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cDNA, genomic sequence cloning and overexpression of giant panda (*Ailuropoda melanoleuca*) mitochondrial ATP synthase *ATP5G1*

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ABSTRACT. The *ATP5G1* gene is one of the three genes that encode mitochondrial ATP synthase subunit c of the proton channel. We cloned the cDNA and determined the genomic sequence of the *ATP5G1* gene from the giant panda (*Ailuropoda melanoleuca*) using RT-PCR technology and touchdown-PCR, respectively. The cloned cDNA fragment contains an open reading frame of 411 bp encoding 136 amino acids; the length of the genomic sequence is of 1838 bp, containing three exons and two introns. Alignment analysis revealed that the nucleotide sequence and the deduced protein sequence are highly conserved compared to *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, and *Sus scrofa*. The homologies for nucleotide sequences of the giant panda ATP5G1 to those of these species are 93.92, 92.21, 92.46, 93.67, and 92.46%, respectively, and the homologies for amino acid sequences are 90.44, 95.59, 93.38, 94.12, and 91.91%, respectively. Topology prediction showed that there is one protein kinase C phosphorylation site, one

casein kinase II phosphorylation site, five N-myristoylation sites, and one ATP synthase c subunit signature in the ATP5G1 protein of the giant panda. The cDNA of *ATP5G1* was transfected into *Escherichia coli*, and the ATP5G1 fused with the N-terminally GST-tagged protein gave rise to accumulation of an expected 40-kDa polypeptide, which had the characteristics of the predicted protein.

Key words: Giant panda; *Ailuropoda melanoleuca*; *ATP5G1*; cDNA cloning; Overexpression

INTRODUCTION

In mammals, the mitochondrial ATP synthase contains 16 different nuclear- or mitochondrial-encoded subunits that are separated into two oligomers. The catalytic F_1 oligomer extends into the mitochondrial matrix and is composed of five subunits that form the "headpiece" (α and β subunits) and central stalk (γ, δ, and ε subunits) (Walker et al., 1985; Abrahams et al., 1994). The Fo oligomer is membrane bound and composed of 10 different subunit types in mammals (a, b, c, d, e, f, g, F6, A6L, OSCP), in which OSCP, F6, b, and d subunits form a peripheral stalk (Walker et al., 1991; Collinson et al., 1994; Carbajo et al., 2005). Mitochondrial ATP synthase subunit c, a highly hydrophobic protein, is one of the chains of the nonenzymatic membrane components that reside in the transmembrane portion of the F_0 complex. The *ATP5G1* gene is one of the three genes (*ATP5G1*, *ATP5G2*, *ATP5G3*) (Gay and Walker, 1985; Farrell and Nagley, 1987; Higuti et al., 1993a,b), that encode subunit c of the proton channel (Fillingame, 1992). These three different genes, which encoded the c-Fo subunit, are translated into the same mature c subunit protein with different mitochondrial import presequences (Dyer and Walker, 1993; Yan et al., 1994; De Grassi et al., 2006). Of these three isoforms, only the *ATP5G1* gene expression is actively regulated in response to various physiological stimuli (such as ontogenic development and cold acclimation), whereas the other isoforms are thought to maintain the basal levels of the c-Fo subunit (Gay and Walker, 1985; Andersson et al., 1997; De Grassi et al., 2006).

The coupled rotation of the subunit c in F_0 appears to be essential for energy coupling between proton transport through F_0 and ATP hydrolysis or synthesis in F_1 (Sambongi et al., 1999). Therefore, subunit c is a key component for F_0 complex function (Groth, 2000), like an energy-driving motor. The *ATP5G1* belongs to the low transcript gene group and its transcriptional regulatory may play a key role in the biogenesis of mammalian H^+-ATP synthase (Houstek et al., 1995; Sangawa et al., 1997). It was also found that the protein levels of the ATP5G1 might be crucial for defining the final content of the ATP synthase in brown adipose tissue (Kramarova, 2008). Furthermore, it was reported that the *ATP5G1* gene of mouse (Kandil et al., 1997) is closely linked to Psme1 and Psme 2, which have been shown to be highly expressed in lung, spleen and liver tissue in pig (Wang et al., 2004), suggesting that ATP5G1 might be related to animal immunity.

