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cDNA Cloning, Prokaryotic Expression of Two Splicing Products of mLRG, a Mouse Gene of Lipopolysaccharide Response

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Abstract

Aim: To clone two splicing products of the mouse mLRG-cDNA and to express mLRG protein. Methods: The sequence obtained was compared human *lrg* to mouse genome with a comparative BLAST genome search and found completely identical. We spliced some fragments to a whole mouse *lrg*-cDNA sequence and designed a pair of primers at completely homologous fragments in 5'-UTR and 3'-UTR, we amplified mouse *lrg*-cDNA by RT-PCR. Then the sequence encoding the mLRG protein was amplified by RT-PCR from the total RNA of NIH3T3 cell stimulated by lps (lipopolysaccharide), and we got two splicing products of mLRG (mLRGW, mLRGS) and two sequences encoding protein were cloned into the prokaryotic expression vector pTAT so as to construct the recombinant expression vector pTAT-MLRGW and pTAT-MLRGS. The proteins were expressed in *E. coli BL*21 (DE3). Results: We got a cDNA fragment with the length of 1905 bp. Its location is at chromosome X qF4 site and we amplified two encoding regions covered 1554 bp and 1404 bp respectively (*mlrgW mlrgS*). His-TAT-mLRGW and His-TAT-mLRGS fusion protein were expressed successfully. *mlrgW* is consist of 10 exons and 9 introns; *mlrgS* is consist of 11exons and 10 introns. Conclusion: Cloning of two splicing products of mouse novel gene MLRG and prokaryotic protein expressions are of help in the further study of this gene.

Keywords

mLRG, Lipopolysaccharide Response Gene, cDNA Cloning, Prokaryotic Protein Expression

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1. Introduction

To date, primary protein lipopolysaccharide responses investigated are lipopolysaccharide (LPS) binding protein (LBP), soluble CD14 and Toll-like receptor 4 (TLR4). The mechanism of these proteins regulate is perplexing.

The binding of LPS to CD14 initiates a wide variety of biological reactions mainly occurring in monocytic cell lineages [1]. Since CD14 is not a trans-membrane protein, it couples with the trans-membrane protein, Toll-like receptor 4 (TLR4) and initiates intracellular signaling cascades [2] [3]. Soluble CD14 (sCD14) complexed with LPS also activates endothelial cells via TLR4 to induce microcirculatory disturbances [4]-[8]. The important roles of CD14 as the LPS recognition molecule *in vivo* have also been explored using CD14 transgenic and knockout mice [9]-[11].

LPS binding protein (LBP) and CD14 play key roles in promoting innate immunity to Gram-negative bacteria by transferring LPS to the signaling receptor complex, MD-2/Toll-like receptor 4 (TLR4). The dual stimulatory and inhibitory mechanisms of sCD14 and LBP may benefit the infected host by promoting inflammation in local sites, where it is needed, while at the same time preventing potentially detrimental systemic responses to LPS [12]. However, the mechanism of LPS-stimulated tissues damage remains obscure.

A number of studies have investigated the impact of LPS and other bacterial products on apoptosis of white blood cells. *In vitro*, LPS has been shown to promote apoptosis in macrophages [13]-[15] but to inhibit apoptosis of neutrophils [16] [17]. *In vivo* LPS has a pro-apoptotic effect on lymphocytes in Peyer's patches and thymocytes, whereas it has anti-apoptotic effects in peritoneal neutrophils [18]-[20]. Mani Alikhani, Zoubin Alikhani *et al*. demonstrated that lipopolysaccharides indirectly stimulate apoptosis and global induction of apoptotic genes in fibroblasts through experiment, but number of gene participate in this procedure is more than forty [21]. So we need to search for upstream gene from cells LPS-stimulated to elucidate the mechanism of LPS damage.

