Molecular Cloning, Genomic Organization, and Expression of Three Chicken 5′-AMP-Activated Protein Kinase Gamma Subunit Genes[1]

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ABSTRACT The 5′-AMP-activated protein kinase (AMPK) plays a key role in regulating cellular energy homeostasis. The AMPK is a heterotrimeric enzyme complex that consists of 1 catalytic (α) and 2 regulatory (β and γ) subunits. Mutations of the γ subunit genes are known to affect AMPK functioning. In this study, we characterized the genomic organization and expression of 3 chicken AMPK γ subunit genes (cPRKAG). Alternative splicing of the second exon of the cPRKAG1 gene resulted in 2 transcript variants that code for predicted proteins of 298 and 276 amino acids. Use of an alternate promoter and alternative splicing of the cPRKAG2 gene resulted in 4 transcript variants that code for predicted proteins of 567, 452, 328, and 158 amino acids. Alternative splicing of exon 3 of the cPRKAG3 gene resulted in the production of "long" and "short" transcript variants that code for predicted proteins of 382 and 378 amino acids, respectively. We found evidence for differential expression of individual γ subunit gene transcript variants and, in some cases, tissue-specific expression was observed. The cPRKAG subunit genes displayed similar structural features and high sequence homology compared with corresponding mammalian γ subunit gene homologues.

Key words: 5′-AMP-activated protein kinase, gamma subunit, alternative splicing, alternate promoter usage, energy homeostasis

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INTRODUCTION

The 5′-AMP-activated protein kinase (**AMPK**) is a serine/threonine kinase and a central component of a kinase-signaling cascade that plays a key role both in sensing energy status and in maintaining energy balance in a wide variety of cells and tissues (Hardie et al., 1998; Carling, 2005). The AMPK exists as a heterotrimeric enzyme complex consisting of 1 catalytic (α) and 2 regulatory (β and γ) subunits (Mitchelhill et al., 1994; Stapleton et al., 1994). In mammals, there are 2 α subunit isoforms, designated α-1 and α-2 (Stapleton et al., 1996); 2 β subunit isoforms, β-1 and β-2 (Stapleton et al., 1997); and 3 γ subunit isoforms, γ-1, γ-2, and γ-3 (Cheung et al., 2000). Each of these subunit isoforms is encoded by a separate gene (Kahn et al., 2005).

The AMPK is activated in response to metabolic and nutritional stresses that cause a depletion of cellular ATP and increase the intracellular AMP:ATP ratio (Hardie et al., 1998). Activation of AMPK requires phosphorylation of a critical Thr residue (Thr 172) located in the activation loop of the N-terminal kinase domain of the α catalytic subunit by an upstream protein kinase such as the tumor suppressor LKB1 or Ca^{2+}/calmodulin dependent protein luinase kinase (Hawley et al., 1996, 2003; Hurley et al., 2005). Once activated, AMPK phosphorylates a variety of downstream protein targets that affect carbohydrate, protein, and lipid metabolism (Kahn et al., 2005).

The γ subunit constitutes the energy-sensing module within the AMPK complex by binding AMP or ATP in a mutually exclusive manner (Scott et al., 2004). Gamma subunit proteins contain 4 repeats of a structural motif consisting of approximately 60 amino acids and classified as a cystathionine β-synthase (**CBS**) domain (Bateman, 1997). Two CBS domains form a functional unit recently designated as a "Bateman domain" (Kemp, 2004), which serves as a cooperative regulatory AMP- and ATP-binding site within the AMPK complex (Scott et al., 2004). Several mutations have been identified in human γ-2 and pig γ-3 subunit genes that occur within or close to a CBS domain. Six different point mutations in the human γ-2 gene are associated with certain disease states (e.g., Wolf-Parkinson-White syndrome) characterized by hypertrophic cardiomyopathy, abnormalities in electrical conductance, glycogen overload, and heart failure (Daniel and Carling, 2002). A nonconservative missense mutation (R225Q) in the porcine γ-3 gene causes a dominant phenotype characterized by abnormally high skeletal muscle glycogen content and significant effects on meat quality (Milan et al., 2000).

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Little is currently known about the structure of chicken AMPK subunit genes. Recently, we reported evidence for 7 chicken AMPK subunit gene homologues located on different chromosomes (Proszkowiec-Weglarz et al., 2006). However, the complete sequence and structural features of individual AMPK subunit genes in chickens, especially those encoding the γ subunit isoforms, remain largely unknown. Because AMPK plays a central role in the regulation of energy balance, AMPK subunit genes are important potential candidates to be used as selection markers for feed efficiency and meat quality traits in commercial livestock populations (Benkel et al., 2005). Knowledge about γ subunit gene structure and expression is also essential to understanding how AMPK influences physiological function. Therefore, the purpose of this work was to clone, sequence, and characterize transcripts from the 3 chicken AMPK γ subunit genes (**cPRKAG**) and to study their expression in different tissues.

MATERIALS AND METHODS

Birds and RNA Isolation

All bird studies were conducted according to research protocols approved by the Institutional Animal Care and Use Committee. Day-old male broiler chicks were purchased from Hubbard-ISA (Duluth, GA) and grown until 3 wk of age. A standard commercial diet and water were provided ad libitum. Samples (n = 6) of liver, brain, heart, kidney, spleen, duodenum, skeletal muscle, abdominal fat, pancreas, and hypothalamic tissue were collected and snap-frozen in liquid nitrogen before RNA isolation. Total RNA was isolated from tissue samples using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Nucleotide Sequencing

Total RNA from liver, heart, and skeletal muscle and a primer-directed reverse transcription PCR (**RT-PCR**)-based strategy were used to clone portions of the chicken AMPK γ-1, γ-2, and γ-3 genes (cPRKAG1, cPRKAG2, and cPRKAG3, respectively). Nucleotide sequences for cPRKAG1, cPRKAG2, and cPRKAG3 cDNA, including the complete coding region and portions of the 5′- and 3′-untranslated regions (**UTR**), were derived using primer sets initially based on predicted genomic sequences, on available EST sequences, or both. With these primers, a series of overlapping PCR products was generated. The overlapping PCR products for each gene were then assembled into a single fragment of contiguous sequence. The presence and identity of unique cPRKAG gene transcript variants were further verified by RT-PCR using a unique 5′-end primer (when possible) and a common downstream 3′-end primer to amplify the entire coding region and portions of the 5′- and 3′-UTR to confirm that all cloned transcript variants corresponded to actual mRNA transcripts. Each PCR product was subjected to bidirectional automated fluorescent DNA sequencing using a Beckman Coulter CEQ 8000XL Genetic Analysis System with the dye terminator cycle sequencing method (Quick Start Kit, Beckman Coulter Inc., Fullerton, CA).

