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Rat tissues express serum amyloid A protein-related mRNAs

(acute-phase protein/high density lipoprotein/lipid)

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ABSTRACT **Serum amyloid A (SAA) is a small (12 kDa) acute-phase apoprotein of high density lipoprotein found in mammals. It is also the precursor to amyloid protein A, the main protein constituent of fibrils found in amyloidosis secondary to chronic or recurrent inflammation—e.g., rheumatoid arthritis. However, rats do not develop amyloidosis and SAA is not an apoprotein of rat high density lipoprotein; thus rats appear to be an exception in regard to expression of SAA genes. We report here that rats do have representatives of the SAA gene family and express two distinct SAA mRNAs. Moreover, the pattern of genes expressed among tissues, and their induction by inflammatory agents, is similar to that of related mouse genes. RNA from various tissues of normal and injured rats was examined by RNA blot hybridization with SAA cDNA and complementary RNA probes for the three murine SAA genes. A SAA mRNA of ≈400 nucleotides related to mouse SAA_1 and SAA_2 mRNAs reached a high level in liver 24 hr after injection of bacterial lipopolysaccharide. No extrahepatic tissues were found to express the SAA_1/SAA_2-related mRNA. Turpentine induced two hepatic SAA_1/SAA_2-related mRNAs of ≈400 and ≈500 nucleotides in length. Liver SAA_1/SAA_2-related mRNA hybrid selected and translated in a wheat germ protein-synthesizing system, from lipopolysaccharide- and turpentine-injected rats, produced a single protein with an estimated molecular mass of 8 kDa. This rat liver SAA-related mRNA appears to lack a highly conserved coding region for portions of two amphipathic helical domains and the joining sequence. An mRNA related to mouse SAA_3 was found expressed at a high level in lung after lipopolysaccharide but not following turpentine injection. This mRNA was also expressed at high levels in ileum and large intestine of control rats and was not found in the liver of control or challenged rats. These observations show that the SAA gene family is present and expressed in rats and that its expression is found under situations similar to those found in mice. This lends support for the importance of the SAA gene family in the response to injury by vertebrates.**

A complex series of systemic and metabolic changes occur in vertebrates in response to injury or infection. These reactions are collectively termed the acute-phase response. Conspicuous among these events is the rise in concentration of certain plasma proteins of hepatic origin termed acute-phase proteins (1). Serum amyloid A (SAA) is a family of apolipoproteins found mainly associated with high density lipoprotein (HDL) (2, 3). Members of this family are acute-phase proteins (1, 4, 5). In addition, part of the SAA is a component of amyloid deposits associated with inflammatory disease. The major protein of these deposits is amyloid A protein (AA), comprising the NH_2-terminal 76 residues of the circulating precursor SAA (6, 7). However, rats do not develop amyloidosis and no SAA has been found in rat HDL following injury. The apparent absence of expression in the rat has been puzzling because of the high degree of conservation of the SAA gene(s) in other mammals and the duck (8–12).

The murine SAA gene family is composed of three transcribed genes, SAA_1, SAA_2, and SAA_3 (13), whose structures are known. Because of the high degree of conservation of this gene family in mammals, we used cDNA and complementary RNA (cRNA) probes derived from mouse SAA gene sequences. With these probes we examined, by RNA blot analysis, different rat tissues for expression of SAA mRNAs after lipopolysaccharide (LPS) or turpentine injection. The data show that at least two SAA-related mRNAs are expressed in rat tissues and give insight into certain structural features.

MATERIALS AND METHODS

RNA Preparation and Blot Analysis. An acute-phase response was elicited in 4-month-old male Sprague–Dawley rats by injection of gum turpentine or bacterial LPS. In one group of rats, 0.5 ml of gum turpentine per 100 g of body weight was injected subcutaneously into several sites in the dorsolumbar region. Another group received 50 μg of LPS (*Escherichia coli* 0111:B4, Difco) per 100 g of body weight by intravenous injection via the tail vein. RNA was prepared from tissues 24 hr after injection and from control unstimulated rats by Polytron homogenization in 5 M guanidinium isothiocyanate and precipitation with LiCl (14) as described elsewhere (15).