Human lipopolysaccharide response gene (*hlrg*) was cloned from LPS-stimulated human dental pulp cell by the method of Improved PCR-based subtractive hybridization strategy [22] by *Ph.D* Chai YB of our teams in 1999 (Accession No. AF143740) [23]. We found a more complete novel human *lrg* cDNA in GenBank database BLAST search (Accession No. AK002071). It may be relative of endotoxin response. Mouse lipopolysaccharide response gene (*lrg*) is not reported. We compared human lipopolysaccharide response gene (*lrg*) for mouse ESTs in GenBank database BLAST search [24] and spliced good homology segments to a predicted mouse lipopolysaccharide response gene (*lrg*) sequence [25]. We designed a set of primers at completely homologous fragments in 5'-UTR and 3'-UTR, and amplified two mouse *lrg*-cDNA sequences by RT-PCR. We got mouse lipopolysaccharide response whole gene (mLRGW) and mouse lipopolysaccharide response splice gene (mLRGS) prokaryotic protein expressed production. Cloning of two splicing products of mouse novel gene mouse lipopolysaccharide response gene (mLRG) and prokaryotic protein expressions are of help in the further study of this gene.

2. Methods and Materials

2.1. Prediction of Mouse *lrg*

Comparing human lipopolysaccharide response gene (*lrg*) to mouse genome with a comparative BLAST genome search and searching completely identical. Splicing some fragments to a whole mouse *lrg*-cDNA sequence and designing a set of primers at completely homologous fragments in 5'-UTR and 3'-UTR (P1: GCTGCCGTCACCTCATGG, P2: TTCACATCAAGGAACCATCG).

2.2. Isolation of RNA

NIH3T3 cells were plated on 150-mm glass culture dishes at a density of ~7×10^6 cells/plate. Cells were treated with (final concentration 100 μg/ml) lps stimulation for approximately 24 h prior to cell lysis. Cell extracts were prepared at 4˚C; the cells were washed twice with phosphate-buffered saline and lysed in Trizol reagent (purchased from Invitrogen company). Total cellular RNAs were extracted from cells according to the manufacturer' instructions.

2.3. Synthesis of cDNA Library and Amply Mouse *lrg*

The extracted RNA (500 ng) was reverse transcribed into cDNA first-strand using 200 U of Moloney Murine

Leukemia Virus Reverse transcriptase (Promega, madison, USA) and 1 mg of oligo (dT) 15 primer (Promega) in a final volume of 25 μl of enzyme buffer (Promega) for 60 min at 42˚C. This cDNA first-strand was taken as template for RT-PCR. PCR was performed with 2 μL of cDNA first-strand and 0.01 pmol of each primer (P1, P2), in a 50 μL reaction volume containing 100 nmol $MgCl_2$, 10 nmol dNTPs, 2.5 U of Taq DNA polymerase (Promega), and 5 μL 10 × Taq DNA polymerase buffer. The reaction mixture was heated at 94˚C for 5 min, followed by amplification through 35 cycles. Each cycle included denaturation at 94˚C for 30 s, annealing at 58˚C for 45 s and extension at 72˚C for 90 s. Then 10 μL of the PCR product was electrophoresed on a 10 g/L agarose gel containing ethidium bromide (0.5 g/mL), and visualized under ultraviolet light.

2.4. Cloning into Vector and Sequencing

The PCR product was recycled with Plamids Gel Recycle Kit (purchased from BioLabs company), T4 DNA Polymerase (purchased from Takara company) blunt, pUC19 vector was digested using restriction enzyme HincII (purchased from Takara company). The recycled plasmid was inserted in the digested pUC19 vector with T4 DNA Ligase (purchased from Takara company). The ligated plasmids were transformed into engineering bacteria TOP10, the transformed TOP10 was tiled in flat plate contained agar and X-gal. Selecting white colony to sequence.

2.5. Cloning to pTAT and Prokaryotic Expression

Designing a set of primers (PF: GCGGGTACCGCGACTCGGCTTGAGGAGGTAACGCGA inlet *Kpn* Ⅰ site, PR: CCGCTCGAGGCCTTAAGCAGGAGAACC, inlet *Xho*I site) and amplifying the sequence enconding using LPS-stimulated NIH3T3 cDNA first-strand construct as template and two franking primers(PF, PR), The PCR reaction was performed using standard protocols with 35 cycles of 30 s at 94˚C, 45 s at 53.5˚C and 90 s at 72˚C. Appropriately sized amplification products were verified by agarose gel electrophoresis. The PCR products were inserted in the multiple cloning site of pTAT (Invitrogen) between *Kpn*I and *Xho*I, transformed into *E. coli* BL21(DE3) (stored in our labs) respectively. The individual clones were sequenced. The transformant *E. coli* BL21(DE3) clones were grown at 37.8˚C to an A600 value of 0.6 in LB/ampicillin (100 μg/ml), induced with 1mM isopropyl thio-*β*-D-galactoside (IPTG) and incubated for 3 h. The induced cells were disrupted by sonication and centrifuged at 12,000 rpm for 10min. Supernatant and sediment were prepared to samples for 12% SDS-PAGE respectively.