Rapid Amplification of cDNA Ends

Rapid amplification of cDNA ends (**RACE**) was successfully used to characterize the 5′-ends of cPRKAG2 and cPRKAG3 cDNA. Total RNA (1.0 μg) was used to prepare 5′-RACE-ready cDNA using the SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA). Polymerase chain reaction was performed using Platinum Taq DNA polymerase with 3.5 m*M* Mg^{+2} (Invitrogen), touchdown PCR, and cPRKAG2 or cPRKAG3 gene-specific 5′-RACE primers based on sequences obtained by prior sequencing of portions of the coding regions. We attempted to use 5′-RACE to determine 5′-end sequence for cPRKAG1, but we were unsuccessful due to high GC content of this region. In addition, 3′-RACE was not successful in generating 3′-end products for any of the cPRKAG transcripts. Instead, we utilized existing EST and genomic sequence in conjunction with a primer-walking strategy to sequence and verify these regions.

Cloning

Amplified cDNA fragments and RACE products containing sequences corresponding to the 5′-end were either subjected to direct sequencing or were subcloned into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) and sequenced using M13 forward and reverse primers.

RT-PCR

Reverse transcription reactions (20 μL) consisted of the following: 1.0 μg of total RNA, 50 units of SuperScript III Reverse Transcriptase (Invitrogen), 40 units of an RNase inhibitor (Invitrogen), 0.5 m*M* dNTPs, and 100 ng of random hexamer primers. Polymerase chain reaction was performed in 25-μL reactions containing the following: 20 m*M* Tris-HCl, pH 8.4; 50 m*M* KCl; 1.0 unit of Platinum Taq DNA Polymerase (Invitrogen); 0.2 m*M* dNTPs; 2.0 m*M* Mg^{2+}; 10 pmol of each gene-specific primer (Table 1); 5 pmol each of an appropriate mixture of primers:competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion Inc., Austin, TX); and 1 μL of the reverse transcription reaction. Thermal cycling parameters were as follows: 1 cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, with a final extension at 72°C for 8 min. For cPRKAG1 (transcript variants 1 and 2 combined), touchdown PCR was used as follows: 1 cycle at 94°C for 2 min, followed by 15 cycles, 94°C for 30 s, 72°C for 30 s, 72°C for 1.5 min (after each cycle, the temperature was decreased 0.5°C from 72°C to 65°C), followed by 25 cycles, 94°C for 30 s, 65°C for 30 s, 72°C for 1.5 min, with a final extension at 72°C for 8 min. The cPRKAG subunit gene transcript

Table 1. Gene-specific oligonucleotide primers used for the analysis of chicken 5′-AMP-activated protein kinase γ subunit gene (cPRKAG) expression by reverse transcription PCR

Gene[1]	Reference Sequence[2]	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
cPRKAG1	DQ133597	GGGGAAGTGGGGCGGTGGT	Forward	305, 200[3]
(T1 and T2)		TCTGCACCATGGGCGACTTG	Reverse	
cPRKAG1	DQ133597	ATGAAGTCACACCGCTGCTA	Forward	400
(T1)		TGTAGAGAGTGTTGCCCGAGT	Reverse	
cPRKAG2	DQ212708	CGAGCTCGAAGATGAAGCAC	Forward	446, 387[4]
(T1 to T3 and T4)		CATTTCCACTGACGGGATCT	Reverse	
cPRKAG2	DQ212708	GATGAGACCGTGGGGATAAG	Forward	397
(T1)		AGACTCCTGGTAGGGGAAGG	Reverse	
cPRKAG2	DQ212709	GTGATTTCTTCCTCGCAACG	Forward	443
(T2)		CTCGAGTTTCTCCAGCATCC	Reverse	
cPRKAG2	DQ212710	GGATGCTCATCACGTCTCCT	Forward	281
(T3)		GCTGCTCTCACACCATTTGC	Reverse	
cPRKAG3	DQ079814	TGTGCCCACAGGGTTCC	Forward	193
(T1, "Long")		GGCATCATAGCAGCAGTGAC	Reverse	
cPRKAG3	DQ079815	GGAAGAAGGGTTCCCAGAG	Forward	498
(T2, "Short")		GAGTAGACGGCGTCGAAGAG	Reverse	

[1]Gene name designation and specific transcript variants (T) are identified for each pair of primers.

[2]GenBank accession no. for sequence from which primers were designed.

[3]The product sizes correspond to T1 and T2, respectively.

[4]The product sizes correspond to T1 to T3 and T4, respectively.

variants were coamplified along with 18S rRNA in a multiplex PCR format for relative quantitative assays. Negative controls were run to ensure PCR accuracy and specificity.

Quantitation of Gene Expression

Relative quantitation of PCR products was accomplished using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF), as described previously (Richards and Poch, 2002). The level of cPRKAG gene expression was determined as the ratio of integrated peak area for each PCR product relative to that of the coamplified 18S rRNA internal standard. Values are presented as the mean ± SEM of 6 individual expression ratio determinations.

