



Regulation of *WNK4* gene transcription in the kidneys

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ABSTRACT. With-no-lysine (K) kinase-4 (*WNK4*) is a newly cloned kinase-encoding gene that plays a crucial role in the maintenance of electrolyte homeostasis. Mutations of *WNK4* can cause pseudohypoaldosteronism type α , an autosomal dominant disease characterized by hyperkalemia, metabolic acidosis and hypertension. We explored the expression and regulatory mechanism of *WNK4* in the human kidneys, which is a key regulator of blood pressure. Expression of *WNK4* was determined by RT-PCR. Transcription initiation site and regulatory elements in the promoter region of *WNK4* were systematically analyzed with a combined set of experimental and bioinformatic methods. Using 5'-RACE, we have determined the transcription initiation site. We identified a number of putative *cis*-acting elements by analysis of the promoter region with the TRANSFAC-TESS software; these were sub-

sequently confirmed with an electrophoresis mobility shift assay. As confirmed by a CAT-ELISA reporter assay, the promoter region of *WNK4* has a high level of transcriptional activity. Several hormones, in particular dexamethasone, can suppress the level of *WNK4* mRNA. These results have shed light on the regulatory mechanism of *WNK4* expression in kidneys, as well as the influence of various hormones on expression levels. This should prove useful for studies on the roles of *WNK4* in the pathogenesis of hypertension.

Key words: Gene expression; Kidney; Promoter region; *WNK4*; Transcription factor

INTRODUCTION

With-no-lysine (K) kinase-4 (*WNK4*) is a newly cloned serine/threonine kinase gene. Mutations of its exons 7 and 17 can cause pseudohypoaldosteronism type α , an autosomal dominant disease featuring hyperkalemia, metabolic acidosis, and hypertension. Expression of *WNK4* has been found in several types of chloride-transporting epithelia, with its protein products located subcellularly near the tight junctions, along the lateral membranes, and within the cytoplasm. Previous studies indicated that *WNK4* can suppress the activities of NaCl co-transporter and potassium channels in the outer layer of renal medulla while enhance paracellular chloride flux (Kahle et al., 2003, 2004; Yamauchi et al., 2004). Therefore, the *WNK4* gene probably plays a crucial role in blood pressure regulation. Of note, Erlich et al. (2003) also found that single nucleotide polymorphisms in the *WNK4* gene can influence the risk of hypertension in Caucasians. Our previous study suggested a similar role for the gene in Chinese hypertensive patients (Sun et al., 2003).

To date, most studies on the *WNK4* gene have focused on its functions, while the regulatory mechanisms of its expression remained poorly understood. The present study therefore aimed to explore the mechanisms of transcriptional modulation of *WNK4*. To explore whether *WNK4* also plays a role in hormone-influenced blood pressure modulation, we have also investigated the influence of various hormones on its transcription.

MATERIAL AND METHODS

Tissue samples and cell culture

With informed consent obtained, tissue samples were obtained from non-viable fetuses from the Shengjing Hospital Affiliated to China Medical University. Human embryo kidney 293 (HEK293) cells were cultured in a minimum essential medium (Gibco, Los Angeles, CA, USA) supplemented with 15% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

RNA preparation and RT-PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

cDNA was synthesized with reverse transcription using random primers following a protocol recommended by the manufacturer (Promega, Madison, WI, USA). PCR was carried out under optimized conditions using the following primers, with β -actin used as an internal control: *WNK4* primer sense: 5'-GTTTCATTCTGCCTTCGGAGCG-3' and antisense: 5'-CAGTCATCA CAGCCAGTGAGA-3'; β -actin primers sense: 5'-AGAGCTACGAGCTGCCTGAC-3' and antisense: 5'-AGTACTTGCGCTCAGGAGGA-3'.

