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Human Serum Amyloid A (SAA): Biosynthesis and Postsynthetic Processing of PreSAA and Structural Variants Defined by Complementary DNA†

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ABSTRACT: To study structural variants of human serum amyloid A (SAA), an apoprotein of high-density lipoprotein, complementary DNA clones were isolated from a human liver library with the use of two synthetic oligonucleotide mixtures containing sequences that could code for residues 33–38 and 90–95 of the protein sequence. The SAA-specific cDNA clone (pA1) contains the nucleotide sequence coding for the mature SAA and 10 amino acids of the 18-residue signal peptide. It also includes a 70 nucleotide long 3′-untranslated region and approximately 120 bases of the poly(A) tail. The derived amino acid sequence of pA1 is identical with the α form of apoSAA1. A fragment of pA1 containing the conserved (residues 33–38) region of SAA also hybridized with RNA from human acute phase liver and acute phase stimulated, but not unstimulated, mouse and rabbit liver. In contrast, a fragment corresponding to the variable region hybridized to a much greater extent with human than with rabbit or murine RNA. Human acute phase liver SAA mRNA ($\sim$600 nucleotides in length) directs synthesis of preSAA (M_r 14000) in a cell-free translating system. In a *Xenopus* oocyte translation system preSAA is synthesized and processed to the mature M_r 12000 product. The complete 18 amino acid signal peptide sequence of preSAA was derived from sequencing cDNA synthesized by "primer extension" from the region of SAA mRNA corresponding to the amino terminus of the mature product. Two other SAA-specific cDNA clones (pA6 and pA10) differed from pA1 in that they lack the internal *Pst*I restriction enzyme site spanning residues 54–56 of pA1. Thus, there are at least two SAA gene products transcribed during the acute phase response.

Serum amyloid A (SAA) is one of two major inducible human acute phase proteins. SAA is named for the insoluble β-pleated sheet fibril protein amyloid A (AA) that is probably derived from SAA by proteolysis at both its amino and carboxyl ends [as reviewed by Kushner et al. (1982), Skinner & Cohen (1983), and Kisilevsky (1983)]. AA varies in length from 45 to 83 residues and is found only in tissues. Originally the low molecular weight (M_r 12000) species of SAA obtained under denaturing conditions was called SAAL (Linke et al., 1975; Benditt et al., 1980) to distinguish it from the approximately 160000-dalton SAA complex detected under physiologic conditions. However, because of association of SAA with the HDL_3 subclass of serum lipoproteins (Benditt & Eriksen, 1977), the term apoSAA is now frequently employed (Benditt et al., 1982; Marhaug et al., 1982).

Two major isotypes of SAA have been recognized in human, rabbit, monkey, mink, and mouse serum; in human four additional minor variants have been described (Benditt & Er-

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iksen, 1977; Benditt et al., 1982; Marhaug et al., 1982; Tobias et al., 1982, Parks & Rudel, 1979; Anders et al., 1977; Hoffman & Benditt, 1982; Bausserman et al., 1980). The entire amino acid sequence of one of the major human SAA polymorphs, isolated from a pool of human serum and designated apoSAA1, has been determined and is similar to that determined for two patients with inflammatory disease (Parmelee et al., 1982; Sletten et al., 1983). The other major subclass of human SAA has not been completely sequenced but is homologous to SAA and AA through residue 20 (Bausserman et al., 1982).

Serum levels of SAA and C-reactive protein are elevated as much as 1000-fold during acute inflammation or tissue injury. Recently, investigation of the cellular and molecular mechanism of acute phase SAA induction has been used as an experimental model for regulation of acute phase reactant synthesis (Sipe et al., 1982). Within 90 min of endotoxin administration, a circulating macrophage-derived mediator, interleukin 1, appears prior to transcription of SAA mRNA and synthesis of SAA in liver (Morrow et al., 1981; Sipe, 1978). More detailed understanding of the molecular mechanisms controlling SAA synthesis will require studies of transcription, translation, and postsynthetic processing. It was the purpose of this study to isolate complementary DNA probes for human SAA in order to obtain additional information about the structure of human SAA isotypes and the biosynthesis and postsynthetic processing of SAA and to compare human SAA to the corresponding protein in other species.

