Expression and Sequence Analyses of Serum Amyloid A in the Syrian Hamster†

C. F. Webb,‡ P. W. Tucker,‡ and S. B. Dowton*,§

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235, and Edward Mallinckrodt Department of Pediatrics and James S. McDonnell Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110

Received October 6, 1988; Revised Manuscript Received January 26, 1989

ABSTRACT: Reactive amyloidosis occurs during chronic inflammation and involves deposition of amyloid A (AA) fibrils in many organs. Amyloid A is derived by proteolysis from serum amyloid A component (SAA), a major acute-phase reactant in many species. Since spontaneous amyloidosis occurs commonly in Syrian hamsters, we have studied the structure and expression of SAA genes during inflammation in these animals. Two cDNA clones and one genomic clone were sequenced, suggesting that Syrian hamster SAA is encoded by at least two genes. Hepatic mRNA analyses showed that SAA was inducible in many hamster organs during acute inflammation. These studies also demonstrated that SAA mRNA for one isotype is maximally expressed at a site of local tissue damage.

During inflammation, alterations occur in the concentration of several plasma proteins—the acute-phase reactants. The concentration of one such protein, serum amyloid A (SAA), may increase several hundredfold to a thousandfold within 12 h following an inflammatory stimulus (Kushner et al., 1982). Human SAA, which circulates in the plasma complexed to a high-density lipoprotein subfraction, was originally identified by its cross-reaction with antisera raised to the amyloid A (AA) protein purified from deposits of secondary amyloidosis (Benditt & Eriksen, 1977). In amyloid deposits, AA protein occurs as a β-pleated sheet fibrillar protein and is derived from SAA by proteolysis of a 28 amino acid peptide from the carboxyl terminus of the molecule (Husebekk et al., 1985; Husby & Sletten, 1986).

Serum amyloid A proteins have been isolated and characterized in many species including duck, mink, rabbit, man, and mouse. Available structural and amino acid sequence data among the variety of animals show that SAA has been phylogenetically conserved (Anders et al., 1977). Protein analyses have demonstrated several isotypic forms of human SAA (Bausserman et al., 1980), and recent studies show that the murine genome contains three SAA genes and one pseudogene (Lowell et al., 1986a). Circulating polypeptides have been identified for two of these three murine SAA genes, one of which appears to be the sole precursor of the AA fibril protein in the mouse (Meek et al., 1986; Shiroo et al., 1987). Although SAA has been shown to enhance the clearance of high-density lipoprotein particles (Hoffman & Benditt, 1982), other functions of SAA remain unknown.

While synthesis of SAA occurs predominantly in the liver, recent studies have demonstrated that murine SAA mRNA may be detected at several extrahepatic sites, including lung, heart, spleen, intestines, and kidney (Ramidori et al., 1985; Meek & Benditt, 1986). The relevance of extrahepatic production of SAA mRNA and the mechanisms of tissue-specific regulation of expression of the SAA genes during acute inflammation are not known. Local control of production of this protein may provide a more finely controlled balance and kinetically more rapid local response than is provided by the intravascular SAA supply. Similarly, the relative involvement of systemically circulating and extrahepatically synthesized SAA in amyloidogenesis remains to be defined.

Experimental amyloidosis has been described in several species. The most extensive data concerning amyloidogenesis have been derived from experiments in mice. Murine models of systemic amyloidosis are characterized by strain specificity of their susceptibility to amyloid deposition and necessity for casein or lipopolysaccharide administration. A high incidence of spontaneously occurring AA amyloidosis has been described in the Syrian hamster, providing an excellent model for studying molecular mechanisms involved in amyloidogenesis (Schmidt et al., 1983). Accordingly, we have studied the structure and expression of SAA genes in the Syrian hamster.

Materials and Methods

Animals and Reagents. Randomly outbred female golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Wilmington, MA). Molecular biologic enzymes were purchased from Bethesda Research Laboratories or New England Biolabs. Bacterial alkaline phosphatase and proteinase K were obtained from Boehringer Mannheim. Radiolabeled nucleotides were purchased from New England Nuclear or ICN.