5'-Rapid amplification of cDNA (5'-RACE) and software analysis

A human kidney Marathon-Ready cDNA amplification kit (Clontech, Mountain View, CA, USA) was used following manufacturer instructions. The forward primers included a 27-mer adaptor primer (AP1) and a 23-mer nested AP2. The reverse primers were gene-specific primers (GSP) for the exon 1 of the *WNK4* gene, which included an outer primer (GSP1, 5'-ACGCGCGGAGTCTCCGCAGCCT-3') and an inner primer (GSP2, 5'-CTGGGACATG AGGACGGTGGTCT-3'). The first round of PCR was carried out with the Marathon-Ready cDNA as the template and AP1 and GSP1 as primers. The product was diluted and used as the template for a second round PCR with AP2 and GSP2 as primers. The PCR product was analyzed by agarose gel electrophoresis. Subsequently, DNA from the major band was cloned into a pMD18-T vector and sequenced. On the basis of the 5'-RACE results, the transcription initiation site and promoter region of *WNK4* were determined.

Electrophoresis mobility shift assay (EMSA)

Nuclear extract from kidney tissues was prepared following standard procedures. To prepare the probe, an oligonucleotide 5'-CGCGGCTGGGCGGGGCGGTGACTAAGGT-3' containing the potential Sp1 and AP1 elements was synthesized, annealed with a complementary fragment, and labeled with [γ - 32 P]-ATP (Furui Biotechnology, Beijing, China). The binding reaction was performed in a volume of 15 μ L containing approximately 5000 cpm of labeled probe and 5 μ g nuclear extract. After incubating on ice for 1 h, the reaction mixtures were loaded onto an 8% polyacrylamide gel and run at 150 V for 4 h. Subsequently, the gels were dried under vacuum and analyzed with autoradiography.

Plasmid construction

A 1275-bp fragment harboring the *WNK4* promoter was obtained through PCR using human genomic DNA as the template and 5'-CACTGACCTCTCCGTTCGGC-3' (forward) and 5'-CAGATCATT CAGGGCAAAGAC-3' (reverse) as primers. The PCR product was cloned into a pDM18-T vector, and subcloned into a unique *Hind* β /*Xba*I site of a pCAT-Basic vector (Promega) in order to generate a reporter construct (pCAT-*WNK4*-promoter). The construct was confirmed with sequencing. Coding frame shift of the *CAT* gene was excluded. For transient transfection, the plasmids were prepared using an Axyprep Plasmid Miniprep kit (Axygen Biosciences, Union City, CA, USA).

Transient transfection and ELISA

HEK293 cells were seeded on 6-well plates and grown to 80-90% confluence. For

transient transfection, the standard medium was replaced with a serum-free medium. To normalize transfection efficiencies, pSV- β -galactosidase (Promega) was used as the internal control. In each experiment, 0.5 μ g pCAT-*WNK4*-promoter construct and 0.05 μ g pSV- β -galactosidase vector were co-transfected into cultured cells using Lipofectamine 2000 (Invitrogen) transfection reagents. Activities were determined from a single sample using a CAT-ELISA kit (Roche, Mannheim, Germany) according to manufacturer instructions.

Northern blotting

Thirty micrograms of total RNA was fractionated by electrophoresis on a 1% agarose-formaldehyde gel, blotted by capillary transfer onto a nylon membrane (Amersham Biosciences, Sweden), and cross-linked through UV irradiation. The membrane was probed with an antisense cRNA from *WNK4* cDNA labeled by random priming using a commercial kit (Amersham Biosciences) and [32 P]-dCTP. The blot was pre-hybridized at 42°C for 2 h in 50% formamide, 0.1% SDS, 2X Denhardt's solution, 5X standard SSPE, and 0.1 mg/mL salmon sperm DNA. Subsequently, probe was added to the pre-hybridization solution, and the blot was hybridized overnight at 42°C, washed twice for 10 min each at 55°C in 2X standard saline citrate and 0.1% SDS, and autoradiographed through an intensifying screen at -70°C. The *WNK4* probe was then removed with boiling, and the same blot was re-hybridized to a cDNA probe encoding for β -actin.