Materials and Methods

Preparation of Synthetic Oligonucleotide Mixtures. Two synthetic oligonucleotide mixtures containing all 17-nucleotide DNA sequences that could code for the amino acid sequence of SAA between residues 33 and 38 and between residues 90 and 95 and subsequently a single sequence oligonucleotide coding for residues 1–8 were prepared by a solid-phase phosphotriester method using a library of dimer anions (Markham et al., 1980; Gait et al., 1980; Edge et al., 1981). Following the removal of protecting groups and cleavage of the product from the poly(dimethylacrylamide) beads, the 17 nucleotide long oligonucleotide mixtures were purified by high-performance liquid chromatography (HPLC) as described by Newton et al. (1983), and the single 24 nucleotide long oligonucleotide was purified by gel filtration on a Sephadex G-50 column and electrophoresis on a 20% polyacrylamide gel.

Identification of SAA cDNA Clones. The preparation of the human liver cDNA library, which contains 230 000 recombinant clones, has been described (Woods et al., 1982). A modification of the Grunstein & Hogness (1975) procedure was used to screen recombinant colonies on 82-mm nitrocellulose filters with the synthetic oligonucleotide mixtures. The oligonucleotide mixtures were 5′ labeled by using adenosine [γ-^{32}P]triphosphate (New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Bethesda Research Laboratories, Rockville, MD) (Maxam & Gilbert, 1977) and hybridized for 16 h at 37 °C to filter-bound DNA at a concentration of 0.25 μg/20 mL in 0.9 M NaCl/90 mM sodium citrate/1 × Denhardt's solution (Denhardt, 1966)/0.05% sodium pyrophosphate/100 μg/mL tRNA. Hybridized filters were washed extensively with 0.9 M NaCl/90 mM sodium citrate/0.05% sodium pyrophosphate at 20 °C and once in the same buffer for 10 min at 42 °C. Hybridization signals were visualized by autoradiography.

Isolation and Sequence Analysis of SAA cDNA. Plasmid DNA was isolated from bacteria by the cleared lysate method of Clewell & Helinski (1969). The SAA-specific cDNA was excised from the plasmid by *Pst*I digestion and purified by agarose and polyacrylamide electrophoresis (Maxam & Gilbert, 1977). DNA sequence analysis was carried out as described by Maxam & Gilbert (1977) after 3′ end labeling with [α-^{32}P]cordycepinyl-ATP (New England Nuclear) and terminal deoxynucleotidyltransferase (New England Biolabs) and by the chain termination procedure of Sanger et al. (1977) after insertion of pA1 into the M13 cloning vector.

Induction of Acute Phase Response. Fresh human liver was obtained from a cadavar donor as previously reported (Woods et al., 1982). The donor had sustained severe injury 48 h prior to death and was undergoing an acute phase response as judged by high levels of serum CRP.

Acute phase reactions were induced in 6-week-old female CBA/J mice by subcutaneous injection of silver nitrate (Sipe et al., 1982) and in rabbits by intramuscular injection of turpentine oil (Kushner & Feldman, 1978).

Messenger RNA Isolation. Total cytoplasmic RNA was isolated from human acute phase liver and from acute phase and control mouse and rabbit livers as described by Woods et al. (1982). Poly(A)+ mRNA was purified by affinity chromatography on oligo(dT)–cellulose (Aviv & Leder, 1972).

Northern Blot Analysis. RNA from human, mouse, or rabbit liver was fractionated by electrophoresis on 1% agarose gels and transferred to nitrocellulose filters (Goldberg, 1980; Lehrach et al., 1977).

Hybridization (Wahl et al., 1979) with single-stranded SAA-specific cDNA pA1 was carried out at 42 °C for 18 h followed by washing in 1 × SSC and 0.1% SDS for 1 h at 55 °C and visualization by autoradiography.

Translation of Messenger RNA. RNA was translated in the presence of [^{35}S]methionine both in a rabbit reticulocyte lysate cell free system (Pelham & Jackson, 1976) and by injection into *Xenopus* oocytes as described by Tucci et al. (1983). The oocytes were washed with phosphate-buffered saline (PBS) and homogenized in 200 μL of PBS containing 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 10 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM phenylmethanesulfonyl fluoride, pH 7.6 (lysis buffer). The intracellular oocyte fraction was obtained by centrifugation at 12000*g* for 5 min followed by addition of an equal volume of 1% sodium dodecyl sulfate (SDS) in PBS.