2.6. Bioinformatics Analysis

The sequences encoding obtained were searched in UCSC database, and analysis chromosome location and exon/intron boundary (http://genome.ucsc.edu/) [26]. All exon/intron boundaries were shown to follow the canonical ag/gt rule of splicing.

3. Results

3.1. Cloning Two Splicing Products of mLRG cDNA

The length of applied MLRG cDNA by RT-PCR is 1907 bp (**Figure 1**), the complete mLRG sequence encoding was known through sequencing, the length of mLRG encoding is 1554 bp (named *mlrgW*), and we got another splicing form sequence encoding about 1407 bp (named *mlrgS*), *mlrgS* lock a fragment about 147 bp between 176th and 322th in *mlrgW* sequence (**Figure 2**).

3.2. Analysis in Murine Genome

We found a g to a change at 229th nucleotide in the first intron (an amino acid G to D change), a t to g change at 1495th nucleotide in the tenth intron (an amino acid L to G change), a c to t change at 1566th nucleotide in the tenth intron, the stop condon antelocate 18 nucleotides through camparing *mlrgW* and *mlrgS* to murine genome in UCSC database. *mlrgW* and *mlrgS* locate chromosome X qF4 site, *mlrgW* and *mlrgS* sequence are 99.8% with homology to murine genome after comparing with sequence in GenBank. Since the genomic organization of *mlrgW* and *mlrgS* has not been investigated, we also examined the exon/intron structure of the gene by combining genomic and RT-PCR. The sequence encoding of *mlrgW* contains 10 exons and 9 introns (**Figure 3**), and the

