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Full Length Research Paper

ZNF 197L is dispensable in mouse development

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The gene trap technique is a newly powerful approach for characterizing and mutating genes in mouse. We used gene trap method to identify mice gene of unknown function and to establish their mouse line. Here, we found one such gene termed as *Ayu17-923* (similar to zinc finger protein 197 transcript variant 2, ZNF 197L), which is located on mouse chromosome 3 encoding 136 amino acids carrying a KRAB domain in its N-terminal. The insertion of trap vector into the first intron of this gene resulted in mutation. Homozygous mice for this mutation have normal survival and have no conspicuous phenotype. These data showed that *Ayu17-923* (ZNF197L) gene was dispensable for mouse survival and development.

Key words: Gene trapping, Ayu17-923, ZNF 197L, KRAB domain, mouse survival.

INTRODUCTION

Gene trapping offered an efficient tool for the identification of gene and production of its mutant (Gossler et al., 1989). Now, it was widely used for a better understanding of the gene function, not only on the isolation of new genes, but also on the production of mutant being important (Lako and Hole, 2000; Carlson and Largaespada, 2005). We previously developed a new gene trap vector, termed as pU17, which was a promoterless vector (Taniwaki et al., 2005). This pU17 contained stop codon in the upstream of ATG codon of *β-geo* and did not contain IRES sequence. Thus, the *β-geo* was expected to be expressed only when the vector was integrated near the ATG codon of mouse endogenous gene (Taniwaki et al., 2005). Using this approach, we identified a novel gene carrying the KRAB domain on mouse chromosome 3 which is Mus musculus, similar to zinc finger protein 197 transcript variant 2 (XM_ 001476346, GI:149251673). KRAB domain is involved in protein-protein interactions (Kim et al., 1996; Friedman et al., 1996), and KRAB-containing proteins are thought to have critical functions in cell proliferation, differentiation, apoptosis and neoplastic transformation (Urrutia, 2003). However, expression pattern and functions of this protein had not been clear yet. Here, we reported that this gene is strongly expressed in mouse ES cells and kidney. Its deficient mice have normal phenotype and life span, indicating that this gene is compensable for mouse survival.

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MATERIALS AND METHODS

Animals

Mice were kept at SPF facility with 24°C and 55% relative humidity on a 08:00 to 19:00 h light/19:00 to 08:00 h dark cycle and were fed water and breeding quality feed *ad libitum*. Chimeric mice were produced by the aggregation method using gene-trap ES clones and morulas of ICR (Charles River) mice, and the chimeric mice were mated with C57BL/6 (CLEA) females to obtain F1 heterozygotes. Heterozygous males were mated with heterozygous females to produce wild-type mice, heterozygous mice, and homozygous mice for the gene trap insertions. Offspring were weaned at 3 weeks of age. At that time, tail biopsies were taken for genotyping.

Trapping vector

The trapping vector, pU17, as described previously by Taniwaki et al. (2005), consists of a mouse En2 splicing acceptor, *lox71* site, *β-*

galactosidase, neomycin phosphotransferase, *loxP*, polyA, and *lox2272* site in pSP73 plasmid. When the pU17 was inserted into mouse endogenous gene locus, the *β-geo* would be expressed under the promoter of trapped gene, thus making it possible to select the trap clones in the presence of G418.

Cell culture and electroporation

The ES cell line, TT2, was grown as described by Yagi et al. (1993). For co-electroporation experiments, a targeting plasmid and pCAGGS-Cre were used together in their circular forms. The ES cells ($1x10^7$ cells/0.8ml in PBS) were electroporated at 200 V and 960 μF, and after 24 or 48 h, they were selected with G418 at 200 μg/mg for 7 days. Colonies were stained with *X-gal* or picked and expanded for DNA analysis. The primers used in DNA analysis are as follows: GF1, 5'-TCACATGCTCACCATGTTGC-3'; GF2, 5'-ATTG ATGTGACCACCTCACC-3'; GR1, 5'-TCTGGAGTCTCATCTGT AGC-3'; Z2, 5'-TGTGAGCGAGTAACAACC-3'; 1R, 5'-GCAGTTATG CCCTATTATGG-3'; 3R, 5'-GATCTCATTCATCAAGC GGT-3'.

