ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(4): 241–247 **CN** 31-1940/Q

Identification of the Alternative Promoters of the *KChIP4* Subfamily

Xiao-Yun DENG, Fang CAI, Kun XIA*, Qian PAN, Zhi-Gao LONG, Ling-Qian WU, De-Sheng LIANG, He-Ping DAI, Zhuo-Hua ZHANG, and Jia-Hui XIA

National Laboratory of Medical Genetics, Central South University, Changsha 410078, China

Abstract The subfamily of voltage-dependent potassium (Kv) channel interacting protein 4 (KChIP4) is made up of the auxiliary interacting protein of voltage-dependent potassium channels. In this study, the structure of four splicing variants of the human *KChIP4* gene was analyzed. Three of the four isoforms of the *KChIP4* gene, *KChIP4.1*, *KChIP4.2* and *KChIP4.4*, were amplified from mouse and human fetal brain tissues by reverse transcription-polymerase chain reaction and then identified. Based on the bioinformatics analysis of the genomic sequences of the gene, we cloned and characterized two promoter fragments from the *KChIP4* gene. One was a 325 bp fragment upstream of the 5′ end of the *KChIP4.1* mRNA sequence and the other was an 818 bp fragment located immediately at the 5′ end of the *KChIP4.4* variant. Both of them can initiate the transcription of the reporter gene in HT1080 cells and Sprague-Dawley (SD) rat fetal brain neurons, and they contain C+G islands, except typical TATA boxes and CAAT boxes. This shows that the *KChIP4* gene expression is regulated by an alternative promoter.

Key words splicing variant; alternative promoter; transcriptional regulation

A-type channels are voltage-dependent potassium (Kv) channels that are activated in the subthreshold range of membrane potentials, and they are completely deactivated during depolarizing pulses when other voltage-dependent potassium channels are just beginning to be activated [1–3]. Voltage-gated K^+ currents are functionally and structurally variable, and are often considered to be major determinants of excitability in excitable cells, such as neurons and cardiac myocytes [4–8]. The Kv channels are tetramers, and heteromeric channels are assembled only from subunits in the same subfamily [7]. Various auxiliary and other channel-interacting proteins have been identified. In 2000, new members of the channel auxiliary subunits for the voltage-gated A-type potassium channel Kv4 family were discovered. These Kv4 channel-interacting auxiliary proteins are called Kv channel-interacting proteins (KChIPs), and they are encoded by at least four genes, *KChIP1–4* [9,10]. Preliminary study has revealed that the *KChIP1–3* genes are highly and specifically expressed in the brain, although *KChIP2* mRNA is also abundant in the heart. *KChIP1–3* increase the total Kv4 current, slow channel inactivation moderately and accelerate recovery from inactivation considerably. Association with KChIPs dramatically increases current amplitude and alters the gating properties of Kv4 channels; Kv4-KChIP complexes exhibit some of the gating and modulation properties, reconstructing native transient Kv currents in cardiac myocytes and neurons [11,12].

KChIP4 was first identified as the interacting protein of presenilin 2 [10]. There are four members in the KChIP4 subfamily called KChIP4.1–KChIP4.4. They have different properties and perform the modulation function in Kv4-KChIP co-transfected cells [13,14]. The heterogeneity in KChIP4 effects on the Kv channel is caused by its alternative N-terminal peptides. Calsenilin-like protein (CALP)/KChIP4.1, which has been cloned as a presenilin 2 co-localized protein in the endoplasmic reticulum, binding to Kv4 and modulating the channel kinetics in the same

Received: December 14, 2004 Accepted: February 25, 2005

This work was supported by the National High Technology Research and Development Program of China (2002BA711A07-08), the Major State Basic Research Development Program of China (2004CB518601), and National Natural Science Foundation of China (30300101, 30123006, and 30400226)

*Corresponding author: Tel, 86-731-4472093; Fax, 86-731-4478152; E-mail, xiakun48@yahoo.com.cn

DOI: 10.1111/j.1745-7270.2005.00034.x

manner, is essential for the facilitation of the functional expression of Kv4 [15]. KChIP4.4, which is also termed KChIP4a, exhibits a unique effect on Kv4 channel gating because of its N-terminal K-channel inactivation suppressor (KIS) domain [16]. KChIP4.4 eliminates fast deactivation in conjunction with changes in other kinetic parameters, and effectively converts the fast-deactivating A-type current to a slow-deactivating delayed rectifier type of current. In general, the different effects of KChIP4.1 and KChIP4.4 on the Kv channel are mainly a result of their diverse N-terminus. Given that the human *KChIP4* gene spans nearly 1,219,331 bp in the chromosome 4, and the physical distance between the first exon of the *KChIP4.1* variant and the first exon of the *KChIP4.4* variant is more than 640 kb, it is very interesting to elucidate the mechanism for the transcription of *KChIP4* and the manner in which it occurs.