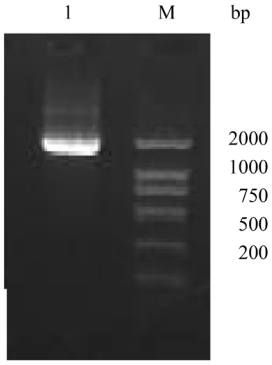

Figure 1. Agrose gel electrophoresis of the PCR product of mLRG cDNA. M: 2000 bp ladder marker; 1: the PCR product of mLRG cDNA.

```
15 atggcgactcggcttgaggaggtaacgcgaggaagaggcggcggt
   M  A  T  R  L  E  E  V  T  R  G  R  G  G  G
60 actgaggaggctagtgagggcggacggggcggacggcgacgtagc
   T  E  E  A  S  E  G  G  R  G  G  R  R  R  S
105 ccccgcagaagtttgaaattggcacaatggaagaagctagaatt
   P  P  Q  K  F  E  I  G  T  M  E  E  A  R  I
150 tgtgggttaggagtaaaagcagacatggtatgtaactctcaagca
   C  G  L  G  V  K  A  D  M  V  C  N  S  Q  A
195 aatgatattcttcaacatcaagaccccagttgtgatggcacaact
   N  D  I  L  Q  H  Q  D  P  S  C  D  G  T  T
240 aagaaacattcactggaaggggatgatggcagtgactttattaca
   K  K  H  S  L  E  G  D  D  G  S  D  F  I  T
285 aagaacagaaatttggtgagctcagtattctgtacacaggagaaa
   K  N  R  N  L  V  S  S  V  F  C  T  Q  E  K
330 agagaagaaattcctggacgagaagctcgaacaggtcctcctgat
   R  E  E  I  P  G  R  E  A  R  T  G  P  P  D
375 ggccagcaagattcagagtgcagcaggaacaaagagaagacctta
   G  Q  Q  D  S  E  C  S  R  N  K  E  K  T  L
420 ggaaaagaagttttattactgatgcaagcgctaaacacccttca
   G  K  E  V  L  L  L  M  Q  A  L  N  T  L  S
465 accccagaggagaagctggcagctctctgtaagaaatatgctgat
   T  P  E  E  K  L  A  A  L  C  K  K  Y  A  D
510 ctcctggaagaaagcaggaatgttcagaaacaaatgaagattctg
   L  L  E  E  S  R  N  V  Q  K  Q  M  K  I  L
555 cagaagaagcaagcccagattgtgaaagagaaagttcaccttcag
   Q  K  K  Q  A  Q  I  V  K  E  K  V  H  L  Q
600 agtgaacacagcaaggccatcttggcaagaagcaaactggaatct
   S  E  H  S  K  A  I  L  A  R  S  K  L  E  S
645 ctttgcagggaacttcagcgtcataataagaccttaaaggaggag
   L  C  R  E  L  Q  R  H  N  K  T  L  K  E  E
690 aatatgcagcaggcacgagaggaggaagaacgacgtaaagaagca
   N  M  Q  Q  A  R  E  E  E  E  R  R  K  E  A
735 acagcacatttccagataactctaaatgaaatccaagctcagttg
   T  A  H  F  Q  I  T  L  N  E  I  Q  A  Q  L
780 gaacaacatgacatccacaatgccaaactgcgacaggagaacatt
   E  Q  H  D  I  H  N  A  K  L  R  Q  E  N  I
825 gaactgggagagaagttgaagaagcttattgagcagtatgcacta
   E  L  G  E  K  L  K  K  L  I  E  Q  Y  A  L
870 agggaagagcatattgataaagtattcaaacacaaggaattgcaa
   R  E  E  H  I  D  K  V  F  K  H  K  E  L  Q
915 caacagcttgtggatgccaaacttcagcaaacaacacagctgata
   Q  Q  L  V  D  G  K  L  Q  Q  T  T  Q  L  I
960 aaagaagctgatgaaaaacatcagagagagagagagttttatta
   K  E  A  D  E  K  H  Q  R  E  R  E  F  L  L
1005 aaagaagcaacagaatccaggcacaaatatgaacaaatgaaacag
   K  E  A  T  E  S  R  H  K  Y  E  Q  M  K  Q
1050 caagaagtacaactaaaacagcagctttctctttatatggataaa
   Q  E  V  Q  L  K  Q  Q  L  S  L  Y  M  D  K
1095 tttgaagaattccagactactatggcaaaaagcaatgaacttttt
   F  E  E  F  Q  T  T  M  A  K  S  N  E  L  F
1140 acaaccttcaggcaggaaatggaaaagatgacaaagaaaattaaa
   T  T  F  R  Q  E  M  E  K  M  T  K  K  I  K
1185 aaactggaaaaagaaacaatatatggcgtaccaaatgggaaaac
   K  L  E  K  E  T  I  I  W  R  T  K  W  E  N
1230 aatataaaagcactttctgcagatggccgaagagaaaactgtccgt
   N  N  K  A  L  L  Q  M  A  E  E  K  T  V  R
1275 gataaagagtacaaggcttttcaaataaaactggaacggttagag
   D  K  E  Y  K  A  F  Q  I  K  L  E  R  L  E
1320 aagctgtgcagggctcttcagacagagagaaatgagctcaacgag
   K  L  C  R  A  L  Q  T  E  R  N  E  L  N  E
1365 aaggtcgaagtcctgaaagagcaggtctctatcaaagcagcagat
   K  V  E  V  L  K  E  Q  V  S  I  K  A  A  D
1410 ggggacttggtgtcacctgcaacgcagccctgtgctgtcctggat
   G  D  L  V  S  P  A  T  Q  P  C  A  V  L  D
1455 tccttcaaagagacttcaagaagaaccctgggaatgcactgggag
   S  F  K  E  T  S  R  R  T  L  G  M  H  W  E
1500 gctagagccaagtcagtgtgtgagaaaagtgctgcacaaaagccc
   A  R  A  K  S  V  C  E  K  S  A  A  Q  K  P
1545 tcatcttcaggttctcctgcttaa 1568
   S  S  S  G  S  P  A  *
```

*:Represent as stop codon the sequence of mLRGS cDNA lock is underlined

Figure 2. ORF sequence and the deduced amino acid sequence of mLRGW and mLRGS cDNA.

sequence of *mlrgS* contains 11 exons and 10 introns (**Figure 4**). *mlrgS* cut out a intron in the second exon of *mlrgW*, so *mlrgS* contains 11 exons and 10 introns, it is internal splicing. The open reading frame (ORF) of these two are correct. It demonstrates that these two splicing products of *mlrg* are correct different expressions of murine *lrg* (**Figure 5**). They maybe acted as two different roles in responsing to LPS-stimulate.