The nuclear genes, *ATP5G1*, encoding the mitochondrial ATP synthase subunit c, have been cloned in human and other animal species. The giant panda (*Ailuropoda melanoleuca*) is a rare species currently found only in China. They are known as a "living fossil". For many years, studies on the giant panda have been mainly concentrated on fields such as breeding and propagation, ecology, morphology, taxology, physiology, and pathological biochemistry. Re-

searches on genetic diversity, parentage and phylogenesis, etc., have been developed (Towbin et al., 1979; Montali, 1990; Wu et al., 1990; Mather et al., 1997; Liao et al., 2003a,b; Du et al., 2007; Hou et al., 2007; Hou et al., 2009). However, the *ATP5G1* gene from the giant panda (*A. melanoleuca*) has not yet been reported.

In the present study, we have amplified the cDNA sequence of the *ATP5G1* gene from the total RNAs extracted from the skeleton muscle of the giant panda, and then analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those of humans and other reported mammalian species. The overexpression was also successfully done in *Escherichia coli* using pGEX4T-1 plasmids. The study provided scientific data for inquiring into the hereditary traits of the gene from giant panda and formulating the protective strategy for the giant panda.

MATERIAL AND METHODS

Materials and RNA isolation

Skeletal muscle was collected from a dead giant panda at the Wolong Conservation Center of the giant panda, Sichuan, China. The collected skeletal muscle was frozen in liquid nitrogen and then used for RNA isolation.

Total RNAs were isolated from about 400 mg muscle tissue using the Total Tissue/ Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to manufacturer instructions. The total RNAs extracted were dissolved in DEPC (diethylpyrocarbonate) water, and kept at -70°C.

Primer design, RT-PCR, cloning of RT-PCR products and sequencing

The PCR primers were designed by Primer Premier 5.0, based on the mRNA sequence of *ATP5G1* from *Homo sapiens* (NM_001002027), *Mus musculus* (NM_001161419), *Rattus norvegicus* (NM_017311), *Bos taurus* (NM_176649), and *Sus scrofa* (NM_001025218). The specific primers of *ATP5G1* are as follows: *ATP5G1*-F: 5'-GGGCAGTGGG AGTGCAGACT GA-3'; *ATP5G1*-R: 3'-CGTTGTGTTTAATGGTAAAGCT-3'.

Total RNAs were synthesized into the first-stranded cDNAs using a reverse transcription kit with Oligo dT as the primers according to manufacturer instructions (Promega, Shanghai, China). The 20-L first-strand cDNA synthesis reaction system included 1 g total RNAs, 5 mM $MgCl_2$, 1 mM dNTPs, 0.5 g Oligo dT_{15}, 10 U/L RNase inhibitor, and 15 U AMV reverse transcriptase, and was incubated at 42°C for 60 min.

The first-strand cDNA synthesized was used as a template. The total reaction volume for DNA amplification was 25 µL. Reaction mixtures contained 1.5 mM $MgCl_2$, 200 µM of each dATP, dGTP, dCTP, and dTTP (Omega, China), 0.3 µM of each primer, 5.0 U Taq plus DNA polymerase (Sangon Co., Shanghai, China). DNA amplification was performed using an MJ Research thermocycler, Model PTC-200 (Watertown, MA, USA) with a program of 4 min at 94°C followed by 30 cycles of 1 min at 94°C, 0.5 min at 45°C and 1.5 min at 72°C, and then ended with a final extension for 10 min at 72°C. After amplification, PCR products were separated by electrophoresis on 1.5% agarose gel with 1X TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide and visualized under UV light. The expected fragments

of PCR products were harvested and purified from gel using a DNA harvesting kit (Omega), and then ligated into a pMD18T vector (Takara, Japan) at 22°C for 12 h. The recombinant molecules were transformed into *E. coli* complete cells (JM109), and then spread on the LB plate containing 50 μg/mL ampicillin, 200 mg/mL IPTG (isopropyl-beta-D-thiogalactopyranoside), and 20 mg/mL X-gal. Five colonies were chosen with a vaccination needle, extraction plasmid. Plasmid DNA was isolated and digested by *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by Invitrogen (Shanghai, China).

Cloning the genomic sequence of *ATP5G1*

The PCR primers were the same as the *ATP5G1*-F and *ATP5G1*-R presented above. The genomic sequence of the *ATP5G1* gene was amplified using touchdown-PCR with the following conditions: 94°C for 30 s, 62°C for 45 s, 72°C for 4 min in the first cycle and the annealing temperature decreased 0.5°C per cycle; after 20 cycles, conditions changed to 94°C for 30 s, 52°C for 45 s, 72°C for 4 min for another 20 cycles. The fragment amplified was also purified, ligated into the clone vector and transformed into *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Invitrogen.