Statistical Analysis

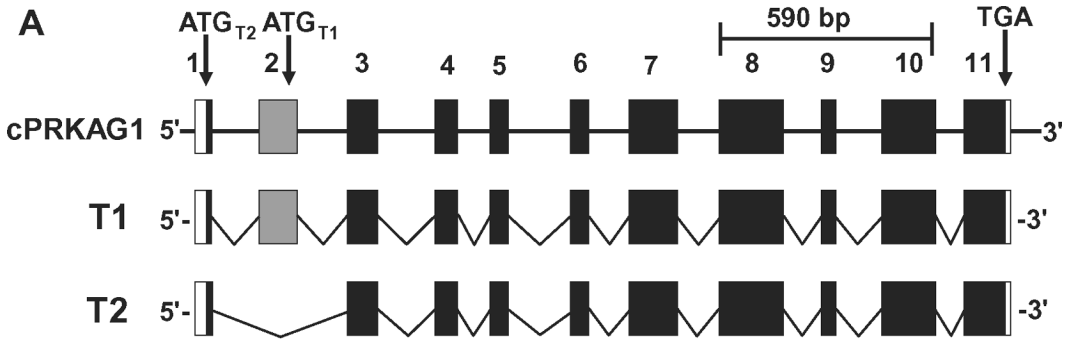
Gene expression data were subjected to ANOVA using the GLM procedure of SAS software (SAS System for Windows, Version 8.2, SAS Institute Inc., Cary, NC). The Duncan's multiple range test option of the GLM procedure for SAS was used to determine significance of mean differences. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

The cPRKAG1 Gene

A molecular-cloning strategy involving primer-directed RT-PCR was used to sequence 991 bp of a liver-derived cDNA corresponding to the complete coding region and portions of the 5′- and 3′-UTR of the cPRKAG1 mRNA. The cPRKAG1 gene consists of 11 coding exons ranging in size from 38 to 166 bp (Figure 1, panel A). Its chromosome location is currently unknown. The lack of relevant sequence due to gaps in the draft of the chicken genome necessitated that we project the size of certain cPRKAG1 exons (exons 3 to 7) based on corresponding sizes determined for mammalian species and as verified by comparison with cPRKAG2 and cPRKAG3 genes (see below). Figure 1, panel B, presents a comparison of the number, order, and size of each coding exon for chicken and mammalian PRKAG1 genes. Mammalian PRKAG1 genes consist of 12 exons, including an additional exon near the 5′-end of the gene (Shamsadin et al., 2001; Benkel et al., 2005) that is not present in the chicken (Figure 1, panel B). A portion of the cPRKAG1 gene consisting of a block of 8 exons (3 to 10) is generally conserved with respect to size and location across different vertebrate species, except for exon 4 in the human PRKAG1 T1 transcript, which is subject to alternative splicing (Figure 1, panel B). These exons code for a major portion of the CBS domain-containing region of the γ-1 subunit protein.

Sequence analysis of products from RT-PCR indicated that there were 2 cPRKAG1 transcripts that shared a common 3′-end but differed at their 5′-ends (GenBank accession no. DQ133597 and DQ133598). These 2 transcript variants are the result of alternative splicing of exon 2. Transcript variants 1 and 2 contain open reading frames of 897 and 831 bp, respectively, that show significant homology at the nucleotide level, ranging from 76 to 79%, compared with corresponding mammalian sequence. Because transcript variant 2 lacks sequence from exon 2, there is a reduction in the open reading frame caused by the use of an alternative translation initiation codon in exon 1 (Figure 2). Two transcript variants have been reported for the human PRKAG1 gene (GenBank accession no. NM_002733 and NM_212461), although the mecha-



B

Exon no.	Chicken (T1)	Chicken (T2)	Human (T1)	Human (T2)	Mouse
	(nucleotides)				
1	>52	>52	78	78	51
–	–	–	49	49	46
2	104	–	110	110	110
3	82	82	131	82	82
4	59	59	59	59	59
5	46	46	46	46	46
6	55	55	55	55	55
7	127	127	127	127	127
8	166	166	166	166	166
9	38	38	38	38	38
10	147	147	147	147	147
11	>115	>115	750	750	684
CDS	897	831	996	744	993

Figure 1. A model depicting the structural organization of the chicken 5′-AMP-activated protein kinase γ-1 gene (cPRKAG1) and its 2 transcript variants (T1 and T2; panel A). Coding exons are depicted as black boxes; noncoding exons are depicted as open boxes; and the alternatively spliced exon (2) is depicted as a gray box. The positions of the 2 translation initiation codons ($ATG_{T1/T2}$) and the termination codon (TGA) are shown. A portion of cPRKAG1 gene for which the length (590 bp) is known from available genomic sequence is indicated. Exon and coding region (CDS) size comparisons for PRKAG1 genes from different species (panel B). GenBank accession no. are as follows: chicken T1, DQ133597; chicken T2, DQ133598; human T1, NM_212461; human T2, NM_002733, and mouse, NM_016781.

nism for their production (i.e., use of alternative splicing sites located at the intron 3-exon 4 junction) differs from that used to produce the 2 transcript variants from the cPRKAG1 gene. Transcript variant 1 in the chicken codes for a predicted protein of 298 amino acids with 4 CBS domains, whereas transcript variant 2 codes for a 276 amino acid protein with 3 complete and 1 partial CBS domains (Figure 2). The homology of the CBS domain-containing region ranged from 88 to 98% at the amino acid level compared with mammalian sequence. Overall, amino acid sequence identity of cPRKAG1 compared with mammalian PRKAG1 subunit proteins ranged from 87 to 91%.

Transcript variants 1 and 2 of the cPRKAG1 gene were expressed in all tissues examined (Figure 3, panels A and B). However, transcript variant 1 was the predominant one. We attempted to develop a relative quantitative assay for both transcript variants, but we were unable to design a unique forward primer spanning the exon 1–exon 2 junction because the GC-rich character of exon 1 precluded using any of this sequence for primer design. Instead, a specific assay for transcript variant 1 (forward primer in exon 2) was used to quantify this predominant form. The highest levels of expression of transcript variant 1 were observed in heart, kidney, spleen, and duodenum (Figure 3, panel B), whereas transcript variant 2 was most highly expressed in skeletal muscle (Figure 3, panel A). Transcript variant 2 displayed a very low level of expression across all tissues. Because this transcript encodes a protein that is truncated within the first CBS domain, it is unlikely to play a significant role in determining AMPK function.