3.3. Prokaryotic Protein Expression

mlrgW and *mlrgS* were cloned to pTAT vector successfully, and results of sequencing were identified with sequence results of *mlrg*-cDNA, pTAT-MLRGW and pTAT-MLRGS were identified using cut with double enzymes (*KpnI* and *XhoI*), monoenzyme (*HindIII*) respectively (**Figure 6**). **Figure 7** shows that mlrgS was divided into two fragments (one is about 680 bp, another is about 730 bp) by *HindIII*. It is not obvious in Agarose gel electrophoresis because two fragments is too close. All identified restriction sites are correct. It demonstrated that mLRGW-pTAT/BL21(DE3) and mLRGS-pTAT/BL21(DE3) we cloned were right, and can be used to express mLRGW and mLRGS proteins correctly. His-TAT-mLRGW protein contained 468 amino acids band at ***M***r 66500, and His-TAT-mLRGS protein contained 518 amino acids band at *M*r 61,000. The result is concordant with predicted molecular weight. His-TAT-mLRGW and His-TAT-mLRGS were existed in inclusion body. His-TAT-mLRGW and His-TAT-mLRGS were not expressed in mLRGW/BL21(DE3) and mLRGS/BL21-(DE3) without induction. The result demonstrates that mLRGW/BL21(DE3) and mLRGS/BL21(DE3) were inducible (**Figure 8**).