Construction of the expression vector and overexpression of recombinant *ATP5G1*

Based on the sequence of *ATP5G1* coding sequences, the primers 5'-CCG**GAA TTC**AC AATGCAGACCACCG-3' (*Eco*RI) and 5'-CCG**GTCGAC**GACCCTCACGGAACCT-3' (*Sal*I) were designed to amplify the cDNA (restriction sites are bold and underlined). The PCR was performed at 94°C for 3 min; 35 cycles of 30 s at 94°C, 45 s at 53°C and 1 min at 72°C, and a final extension of 10 min at 72°C. The amplified PCR product was cut and ligated into corresponding site of pGEX 4T-1 vector (Stratagen, Shanghai, China). The resulting construct was transformed into *E. coli* BL21 strain (Novagen, Shanghai, China) and used for the induction by adding IPTG at an OD_{600} of 0.6 and culturing further for 4 h at 37°C using the empty vector transformed BL21 as a control. The culture was centrifuged at 10,000 *g* for 5 min at room temperature after it was induced for 0.5, 1, 2, 3, and 4 h, and then separated by SDS-PAGE and stained with Coomassie blue R 250.

After 4 h of IPTG induction the cell suspension was sonicated in ice-water for a total of 15 min with a microsonicator (Sonics Inc., CA, USA), then the cells were centrifuged at 10,000 g for 30 min at 4°C, and the supernatant and pellet treated in lysis buffer containing 6.25 mM Tris-HCl, pH 6.8, 2 mM EDTA, 15% sucrose, 10% glycerol, 3% SDS, and 0.7 M β-mercaptoethanol, were also separated by SDS-PAGE to test the solubility of recombinant ATP5G1.

Data analysis

The sequence data were analyzed by the GenScan software (http://genes.mit.edu/GENSCAN.html). Homology research of the giant panda *ATP5G1* compared with the gene sequences of other species was performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). ORF of the DNA sequence was searched using the ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein structure of the *ATP5G1* sequence cloned was deduced using the PredictProtein software (http://cubic.bioc.columbia.Edu/predictprotein/).

RESULTS

Analysis of the cDNA of *ATP5G1* from the giant panda

About 500 bp of cDNA fragment was amplified from the giant panda with the primers *ATP5G1*-F and *ATP5G1*-R (Figure 1). The length of the cDNA cloned is 530 bp. BLAST research showed that the cDNA sequence cloned is highly homologous with the *ATP5G1* from *H. sapiens* (NM_001002027) and some other mammals reported, including *M. musculus* (NM_001161419), *R. norvegicus* (NM_017311), *B. taurus* (NM_176649), and *S. scrofa* (NM_001025218). On the basis of the high identity, we concluded that the cDNA isolated is the cDNA encoding the giant panda ATP5G1 protein. The *ATP5G1* sequence has been submitted to GenBank (accession No. GU338364). An ORF of 411 bp encoding 136 amino acids was found in the cDNA sequence (Figure 2).

