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Diversity and Complexity of the Mouse *Saa1* and *Saa2* genes

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Abstract: Mouse strains show polymorphisms in the amino acid sequences of serum amyloid A 1 (SAA1) and serum amyloid A 2 (SAA2). Major laboratory mouse strains are classified based on the sequence as carrying the *A* haplotype (e.g., BALB/c) or *B* haplotype (e.g., SJL/J) of the *Saa1* and *Saa2* gene unit. We attempted to elucidate the diversity of the mouse *Saa1* and *Saa2* family genes at the nucleotide sequence level by a systematic survey of 6 inbred mouse strains from 4 *Mus* subspecies, including *Mus musculus domesticus*, *Mus musculus musculus*, *Mus musculus castaneus*, and *Mus spretus*. *Saa1* and *Saa2* genes were obtained from the mouse genome by PCR amplification, and each full-length nucleotide sequence was determined. We found that *Mus musculus castaneus* mice uniquely possess 2 divergent *Saa1* genes linked on chromosome 7. Overall, the mouse strains had distinct composite patterns of amino acid substitutions at 9 positions in SAA1 and SAA2 isoforms. The mouse strains also had distinct composite patterns of 2 polymorphic upstream regulatory elements that influenced gene transcription in *in vitro* reporter assays. *B* haplotype mice were revealed to possess an LTR insertion in the downstream region of *Saa1*. Collectively, these results indicate that the mouse *Saa* genes hold broader diversity and greater complexity than previously known, and these characteristics were likely attained through gene duplication and repeated gene conversion events in the *Mus* lineage.

Key words: gene conversion, gene duplication, *Mus* subspecies, polymorphism, serum amyloid A

Introduction

Serum amyloid A 1 (SAA1) and serum amyloid A 2 (SAA2) are acute phase apolipoprotein reactants that are concomitantly expressed, mainly in the liver, in response to inflammatory stimuli [9]. The *Saa1* and *Saa2* genes are a gene family that has been found in all mammalian species examined thus far. The homologous *Saa1* and *Saa2* genes are thought to have been formed through gene duplication, and gene conversion between the duplicated genes contributed to the preservation of their mutually high similarity. The mouse *Saa1* and *Saa2* genes are ~3.5 kilobase (kb) pairs in length and are lo-

cated within an interval of ~8 kb on chromosome 7 [8]. The overall exon/intron organization of the 2 genes is highly similar. Furthermore, the *Saa1* and *Saa2* genes of the reference C57BL/6J mouse strain (*Mus musculus domesticus*) retain 96% nucleotide sequence identity over their entire length. At the protein level, the reference SAA1 (accession number NP_033143) and SAA2 (NP_035444) proteins show 122 amino acid-long similar sequences, with differences at 9 positions (25th, 26th, 46th, 49th, 50th, 79th, 82nd, 95th, and 120th).

In addition to the C57BL/6J strain, amino acid sequences have been known for SAA isoforms from other mouse strains. Interestingly, these isoforms show poly-

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Fig. 1. Structure of the mouse *Saa1* and *Saa2* genes. Exons are indicated by boxes. Solid and broken lines indicate regions of high and low nucleotide sequence similarity between the genes, respectively. A C/EBP β -binding motif and an NF- κ B-binding motif located at upstream of the first non-coding exon of the genes are indicated by circles. Approximate positions of a starting methionine codon (ATG) in exon 2, a stop codon (TGA) in exon 4, a polyadenylation signal sequence (aataaa), and an ERVK LTR insertion are shown. Approximate positions of the oligonucleotide primers used in this study are indicated by horizontal arrows. The nucleotide sequences of the oligonucleotide primers are shown at the bottom. The sizes of exons, introns, and upstream and downstream regions are not drawn to the same scale.

morphisms in the amino acid sequence. Major laboratory mouse strains of *M. m. domesticus* are classified into A haplotype (e.g., C57BL/6J and BALB/c) or B haplotype (e.g., SJL/J and 129/SvJ) for the *Saa1* and *Saa2* gene unit [16]. Compared to C57BL/6J strain, SJL/J of B haplotype expressed an SAA2 isoform with a substitution of aspartic acid for alanine at position 120 [2, 3]. In addition to *M. m. domesticus*, the house mouse includes other subspecies *M. m. musculus* and *M. m. castaneus* and another more remotely related species *Mus spretus* [1, 11, 15]. The SAA1 and SAA2 isoforms of *M. m. musculus* have complex composite patterns of amino acid substitutions between reference SAA1 and SAA2 at 9 positions [2]. The SAA1 and SAA2 amino acid sequences of *M. m. castaneus* and *M. spretus* have not been reported. However, it was demonstrated that *M. spretus* expresses SAA isoforms with distinct isoelectric points [14], suggesting that the mice have different amino acid sequences for the SAA isoforms. Thus, these mouse (sub)species provide a good opportunity to resolve diversity, complexity, and evolutionary characteristics of the *Saa* genes.