In this study, we identified three of the four splicing variants of the *KChIP4* gene from mouse and human fetal brain tissues. On the basis of bioinformatics prediction, the promoter sequence of the *KChIP4.1* and *KChIP4.4* splicing variants was isolated and cloned into the chloramphenicol acetyltransferase (CAT) reporter gene vector respectively. Our results indicate that the promoter sequence of both variants can significantly drive the expression of the reporter gene in the HT1080 cell line and mouse fetal brain primary neurons. No cell-type specificity was found.

Materials and Methods

Isolation of total RNA

The human fetal brain tissues were obtained from aborted fetuses. The mice were purchased from the Department of Animal Breeding (Central South University, Changsha, China). Total RNA was prepared from fresh tissues using the ready-to-use Trizol reagent (Gibco, Los Angeles, USA) according to the manufacturer's instructions. The total RNA samples were verified by 1.0% agarose gel electrophoresis.

The identification of the *KChIP4* gene subfamily

For reverse transcription, 5–10 μg of the total RNA was used in 20 μl of reaction mixture. The first cDNA strand was synthesized with Avian myeloblastosis virus reverse transcriptase (Promega, Madison, USA) and oligo$(dT)_{15}$. To determine whether the four variants of *KChIP4* are expressed in mouse or human fetal brain tissue, the primers were designed as following. The sense primer for mouse *KChIP4.1* (*mKChIP4.1*) cDNA amplification was 5′-CCA GCTCCCAGCGTCTTGGTCG-3′, and the sense primer for *mKChIP4.4* was 5′-GAC-CTAGCTGACCATGAACTTGGAG-3′. Given that the first exon of the *KChIP4.1* is identical to the first exon of *KChIP4.2* and *KChIP4.3*, and the expected lengths of their sequences are different from each other, the sense primers for *mKChIP4.2* and *mKChIP4.3* were the same as that of *mKChIP4.1*. The antisense primer for the amplification was 5′-GTGGTCCGTGTCGAATGCATTGAAGAG-3′. The sense primer for the human *KChIP4.1* (*hKChIP4.1*) and *hKChIP4.2* cDNA amplification was 5′-GACATG-AATGTGAGGAGGGTGGAAAg-3′; the sense primer for *hKChIP4.3* was 5′-CATTCGCTGTGTACCAAG-TGCTGTCC-3′; the sense primer for *hKChIP4.4* was 5′-ATGAACTTGGAAGGGCTTGAAATGATAG-3′. The antisense primer for the four *hKChIP4* splicing variants was 5′-GTCTGTTGGATTCAGGATCTATTTGACAAG-3′. The polymerase chain reaction (PCR) process included 28 cycles of denaturing for 30 s at 95 °C, 30 s of annealing at 58 °C and 90 s of extension at 72 °C. The amplified cDNAs were analyzed on 6% polyacrylamide gel. After being stained with ethidium bromide, the gel images under ultraviolet light were captured. The identity of each resulting product corresponding to a particular cDNA was confirmed by DNA sequencing. β-actin gene was used as an internal control with sense primer 5′-CCCAGATCA-TGTTTGAGACCTTC-3′ and antisense primer 5′-GTCAGGTCCCGGCCAGCCAGGTCC-3′. The PCR was performed using 22 cycles, and the length of amplified β-actin cDNA was 199 bp.