RESULTS

Expression of the *WNK4* gene in various tissues

To assess expression of the *WNK4* gene, total RNA from fetal kidney, heart, brain, small intestine, spleen, liver, and lung was extracted and assayed with RT-PCR. As shown, *WNK4* was expressed in all of the samples except liver. The most prominent expression was found in the kidneys (Figure 1).

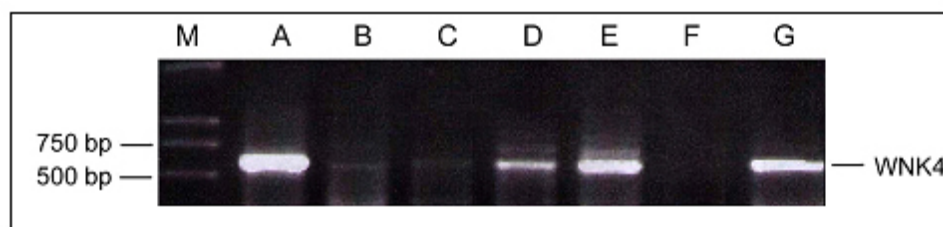


Figure 1. Expression of the *WNK4* gene in various tissues by RT-PCR analysis. Lane M = DNA marker; lane A = kidney; lane B = heart; lane C = brain; lane D = intestine; lane E = spleen; lane F = liver; lane G = lung.

Identification of the *WNK4* transcription initiation site

5'-RACE was performed in order to identify the transcription initiation site of the

WNK4 gene. For the first round of PCR, a primer binding with the first exon and human kidney Marathon-Ready cDNA were used, and a 442-bp band was derived. With diluted PCR product as the template, a 102-bp band was obtained with 5'-nested RACE (Figure 2). DNA from the 2 bands was then cloned into a pMD18-T vector and sequenced. BLAST analysis of the 2 sequences indicated a same transcription initiation site.

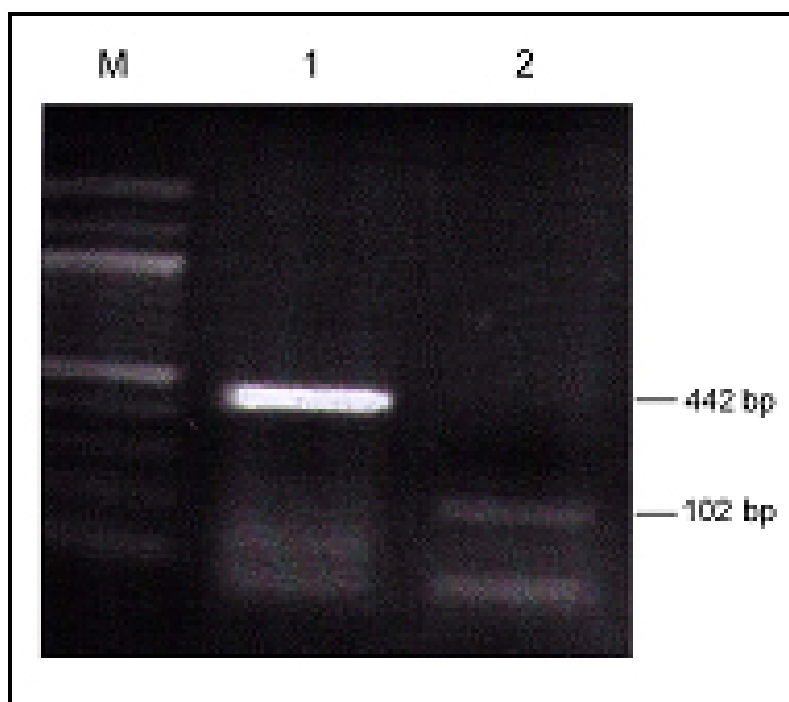


Figure 2. Identification of the transcription initiation site by 5'-RACE. A 442-bp band was seen after the first round PCR and the product of nested PCR was a 102-bp band. *Lane M* = DNA marker; *lane 1* = product of first round PCR; *lane 2* = product of nested PCR.