The cPRKAG2 Gene

The cPRKAG2 gene, located on chromosome 2, consists of 18 coding exons ranging in size from 38 to >743 bp and spans in excess of 180 kb, with the first intron (61 kb) accounting for approximately 30% of the entire gene sequence (Figure 4, panel A). Figure 4, panel B, presents a size comparison for exons comprising PRKAG2 genes for different species. The organization of cPRKAG2 is similar to the human PRKAG2 gene, which is located on chromosome 7 (7q36) and spans 320 kb with the first (90 kb) and third (105 kb) introns accounting for nearly two-thirds of the total gene size (Lang et al., 2000). Interestingly, the cPRKAG2 gene appears to exhibit syntenic conservation compared with human and mouse counterparts, based on a consensus linkage map of the chicken genome (Groenen et al., 2000). Another gene, RAS homologue enriched in the brain (Rheb), was found to be closely linked to the cPRKAG2 gene, as has been observed in humans (Lang et al., 2000). Exons 9 to 16, which code for a major portion of the CBS domain-containing region of

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                                                   M  E↓
  1  GGGGAAGTGGGGCGGTGGTGGCGGCAGCGGCGGCGGACCCGGGGCCATGGAGGGGAGCCC

                              M  K  S  H  R  C  Y  D  L  I  P  T   12
 61  CTCCGTGGCGCCTACACCGCCTTCATGAAGTCACACCGCTGCTACGACCTCATCCCCACC
                                        CBS domain 1
      S  S  K  L  V  V  F  D  T  S  L  Q↓V  K  K  A  F  F  A  L   32
121  AGCTCCAAACTCGTCGTCTTCGACACTTCCCTTCAGGTGAAGAAGGCTTTCTTTGCACTG

      V  T  N  G  V  R  A  A  P  L  W  D  S  K  K  Q  S  F  V  G   52
181  GTCACCAATGGCGTGCGGGCAGCCCCGCTGTGGGACAGCAAAAAGCAGAGCTTTGTGGGC

      M  L  T  I  T  D  F  I  N  I  L  H  R  Y  Y  K  S  P  M  V   72
241  ATGCTGACCATCACTGACTTCATCAACATCCTGCACCGCTACTACAAGTCGCCCATGGTG

      Q  I  Y  E  L  E  E  H  K  I  E  T  W  R  E  V  Y  L  Q  D   92
301  CAGATCTATGAGCTGGAGGAGCACAAAATCGAGACGTGGAGAGAGGTGTACCTGCAGGAC
                                        CBS domain 2
      S  F  K  P  L  V  C  I  S  P  N  A  S  L  F  D  A  V  S  S  112
361  TCCTTCAAGCCGTTGGTCTGCATCTCCCCCAATGCCAGCCTTTTTGATGCCGTCTCCTCC

      L  I  R  N  K  I  H  R  L  P  V  I  D  P  D  S  G  N  T  L  132
421  CTGATCCGCAATAAGATCCACCGCCTCCCCGTCATCGACCCCGACTCGGGCAACACTCTC

      Y  I  L  T  H  K  R  I  L  K  F  L  K  L  F  I  A  E  V  P  152
481  TACATCCTCACCCACAAACGCATCCTCAAGTTCCTCAAACTCTTTATTGCAGAGGTCCCA

      K  P  E  F  M  A  R  T  L  E  E  L  Q  I  G  T  Y  S  N  I  172
541  AAGCCTGAGTTTATGGCCCGGACTTTGGAGGAGCTGCAGATCGGTACCTACAGCAACATC
                                        CBS domain 3
      A  V  V  S  T  S  T  P  I  Y  V  A  L  G  I  F  V  Q  H  R  192
601  GCCGTGGTGAGCACCAGCACTCCCATCTATGTGGCTCTGGGCATCTTCGTGCAGCACCGC

      V  S  A  L  P  V  V  D  D  S  G  R  V  V  D  I  Y  S  K  F  212
661  GTCTCCGCTCTGCCCGTGGTCGATGATTCGGGGCGGGTGGTGGATATCTACTCCAAGTTC

      D  V  I  N  L  A  A  E  K  T  Y  N  N  L  D  V  T  V  T  R  232
721  GACGTTATCAATTTGGCAGCTGAGAAGACCTACAACAACCTGGACGTGACGGTGACGCGG
                                        CBS domain 4
      A  L  Q  H  R  S  H  Y  F  E  G  V  L  K  C  Y  K  H  E  T  252
781  GCGCTGCAGCACCGCTCCCATTACTTCGAGGGCGTCCTCAAATGCTACAAACACGAAACC

      L  E  A  I  I  N  R  L  V  E  A  E  V  H  R  L  V  V  V  D  272
841  TTGGAAGCCATCATCAACCGCCTGGTGGAGGCCGAGGTGCACCGTTTGGTGGTGGTGGAT

      E  S  D  V  V  K  G  I  V  S  L  S  D  I  L  Q  A  L  V  L  292
901  GAGAGCGACGTGGTGAAGGGCATCGTCTCCCTCTCGGATATCCTCCAAGCCCTGGTTCTC

      P  E  G  S  E  P  *                                          298
961  CCAGAGGGCTCTGAGCCCTGACTGATGCTGT
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Figure 2. Nucleotide and predicted amino acid sequence of the complete coding region for the chicken 5′-AMP-activated protein kinase γ-1 gene (cPRKAG1). The position of the 4 cystathionine β-synthase (CBS) domains is indicated by underlined portions of the nucleotide sequence. The second exon, which is spliced out of transcript variant 2, is indicated by gray highlighting, with the arrows indicating the splice points. The position of both translation initiation codons (ATG) is indicated by boxes, as is the position of the common termination codon (TGA). Nucleotide numbers are listed to the left and amino acid numbering is included on the right.

the cPRKAG2 gene, are conserved with respect to size and location across different animal species (Figure 4, panel B).

Four different cPRKAG2 cDNA containing complete coding regions and portions of the 5′- and 3′-UTR were obtained from heart, liver, and skeletal muscle (GenBank accession no. DQ212708 to DQ212711). Each of the 4 transcript variants was found to have a similar 3′-end, with a unique 5′-end giving rise to transcripts ranging in size from 1,731 to 2,488 bp (Figure 4, panel A). Transcript