5'-RACE and sequence analysis

5'-RACE was performed using the method described by Frohman et al. (1988). In brief, total RNA was isolated from G418-selected ES cells. Total RNA was poly-A selected by passing it through a poly-dT column twice (mRNA purification kit; Pharmacia, Freiburg, Germany). First-strand cDNA synthesis using one of two *β*-galactosidase-specific primers (5'-CTGCAAGGCGATTAAGTTGG-3' or 5'-ATT CAGGCTGCGCAACTGTTG G-3'), RNase H treatment, spin-column purification, and dCTP tailing were performed (5'-RACE system; Gibco BRL, Gaithersburg, MD, USA). The cDNA was amplified with two rounds of PCR reaction. For the first PCR, a forward primer specific to the *β*-galactosidase sequence (see above) and a reverse primer annealing to the poly-dC tail (Gibco BRL, Gaithersburg, MD, USA) or to the ligated anchor primer (Clontech, Mountain View, CA, USA) were used. The products of the first PCR either were, or were not size selected by agarose gel electrophoresis and were reamplified in a second round of PCR using a nested *β*-galactosidase primer (5'-TAACGCCAGGGT TTTCCCAG-3') and a non-nested anchored primer (Gibco BRL, Gaithersburg, MD, USA) or a nested anchor primer (Clontech, Mountain View, CA, USA). PCR products were cloned into *E.coli*. DNA was isolated from positive colonies and sequenced by using the dideoxynucleotide termination method. Sequences were compared with published sequences in EST and Celera database.

Southern blotting

Genomic DNA from Ayu17-923 ES cells was extracted with standard procedure. Each 20 μg of genomic DNA was digested with *Pst*I, *Hin*dIII, *Sph*I, *Eco*RV, respectively, and loaded onto 0.8% agarose gel for electrophoresis, and then transferred onto Hybond N membranes (Amersham Biosciences, Uppsala, Sweden) and hybridized to ^{32}P-labeled pU17 vector probe. Blots were exposed to Fuji autoradiography film (Fuji Film, Tokyo, Japan).

PCR genotyping

DNA was isolated from tail biopsies, as described previously. To genotype offspring of heterozygous, PCR amplification of 506 bp of the neomycin gene was performed by using NeoF: 5'-AGAGGCTATTCGGCTATGAC-3' as a forward primer and NeoR: 5'-CACCAT GATATTCGGCAAGC-3' as a reverse primer (Tang et al., 2008). For genotype offspring of wild type, the primers on mouse genome located on the flanks of the trapping vector were used: Gf3, 5'-TGCCTCAGAGGAATTCTGGC-3' as a forward primer and Gr2, 5'-ATGGTTCACTCTGATCCAGG-3' as a reverse primer. PCR amplification of 747 bp was performed under the following PCR conditions: 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs; 1) denaturing at 94°C for 5 min; 2) denaturing at 94°C for 1 min; 3) annealing at 60°C for 1 min; 4) extension at 72°C for 1 min; 30 cycles to 2, and 5) final extension at 72°C for 10 min. PCR products were separated on a 1.0% agarose gel and visualized with ethidium bromide.

RT-PCR

In the RT-PCR assays, RNA samples were reverse transcribed according to the manufacturer's instructions for ThermoScriptTM RT-PCR System (InvitrogenTM), and gene-specific primers (923F: 5'-CTTAGGGCAATGTCAGCG-3', 455R1: 5'-CAGTGTCCAGCC TTGCCTC-3', CF3: 5'-GAGCAAGCTGGAAAGATTGG-3', CR2: 5'-ACTGTAGCTGACTTCAGACG-3') were used to amplify selected region of each target moiety. HPRT (HPRT-F: 5'-CACAGGACT AGAACACCTGC-3', HPRT-R: 5'-GCTGGTGAAAAGGACCTCT-3') was used as an internal standard. To amplify fusion RNA of ZNF 197L mRNA and beta-gal mRNA, primers were: CF1, TTTTGA TCTCTGCTGCCAGG; Z2, TGTGAGCGAGTAACAACC; Z5, ACGGCGGATTGACCGTAATG.