Isolation of SAA cDNA Clones. SAA cDNA clones were isolated from a hamster hepatic cDNA library (Dowton et al., 1985). Thirty thousand bacterial colonies containing recombinant plasmids were screened by colony hybridization using a modification of the method of Grunstein and Hogness (1975) with a human SAA cDNA insert, pA1 (Sipe et al., 1985). Hybridizations were carried out at 65 °C, and washes were performed under conditions of high stringency as previously described (Woods et al., 1982). Hybridization signals were visualized by autoradiography using Kodak XAR-5 film. The insert sizes of positive clones were studied by agarose gel electrophoresis following excision of SAA-specific inserts from plasmid using *Pst*I.

Isolation of Genomic Clones for Hamster SAA. A Syrian hamster genomic library, constructed in the bacteriophage Charon 4A (McGuire et al., 1985), was screened with a

† This work was supported by USPHS Grants AI20959 and AI 24835. S.B.D. was supported by an Amy Clare Potter fellowship.

* Address correspondence to this author at the Division of Medical Genetics, Department of Pediatrics, Washington University School of Medicine, 400 S. Kingshighway, St. Louis, MO 63110.

‡ University of Texas Southwestern Medical Center.

§ Washington University School of Medicine.

0006-2960/89/0428-4785$01.50/0

hamster SAA-specific cDNA clone ($phSAA_2$). The identity of the cDNA clone used to screen the genomic library has been confirmed by nucleotide sequence analysis. Approximately 10^6 recombinant plaques were screened in duplicate on nitrocellulose filters (Maniatis et al., 1982). Positive clones were initially studied by restriction mapping and DNA blot hybridization studies using the cDNA clone $phSAA_2$ as a hybridization probe. DNA blots were performed as described below. One genomic clone ($ghSAA_3$) was selected for detailed structural analysis.

Analysis of SAA Gene Structure. (A) Southern Blot Hybridization of Genomic Hamster DNA with SAA-Specific cDNA Probe. High molecular weight genomic DNA was isolated from a hamster liver which had been pulverized in liquid nitrogen (Blin & Stafford, 1976). After restriction endonuclease digestion, 10-μg aliquots of genomic DNA were subjected to agarose gel electrophoresis and Southern blotting (Southern, 1975). The blot was hybridized with an SAA-specific cDNA probe ($phSAA_2$) which had been radiolabeled by nick translation.

(B) Nucleotide Sequence Analyses. The SAA-specific cDNA inserts of clones isolated from the cDNA library were subcloned into M13mp11 or $pGEM_3$. SAA-specific synthetic oligonucleotides were used as primers for sequence reactions. Sequence analyses were performed by techniques described by Maxam and Gilbert (1977) and Sanger et al. (1977). A 3.8-kb *Eco*RI–*Hin*dIII fragment of the genomic clone ($ghSAA_3$), which was demonstrated to hybridize with nick-translated $phSAA_1$, was subcloned into pUC18. This fragment was digested with *Sau*3AI and shotgun-cloned into M13mp18. All sequence data were confirmed by sequencing both strands or using the alternative method.

Oligonucleotide Synthesis. Primers for sequencing were generated on an LKB oligonucleotide synthesizer. The oligonucleotide for RNA blot analysis was synthesized by using an Applied Biosystems 360B synthesizer.

RNA Analysis. For studies of extrahepatic production of SAA mRNA and kinetics of SAA induction during inflammation, RNA was isolated from hamster organs (liver, lungs, muscle, lungs, muscle, ovary, spleen, testis, adrenal, uterus, brain, urinary bladder, diaphragm, esophagus, heart, intestine, and kidney) by guanidinium isothiocyanate extraction and ultracentrifugation through 5.7 M cesium chloride (Woods et al., 1982). For the kinetic experiment, RNA was isolated from hamster livers at varying time points following induction of a sterile abscess by subcutaneous turpentine injection. RNA isolated from hamsters without administration of turpentine (unstimulated) served as a base-line control. Dot blots were prepared by depositing 5 μg of RNA from each point onto a nylon membrane and hybridization with nick-translated $phSAA_1$ using the conditions outlined above. Nonspecific hybridization of $phSAA_1$ with pBR322 DNA was not observed when pBR322 DNA was immobilized on the nylon membrane and hybridized under similar conditions (data not shown).

