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Research report

Cloning and developmental expression of *Shaker* potassium channels in the cochlea of the chicken☆

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Abstract

Signal coding by the receptor and neuronal cells of the auditory system involves various ion channels that modulate a sound stimulus. The genes that encode a number of these ion channels and their accessory subunits are presently unknown for channels found in the sensory epithelium and cochlear nerve. Among these genes are those that encode delayed rectifier and transient type potassium channels found in both the sensory cells and the ganglion. Here, we report the cloning and developmental expression of *Shaker* family members that include cKv1.2, cKv1.3, cKv1.5, and the *Shaker*-related cGMP-gated potassium channel cKCNA10. Clones were obtained by screening a chicken embryonic cochlea cDNA library using, as a probe, a mixture of two DNA fragments of cKv1.2 and cKv1.3 obtained by the reverse transcription polymerase chain reaction (RT-PCR). Sequence analysis revealed chicken homologues of Kv1.2, Kv1.3, Kv1.5 and cGMP-gated potassium channels with a deduced amino acid homology of 96–98%, 82–84%, 67–71% and 67–79% to correspondent mammalian homologues. During development of chicken inner ear, RT-PCR studies show expression of cKv1.2, cKv1.3 and cKv1.5 as early as Embryonic Day (ED) 3, while cKCNA10 was detected at low levels beginning on ED6 and was highly expressed by ED9. Additionally, analysis of expression in different parts of the cochlea showed that these genes were co-expressed in different regions of the cochlea, including the cochlear ganglion, sensory epithelium, lagena, and tegmentum. This expression pattern suggests the potential for the formation of heteromeric channels from the corresponding α-subunits in these various tissues.

Theme: Cellular and molecular biology
Topic: Gene structure and function: general

Keywords: Chicken; *Shaker* potassium channel; Development; cDNA cloning

1. Introduction

Voltage-gated potassium channels of the delayed rectifier type play a role in coding auditory and vestibular stimuli in a number of different vertebrates [15,16,22]. Previously, an outwardly rectifying type current (I_K) was reported in hair cells of the adult chick cochlea in regions where there are low frequency, calcium-generated action potentials [5]. Frequency coding at this end of the epithelium is towards the low end of the spectrum. More recently, a delayed rectifier type current, which was tetra ethylammonium (TEA)-sensitive, was reported in Embryonic Day 3 (ED3) cochlear ganglion cells [26]. These channels are the first conductances acquired during the development of this system, as observed in whole-cell tight-seal recordings made from both isolated hair cells and ganglion cells [6,26]. Thus, their contribution in forming action potentials may be crucial to maintaining cellular homeostasis during early development. Presently, however, we are only beginning to identify the genes that encode these channel subtypes.

Several gene subfamilies encode voltage-gated channels with outwardly rectifying characteristics, as shown by their expression in *Xenopus* oocytes [28,31]. These include members of the gene subfamilies *Shaker*, *Shab*, and *Shaw*. All members of the mammalian *Shaker* subfamily, except Kv1.4, demonstrate delayed rectifier type of current kinetics, when expressed alone, but switch to transient type when

☆ Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s): cKv1.2—AY329361, cKv1.3—AY329363, AY329366, cKv1.5—AY329364, AY329365, cKcna10—AY329362.

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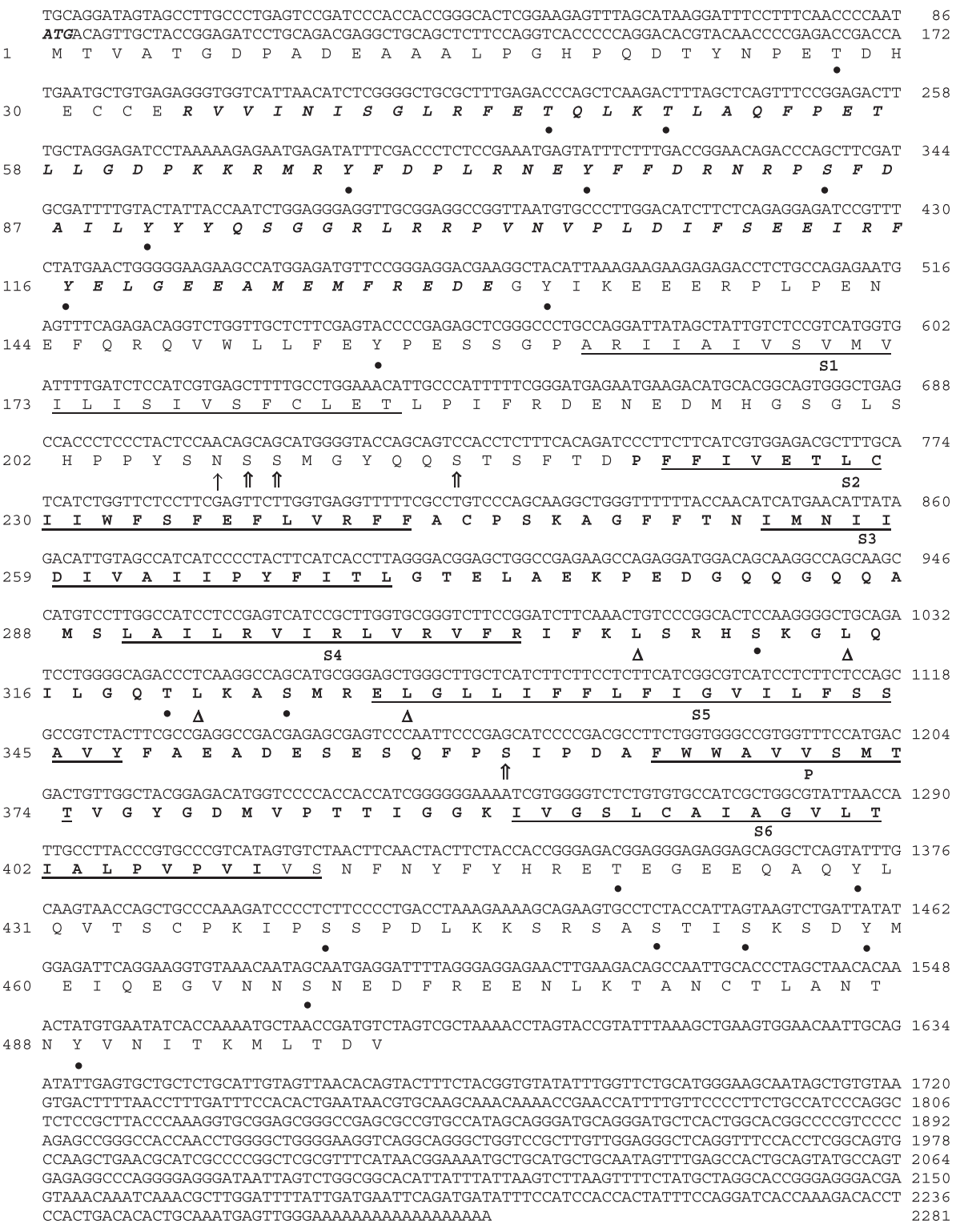
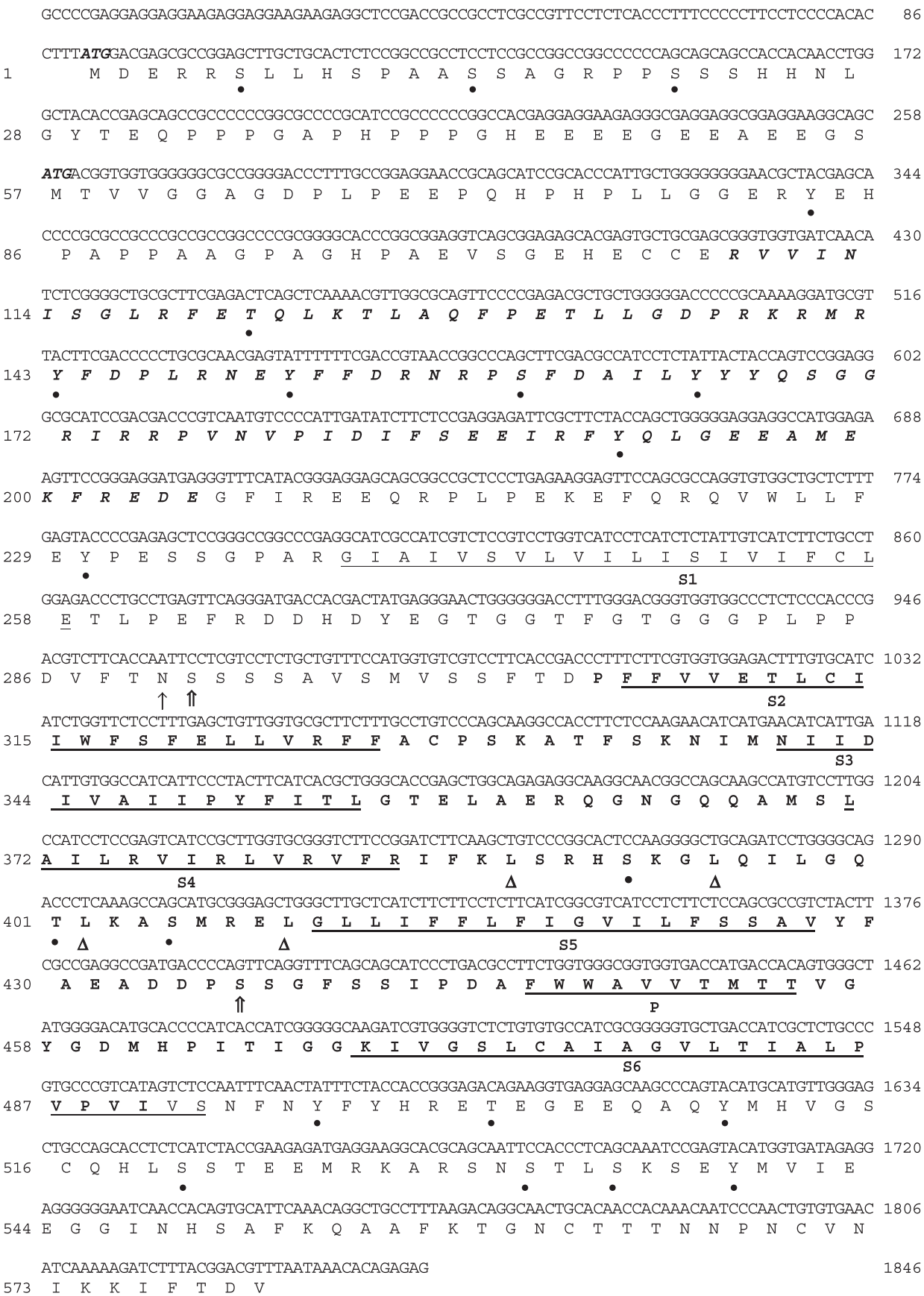


