Full Length Research Paper

Sequence analysis and over-expression of ribosomal protein S28 gene (*RPS28*) from the Giant Panda

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RPS28 is a component of the 40S small ribosomal subunit encoded by *RPS28* gene, which is specific to eukaryotes. The cDNA and the genomic sequence of *RPS28* were cloned successfully from the Giant Panda using RT-PCR technology and Touchdown-PCR, respectively. Both sequences were analyzed preliminarily and the cDNA of the *RPS28* gene was also overexpressed in *Escherichia coli* ER2566 and immunoblotted. The cDNA of the *RPS28* cloned from Giant Panda is 216 bp in size, containing an open reading frame of 210 bp encoding 69 amino acids. The length of the genomic sequence is 790 bp, which was found to possess three exons and two introns. Alignment analysis indicated that the nucleotide sequence of the coding sequence shows a high homology to those of *Homo sapiens*, *Bos Taurus, Mus musculus, Rattus norvegicus* and *Sus scrofa* (92.4, 92.4, 87.1, 86.7 and 89.5%, respectively) as determined by Blast analysis.. The amino acid sequence encoded by *RPS28* gene of the Giant Panda shared a high homology (100%) with those of *Homo sapiens*, *B. Taurus, M. musculus, R. norvegicus* and *S. scrofa*. Primary structure analysis revealed that the molecular weight of the putative RPS28 protein is 7.841 kD with a theoretical pI 10.70. Topology prediction showed there is one ribosomal protein S28e signature site, three protein kinase C phosphorylation site and two casein kinase II phosphorylation site in the RPS28 protein of the Giant Panda. The *RPS28* gene can be really expressed in *Escherichia coli* and the RPS28 protein fusioned with the N-terminally GST -tagged protein gave rise to the accumulation of an expected 34 KDa polypeptide. The expression product obtained could be used for purification and study of its function further.

Key words: *RPS28* gene, ribosomal protein S28 (RPS28), giant panda, (*Ailuropoda melanoleuca*), genomic cloning, overexpression.

INTRODUCTION

The ribosome, a compact ribonucleoprotein (RNP), which plays an important role in the translational mechanism, is universal to all organisms. Biochemically, the eukaryotic ribosome is composed of four ribosomal RNA molecules and over 70 ribosomal proteins (RPs) (Wool et al., 1979; Wool, 1990). In mammals, the 60S and 40S subunits are composed of 47 and 32 RPs, respectively (Wool et al., 1995). Ribosome biogenesis is coordinately controlled by a variety of mechanisms including transcriptional and post-transcriptional regulation in response to changes within or outside the cell such as carbon source and nut-

rient availability (Mager, 1988).

Ribosomal protein S28 (RPS28) is a component of the 40S small ribosomal subunit encoded by *RPS28* gene, which is specific to eukaryotes. The *RPS28* was the smallest in the 40S small ribosomal subunit (Yoshihama et al., 2002). Three small-subunit ribosomal proteins shown to influence translational accuracy in *Saccharo-myces cermisiae* are conserved in structure and function with their procaryotic counterparts. One of these three small-subunit ribosomal proteins is encoded by *RPS28A* and *RPS28B* (RPS28) (Anthony and Liebman, 1995).

The sequence information about *RPS28* gene from mammalian is focused on *Homo sapiens*, *Mus musculus,* and *Rattus norvegicus* etc. (Chan et al., 1991; Hassouna et al., 1984; Kenmochi et al., 1998; Wool et al., 1995; Yoshihama et al., 2002). However, *Rps28* gene from the

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Giant Panda (*Ailuropoda melanoleuca*) has not been reported yet.

The Giant Panda, *A. melanoleuca*, is one of the most endangered animals in the world and lives only in the remote high mountains in the Sichuan, Shanxi and Gansu provinces of China. They are known as a "living fossil". So far most studies have focused on genetic diversity, parentage and phylogenies etc, while reports on functional gene are handful (Du et al., 2007; Hou et al., 2007a, b; Hou et al., 2008; Peng et al., 2007). It would further our understanding of this rare species by molecular studies of genes such as *RPS28*.

This study was conducted using RT-PCR technique to amplify the cDNA of *RPS28* gene from the total RNA and Touchdown-PCR technique to amplify the genomic sequence of the *RPS28* from DNA which from the skeleton muscle of the Giant Panda. The sequence characteristics of the protein encoded by the cDNA was compared with those of human and other mammalian species reported. We also overexpressed it in *Escherichia coli* using pGEX 4T-2 plasmids. The study provides scientific data for inquiring into the hereditary traits of the gene from Giant Panda and formulating the protective strategy for the Giant Panda.