RESULTS

Creating Ayu17-923 ES clone

The aim of the procedure was to insert the pU17 β-geo gene trap vector downstream of a cellular gene promoter that is specifically expressed in undifferentiated ES cells. Normal undifferentiated ES cells were electroporated with the linearized plasmid and then selected with G418. One clone (termed: Ayu17-923) containing cells with the typical morphology of undifferentiated mouse ES cell showing positive for the β-galactosidase activity (date not shown) was isolated and further propagated.

Single integration of trap vector in Ayu17-923 ES cell genomic DNA

As a first step toward identifying the integration site of the pU17 β-geo insert, a Southern blotting analysis was performed on the Ayu17-923 ES cell genomic DNA. By using *Pst*I, *Hin*dIII, *Sph*I, and *Eco*RV that cut only once in the PU17 vector, a pU17 probe revealed a single integration locus for the trap vector (Figure 1).

Characterization of the integration site of the trap vector

When the information of the trapping vector and Ayu17-923 ES cell genome DNA was analyzed with plasmid rescue, no deletion was found on the mouse genome

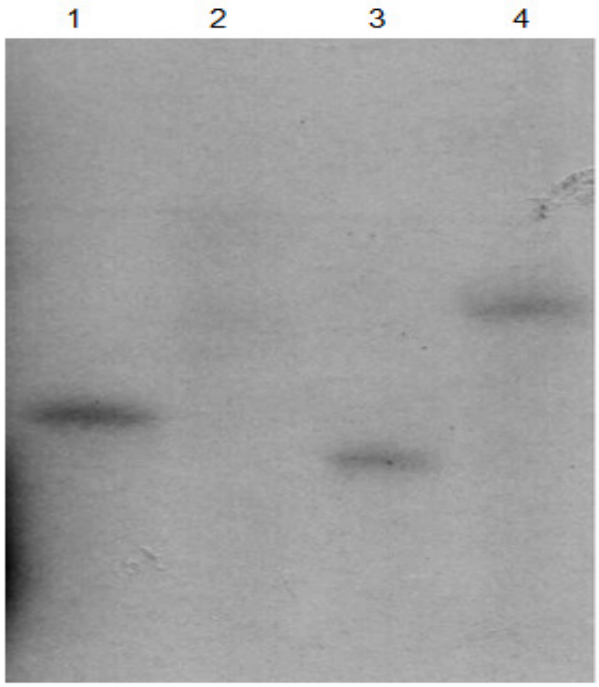

Figure 1. Southern blot analysis for single integration. 20 ug of Ayu17-923 ES genomic DNA was digested with several restriction enzymes. A fragment of pU17 vector was used as a probe to detect the trapping vector. 1: *Pst*I, 2: *Hin*dIII, 3: *Sph*I, 4: *Eco*RV.