Fig. 1. Nucleotide and deduced amino acid sequence of cKv1.2. Amino acids from the cytoplasmic tetramerization domain and the start codon are shown in bold italics, and the ion transport domain in boldface. Six transmembrane alpha-helices S1 to S6 and the pore region P are underlined. Potential phosphorylation sites are marked with closed circles, as follows: protein kinase CaMII phosphorylation sites at 46 and 451; casein kinase II phosphorylation sites at 27, 324, 421, 440, 454, 468; protein kinase A phosphorylation sites at 46, 84, 311, 421, 451; protein kinase C phosphorylation sites at 320 and 324, tyrosine kinase phosphorylation sites at 68, 76, 90, 116, 132, 429, 458 and 489. Triangles mark leucine in the leucine zipper region between S4 and S5, ↑ refers to putative N-linked glycosylation site at 207, ⇑ indicate O-linked glycosylation sites at 208, 209, 215 and 360.


S1
S2
S3
S4
S5
S6
P

coexpressed with Kvβ-subunits. More recently, other *Shaker*-related channels (rbKcn1, hKCNA10) were discovered with characteristics similar to nucleotide-gated channels [33,19]. The ability of different *Shaker* members to form heteromeric channels multiplies the variety of current characteristics that can be expressed by such channels, providing modulation of sensory stimuli from hair cells to afferent terminals [32].

Previously, we revealed the expression of potassium channel genes in the cochlea that are members of the *Shal* (cKv4.2) and *Shaker* subfamilies (cKv1.4) [21]. Here, we screen chick genomic and cDNA libraries using low stringency hybridization with probes containing parts of coding regions for *Shaker* subfamily members Kv1.2 and 1.3. These screenings resulted in the cloning of *Shaker*-family members cKv1.2, cKv1.3, cKv1.5, and *Shaker*-like channel cKCNA10. Expression of mRNA showed some similarities and differences during the course of embryonic development in the cochlea. Additionally, expression was localized to similar regions of the cochlea, suggesting the potential for forming delayed rectifier type potassium channels composed of heteromultimeric subunits.

2. Materials and methods

2.1. Chick DNA libraries

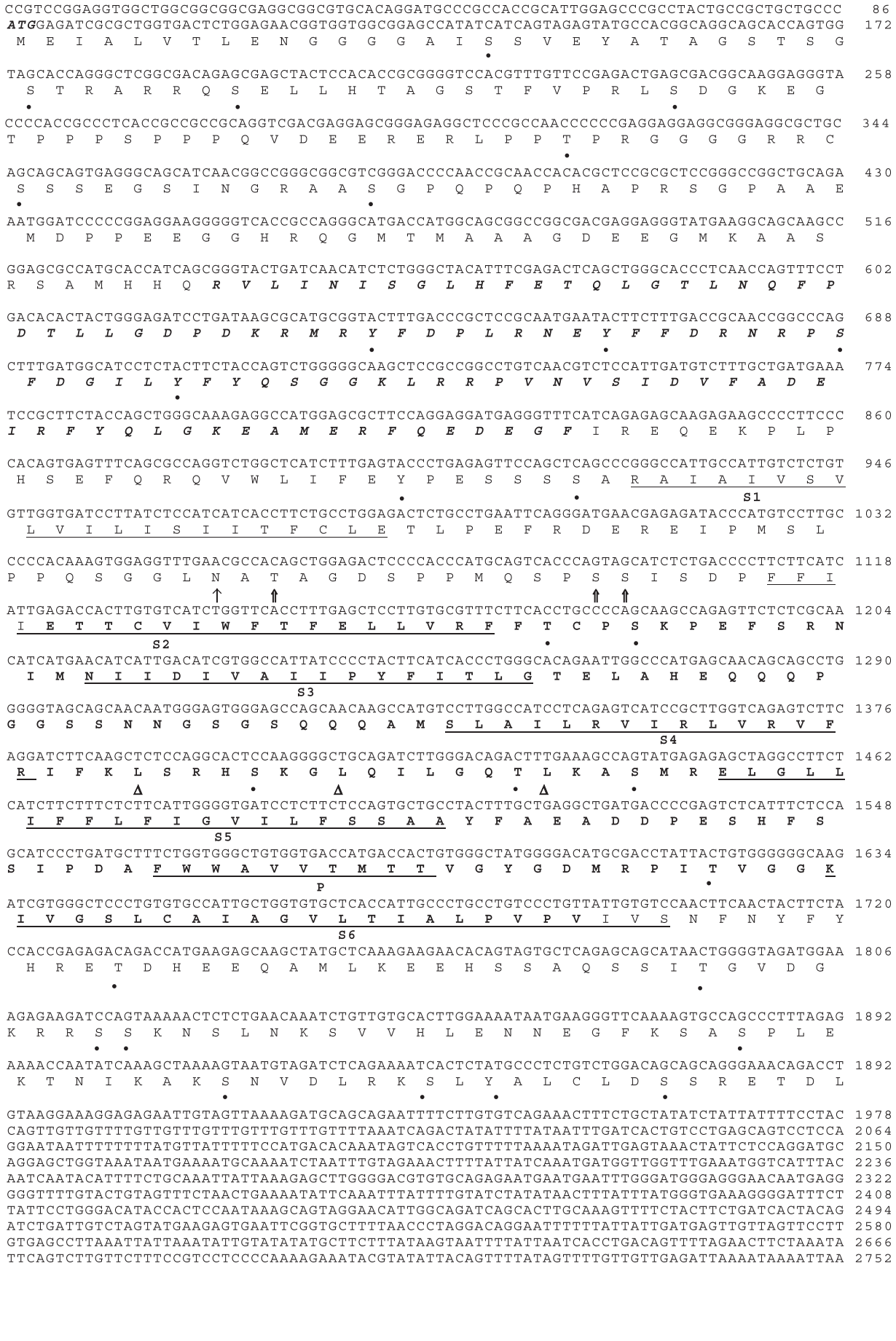
We obtained genes that encoded members of the *Shaker* subfamily by screening two different DNA libraries. The first was a cDNA library made from cochlear epithelial tissue of late embryonic chicken (days 14–19), *Gallus gallus* (gift from S. Heller, Massachusettes Eye and Ear Infirmary, Boston, MA, USA). This library was constructed by cloning cDNA fragments into the ZAPII vector by *Eco*RI and *Xho*I restriction sites (Stratagene, La Jolla, CA, USA). The second was made from genomic DNA using liver tissue from adult male chicken, *G. gallus* (Clontech, Palo Alto, CA, USA). This library was constructed by cloning genomic DNA fragments into the *Bam*HI restriction site of the EMBL3 SP6/T7 vector.