In order to address these issues, we conducted PCR cloning and nucleotide sequencing of the entire lengths of the *Saa1* and *Saa2* genes from various strains of *Mus* (sub)species, including *M. m. castaneus* and *M. spretus*.

Materials and Methods

Mouse strains

Genomic DNAs from SJL/J (*M. m. domesticus*), CAST/EiJ (*M. m. castaneus*), and SPRET/EiJ (*M. spretus*) were obtained from the Jackson laboratory. BLG2/Ms (RBRC00653; *M. m. musculus*), NJL/Ms (RBRC00658; *M. m. musculus*), and HMI/Ms (RBRC00657; *M. m. castaneus*) were obtained from RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. All experimental procedures involving mice were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University.

PCR cloning and nucleotide sequence analysis of mouse *Saa1* and *Saa2* genes

Genomic DNA was isolated from the liver of mice by standard methods. To ensure specific, full-length PCR cloning of the *Saa1* and *Saa2* genes, oligonucleotide primers were designed so that the primers could anneal to the non-homologous 5'-upstream and 3'-downstream regions of the genes (Fig. 1). PCR amplification was performed using a TaKaRa LA *Taq* DNA polymerase (TAKARA BIO INC., Otsu, Japan) following the manufacturer's instructions with modifications. 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, NA) and an ABI 310 automated sequencer. Entire nucleotide sequences of the PCR products were determined by sequence walking. As for the *Saa1* PCR products from *M. m. castaneus* mice, overlaps of the 2 peaks were observed for the sequence chromatogram. These PCR products were then cloned into the pCR2.1 vector (Life Technologies) and nucleotide sequences of the cloned DNAs were determined.

Reverse transcription-PCR analysis of Saa1 in HMI/Ms mice

Three HMI/Ms mice were subcutaneously administered 0.5 ml of 2% silver nitrate solution. The 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 Life Sciences, Piscataway, NJ). First-strand cDNA was synthesized using a First-Strand cDNA Synthesis Kit (GE Healthcare Life Sciences). cDNA fragments for the *Saa* genes were then amplified by PCR with the primer pair Saa-exon2F and Saa-DownR2 (Fig. 1). The PCR products were purified and directly sequenced with Saa-exon2F.

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 mouse strains with the Saa1-p1 and Saa-p2 primer pair (Fig. 1). For *Saa2*, the primer Saa2-p1 was paired with Saa-p2. The PCR products were then digested with *KpnI* and *BglII* and cloned into the *KpnI/BglII* site of the pGL4.10 basic plasmid vector (Promega, Madison, WI) to generate luciferase reporter plasmid constructs. Promoter/enhancer activity assays were performed as described [20]. Analysis of variance was used to examine any significant differences in promoter/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

The entire *Saa1* (approximately 3.6 kb) or *Saa2* gene (4.1 kb), from the 5' upstream regulatory region to the polyadenylation signal, was obtained by PCR amplification from genomic DNA samples by using primers specific for the genes. Comparison of the nucleotide sequences between the mouse strains revealed polymorphisms (substitutions, insertion/deletions, and variable number of repeats) at 194 and 216 positions in the *Saa1* and *Saa2* genes, respectively. Among the polymorphisms, 43 of *Saa1* and 91 of *Saa2* were found in the Mouse SNP Database v1.2 (http://cgd.jax.org/cgdsnpdb/build_36/), while others were novel polymorphisms. Profiles of the mouse strains for the *Saa1* and *Saa2* gene polymorphisms are available upon request.