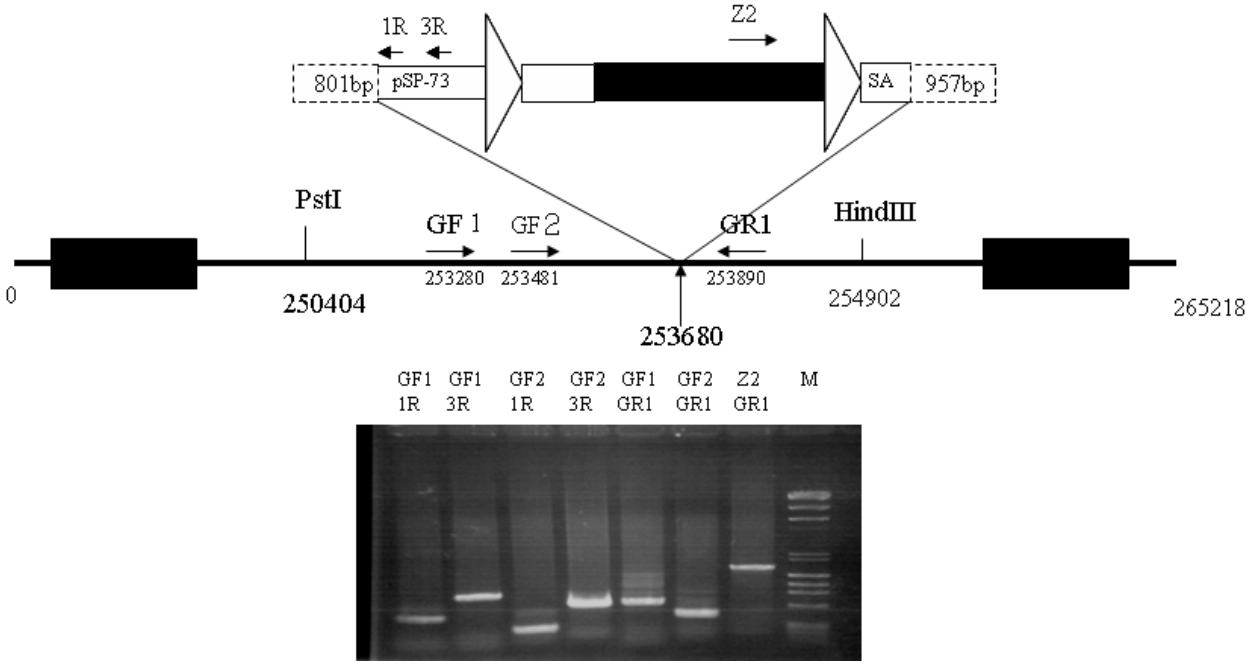

Figure 2. Location of trapping vector in Ayu17-923. gene primers on genomic DNA (GF1, GF2, GR1) and trapping vector(1R, 3R, Z2) was indicated. PCR was performed to show the correction of trapping vector location.

DNA but the trapping vector had a 957 bp deletion just at its 5'terminals, and 801 bp at its 3'terminals (Figure 2).