RNA blot analysis was performed as described (16). Briefly, 10 μg of total RNA was denatured and electrophoresed through 1.5% agarose/2.2 M formaldehyde gels (17) and transferred to GeneScreen*Plus* nylon membranes (New England Nuclear) (18). Blots were hybridized in 0.25 M Na_2HPO_4, adjusted to pH 7.2 with HCOOH, 0.25 M NaCl, 7% (wt/vol) sodium dodecyl sulfate (SDS), 50% (vol/vol) deionized formamide, 10% (wt/vol) polyethylene glycol 8000, and 20 μg of denatured and sheared calf thymus DNA per ml (16, 19) with various cDNA and cRNA probes at 1×10^6 cpm/ml.

cDNA and cRNA Probes. A partial cDNA of mouse SAA_1 (16) was labeled with ^{32}P by nick-translation (20) to a specific activity of $\approx 2 \times 10^6$ cpm/ng. SAA_1 and SAA_3 cDNAs [SAA_3 cDNA in pRS48 (21) was a gift of J. Morrow, Johns Hopkins University] were cloned into transcription plasmids pGEM-1 and pGEM-2, respectively. Single-stranded cRNA probes were transcribed in the presence of [α-^{32}P]UTP according to the manufacturer's directions (Promega Biotec). Two cRNA probes were transcribed from mouse SAA_1 cDNA (Fig. 1*B*). One cRNA was transcribed from a 110-bp sequence of *Hin*dIII-linearized p125 with T7 RNA polymerase. This region encodes amino acid residues 30–66 of SAA_1 and hybridizes to all three mouse SAA mRNAs and human SAA mRNA (unpublished observation). The other cRNA was

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Abbreviations: SAA, serum amyloid A; AA, amyloid A; LPS, lipopolysaccharide; HDL, high density lipoprotein; cRNA, complementary RNA; apo, apolipoprotein.