For studies of differential tissue expression of SAA genes, RNA (liver, lungs, spleen, kidney, and muscle at the abscess site) from two experimental groups of hamsters was isolated—a control (unstimulated) group and hamsters 24 h following induction of a sterile abscess by subcutaneous turpentine injection. RNA from individual hamsters was fractionated by agarose–formaldehyde gel electrophoresis and transferred to nylon membranes. Uniformity of sample application to the gel was checked by ultraviolet visualization of the ethidium bromide stained gel. The RNA blot was hybridized with a radiolabeled synthetic 21-mer oligonucleotide for SAA_3 [residues 1494–1514 (Figure 3)] at 42 °C for 48 h in 10× Denhardt's solution, 0.05 M Tris (pH 7.5), 1.0 M sodium chloride, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate, and 0.1 mg/mL tRNA. Following hybridization, the RNA blot was washed in 0.9 M sodium chloride/60 mM sodium citrate for 10 min and exposed to Kodak XAR-5 film with an intensifying screen. The blot was then stripped by boiling and rehybridized with a probe ($phSAA_1$) representing a different SAA mRNA. This probe was radiolabeled by nick translation. Following prehybridization, hybridization was performed at 42 °C for 18 h in 50% formamide, 5× Denhardt's solution, 0.05 M sodium phosphate (pH 6.5), 0.75 M sodium chloride, 5 mM EDTA, 250 μg/mL denatured salmon sperm DNA, and 500 μg/mL tRNA. The blot was washed in 15 mM sodium chloride/1.5 mM sodium citrate/0.1% sodium dodecyl sulfate for 45 min at 65 °C and exposed to Kodak XAR-5 film.

Results

Identification of Hamster SAA-Specific cDNA Clones. Following hybridization of the human pA1 clone with membrane-bound hamster hepatic cDNA library, nine SAA clones were identified and colony purified. Insert sizes ranged from 450 base pairs to 612 base pairs. The clones, $phSAA_1$ and $phSAA_2$, containing the two longest SAA inserts, 612 base pairs and 544 base pairs, respectively, were selected for further analysis.

Identification of Hamster SAA Genomic Clones. Filter hybridization screening of the hamster genomic DNA library yielded 11 positive clones, which were divided into 3 groups on the basis of restriction mapping (Figure 1). DNA blot analysis of these clones demonstrated that the cDNA probe ($phSAA_1$) hybridized with a single 8.3-kb *Eco*RI fragment in one set of genomic SAA clones. The size of this fragment corresponded directly with an *Eco*RI fragment detected when the same probe ($phSAA_1$) was used in hybridization studies of genomic hamster DNA (Figure 2). This clone, $ghSAA_3$, was selected for sequence analysis.

Analysis of SAA Gene Structure. Hybridization of the probe $phSAA_2$ with genomic hanster DNA reveals multiple restriction fragments with each enzyme (Figure 2). These data imply the existence of at least two SAA genes in the hamster. The exon structure and sequencing strategy for the SAA genomic clone ($ghSAA_3$) are depicted in Figure 1. The nucleotide sequences for $phSAA_1$, $phSAA_2$, and $ghSAA_3$ are shown in Figure 3 accompanied by the derived amino acid sequences for the relevant predicted SAA isotypes. Both cDNA clones included sequences for all coding regions of SAA polypeptides including a 19 amino acid signal peptide as well as polyadenylation signals. Clone $phSAA_1$ included 108 nucleotides of 5′ untranslated region and does contain a poly(A) tail. Clone $phSAA_2$ only had nine nucleotides of 5′ untranslated region. Coding sequences were identified in three exons of the clone $ghSAA_3$.