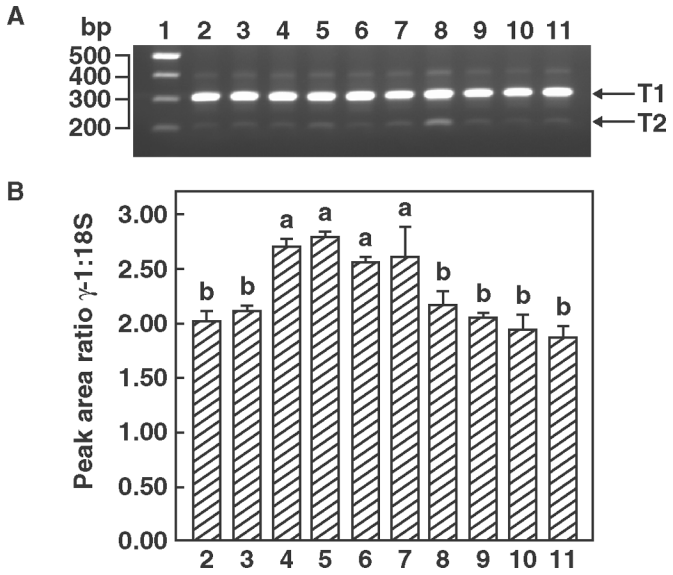


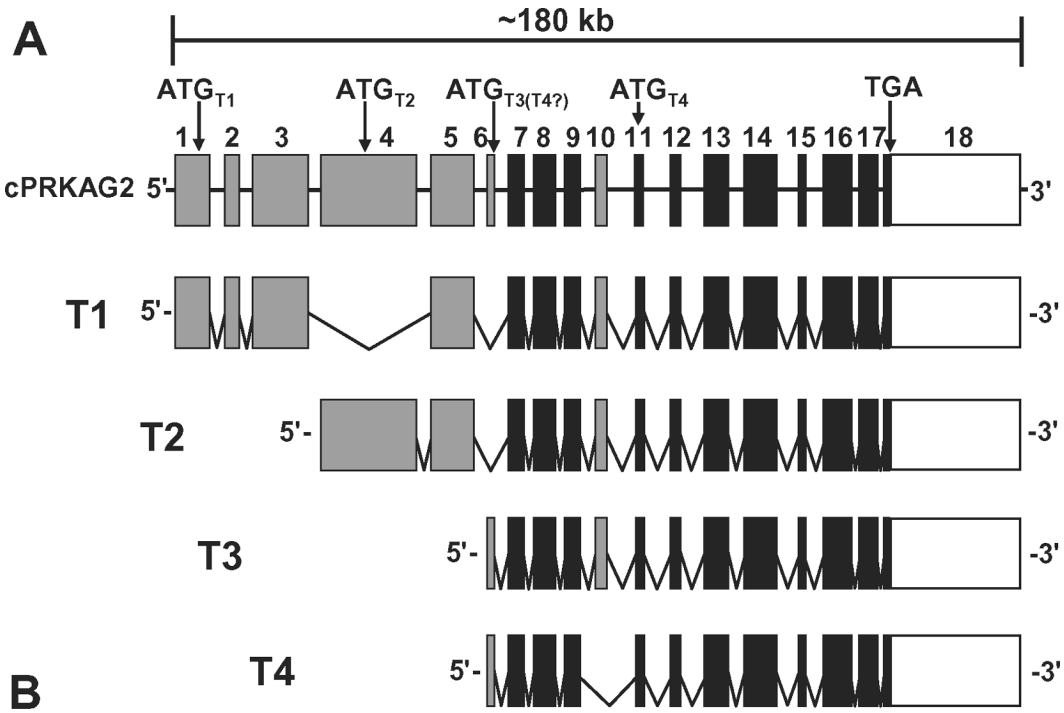
Figure 3. Expression of 2 chicken 5′-AMP-activated protein kinase γ-1 gene (cPRKAG1) transcript variants (T1 and T2) in different tissues from 3-wk-old broiler chickens. Typical separation of PCR products for T1 and T2 on a 1.5% agarose gel stained with ethidium bromide (panel A). Reverse transcription PCR and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection were used to quantify the level of expression of T1 relative to an 18S rRNA internal standard. Expression ratio (T1:18S) values represent the mean ± SEM of 6 determinations. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons. Samples are identified as follows: 1) 100 bp of DNA ladder, 2) liver, 3) brain, 4) heart, 5) kidney, 6) spleen, 7) duodenum, 8) skeletal muscle, 9) abdominal fat, 10) pancreas, and 11) hypothalamus.

variant 1, the longest of the 4 transcripts (2,488 bp), is similar to mouse PRKAG2 (GenBank accession no. NM_145401). Transcript variant 2 is shorter (2,448 bp) due to the use of an alternate first exon (exon 4). Both transcripts demonstrated alternative splicing, resulting in the loss of sequence contained in exon 6. For transcript variants 3 and 4, it appears that a combination of alternative splicing of exon 10 and alternate promoter usage results in 2 transcripts whose 5′-ends derive their sequence from exon 6 (Figure 4, panel A). Two distinct transcript variants have been reported for human PRKAG2 that correspond to cPRKAG2 transcript variants 1 and 3 (Cheung et al., 2000; Lang et al., 2000). The nucleotide homology of the cPRKAG2 coding region for all 4 transcript variants ranged from 41 to 87%, compared with mammalian gene counterparts. Transcript variants 1 to 4 code for predicted proteins of 567, 452, 328, and 158 amino acids, respectively. A comparison of the predicted amino acid sequence between chicken and mammalian γ-2 subunit proteins ranged from 51 to 95% amino acid identity, whereas the identity specifically within the CBS domain-containing portion of the subunits ranged from 84 to 100%. The predicted proteins from transcript variants 1 to 3 each contain 4 consecutive CBS domains. Use of an internal translation initiation codon (ATG_{T4} in exon 11) in transcript variant 4 would produce a predicted protein (158 amino acids) that contains only the last 2 CBS domains due to truncation at its N-terminal end. However, it must be noted that transcript 4 possesses 2 potential translation initiation sites ($ATG_{T3,T4}$). The use of the upstream translation initiation codon (ATG_{T3} located in exon 6) would produce a more severely truncated γ-2 subunit protein (78 amino acids) due to the introduction of a premature termination codon. This truncated protein is predicted to contain some N-terminal sequence and only a portion of the first CBS domain. It is not possible to determine whether the upstream, the downstream, or both translation initiation codons would be utilized for transcript variant 4. In eukaryotic species, a scanning mechanism that recognizes sequence flanking the AUG codon determines the site(s) of translation initiation, and this process does permit the production of multiple proteins from a single mRNA (Kozak, 2001). The production of 2 different size peptides from a single mRNA by 2 initiation sites has recently been reported for the human neuropeptide Y gene (Kaipio et al., 2005). Moreover, the use of alternative initiation of translation in humans has been reported to occur most frequently in gene transcripts coding for regulatory proteins (Prats and Prats, 2002). However, it is not known to what extent this applies to cPRKAG2 transcript variant 4, because it is generally not highly expressed (see below) and both predicted proteins are severely truncated and presumably functionally impaired.