2.2. Screening for Shaker α-subunits

We screened the cDNA library, using a mixture of two DNA fragments labeled with ^{32}P. Using RNA isolated from chicken brain and primers, whose design was based on the rat cDNA sequence for the K^+ channel, RCK1 (accession no. X12598.1), we generated a fragment of approximately 0.9 kb in length using reverse transcription polymerase chain reaction (RT-PCR). The forward and reverse primers were: ATCAACATCTCCGGGCTGCGCTT (368–390 bp) and GTCTTGCTGGGGCAGGCGAAGAA (965–987 bp), respectively. The sequence of the derived fragment was homologous to the mammalian K^+ channel subunit, Kv1.2, which contained most of the region coding for the conservative tetra binding and ion transport domains of the *Shaker* subfamily. We generated a second fragment of approximately 0.6 kb in length using PCR. The primers for PCR were chosen based on a partial sequence, that was obtained previously in our laboratory for chick Kv1.3 (accession no. AF140426). The forward and reverse primers were GGGCGCATCCGACGACCGTCAAT and ACAGGCAAAGAAGCGCACCAACA, respectively. The sequence of this second fragment covered most of the region coding for the ion transport domain of Kv1.3. Fragments were purified from gel slices, using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and labeled with ^{32}P using a random priming kit (Invitrogen, Carlsbad, CA, USA). These fragments were used at a concentration of 1×10^6 dpm/ml for screening approximately 1.6×10^6 plaques from an amplified library. Hybridization was performed at 50 °C (calculated criterion of T_m − 30 °C assuming a 20% mismatch and 50% G + C content based on human and rat sequences) for 16 h in aqueous solution, containing 5 × Standard Sodium Citrate (SSC) (1 × SSC = 0.15 M NaCl, 15 mM Na citrate pH 7.0), 5 × Denhardt's, 0.5% sodium dodecyl sulfate (SDS) and 40 μg/ml sheared salmon sperm DNA (Invitrogen). Filters were washed three times for 30 min each in 2 × SSC/0.5% SDS at 50 °C, and one time in 0.1 × SSC for 15 min at 50 °C, air dried and exposed to Fuji X-ray film for 40–44 h at − 70 °C. Isolates with strong or moderate signals were plaque purified through three successive rounds of screening in the above-mentioned conditions. Plasmid clones were excised in vivo with the aid of ExAssist® helper phage (Stratagene) and analyzed further by Southern blot hybridization.

Based on sequencing data, primer pairs were designed to generate DNA fragments that had specific sequences for chicken Kv1.2, Kv1.3, Kv1.5 and KCNA10. The pairs of forward and reverse primers were, respectively, CTTTAGCTCAGTTCCGGAGACTT (nucleotides 235–258) and CCACTGCCGTGCATGCTTCATTC (nucleotides 659–682) for Kv1.2 (Fig. 1), GGGCGCATCCGACGACCGTCAAT (nucleotides 601–624) and ACAGGCAAAGAAGCGCACCAACA (nucleotides 1052–1074) for Kv1.3 (Fig. 2), CAAGCCAGAGTTCTCTCGCAACA (nucleotides 1184–1206) and GGTCATCAGCCTCAG-

Fig. 2. Nucleotide and deduced amino acid sequence of cKv1.3. Amino acids from the cytoplasmic tetramerization domain and the start codons for two ORFs are shown in bold italics, and the ion transport domain in boldface. Six transmembrane alpha-helices S1 to S6 and pore region P are underlined. Potential phosphorylation sites are marked with closed circles, as follows: protein kinase CaMII phosphorylation sites 21, 121 and 532; casein kinase II phosphorylation sites at 405, 502, 520, 535; protein kinase A phosphorylation sites at 6, 21, 121, 159, 392, 502, 532; protein kinase C phosphorylation sites at 401 and 405, tyrosine kinase phosphorylation sites at 83, 143, 151, 165, 191, 230, 496, 510 and 539. Triangles mark leucine in the leucine zipper region between S4 and S5, ↑ refers to putative N-linked glycosylation site at 290, ⇑ indicate putative O-linked glycosylation sites at 291 and 436.



CAAAGTAG (nucleotides 1508–1530) for Kv1.5 (Fig. 3) and ACTGGAGGATCCTCATCAACAGT (nucleotides 241–263) and AGGTGATTTCATCAGCAAAGACA (nucleotides 581–603) for KCNA10 (Fig. 4). The fragments generated (~ 0.3–0.5 kb) were labeled with ^{32}P and used for screening a genomic library. For each channel, approximately 450,000 plaques were screened, of which four isolates gave a strong signal. These isolates were purified through two additional rounds of screening. Hybridization of filters, containing λ phage plaques with ^{32}P labeled fragments during screening of the genomic library, was done at 65 °C. All other conditions were the same as for screening the cDNA library. Three to four isolated phage plaques for each unique probe were taken for phage amplification. Phage DNA was isolated using Qiagen kit and analyzed by Southern blot hybridization.

2.3. Sequence determination of selected isolates

Bluescript plasmids containing cDNA sequences and λ phage DNA containing genomic sequences of the corresponding channels were isolated using QIAGEN kits for plasmid or λ phage DNA preparation, respectively. Both strands of selected DNA were sequenced (Biotechnology Center, University of Florida, Gainesville, FL, USA) using fluorescent-labeled dideoxynucleotides and the AmpliTaq DNA polymerase, according to ABI Prism Dye Terminator cycle sequencing protocols (part #402078) developed by Applied Biosystems (Perkin-Elmer, Foster City, CA). The labeled extension products were analyzed on an Applied Biosystems Stretch DNA sequencer (Model 373, Perkin-Elmer). Oligonucleotide primers were designed using OLIGO 4.0 software (National Biosciences, Plymouth, MN, USA) and synthesized at the DNA Synthesis Core Laboratory (University of Florida). Nucleotide sequences were aligned and assembled using programs in the Sequencer 3.0 software package (Gene Codes, Ann Arbor, MI, USA). Lasergene software was used to determine multiple alignment of sequences and predict translation products. Transmembrane regions were predicted using the Dense Alignment Surface method [4] provided by the Transmembrane Prediction Protein Server (Stockholm Bioinformatics Center, Stockholm, Sweden). Putative phosphorylation and glycosylation sites were determined using the database from PhosphoBase 2.0, NetNGlyc 1.0, and NetOGlyc 2.0 provided by the Center for Biological Sequence Analysis (BioCentrum-DTU, Lyngby, Denmark). Tyrosine phosphorylation sites were determined manually based on the tyrosine kinase substrate sequence [27]. Tyrosine residues were counted as putative phosphorylation sites, if the surrounding sequence from −4 amino acids to +4 amino acids contained at least two specific amino acids with a score greater or equal to 1.5. The results obtained using these methods were verified using the PredictProtein Server of the Columbia University Bioinformatics Center (Columbia University, Department of Biochemistry and Molecular Biophysics, New York, NY).