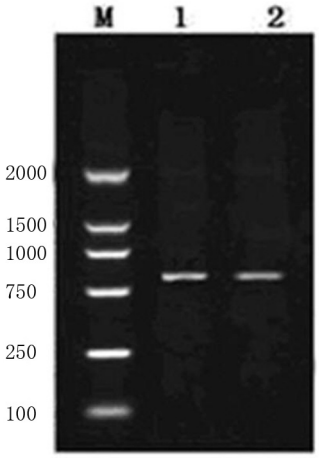

Figure 1. Reverse transcription-polymerase chain reaction products of the giant panda *ATP5GI. Lane M* = Molecular ladder DL2000; *lanes 1* and *2* = amplified *ATP5G1*.

```
1     GGGCAGTGGGAGTGCAGACTGAGACA  ATG CAG ACC ACC GGG GCA CTA CTC ATT TCT CCG
1                                 M  Q  T  T  G  A  L  L  I  S  P
60    GCT CTG ATC CGC TGT TGT ACC AGG GAT CTA ATC AGG CCT GTG TCT GCC TCC CTC
12     A  L  I  R  C  C  T  R  D  L  I  R  P  V  S  A  S  L
114   TTG AGT AGG CCA GAG ATC CCA GCT AAA CAG CCA TCC TGC AGC AGC TCC CCG CTC
30     L  S  R  P  E  I  P  A  K  Q  P  S  C  S  S  S  P  L
168   CAG GTG GCC CGA CGG GAG TTC CAG ACC AGT GTT GTC TCC CGG GAC ATT GAC ACA
48     Q  V  A  R  R  E  F  Q  T  S  V  V  S  R  D  I  D  T
222   GCA GCC AAG TTT ATT GGC GCT GGG GCT GCC ACA GTT GGT GTG GCT GGT TCA GGG
66     A  A  K  F  I  G  A  G  A  A  T  V  G  V  A  G  S  G
276   GCT GGC ATT GGA ACA GTG TTT GGC AGC TTG ATC ATT GGC TAT GCC AGG AAC CCA
84     A  G  I  G  T  V  F  G  S  L  I  I  G  Y  A  R  N  P
330   TCT CTC AAG CAG CAG CTC TTC TCC TAT GCC ATT CTG GGC TTT GCC CTG TCT GAG
102    S  L  K  Q  Q  L  F  S  Y  A  I  L  G  F  A  L  S  E
384   GCC ATG GGG CTC TTC TGT TTG ATG GTC GCC TTC CTC ATC CTC TTC GCC ATG TGA
120    A  M  G  L  F  C  L  M  V  A  F  L  I  L  F  A  M  *
438   GGTTCCGTGAGGGTCACCTACCTGTCCCTGCTG CTTCGACTTCAGGCCATGCCTGGTGCTGGAGTATGCC
508   AAG CTTTACCATT AAACACAACG
```

Figure 2. Nucleotide and deduced amino acid sequences of cDNA encoding the giant panda *ATP5G1*.

Analysis of the genomic sequence of *ATP5G1* from the giant panda

A fragment of about 1800 bp was amplified from genonic DNA of the giant panda using primers *ATP5G1*-F and *ATP5G1*-R (Figure 3). The length of the DNA fragment cloned was 1,838 bp. Comparison between the cDNA sequence and this DNA fragment indicated that the cDNA sequence is a full cDNA corresponding to four exons in the the *ATP5G1* genomic sequence of the giant panda. The genomic sequence of the *ATP5G1* gene has been submitted to GenBank (accession No. GU338365).

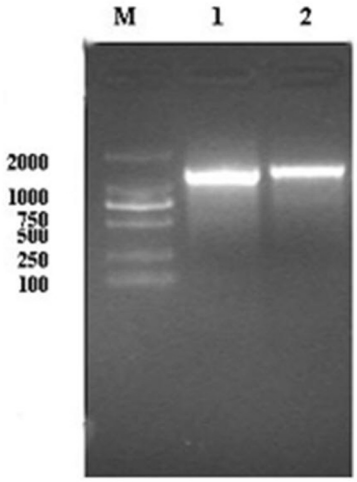

Figure 3. PCR products of complete genomic sequence of the giant panda *ATP5G1*. *Lane M* = Molecular ladder DL2000; *lanes 1* and *2* = amplified *ATP5G1* genomic sequences.

Prediction and analysis of protein functional sites in the ATP5G1 protein of the giant panda

Primary structure analysis revealed that the molecular weight of the putative ATP5G1 protein of the giant panda is 14.2147 kDa with a theoretical pI = 9.43 (Table 1). Topology prediction shows there is one protein kinase C phosphorylation site, one casein kinase II phosphorylation site, five N-myristoylation sites, and one ATP synthase c subunit signature in the ATP5G1 protein of the giant panda (Figure 4).

Overexpression of the *ATP5G1* gene in *E. coli*

The *TP5G1* gene was overexpressed in *E. coli* and amplified individually by PCR, then cloned in a pGEX 4T-1 plasmid, resulting in a gene fusion coding for a protein bearing a GST-tag extension at the N-terminus. Expression was tested by SDS-PAGE analysis of protein extracts from recombinant in *E. coli* BL21 (Figure 5).

The results indicated that the protein *ATP5G1* fusion with the N-terminally GST-tagged form gave rise to the accumulation of an expected 40-kDa polypeptide that formed

inclusion bodies. Apparently, the recombinant protein was expressed after half an hour of induction and after 3 h reached the highest level.

Table 1. Molecular weight and isoelectric point of ATP5G1 of the giant panda (*Ailuropoda melanoleuca*) and other five mammals.

