RESEARCH ARTICLE

AA amyloidosis-resistant CE/J mice have *Saa1* and *Saa2* genes that encode an identical SAA isoform

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Running head: CE/J mouse *Saa1* and *Saa2* genes

Abstract

The CE/J mouse strain is resistant to Amyloid A protein (AA) amyloidosis. In contrast to AA amyloidosis-susceptible mouse strains that concomitantly express serum amyloid precursor protein (SAA) type 1 and 2 isoforms encoded by the *Saa1* and *Saa2* genes, respectively, in response to inflammatory stimulation from the liver, CE/J mice express only a single SAA isoform named SAA2.2. In addition, CE/J mice uniquely possess a Q30L amino acid substitution in SAA2.2 that inhibits amyloidogenesis. To elucidate the genetic basis underlying the expression of only a single SAA isoform in this strain, we conducted PCR cloning and nucleotide sequencing of the *Saa1* and *Saa2* genes from the CE/J genome. We revealed that CE/J mice possess functional *Saa1* and *Saa2* genes. Intriguingly, the 2 genes were identical with respect to amino acid sequence, each encoding the SAA2.2 isoform. RT-PCR analysis of inflamed liver tissue from CE/J mice demonstrated that both genes are expressed at equivalent levels. Reporter assays revealed that promoter/enhancer sequences of *Saa1* and *Saa2* genes in CE/J are also functional. These results indicate that the SAA2.2 isoform in CE/J is a mixture of *Saa1* and *Saa2* gene products.

Keywords: mouse strain, gene family, gene conversion, nucleotide substitution, amino acid substitution

Abbreviations: ApoAII = apolipoprotein A-II; bp = base pair; C/EBPβ = CAAT enhancer-binding protein β; IL-1β = interleukin-1β; IL-6 = interleukin-6; NF-κB = nuclear factor κB; PCR = polymerase chain reaction; RT-PCR = reverse transcription polymerase chain reaction; SAA = serum amyloid A; SNP = single nucleotide polymorphism; TNF-α = tumor necrosis factor-α

Introduction

Mice provide a useful model system for the study of Amyloid A protein (AA) amyloidosis, a disorder characterized by the buildup of insoluble serum amyloid A precursor proteins (SAA) following inflammation. In susceptible mouse strains such as BALB/c and C57BL/6J, experimental AA amyloidosis can be conventionally induced within 1 week after the administration of an inflammatory agent and an amyloid enhancing factor. In contrast, the CE/J strain is resistant to AA amyloidosis [1]. It has been shown that AA amyloid deposits are absent in CE/J mice even after 50 injections of an inflammatory agent administered over 60 days [1].

Whereas AA amyloidosis-susceptible mouse strains codominantly express SAA1 and SAA2 isoforms in response to inflammatory stimulation, the resistance of CE/J mice to AA amyloidosis is considered to be due to the expression of a single unique SAA isoform [2]. SAA proteins are encoded by the *Saa1* and *Saa2* genes, which belong to an ancient gene family found in all mammalian species examined thus far. *Saa1* and *Saa2* genes are thought to have been formed through gene duplication, and gene conversion between the 2 duplicated loci has contributed to a high degree of sequence similarity. The mouse *Saa1* and *Saa2* genes are ~3.5 kilobase (kb) pairs in length and are located within an interval of ~8 kb on chromosome 7 [3]. The overall exon/intron organization of the 2 genes is highly similar. Furthermore, the reference *Saa1* and *Saa2* genes of the C57BL/6J mouse strain retain 96% nucleotide sequence identity over their entire length. At the protein level, the 122 amino acid-long reference SAA1.1 (accession number NP_033143) and SAA2.1 (NP_035444) proteins are similar, with differences at only 9 positions (25th, 26th, 46th, 49th, 50th, 79th,

82nd, 95th, and 120th) [4] (Figure 1). Unlike AA amyloidosis-susceptible mouse strains, only a single SAA (named SAA2.2) has been observed in the plasma of acute phase CE/J mice [1]. The SAA2.2 has a complex composite pattern of the 9 polymorphic amino acids between the reference SAA1.1 and SAA2.1 sequences noted above [2] (Figure 1). Most importantly, SAA2.2 also possesses a unique leucine residue at position 30; a glutamine residue is present at this position in the conventional mouse SAA1 and SAA2 isoforms (Figure 1). It has been postulated that this Q30L amino acid substitution inhibits amyloidogenesis of the SAA2.2 isofom [2].

Thus, even though the biochemical basis of resistance to AA amyloidosis in CE/J has been well characterized, the primary genetic cause for the apparent absence of one of the SAA isoforms in the CE/J plasma has not yet been determined. One possibility is that one of the *Saa* genes of the strain is transcriptionally silent. Consistent with this hypothesis, a previous study resulted in the isolation of a cDNA clone for SAA2.2, but not for the putative SAA1 isoform [2]. Transcriptional induction of the mouse *Saa1* and *Saa2* genes is regulated by 2 *cis*-acting elements of a CAAT enhancer-binding protein β (C/EBPβ)-binding motif and a nuclear factor κB (NF-κB)-binding motif located in tandem within 260 bp upstream of the first non-coding exon of the genes [5]. A proinflammatory cytokine IL-6 enhances gene transcription via the C/EBPβ-binding motif, whereas IL-1β and TNF-α enhance transcription via the NF-κB-binding motif. We postulated that CE/J mice have a defect in these elements, or, that one of the *Saa* genes has been lost from the CE/J genome. To explore these possibilities, we cloned and characterized complete sequences of the *Saa1* and *Saa2* genes from the CE/J genome, including all exons and introns, the upstream enhancer/promoter

regions, and downstream polyadenylation signal sequences.