Isolation and characterization of promoter sequence

Using bioinformatics prediction, a 325 bp promoter fragment (which was called P520) of *hKChIP4.1* and an 818 bp promoter fragment (which was called P183) of *hKChIP4.4* were amplified by PCR, respectively. "GC" was added to the 5′ end of the primers for protection. The *Kpn*I restriction enzyme recognition site (*GGTACC*) on the sense primers and the *Bgl*II restriction enzyme recognition site (*AGATCT*) on the antisense primers were designed to facilitate the subcloning of the promoter fragments into the reporter gene vector. For P520, the sense primer was 5′-CC*GGTACC*GTCCTTCG-AACTCAGCCTAACTTTGTGC-3′ and the antisense primer was 5′-CG*AGATCT*CTGCCAAGTCACCGGAC-3′; for P183, the sense primer was 5′-CC*GGTACC*C-CAGAATATTAGTGCTCAGTGAC-3′ and the antisense primer was 5′-CG*AGATCT*CTGAGAAGTGCTC-

CTCTGCCTGGC-3′. The primers were synthesized by the BioAsia Company (Shanghai, China). The template for the PCR reaction was the genomic DNA extracted from normal human tissue preserved in our laboratory. The *LA Taq* amplification system with GC buffer (TaKaRa, Shiga, Japan) was used because the total content of G and C is more than 60% within the sequences. The restriction enzymes used in the experiments were purchased from TaKaRa.

The PCR product was ligated into the pGEM T-vector (Promega) and confirmed by sequencing. The identified fragments with the promoter sequence were used to construct the pCAT-enhancer vector (Promega), which contained an SV40 enhancer sequence and was able to detect the infirmable promoter activity through its reporter system. The plasmids used for cell transient transfection were extracted using the Tip-100 system (Qiagen, Netherlands/Germany).

Cell culture and transient transfection

The HT1080 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (FBS), 100 u/ml penicillin and 100 u/ml streptomycin (Invitrogen, Carlsbad, USA). The primary neuron cultures were prepared from the brains of mouse embryos aged 17–18 days as described by Ayoubi and van de Ven [16]. The neuronal cells were grown in a neurobasal medium supplemented with B27 (Life Technologies) and 20 ng/ml of basic fibroblast growth factor (Sigma). The HT1080 cells were plated to 60%–70% confluence in 24-well tissue culture dishes and transfected on the following day. The primary cells were plated at a concentration of $2–3\times10^6$ cells/ml in the 12-well tissue culture dishes covered with poly-*D*-lysine substrate (135 kDa, Sigma). All the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The promoter activity of the P520- and P183-CAT-enhancer constructs was tested in the HT1080 cells and the primary neuronal cells. The constructed plasmids (1.5 μg/well) were transiently transfected (in triplicate) into each type of cell in serum-free medium using 2–3 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were further cultured at 37 °C for 24–48 h. For the HT1080 cells, the pRSV-galactosidase reporter vector (Promega) was co-transfected as an internal control. In the primary neuronal cells, the expression of β-gal was too low to be detected using the β-galactosidase reporter system, so the single-plasmid transfection method was applied.

CAT assays, β-galactosidase assays and detergent compatible (DC) protein assays

The transfected cells were harvested after two washes with cold phosphate-buffered saline (pH 7.4), and then incubated with 100 μl of reporter lysis buffer (Promega) for 15 min at room temperature. The cell extracts were incubated in a reaction mix containing ^{14}C-labeled chloramphenicol and *n*-butyryl coenzyme A. Then the CAT activities of the extracts were determined. The β-galactosidase activities of the transfected cells were measured by the luminescent β-galactosidase genetic reporter system (Promega). CAT activities of the primary neuronal cells were normalized by total protein concentration with the DC protein assay reagent (Bio-Rad, Hercules, California, USA), and those of the HT1080 cells were revised by β-galactosidase activities. Each assay was performed in triplicate.

Microscope observation

To determine whether both the promoter sequences can initiate the transcription of the reporter gene, the *CAT* gene in the previous constructs was replaced by the green fluorescent protein (GFP) gene. The *GFP* gene was recovered from the pEGFP-N1 expression vector that was digested with *Nco*I and *Xba*I. The new constructs, which contained the promoter of the *KChIP4* gene and the GFP coding sequence (CDS) fragment, were called P520-GFP and P183-GFP, respectively. The *CAT* gene fragment of the pCAT-control plasmid and the pCAT-enhancer plasmid was also replaced by the GFP CDS fragment, and termed as the pGFP-positive control plasmid and pGFP-enhancer vector. They were used as the positive control and the negative control, respectively. The plasmids were transfected into the HT1080 cells using Lipofectamine 2000. We observed the live transfected HT1080 cells using a Zeiss IM 35 microscope with a 10× objective (Plan-Neofluar, NA 0.75, Newark, USA) and photographed the cells.

