mRNA. Poly(A) tails may affect the translation and stability of the mRNA (6). WNK1 transcripts with the longer, 2.6-kb 3' untranslated region are the most abundant. This is particularly striking in tissues in which the human WNK1 gene is highly expressed, such as kidney, skeletal muscle, heart, and brain.

The detection of WNK1 transcripts displaying alternative splicing of exons 9, 11, and 12, which encode nonidentical proteins, confirms previous reports (17, 22). These three exons are conserved between human, mouse, and rat. In rat, the first published WNK1 sequence lacked exons 11 and 12 (20). Our RT-PCR analysis reveals that these exons are not frequently used, suggesting that they may have special roles only in small cell populations or during certain developmental stages. We also found a novel splicing site in intron 3, 83 bp upstream from exon 4. The corresponding isoform is produced in only small amounts and contains a premature stop codon, leading to a predicted protein of 394 residues that is truncated after catalytic kinase subdomain VII, which should therefore be inactive. Such rare alternative splicing events, leading to the introduction of a premature stop codon and the production of a noncatalytic enzyme have been described for the human

α -galactosidase A gene, which is responsible for Fabry disease (7). Abnormal regulation of the production of this transcript by mutation within an intron leads to a particular cardiac phenotype of Fabry disease. Preliminary data indicate that production of this truncated human WNK1 isoform is not affected in the leukocytes of affected individuals from our PHA2 kindred with deletions in intron 1 (data not shown).

The existence of two proximal promoters and alternative splicing events cannot account for the molecular structure of the kidney-specific WNK1 transcript. We demonstrate in this study that the renal isoform, which in length is undistinguishable from the short 9-kb ubiquitous isoform, is kidney specific. It has the long, 2.6-kb 3' untranslated region but lacks the kinase subdomains encoded by the 5' part of the gene. Another novel alternative promoter within intron 4 mediates the specific production of this variant. We identified a single transcription start site within exon 4a and thus the structure of this exon, which is present in human, mouse, and rat, with high levels of similarity over the 90 bp of the coding sequence. The predicted human renal WNK1 protein sequence lacks the first 437 residues of the kinase-active WNK1 isoform but contains a novel cysteine-rich region at the N terminus. We characterized the minimal promoter sequence of this transcript, since the region immediately upstream from exon 4a has transcriptional activity *in vitro*, in both renal and nonrenal cell lines, and we discovered an enhancer element far upstream from the transcription start site (nucleotide -4284 to -3447) that conferred a ~20-fold activation selectively in renal MDCK cells. Preliminary *in situ* hybridization experiments with embryonic mouse kidney indicate a late expression of this renal isoform that may explain why it is not expressed in human embryonic kidney (HEK 293) cells and thus why the enhancer had no effect in these cells (not shown). The enhancer region was restricted to a 157-bp fragment that contains sequences highly homologous to 5' flanking sequences of the human NCC and kallikrein genes. Both share a similar expression profile restricted to the distal convoluted tubule. These results suggest that the renal-specific production of the kinase-deficient WNK1 isoform is due to a specific regulatory region mediating high levels of transcription in the distal tubule. Isolation of the transcription factors involved in the regulation of this enhancer might provide important new insights into the molecular events responsible for the specific expression of this isoform in the distal convoluted tubule and into the understanding of the physiopathological mechanism of the human WNK1 mutations responsible for PHA2.

The renal WNK1 isoform is the first reported isoform of the WNK family to be generated by the use of an alternative promoter and directly translated in a form predicted to be devoid of serine-threonine kinase activity. The existence of noncatalytic isoforms of other members of the kinase superfamily has been reported. A particular analogy can be made with the TrkB gene, which also spans a large region and generates a large number of different isoforms from alternative promoters, splicing sites, and polyadenylation sites (15). In this gene, a specific brain isoform, devoid of the tyrosine kinase domain, is generated by the use of an alternative exon 19 (8, 15). As for this gene, the WNK1 kinase-defective isoform is conserved in humans and rodents, highlighting its probable physiological importance. Within the kidney, this truncated isoform is produced in very large amounts, about 10 times

larger than those for the ubiquitous isoforms, in analyses of total mouse kidney extracts. In situ hybridization showed even more striking features, with exclusive, high-level production of this transcript in the distal convoluted tubule but only very weak and diffuse staining for the kinase domain-containing isoforms.