Materials and Methods

PCR cloning and nucleotide sequence analysis of the *Saa1* and *Saa2* genes from a CE/J mouse

All experimental procedures involving mice were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University. CE/J mice were purchased from the Jackson laboratory. Genomic DNAs were isolated from the liver by using a standard method. The mouse *Saa1* and *Saa2* genes have high nucleotide sequence similarity in the region between approximately 290 base pairs (bp) 5′ to the transcription start site and 450 bp 3′ to the polyadenylation signal of the genes [3]. To ensure specific, full-length PCR cloning of *Saa1* and *Saa2*, oligonucleotide primers (mgSaa1-F, mgSaa1-R, mgSaa2-F, and mgSaa2-R) were designed such that the primers could anneal to the non-homologous 5′-upstream and 3′-downstream regions of the genes. mgSaa1-F and mgSaa1-R are located at 603 bases upstream of the transcription start site and 504 bases downstream of the polyadenylation signal of *Saa1*, respectively. mgSaa2-F and mgSaa2-R are located at 604 bases upstream of the transcription start site and 1018 bases downstream of the polyadenylation signal of *Saa2*, respectively. The nucleotide sequences and locations of the primers in the mouse genome assembly (GRCm37/mm9) were as follows:

mgSaa1-F: 5′-CCCAAGAGCTGAGAACTTATGCTAG-3′ (53998977-53998953)

mgSaa1-R: 5′-ACTGCACAGGACTTGCCCAATC-3′ (53995360-53995381)

mgSaa2-F: 5′-CCCTCTCCATATTAGTGTGGATTGTC-3′ (54006575-54006599)

mgSaa2-R: 5′-GGACTCAGTGCAAGTCAAACAG-3′ (54010683-54010662)

The expected sizes of the PCR products were 3618 bp for *Saa1* and 4109 bp for *Saa2*. PCR amplification was performed using a TaKaRa LA *Taq* DNA polymerase (TAKARA BIO INC., Otsu, Japan) following the manufacturer's instructions with modifications. A 25-μL reaction volume was used for PCR. The cycling parameters for PCR consisted of an initial denaturation for 1 min at 94°C, followed by 35 cycles of 20 s at 94°C, 15 s at 55°C, and 4 min at 72°C. The PCR products were purified using an UltraClean PCR Clean Up Kit (Mo Bio Laboratories, Carlsbad, CA) and sequenced using a BigDye Cycle Sequencing FS Ready Reaction Kit (Life Technologies, Grand Island, NE) and an ABI 310 automated sequencer (Life Technologies). Entire nucleotide sequences of the PCR products were determined by sequence walking.

Reverse Transcription PCR analysis for *Saa* gene expression

Two-month-old CE/J mice (n=6) were subcutaneously administered 0.5 mL of 2% silver nitrate solution and 100 μg of AA amyloid fibrils intravenously. Three mice were euthanized the next day, and the livers were collected. mRNA was extracted from the livers with the QuickPrep Micro mRNA Purification Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). First-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (GE Healthcare Bio-Sciences). cDNA fragments for the *Saa* genes were then amplified by PCR by using the primer pair Saa-exon2F (5′-GGTTTTTTTCATTTGTTCACGAGGC-3′) and Saa-DownR2 (5′-ACCCTCTCCTCCTCAAGCAG-3′) (Figure 2). The reaction volume used for PCR was 25 μL, and the cycling parameters included an initial denaturation for 1 min at 94°C, followed by 20 cycles of 20 s at 94°C, 15 s at 55°C, and 45 s at 72°C. The PCR products were purified

and directly sequenced with the primers. The PCR products from a CE/J mouse were ligated to the pCR2.1 plasmid vector (Life Technologies), introduced into the *E. coli* DH5α, and spread on Luria Broth agar plates. Recombinant plasmids were isolated from bacterial colonies and their nucleotide sequences were determined.

The remaining 3 mice were sacrificed 10 days after administration of silver nitrate and AA fibrils. Major tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 4-μm-thick sections for Congo Red staining and immunohistochemistry for diagnosis of AA amyloidosis.

Promoter/enhancer activity assays for the mouse *Saa1* and *Saa2* genes

The chromosomal DNA sequence of approximately 640 bp that encompasses the putative regulatory region and untranslated exon 1 of the mouse *Saa1* gene was obtained by PCR from genomic DNA of CE/J and BALB/c mice by using the Saa1-p1 (5′-AGGTACCCAAGAGCTGAGAACTTATGCTAG-3′) and Saa-p2 (5′-CAATCAATCAGTTTCTGTGC-3′) primer pair. For *Saa2*, the primer Saa2-p1 (5′-AGGTACCCTCTCCATATTAGTGTGGATTGTG-3′) was paired with Saa-p2. The PCR products were then digested with *Kpn*I and *Bgl*II and cloned into the *Kpn*I/*Bgl*II site of the pGL4.10 basic plasmid vector (Promega, Madison, WI) to generate luciferase reporter plasmid constructs. The human hepatoma cell line HepG2 was obtained from RIKEN BRC (via the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan) and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. The cells were grown in 24-well plates and then co-transfected

