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Characterization of the gene encoding mouse serum amyloid P component

Comparison with genes encoding other pentraxins

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A CBA/J-strain mouse serum amyloid P component (SAP) genomic clone was isolated and analysed. The clone contains the entire SAP gene and specifies a primary transcript of 1065 nucleotide residues. This comprises a first exon of 206 nucleotide residues containing the mRNA 5'-untranslated region and sequence encoding the pre-SAP leader peptide and the first two amino acid residues of mature SAP separated by a single 110-base intron from a 749-nucleotide-residue second exon containing sequence encoding the bulk of the mature SAP and specifying the mRNA 3'-untranslated region. The overall organization is similar to that of the human SAP gene, and the coding region and intron sequences are highly conserved. The SAP RNA cap site was defined by primer extension analysis of polyadenylated acute-phase liver RNA. The 5'-region of the mouse SAP gene contains modified CAAT and TATA promoter elements preceded by a putative hepatocyte-nuclear-factor-1-recognition site; these structures are in a region that is highly homologous to the corresponding region of the human SAP gene. Comparisons of the mouse SAP gene structure and derived amino acid sequence with those of other mammalian pentraxins were made.

INTRODUCTION

The acute-phase response is a systemic reaction to an inflammatory stimulus and comprises a spectrum of physiological changes (reviewed in ref. [1]). Among these are alterations in the concentration of a wide range of serum proteins known as acute-phase reactants. It is likely that the concentrations of acute-phase reactants present during the course of systemic inflammation contribute more effectively to early host defence than do the concentrations present at homoeostasis. There are a limited number of acute-phase reactants, synthesized principally in the liver, that increase dramatically, by up to a 1000-fold, during inflammation. In mouse, these include serum amyloid P component (SAP) [2,3]. The SAP analogue C-reactive protein (CRP) has been identified in mouse as a low-concentration constitutive serum protein [4,5]. Paradoxically, in man (and rabbit) it is CRP that is an acute-phase reactant (reviewed in ref. [6]) and SAP that is present constitutively at relatively low concentrations (reviewed in ref. [7]).

SAP and CRP are pentraxins, proteins with a discoid organization of five non-covalently bound monomeric subunits. Native SAP is arranged as two discs oriented face to face [8], whereas native CRP is a single disc [9]. Gross structural similarities and striking amino acid sequence homologies indicate that SAP and CRP are products of an ancestral gene-duplication event. This hypothesis is strengthened by the location of the mouse SAP gene in a region of mouse chromosome 1 [10] that is syntenic with the portion of human chromosome 1 containing band q2.1, to which both SAP and CRP have been mapped [11]; in both species this evolutionarily conserved part of the genome contains a considerable number of immunologically important loci, including genes encoding F_c receptors, lymphocyte surface antigens and inflammation-associated macrophage products (reviewed in ref. [12]).

The magnitude of the increase in the concentrations of acute-phase pentraxins in serum during inflammation suggests an important role for these proteins in early host defence. However, the precise function of SAP and CRP during the acute-phase response remains unclear. SAP is a precursor of amyloid P component, which is found associated with the amyloid deposits that are the occasional consequence of a number of inflammatory diseases (reviewed in ref. [7]). It has been suggested that SAP can act as an elastase inhibitor [13], thereby aberrantly protecting amyloid deposits from degradation in a manner similar to its possible true role in protecting the host from damage by inflammation-associated proteinases. CRP has long been known to promote a range of immune functions, including agglutination, phagocytosis and complement fixation (reviewed in ref. [1]). Recently, both SAP [14] and CRP [15] have been shown to be capable of high-affinity binding to chromatin, indicating that their major role during inflammation may be the clearance of nuclear material released from damaged tissue.

The genes for human SAP [16] and CRP [17,18] have

Abbreviations used: SAP, serum amyloid P component; CRP, C-reactive protein.