2.4. Confirmation of intronless organization of the cKv1.5

To confirm the intronless organization of the cKv1.5 gene, we designed four pairs of primers (based on the received cDNA and part of the genomic sequence) so that sequences of PCR-generated DNA fragments would overlap and cover the coding region of this gene. These fragments covered the 75–531, 494–922, 556–1530, and 1483–1898 bp of the presented sequence (Fig. 3).

2.5. Detection of mRNA transcripts for Shaker α-subunits

The whole cochlear duct was excised from chicks aged ED 3, 6, 9, 10, 14 and post-hatched day 3 and 17. Tissues were dissected in sterile divalent saline containing (mM): NaCl 154, KCl 6, $MgCl_2$ ($6H_2O$) 2.3, $CaCl_2$ ($2H_2O$), 5.6, and buffered to pH 7.4 with 4-2-hydroxyethyl1-1-piperazine ethane-sulfonic acid (HEPES) titrated with 1 M NaOH. Also, the cochlear duct and its ganglion was microdissected into different tissue types that included the cochlear epithelium, lagena, cochlear ganglion, and tegmentum vasculosum. The cochlear epithelium was collected by stripping off the tectorial membrane and aspirating the sensory and supporting cells using a glass borosilicate micropipette. The remaining tissues were cut away from the cochlear duct using irridectomy scissors. Additionally, we removed an approximately 2 × 2 mm sample from the chicken cerebellum. Samples were frozen and stored at −70 °C.

Total RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. Intact cochlea or micro-dissected parts were homogenized in Tri Reagent in a Duall homogenizer with Teflon pestle (Fisher Scientific, Suwanee, GA, USA) or by pipetting, respectively. Yeast tRNA (Sigma, St. Louis, MO, USA) (10–20 μg per 1 ml of Tri Reagent) was added to tissue samples before homogenization for better quantitative recovery of RNA. DNA contaminants were removed using the DNaseI kit and protocol from

Fig. 3. Nucleotide and deduced amino acid sequence of cKv1.5. Amino acids from the cytoplasmic tetramerization domain and the start codon are shown in bold italics, and the ion transport domain in boldface. Six transmembrane alpha-helices S1 to S6 and pore region P are underlined. Potential phosphorylation sites are marked with closed circles, as follows: protein kinase CaMII phosphorylation sites 37, 87, 99, 363, 512, 578; casein kinase II phosphorylation sites 17, 87, 366, 452, 549, 569, 599, 610, 625; protein kinase A phosphorylation sites 37, 52, 87, 99, 201, 363, 439, 549, 578, 617; protein kinase C phosphorylation sites 30, 77, 278, 448, 452, 577, 625; PKG phosphorylation sites 37, 87, 578 and tyrosine kinase phosphorylation sites at 185, 193, 207, 272 and 619. Triangles mark leucine in the leucine zipper region between S4 and S5, ↑ refers to putative N-linked glycosylation site at 323, ⇑ indicate putative O-linked glycosylation sites at 336 and 337.

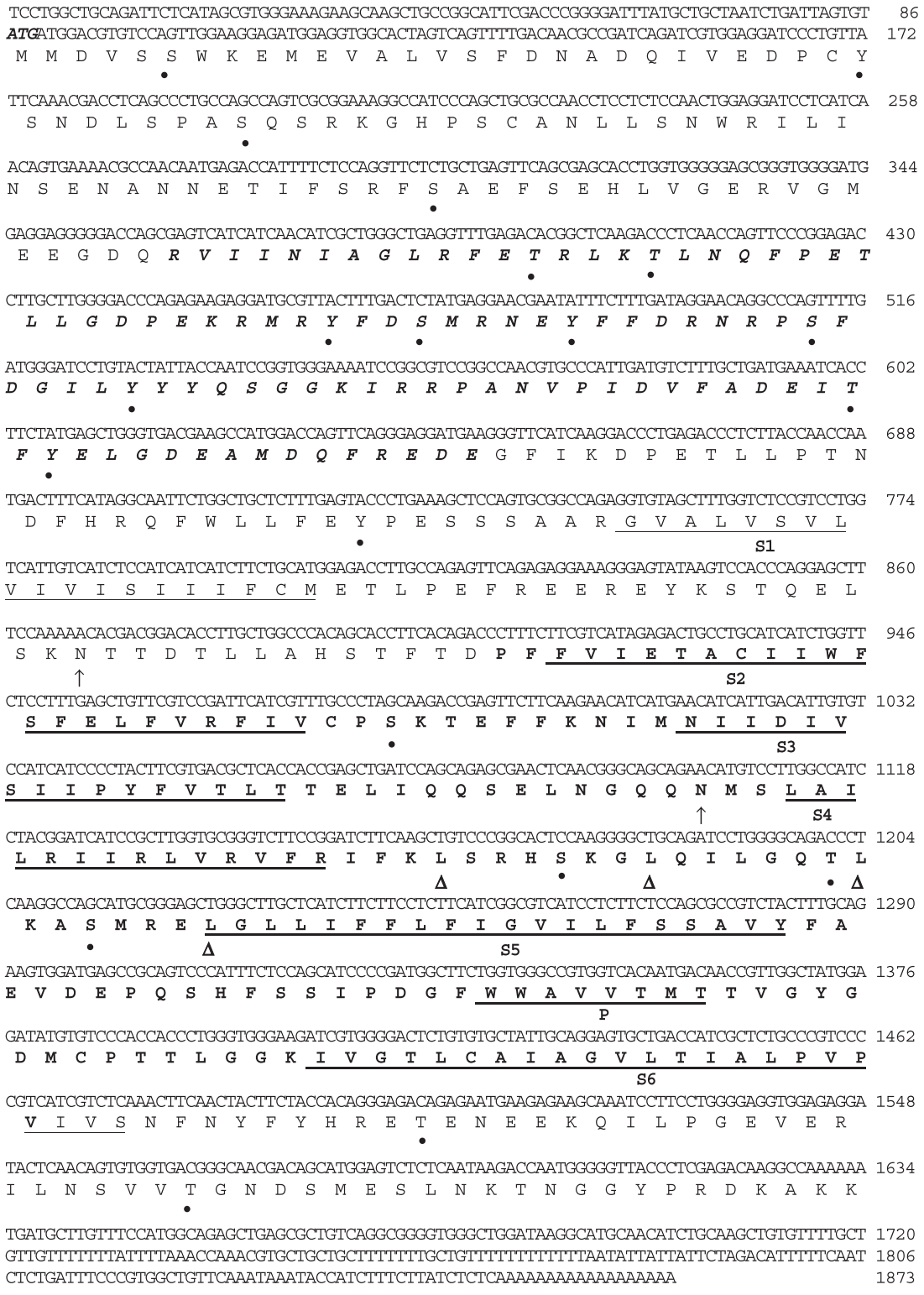


Fig. 4. Nucleotide and deduced amino acid sequence of cKCNA10. Amino acids from the cytoplasmic tetramerization domain and the start codon are shown in bold italics, and the ion transport domain in boldface. Six transmembrane alpha-helices S1 to S6 and pore region P are underlined. Potential phosphorylation sites are marked with closed circles, as follows: protein kinase CaMII phosphorylation sites 104 and 108; casein kinase II phosphorylation sites 6, 172, 300, 376, 473, 494; protein kinase A phosphorylation sites 72, 104, 108, 142, 363, 473; protein kinase C phosphorylation sites 6, 39, 129, 372, 376; tyrosine kinase phosphorylation sites at 29, 126, 134, 148, 174 and 213. Triangles mark leucine in the leucine zipper region between S4 and S5, ↑ refer to putative N-linked glycosylation sites at 261 and 339. Potential O-linked glycosylation sites have not been found.

GenHunter (GenHunter, Nashville, TN, USA) or TURBO DNase (Ambion, Austin, TX). Following phenol/chloroform (3:1) and chloroform extraction, RNA was ethanol precipitated for 30 min at −80 °C, spun at 12,000 × *g* for 10 min at 4 °C, washed with 75% ethanol, dried and dissolved in 20 μl DEPC-treated H_2O. Total RNA, in a quantity equivalent to one cochlea or four micro-dissected epithelia, ganglia, tegmenta or lagena was used in an RT reaction that was set at 58 °C for 1 h in a volume of 20 μl using a reverse primer specific for each *Shaker* isoform sequence (Section 2.2) and the Thermoscript™ reverse transcription system (Invitrogen).