with the reporter construct (0.8 μg/well) and the control reporter plasmid phRL-TK (0.016 μg/well; Promega) by using Lipofectamine 2000 (Life Technologies). At 24 h after transfection, recombinant human IL-1β (Peprotech, Rocky Hill, NJ) and IL-6 (HumanZyme, Chicago, IL) were each added to the medium at a final concentration of 10 ng/mL. After 4 h, the cells were lysed in a passive lysis buffer, and the luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega). Analysis of variance was used to examine any significant differences in enhancer activity among the subjects. Subsequent post-hoc tests to determine significant differences in the pair-wise comparisons were performed using the Tukey-Kramer test. Assays were conducted in triplicate, and the experiments were repeated 3 times.

Results

CE/J mice have *Saa1* and *Saa2* genes that encode identical SAA2.2 isoforms

We obtained PCR products for both *Saa1* and *Saa2* from the CE/J genome by using primers specific for each gene. The PCR products contained putative upstream regulatory elements (see the next section), all exons and introns, and downstream polyadenylation signal sequences. Comparison of the nucleotide sequences of the PCR products to the C57BL/6 mouse genome assembly revealed polymorphisms (substitutions and insertion/deletions) at 80 and 114 positions in the *Saa1* and *Saa2* genes, respectively (Tables 1 and 2). Among the polymorphisms, 21 of *Saa1* and 43 of *Saa2* were found in the Mouse SNP Database v1.2 (http://cgd.jax.org/cgdsnpdb/build_36/), while others were novel polymorphisms (shown without SNP reference ID in Tables 1 and 2). The nucleotide sequence of *Saa1* and *Saa2* of

CE/J showed high overall similarity (Figure 2A). Intriguingly, the coding sequences of the *Saa1* and *Saa2* PCR products were identical except for one silent nucleotide substitution in the 59th codon (AAC in *Saa2* and AAT in *Saa1*). Both *Saa1* and *Saa2* PCR products had a strain-specific nucleotide substitution that gave rise to the Q30L amino acid substitution unique to the SAA2.2 isoform [2]. However, the *Saa1* and *Saa2* PCR products from CE/J showed differences at 15 positions in the sequence between the termination codon and polyadenylation signal (Figure 2A).

These nucleotide differences allowed us to distinguish transcripts derived from the 2 genes. Indeed, RT-PCR products obtained from the acute phase liver of all 3 CE/J mice contained both *Saa1* and *Saa2* sequences (Figure 2B), indicating that the *Saa1* gene was actually transcribed in the liver of the CE/J mice under inflammatory conditions. The peak heights of chromatograms were almost equal for the *Saa1* and *Saa2* products. Furthermore, when the PCR products were TA-cloned into the plasmid vector, 27 and 21 of 48 recombinant plasmids contained PCR products for *Saa1* and *Saa2*, respectively, suggesting that the 2 genes were expressed at equivalent levels. These results indicate that the seemingly single SAA2.2 isoform of the CE/J mice is likely a mixture of *Saa2* and *Saa1* gene products that have identical amino acid sequences.

Despite the observed expression of both *Saa2* and *Saa1*, CE/J mice did not have AA amyloidosis (data not shown); this finding is consistent with a previous report [1].

Promoter/enhancer sequence of *Saa1* and *Saa2* genes of CE/J are functional

To confirm the transcriptional capacity of *Saa1* and *Saa2* in CE/J mice, we characterized the

upstream promoter/enhancer regions of the genes. Major laboratory mouse strains are classified as carrying the A haplotype (e.g., BALB/c) or B haplotype (e.g., 129/Ola) of the *Saa1* and *Saa2* gene unit [6]. A previous study revealed nucleotide sequence polymorphisms (either ggagtTttCc or ggagtAttAc) in the NF-κB-binding motif of the mouse *Saa1* and *Saa2* genes that influenced the effects of IL-6 and IL-1β in a promoter/enhancer reporter assay *in vitro* [5]. Strains of A haplotype have the ggagtTttCc and ggagtAttAc sequences in *Saa1* and *Saa2* genes, respectively. On the other hand, strains of B haplotype have the ggagtTttCc and ggagtAttAc sequences in *Saa2* and *Saa1* genes, respectively. In contrast to these mouse strains, it was found that CE/J mice had the ggagtAttAc sequence in both the *Saa1* and *Saa2* genes (Figure 3A). In addition, we found a nucleotide substitution (Gattgcacaatga) in the C/EBPβ-binding motif of the *Saa2* gene of CE/J (Figure 3A).

In a previous study, the use of promoter/enhancer reporter assay demonstrated that BALB/c *Saa1* gene expression was synergistically upregulated by IL-1β working on the NF-κB-binding motif and IL-6 working on the C/EBPβ-binding motif [5] (Figure 3B). We prepared reporter plasmid constructs for *Saa2* (Type-2.2) and *Saa1* (Type-2.1) upstream sequences of CE/J, and the enhancer activity was compared to that of BALB/c *Saa1* (Type-1). Synergistic upregulation by IL-1β and IL-6 were not observed for the Type-2.2 and Type-2.1 constructs. Both constructs, however, showed moderate responses to IL-6 similar to the Type-1 construct (Figure 3B). These results indicate that the promoter/enhancer sequence of *Saa1* and *Saa2* of CE/J are functional, and are consistent with the fact that we observed similar levels of *Saa1* and *Saa2* transcripts by RT-PCR in the CE/J inflamed liver. In addition, these results suggest that the nucleotide substitution in the C/EBPβ-binding motif found in the

Saa2 gene of CE/J does not influence enhancer activity.