Currently, we can only speculate on the function and mechanism of action of the various WNK1 isoforms. It has been demonstrated in vitro that the complete rat protein is capable of autophosphorylation (20, 21), but no specific substrate has yet been identified. Similarly, the regulators of WNK1 expression and the signal transduction pathways activated by this protein remain to be determined. However, we know that changes in NaCl concentration may result in the activation of WNK1 in vitro (20). Furthermore, the ubiquitous presence of this protein in several epithelia, such as pancreatic ducts, epididymis, sweat ducts, and colonic crypts, is consistent with a role for WNK1 in the regulation of sodium and chloride reabsorption (2). However, this function cannot account for the strong expression of the full-length WNK1 isoforms in tissues such as heart and skeletal muscle, as observed in Northern blots with various species. It is possible that the WNK1 produced in tissues not involved in ion transport (heart and skeletal muscle) has another, as-yet-unknown, function. Rat WNK1 was the first mammalian member of this kinase family to be cloned and characterized (20), and 18 WNK-like kinases in seven species have been identified from in silico alignment, with high levels of similarity in the kinase motif (17). Several lines of evidence suggest that WNK kinases may play a role in signaling related to cell contact or adhesion: (i) WNK1 is present exclusively in multicellular organisms (17), (ii) WNK4 is present in the tight junctions of the renal distal tubule (18), (iii) in vitro studies suggest that WNK1 undergoes oligomerization (21), and (iv) partial WNK1 clones have been isolated from prostatic carcinoma and colorectal cell lines (11, 17). Further investigation is required to determine whether the two proximal promoters and the alternative splices described here can account for the differences in WNK1 gene expression, protein structure, and distribution.

The renal isoform lacks most of the N-terminal kinase domain but does contain, in addition to the two coiled-coil domains and the PXXP motifs, an autoinhibitory domain recently described by Xu et al. (21). This domain is conserved among the members of the WNK family and blocks the activity of the WNK1 kinase domain (21). In many protein kinases, this domain is used to regulate kinase activity, by a mechanism involving its removal from the inhibitory site by activating signals (9). WNK1 autoinhibition could be blocked, at least partially, by the formation of oligomers via the coiled-coil domains (21). It therefore seems likely that, as for the TrkB long and short isoforms (15), the full-length WNK1 isoform can bind the short renal isoform but not phosphorylate it, due to the absence of the critical serine residues. The renal kinase-defective WNK1 could therefore act as a dominant negative form of the catalytic WNK1 isoform. Alternatively, the marked predominance of the kinase-defective isoform within the distal convoluted tubule may reflect this isoform having a specific function in the kidney, regulating other kinases such as WNK4. The recent demonstration of WNK1 inhibition of the effect of WNK4 to reduce NCC-mediated Na uptake in *Xenopus* oocytes suggests

that the long WNK1 isoform may bind WNK4 within the cell, preventing its phosphorylation and/or migration to the membrane, thereby playing the role of a negative regulator (13, 23). If this is also true for the kidney-specific kinase-deficient isoform, interactions with WNK4, and possibly with other proteins, would then result in physiological regulation of the signaling pathways controlling the ion permeability of nephron epithelia.

Human WNK1 mutations causing PHA2 have been shown to be associated with overproduction of the short WNK1 transcript in the leukocytes of affected subjects. Therefore, they might increase NCC activity by inhibiting the negative physiological effects of WNK4 on the NCC leading to sodium retention and hypertension. This interaction model may account for human PHA2-causing mutations of the WNK1 and WNK4 genes being undistinguishable in terms of biological and clinical features. Analysis in vitro and in vivo of the consequences of the PHA2-causing deletions of intron 1 for WNK1 gene expression and protein structure should benefit from the results of this study, making it possible to obtain further insight into the molecular mechanisms of this particular form of human hypertension.

ACKNOWLEDGMENTS

We thank Juliette Hadchouel for critical reading and comments on the manuscript and Marie-Thérèse Morin for assistance with in situ hybridization.

This work was supported by grants from INSERM, Association Claude Bernard, Bristol Myers Squibb, and the ACI Integrative Biology Programme of the MJER. C.D. received a fellowship from the MJER.