Functional analysis of the *WNK4* promoter

By analyzing a 1000-bp sequence upstream of the *WNK4* transcription initiation site with the TRANSFAC-TESS software, a number of cis-acting elements, including AP1, AP2, Sp1, and GRE, were discovered (part of the results are shown in Figure 3A), which confirmed the region to be the promoter of the *WNK4* gene.

An EMSA assay was performed to verify the results of the above prediction. Nucleotides (named SA) containing the potential Sp1 and AP1 elements (Figure 4) were synthesized and labeled as probe. As demonstrated by EMSA, nuclear extract from kidney tissues could form specific complexes with the SA probe, and this can be competed by a cold SA sequence, which confirmed that sequence from the *WNK4* promoter can bind specifically with transcription factors Sp1 and AP1 (Figure 3B).

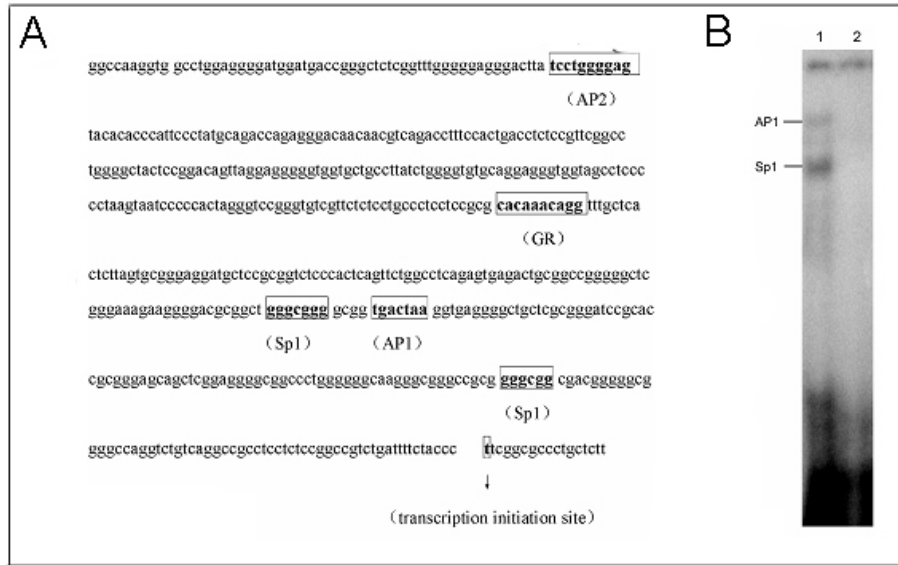


Figure 3. Analysis of the *WNK4* promoter by TRANSFAC-TESS search and electrophoresis mobility shift assay (EMSA). **A.** Predicted binding sites in the promoter region of the *WNK4* gene. **B.** Binding of nuclear extract from kidney to the *WNK4* promoter as shown with EMSA. Lane 1 = SA + nuclear extract; lane 2 = SA + nuclear extract + cold SA competition.

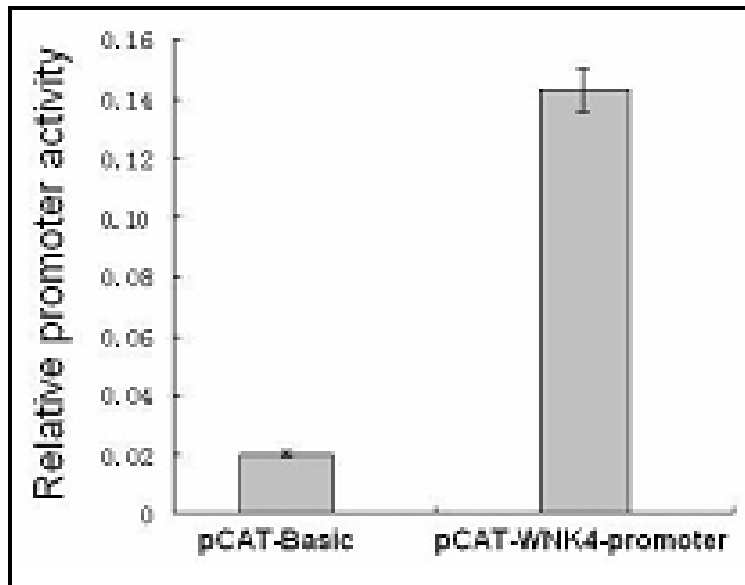


Figure 4. *WNK4* promoter activity by CAT-ELISA. Compared with pCAT-Basic, pCAT-*WNK4*-promoter showed high transcriptional activity.