Results

By correlating our results with the National Center for Biotechnology Information (NCBI) database, the present study indicates that there are at least four splicing variants, *KChIP4.1*, *KChIP4.2*, *KChIP4.3* and *KChIP4.4*, for the human *KChIP4* gene, and the N-terminus of the proteins that these variants transcribed varies from one to the other. For the mouse *KChIP4* gene, only the *mKChIP4.1* and

mKChIP4.4 cDNA sequences have been recorded in the NCBI database, which are different from those of human beings. The human *KChIP4* gene was located at chromosome 4p15.31 (accession No. NT_006316.16) and spanned over 1,219,331 bp in the genome. There were 11 exons appearing in the *hKChIP4* gene (**Fig. 1**) and exons 5–11 were found to be expressed at the C-terminus of all the four isoforms. The analysis of the gene structure showed that the physical distances between two neighboring exons of exons 1–4, such as exon 1 and 2, exon 2 and 3, and exon 3 and 4, in the human genome were about 185 kb, 458 kb, and 421 kb, respectively. The physical distance between the remaining immediate exons was no longer than 100 kb. Although they have the common exons 5–11 in their ORFs, which indicats that the expressed isoforms have same C-termini, the exons at the 5′ of these variants are different from each other. *KChIP4.1* contained exon 1 and 4; *KChIP4.2* contained exon 1; *KChIP4.3* contained exon 1 and 2; and *KChIP4.4* contained only exon 3 (**Fig. 1**). To date, in the mouse *KChIP4* gene, only the *mKChIP4.1* and *mKChIP4.4* isoforms have been reported in the NCBI database. On the assumption that the human and mouse genes might have a similar gene structure, the cDNA structure of the *mKChIP4.2* variant can be deduced as shown in **Fig. 1(C)**. Therefore, the sequence of the *mKChIP4.2* does not contain the second exon (exon 4) of the *mKChIP4.1*. Nevertheless, this kind of analogy can not be extended to the *mKChIP4.3* because the second exon of *hKChIP4.1* is replaced by another fragment (exon 2) in the human genome, and this exon of human *KChIP4.3* can not be in homologous alignment with a fragment in the mouse genome. Because of the various combinations of the exons in the gene structure, these variants showed distinct functions in the physiologic and biochemical process.

RT-PCR revealed that three of the four splicing variants, *KChIP4.1*, *KChIP4.2* and *KChIP4.4*, were expressed in the mouse and human fetal brain tissues (**Fig. 2**). Our study is the first to identify the expression of *KChIP4.2* in the mouse fetal brain. However, RT-PCR could not detect the *KChIP4.3* isoform in mouse or human fetal brain tissues. This might be because the abundance of the mRNA is too low to be detected by RT-PCR, or that this isoform is expressed in a spatio-temporal manner different from those of the other three isoforms.

In accordance with the predictive analysis of the genomic sequence of *KChIP4* using the Promoter inspector software (Genomatrix, Munich, Germany), we isolated and cloned two promoters of the *KChIP4* gene. One was a 325 bp fragment at the 5′ UTR of the *KChIP4.1* mRNA sequence, and the other was an 818 bp fragment at the 5′ UTR of the *KChIP4.4*. Both of them were used to construct the pCAT-enhancer vector and transfected into the HT1080 cell line and primary neurons of the fetal rat brain by means of Lipofectamine 2000. The pCAT-control plasmid was used as the positive control and the