The three hamster sequences exhibited a high degree of homology to other known SAA sequences. Comparison of the predicted amino acid sequences from the hamster clones with SAA sequences from the mouse and human (Table I) showed that the hamster genomic clone ($ghSAA_3$) was most highly homologous to mouse SAA_3 (Lowell et al., 1986a). Consequently, this clone was designated $ghSAA_3$, and the two cDNA clones have been named $phSAA_1$ and $phSAA_2$ according to their homology with the murine sequences. All three hamster sequences were found to be more highly related to the mouse than human sequences, although pA1 exhibited the highest degree of identity with $ghSAA_3$ and pSAA82 was most ho-

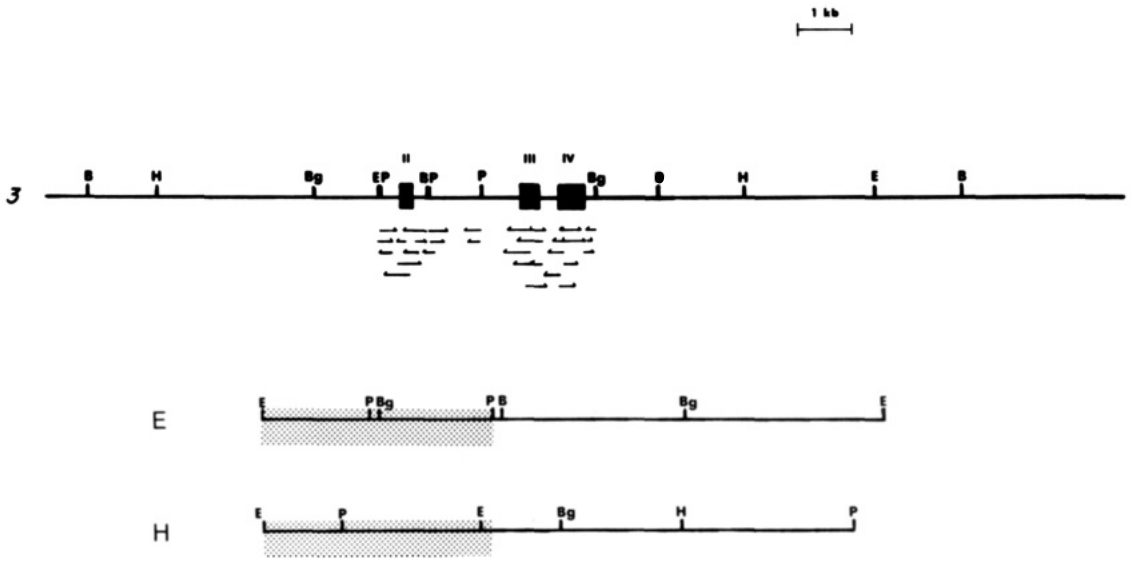

FIGURE 1: Restriction maps of genomic hamster SAA clones $ghSAA_3$, ghSAA-E, and ghSAA-H (B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I). Solid boxes indicate exons II, III, and IV of $ghSAA_3$, while shaded regions denote fragments which hybridized with $phSAA_2$. Arrows show sequencing strategy.

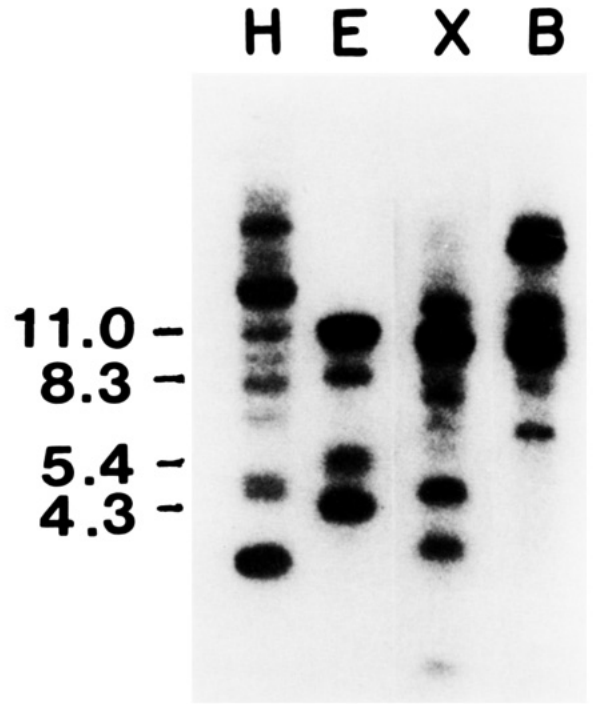

FIGURE 2: Autoradiogram of Southern blot of hamster hepatic DNA digested with four restriction enzymes (H, *Hin*dIII; E, *Eco*RI; X, *Xba*I; B, *Bam*HI) and hybridized with hamster SAA cDNA probe ($phSAA_2$).

mologous to $phSAA_1$, and $phSAA_2$ (Sipe et al., 1985; Kluve-Beckerman et al., 1986).