Discussion

The data presented in this study indicate that the apparent absence of one of the SAA isoforms in the CE/J plasma is because both *Saa1* and *Saa2* genes in the strain code for the identical SAA2.2 isoform. To the best of our knowledge, this is the first example of an *Saa1* and *Saa2* gene unit/ haplotype that encodes 2 identical SAA isoforms. This is in contrast to the SAA1 and SAA2 isoforms described in standard laboratory mouse strains, which have amino acid differences at 9 positions. Moreover, human SAA1 and SAA2 isoforms have amino acid differences at 6 positions.

The extremely high similarity of *Saa1* and *Saa2* coding sequences of CE/J has likely been attained through gene conversion. It has been postulated that a Q30L amino acid substitution in the SAA2.2 isoform inhibits its amyloidogenesis [2]. The nucleotide substitution for the Q30L amino acid substitution is present in both *Saa1* and *Saa2* of CE/J. It is highly unlikely that identical nucleotide substitution occurred independently in 2 genes. Rather, it is reasonable to consider that the nucleotide substitution was transferred from one of the genes (*Saa2*) to the other (*Saa1*) through a gene conversion event.

Whether CE/J mice are advantaged or disadvantaged as a result of possessing only a single SAA isoform is not clear. The SAA proteins are an acute phase apolipoprotein reactant. However, the precise physiological/functional differences between SAA1 and SAA2 are not well understood. An advantage of CE/J mice in experimental medicine is that they do not develop AA amyloidosis [1]. Thus, the mouse strain provides an excellent model system for

the study of amyloid enhancing factors other than AA fibril in AA amyloidosis [7]. Furthermore, it has been shown that AA can cross-seed and cross-compete with other amyloid fibrils, such as that observed for the AApoAII in an induced amyloidosis mouse model [8]. The interference by AA could, in some cases, impede the correct interpretation of data obtained from these models. CE/J or congenic strains in which the *Saa* gene unit of CE/J is introduced onto the background of different mouse strains should be potentially more relevant models for studies where such interference by AA should be avoided. It is necessary to discriminate heterozygous carrier mice of the CE/J *Saa* allele to breed congenic strains. The nucleotide sequence data of *Saa* genes of CE/J should be useful for breeding such congenic mouse strains.

Declaration of interests

The authors report no conflicts of interest. This work was supported in part by a Grants-in-Aid for Priority Areas (grant number 22020015) and Scientific Research (B) (grant number 20300144) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Intractable Disease Division, the Ministry of Health, Labor and Welfare of Japan to the Research Committees for Amyloidosis and for Epochal Diagnosis and Treatment of Amyloidosis in Japan.

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Figure legends

Figure 1. Alignment of amino acid sequence of the mouse SAA isoforms. Identical residues to the SAA1.1 are indicated by dots.

Figure 2. (A) Pair-wise alignment of nucleotide sequences of the CE/J *Saa1* and *Saa2* genes. Only sequences from exon 2 to the AATAAA polyadenylation signal are shown. Most intron sequences are omitted. Coding regions are denoted by uppercase letters. Intron and untranslated sequences are denoted by lowercase letters. Start and stop codons are boxed. Different residues between the 2 sequences are indicated by dots. Nucleotide gaps are indicated by dashes. Positions of the oligonucleotide primers used for RT-PCR analysis (Saa-exon2F and Saa-DownR2) are indicated by arrows. The leucine codon at the 30th amino acid residue unique to CE/J is indicated by asterisks. The nucleotide substitution at the 59th codon is indicated by a white arrowhead. Five nucleotide substitutions in the 3′-untranslated sequence are indicated by black arrowheads. (B) Sequence chromatograms for the RT-PCR products from CE/J acute-phased liver. Overlaps of 2 different nucleotides at the 59th codon and in the 3′-untranslated sequence are indicated by white and black arrowheads, respectively.

Figure 3. Enhancer/promoter activities of the upstream regulatory regions of *Saa1* and *Saa2* genes of CE/J. (A) Comparison of the nucleotide sequences of upstream enhancer elements of the *Saa1* and *Saa2* genes of mouse strains. Data for 129/Ola are from a previous study [5]. Polymorphic residues are denoted by uppercase letters. The upstream regulatory sequences are classified into Type-1, Type-2.1, or Type-2.2 based on composite patterns of the 2

polymorphic regulatory elements. (B) The relative luciferase activities are presented as ratios of the cytokine-treated value to that of the cytokine-untreated control value (mean ± SD; n = 3). $^{**}P < 0.01$ compared to Type-1. Data presented here represent 3 independent experiments.