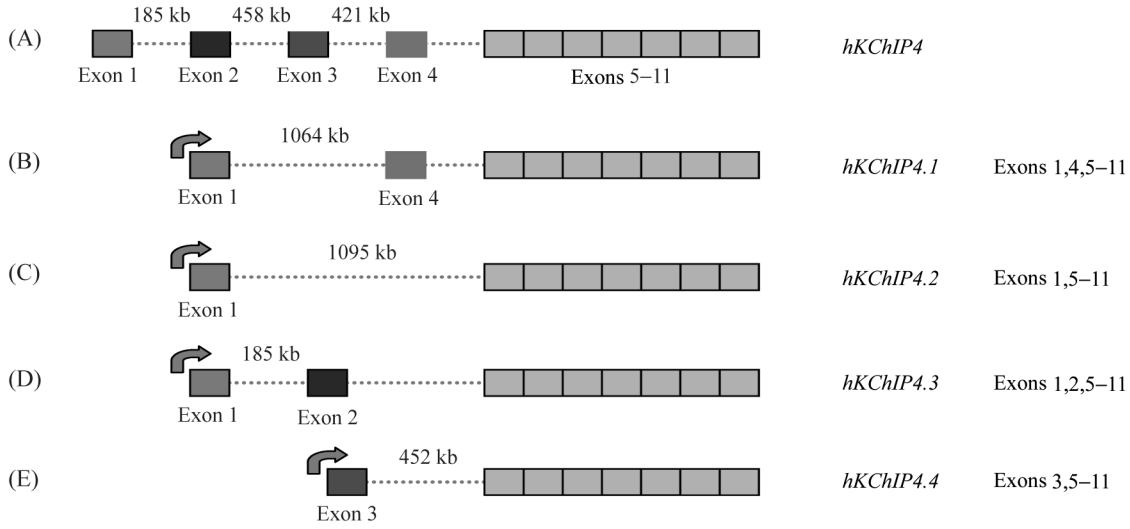

Fig. 1 The structure of the human *KChIP4* gene

(A) Structure of the gene in the genome. Exons are represented as open boxes, and introns are depicted as horizontal lines. (B–E) Structures of *KChIP4.1*, *KChIP4.2*, *KChIP4.3* and *KChIP4.4*, respectively.

blank pCAT-enhancer vector was used as the negative control in the cell transfection experiments. The alternative promoter activities were detected both in the HT1080 cell line [**Fig. 3(A)**] and primary neurons of the fetal rat brain [**Fig. 3(B)**]. The biostatistical analysis of the transfectional experiments showed that the promoter activity of the P520 fragment was 46.20%±2.92% (n=3, P<0.01) of that of the positive control, and 42.78 times that of the negative control. The promoter activity of the P183 fragment was 35.43%±1.25% (n=3, P<0.01) of that of the positive control, and 31.97 times that of the negative control. In primary neurons, the promoter activity of the P520 fragment was 22.72%±1.22% (n=3, P<0.01) of that of the positive control and 10.7 times that of the negative control, while the activity of the P183 promoter was 16.00%±0.31% (n=3, P<0.01) of that of the positive control and 7.54 times that of the negative control. These results suggest that both of the promoters can significantly initiate the reporter gene expression in HT1080 cells and primary neurons without cell-type restriction.

Further evidence that can confirm the transcriptional activity of the isolated fragments was also obtained in the cell transfection experiments. The *GFP* reporter genes driven by both the P520 and P183 promoters were all expressed in the HT1080 cells [**Fig. 4(A–D)**].

Discussion

It has been reported that the KChIP4 isoforms act as the auxiliary proteins to regulate the properties of the

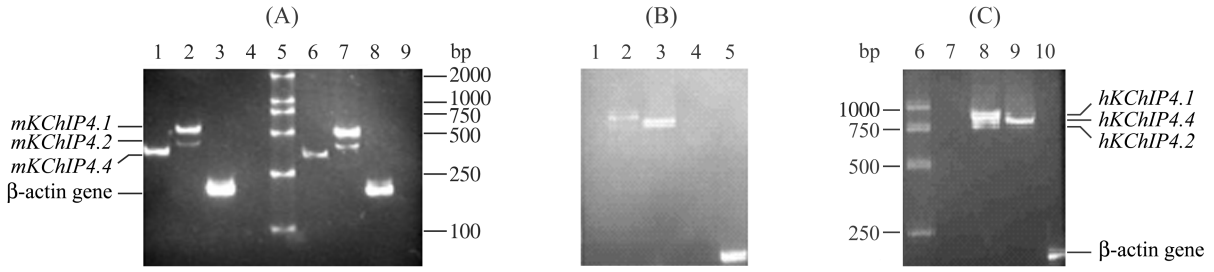

Fig. 2 Identification of the isoforms of *KChIP4* genes in mouse and human fetal brain tissue