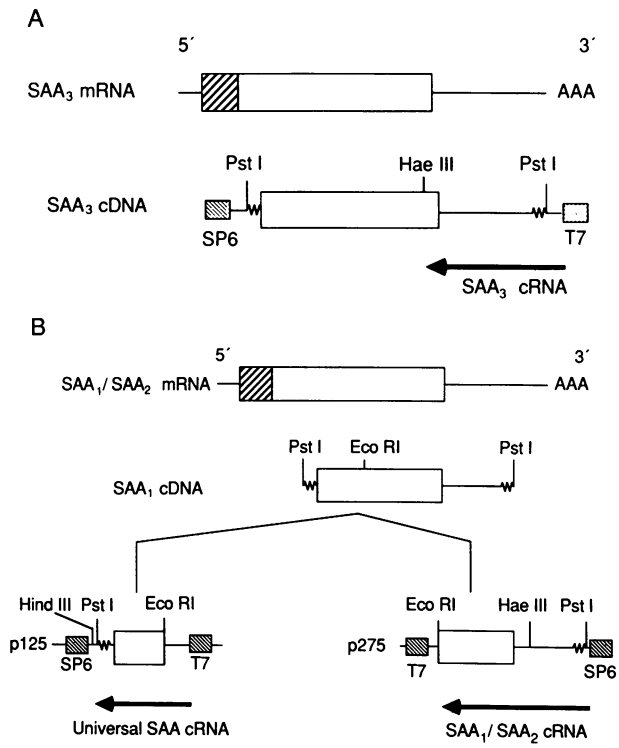

FIG. 1. Diagram of transcription plasmids. A diagram of the SAA mRNAs is shown at the top. The 5′ and 3′ untranslated sequences are shown as a line (—); the coding region is shown as a box (□) with the signal peptide indicated with stripes (▨). The coding sequences are 369 base pairs (bp) in length (6). (*A*) SAA_3 cDNA was excised from the plasmid pRS48 (a gift from J. Morrow) with *Pst* I and cloned into pGEM-2. SAA_3-specific cRNA was transcribed with T7 RNA polymerase from 122 bp of 3′ noncoding and 18 bp of 3′ coding sequences after digestion of the plasmid with *Hae* III. (*B*) One transcription vector was constructed by subcloning the 3′ *Eco*RI/*Pst* I fragment of a partial SAA_1 cDNA into the plasmid, pGEM-1, designated p275. cRNA was synthesized by transcription with SP6 RNA polymerase in the presence of [^{32}P]UTP from 120 bp of 3′ untranslated and 110 bp of translated sequence from *Eco*RI-cleaved p275. The transcribed SAA_1 sequence has 90% homology with SAA_2 mRNA but only 50% homology with SAA_3 mRNA. Another transcription vector was constructed by subcloning the 5′ *Pst* I/*Eco*RI fragment of the partial SAA_1 cDNA into pGEM-1, designated p125. This region of the SAA_1 cDNA codes for amino acid residues 30–66 and is conserved among the three murine SAA sequences: 94% homology with SAA_2 and 85% with SAA_3. The cRNA was transcribed with T7 RNA polymerase from *Hin*dIII-cleaved p125.

transcribed from a 230-bp sequence of *Eco*RI-linearized p275 utilizing SP6 RNA polymerase. This sequence contains 120 bp of 3′ noncoding and 110 bp of 3′ coding sequence and hybridizes to mouse SAA_1 and SAA_2 but not to mouse SAA_3 mRNA. SAA_3-specific cRNA (Fig. 1*A*) was transcribed from a sequence consisting of a 122-bp 3′ untranslated and 18-bp 3′ translated sequence of SAA_3 cDNA after digestion of the plasmid with *Hae* III restriction enzyme. This 140-bp sequence is only 35% homologous to SAA_1 and does not hybridize to SAA_1 or SAA_2 mRNA. Hybridization specificity was confirmed by RNA blot analysis of several murine RNAs and blots of mouse genomic DNA (data not shown).

Hybrid Selection of mRNA and Cell-Free Translation. Poly(A)$^+$ RNA was prepared by chromatography with oligo(dT)-cellulose (20) from rat liver total RNA isolated following LPS or turpentine injection (described above).

SAA mRNA was hybrid selected essentially as described elsewhere (15). GeneScreen*Plus* (New England Nuclear) membranes were used instead of nitrocellulose. Twenty micrograms of $pSAA_1$ plasmid DNA (6, 16) in 25 μl of 0.1 M NaOH/2.0 M NaCl was boiled for 1 min and cooled, and 5 μl was applied to a 4 mm × 4 mm square of nylon membrane. After rinsing, baking, and washing, the filters each received 10 μl of RNA hybridization solution containing 6.5–9 μg of RNA. The filters were incubated at 45°C for 2 hr and washed, and the hybridized RNA was recovered and translated as described below.

Cell-free translation of RNA was carried out in a wheat germ translation system in the presence of [^{35}S]methionine (New England Nuclear) according to the manufacturer's directions (Amersham). Translation products were separated on polyacrylamide/SDS/urea gels (4), stained with Coomassie blue R-250 treated with EN^{3}HANCE (New England Nuclear), dried, and apposed to x-ray film (Kodak XAR-5) at −80°C. The molecular mass of the rat SAA translation product was estimated from the mobilities of known protein standards (Pharmacia) and pancreatic trypsin inhibitor.

RESULTS

RNA blots hybridized with mouse SAA_1 cDNA (Fig. 2) showed the following. Several patterns of expression of SAA mRNA were evident. LPS and turpentine induced high levels of SAA-related mRNA in liver (panel 1) not detected in the control. A 5- to 10-fold greater induction was seen after turpentine when compared with LPS. SAA-related mRNA of rat liver is smaller [0.4–0.5 kilobase (kb)] than mouse SAA mRNA (lane 5). In contrast to liver, lung (panel 2) expressed SAA mRNA after LPS injection but not after turpentine injection. The lung SAA mRNA was larger (≈0.6 kb) than rat liver SAA mRNA but is similar in size to mouse SAA mRNA (lane 5). Kidney and adrenal gland RNAs were examined and found to express SAA-related mRNA at very low levels after LPS injection and not after turpentine injection (data not shown). Control rats were found to express substantial amounts of SAA-related mRNA in ileum (panel 3) and large intestine (panel 4); treatment with turpentine (not shown) or LPS did not further elevate SAA mRNA.