MATERIALS AND METHODS

Materials

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 DNA and RNA isolation.

DNA and RNA isolation

The genomic DNA was isolated from Giant Panda muscle tissue according to the literature (Sambrook et al., 1989). The DNA obtained was dissolved in TE buffer and kept at -20°C. Total RNAs were isolated from about 400 mg of muscle tissue using the Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to the manufacturer's instructions and then dissolved in RNase-free ddH$_2$O, and kept at -70°C. DNA and RNA sample quality was checked using Experion (Bio-Rad) and quantification was performed spectrophotometrically.

Primers design, RT-PCR, Cloning of cDNA sequence and sequencing

The PCR primers were designed by Primer Premier 5.0, basing on the mRNA sequence of *RPS28* from *H. sapiens* (NM_001031), *M. musculus* (NM_016844), *R. norvegicus* (NM_001105730), *Bos taurus* (NM_001025316) and *Sus scrofa* (NM_001001587). The specific primers of cDNA sequence are as follows:

RPS28-F: 5'- GCCATCATGG ACACGAGCCG-3'
RPS28-R: 5'-TCAACGCAGC CTCCGGGCTT-3'

Total RNAs (1 µg) were synthesized into the first-stranded cDNAs using a reverse transcription kit with Oligo dT as the primers followed by PCR amplification according to the manufacturer's instructions (Promega). Reverse transcription reactions were performed in duplicate. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis. After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1× TAE 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 pMD18-T vector (TaKaRa) at 16°C for 2 h. The recombinant molecules were transformed into *E. coli* competent cells (DH5α) and then spread on the LB-plate containing 50 µg/mL ampicillin, 200 mg/mL IPTG and 20 mg/mL X-gal. Plasmid DNA was isolated and digested by *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by Huada Zhongsheng Scientific Corporation (Beijing, China).

Cloning the genomic sequence of *RPS28*

The PCR primers were above (*RPS28*-F and *RPS28*-R). The genomic sequence of the *RPS28* gene was amplified using Touchdown-PCR with the following conditions: 94°C for 30 s, 62°C for 45 s, 72°C for 3 min in the first cycle and the anneal temperature deceased 0.5°C per cycle; after 20 cycles conditions changed to 94°C for 30 s, 52°C for 45 s, 72°C for 3 min for another 20 cycles. The fragment amplified was also purified, ligated into the clone vector and tansformed into the *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Huada Zhongsheng Scientific Corporation.

Construction of the expression vector and overexpression of recombinant RPS28

PCR fragment corresponding to the RPS28 polypeptide was amplified from the RPS28 cDNA clone with the forward primer, 5'- cggatccATGGACACGAGCCGTGTG -3' (BamHI) and reverse primer, 5'-cgaattcttaTCAACGCAGCCTCCGG -3' (EcoRI), respectively. The PCR was performed at 94°C for 2 min; 35 cycles of 30 s at 94°C, 45s at 56°C and 1 min at 72°C; 7 min at 72°C. The amplified PCR product was cut and ligated into corresponding site of pGEX 4T-2 vector (Stratagen). The resulting construct was transformed into *E. coli* ER2566 strain (Novagen) and used for the induction by adding IPTG (isopropyl-b-D-thiogalactopyranoside) at an OD600 of 0.6 and culturing further for 4 h at 37°C, using the empty vector transformed ER2566 as a control. The recombinant protein samples were induced after 1, 2 and hours, and then separated by SDS-PAGE and stained with Coomassie Brilliant Blue dye.

SDS-PAGE and western blotting analysis

The culture was centrifuged at 10000 g for 5 min at room temperature after it was induced for 0.5, 1, 2, 3 and 4 h, respectively. The culture supernatant was concentrated with methanol and chloroform (3:1,v/v), and SDS–PAGE (SDS polyacrylamide gel electrophoresis) was performed to investigate protein production and purity using slab gels containing 12% (w/v) polyacrylamide on a mini-protean II slab cell apparatus (Bio-Rad,Hercules,CA). Protein samples were visualized by Coomassie brilliant blue R-250 staining. The samples from the SDS–PAGE gels were transferred to a membrane of polyvinylidene difluoride (PVDF, Millipore, Billerica, MA), as previously described (Towbin et al., 1979). Western blotting analysis was performed using mouse monoclonal antibody against GST-tag as primary antibody and peroxidase-conjugated goat anti-mouse IgG as secondary antibody.