Antiserum to Human AA and Rabbit SAA. New Zealand white rabbits were injected with amyloid A (AA) protein (Skinner & Cohen, 1971) in complete Freund's adjuvant via foot pad and intradermal routes as described by Linke et al. (1975). The animals were boosted intradermally in 2- or 3-week intervals and bled 1 week after the last boost. The resulting antiserum did not react with normal serum but formed a precipitin line with acute phase serum containing SAA. Guinea pig antiserum to rabbit SAA was generously provided by Dr. Peter S. Tobias, Research Institute of Scripps Clinic, La Jolla, CA.

Immunoprecipitation and Analysis of Messenger RNA Translation Products. Immunoprecipitation was carried out by adding 3 μL of antiserum to each sample and incubating the mixture at 0 °C overnight. Fifty microliters of a washed 10% IgG Sorb suspension (Enzyme Center, Cambridge, MA) was added, and the immunoprecipitates were centrifuged and washed as described by Tucci et al. (1983). Samples were suspended in Laemmli (1970) sample buffer, boiled for 2 min, and centrifuged at 12000*g* for 2 min. Supernatants were removed and analyzed by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were stained with Coomassie

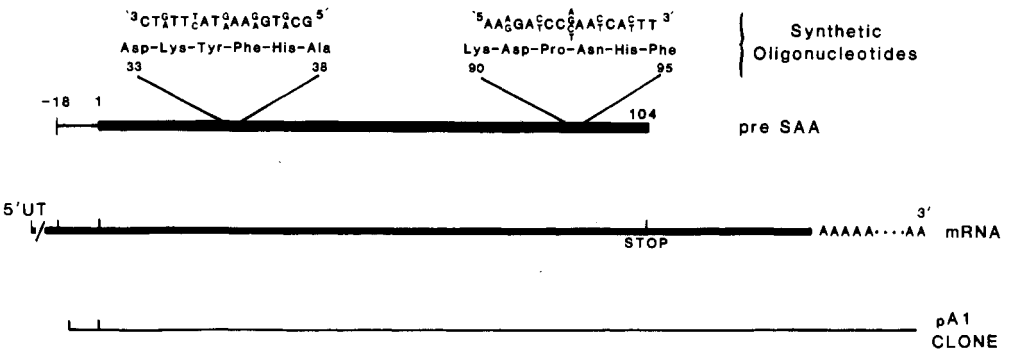

FIGURE 1: Relation between clone pA1, preSAA, its mRNA, and the synthetic oligonucleotides. The 5′-untranslated region is designated UT. Slash signifies that this region is of unknown length. Note, the N-terminal oligonucleotide was constructed to give anticodon sequence based on amino acids 33–38; the C-terminal oligonucleotide was constructed to give codon sequence based on amino acids 90–95.

A

```
-10                                   1                                  10
TTC TGC TCC TTG GTC CTG GGT GTC AGC AGC CGA AGC TTC TTT TCG TTC CTT GGC GAG GCT
 F   C   S   L   V   L   G   V   S   S   R   S   F   F   S   F   L   G   E   A

                                                                          30
TTT GAT GGG GCT CGG GAC ATG TGG AGA GCC TAC TCT GAC ATG AGA GAA GCC AAT TAC ATC
 F   D   G   A   R   D   M   W   R   A   Y   S   D   M   R   E   A   N   Y   I

                                                                          50
GGC TCA GAC AAA TAC TTC CAT GCT CGG GGG AAC TAT GAT GCT GCC AAA AGG GGA CCT GGG
 G   S   D   K   Y   F   H   A   R   G   N   Y   D   A   A   K   R   G   P   G

                                                                          70
GGT GTC TGG GCT GCA GAA GCG ATC AGC GAT GCC AGA GAG AAT ATC CAG AGA TTC TTT GGC
 G   V*  W   A   A   E   A*  I   S   D   A   R   L   N   I   Q   R   F   F   G

                                                                          90
CAT GGT GCG GAG GAC TCG CTG GCT GAT CAG GCT GCC AAT GAA TGG GGC AGG AGT GGC AAA
 H   G   A   E   D   S   L   A   D   Q   A   A   N   E   W   G   R   S   G   K

                                                  104
GAC CCC AAT CAC TTC CGA CCT GCT GGC CTG TCT GAG AAA TAC TGA . . .
 D   P   N   H   F   R   P   A   G   L   S   E   K   Y  STOP
```