Identification and Expression of SAA-Specific mRNA. Hybridization of RNA isolated from many hamster organs with $phSAA_1$ revealed the presence of a 1.1-kb SAA-specific mRNA which is inducible following induction of an acute inflammatory response (Figure 4A). A less abundant, higher molecular mass mRNA species, which was also inducible, was noted. Hybridization of the dot blot of RNA samples isolated at varying time points after the onset of inflammation revealed that SAA mRNA concentration rises quickly and remains elevated from 12 h after stimulation to 48-h postinjury after which a return toward resting levels is documented (Figure 4B). This observation is similar to that described for the mouse (Lowell et al., 1986b).

Table I: Comparison of Amino Acid Sequence Homologies[a]

		percent		
		$hSAA_1$	$hSAA_2$	$hSAA_3$
mouse	SAA_1	72.8	72.8	70.9
	SAA_2	71.8	71.8	68.0
	SAA_3	84.5	84.5	88.3
human	pA1	69.7	69.7	72.9
	$pSAA8_2$	68.0	68.0	71.3
hamster	$hSAA_1$	100	96.7	88.5
	$hSAA_2$	96.7	100	87.7
	$hSAA_3$	88.5	87.7	100

[a] Amino acid sequences used in calculations were derived from nucleotide sequences for hamster (Figure 3), mouse (Lowell et al., 1986a), and man [pAl (Sipe et al., 1985); pSAA82 (Kluve-Beckerman et al., 1986)].

The differential tissue expression of the SAA genes is shown in Figure 5. In this figure, the difference in tissue expression of SAA genes is demonstrated by autoradiography of a blot of RNA isolated from different hamster organs sequentially hybridized with two probes generated from different SAA-specific mRNAs. The RNA species detected by the two probes are of identical size. Specificity of the oligonucleotide for $ghSAA_3$ sequence was demonstrated by successful hybridization with $ghSAA_3$ DNA on a Southern blot and failure of hybridization of the oligonucleotide with $phSAA_1$ and $phSAA_2$ under similar hybridization and washing conditions (data not shown).

Discussion

The structures of the hamster SAA genes and their products reported here resemble those described in man and mouse (Lowell et al., 1986a; Woo et al., 1987). A hamster SAA pre-apoprotein sequence of 122 amino acids is predicted by nucleotide sequence of 2 different cDNA clones and 3 exons present in the hamster genomic clone. The nucleotide sequence for the hamster SAA gene described herein is divergent from the reported human SAA gene sequence, largely due to differences in intronic and untranslated regions (Woo et al., 1987). However, when coding regions alone are considered, hamster apoSAA shares ~73% amino acid sequence homology with the corresponding region of a human SAA gene.

SAA gene sequences within a species have been most extensively studied in the mouse (Lowell et al., 1986a). While