Further detail of the homology of the several rat SAA RNAs with the several mouse genes was examined as follows. Mouse SAA_1 and SAA_2 mRNAs are 96% homologous with each other, whereas mouse SAA_3 mRNA is only 76% homologous to SAA_1 mRNA (13). One specific cRNA probe was designed to recognize SAA_1 and SAA_2 mRNA (Fig. 1*B*); another was designed to hybridize selectively with SAA_3 mRNA (Fig. 1*A*). Each probe was hybridized to blots of rat RNAs. The SAA_1/SAA_2-specific probe hybridized to turpentine- and LPS-induced liver SAA mRNAs (Fig. 3,

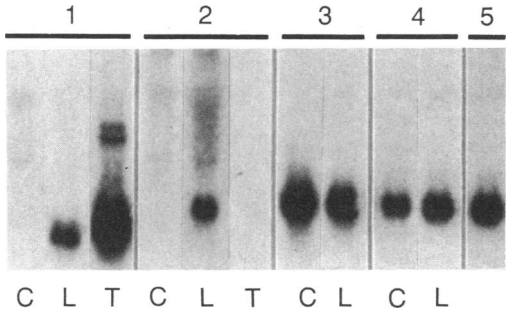

FIG. 2. Expression of SAA mRNA in rat tissues. Total RNA (10 μg) extracted from rat tissues was separated on denaturing agarose gels, transferred to nylon membranes, and hybridized with a mouse ^{32}P-labeled SAA_1 cDNA. RNA sources were as follows: control tissue (C) or 24 hr after injection with LPS (L) or turpentine (T). Mouse liver RNA (0.5 μg), extracted 18 hr after LPS injection, was used as a control (lane 5). Autoradiographs show hybridization of RNA from liver (panel 1), lung (panel 2), ileum (panel 3), and large intestine (panel 4). Exposure times of samples in panels 3 and 4 were four times longer than those in panels 1 and 2 and in lane 5.

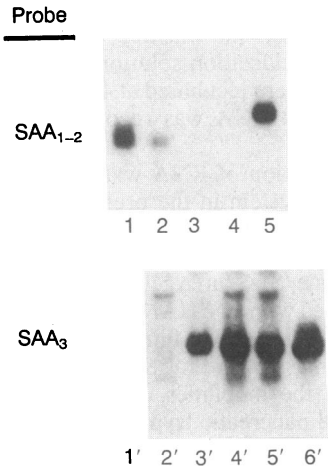

FIG. 3. Analysis of rat SAA mRNA for SAA_1/SAA_2-related or SAA_3-related sequences. RNAs (10 μg) were analyzed by RNA blot hybridization as in Fig. 2 with ^{32}P-labeled single-stranded cRNA probes. One is specific for mouse SAA_1 and SAA_2 mRNAs and the other is specific for mouse SAA_3 mRNA. Autoradiograph exposure of the lower panel was three times longer than that of the upper panel. RNA samples are as follows: rat liver RNA after turpentine injection (lanes 1 and 1') and LPS injection (lanes 2 and 2'), LPS ileum (lanes 3 and 4'), LPS lung (lanes 4 and 5'), 2 μg of mouse LPS liver RNA (lanes 5 and 6'), and LPS large intestine (lane 3').

lanes 1 and 2) but not ileum or lung (lanes 3 and 4) or large intestine (not shown). The SAA_3 cRNA hybridized to RNA from lung, ileum, and large intestine (Fig. 3, lanes 3', 4', and 5') but not to liver RNA (Fig. 3, lanes 1' and 2').