B

```
9                                                                   27
 E   A   F   D   G   A   R   D   M   W   R   A   Y   S   D   M   R   E   A    Derived human SAA
GAG GCT TTT GAT GGG GCT CGG GAC ATG TGG AGA GCC TAC TCT GAC ATG AGA GAA GCC    pA1
  A     GG  C A     T   A A             C                       AG A     T    pRS48
        G   Q       S                                           K   K         Derived mouse SAA

28                                                                  46
 N   Y   I   G   S   D   K   Y   F   H   A   R   G   N   Y   D   A   A   K    Derived human SAA
AAT TAC ATC GGC TCA GAC AAA TAC TTC CAT GCT CGG GGG AAC TAT GAT GCT GCC AAA    pA1
  C   GG  AA AA                                                   CGG   pRS48
      W   K   N                                                   R     Derived mouse SAA
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FIGURE 2: (A) Nucleotide and derived amino acid sequence of pA1. Published amino acid sequence of human apoSAA1 (Parmelee et al., 1982) specifies at positions 52 and 57 valine and alanine, respectively, for the α form and alanine and valine, respectively, for the β form. (B) Comparison of mouse and human SAA cDNA nucleotide and derived amino acid sequences. Nucleotide and derived amino acid differences are shown for the mouse. Gaps signify identity. Nucleotide and derived mouse SAA sequence from Stearman et al. (1982).

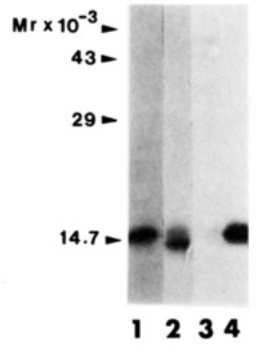

FIGURE 3: Translation and postsynthetic processing of an inducible SAA mRNA. Lane 1, immunoprecipitated SAA synthesized in a reticulocyte cell-free system programmed with acute phase human liver mRNA; lane 2, intracellular ^{35}S-labeled SAA immunoprecipitated after injection of oocytes with poly(A)+ acute phase human liver mRNA; lanes 3 and 4, immunoprecipitation with anti-SAA antiserum of rabbit reticulocyte lysate cell-free systems programmed with mRNA from unstimulated (lane 3) and stimulated (lane 4) rabbits.

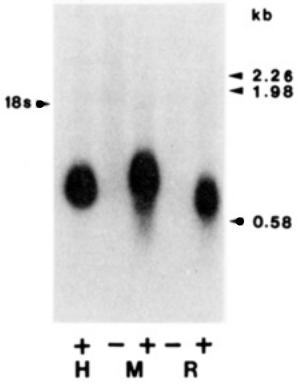

FIGURE 4: Northern blot analysis of RNA from acute phase human (H) and from unstimulated (−) and stimulated (+) mouse (M) and rabbit (R) liver hybridized with the 5′ and the 3′ *Pst*I fragments of pA1 (see also legend to Figure 6). Ten micrograms of cytoplasmic RNA was size fractionated by electrophoresis on agarose gels and transferred to nitrocellulose filters. Filters were hybridized with radiolabeled SAA-specific single-stranded cDNA.

blue, soaked in EN^{3}Hance (New England Nuclear, Boston, MA), dried, and exposed to medical X-ray film (Kodak XAR-5) at −80 °C.

RESULTS

Identification of SAA-Specific cDNA Clones and Sequence Analysis of pA1. The synthetic oligonucleotide mixtures used to screen 25 000 clones of the human cDNA acute phase liver library and the amino acid sequences from which the oligonucleotides were derived are shown in Figure 1.