	A. melanoleuca	*Homus sapiens*	*Mus musculus*	*Rattus norvegicus*	*Bos taurus*	*Sus scrofa*
Molecular weight (kDa)	14.21476	14.27673	14.19973	14.24389	14.22266	14.21476
Isoelectric point	9.43	9.81	10.05	9.96	9.89	9.41

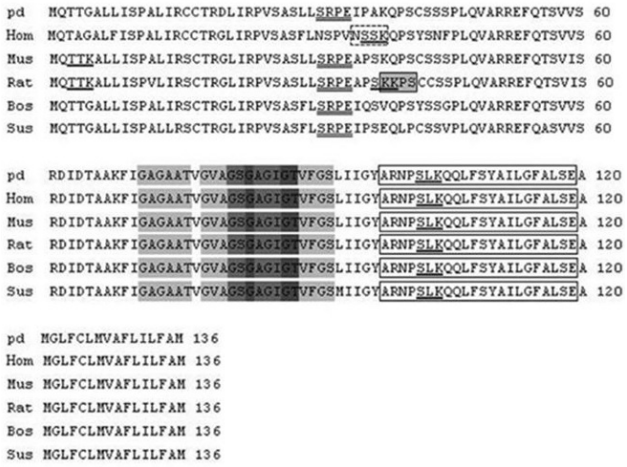

Figure 4. Comparison of the amino acid sequence of ATP5G1 between the giant panda and other five mammalian species. pd = giant panda; Hom = *Homo sapiens*; Mus = *Mus musculus*; Rat = *Rattus norvegicus*; Bos = *Bos taurus*; Sus = *Sus scrofa*. ▬ = protein kinase C phosphorylation site; ▬ = casein kinase II phosphorylation site; ▭ = N-myristoylation site; ⬚ = N-glycosylation site; ▭ = ATP synthase c subunit signature; ▭ = cAMP- and cGMP-dependent protein kinase phosphorylation site.

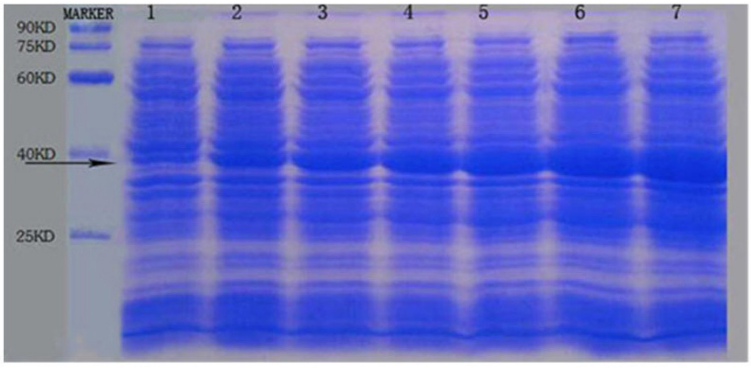

Figure 5. Protein extracted from *Escherichia coli* BL21 strains were analyzed by SDS-PAGE gel stained with Commassie blue R250. The arrow indicates the recombinant protein bands induced by IPTG at 0, 0.5, 1, 2, 3, and 4 h (*lanes 2-7*), respectively. *Lane 1* = Products of the *E. coli* strains with the empty vectors.

The supernatant and pellet from the sonicated *E. coli* cells were also tested by SDS-PAGE analysis after 4 h of IPTG induction. The results showed that about 80% of the recombinant ATP5G1 were soluble and thus can be used to further functional studies.

DISCUSSION

Alignment analysis of the cDNA sequence of *ATP5G1* and the deduced amino acid sequence between the giant panda with other mammals reported, including *H. sapiens* (NM_001002027), *M. musculus* (NM_001161419), *R. norvegicus* (NM_017311), *B. taurus* (NM_176649), and *S. scrofa* (NM_001025218), was performed by DNAMAN 6.0. The homologies for coding sequence between the giant panda with the five mammals cited above were 93.92, 92.21, 92.46, 93.67, and 92.64%, respectively; the homologies for deduced amino acid sequence are 90.44, 95.59, 93.38, 94.12, and 91.91%, respectively. These results indicated that the coding sequence of *ATP5G1* and the deduced amino acid sequence are highly conserved. Further analysis showed that the molecular weight and theoretical pI of the putative protein of ATP5G1 among its mammalian homologues were very close (Table 1).