Separation of rat liver RNA through a 2% agarose gel (Fig. 4) revealed that turpentine injection induced accumulation of two SAA mRNAs (arrows) of ≈0.4 and ≈0.5 kb in length. However, LPS treatment induced only a single 0.4-kb species. All of the mouse SAA mRNAs are ≈0.6 kb long (8, 22) and code for preproteins of ≈14 kDa (6). To determine the size of protein(s) encoded by rat liver SAA mRNAs, rat liver RNAs from LPS- and turpentine-treated rats were hybrid selected with the $pSAA_1$ plasmid and translated in a cell-free translation system in the presence of [^{35}S]methionine. The translation products were separated on polyacrylamide/SDS/urea gels (Fig. 5). Material treated with pBR322 as a control is shown in lane 2. A protein of ≈8 kDa is translated

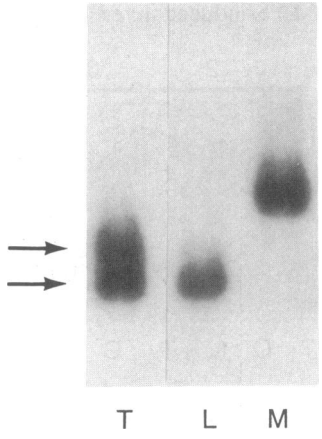

FIG. 4. Turpentine injection stimulates two sizes of liver SAA mRNA. RNA (10 μg) from rat liver after turpentine (T) and LPS (L) injection and mouse LPS liver RNA (M) (2 μg) were separated through denaturing 2% agarose, transferred to nylon membrane, and hybridized as in Fig. 1. The exposure of lane L was three times longer than that of the others. Arrows indicate rat ≈400- and ≈500-nucleotide SAA mRNAs.

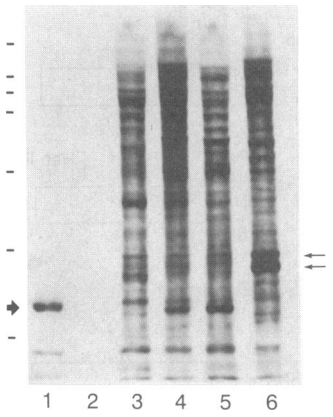

FIG. 5. Cell-free translation of hybrid-selected and total RNA from rat liver. Hybrid-selected (lanes 1 and 2) and total RNA (lanes 3–6) was translated in a wheat germ cell-free translation system in the presence of [^{35}S]methionine. Total translated products were separated on a polyacrylamide/urea/SDS gel and fluorographs were prepared. Lane 1, turpentine liver RNA hybrid selected with $pSAA_1$ cDNA; lane 2, RNA as in lane 1 except hybrid selected with pBR322; lanes 3, 4, and 5, total liver RNA from uninjected controls, LPS-injected rats, and turpentine-injected rats, respectively; lane 6, mouse LPS liver RNA (16). Molecular mass protein standards are indicated on the left margin (from top to bottom): phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean tryspin inhibitor (20 kDa), α-lactalbumin (14.4 kDa), and apoprotinin (6.5 kDa). Mouse $pre\text{-}SAA_1$ (upper arrow) and $pre\text{-}SAA_2$ (lower arrow) are indicated in the right margin. The rat liver SAA translation product is indicated in the left margin (bold arrow).

from rat liver SAA mRNA after either LPS (not shown) or turpentine (lane 1) injection. This is significantly smaller than the 14-kDa pre-SAA translated from mouse liver RNA (lane 6). In addition, only a single SAA protein is identified from the 0.4- and 0.5-kb SAA mRNAs induced by turpentine injection (lane 1). Translation of total RNA also shows that LPS (lane 4) and turpentine (lane 5) induce the expression of an 8-kDa protein that is not present in control liver RNA (lane 3).

We estimate that rat liver SAA mRNA (Fig. 3) is 0.2 kb shorter than mouse SAA mRNA, and the translated rat liver SAA (Fig. 5) is ≈6 kDa smaller than mouse pre-SAA. The question of what specifically, when compared to mouse, is missing in the rat SAA mRNA sequence was addressed as follows. Our SAA_1/SAA_2 cRNA, encompassing the COOH-terminal 38 amino acid residues (67–104) and a portion of 3' noncoding sequence of mouse SAA_1 had been found to hybridize to rat liver SAA mRNA (Fig. 3). Blots of rat RNAs were hybridized to a cRNA probe (Fig. 1*B*) encompassing an