M. m. castaneus mice are unique in possessing 2 linked but divergent Saa1 genes

In the sequence chromatograms for the *Saa1* PCR products from CAST/EiJ and HMI/Ms strains, overlaps of 2 signal peaks were observed at multiple positions (Fig. 2A), suggesting heterogeneity of the PCR products. The PCR products were then cloned and their nucleotide sequences were confirmed. Two types of sequences were obtained for the clones (Figs. 2B and C). All overlaps in the sequence chromatograms of the primary *Saa1* PCR products of CAST/EiJ and HMI/Ms could be accounted for by superimposition of the 2 sequences. Similar results were obtained for 4 additional *M. m. castaneus* mice caught in the Philippines [7]. Because CAST/EiJ and HMI/Ms are inbred strains and are assumed to be homozygous for the *Saa1*, these results suggested that *M. m. castaneus* mice have 2 *Saa1* genes. To confirm this point, (C57BL/6 $\times$ *M. m. castaneus*) F₁ mice were examined. If the 2 types of *Saa1* PCR products of *M. m. castaneus* mice were derived from alleles of a single *Saa1* locus, we would expect that the F₁ mice would yield only 1 of the 2 types of *Saa1* PCR products characteristic of *M. m. castaneus*. Actually, 3 types of *Saa1* PCR products were obtained from the F₁ mice: 2 types of PCR products of *M. m. castaneus* and one of C57BL/6 type. Collectively, these results were consistent with the hypothesis that *M. m. castaneus* mice have 2 *Saa1* genes. These genes are provisionally named *Saa1a* and *Saa1b*. Amino acid sequence of the deduced *Saa1a* product was highly similar to that of reference SAA1 (Fig. 3). On the contrary, the deduced *Saa1b* product had a chimeric structure

Fig. 2. Sequence chromatograms of PCR products for the *Saa1* gene from a *M. m. castaneus* HMI/Ms mouse (A), a *Saa1a* clone (B), and a *Saa1b* clone (C). The sequences correspond to a part of exon 3 of the gene. (D) A sequence chromatogram of the RT-PCR product for the *Saa1* gene from an HMI/Ms mouse. The sequence corresponds to a part of exon 4 of the gene. Overlaps of different nucleotides are indicated by arrowheads.

with the reference SAA2.

Saa1a and *Saa1b* PCR products from *M. m. castaneus* showed nucleotide differences in the exons 3 and 4 sequences (Fig. 2). These differences allowed us to distinguish transcripts derived from the 2 genes. RT-PCR products obtained from the HMI/Ms mouse acute phase liver contained both *Saa1a* and *Saa1b* sequences (Fig. 2D), indicating that both genes were actually transcribed in the liver of the HMI/Ms mice during inflammatory conditions.

The mouse *Saa1* (30.54 cM) and *Saa2* (30.56 cM) are linked on chromosome 7. To examine if the *Saa1a* and *Saa1b* genes are linked, 2 congenic mouse strains, in which a segment of chromosome 7 (23.9 cM ~ 43.7 cM) from *M. m. castaneus* was introduced into the background of a C57BL/6 strain (A. Ishikawa, personal communication), were examined. PCR products for both *Saa1a* and *Saa1b* were obtained from the mice, indicating that the 2 genes are linked on chromosome 7 of *M. m. castaneus*. It is highly likely that gene duplication of the *Saa1* gene occurred after the *M. m. castaneus* subspecies was separated from other lineages, and a gene con-

version took place subsequently in the *Saa1b* gene.

Mouse strains have distinct composite patterns for amino acid substitutions in SAA1 and SAA2

Previous studies revealed amino acid substitutions at 9 positions in the mouse SAA1 and SAA2 isoforms [2, 3] (Fig. 3). In this study, nucleotide changes, which would lead to any additional novel amino acid substitutions not previously reported, were not found in the mouse strains examined. Intriguingly, however, we found that the amino acid substitutions at 9 positions prevailed in 4 *Mus* subspecies (Fig. 3). This result suggested that the nucleotide substitutions associated with the amino acid substitutions occurred before the divergence of the (sub)species approximately 2–4 million years ago [15, 19]. In addition, we found that the mouse strains had distinct composite patterns of the 9 polymorphic amino acids for SAA1 and SAA2 isoforms (Fig. 3). This result suggests that gene conversion events occurred repeatedly between *Saa* genes. In the composite patterns, those at the 25th and 26th positions were always concomitant, namely, either valine-histidine or isoleucine-glycine. These amino acids are encoded in exon 2 of the genes. Also, substitutions at the 46th, 49th, and 50th positions (asparagine-asparagine-serine or glycine-aspartic acid-glycine) and at the 79th and 82nd positions (glycine-alanine or alanine-serine) were concomitant. The former 3 and latter 2 amino acids are encoded in the exon 3 and exon 4, respectively.