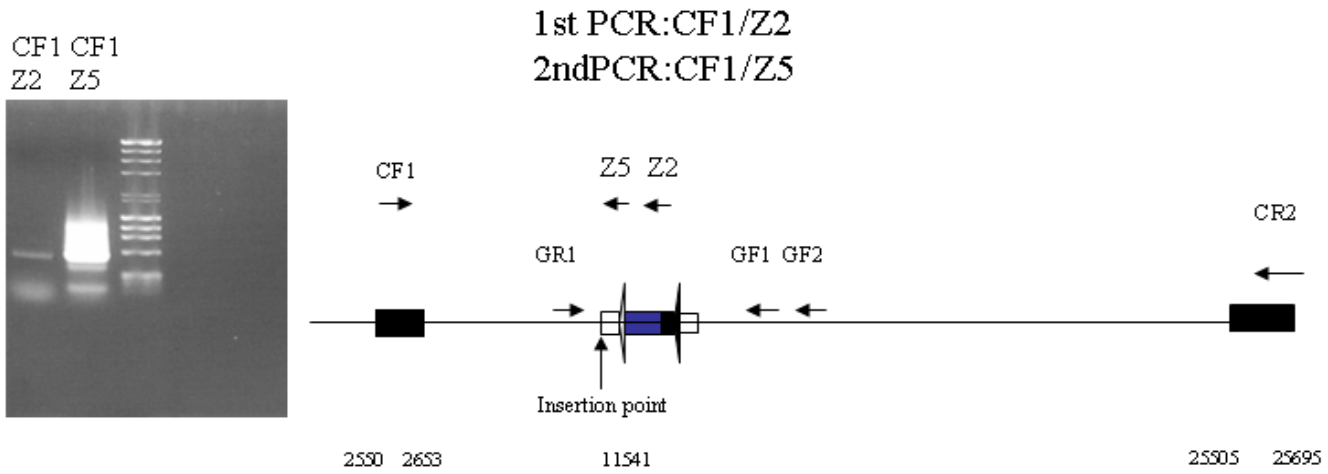

Figure 3. Fusion mRNA of ZNF 197L mRNA and beta-gal mRNA. RT-PCR analysis was performed to detect fusion mRNA existence. Primer of ZNF 197L (CF1) and primer of beta-gal mRNA (Z2) were used to amplify.

The trap vector was inserted in intron between exon 1 and exon 2 after the transcription start code. This information was further demonstrated by PCR analysis with the primers locating on trapping vector and cell genomic DNA. The relationship between integrated vector DNA and Ayu17-923ES cell genomic DNA is described in Figure 2. The location of trapping vector was further demonstrated by PCR using some primers on ES cell genomic DNA and trapping vector (Figure 2).

Furthermore, we confirmed trap vector integrated site on RNA level. When we used a primer on ZNF 197L, another primer on beta-gal, cDNA of Ayu17-923 ES cells as templates, RT-PCR result showed that ZNF 197L mRNA and beta-gal mRNA could generate a fusion RNA (Figure 3). This data demonstrated that the trap vector integrated in ZNF 197L gene in a right site as described.

Identification of Ayu17-923 gene

The mouse gene was localized at the chromosome 3. We termed this gene as *Ayu17-923*. When its 5'RACE sequence was blasted in PubMed database, a homologue sequence, Mus musculus similar to zinc finger protein 197, transcript variant 2 (accession number: XM_001476346) was found. Analysis of the genomic region around the insertion site revealed that the pU17 was inserted into the 1st intron, 13.9 kb upstream of exon 2 (Figure 2). Using PCR amplification on genomic DNA samples, the genotype of offspring was easily determined (Figure 4).

Inhibited expression of Ayu17-923 in Ayu17-923 ES cells

In order to detect whether *Ayu17-923* expression was inhibited or not, RT-PCR array was carried out. RNAs from *Ayu17-923* ES cells and its derived TT2 ES cells were extracted and transcribed into cDNA. *Ayu17-923* special primers (923F and 445R1) were used to check *Ayu17-923* expression in these two cell lines. Compared with TT2 ES cells (control), the expression of Ayu17-923 was obviously decreased at different cDNA concentration (Figure 5). So, *Ayu17-923* ES cell clone could be used to generate Ayu17-923 knock out mice line.

Generation of mutant mice

To analyze the function of *Ayu17-923* gene, Ayu17-923 ES cells were injected into blastocysts to generate germ line chimeras. Genotyping was done by PCR using sets of primers as descried earlier. Heterozygous mice were fertile with no morphological abnormalities and had a normal life span. Before birth, homozygous mice showed no obviously phenotype.

Expression pattern of ZNF 197L in mouse

RNA was isolated from adult mouse brain, heart, lung, liver, spleen, kidney and Ayu17-923 ES cells (positive control). ZNF197L cDNA primers (CF3,CR2) that were used to do RT-PCR. ZNF197L expression could be detected weakly in mouse brain, heart, lung and spleen, highly in kidney. However it could not be expressed in liver (Figure 6).