Individual cPRKAG2 gene transcript variants were expressed in all tissues, and some exhibited a tissue-specific expression pattern (Figure 5, panels A to F). Transcript variant 1 was preferentially expressed in liver (Figure 5, panel C). The highest level of expression of transcript variant 2 was observed in liver and heart tissue (Figure 5, panel D). Brain, heart, kidney, skeletal muscle, abdominal fat, and hypothalamus preferentially expressed transcript variant 3 (Figure 5, panel E), whereas, in spleen and duodenum, transcript variants 1 to 3 were expressed at similar levels (Figure 5, panels B to E). The lowest expression of all 4 transcripts for cPRKAG2 was observed in pancreas (Figure 5, panels B to F). Transcript variant 4 was most highly expressed in skeletal muscle tissue (Figure 5, panel F), albeit at a reduced level compared with transcript variants 1 to 3 (Figure 5, panel B).

The cPRKAG3 Gene

A cDNA derived from the cPRKAG3 gene, consisting of 1,788 bp, was initially cloned from chicken skeletal muscle total RNA using a primer-directed RT-PCR-based strategy and 5′-RACE. Genomic sequence (GenBank accession no. DQ280152) was used to determine exon-intron boundaries and to accurately establish features of the cPRKAG3 gene organization. The cPRKAG3 gene is the best defined of the 3 chicken γ subunit genes. It consists of 12 coding exons and 1 noncoding exon, ranging in size from 38 to >577 bp, and 12 introns, ranging in size from 73 to 759 bp, with the first intron being the largest (Figure 6, panel A). Nucleotide sequence homology within the coding region ranged from 62 to 71% for the chicken as compared with mammalian PRKAG3 genes. The number,



B

Exon no.	Chicken (T1)	Chicken (T2)	Chicken (T3)	Chicken (T4)	Human (T1)	Human (T2)	Mouse
	(nucleotides)						
1	>172	–	–	–	204	–	420
2	72	–	–	–	72	–	72
3	280	–	–	–	280	–	280
4	–	>484	–	–	–	–	–
5	215	215	–	–	218	–	215
6	–	–	>41	>41	–	151	–
7	82	82	82	82	70	–	70
8	110	110	110	110	112	110	104
9	82	82	82	82	82	82	82
10	59	59	59	–	59	59	59
11	46	46	46	46	46	46	46
12	55	55	55	55	55	55	55
13	127	127	127	127	127	127	127
14	166	166	166	166	166	166	166
15	38	38	38	38	38	38	38
16	147	147	147	147	147	147	147
17	94	94	94	94	94	94	94
18	>743	>743	>743	>743	292	1,108	1,131
CDS	1,704	1,359	987	477	1,710	987	1,701

Figure 4. A model depicting the structural organization of the chicken 5′-AMP-activated protein kinase γ-2 gene (cPRKAG2), spanning approximately 180 kb on chromosome 2, and its 4 transcript variants (T1 to T4; panel A). Coding exons are depicted as black boxes; noncoding exons are depicted as open boxes; and the alternatively spliced exons are depicted as gray boxes. The positions of the 4 putative translation initiation codons used by each of the transcript variants (ATG_{T1} to ATG_{T4}) and the common termination codon (TGA) are shown. Exon and coding region (CDS) size comparisons for PRKAG2 genes from different species (panel B). GenBank accession no. are as follows: chicken T1, DQ212708; chicken T2, DQ212709; chicken T3, DQ212710; chicken T4, DQ212711; human T1, NM_016203; human T2, AF087875; and mouse, NM_016781.

size, and order of cPRKAG3 coding exons showed a high degree of similarity to mammalian PRKAG3 genes (Figure 6, panel B). However, cPRKAG3 apparently lacks an exon present at the 5′-end of human and mouse PRKAG3 genes, and this accounts for the shorter N-terminal region of the chicken γ-3 subunit protein as compared with mammalian proteins. The core group of 8 exons (4 to 11) that encode a significant portion of the CBS domain region of the γ-3 isoforms is totally conserved with respect to size and order when compared with mammalian PRKAG3 counterpart genes, as well as with cPRKAG1 and cPRKAG2 genes. Amarger et al. (2003) compared the genomic organization of PRKAG3 genes of the pig, human, and mouse and observed that exon size was perfectly