Reporter assay for the *WNK4* promoter

To explore the activity of the *WNK4* promoter, a pCAT-*WNK4*-promoter plasmid was constructed and transiently transfected into HEK293 cells. Using β -gal as the internal control, ELISA was carried out to detect the promoter activities. As shown in Figure 4, compared with pCAT-Basic, the pCAT-*WNK4*-promoter showed a high activity, which confirmed a transactivation function of the *WNK4* promoter.

Influence of various hormones on *WNK4* expression

To assess the influence of various hormones on the expression of *WNK4* in the kidney, HEK293 cells were treated respectively with 5 M angiotensin α , 1 μ M estrogen, 1 nM dexamethasone, 100 nM insulin, and 6 nM growth hormone. The results of RT-PCR are shown in Figure 5A. Compared with untreated controls, all of the above hormones suppressed the expression of *WNK4*, and among these, the effect of dexamethasone was the most pronounced. Northern blotting analysis also confirmed suppression of *WNK4* gene expression by these hormones (Figure 5B).

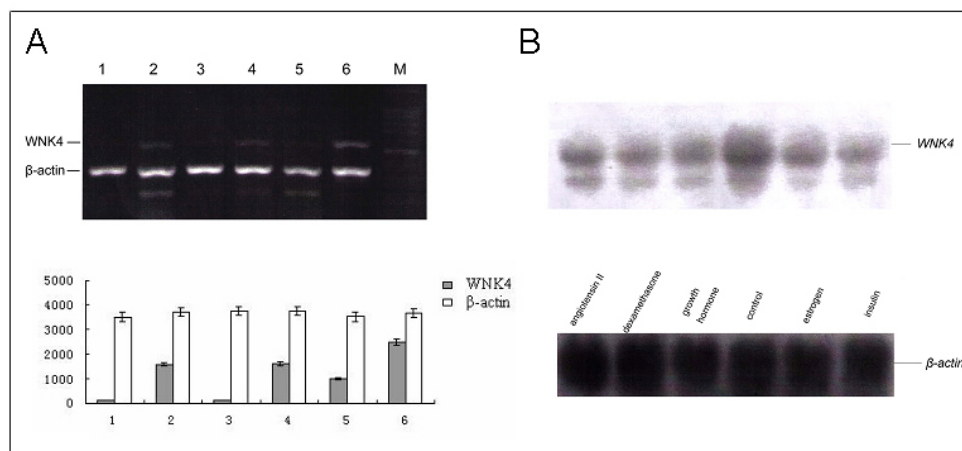


Figure 5. Regulation of the *WNK4* mRNA level by various hormones by RT-PCR assay and Northern blotting. **A.** Lane 1 = estrogen; lane 2 = insulin; lane 3 = dexamethasone; lane 4 = angiotensin II; lane 5 = growth hormone; lane 6 = control; lane M = DNA marker. **B.** Expression of the *WNK4* mRNA level repressed by hormones.

Dexamethasone regulation of *WNK4* promoter activity

To assess the effect of particular hormones on *WNK4* transcription, CAT-ELISA was carried out after co-transfection of pCAT-*WNK4*-promoter and pSV- β -galactosidase into the HEK293 cells. As shown in Figure 6, under basal conditions, addition of dexamethasone suppressed the activity of the *WNK4* promoter in a dose-dependent manner, with the lower concentration of dexamethasone (1 nM) showing a more prominent effect than the higher one (10 nM).