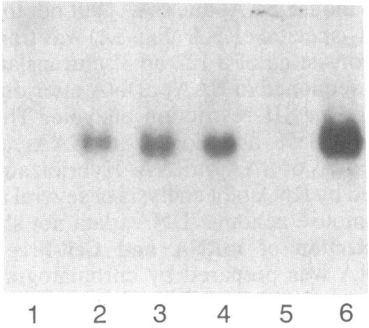

FIG. 6. Analysis of rat SAA mRNAs with a probe for a highly conserved coding sequence. Northern blots of RNA (10 μg) from rat turpentine liver (lane 1), LPS large intestine (lane 2), ileum (lane 3), lung (lane 4), and liver (lane 5), and mouse LPS liver (2 μg; lane 6) were hybridized with ^{32}P-labeled cRNA from p125.

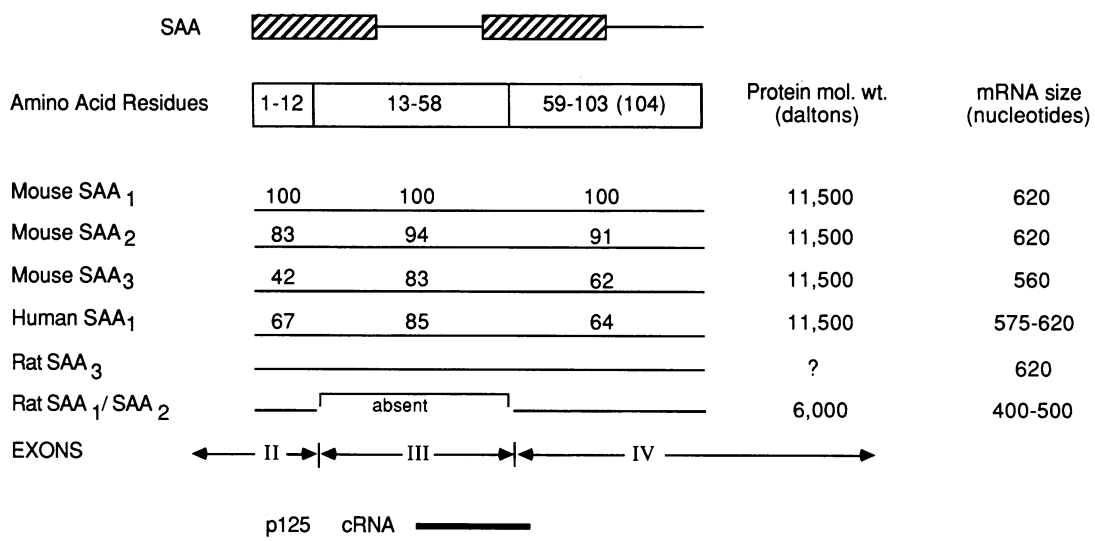

FIG. 7. Schematic illustration of SAA structure. Amphipathic α-helices are shown by striped boxes (▨) (28, 29). The numbers along horizontal lines are the % homology of amino acid residues encoded by mouse SAA_1 exon II, exon III, and exon IV (13, 22). Note the high degree of homology within exon III. The approximate molecular masses and mRNA lengths are indicated along the right margin (13, 17, 30). The cRNA probe (p125) for exon III is shown at the bottom. Rat SAA_1/SAA_2 length (structure) is estimated from the report of Liao *et al.** and data of this report. Rat SAA_3 is assumed by analogy to be equivalent in size to other SAAs since the mRNA is of similar length.

adjacent region of the mouse coding sequence from amino acid residue 30 to 66 (Fig. 6). Rat liver SAA mRNA did not hybridize with this probe. On the other hand, the 0.6-kb SAA-related RNA of lung (lane 4), large intestine (lane 2), and ileum (lane 3) did hybridize with the 30- to 66-residue probe. Furthermore, a SAA_3 cDNA, which contains the 5′ coding sequence of murine SAA_3 beginning at residue 8 (21), did not hybridize to liver SAA mRNA (data not shown). Taken altogether the data indicate that rat liver SAA mRNA lacks a portion of sequence coding for ≈50 residues of the NH_2-terminal half of the protein.