RT products were amplified by PCR containing 2 μl of RT reaction mixture, 2 mM $MgCl_2$, 0.3–0.5 U Taq DNA polymerase (Fisher Scientific, GA), and 200 nM of primers mixed in a buffer containing 50 mM KCl and 10 mM Tris (pH 8.3), for a total volume of 25 μl. The forward and reverse primers (Integrated DNA Technologies, IA) for the channel genes were the same as those used for screening the genomic library. RT-PCR with primers for chick β-actin mRNA served as a positive control. The forward and reverse primers for chick β-actin were TGGATGATGATATTGCTGCG (nucleotides 2–18) and CTCCATATCATCCCAGTGG (nucleotides 248–229), respectively. PCR, where RNA was added instead of cDNA template, served as a negative control. Reactions were performed using a Perkin Elmer 2400 or PTC 200 (MJ Research, Waltham, MA) thermalcyclers. For cDNA samples obtained from total RNA from the cochlea, PCR was performed at 94 °C for 2 min, 28–40 cycles at 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and one cycle at 72 °C for 7 min. For cDNA samples made with RNA isolated from brain or micro-dissected parts, PCR was accomplished at 94 °C for 1 min, 35–37 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min, and one cycle at 72 °C for 10 min. Expected PCR products 448 (cKv1.2), 474 (cKv1.3), 347 (cKv1.5), 364 (cKCNA10) and 247 bp (β-actin) were observed on a 1% agarose gel, containing 0.5 μg/ml ethidium bromide. A 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a DNA size standard. The identity of these fragments was verified by sequencing.

3. Results

3.1. Isolation and sequencing analysis of chick Shaker channels

Primary screening of 1.6×10^6 phage plaques from the amplified cDNA library with probes homologous to mammalian Kv1.2 and Kv1.3 gene sequences produced 18 isolates with weak to strong signals. Eleven isolates with moderate to strong signals were purified by an additional two rounds of screening using the same probe. Plasmids containing the cloned fragments were excised in vivo from the λ phage vector and further analyzed by double restriction digestion with *Eco*RI and *Xho*I, followed by Southern blot hybridization using the same probe. Estimated sizes of the plasmid DNA inserts varied from 1.6 to 4 kb. All cloned fragments at this point were subjected to one step sequencing from the 5′ end of the cDNA. Also, one step sequencing from the 3′ end was performed for the three longest (i.e., longer than 3 kb) cDNA fragments. Homology search revealed that the sequences represented chicken homologues of the mammalian *Shaker* subfamily ion channels. Nine of them were homologous to the mammalian channel Kv1.2, one was homologous to the Kv1.5, and one was homologous to the cGMP-gated *Shaker*-like channel KCNA10. Based on the GenBank homology search results, cDNA sequences for cKv1.2 and cKCNA10 clones seemed to contain complete coding sequences (cds). In the event that our cDNA clones were not full-length, we screened a genomic chicken library to reconstitute the longest possible cKv1.2 and cKCNA10 open reading frames (ORFs). Additionally, we cloned, from the genomic library, cKv1.3 and cKv1.5 sequences containing complete cds. With probes specific for each channel, we screened approximately 450,000 plaques of amplified genomic library and obtained six primary isolates with strong signals. Three to four primary isolates were purified further through two additional screenings and checked with Southern blot hybridization. All of them contained inserts that were 8 to 20 kb in length. One or two final isolates were used for λ DNA isolation and sequencing. As a result, the longest ORF was defined for each of the cloned *Shaker* channel subunits.

For confirmation of intronless organization of one of the cloned genes (cKv1.5), we designed four pairs of primers (based on the received cDNA and part of the genomic sequence) in such a way, that sequences of PCR-generated DNA fragments would overlap and cover the coding region of this gene. These fragments covered the 75–531, 494–922, 556–1530, and 1483–1898 bp of the presented sequence (Fig. 3). Generated fragments appeared to have the same size and sequence no matter what template was taken for PCR: genomic DNA or cDNA.

3.2. Primary sequence analysis

The nucleotide and deduced amino acid sequences for the chicken Kv subunits are shown in Figs. 1–4, while protein sequence alignments are presented in Figs. 5–8. After analysis of the genomic sequences that lie upstream of the cDNA sequences that were determined previously, it was confirmed that the longest reconstituted ORFs for cKv1.2 (Fig. 1) and cKCNA10 (Fig. 4) are the same as those deduced from the cDNA sequences. The longest ORFs for cKv1.3 (Fig. 2) and cKv1.5 (Fig. 3) were reconstituted on the basis of their genomic sequences and partial cDNA sequence (for cKv1.5). Sequences CC uuuAUGG and gCAgCAUGa, which flank the AUG initi-

Fig. 5. Alignment of the deduced amino acid sequence of cKv1.2 with mammalian Kv1.2 channels. Mammalian homologues include human hKv1.2 (accession no. NP_004965), rabbit rbKv1.2 (accession no. AAF91476), mouse mKv1.2 (accession no. NP_032443), rat rKv1.2 (accession no. NP_037102) and dog dKv1.2 (accession no. A48672). ClustalW analysis revealed cKv1.2 has homology of 97.8% to hKv1.2, 97.6% to rbKv1.2, 97.2% to mKv1.2, 97.0% to rKv1.2, and 96.6% to dKv1.2.

ator codon in the first and second longest ORFs for cKv1.3 (Fig. 2), are similar to the Kozak sequence CC(A/G) CCAUGG, which is the eukaryotic consensus site for the initiation of translation [14]. Both sequences could contain the first AUG codons for translation initiation, if the corresponding mRNAs are transcribed from different promoters, as is the case for the Kv3 channel [7]. Also, the second longest ORF for cKv1.3 is considered a putative coding region, because the first few N-terminal amino acids constitute a block that is homologous to the amino acids at the very N-terminal end of the known mammalian Kv1.3 subunits (Fig. 6). The very N-terminal sequence of the longest ORF for cKv1.5 shows homology to the N-terminal sequences of mammalian cKv1.5 subunits (Fig. 7). The sequence ugcCCAUGG, which flanks the initiator codon AUG for cKv1.5, is partially homologous to the consensus sequence CC(A/G)CCAUGG [14].

Hydropathy analysis of all cloned cKv subunits (data not shown) revealed six potential membrane-spanning domains and one pore-forming domain similar to those found in other K^+ channels. The amino- and carboxyl-terminal ends for deduced protein sequences are proposed to be intracellular in accordance with prevailing models of K^+-channel structure [9]. Cytoplasmic tetramerization domain T1 (N-terminal end), and ion transport domains (region, including S2 to S6 transmembrane domains) compose the central, most conservative part of the sequences (Figs. 1–8). In all protein sequences, the fourth hydrophobic domain, S4, is considered the voltage-sensor in the voltage-gated potassium channel subfamily. This region contains seven basic amino acids that are number-specific for the *Shaker* subfamily ion channels [31]. Leucine zipper regions between the S4 and S5 transmembrane domains determine the tertiary structure of the channels, and the GYG signature sequences in the

Fig. 6. Alignment of the deduced amino acid sequence of the second longest ORF for cKv1.3 with mammalian Kv1.3 channels. Mammalian homologues include rat rKv1.3 (accession no. NM_019270), mouse mKv1.3 (accession no. 184205), human hKv1.3 (accession no. AAH35059), and rabbit rbKv1.3 (accession no. AAC24718). ClustalW analysis revealed cKv1.3 has homology of 84.4% to rKv1.3, 83.6% to mKv1.3, 81.6% to hKv1.3, and 81.6% to rbKv1.3.

pore regions determine specificity for potassium ions [10]. Both are present in all four protein sequences.