The upstream regulatory regions of the mouse Saa1 and Saa2 also show considerable diversity

We also investigated the upstream regulatory regions of *Saa1* and *Saa2*. Transcriptional induction of these mouse genes is regulated by 2 putative *cis*-acting elements of a CAAT enhancer-binding protein β (C/EBP β)-binding motif (cccattgcacaatga) and a nuclear factor κ B (NF- κ B)-binding motif located in tandem within 260 bp upstream of the first non-coding exon of the genes [17] (Fig. 1). A proinflammatory cytokine IL-6 enhances gene transcription via the C/EBP β -binding motif, whereas IL-1 β and TNF- α enhance transcription via the NF- κ B-binding motif. A previous study revealed nucleotide sequence polymorphisms (either ggagtTttCc or ggagtAttAc) in the NF- κ B-binding motif of the mouse *Saa1* and *Saa2* genes that influenced the effects of IL-6 and IL-1 β in an enhancer/promoter reporter assay *in vitro* [17].

In this study, screening of various mouse strains led to the identification of a new variant sequence (ggagCAT-

Subspecies	Strain		Amino acid position								
			25	26	46	49	50	79	82	95	120
<i>domesticus</i>	C57BL/6J	SAA1	V	H	N	N	S	G	A	I	D
		SAA2	I	G	G	D	G	A	S	M	A
	SJL/J	SAA1	V	H	N	N	S	G	A	M	D
		SAA2	I	G	G	D	G	A	S	M	D
<i>musculus</i>	BLG2/Ms & NJL/Ms	SAA1	V	H	G	D	G	G	A	M	D
		SAA2	I	G	G	D	G	G	A	M	D
<i>castaneus</i>	CAST/EiJ & HMI/Ms	SAA1A	V	H	N	N	S	G	A	M	D
		SAA1B	V	H	G	D	G	A	S	M	D
	CAST/EiJ HMI/Ms	SAA2	I	G	G	D	G	G	A	M	D
		SAA2	I	G	G	D	G	A	S	M	D
<i>Mus spretus</i>	SPRET/EiJ	SAA1	I	G	G	D	G	G	A	I	D
		SAA2	I	G	G	D	G	A	S	M	D

exon 2 ▲ exon 3 ▲ exon 4

Fig. 3. Comparison of composite patterns of amino acid substitutions at 9 positions of the mouse SAA1 and SAA2 isoforms. Two *M. m. musculus* strains, BLG2/Ms and NJL/Ms, have identical amino acid sequences for both SAA1 and SAA2 isoforms. In addition, *M. m. castaneus* strains CAST/EiJ and HMI/Ms have identical amino acid sequences for both SAA1A and SAA1B isoforms. Arrowheads indicate the boundaries of corresponding exons.

tAc) with respect to the NF- κ B-binding motif in the *Saa1b* gene of *M. m. castaneus* CAST/EiJ and HMI/Ms strains (Fig. 4A). In addition, we found a variant sequence (Gattgcacaatga) in the C/EBP β -binding motif of the *Saa2* gene of NJL/Ms and *Saa1b* of *M. m. castaneus*, for which nucleotide changes have not yet been reported. These variant sequences formed 2 novel composite patterns of the 2 polymorphic regulatory elements (Type-2.2 and Type-2.3; Fig. 4A) in addition to the previously known Type-1 and Type-2.1 [17].

Reporter plasmid constructs were then prepared for the 4 types of the mouse *Saa1* and *Saa2* upstream sequences (Type-1: C57BL/6J *Saa1*; Type-2.1: SJL/J *Saa1*; Type-2.2: NJL/Ms *Saa2*; Type-2.3: CAST/EiJ *Saa1b*), and the enhancer activity was compared. Consistent with the previous report [17], reporter expression was moderately enhanced by IL-6 alone, but not by IL-1 β alone in the Type-1 and Type-2.1 constructs (Fig. 4B). Moreover, synergistic upregulatory effects of IL-1 β and IL-6 were observed for the Type-1 regulatory sequence. Type-2.2 and Type-2.3 constructs showed a similar response to Type-2.1. These results suggest that the newly identified nucleotide alterations in the C/EBP β -binding and NF- κ B-

binding motifs do not influence their enhancer activities.

B haplotype mice possess an LTR insertion in the downstream region of Saa1

The downstream regions of *Saa1* and *Saa2* also contained nucleotide polymorphisms between the mouse strains. In particular, an endogenous retrovirus (ERV) long terminal repeat (LTR) sequence (507 bp in length) was found at approximately 420 bp downstream of the polyadenylation signal sequence of *Saa1* in SJL/J (Figs. 1 and 5A). To examine if the LTR insertion is unique to SJL/J, additional mouse strains were analyzed. PCR amplification and nucleotide sequencing of the *Saa1* downstream region with the primer pairs of Saa1-Down-F and mgSaa1-R revealed that 129/SvJ also had the insertion (Fig. 5B). Both SJL/J and 129/SvJ have *B* haplotype for the *Saa1* and *Saa2* gene unit [16]. Thus, our results suggest that the LTR insertion is specific to *B* haplotype strains.