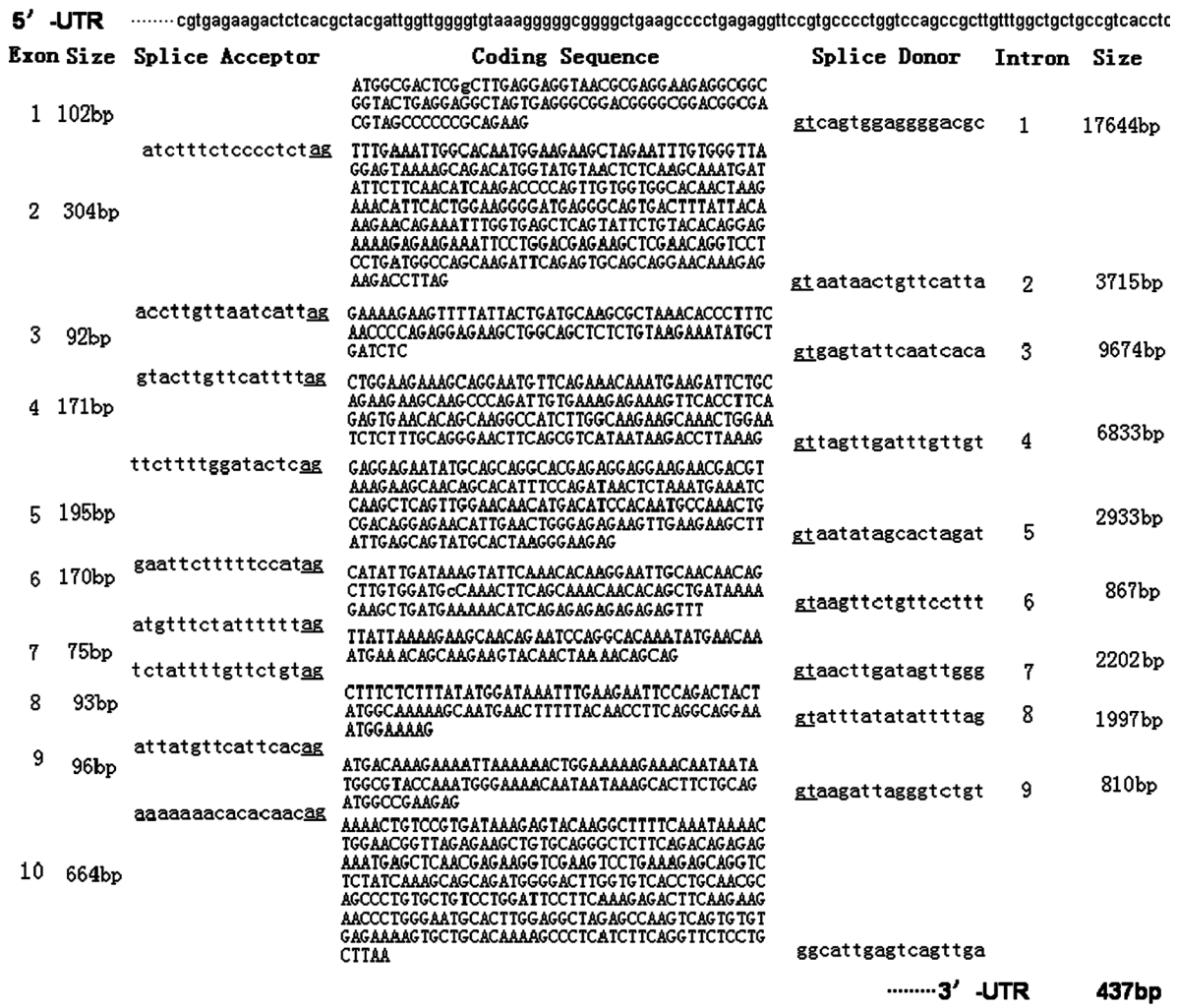

Figure 3. Exon/intron structure of *mlrgW*. Exon bases are given in capital letters, intron bases in small letters. Canonical ag/gt bases at the exon/intron boundaries are underlined.

5′ -UTR ·····cacgctacgattggttggggtgtaaaggggcggggctgaagcccctgagaggttccgtgcccctggtccagccgcttgtttggctgctgccgtcacctc					
Exon	**Size**	**Splice Acceptor**	**Coding Sequence**	**Splice Donor**	**Intron**	**Size**
1	102bp		ATGGCGACTCGgCTTGAGGAGGTAACGCGAGGAAGAGGCGGC GGTACTGAGGAGGCTAGTGAGGGCGGACGGGGCGGACGGCGA CGTAGCCCCCCGCAGAAG	gtcagtggaggggacgc	1	17644bp
2	60bp	atctttctcccctctag	TTTGAAATTGGCACAATGGAAGAAGCTAGAATTTGTGGGTTA GGAGTAAAAGCAGACATG	gtatgtaactctcaagc	2	147bp
3	97bp	accttgttaatcattag	GAGAAAAGAGAAGAAATTCCTGGACGAGAAGCTCGAACAGGT CCTCCTGATGGCCAGCAAGATTCAGAGTGCAGCAGGAACAAA GAGAAGACCTTAG	gtaataactgttcatta	3	3715bp
4	92bp	cagtattctgtacacag	GAAAAGAAGTTTTATTACTGATGCAAGCGCTAAACACCCTTTC AACCCCAGAGGAGAAGCTGGCAGCTCTCTGTAAGAAATATGCT GATCTC	gtgagtattcaatcaca	4	9674bp
5	171bp	gtacttgttcattttag	CTGGAAGAAAGCAGGAATGTTCAGAAACAAATGAAGATTCTGC AGAAGAAGCAAGCCCAGATTGTGAAAGAGAAAGTTCACCTTCA GAGTGAACACAGCAAGGCCATCTTGGCAAGAAGCAAACTGGAA TCTCTTTGCAGGGAACTTCAGCGTCATAATAAGACCTTAAAG	gttagttgatttgttgt	5	6833bp
6	195bp	ttcttttggatactcag	GAGGAGAATATGCAGCAGGCACGAGAGGAGGAAGAACGACGT AAAGAAGCAACAGCACATTTCCAGATAACTCTAAATGAAATC CAAGCTCAGTTGGAACAACATGACATCCACAATGCCAAACTG CGACAGGAGAACATTGAACTGGGAGAGAAGTTGAAGAAGCTT ATTGAGCAGTATGCACTAAGGGAAGAG	gtaatatagcactagat	6	2933bp
7	170bp	gaattctttttccatag	CATATTGATAAAGTATTCAAACACAAGGAATTGCAACAACAG CTTGTGGATGcCAAACTTCAGCAAACAACACAGCTGATAAAA GAAGCTGATGAAAAAACATCAGAGAGAGAGAGAGTTT	gtaagttctgttccttt	7	867bp
8	75bp	atgtttctatttttag	TTATTAAAAGAAGCAACAGAATCCAGGCACAAATATGAACAA ATGAAACAGCAAGAAGTACAACTAAAACAGCAG	gtaacttgatagttggg	8	2202bp
9	93bp	tctattttgttctgtag	CTTTCTCTTTATATGGATAAATTTGAAGAATTCCAGACTACT ATGGCAAAAAGCAATGAACTTTTTACAACCTTCAGGCAGGAA ATGGAAAAG	gtatttatatattttag	9	1997bp
10	96bp	attatgttcattcacag	ATGACAAAGAAAATTAAAAAACTGGAAAAAGAAACAATAATA TGGCGTACCAAATGGGAAAACAATAATAAAGCACTTCTGCAG ATGGCCGAAGAG	gtaagattagggtctgt	10	810bp
11	664bp	aaaaaaacacacaacag	AAAACTGTCCGTGATAAAGAGTACAAGGCTTTTCAAATAAAAC TGGAACGGTTAGAGAAGCTGTGCAGGGCTCTTCAGACAGAGAG AAATGAGCTCAACGAGAAGGTCGAAGTCCTGAAAGAGCAGGTC TCTATCAAAGCAGCAGATGGGGACTTGGTGTCACCTGCAACGC AGCCCTGTGCTGTCCTGGATTCCTTCAAAGAGACTTCAAGAAG AACCCTGGGAATGCACTTGGAGGCTAGAGCCAAGTCAGTGTGT GAGAAAAGTGCTGCACAAAAGCCCTCATCTTCAGGTTCTCCTG CTTAA	ggcattgagtcagttgac ····· 3′ -UTR		103bp