The genomic sequence of the ATP5G1 cloned is 1838 bp in size, containing 3 exons and 2 introns. Compared with some mammals, including *H. sapiens* (NC_000017), *M. musculus* (NC_000077), *R. norvegicus* (NC_005109), *B. taurus* (NC_007317), *Pan troglodytes* (NC_006484), *Macaca mulatta* (NC_007873), *Canis familiaris* (NC_006591), and *Gallus gallus* (NC_006114), the length of the genomic sequence, number of exons, sites of CDS joined, 5'- and the 3'-untranslated sequences are different (see Table 2).

Table 2. Comparison of the *ATP5G1* genomic sequence among 9 mammal species.

	Genomic sequence length (bp)	Number of exons	Number of introns	Length of 5'-untranslated sequence (bp)	Length of 3'-untranslated sequence (bp)	CDS join	GenBank accession Nos.
Ailuropoda melanoleuca	1838	3	2	26	93	27..65, 884..961, 1452..1745	GU33836
Homo sapiens	3086	4	3	632	102	633..671, 1587..1664, 2371..2549, 2870..2984	NC_000017
Mus musculus	6186	4	3	13	4252	13..51, 999..1076, 1337..1515, 1820..1934	NC_000077
Rattus norvegicus	2581	4	3	565	99	566..604, 1531..1608, 1917..2095, 2370..2484	NC_005109
Bos taurus	2124	4	3	40	103	41..79, 973..1050, 1428..1606, 1907..2021	NC_007317
Pan troglodytes	3106	4	3	653	101	654..692, 1607..1684, 2419..2570, 2891..3005	NC_006484
Macaca mulatta	3044	4	3	629	100	630..668, 1573..1647, 2328..2506, 2830..2944	NC_007873
Canis familiaris	2714	4	3	642	101	643..681, 1424..1501, 1980..2158, 2499..2613	NC_006591
Gallus gallus	3447	4	3	399	371	400..441, 1769..1846, 2116..2291, 2962..3076	NC_006114

The deduced protein sequence of ATP5G1 is composed of a signal peptide of 61 amino acids and a mature subunit protein of 75 amino acids (Gay and Walker, 1985; Higuti et al., 1993a,b; Piko et al., 1994). The cDNA of *ATP5G1* cloned here is 530 bp in length and contains an open reading frame of 411 bp encoding 136 amino acids. The 5'-noncoding region is 26 bp in length and the 3'-noncoding region consists of 96 nucleotides, excluding the poly (A) tail.

A polyadenylation signal (ATTAAA), which is common in eukaryotic mRNAs (Wickens and Stephenson, 1984; Fearnley et al., 1990), was located 8 nucleotides upstream from the poly (A) addition site (Figure 4). Part of the poly (A) sequence was present in the 3'-end of this cDNA clone. The mature protein coding region in ATP5G1 is 225 nucleotides long, is capable of encoding a protein of 75 amino acids with a molecular weight of 7.608 kDa, and extends from GAC at nucleotide position 210-212 to nucleotide position 434. This coding sequence is followed by the termination codon TGA. Interestingly, the sequence of the mature human subunit c is completely identical with those of the mature protein of the other five species compared. The possible coding region in *ATP5G1* for the import signal peptide is located upstream from the mature protein coding region from only one ATG codon at nucleotide position 27-29 to nucleotide position 209, which is capable of encoding 61 amino acids. In contrast to the mature proteins, the pre-sequences are not conserved.

Our analysis revealed that there is one protein kinase C phosphorylation site, one casein kinase II phosphorylation site, five N-myristoylation sites, and one ATP synthase c subunit signature in the ATP5G1 protein of the giant panda. It is known that the functional motif (ATP synthase c subunit signature) as well as the highly conserved protein sequences occurring across various species of subunit c play a vital role in maintaining the normal mitochondrial physiological function (Palmer et al., 1989; Li et al., 2001).

In conclusion, the cDNA and the complete coding sequence of the *ATP5G1* gene have been cloned and the *ATP5G1* cDNA is expressed efficiently in prokaryotic organism using pGEX 4T-1 plasmids. The gained fusion protein is in accordance with the expected 40-kDa polypeptide. These results suggest that the protein is active and is exactly the protein encoded by the *ATP5G1* from the giant panda. Further study on the ATP5G1 protein will contribute to the genetic polymorphism and the protection for gene resources of the giant panda.

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