(A) RT-PCR analysis results of the fetal brain tissue total RNA from two mice. 1–4, results from one mouse; 6–9, results from another mouse. 1 and 6, represent the amplified products of *mKChIP4.4*; 2 and 7, represent the amplified products of *mKChIP4.1* and *mKChIP4.2*, respectively; 3 and 8, represent the β-actin gene as the internal control; 4 and 9, were the negative controls; 5, marker. (B,C) Analysis results of the fetal brain tissues from the human being. (B) PCR results from the human fetal brain cDNA library. (C) RT-PCR analysis results of the fetal brain tissue total RNA from the human being. 1, the negative control; 5 and 10, the β-actin gene as the internal control; 2 and 8, *hKChIP4.1* and *hKChIP4.2*, respectively; 3 and 9, *hKChIP4.4*; 4 and 7, amplified results of *hKChIP4.3*; 6, marker.

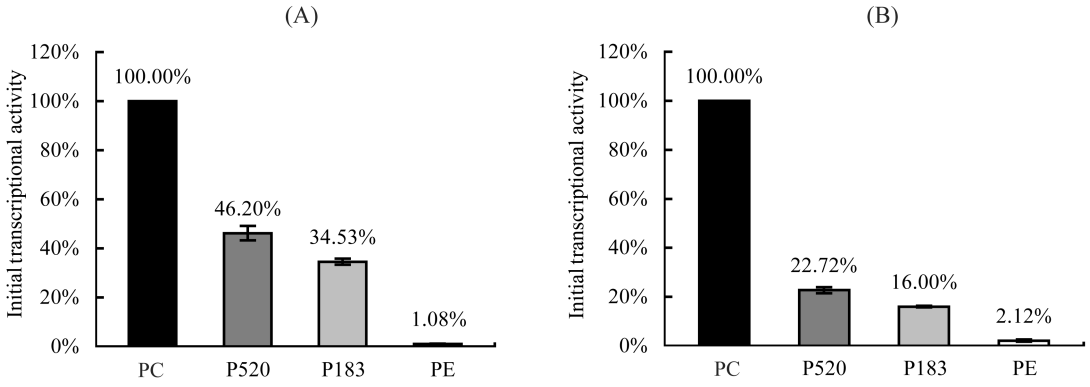

Fig. 3 The initial transcriptional activity of the P520 and P183 fragments in the HT1080 cells and the primary neurons

(A,B) The initial transcriptional activity of P520 and P183 fragments in the HT1080 cells and the primary neurons, respectively. PC, represents the relative activity of pCAT-control, PE, represents the relative activity of pCAT-enhancer.

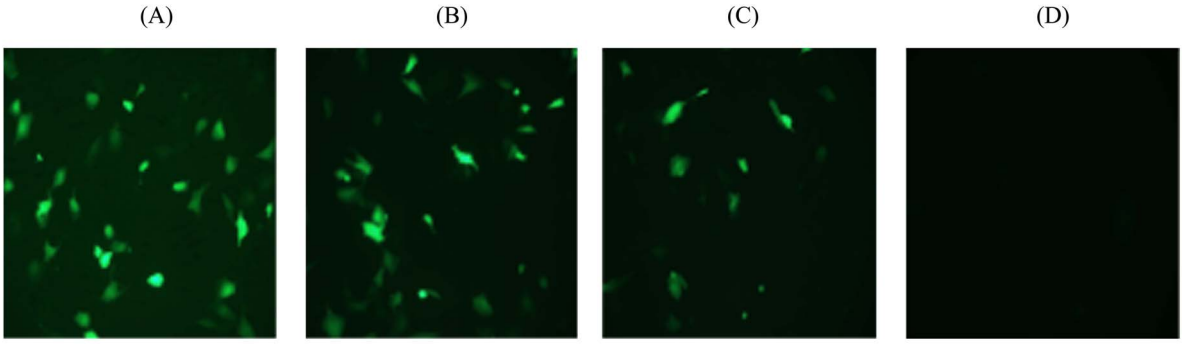

Fig. 4 Promoter fragments initiating the expression of the GFP in the HT1080 cells