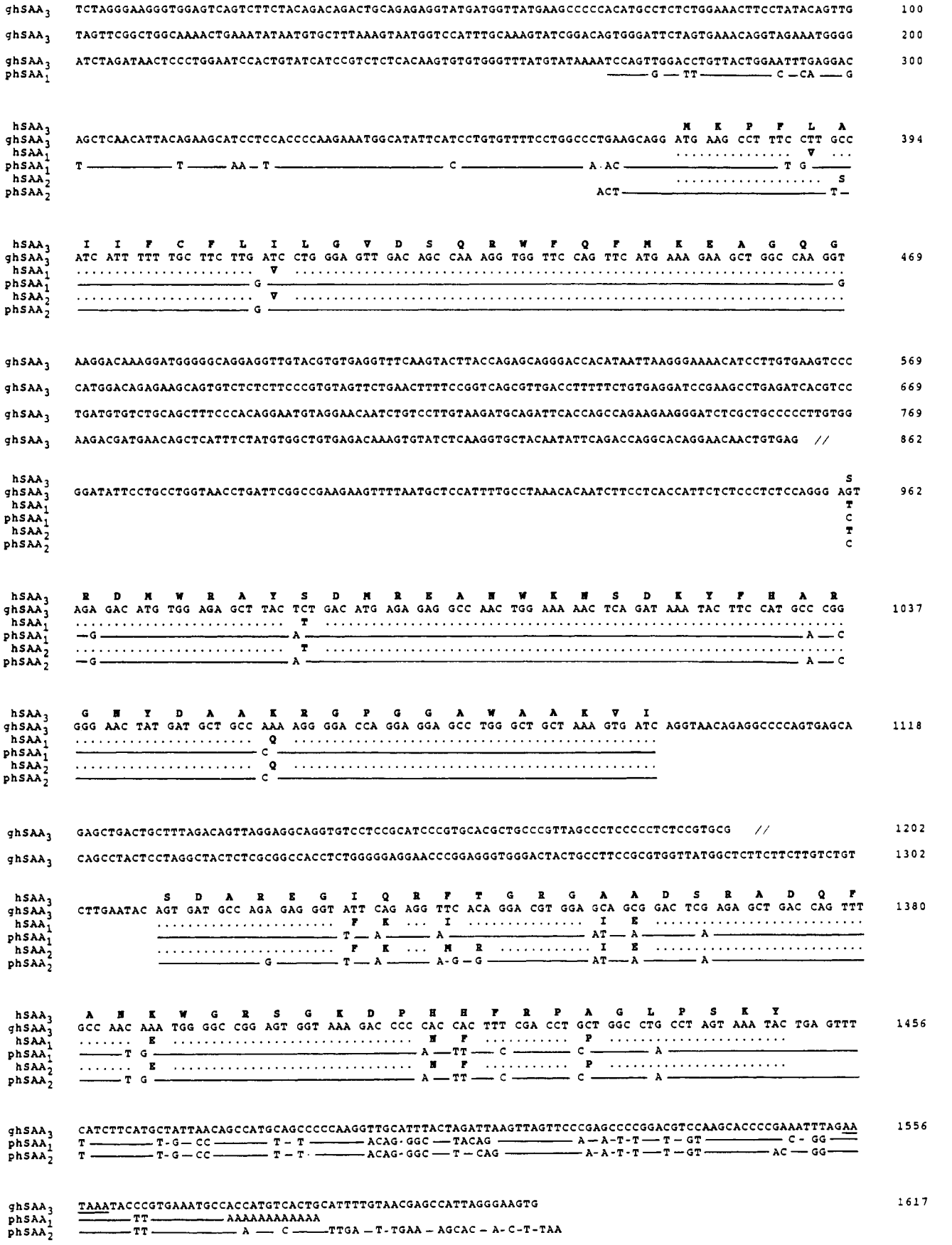

FIGURE 3: Nucleotide sequence of ghSAA$_3$, phSAA$_1$, and phSAA$_2$. Dashes within nucleotide for phSAA$_1$ and phSAA$_2$ indicate identity with the corresponding region of ghSAA$_3$ sequence. Homologous regions in derived polypeptide sequences are indicated as a dotted line. Two slants indicate a gap in the sequence data in intronic regions. The polyadenylation signal is underlined, and putative 5′ and 3′ consensus splice junctions are overlined.