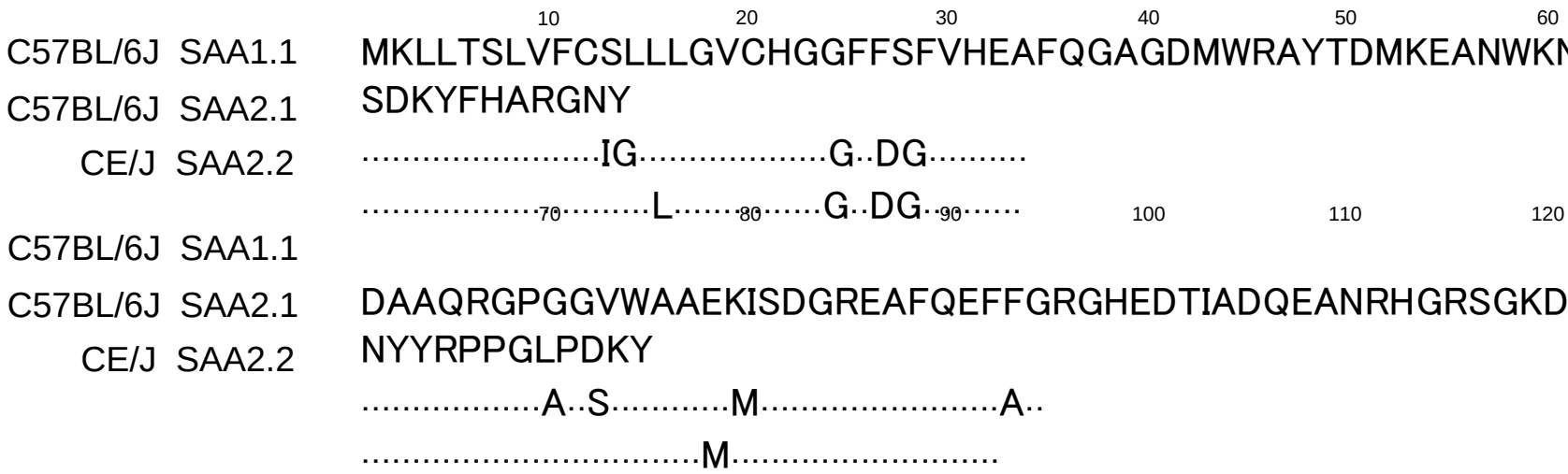
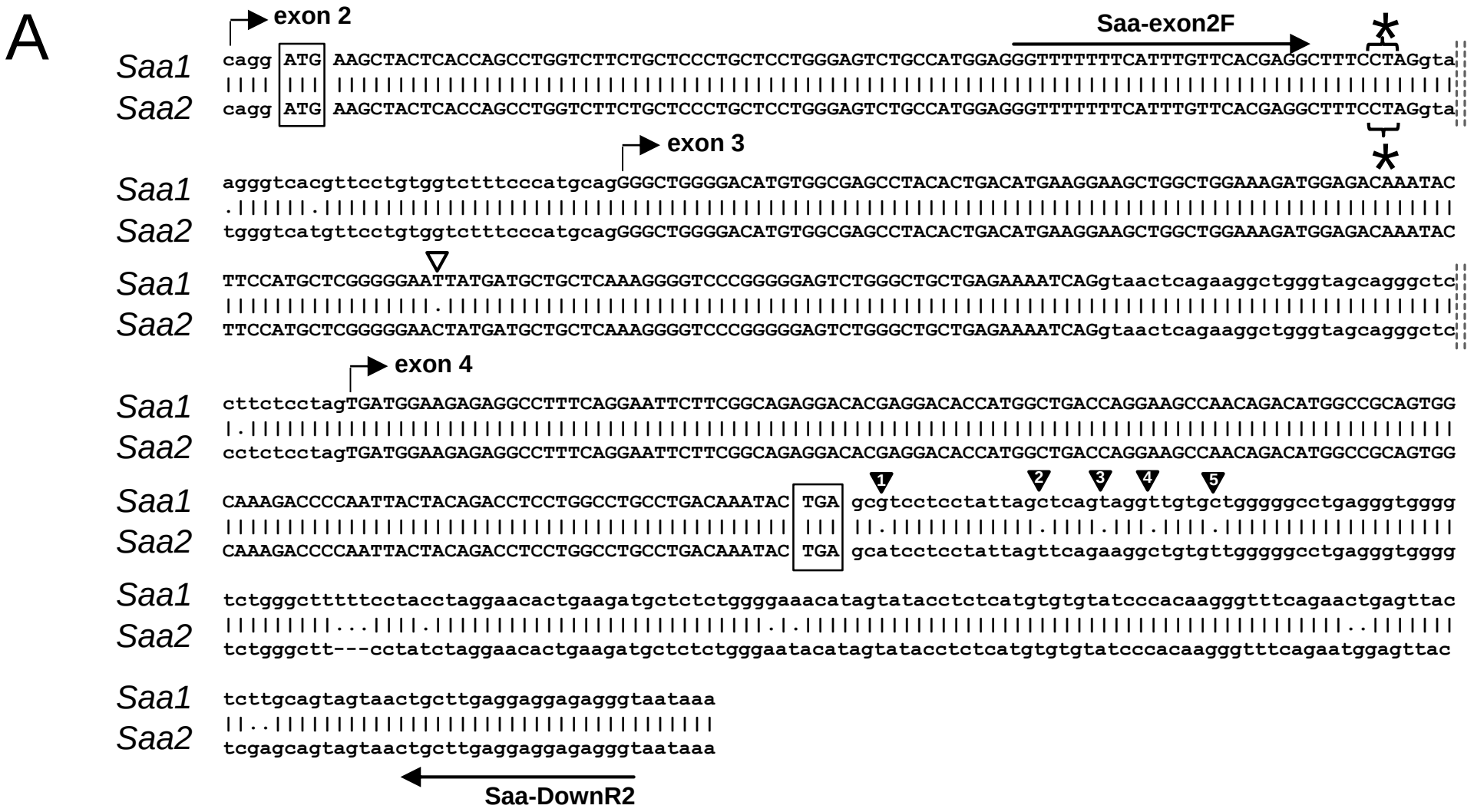