Data analysis

The sequence data were analyzed by GenScan software
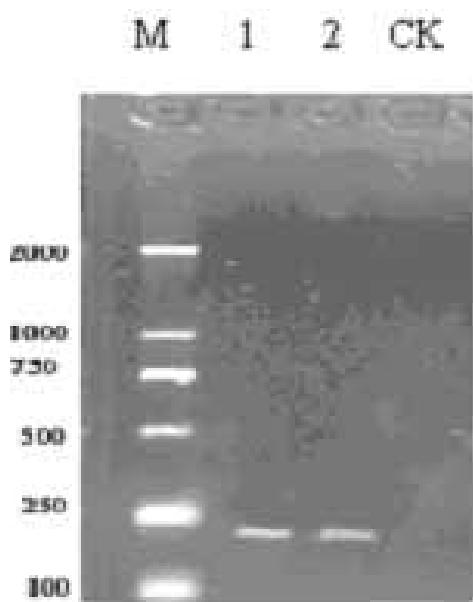
Figure 1. Reverse Transcription Polymerase Chain Reaction Products of the Giant Panda *RPS28*. M: Molecular ladder DL2000; 1, 2: the amplified *RPS28*.
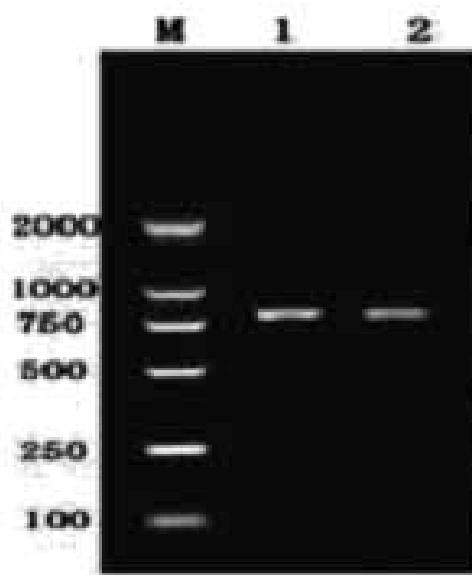
Figure 2. Polymerase Chain Reaction Products of complete genomic sequence of *RPS28* from Giant Panda. M: Molecular ladder DL2000; 1, 2: the amplified *RPS28* complete sequences.

(http://genes.mit.edu/GENSCAN.ht ml). Homology research of the Giant Panda *RPS28* compared with the gene sequences of other species ware performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). ORF of the DNA sequence was searched using ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein structure of the *RPS28* sequence cloned was deduced

using PredictProtein software (http://cubic.bioc.columbia.edu/predictprotein/). Multiple Sequence Alignment was performed by software DNAstar Lasergene and DNAMAN 6.0.

RESULTS

Analysis of the cDNA of *RPS28* from the Giant panda

A cDNA fragment of about 200 bp was amplified from the Giant Panda with primers *RPS28*-F and *RPS28*-R (Figure 1). The length of the cDNA cloned is 216 bp. Blast research showed that the cDNA sequence cloned shares a high homology with the *RPS28* from some mammals reported, including *H. sapiens*, *B. Taurus, M. musculus, R. norvegicus* and *S. scrofa*. On the basis of the high identity, we concluded that we had cloned the cDNA encoding the Giant Panda RPS28 protein. The *RPS28* cDNA sequence was submitted to Genbank (accession number: FJ169481), containing the 5'-untranslated sequence in size of 6 bp. An ORF of 210 bp encoding 69 amino acids was found in the cDNA.

Analysis of the genomic sequence of *RPS28* from the Giant Panda

A DNA fragment of about 800 bp was amplified with primers *RPS28*-F and *RPS28*-R (Figure 2). The length of the DNA fragment cloned is 790 bp. Comparison between the cDNA sequence and the DNA fragment sequence of the *RPS28* amplified from Giant Panda was performed by software Lasergene. The result indicated that the cDNA sequence is in full accord with three fragments in the DNA fragment, which manifests that the DNA fragment amplified is the genomic sequence of the *RPS28* from Giant Panda (Figure 3). The genomic sequence of the *RPS28* has been submitted to Genbank (accession number: FJ169482).

Prediction and analysis of protein functional sites

Primary structure analysis revealed that the molecular weight of the putative RPS28 protein is 7.841 kD with a theoretical pI 10.70. Topology prediction showed there is one ribosomal protein S28e signature site, three protein kinase C phosphorylation sites and two casein kinase II phosphorylation sites in the RPS28 protein of the Giant Panda (Figure 4).