Ayu17-923 was not essential for development

Many *in vitro* studies indicates that zinc finger protein 197 plays an important role in signal transduction pathways,

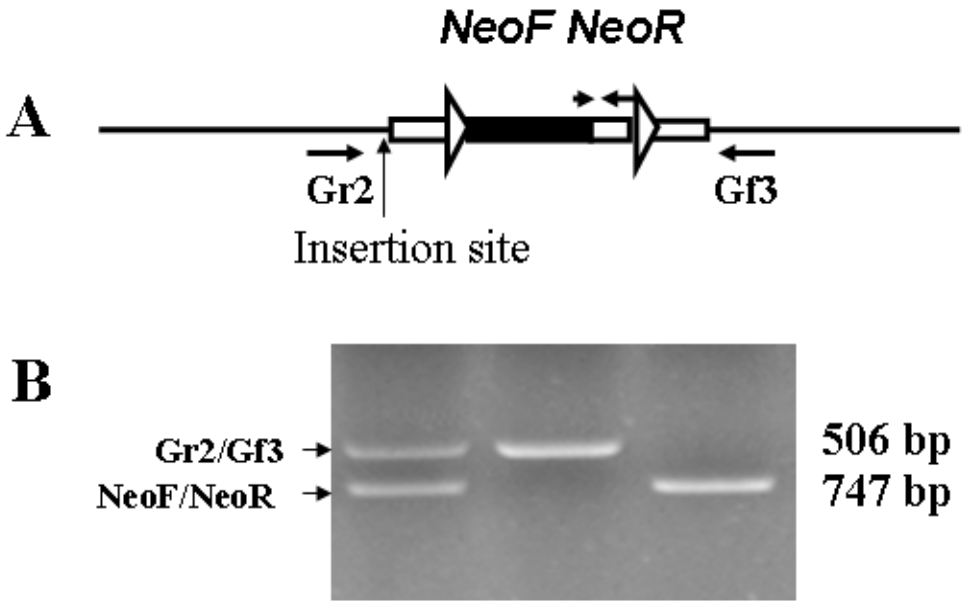

Figure 4. PCR genotyping. A: *NeoF* and *NeoR* were primers on trapping vector. *Gr2* and *Gf3* were primers on *Ayu17-923* gene, flanking the trapping vector. B: PCR results of *Ayu17-923* genotyping.

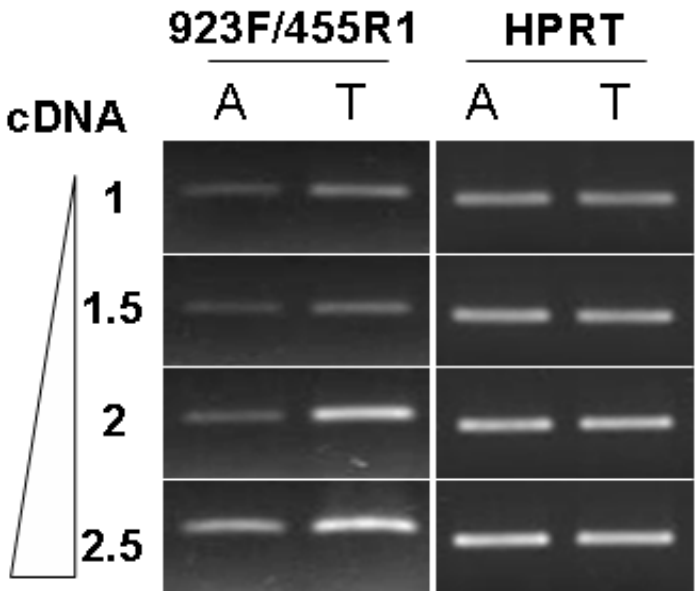

Figure 5. Ayu17-923 expression was analyzed with RT-PCR in mutant ES cells. RNA from Ayu17-923 ES cells and normal ES cells (TT2) were transcribed into cDNA. ZNF197L primers (923F and 445R1) were used to amplify cDNA in different concentrations. HPRT was used as an internal standard. A, Ayu17-923 ES cells; T, normal ES cells.

but homozygous mutant mice obtained from the crosses of F1 heterozygotes did not show any obvious abnormalities. They did not exhibit growth retardation, were fertile, and took care of their offspring. Histological analysis did not identify any abnormalities in the main organs including the brain, heart, lung, liver, spleen, kidney, testis, and intestine. The frequency of sudden death in the homozygotes was not different from that in