By use of the 33–38-oligonucleotide mixture as probe, approximately 300 clones were identified. Eight clones were colony purified and rescreened with the 90–95-oligonucleotide mixture. The largest clone detected with both oligonucleotides (pA1) was approximately 550 base pairs in length and was selected for further study. The nucleotide sequence of pA1 was determined, and its derived amino acid sequence (Figure 2a) corresponds to the α species of apoSAA1 (Parmelee et al., 1982; Sletten et al., 1983) in that a valine at position 52 and alanine at 57 is specified (Parmelee et al., 1982). The pA1 clone also has a 3′-untranslated region of approximately 70 nucleotides and a poly(A)+ tail of approximately 120 bases (Figure 1). At the 5′ end are 30 nucleotides corresponding to 10 amino acid residues of the leader sequence. Of an additional six SAA-specific clones analyzed, two were found to be approximately 600 bases and to lack the internal *Pst*I site contained in pA1.

Biosynthesis and Postsynthetic Processing of SAA. Translation of human acute phase liver mRNA in a cell-free system resulted in synthesis of a 14 000-dalton protein that precipitated with antiserum to SAA. Translation of an aliquot of the same mRNA in *Xenopus* oocytes resulted in synthesis of SAA with M_r 12 000, the size of apoSAA isolated from plasma (see Figure 3). These results are consistent with the presence of a leader sequence of between 15 and 20 amino acids. Liver mRNA from stimulated (turpentine injection) but not unstimulated rabbits directed synthesis of an anti-AA precipitable protein of molecular mass similar to that produced by human mRNA under cell-free conditions. Northern blot analysis of the human, mouse, and rabbit mRNA probed with the SAA cDNA clone is shown in Figure 4. These data suggest that murine SAA mRNA is slightly larger than either human or rabbit SAA mRNA.

To obtain the complete sequence of the signal peptide, a single 24 nucleotide long oligonucleotide ($^{3'}$GCT TCG AAG AAA AGC AAG GAA CCG$^{5'}$) corresponding to the nucleotides that encode the first 8 amino acid residues of mature SAA was synthesized. This was radiolabeled at the 5′ end, hybridized with poly(A)+ human liver RNA, and used as a priming site for cDNA synthesis with AMV reverse transcriptase with SAA mRNA as template. The cDNA was purified by polyacrylamide gel electrophoresis and sequenced by the method of Maxam & Gilbert (1977). The sequence corresponds to the 5′ end of pA1, and an additional 36 nucleotides extending through and beyond the region of the mRNA specifying the signal peptide were determined. The derived amino acid sequence of the signal peptide consists of 18 residues beginning with an N-terminal methionine and includes 12 hydrophobic residues (Figure 5). This sequence is similar in composition to the signal peptides determined for other secreted products (Von Heijne, 1982).

Cross-Hybridization of pA1 with Mouse and Rabbit mRNA. The amino acid sequences of both SAA and AA include a region from residues 33 to 45 that is conserved in human (Parmelee et al., 1982; Sletten et al., 1983), mouse (Hoffman et al., 1984a,b; Gorevic et al., 1978), monkey (Hermodson et al., 1972), guinea pig (Skinner et al., 1974), duck (Gorevic et al., 1977), and mink (Waalen et al., 1980). In the cDNA clone pA1, the nucleotide sequence coding for this region is separated from that coding for the more variable C-terminal half of SAA by a *Pst*I restriction site spanning residues 54–56 (−AAE−) (Figure 2A). *Pst*I-digested pA1 was 3′ end labeled, and the strands of each insert fragment were separated by polyacrylamide gel electrophoresis (Maxam & Gilbert, 1977). The strands complementary to the SAA mRNA (determined by nucleotide sequencing) were isolated