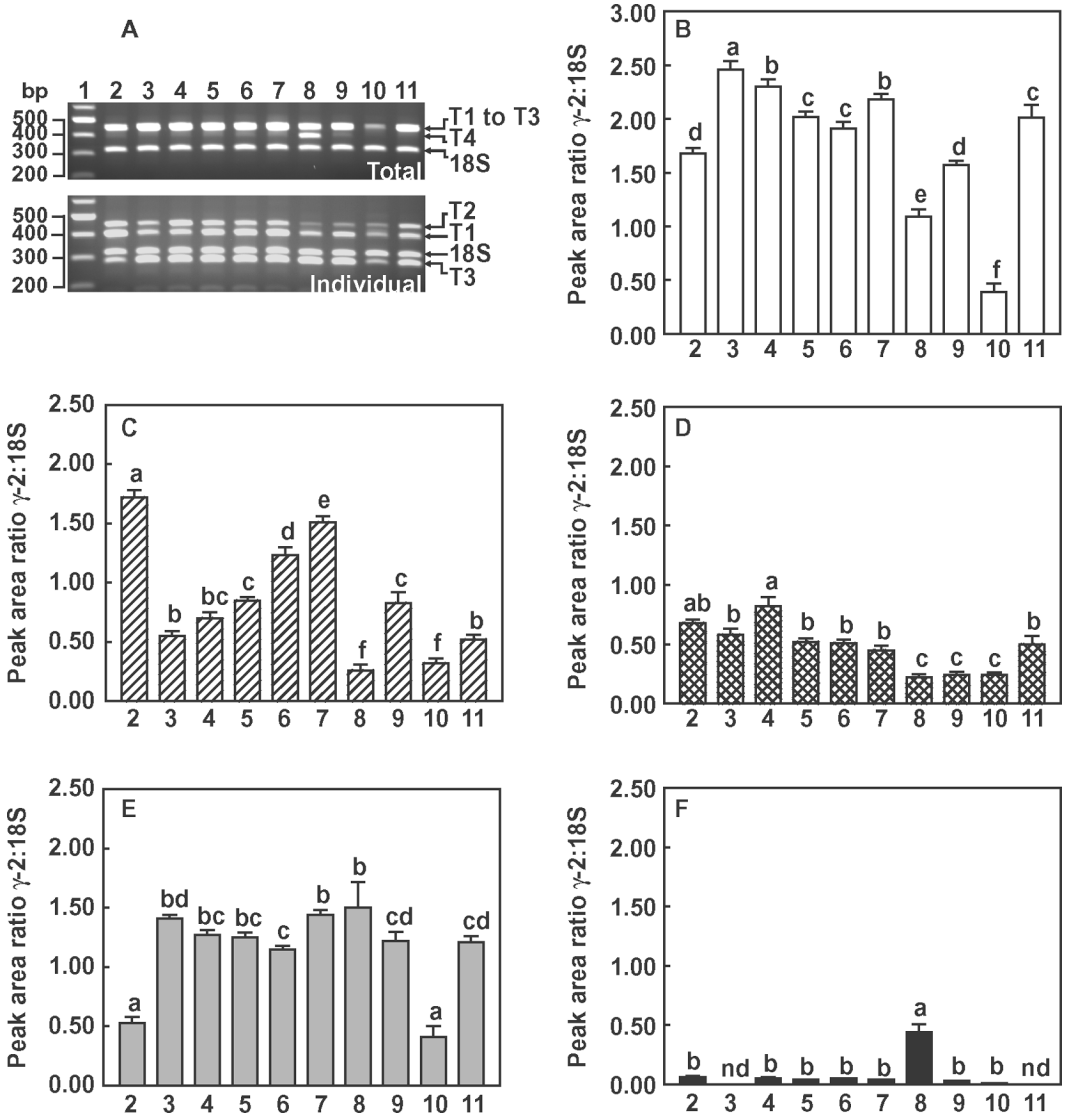
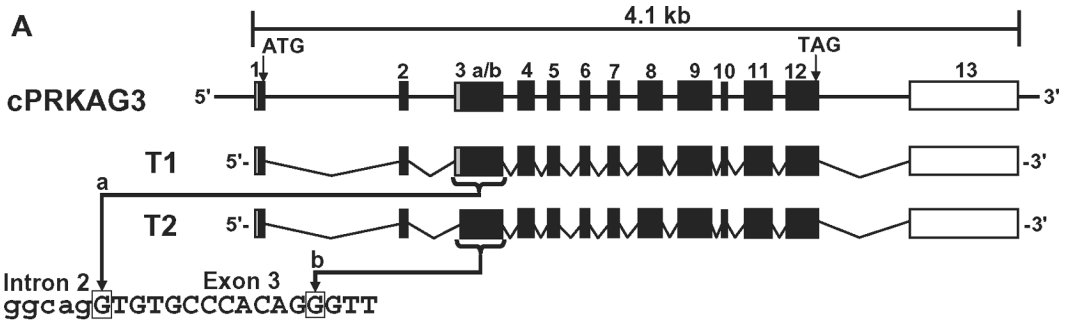


Figure 5. Expression of 4 chicken 5′-AMP-activated protein kinase γ-2 gene (cPRKAG2) transcript variants (T1 to T4) in different tissues from 3-wk-old broiler chickens. A typical separation of PCR products for T1 to T3 and T4 on a 1.5% agarose gel stained with ethidium bromide (panel A). Reverse transcription PCR and capillary electrophoresis with laser-induced fluorescence (CE-LIF) were used to quantify the level of expression of T1 to T3 combined (panel B) and individual transcript variants, T1 (panel C), T2 (panel D), T3 (panel E), and T4 (panel F) relative to an 18S rRNA internal standard. Expression ratio (T1 to T4:18S) values represent the mean ± SEM of 6 determinations. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons. Samples are identified as follows: 1) 100 bp of DNA ladder, 2) liver, 3) brain, 4) heart, 5) kidney, 6) spleen, 7) duodenum, 8) skeletal muscle, 9) abdominal fat, 10) pancreas, and 11) hypothalamus.

conserved, with the exception of the first exon. Similar findings were reported for the equine and zebrafish PRKAG3 genes (Park et al., 2003). The cPRKAG3 gene, located on chromosome 7, spans 4.1 kb, making it much more compact than cPRKAG2. This organization is similar to the human PRKAG3 gene, which is located on chromosome 2 (2q35). The cPRKAG3 gene exhibits syntenic conservation as compared with its human counterpart, based on a consensus linkage map of the chicken genome (Groenen et al., 2000). Park et al. (2003) reported the presence of a conserved block of synteny involving the linkage of PRKAG3 with the KIAA0173 and serine/threonine kinase genes in mammals and 2 fish species. We have found the same linkages to occur on chromosome 7 in the chicken as well.

During cloning and sequencing of 5′-RACE-derived PCR products of the cPRKAG3 gene, it was determined that there were 2 transcript variants that were identical at their 3′-ends but which had slightly different 5′-ends (GenBank accession no. DQ079814 and DQ079815). These 2 transcript variants, also called "long" and "short" forms, are produced by alternative splicing at the 5′-end of exon 3 that results in the inclusion or deletion of a 12-bp segment of sequence located at the intron 2–exon 3 boundary. This is due the presence of 2 potential splice sites located 12 bp apart (Figure 6, panel A). Roux et al. (2006) reported a similar situation for the bovine PRKAG3 gene, with the last 18 bp of intron 1 being subject to alternative splicing to produce "short" or "long" transcripts. They also found 2 additional alternative splicing sites separated by 3 bp



B

Exon no.	Chicken (T1)	Chicken (T2)	Human	Mouse (T1)	Mouse (T2)
	(nucleotides)				
1	>42	>42	52	45	-
2	40	40	40	40	106
3	239	227	156	153	153
–	–	–	404	407	407
4	82	82	82	82	82
5	59	59	59	59	59
6	46	46	46	46	46
7	55	55	55	55	55
8	127	127	127	127	127
9	166	166	166	166	166
10	38	38	38	38	38
11	147	147	147	147	147
12	170	170	233	182	182
13	>576	>576	694	–	–
CDS	1,149	1,137	1,470	1,470	1,395