10 20 30 40 50 60
C57BL/6J SAA1.1 MKLLTSLVFCSLLLGVCHGGFFSFVHEAFQGAGDMWRAYTDMKEANWKN
C57BL/6J SAA2.1 SDKYFHARGNY
CE/J SAA2.2 .....IG.....G..DG.....
70 80 90 100 110 120
.....L.....G..DG.....
C57BL/6J SAA1.1
C57BL/6J SAA2.1 DAAQRGPGGVWAAEKISDGREAFQEFFGRGHEDTIADQEANRHGRSGKDP
CE/J SAA2.2 NYYRPPGLPDKY
.....A..S.....M.....A..
.....M.....

Figure 1


A
exon 2
Saa-exon2F
Saa1
Saa2
cagg ATG AAGCTACTCACCAGCCTGGTCTTCTGCTCCCTGCTCCTGGGAGTCTGCCATGGAGGGTTTTTTTCATTTGTTCACGAGGCTTTCCTAGgta
exon 3
agggtcacgttcctgtggtctttcccatgcagGGGCTGGGGACATGTGGCGAGCCTACACTGACATGAAGGAAGCTGGCTGGAAAGATGGAGACAAATAC
tgggtcatgttcctgtggtctttcccatgcagGGGCTGGGGACATGTGGCGAGCCTACACTGACATGAAGGAAGCTGGCTGGAAAGATGGAGACAAATAC
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TTCCATGCTCGGGGGAACTATGATGCTGCTCAAAGGGGTCCCGGGGGAGTCTGGGCTGCTGAGAAAATCAGgtaactcagaaggctgggtagcagggctc
exon 4
cttctcctagTGATGGAAGAGAGGCCTTTCAGGAATTCTTCGGCAGAGGACACGAGGACACCATGGCTGACCAGGAAGCCAACAGACATGGCCGCAGTGG
cctctcctagTGATGGAAGAGAGGCCTTTCAGGAATTCTTCGGCAGAGGACACGAGGACACCATGGCTGACCAGGAAGCCAACAGACATGGCCGCAGTGG
CAAAGACCCCAATTACTACAGACCTCCTGGCCTGCCTGACAAATAC TGA gcgtcctcctattagctcagtaggttgtgctgggggcctgagggtgggg
CAAAGACCCCAATTACTACAGACCTCCTGGCCTGCCTGACAAATAC TGA gcatcctcctattagttcagaaggctgtgttgggggcctgagggtgggg
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tcttgcagtagtaactgcttgaggaggagagggtaataaa
tcgagcagtagtaactgcttgaggaggagagggtaataaa
Saa-DownR2
B
TCGGGGGAANTATGATGC
TGAGCNTCCTCCTATTAGNTCAGNAGGNTGTGNTGGGGG

Figure 2

A

Strain	*Saa1* C/EBPβ-binding	NF-κB-binding	Type	*Saa2* C/EBPβ-binding	NF-κB-binding	Type
C57BL/6J	CCCattgcacaatga…ggagtTttCc		1	CCCattgcacaatga…ggagtAttAc		2.1
129/Ola	CCCattgcacaatga…ggagtAttAc		2.1	CCCattgcacaatga…ggagtTttCc		1
CE/J	CCCattgcacaatga…ggagtAttAc		2.1	Gattgcacaatga…ggagtAttAc		2.2