Reconstituted protein sequences of chicken Kv subunits showed different rates of divergence from their mammalian homologues (Figs. 5–8). cKv1.2 appeared to be the most conservative, with 97–97.8% homology to mammalian Kv1.2 subunits. In decreasing homology, cKv1.3 had 81.6–84.4%, cKv1.5 showed 67.3–70.5%, and cKCNA10 had 66.9–79.1% identity to corresponding mammalian homologues. Differences along the protein sequences were distributed unevenly. Most of the differences occur in the N-terminal end of the protein sequence (excluding the T1 domain), the C-terminal end, and the extracellular loops between transmembrane regions. A comparison of these regions between the chicken and mammalian homologues shows differences that gradually increase as follows: cKv1.2 < cKv1.3 < cKv1.5 < cKCNA10. For cKv1.2, these differences include two amino acids in the N-terminal end, one amino acid in the S3–S4 extracellular loop, and two amino acids between the S5 and P regions (Fig. 5). However, the main differences between chicken and mammalian sequences for this subunit lie in the extracellular loop between S1 and S2 transmembrane domains (amino acids 198–210), in which eight of 13 amino acids are different than in mammalian homologues (Fig. 5). This region is not important for the channel's current conductance, but it is needed for proper membrane localization. This region contains one N- and two O-putative glycosylation sites.

For cKv1.3, the most extensive differences in amino acid sequence lie in the N-terminal end upstream to the T1

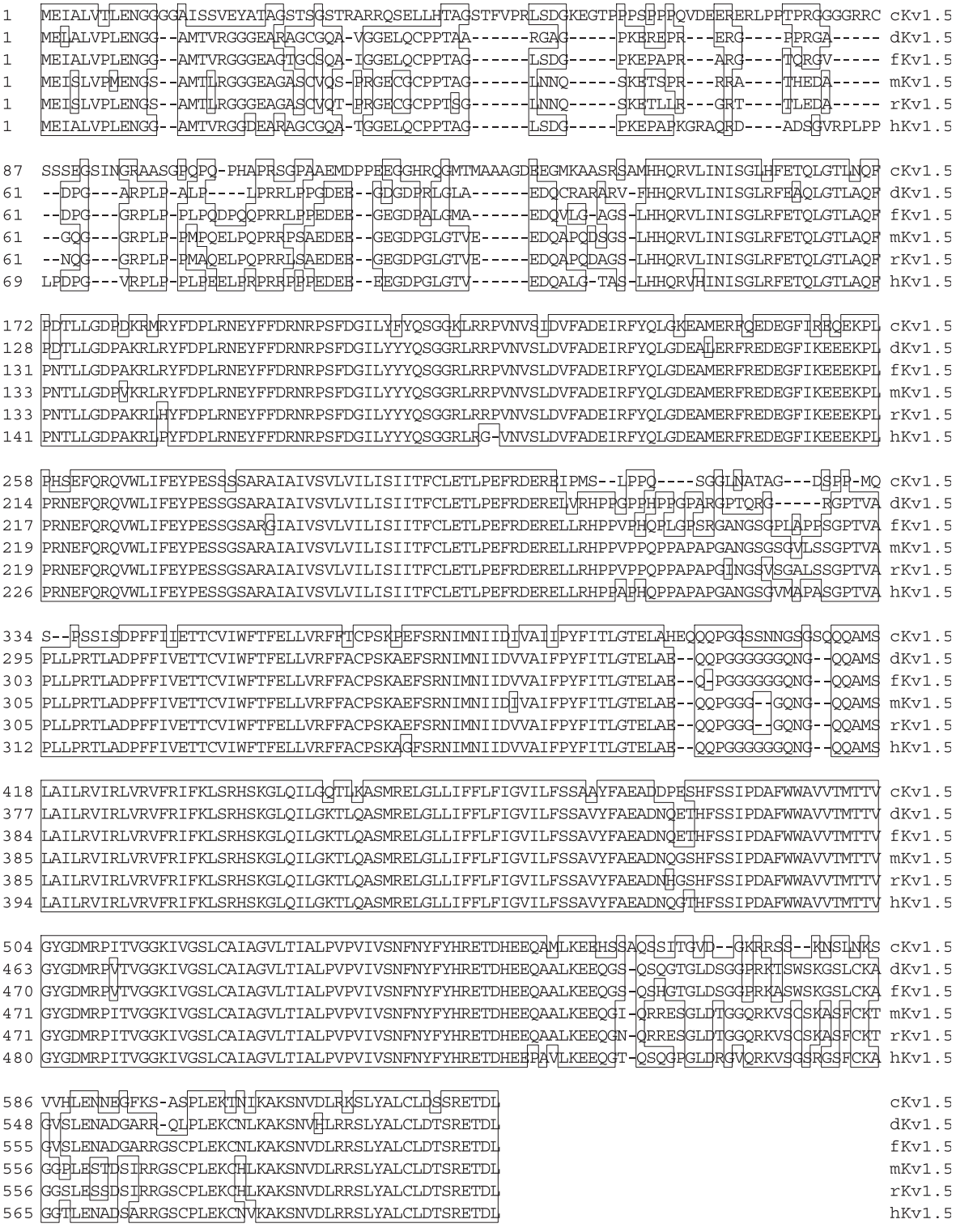


Fig. 7. Alignment of the deduced amino acid sequence of cKv1.5 with mammalian Kv1.5 channels. Mammalian homologues include dog dKv1.5 (accession no. AAA57320), ferret fKv1.5 (accession no. P79197), mouse mKv1.5 (accession no. AAG40241), rat rKv1.5 (accession no. NP_037104), and human hKv1.5 (accession no. AAA61276). ClustalW analysis revealed cKv1.5 has homology of 70.5% to dKv1.5, 68.4% to fKv1.5, 68.1% to mKv1.5, 68.1% to rKv1.5, and 67.3% to hKv1.5.

domain, the extracellular loop between S1 and S2 transmembrane domains, and the C-terminal end (Fig. 6). The longest cKv1.3 ORF shows no homology to any of the mammalian Kv1.3 subunits at the N-terminal end, from the first to the fifty-sixth amino acid. The fifty-seventh amino acid of the longest ORF, Methionine, is also a first amino