Figure 6. A model depicting the structural organization of the 5′-AMP-activated protein kinase γ-3 gene (cPRKAG3) spanning 4.1 kb on chromosome 7 and its 2 transcript variants (T1 and T2; panel A). Coding exons are depicted as black boxes; noncoding exons are depicted as open boxes; and the alternatively spliced exons are depicted as gray boxes. The positions of the translation initiation codon (ATG) and the termination codon (TAG) are shown. The 2 potential 5′-ends of exon 3 (3 a/b) are highlighted by gray or black boxes, respectively. A portion of the sequence corresponding to the intron 2–exon 3 junction is shown, as are the 2 potential splice points (box), located 12 bp apart. Exon and coding region (CDS) size comparisons for PRKAG3 genes from different species (panel B). GenBank accession no. are as follows: chicken T1, DQ079814; chicken T2, DQ079815; human, NM_017431; mouse T1, AF525500; and mouse T2, AF525501.

(1 codon) at the 3′-end of intron 9, which resulted in the inclusion (or exclusion) of an additional amino acid in the area of the protein located between the second and third CBS domains. The net effect is the production of 4 transcript variants from the bovine PRKAG3 gene. We analyzed the equivalent region in the cPRKAG3 gene (i.e., 3′-end of intron 8) and found no evidence for an alternative splice site. Thus, there are apparently only 2 transcript variants for the cPRKAG3 gene, as appears to be the case for human and mouse PRKAG3 genes (Roux et al., 2006). The cPRKAG3 transcript variant 1 (“long” form) codes for a predicted protein of 382 amino acids, whereas transcript variant 2 (“short” form) codes for a predicted protein of 378 amino acids. Both predicted proteins contain 4 complete CBS domains. A comparison of predicted amino acid sequence between chicken and mammalian γ-3 subunit proteins ranged from 65 to 67%, whereas the sequence identity specifically within the CBS domain-containing portion of the subunits ranged from 68 to 88%.

The cPRKAG3 gene is expressed exclusively in heart and skeletal muscle. Both of these tissues demonstrated a predominance of transcript variant 2 (“short” form), which is expressed at a significantly higher level (1.5-fold) compared with transcript variant 1 (“long” form). Moreover, the expression of both transcripts was higher (2-fold) in skeletal muscle as compared with heart tissue (Figure 7). In contrast, significant expression of PRKAG3 in mammals has only been observed in skeletal muscle, but not in heart (Cheung et al., 2000). Moreover, Roux et al. (2006) observed preferential expression of the “short” transcript of the bovine PRKAG3 gene.

In conclusion, we reported the first evidence characterizing the genomic organization and expression of 3 γ subunit genes in the chicken. The combined effects of alternative splicing and alternate promoter usage resulted in the production of multiple transcripts for each of the cPRKAG genes and, in some cases, tissue-specific expression indicative of additional levels of complexity in the regulation of these genes. Lareau et al. (2004) have suggested that alternative splicing, common in many eukaryotes, may play an important role in regulating protein expression, thus contributing substantially to biological complexity. Although multiple cPRKAG subunit gene transcripts are expressed, it remains to be shown that the proteins encoded by them are actually produced and are indeed functional, especially those that contain missing or truncated CBS domains. This will require further study with the production and use of specific antibody reagents that recognize each of the γ subunit proteins. Each of the cPRKAG genes showed a high degree of similarity to mammalian counterparts, indicative of a conserved func-

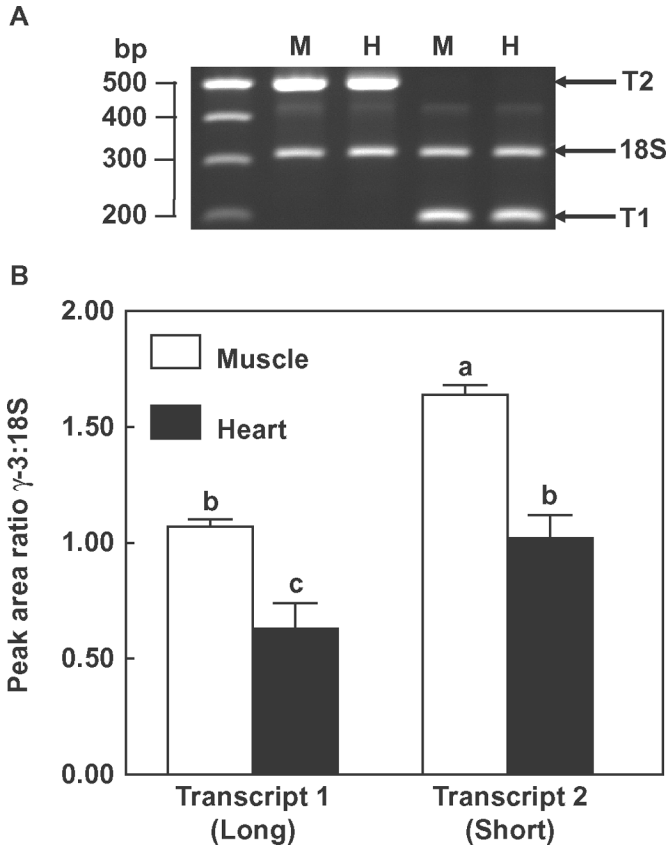


Figure 7. Expression of 2 5′-AMP-activated protein kinase γ-3 gene (cPRKAG3) transcripts in different tissues from 3-wk-old broiler chickens. A typical separation of PCR products for transcript variants 1 ("long" form) or 2 ("short" form) and 18S on a 1.5% agarose gel stained with ethidium bromide (panel A). Reverse transcription PCR and capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection were used to quantify the level of expression of T1 and T2 relative to an 18S rRNA internal standard. Expression ratio (T1 and T2:18S) values represent the mean ± SEM of 6 determinations. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons. Samples are identified as follows: skeletal muscle (M) and heart (H).

tion for the γ subunit proteins and possibly reflecting their derivation by duplication of a common ancestral gene, as has been suggested by Park et al. (2003) for mammalian PRKAG genes. Our findings offer new insights into chicken AMPK subunit gene structure and expression.

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