B

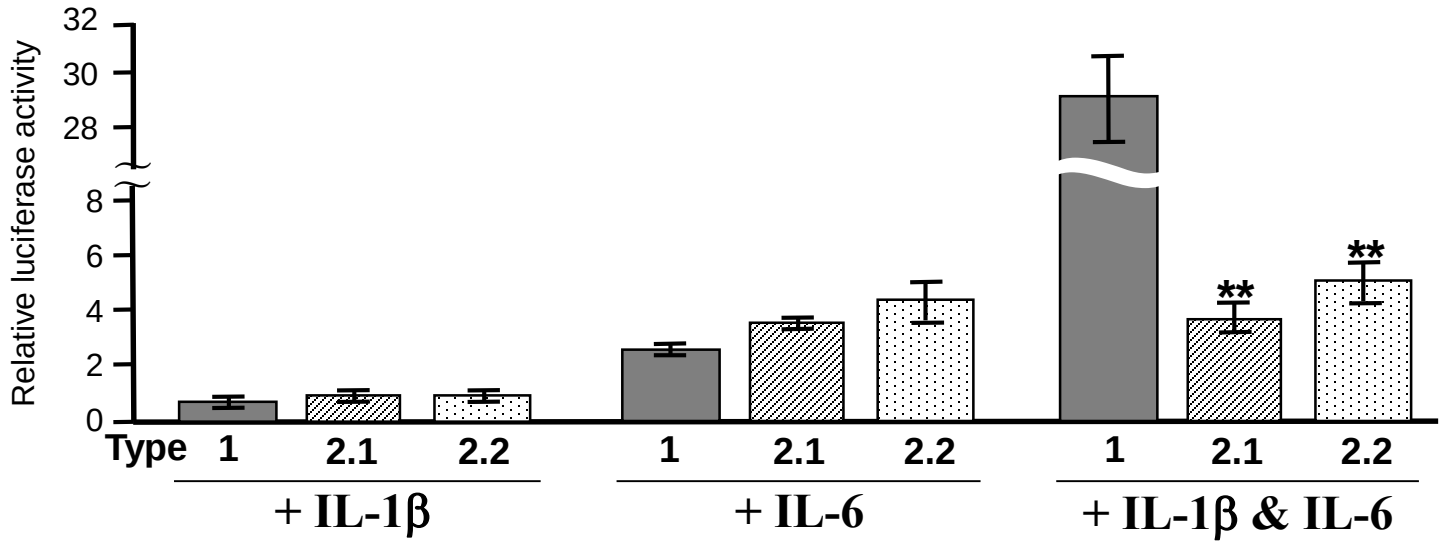

Figure 3

Table 1. Nucleotide polymorphisms of the *Saa1* gene between C57BL/6 and CE/J

RGCm37 coordinate	Position in the gene	C57BL/6	CE/J	SNP reference ID	RGCm37 coordinate	Position in the gene	C57BL/6	CE/J	SNP reference ID
53,995,414	Downstream	T	C	rs51615516	53,997,747	Intron 2	C	T	
53,995,515	Downstream	T	G		53,997,787	Intron 2	G	T	
53,995,665	Downstream	A	G	rs8266333	53,997,804	Exon 2	T	A	
53,995,751	Downstream	A	G		53,997,939	Intron 1	T	C	
53,995,967	3' UTR	A	G	rs3023127	53,997,994	Intron 1	A	G	
53,995,973	3' UTR	A	T	rs8266329	53,998,035	Intron 1	A	T	
53,996,015	3' UTR	G	A		53,998,042	Intron 1	C	T	
53,996,092	Exon 4	T	G	rs8236663	53,998,051	Intron 1	A	G	
53,996,158	Exon 4	A	C	rs8236664	53,998,139-53,998,129	Intron 1	ACCCCTCTCTA	TGCCTCACTG	
53,996,170	Exon 4	A	G	rs8266437	53,998,142	Intron 1	G	T	
53,996,485	Intron 3	C	T		53,998,156	Intron 1	A	C	
53,996,491	Intron 3	T	C	rs8236665	53,998,171	Intron 1	T	C	rs32823326
53,996,679	Exon 3	G	A		53,998,216	Intron 1	T	C	rs8266415
53,996,707	Exon 3	G	C	rs16794336	53,998,233	Intron 1	A	T	
53,996,708	Exon 3	A	C		53,998,246	Intron 1	G	A	
53,996,709	Exon 3	G	A	rs16794335	53,998,306	Intron 1	A	G	
53,996,711	Exon 3	T	C	rs16794334	53,998,328	Exon 1	C	G	
53,996,719	Exon 3	T	C		53,998,345	Exon 1	A	G	
53,996,720	Exon 3	T	C		53,998,355	Upstream	G	T	
53,996,789	Intron 2	T	G		53,998,391	Upstream	C	C	
53,996,796	Intron 2	A	T		53,998,407-5,3998,406	Upstream	del	G	
53,996,830	Intron 2	A	G		53,998,425	Upstream	A	G	
53,996,854	Intron 2	G	A		53,998,433	Upstream	G	T	
53,997,000	Intron 2	G	C	rs32503036	53,998,436	Upstream	A	T	
53,997,038	Intron 2	A	G	rs8266431	53,998,444	Upstream	A	G	
53,997,243	Intron 2	A	G	rs8266354	53,998,505	Upstream	C	G	rs32823332
53,997,326	Intron 2	C	A		53,998,581	Upstream	T	C	
53,997,353	Intron 2	G	A		53,998,645	Upstream	T	del	
53,997,373	Intron 2	A	G		53,998,647	Upstream	G	G	
53,997,390	Intron 2	T	C	rs32268894	53,998,657	Upstream	C	T	
53,997,458	Intron 2	A	G		53,998,682-53,998,683	Upstream	TG	CC	
53,997,526	Intron 2	A	G		53,998,694	Upstream	T	C	
53,997,528	Intron 2	C	A		53,998,709	Upstream	C	G	
53,997,536	Intron 2	G	A		53,998,755	Upstream	A	C	
53,997,551	Intron 2	A	T		53,998,783	Upstream	A	T	
53,997,554	Intron 2	G	A	rs32822516	53,998,788	Upstream	A	del	
53,997,624-53,997,623	Intron 2	del	G		53,998,796	Upstream	G	A	
53,997,632	Intron 2	T	C		53,998,819	Upstream	T	A	
53,997,633	Intron 2	C	A		53,998,827	Upstream	A	G	rs47050954
53,997,634	Intron 2	A	G		53,998,904	Upstream	G	C	rs32824058