Discussion

A previous study of the BALB/c *Saa1* and *Saa2* genes revealed high similarity of the genes that were likely to

Fig. 4. Comparison of the enhancer activities of the upstream regulatory regions of mouse *Saa1* and *Saa2* genes. (A) Comparison of the nucleotide sequences of upstream enhancer elements of the mouse *Saa1* and *Saa2* genes. Polymorphic residues are denoted with uppercase letters. CAST/EiJ and HMI/Ms have identical types of upstream enhancer elements for *Saa1a* (Type-2.1), *Saa1b* (Type-2.3), and *Saa2* (Type-1). (B) The relative luciferase activities are presented as ratios of the cytokine-treated value to that of the cytokine-untreated control value (mean $\pm$ SD; n=3). ** P <0.01 compared to Type-1. Data presented here represent 3 independent experiments.

Fig. 5. (A) Nucleotide sequence of the ERVK LTR (uppercase letters) in the *Saa1* downstream region of SJL/J. Numbers indicate the nucleotide positions in the mouse draft genome sequence data (RGCm38). (B) An agarose gel electrophoresis of PCR products for the *Saa1* downstream region of mouse strains showing an increased product size in SJL/J and 129/SvJ due to the LTR insertion.

be attained through gene conversion [8]. The data obtained in this study revealed that the mouse *Saa* genes hold broader diversity and greater complexity than previously known. These characteristics were likely attained

through repeated gene duplication and conversion events in the *Mus* lineage. The composite pattern of amino acid substitutions in SAA1 and SAA2 isoforms (Fig. 3) suggests that gene conversions occurred between homolo-

gous exons. In addition, distribution of the 4 types of regulatory sequences was independent not only of gene type (*Saa1* or *Saa2*) but also of subspecies, indicating that multiple gene conversion events occurred also in the upstream regulatory regions of *Saa1* and *Saa2*. The physical proximity of the 2 genes (~8 kb) might have contributed to the high incidence of gene conversion. Examination of other mouse strains should give further information on evolutionary characteristics of the mouse *Saa* genes.

Whether *M. m. castaneus* mice are advantaged as a result of possessing 2 *SAA1* isoform is not clear. The *SAA* proteins are an acute phase apolipoprotein reactant. The precise physiological function of *SAA1* and *SAA2*, or functional relevance of the amino acid sequence polymorphisms in *SAA1* and *SAA2* isoforms of mice are not well understood. Rather, the pathological role of *SAA* as precursor proteins for secondary systemic amyloidosis (AA amyloidosis) is well documented in various mammalian species including humans and mice [5, 9]. In mice, *SAA2* is predominantly deposited as amyloid [12]. Relevance of *Saa2* polymorphisms in susceptibility to AA amyloidosis awaits further study. The A/J strain is relatively resistant, while the CE/J strain is extremely resistant to AA amyloidosis [13, 18]. The cause of extreme resistance of CE/J strain to AA amyloidosis is definitely an amino acid substitution in *SAA2* [4]. The basis of resistance of the A/J strain to the development of AA amyloidosis is not clear but may be related to a difference in the ability of macrophages to degrade *SAA* [6]. Other mouse strains also show various degrees of susceptibility to induction of AA amyloidosis [13, 18]. However, the basis for the strain difference has scarcely been addressed [10]. SJL/J mice of the *B Saa* haplotype developed AA amyloidosis in a manner comparable to C57BL/6J mice of *A* haplotype [13]. Thus, it was unlikely that the functional promoter/enhancer polymorphism of *Saa2* is associated with the susceptibility to AA amyloidosis. Even though *M. m. musculus*, *M. m. castaneus*, and *M. spretus* mice are scarcely used to model inflammatory conditions, there is the possibility that the mouse strains with *Saa1* and *Saa2* alleles with different amino acid sequences or differential transcriptional regulation exhibit different responses to measures to reproduce or cure the inflammatory conditions. It is important to point out that such occurrences could, in some cases, impede the correct interpretation of data obtained from these models. Our findings mandate the notion that

strain differences such as those defined here should be taken into account in these studies.

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