Overexpression of the *RPS28* gene in *E. coli*

The *RPS28* gene was overexpressed in *E. coli* and amplified individually by PCR, then cloned in a pGEX 4T-2 plasmid, resulting in a gene fusion coding for a protein bearing a GST-tag extension at the N terminus. Expres-

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GCCATCATGGACACGAGCCGTGTGCAGCCCATCAAGTTGGCCAGGGTGAGGTGGGGGTCC 60
        M   D   T   S   R   V   Q   P   I   K   L   A   R
TGATTTGGGGGCCGGGATGAAGAAAGATGGGGTAAGGGGGTCAAGCTGCCCAGGTCTGAT 120

CCCGGCTTCTCTCCGCCAGGTCACCAAGGTTCTGGGCAGGACTGGCTCACAGGGACAGTG 180
                    V   T   K   V   L   G   R   T   G   S   Q   G   Q   C
CACGCAGGTGAGCGCTTGGGTGGGGACGGCCCGGGGTCTGAGCCCTGTTGACTGCCTGGC 240
    T   Q
CAGCTAGACCCTGAGAACCGGAAGCCTTATATCCCAGGGCTCCCAGAGTGCCCAGAGACC 300

TCACTAGAGCTCTGCCTACAGGGTCTGAGCTCCGTCGACCTCACTCCCCGGGCTCTTACG 360

TTCCTCTTTCTCGTGGCTTAGGCCATCCTAACGACCTCTTTGTGGCCATGCCCTGCCGAG 420

TGTGCTTCCCTTCCGAACGGGACTAGAGCTGCAGCTGTCCCTCCTGGTGGCTGCTTTTGC 480

CGCTCCAGAACTTTGTTCTCCACGACTTGGCTCTCTTGGGCTTCTCTCCTGGCTCAGTTC 540

TGCCTCCCAGACTCAGTCCCCTTTTTAAGTATCTCTGGGCTGGTCCCAACACGTTCTCCG 600

AATCGAAACCTGCTGTCCAGCTGTAGCGGGCCCTAACTCGTTCGCGTCCCCCCGATGGGC 660

CCCGTAGGTGCGCGTGGAGTTCATGGACGACACCAGCCGCTCCATCATCCGCAACGTGAA 720
        V   R   V   E   F   M   D   D   T   S   R   S   I   I   R   N   V   K
AGGCCCTGTGCGCGAGGGCGACGTGCTCACCCTATTGGAGTCAGAGCGAGAAGCCCGGAG 780
    G   P   V   R   E   G   D   V   L   T   L   L   E   S   E   R   E   A   R   R
GCTGCGTTGA                                                   790
    L   R   ✳
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Figure 3. The Giant Panda *RPS28* genomic sequence encoding RPS28 protein. Nucleotides are numbered in the 5'-to-3' direction. The predicted amino acid sequence of the inhibitor and its possible pre-sequence are shown under the nucleotide.

MD TSR VQPIKLARVTKVLGRTGSQGQCTQVRVEFMDD TSR SIIRNVKGPVR

EGDVL TLLE SER EARRLR

Figure 4. Analyzed the functional sites of the amino acid sequence encoded by *RPS28* gene of the Giant Panda. ☐: Protein kinase C phosphorylation site. ▨ : Casein kinase II phosphorylation site. ___: Ribosomal protein S28e signature.

sion was tested by SDS-PAGE analysis of protein extracts from recombinant in *E. coli* ER2566 strains (Figure 5).

The results indicated that the protein RPS28 fusion with the N-terminally GST-tagged form gave rise to the accumulation of an expected 34 kDa polypeptide that formed inclusion bodies. Apparently, the recombinant protein was expressed after half an hour of induction and reached the highest level after 2 h. The Western blotting experiment demonstrated the *RPS28* gene can really ex-

press in *E. coli* ER2566 strains (Figure 6).