Figure 4. Exon/intron structure of *mlrqS*. Exon bases are given in capital letters, intron bases in small letters. Canonical ag/gt bases at the exon/intron boundaries are underlined.

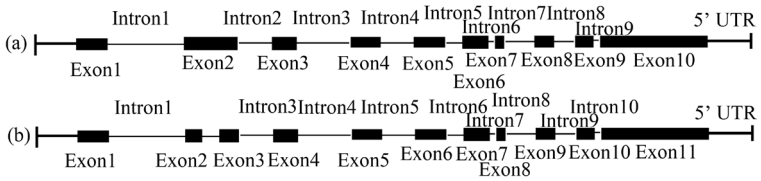

Figure 5. Genomic structure of mLRGW and mLRGS. Introns are represented by a thin line. Exons are depicted by a filled box. (a) Represents mLRGW; (b) Represents mRLGS.

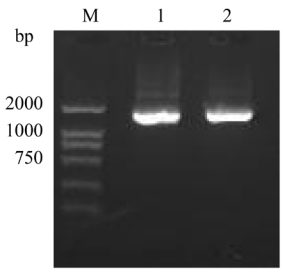

Figure 6. Agarose gel electrophoresis of the PCR product of mLRGW and mLRGS cDNA ORF. M: DNA ladder marker; 1: the PCR product of encoding the mLRGS protein; 2: the PCR product of encoding the mLRGW protein.

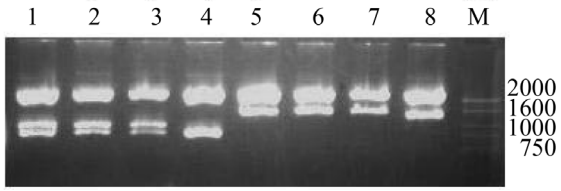

Figure 7. Enzyme digestion analysis of pTAT-MLRGW and pTAT-MLRGS. M: DNA ladder marker; 1-3: pTAT-MLRGW cut with *HindIII*; 4: pTAT-MLRGS cut with *HindIII*; 5-7: pTAT-MLRGW cut with *KpnI* and *XhoI*; 8: pTAT-MLRGS cut with *KpnI* and *XhoI*.