```
-18 -17 -16 -15 -14 -13 -12 -11 -10  -9  -8  -7  -6  -5  -4  -3  -2  -1   1   2   3   4   5   6   7   8
ATG AAG CTT CTC ACG GGC CTG GTT TTC TGC TCC TTG GTC CTG GGT GTC AGC AGC CGA AGC TTC TTT TCG TTC CTT GGC
 M   K   L   L   T   G   L   V   F   C   S   L   V   L   G   V   S   S   R   S   F   F   S   F   L   G
```

FIGURE 5: Derived amino acid sequence of signal peptide of human SAA. The leader sequence of human SAA primary translation product was derived from sequencing cDNA synthesized by "primer extension" from the region of SAA mRNA corresponding to residues 1–8 of the mature product and was derived in part from the nucleotide sequence of cDNA clone pA1 (Figure 2).

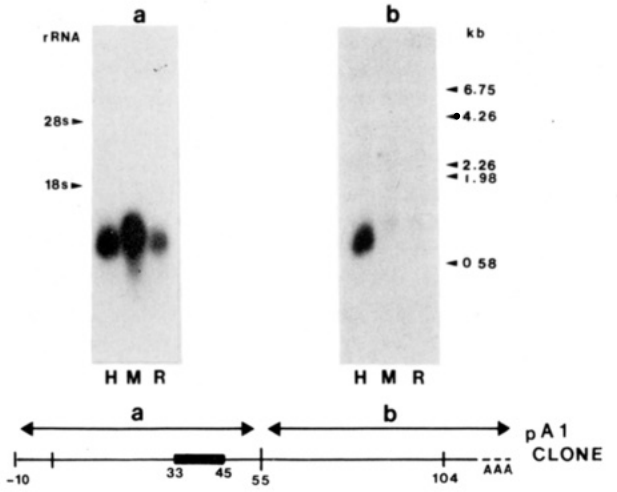

FIGURE 6: Northern blot analysis of human (H), mouse (M), and rabbit (R) acute phase mRNA with invariant (a) and variant (b) fragments of SAA-specific cDNA, pA1. Filter blots of 10 μg of cytoplasmic RNA fractionated on agarose gels were hybridized with the single-stranded cDNA specific for SAA mRNA (a) from residues −10 to 55 and (b) from 55 to 104.

and used as probes in RNA blotting experiments with RNA from acute phase human, rabbit, and mouse liver (Figure 6). The variable (3′-end) probe hybridized strongly to human RNA but in only trace amounts to stimulated rabbit and mouse RNA (Figure 6, right) whereas the conserved (5′-end) probe hybridized strongly to the acute phase RNA from all three species (Figure 6, left). Neither the variable nor the conserved probe hybridized to liver RNA from unstimulated rabbits and mice (Figure 4). Mouse SAA mRNA appears slightly larger than the approximately 600-nucleotide human and rabbit SAA mRNAs; this is in agreement with the 650-nucleotide length reported by Morrow et al. (1981) for SAA mRNA from mice treated with lipopolysaccharide.

Discussion

Two major (apoSAA1 and apoSAA2) and four minor isotypes or polymorphs have been described for human SAA (Benditt et al., 1982; Bausserman et al., 1980). There are two forms of apoSAA1, an α which has valine at position 52 and alanine at position 57 and a β which has alanine at 52 and valine at 57. Clone pA1 therefore corresponds to the α form of SAA1.

A recombinant plasmid, pRS48, homologous to mouse SAA mRNA (Morrow et al., 1981; Stearman et al., 1982) starts at residue 8 of mature mouse SAA. Although pRS48 contains a nucleotide sequence specifying the 13 amino acid constant region common to all known AA and SAA sequences and to pA1, it exhibits only 71% homology to a previously published partial amino acid sequence of mouse SAA (Gorevic et al., 1977). The human clone, pA1, is completely homologous to the sequence of human α apoSAA1.

When the mouse pRS48 and human pA1 sequences are aligned, they differ by 23 of 114 nucleotides. These differences are limited to 14 codons, and nine instances result in amino acid substitutions. Thus, between residues 8 and 45 of human and mouse SAA, there is 75% identity (Figure 2B).

Immunoprecipitation of the cell-free translation products of human acute phase liver mRNA reveals a SAA species of M_r 14000. In a *Xenopus* oocyte translation system using the same mRNA preparation, this species is synthesized and processed to a 12000-dalton product that comigrates with mature SAA in SDS–polyacrylamide gels, indicating the presence of a leader sequence of approximately 2000 daltons that is cleaved prior to or coincident with export from the cell. Nucleotide sequencing of the 5′ end of clone pA1 and of cDNA synthesized by primer extension of SAA mRNA identified a hydrophobic 18-residue leader sequence beginning with an N-terminal methionine. This sequence is analogous to that described for other secreted proteins (Von Heijne, 1982).

While the size of human, mouse, and rabbit preSAA is similar, RNA blot analysis suggests that mouse SAA mRNA is slightly longer than rabbit and human SAA mRNAs (600 nucleotides) in agreement with the earlier report that mouse SAA mRNA is 650 nucleotides long (Morrow et al., 1981). These data also demonstrate that induction of SAA synthesis in rabbit as well as mouse following an acute phase stimulus is the result of an increase in specific SAA mRNA, i.e., is due to a pretranslational induction mechanism.

The derived amino acid sequence between residues 33 and 45 of the SAA-specific cDNA prepared in this study corresponds exactly to that of the invariant region of SAA and AA now recognized to be common to six species. Therefore, it may be used to study the kinetics of transcription of messenger RNA during induction and deinduction of SAA synthesis and possible extrahepatic sites of SAA synthesis and to permit a detailed description of the structure, chromosomal localization, and number of SAA genes in these species. The human SAA cDNA will be used to explore the pathogenesis of AA amyloidosis in experimental models and will permit genetic analysis to determine the role of structural variants in the pathogenesis of secondary amyloidosis.

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