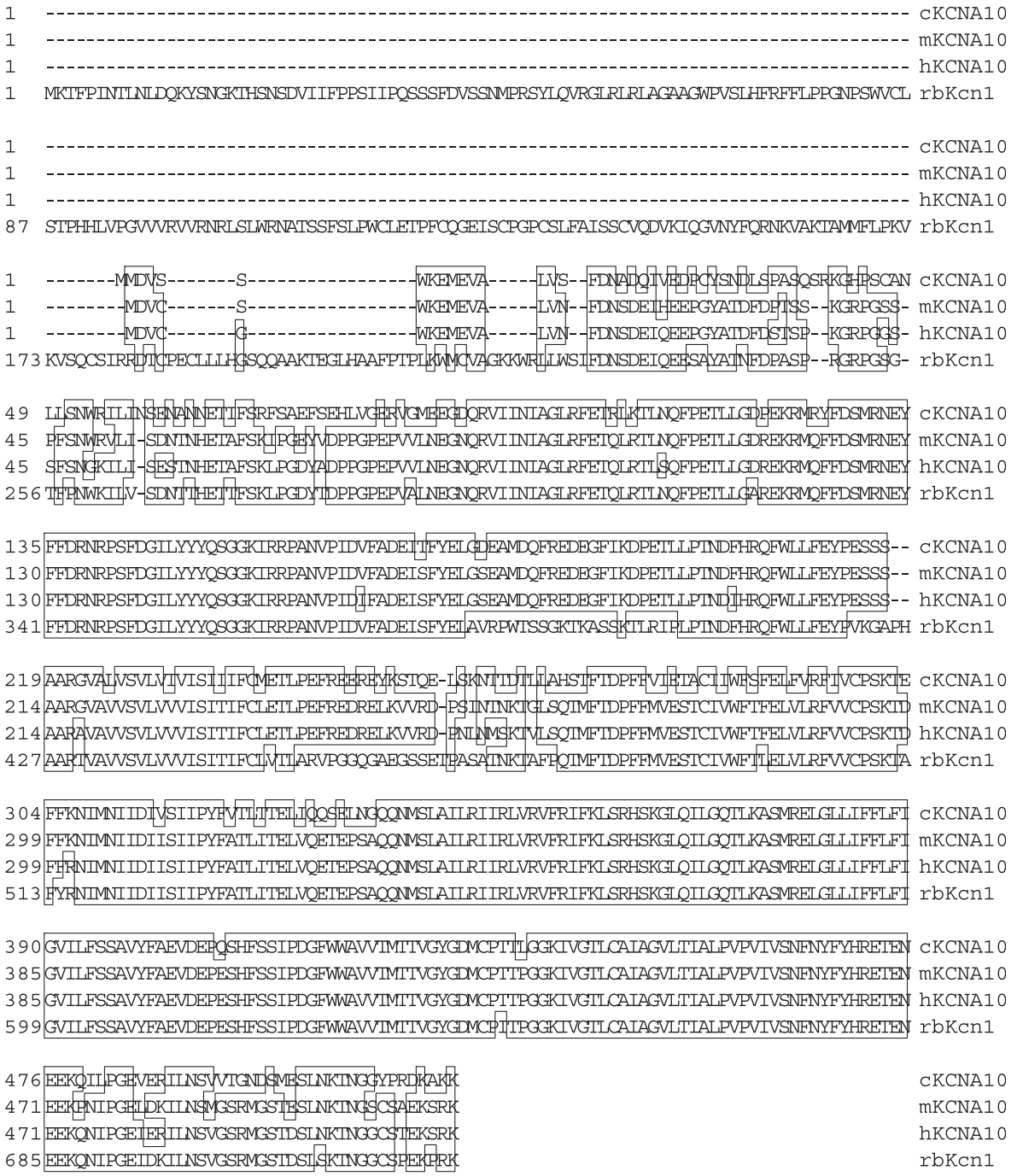


Fig. 8. Alignment of the deduced amino acid sequence of cKCNA10 with mammalian KCNA10 channels. Mammalian homologues include mouse mKCNA10 (accession no. XP_143471), human hKCNA10 (accession no. NP_005540), and rabbit rbKcn1 (accession no. AAA92054). ClustalW analysis revealed cKCNA10 has homology of 79.1% to mKCNA10, 76.3% to hKCNA10, and 66.9% to rbKcn1.

acid for the second longest cKv1.3 ORF, where the first several amino acids, MTVVXG, constitute a conservative block as in mammalian homologues. It suggests that the second largest ORF may code the real Kv1.3 subunit (Figs. 2 and 6). Still, the homology between the N-terminal end of the second largest cKv1.3 ORF (first 52 amino acids) and the correspondent mammalian homologues is much lower than between the central parts of the cds; specifically, it is 15% homologous to the human sequence and 33% homologous to the mouse sequence. In comparison, the chicken–mammalian homology is 82–84% for the full sequence. The homology between the cKv1.3 C-terminal end (amino acids 465–524) and the corresponding mammalian sequences is 80–82%. This homology is lower than between the mammalian C-terminal sequences (98.5–100%).

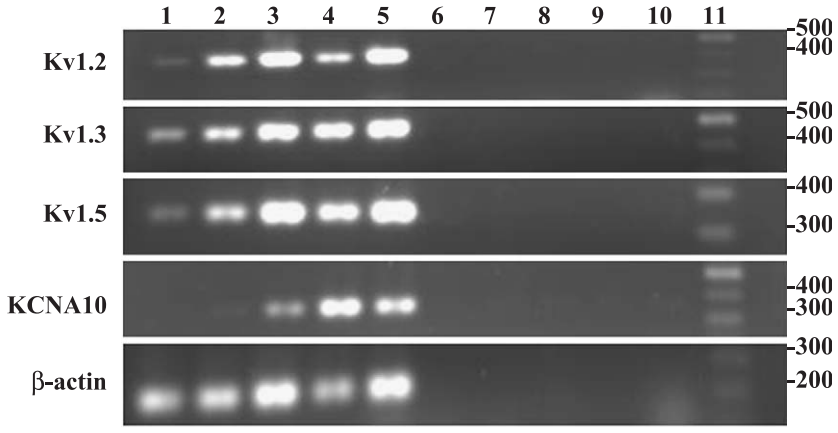


Fig. 9. RT-PCR products from cochlear tissues for various *Shaker* genes. Lanes 1–5 show mRNA expression for Embryonic Days (ED) 3, 6, 9, 14, and post-hatched day 3, with expected products of 448, 474, 347, 364, and 247 bp, respectively. Kv1.2, Kv 1.3 and 1.5 mRNA are expressed as early as ED3, whereas KCNA10 show expression by ED9. Lanes 6–10 are the results in the absence of RT and lane 11 is a DNA size standard consisting of a 100 bp DNA ladder.

In the case of cKv1.5, the deviation in the amino acid sequence from mammalian homologues is similar to Kv1.3 (Fig. 7). In addition, cKv1.5 has a nonhomologous sequence in the extracellular loop between transmembrane regions S3 and S4, including a six amino acid stretch (Figs. 3 and 7). The homology between full-length cKv1.5 and the mammalian homologues is 67–70.5%. The N- (first 148 amino acids) and C-terminal (amino acids 554–631) sequences of cKv1.5 show homology to mammalian sequences of only 26–28% and 46–50%, respectively.

For cKCNA10, the homology profile is similar to cKv1.5 (Fig. 8). In addition, the S1 and S3 transmembrane regions contain evenly distributed single mismatches when compared to the mammalian homologues. Comparison of full-length cKCNA10 and mammalian protein sequences reveals a homology of 67–79%. The N- (first 91 amino acids) and C-terminal (amino acids 479–516) sequences show a homology of up to 33–50% and 49–59%, respectively (i.e., 49% for mouse and 59% for human). Multiple alignments of the C-terminal ends of known KCNA10 family members with the sequences of the cGMP binding domains of the cyclic nucleotide-gated (CNG) channels [8] revealed a less then 20% homology between these two channel families (data not shown). However, within each family the homology is much greater when comparing the full-length C-terminal end: 62–70% for KCNA10 and 78% for CNG channels. Nonetheless, KCNA10 channels contain an 18 amino acid stretch (G473–N490 for cKCNA10) (Fig. 4), where five to six are the same as those found in CNG channels. The first two of these amino acids (G, E) are present also in the catabolite gene activator protein (CAP). They are assumed to form hydrogen bonds with cAMP when the latter binds to CAP [30].

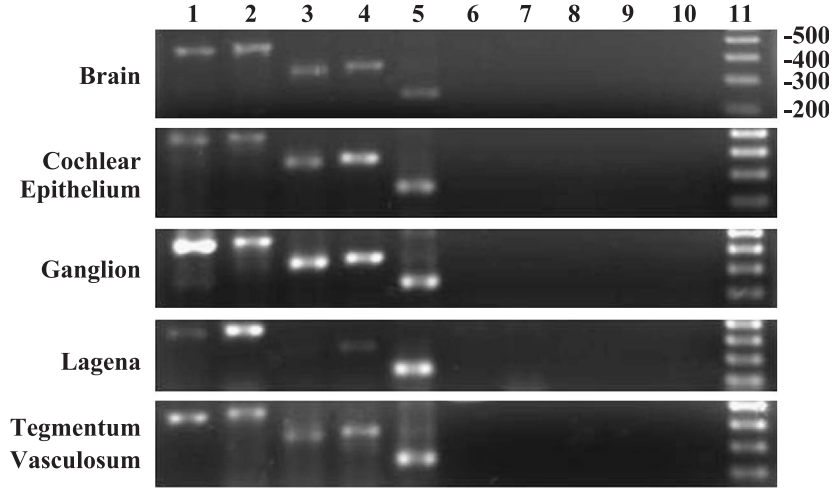


Fig. 10. RT-PCR products for various *Shaker* genes expressed in different parts of the cochlea. Lanes 1–5 show mRNA expression for cKv1.2, cKv1.3, cKv1.5, cKCNA10 and β-actin with expected products of 448, 474, 347, 364 and 247 bp, respectively. Lanes 6–10 are the results in the absence of RT and lane 11 is a DNA size standard consisting of a 100 bp DNA ladder.

3.3. Developmental and differential expression of Shaker in chick inner ear

Analysis of mRNA expression using RT-PCR revealed that potassium channels cKv subunits are expressed in cochlear tissues during chick embryonic development and after hatching (Fig. 9). Expression of cKv1.2, cKv1.3 and cKv1.5 was detected as early as ED3, an age when there is neither organotypic nor morphologic development of the sensory cells. Expression of cKCNA10 was detected at low levels beginning on ED6 and at higher levels by ED9. At this point in time, the cochlea and the ganglion are well into the stages of formation.