(A) The positive control. (B,C) Initiation of the transcriptional activity of the P520 and P183 fragments, respectively. (D) The negative control. All the photographs were taken at 100× magnification.

voltage-dependent potassium channels [10,13,14]. The effects of KChIP4.1 and KChIP4.4 on the Kv4 channels are different [13,14]. One hypothesis is that the difference in the transcriptional splicing at the 5′ end leads to the different N-terminus of the KChIP4.1 and KChIP4.4 isoforms. In the present study, we first analyzed the expression of different variants or the *KChIP4* gene in the fetal brain tissues of mouse and human being. Three of the four splicing variants were identified to be expressed in the mouse and human fetal brain tissues. However, the *KChIP4.3* variant could not be detected either in the human fetal brain tissue or the mouse fetal brain tissue. Possible reasons are that the abundance of this isoform is too low to be detected by RT-PCR, or that it is expressed in a different spatio-temporal manner.

Promoter prediction was carried out using the Promoter inspector software, and the proximal promoter regions of the *KChIP4* gene were cloned and characterized. Our results indicate that an alternative promoter for the *KChIP4* gene exists. The promoter of the *KChIP4.1* variant is located at nucleotide –309 to +176 corresponding to the transcription-initiating site, which contains a CpG island located at nucleotide –370 to +287. Similar to many eukaryotic promoters, this promoter lacks the typical TATA and CAAT boxes. However, it contains four potential Sp1-binding elements, including 5′-TCTGGGGAGGGGCTC-3′ at position –203, 5′-CGGGGGGCGGGGTGC-3′ at position –24, 5′-GCAGGGGCGGCGCTC-3′ at position +24 and 5′-AAGCGGGCGGGCTGC-3′ at position +81, and a core promoter-binding protein (CPBP) element, 5′-GTGAGCGCCGCCCCTGCCACCTG-3′ at position +18 (MatInspector release professional 7.2.2, 2004; Genomatrix, Munich, Germany). The promoter of the *KChIP4.4* variant lacks the canonical TATA and CAAT motifs, but has a high C+G content (approximately 60%) in the promoter region. Surprisingly, it contains more than 40 putative GAGA box elements, 5′-GAGAGGGAG-AGAGAGAG-3′, between position –160 and +18. Furthermore, we tested the activity of the two promoter fragments. Interestingly, the results indicate that the two promoter fragments promote dramatic transcriptional initiation activities in both HT1080 cells and the mouse fetal brain primary neurons as shown by the CAT assay system and GFP reporter gene expression (**Figs. 3, 4)**. These findings indicate that the expression of the *KChIP4* gene may be tightly regulated at the transcriptional level. Additionally, it has been reported that the *KChIP4* gene is specifically expressed in the central nervous system of human beings and other mammalians [13], but in our study, it is obvious that both promoter fragments do not show any significant transcriptional difference in neuronal and non-neuronal cells. The reason may be that the promoter fragments we isolated did not contain the elements for tissue-specific transcription, or there may be other regulatory factors involved in the transcription initiation process.

The utilization of multiple promoters and transcription start sites is a frequently used method to create diversity and flexibility in the regulation of gene expression [16]. The level of transcription initiation varies between different promoters. The turnover or translation efficiency of mRNA isoforms with different leader exons can differ, alternative promoters have different tissue specificity and react differently to growth signals, and alternative promoter usage can lead to the generation of protein isoforms that differ in the amino acid sequence. The use of alternative exons at the 5′ end of the *KChIP4* splicing variants seems

to be suitable for the different regulation of the gene to the Kv4 channel. According to the histone code hypothesis that different histone amino-terminal modifications can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn direct dynamic transitions between transcriptionally active and silent chromatin states [17], it is possible that some elements of the alternative promoter sequences which are responsible for the cell type-specific may locate out of the region. Unlike the CpG sites in the remainder of the genome, CpG islands in the promoter region are almost always maintained in unmethylated states, although the methylation of CpG islands occur on an inactive X chromosome, in promoters of imprinted genes, with oncogenesis, and during aging. In all of these cases, methylation of CpG islands spanning the promoter regions is strongly associated with transcriptional silencing. In addition to the effect of the CpG islands on gene transcriptional activities, there may be other factor(s) affecting the regulation of *KChIP4* gene expression.

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Edited by
Nai-He JING