Table 2. Nucleotide polymorphisms of the *Saa2* gene between C57BL/6 and CE/J

RGCm37 coordinate	Position in the gene	C57BL/6	CE/J	SNP reference ID	RGCm37 coordinate	Position in the gene	C57BL/6	CE/J	SNP reference ID
54,006,611	Upstream	A	T	rs3023119	54,008,468	Intron2	T	A	
54,006,728	Upstream	G	A	rs32823438	54,008,473	Intron2	T	G	
54,006,764	Upstream	A	G	rs8266413	54,008,476	Intron2	C	T	
54,006,796	Upstream	A	del		54,008,503	Intron2	A	T	
54,006,797-54.006.798	Upstream	AA	del		54,008,512	Intron2	C	T	rs46635498
54,006,830	Upstream	G	A		54,008,515	Intron2	T	C	
54,006,831	Upstream	C	G		54,008,526	Intron2	G	C	
54,005,868	Upstream	G	T		54,008,553	Intron2	C	G	rs49644691
54,006,886	Upstream	T	A	rs8266406	54,008,555	Intron2	C	G	rs50321441
54,006,937	Upstream	C	T		54,008,596	Intron2	T	C	rs32820020
54,006,939	Upstream	C	T		54,008,673	Intron2	A	T	rs32820022
54,006,943	Upstream	C	G		54,008,674	Intron2	G	C	
54,006,946	Upstream	C	T		54,008,695	Intron2	del	C	
54,006,947	Upstream	C	T		54,008,713	Intron2	C	G	
54,006,972	Upstream	C	T		54,008,795	Exon 3	A	G	
54,006,973	Upstream	A	G	rs8266404	54,008,885	Exon 3	C	T	
54,007,002	Upstream	T	C	rs32824395	54,009,000	Intron 3	C	T	rs32821027
54,007,014-54,007,018	Upstream	CTCCC	G		54,009,047	Intron 3	C	T	
54,007,035	Upstream	A	G	rs8266402	54,009,166	Intron 3	T	G	
54,007,050	Upstream	A	G	rs8266400	54,009,249	Intron 3	G	A	
54,007,108	Upstream	T	C	rs8266399	54,009,295	Intron 3	C	T	
54,007,127	Upstream	T	C	rs8266396	54,009,332	Intron 3	T	C	
54,007,156	Upstream	G	A		54,009,346	Exon 4	C	G	
54,007,198	Upstream	C	A	rs8266391	54,009,354	Exon 4	A	G	
54,007,225	Exon 1	G	C	rs8266389	54,009,355	Exon 4	G	C	
54,007,247	Intron 1	T	C	rs8266387	54,009,461	Exon 4	A	C	
54,007,327	Intron 1	C	del		54,009,469	Exon 4	C	A	
54,007,382	Intron 1	A	G	rs32825498	54,009,481	3' UTR	A	C	rs32821755
54,007,397	Intron 1	T	G		54,009,482	3' UTR	G	A	
54,007,411	Intron 1	C	A		54,009,515	3' UTR	T	G	
54,007,413-54,007,441	Intron 1	ACCCCTCTCTACTGCCTGCATGCCTTCT	TGCCTCACTGCTGCCTGCCTGCCT		54,009,570	3' UTR	G	A	
54,007,433	Intron 1	A	del		54,009,571	3' UTR	C	A	rs32821757
54,007,502	Intron 1	T	C		54,009,572	3' UTR	A	T	
54,007,614	Intron 1	A	G		54,009,672	3' UTR	C	T	
54,007,733	Exon 2	A	G		54,009,695	Downstream	G	A	rs32821759
54,007,736	Exon 2	G	C		54,009,766	Downstream	G	T	
54,007,737	Exon 2	G	A		54,009,816	Downstream	T	C	rs32822617
54,007,738	Exon 2	G	C		54,009,968	Downstream	G	T	rs32822621
54,007,766	Intron2	C	A	rs32819115	54,010,131-54,010,135	Downstream	ACAGG	del	
54,007,861	Intron2	T	A	rs32819117	54,010,180	Downstream	A	G	
54,007,908	Intron2	G	T	rs8236441	54,010,196	Downstream	A	T	rs49093588
54,007,936	Intron2	G	A	rs8236442	54,010,219	Downstream	A	G	
54,007,951	Intron2	C	A	rs8266424	54,010,226	Downstream	C	T	
54,007,999	Intron2	C	T		54,010,228	Downstream	C	T	rs47642783
54,008,002	Intron2	T	A		54,010,233	Downstream	G	A	
54,008,081	Intron2	G	A		54,010,242	Downstream	T	C	
54,008,168	Intron2	C	A	rs8266422	54,010,269	Downstream	A	T	
54,008,210	Intron2	C	T		54,010,413	Downstream	C	T	rs49403762
54,008,239	Intron2	C	GGG		54,010,418	Downstream	C	T	rs46909412
54,008,382	Intron2	A	G	rs8266417	54,010,453	Downstream	A	G	rs49500822
54,008,389	Intron2	T	A	rs8266356	54,010,456	Downstream	C	A	rs47062754
54,008,408	Intron2	A	G	rs32820017	54,010,468	Downstream	G	T	
54,008,441	Intron2	G	A		54,010,609	Downstream	C	T	
54,008,446	Intron2	T	A		54,010,618-54.010.622	Downstream	ATCTT	del	
54,008,447	Intron2	C	T		54,010,658	Downstream	T	A	rs46387158
54,008,448	Intron2	A	G		54,010,659	Downstream	G	C	rs32822623
54,008,459	Intron2	C	G	rs46155987	54,010,660	Downstream	G	A	