DISCUSSION

An important role for apolipoprotein-SAA (apo-SAA) in response to injury is suggested by (*i*) the highly elevated plasma SAA levels following acute injury, (*ii*) the persistent high levels in chronic inflammatory states, and (*iii*) the conserved amino acid sequence among a wide variety of mammals and White Peking ducks (8–12). There are three SAA genes in the mouse, and expression of individual members varies with different injurious agents and among different tissues (16). The three SAA mRNAs are expressed in liver at high levels (20- to 1000-fold greater than control) after LPS challenge (6, 16, 23, 24). Treatment with casein induces an equally vigorous expression of SAA_1 and SAA_2 mRNA, but SAA_3 mRNA is only slightly elevated in liver (16). SAA mRNAs are also expressed in extrahepatic tissues of mice after LPS injection. But only the SAA_3 gene is expressed in every organ examined thus far (16, 25). In contrast to LPS, injection of casein into mice does not elicit any extrahepatic expression of SAA mRNA (16).

Rats differ from mice in that no apo-SAA has been found to be associated with rat HDL isolated from acute-phase plasma (ref. 26; E.P.B. and N. Eriksen, unpublished). Moreover, rats do not develop AA amyloidosis either as a natural occurrence or under experimental conditions. As shown above, at least two SAA-related genes are expressed in rat tissues: one related to the mouse SAA_1 and SAA_2 and the other related to mouse SAA_3 mRNA. Expression of the former is induced by LPS and turpentine in liver but not in extrahepatic tissues, and expression of the latter is induced in some extrahepatic tissues by LPS but not by turpentine and is not expressed in liver.

Apo-SAA protein sizes from all species examined to date are about 11.5 kDa (103 or 104 amino acid residues). They are secreted proteins, and their primary translation product *in vitro* has an NH_2-terminal signal peptide of 18 or 19 residues and is ≈14 kDa in size in the mouse. The SAA-related preprotein translated from rat liver SAA mRNA is only 8 kDa (Fig. 5), 50 amino acid residues shorter than mouse SAA preproteins. All known SAAs contain a highly (95%) conserved region spanning amino acid residues 32–50. Yet, a specific cRNA probe, which encompasses the region for residues 30–66, does not hybridize to the rat liver SAA_1/SAA_2-related mRNA (Fig. 6). Rat SAA_3-related mRNA, however, does hybridize with this probe. These data indicate that rat liver SAA-related mRNA lacks a significant portion of this highly conserved sequence. In a recent brief report, some data on the structure of rat liver SAA-related mRNA were presented.* Several rat liver SAA cDNAs were sequenced and compared to the mouse SAA_1 gene. The rat cDNAs were all missing the nucleotides of the third exon, which codes for amino acid residues 12–58 (13). These data are consistent with our findings in rats.

Fig. 7 illustrates structural features of apo-SAA. Residues 1–24 and 50–74 are potential amphipathic helices (31, 32). Residues 25–49 connect the two helices and contain a β-hairpin turn (28). Apoliproteins are bound to lipid particles via their amphipathic helical domains (29). Since rat liver SAA appears to be missing a significant portion of both amphipathic helical domains as well as the highly conserved sequence between them, it may not associate with the HDL particle surface. However, it may have sufficient amphipathic structure to be able to associate with lipids intracellularly or at other sites.

The SAA_3-related mRNA is found only in extrahepatic tissues. Lung SAA_3 mRNA is significantly induced by injection of LPS but not by turpentine, even though turpentine is an effective inflammatory agent in rats (27) and induces a high level of SAA_1/SAA_2-like mRNA in liver (Fig. 2). The differential response of rat SAA_3-related mRNA to inflammatory stimuli resembles that found in mice, where SAA_3 mRNA is induced by LPS but not casein injection (16).

*Liao, W. S., Li, X. & Caldwell, C. K., Regulation of Liver Gene Expression, April 29–May 3, 1987, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, abstr. 182.

The findings presented here show that the rat expresses genes homologous to SAAs of other mammals. This fortifies the other available evidence indicating that this gene family is important in response to injury. The elevated expression of the SAA gene family as part of an acute-phase response suggests that they are part of a network of defensive molecules activated early after injury that may convey necessary lipids, including cholesterol, to needed locations. These proteins may aid also in removal of lipid debris derived from injured cells.

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