RT-PCR using RNA isolated from micro-dissected parts of the cochlea showed that all cloned channels are expressed in the sensory epithelium, ganglion and tegmentum (Fig. 10). cKv1.3 showed noticeably higher level of expression in the lagena, compared to cKv1.2 and cKCNA10, whereas expression of cKv1.5 in this region was barely detected (Fig. 10).

4. Discussion

4.1. Intronless organization of Shaker-family genes in chicken

In mammals, *Shaker* genes are intronless, but in *Drosophila*, *Shaker* channel isoforms are formed as a result of an alternative splicing of the pre-mRNA. During such splicing, the first 5′ and the last 3′ exons vary while the central core region stays the same, so that protein isoforms vary mainly in their N- and C-terminal sequences [23]. Initially, conclusions could not be reached about the intron–exon organization of these genes because the putative protein sequence for cKv1.3 and N-terminal sequence for cKv1.5 were produced based on genomic DNA sequences, while protein sequences for cKv1.2 and cKCNA10 were based on cDNA sequences. We hypothesize that chicken *Shaker* genes are intronless, based on the very few differences in the protein sequences between cKv1.2 and mammalian homologues, which are encoded by intronless genes. For further confirmation, we designed primers and generated, using PCR, DNA fragments overlapping in sequence and covering the coding region for cKv1.5. These fragments appeared to have the same size and sequence whether cDNA or genomic DNA was used as the template for PCR.

Variability in chicken and mammalian *Shaker* channel sequences lies mainly in the N- and C-terminal domains, as in *Drosophila Shaker* channel isoforms. If one assumes chicken and mammalian *Shaker* genes originate from an ancestor gene, with a structure similar to that found in *Drosophila*, variability may occur through the mechanism of reverse transcription of similar mRNA isoforms followed by integration of the formed cDNAs into the genome [1]. Taking this process into account, the high homology between cKv1.2 and mammalian Kv1.2 subunits might reflect a more recent point of origin in evolution. In contrast, the lower homology between cKv1.5 and mammalian Kv1.5 subunits may reflect an earlier point of origin.

4.2. Preservation of important protein binding sites in chicken Shaker-family channels and differences between chicken and mammalian homologues

The most important structural domains (i.e., the tetramerization domain T1 and ion transport domains) are well preserved in the cloned subunits. The sequence for the consensus Kvβ1 binding site, FYXLGXEAM (amino acids 232–240 for Kv1.5), is preserved in the chicken *Shaker* sequences. The β-subunit is important for converting the delayed rectifier type current to an A-type transient current when the Kvα-subunit is co-expressed with Kvβ1 [25]. Despite variations in the C-terminal sequences of cKv1.2, cKv1.3 and cKv1.5, the last several amino acid residues (TDV/L) remain conserved. In mammalian channels, these residues are important for interactions with PDZ domain-containing proteins [13,17]. In comparison, the last eight amino acids of cKCNA10 do not have any homology with mammalian C-terminal sequences and do not share the consensus C-terminal sequence TDV/L for binding PDZ domain-containing proteins. A bit upstream of the C-terminal end, all members of the KCNA10 family contain the homologous sequence GEXXXILN, which is present in cyclic nucleotide-gated (CNG) channels and where it is part of the cNMP binding domain [8,30].

As mentioned previously, the N-terminal sequences for cKv1.3, cKv1.5 and cKCNA10 have very little homology with the corresponding mammalian sequences. Within the first 51 amino acids of the N-terminal end of cKv1.3, only three blocks, 3–4 amino acids in length, were similar to mammalian homologues. For cKv1.5, only the first 12 out of 147 amino acids were identical to mammalian homologues. Similarly, only the first 17 out of 90 amino acids of cKCNA10 contain blocks of homologous amino acids greater than 4. Because of these differences in sequence, one would expect to find differences in the efficiency of membrane localization [24], and the ability to interact with tyrosine protein kinases and auxiliary proteins [3].

Src tyrosine kinase binds by its SH3-domain to the proline-rich motifs, RPLPXXP, of the hKv1.5 [11]. The hKv1.5 subunit contains two such motifs in its N-terminal end, where the cKv1.5 subunit has four minimally recognized PXXP motifs, two of which are parts of the bigger motif PXPXXP. The canonical motif PLPXXP, which is found in the human, rat, and mouse Kv1.3 N-terminal end, is present in cKv1.3. However, this motif is found in different parts of the N-terminal sequence of cKv1.3 compared to the mammalian subunits. Also, cKv1.3 has two or four (i.e., longest cKv1.3 ORF sequence) minimal SH3 binding motifs PXXP, one of which is inside the longer

consensus motif PXPXXP. Besides SH3 binding motifs, which are found in the proline-rich N-terminal region of cKv1.3 and 1.5, there are SH2 putative binding motifs YXXP/I in these protein sequences. These regions are known to enhance processive Tyr phosphorylation [20]. cKv1.3 has two SH2 putative binding motifs in its N-terminal region and one in its C-terminal end. cKv1.5 has one such motif in its N-terminal end. Finally, in the N-terminal end of cKv1.2 there is only one minimal SH3 PXXP motif, whereas in the C-terminal end there are two. The three putative SH2 binding motifs, YXXP/I, are distributed the same way, one in the N-terminal end and two in the C-terminal end. The cKCNA10 protein sequence lacks any SH2 or SH3 binding motifs with even distribution of proline residues along the sequence.

Multiple putative phosphoryration sites (PPS) for other protein kinases were found in all cloned Kv-subunits (Figs. 1–4). These sites are involved in channel regulation [12]. Most phosphorylation sites for cKv1.2 and cKv1.3 are located within regions that are homologous to mammalian channels. These sites include the T1 domain at the N-terminal end, the intracellular region between the S4 and S5 transmembrane domains, and the C-terminal region of the sequence closest to the S6 domain. Only one PPS was found in the nonhomologous N-terminal region of these sequences. For cKv1.5, seven PPS out of twenty at the N-terminus and four PPS out of nine at the C-terminus, are absent when compared to the homologous mammalian sequences. For cKCNA10, the picture is similar. Six PPS out of a total of 15, in the very N-terminal portion of the sequence, and one site in the C-terminal portion, are missing in the mammalian homologues.

4.3. Expression of Shaker mRNA starts at early embryonic stages and is localized in different parts of the cochlea

Previously, we reported a possible candidate for a *Shaker* subfamily channel member that conducts a transient type current found in the chicken cochlea, cKvα1.4. However, we showed that cKvα1.4 mRNA is expressed in cochlear tissues beginning on ED7 [21], whereas a cKvβ1.1 subunit is expressed as early as ED3. This finding led to the premise that while functional A-type channels from the *Shaker* subfamily could be assembled in the membrane of excitable cells of the cochlea as early as ED3, the corresponding α partner was absent from this developmental profile. Here, we show that cKv1.2, cKvα1.3 and cKvα1.5 subunits are expressed at the mRNA level as early as ED3, suggesting that these channels might establish functional *Shaker* A-type channels with the cKvβ1.1 subunit. Moreover, delayed rectifier type potassium current is found predominantly in the tall hair cells of the cochlear epithelium and the chicken cochlear ganglion cells [5,18,29]. Given the developmental profiles of cKv1.2, cKv1.3 and cKv1.5, and evidence for their co-expression in the epithelium and ganglion cells, the delayed rectifier channels may consist of heteromultimers. Similar subunits compose these ion channels in human CNS [2]. In comparison, presently, there is no functional evidence for KCNA10 in either hair cells or ganglion cells, although our data suggest mRNA expression in these cells. Localization studies at the cellular level and quantitative analysis of expression will reveal more information about the functional importance of these channels and their potential in forming a variety of multiheteromeric potassium channels in different tissue types of the cochlea.

In summary, we have revealed the molecular structure of four *Shaker* potassium channels from chicken and analyzed their similarity to known mammalian homologues. Our expression studies show that these channels appear at different stages of inner ear development, beginning with the stage of otocyst formation, are co-expressed in different regions of the cochlea, and thus may contribute to transient and delayed rectifier type potassium currents in ganglion and sensory cells at later stages. Further expression and functional studies are necessary to provide a detailed picture of the contribution of each channel to sensory signal transduction in the cochlea.

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