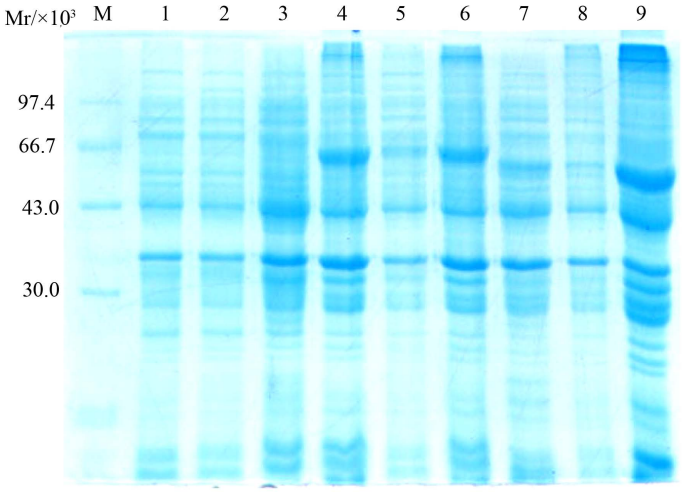

Figure 8. SDS-PAGE analysis of the expression of 6His-TAT-mLRGW and 6His-TAT-mLRGS fusion protein. M: Protein marker; 1: *BL*21 transformed by pTAT without induction; 2: *BL*21 transformed by pTAT induced with IPTG; 3: *BL*21 transformed by pTAT-mLRGW without induction;4: *BL*21 transformed by pTAT-mLRGW induced with IPTG; 5: Supernatant of *BL*21 transformed by pTAT-mLRGW induced with IPTG; 6: Precipitation of *BL*21 transformed by pTAT-mLRGW induced with IPTG; 7: *BL*21 transformed by pTAT-mLRGS induced with IPTG; 8: Supernatant of *BL*21 transformed by pTAT-mLRGS induced with IPTG; 9: Precipitation of *BL*21 transformed by pTAT-mLRGS induced with IPTG.

4. Conclusions

To date, we haven't found the investigation of murine gene of lipopolysaccharide response (*mlrg*).

We predicted the sequence of mLRG cDNA which length is 1905 bp through splicing combined the method of BLASTZ [24] and GenBank database. We demonstrated the sequence of mLRG cDNA through RT-PCR from LPS-stimulated NIH3T3 cells. We synthesized mouse cDNA first-strand from LPS-stimulated NIH3T3 as template again for amplifying mLRG encoding, and we got two splicing products of mLRG encoding (named *mlrgW* and *mlrgS*). Open Reading Frames (ORF) of *mlrgW* and *mlrgS* are right. mLRGW contains 518 amino acids, and mLRGS contains 468 amino acids. Calculated molecular mass of mLRGW is 66,500 Da, and that of mLRGS is 61,000 Da.

mlrgW and *mlrgS* are located at chromosome X qF4 site, and the sequence of them are 99.8% homology with murine genome through comparing the sequence of them to the sequence of murine genome in GenBank database. We found that *mlrgW* contained 10 exons and 9 introns, and *mlrgS* contained 11 exons and 10 introns through synthesized exon/intron boundary of *mlrgW* and *mlrgS* in UCSC database. The second and third exons of *mlrgS* are contained in the second exons of *mlrgW*, and *mlrgS* locks a fragment as an intron in the second exon of *mlrgW*. This form of splicing is internal splicing. Cloning and expressing *mlrgW* and *mlrgS* successfully is

help of study function of human *lrg*.

Further bioinformatics analysis shows *mlrgW* and *mlrgS* contain a Leucine Zipper domain between 431th and 468th amino acids motif at *mlrgW* and a domain between 606th and 807th nucleotide motif at *mlrgW* matches Pfam-B 27780 associated with Zinc Finger Protein (ZIP) C_2H_2 type in the Protein families database (Pfam database). The GAG (=CTC) repeat among Zinc Finger Protein (ZIP) C_2H_2 type constitutes a high-affinity site for Sp1 binding to the wt1 remoter. These manifest that *mlrg* maybe involved in transcription regulation. Leucine zipper domain is the property domain of DNA binding protein [27], but it is riddle which proteins *mlrg* bind with.

Former studies show over-expression human *lrg* can inhibit at G_0 stage of cell cycle in *lrg*-transfected human embryo kidney cells and HepG2 cells. *mlrg* maybe involved in the procedure of oxidative stress, and then impact cell cycle. We are going to demonstrate the mechanism of *lrg* impact cell cycle through further experiment.

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