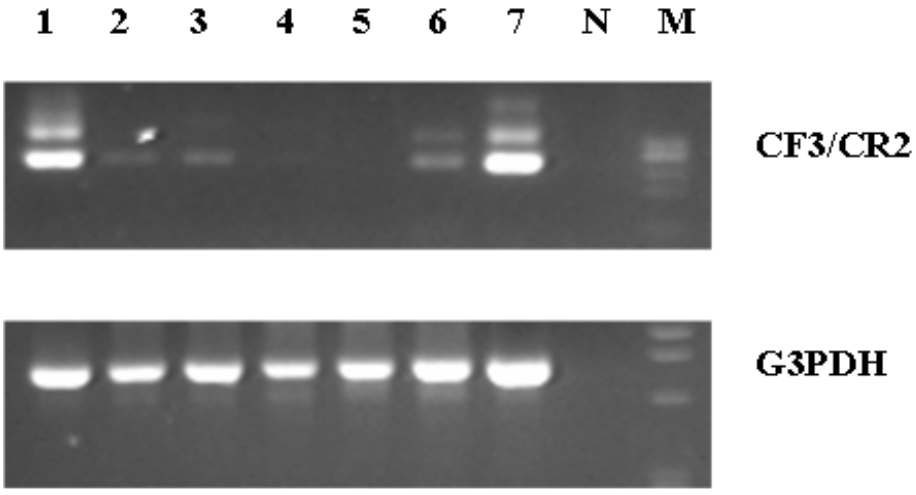

Figure 6. Analysis of ZNF197L expression pattern with RT-PCR. RNA from Ayu17-923 ES cells and mouse tissues were transcribed into cDNA. ZNF197L primers (CF3 and CR2) were used to amplify cDNA. 1-7 are RNA from Ayu17-923 ES cells, adult mouse brain, heart, lung, liver, spleen, and kidney, respectively. RNA of Ayu17-923 ES cells were used as positive control. Irrelevant DNA was used as negative control. GAPDH was used as an internal standard.

the wild-type mice within three months (data not shown).

DISCUSSION

We first created a mouse line with loss of function of zinc finger protein 197 by using trap method (Tang et al., 2009), and confirmed the insertion point of trap vector in zinc finger protein 197 gene. In mutant ES cells, the expression of zinc finger protein 197 was decreased. So the mutant mouse line is useful for functional analysis of zinc finger protein 197 gene in mouse development.

Zinc finger protein 197 belongs to the zinc finger protein super family, members of which are regulatory proteins characterized by nucleic acid-binding zinc finger domains. This protein contains 20 tandemly arrayed C2H2-type zinc fingers, a Kruppel-associated box (KRAB) domain, and a SCAN box (Li et al., 2003). The KRAB domain is found to have function in protein-protein interactions. KRAB-proteins are thought to have critical functions in cell proliferation and differentiation, apoptosis and neoplastic transformation (Urrutia, 2003). The finger-like protrusions make tandem contacts with their target molecule, including DNA-binding motif in transcription factor TFIIIA, RNA, protein and/or lipid substrates (Klug, 1999; Hall, 2005; Brown, 2005; Gamsjaeger et al., 2007; Matthews and Sunde, 2002). Thus, Znf-containing proteins function in gene transcription, translation, mRNA trafficking, cytoskeleton organization, epithelial development, cell adhesion, protein folding, chromatin remodeling and zinc sensing (Laity et al., 2001). However, Zinc finger protein197 functions in development were still unknown. This study is the first to describe its function in development by using knock out mice. Our result showed that the Zinc finger protein197 (Ayu17-923) is compensable in mouse survival.

ZNF 197 deficient mice will be essential material for analyzing the function of ZNF 197 *in vivo*. We will find which genes are being influenced with DNA microassay by comparison with normal mice. Also, we will search the mechanism of ZNF 197 involved in cellular metabolism with these mutant mice.

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