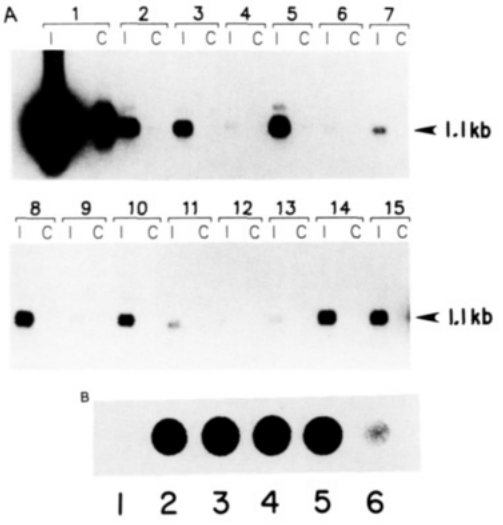

FIGURE 4: (A) RNA blot analyses demonstrating sites of expression of SAA genes. Organs: (1) liver; (2) lungs; (3) muscle; (4) ovary; (5) spleen; (6) testis; (7) uterus; (8) heart; (9) intestine; (10) kidney; (11) adrenal; (12) brain; (13) urinary bladder; (14) diaphragm; (15) esophagus. C, control (unstimulated); I, RNA sample isolated 24 h following induction of acute inflammatory stimulus. (B) Kinetics of accumulation of hepatic SAA-specific mRNA following induction of an acute-phase response by subcutaneous turpentine injection. Five micrograms of RNA isolated at varying time points (2–12, 3–18, 4–24, 5–48, and 6–68 h) following induction of inflammation was deposited on a membrane and hybridized with $phSAA_1$. Five micrograms was also isolated from an unstimulated animal (sample 1).

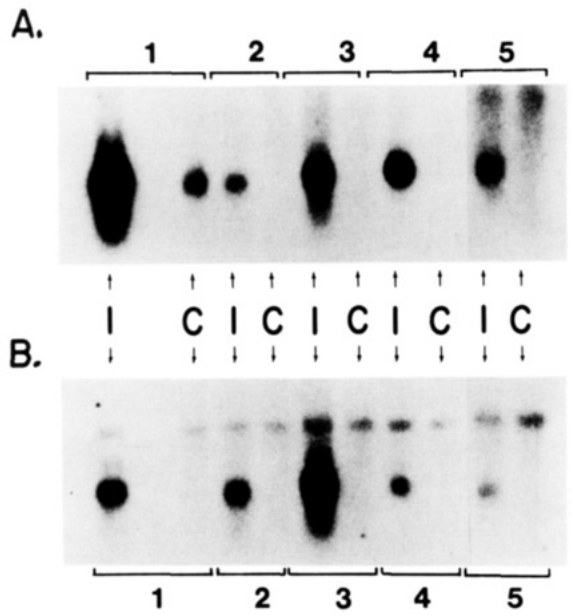

FIGURE 5: Differential tissue expression of SAA genes. Northern blot analysis of RNA isolated from different organs (1, liver; 2, lungs; 3, muscle at abscess site; 4, spleen; 5, kidney) following sequential hybridization with $phSAA_1$ (panel A), and a $ghSAA_3$-specific oligonucleotide representing residues 1494–1514 (panel B). C, control; I, RNA samples 24 h following stimulation with subcutaneous turpentine.

murine SAA_1 and SAA_2 genes demonstrate extensive nucleotide sequence homology throughout, comparison of mouse SAA_3 sequences reveals reduced homology with SAA_1 and SAA_2 in many exonic and intronic segments. Comparison of nucleotide homology between hamster and mouse reveals maximal overall homology of $ghSAA_3$ with the murine SAA_3 gene (88.3%), but poor conservation of sequences in untranslated regions between hamster and murine SAA genes is noted. Like the mouse, three exons encode the sequences which are translated. There may be another more 5′ exon containing 5′ untranslated sequences as has been found in both mouse and human SAA genes (Lowell et al., 1986a; Woo et al., 1987), but it is not contained within this clone. This is not surprising since the intronic distance between the first and second exons of murine and human SAA genes is more than 400 base pairs, and the hamster genomic clone begins about 375 bases 5′ to the splice junction at the beginning of the putative second exon. The absence of relevant transcriptional control signals from the hamster clone is also suggested by the failure of production of SAA mRNA in cell lines transfected with this clone (data not shown).

We have also characterized two different hamster SAA cDNA clones encoding mRNA species which are distinct from that predicted by the nucleotide sequence of the genomic clone. The nucleotide sequences of the two mRNA species represented by these clones are very conserved (97%), and the derived amino acid sequences from the two cDNA clones demonstrate 96.7% homology. The derived amino acid sequences from the cDNA clones share >85% homology with the polypeptide sequence predicted from the coding regions of the genomic clone ($ghSAA_3$). Hence, it seems likely that Syrian hamsters may have three SAA genes although allelic variation cannot be excluded.