REFERENCES

- Achard, J. M., D. G. Warnock, S. Disse-Nicodeme, B. Fiquet-Kempf, P. Corvol, A. Fournier, and X. Jeunemaitre. 2003. Familial hyperkalemic hypertension: phenotypic analysis in a large family with the WNK1 deletion mutation. *Am. J. Med.* **114**:495–498.
- Choate, K. A., K. T. Kahle, F. H. Wilson, C. Nelson-Williams, and R. P. Lifton. 2003. WNK1, a kinase mutated in inherited hypertension with hyperkalemia, localizes to diverse Cl-transporting epithelia. *Proc. Natl. Acad. Sci. USA* **100**:663–668.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159.
- Disse-Nicodeme, S., J. M. Achard, J. Potier, M. Delahousse, B. Fiquet-Kempf, N. Stern, A. Blanchard, J. C. Guilbaud, P. Niaudet, D. Chauveau, B. Dussol, Y. Berland, P. Dequiedt, J. L. Ader, M. Pailard, J. P. Grunfeld, A. Fournier, P. Corvol, and X. Jeunemaitre. 2001. Familial hyperkalemic hypertension (Gordon syndrome): evidence for phenotypic variability in a study of 7 families. *Adv. Nephrol. Necker Hosp.* **31**:55–68.
- Gordon, R. D., S. A. Klemm, T. J. Tunny, and M. Stowasser. 1995. Gordon's syndrome: a sodium-volume-dependent form of hypertension with a genetic basis, p. 2111–2123. *In* J. H. Laragh and B. M. Brenner (ed.), *Hypertension: pathophysiology, diagnosis and management*, 2nd ed. Raven Press, New York, N.Y.
- Hall, T. M. 2002. Poly(A) tail synthesis and regulation: recent structural insights. *Curr. Opin. Struct. Biol.* **12**:82–88.
- Ishii, S., S. Nakao, R. Minamikawa-Tachino, R. J. Desnick, and J. Q. Fan. 2002. Alternative splicing in the alpha-galactosidase A gene: increased exon inclusion results in the Fabry cardiac phenotype. *Am. J. Hum. Genet.* **70**:994–1002.
- Klein, R., D. Conway, L. F. Parada, and M. Barbacid. 1990. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**:647–656.
- Lei, M., W. Lu, W. Meng, M. C. Parrini, M. J. Eck, B. J. Mayer, and S. C. Harrison. 2000. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* **102**:387–397.
- McKnight, S. L., and R. Kingsbury. 1982. Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217**:316–324.
- Moore, T. M., R. Garg, C. Johnson, M. J. Coptcoat, A. J. Ridley, and J. D. Morris. 2000. PSK, a novel STE20-like kinase derived from prostatic carcinoma.

- noma that activates the c-Jun N-terminal kinase mitogen-activated protein kinase pathway and regulates actin cytoskeletal organization. *J. Biol. Chem.* **275**:4311–4322.
12. **Norton, P. A., and J. M. Coffin.** 1985. Bacterial beta-galactosidase as a marker of Rous sarcoma virus gene expression and replication. *Mol. Cell. Biol.* **5**:281–290.
 13. **Rossier, B. C.** 2003. Negative regulators of sodium transport in the kidney: key factors in understanding salt-sensitive hypertension? *J. Clin. Investig.* **111**:947–950.
 14. **Sibony, M., F. Commo, P. Callard, and J. M. Gasc.** 1995. Enhancement of mRNA in situ hybridization signal by microwave heating. *Lab. Invest.* **73**: 586–591.
 15. **Stoilov, P., E. Castren, and S. Stamm.** 2002. Analysis of the human TrkB gene genomic organization reveals novel TrkB isoforms, unusual gene length, and splicing mechanism. *Biochem. Biophys. Res. Commun.* **290**: 1054–1065.
 16. **Vasseur-Cognet, M., and M. D. Lane.** 1993. CCAAT/enhancer binding protein alpha (C/EBP alpha) undifferentiated protein: a developmentally regulated nuclear protein that binds to the C/EBP alpha gene promoter. *Proc. Natl. Acad. Sci. USA* **90**:7312–7316.
 17. **Verissimo, F., and P. Jordan.** 2001. WNK kinases, a novel protein kinase subfamily in multi-cellular organisms. *Oncogene* **20**:5562–5569.
 18. **Wilson, F. H., S. Disse-Nicodeme, K. A. Choate, K. Ishikawa, C. Nelson-Williams, I. Desitter, M. Gunel, D. V. Milford, G. W. Lipkin, J. M. Achard, M. P. Feely, B. Dussol, Y. Berland, R. J. Unwin, H. Mayan, D. B. Simon, Z. Farfel, X. Jeunemaitre, and R. P. Lifton.** 2001. Human hypertension caused by mutations in WNK kinases. *Science* **293**:1107–1112.
 19. **Wilson, F. H., K. T. Kahle, E. Sabath, M. D. Lalioti, A. K. Rapson, R. S. Hoover, S. C. Hebert, G. Gamba, and R. P. Lifton.** 2003. Molecular pathogenesis of inherited hypertension with hyperkalemia: the Na-Cl cotransporter is inhibited by wild-type but not mutant WNK4. *Proc. Natl. Acad. Sci. USA* **100**:680–684.
 20. **Xu, B., J. M. English, J. L. Wilsbacher, S. Stippec, E. J. Goldsmith, and M. H. Cobb.** 2000. WNK1, a novel mammalian serine/threonine protein kinase lacking the catalytic lysine in subdomain II. *J. Biol. Chem.* **275**:16795–16801.
 21. **Xu, B. E., X. Min, S. Stippec, B. H. Lee, E. J. Goldsmith, and M. H. Cobb.** 2002. Regulation of WNK1 by an autoinhibitory domain and autophosphorylation. *J. Biol. Chem.* **277**:48456–48462.
 22. **Xu, Q., B. Modrek, and C. Lee.** 2002. Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. *Nucleic Acids Res.* **30**:3754–3766.
 23. **Yang, C. L., J. Angell, R. Mitchell, and D. H. Ellison.** 2003. WNK kinases regulate thiazide-sensitive Na-Cl cotransport. *J. Clin. Investig.* **111**:1039–1045.