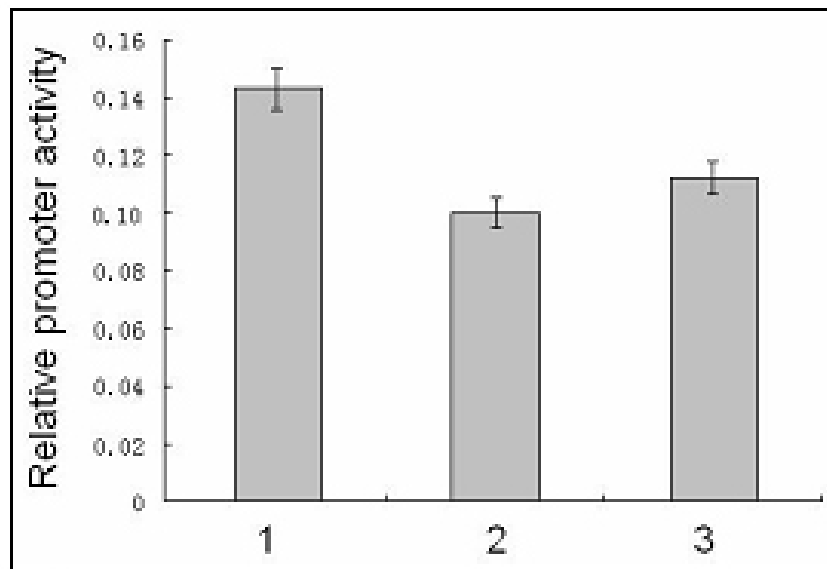


Figure 6. Effect of dexamethasone on the *WNK4* promoter activity. 1 = pCAT-*WNK4*-promoter; 2 = pCAT-*WNK4*-promoter + 1 nM dexamethasone; 3 = pCAT-*WNK4*-promoter + 10 nM dexamethasone.

DISCUSSION

In the present study, we explored tissue-specific expression of the *WNK4* gene and found an abundant expression in the kidney. The *WNK4* protein has been shown to regulate trans- and paracellular ion flux across diverse epithelia. Regulation of *WNK4* expression may therefore be crucial for the maintenance of electrolyte homeostasis. The wide range of *WNK4* expression, as discovered here, seems to suggest a role for the gene in ion transportation regulation. Considering its abundant expression in the kidneys, we utilized kidney tissues to explore the regulation mechanism of *WNK4* transcription. Using a 5'-RACE assay, we determined the transcription initiation site. With a number of transcription factor binding sites from the promoter region predicted, EMSA assay confirmed that at least the AP1 and Sp1 elements are functional. CAT-ELISA assay also confirmed a strong transcription activity for the promoter region.

Similar to other kinases, *WNK4* is a critical signal-regulating enzyme. When activated by upstream signals, protein kinases can up- or downregulate the activities of downstream proteins and participate in various cell activities. Of note, many of such processes are modulated by hormones and cytokines (Dhanasekaran, 1998; Dhanasekaran and Premkumar Reddy, 1998; Mader et al., 1993). For example, as a signal molecule, a steroid hormone may enter the nucleus and bind with a *cis*-acting element to regulate gene expression. Previous studies suggested that several hormones, including estrogen and growth hormone, may participate in the regulation of blood pressure (Bielohuby et al., 2009; Vongpatanasin, 2009). Furthermore, a putative glucocorticoid response element has been identified in the *WNK4* promoter. As confirmed by our study, estrogen, insulin, dexamethasone, angiotensin II, and growth hormone

can all suppress the expression of *WNK4*, with dexamethasone showing the most prominent effect and in a concentration-dependent manner. The stronger effect of the lower concentration may have been due to a negative feedback mechanism. Our study has confirmed the wide range of tissue expression of *WNK4* and delineated the regulatory mechanism of its transcription. It is likely that various hormones can influence blood pressure by modulating *WNK4* expression. The above results may provide a basis for further delineation of the role of *WNK4* in the regulation of blood pressure.

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