The major protein component of secondary amyloid deposits, amyloid A (AA), is presumed to arise from proteolytic cleavage of SAA, a long-standing hypothesis which has recently been supported by demonstration of a precursor–product relationship between these two proteins (Husebekk et al., 1985). Recent studies have also shown that selective deposition of a single SAA isotype occurs in murine amyloidosis (Meek et al., 1986; Shiroo et al., 1987). Murine SAA apoproteins are encoded by three genes, all of which are expressed as mRNA. Hepatocytes synthesize abundant quantities of SAA_1 and SAA_2, but the polypeptide predicted by SAA_3 nucleotide sequence analyses has not been identified. The role of tissue-specific regulation of SAA gene expression in the amyloidogenicity of particular SAA isotypes remains to be elucidated. Extrahepatic synthesis of SAA has been demonstrated in many organs of the mouse including heart, lung, intestine, kidney, spleen, and the adrenal gland (Ramidori et al., 1985; Meek & Benditt, 1986). Differential expression of murine SAA_1, SAA_2, and SAA_3 genes has been demonstrated by using gene-specific oligonucleotide probes following exposure to LPS (Meek & Benditt, 1986). Our studies demonstrate that SAA genes are also expressed in many organs of the hamster. In addition to those organs listed above, SAA-specific mRNA was detected in the following hamster organs—ovary, testis, uterus, spleen, urinary bladder, and esophagus. Abundant accumulation of an SAA-specific mRNA also occurred at the site of a sterile abscess induced by turpentine injection.

The hamster genomic clone $ghSAA_3$ is expressed at the mRNA level in induced tissues. No detectable levels of this isotype were observed in tissues from control animals, even though constitutive expression of SAA was observed in the liver using a probe which cross-hybridizes with all three isotypes. RNA obtained from the site of injury hybridized much more strongly to the SAA_3 probe than did RNA from other tissues, including the liver. Murine SAA_3 is produced at most extrahepatic sites as well as in the liver. It is of interest to note that of the extra hepatic sites studied, elicited peritoneal macrophages produced the greatest amount of SAA_3 in mice injected intraperitoneally with LPS (Meek & Benditt, 1986). Resident macrophages or cells recruited to the site of inflammation caused by turpentine injection may be responsible for

SAA_3 production in our studies. Others have suggested that endothelial or vascular smooth muscle cells may be involved in SAA_3 mRNA production (Meek & Benditt, 1986). In situ hybridization studies will be necessary to define the cellular origin of the different SAA isotype mRNAs. Differential oligonucleotide hybridization studies show that while the modulatory functions of SAA in host defense mechanisms at sites of tissue necrosis and repair remain to be elucidated, the observation that SAA_3 is expressed abundantly at the abscess site suggests that tissue-specific expression of different SAA isotypes may be important at sites of inflammation and tissue injury.

ACKNOWLEDGMENTS

We thank Dr. H. R. Colten for helpful discussions. We thank C. Das, M. Howard, D. J. Waggoner, and J. Morrissey for excellent technical assistance.

Registry No. DNA (*Mesocricetus auratus* clone $ghSAA_3$ protein SAA_3 gene coding region), 120523-65-1; preprotein SAA_3 (*Mesocricetus auratus* clone $ghSAA_3$), 120523-73-1; protein SAA_3 (*Mesocricetus auratus* clone $ghSAA_3$), 120523-72-0; DNA (*Mesocricetus auratus* clone $phSAA_1$ protein SAA_1 messenger RNA complementary), 120523-66-2; preprotein SAA_1 (*M.a.* clone $phSAA_1$), 120523-74-2; protein SAA_1 (*M.a.* clone $phSAA_1$), 120523-69-5; DNA (*M.a.* clone $phSAA_2$ protein SAA_2 messenger RNA complementary), 120523-67-3; preprotein SAA_2 (*M.a.* clone $phSAA_2$), 120523-71-9; protein SAA_2 (*M.a.* clone $phSAA_2$), 120523-70-8.

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