DISCUSSION

Alignment analysis of the cDNA sequence of *RPS28* and the deduced amino acid sequence between the Giant Panda and some mammals reported including *H. sapiens*, *B. Taurus, M. musculus, R. norvegicus* and *S. scrofa* was performed by DNAMAN version 6.0. The
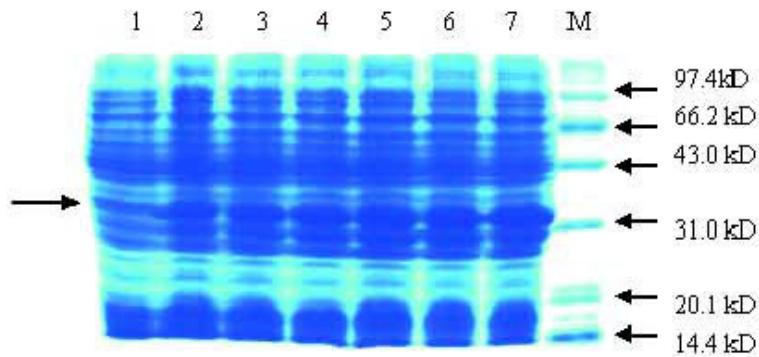
Figure 5. Protein extracted from recombinant *E. coli* ER2566 strains were analyzed by SDS-PAGE gel stained with Commassie blue R250. Numbers on right shows the molecular weight, and the arrow indicates the recombinant protein bands induced by IPTG with 0.5, 1, 1.5, 2, 2.5, and 3 hours (lanes 2-7, respectively). Lane 1 represents the products of the *E. coli* strains with the empty vectors. Lane 8 is marker.
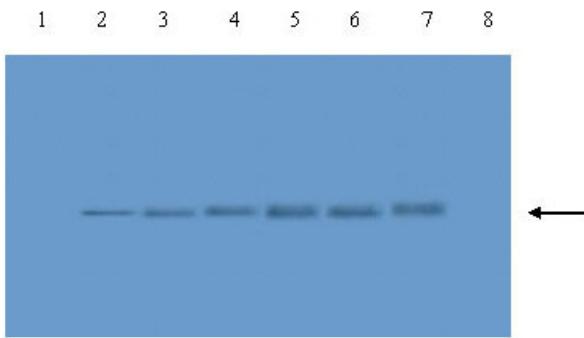
Figure 6. The arrow indicates immunoblot of RPS28 protein induced by IPTG with 0, 0.5, 1, 2, 3 and 4 h (lanes 2-7, respectively). Lane 1 is the control.

homologies for coding sequence between the Giant Panda and the five mammals above are 92.4, 92.4, 87.1, 86.7 and 89.5%, respectively. The amino acid sequence encoded by *RPS28* gene of the Giant Panda shared a high homology (100%) with those of *H. sapiens*, *B. Taurus, M. musculus, R. norvegicus* and *S. scrofa* and indicated the same molecular weight and isoelectric point. That is to say, the deduced amino acid sequence is highly conserved and the functional sites are entirely identical in RPS28 proteins of these mammals.

The genomic sequence of *RPS28* is 790 bp in size. A comparison of the nucleotide sequences of the genomic and cDNA sequences indicated that the genomic suquence of *RPS28* possesses three exons and two introns, which is also supported by restriction mapping of the genomic and cDNA sequences. Compared with some mammals including *H. sapiens* (NC_000019)*, B. taurus* (NC_007305)*, M. musculus* (NC_000083) and *R. norvegicus* (NC_005106)*, the three exons, which comprise the cDNA sequence of *RPS28* gene after RNA spli-

cing, is highly conserved and remain essentially the same. The restriction sites in the exons are the same in both the cDNA and the genomic sequences. On the contrary, the two introns are different in length. The first intron of the *RPS28* gene from Giant panda is 94 bp, while the length ranges from 86 bp (*H. sapiens*) to 94 bp (*B. Taurus* and *A. melanoleuca*) and the length of the second intron of the Giant Panda is 480 bp, while the length ranges from 249 bp (*H. sapiens*) to 968 bp (*R. norvegicus*). The variations in lengths of the introns determine the lengths of the *RPS28* genes (Figure 7).

The *RPS28* gene obtained is expressed efficiently in prokaryotic organism such as the *E. coli* using pGEX 4T-2 plasmids, and the gained fusion protein is in accordance with the expected 34 kDa polypeptide. These results suggest that the protein is active and it is just the protein encoded by the *RPS28* from the Giant Panda. The expression product obtained could be used for purification and study of its function further.

The cDNA and the genomic sequence of *RPS28* were cloned successfully for the first time from the Giant Panda, respectively, which were both sequenced and analyzed preliminarily and the cDNA of the *RPS28* gene was also overexpressed in *E. coli* ER2566 strains and immunoblotted, which is the first report on the *RPS28* gene from the Giant Panda. The data will enrich and supplement the information about *RPS28.* In addition, it will contribute to the protection for gene resources and the discussion of the genetic polymorphism.

mittee of SiChuan Province (07ZA120) and Key Discipline Construction Project in Sichuan Province (SZD0420).

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