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These sequence data have been submitted to the EMBL/GenBank Data Libraries.

been sequenced and characterized. Both have a relatively simple structure with a single intron separating the first exon containing sequence specifying the mRNA 5′-untranslated region and sequence encoding a leader peptide from the second exon containing sequence encoding the bulk of the mature protein and the mRNA 3′-untranslated region. The CRP gene differs as follows: its intron is considerably larger than that of the SAP gene and contains a pyrimidine–purine repeat sequence capable, in theory, of adopting a Z-DNA conformation; the region specifying the mRNA 5′-untranslated region contains three heat-shock (Pelham) consensus [19] elements; and the region specifying the mRNA 3′-untranslated region is unusually large. It has been suggested [12,17] that all or some of the above features, which are shared by the rabbit CRP gene [20], are important to the acute-phase expression of the human CRP gene. Recently, promoter elements that confer the capacity to respond to inflammatory cytokines have been identified in the region upstream from the CRP mRNA cap site [21].

It has been a matter of speculation whether the mouse SAP gene more closely resembles the human SAP gene, as the characteristics of its gene product would suggest, or the human CRP gene, as the characteristics of its dramatic induction during the acute phase of inflammation would suggest. In the present paper we describe the cloning and characterization of the mouse SAP gene and compare its sequence and the sequence of its gene product with those of other pentraxins.

MATERIALS AND METHODS

Mouse genomic DNA library

High-M_r genomic DNA was isolated from a female CBA/J-strain (Jackson Laboratories, Bar Harbor, ME, U.S.A.) mouse liver by the method of Blin & Stafford [22]. DNA (1 mg) was digested to completion with an excess of *Bam*HI (Amersham, Arlington Heights, IL, U.S.A.) and size-fractionated on a 1 % low-melt agarose gel. A gel slice encompassing *Bam*HI DNA fragments from 8 to 10 kb was excised and extracted twice with phenol, once with phenol/chloroform (1:1, v/v) and once with chloroform/isopentanol (24:1, v/v) before ethanol precipitation. The concentration of purified DNA was estimated by u.v. inspection of a sample that had been treated with ethidium bromide and comparison with a similarly treated serial dilution of DNA of known concentration. DNA was ligated to phosphatase-treated *Bam*HI/*Eco*RI-digested EMBL3 bacteriophage arms (Promega Biotech, Madison, WI, U.S.A.) at insert/bacteriophage-arm molar ratios of 1:1, 2:1 and 4:1 according to the manufacturer's instructions. Recombinant bacteriophages were packaged by using Gigapack Plus (Stratagene, La Jolla, CA, U.S.A.) packaging extracts. The three library samples yielded a total of 1.3×10^6 independent recombinants.

Isolation of SAP genomic clones

The CBA/J-strain mouse genomic library was screened by the method of Benton & Davis [23]. Briefly, 5×10^5 recombinant bacteriophages were plated at high density with *Escherichia coli* MB406 as the host bacterial strain and transferred to nitrocellulose filters. Filters were denatured, neutralized, baked and hybridized under standard conditions [24] to a mouse SAP cDNA clone MSAP5 [10] that had been radiolabelled to high specific radioactivity with [α-^{32}P]dCTP (Amersham) with the use of an oligolabelling kit (Pharmacia, Piscataway, NJ, U.S.A.). Following hybridization, filters were washed in 30 mM-NaCl/3 mM-sodium citrate buffer, pH 6.8, containing 0.1 % SDS at 65 °C for 2 h, dried and examined by autoradiography. Three positive clones were identified. One clone, MSAPg1, was plaque-purified with the use of *E. coli* KW251 (Promega Biotech), a *recA*− strain, as the bacterial host to avoid insert loss. MSAPg1 DNA was prepared in bulk and the insert was excised and subcloned into the plasmid pUC18 for bulk preparation.

Sequence analysis

MSAPg1 insert was prepared in bulk and digested with *Pst*I before being subcloned into the *Pst*I site of the polylinker region of the bacteriophage sequencing vector M13mp18 (New England Biolabs, Beverly, MA, U.S.A.) according to the manufacturer's instructions. In addition, MSAPg1 insert was digested with *Pvu*II and subcloned into M13mp18 from which the region between the *Pvu*II sites present in the *laci* and *lacZ* elements had been removed. Sequence was obtained by using the dideoxy chain termination method of Sanger *et al.* [25].

Oligonucleotide synthesis

Unique-sequence oligonucleotides for use as sequencing primers and hybridization probes were synthesized on an Applied Biosystems 380B DNA synthesizer.

Primer extension analysis

Polyadenylated mRNA was isolated from total CBA/J mouse liver RNA with the use of Hybond messenger affinity paper (Amersham) according to the manufacturer's instructions. Specific 18-base oligonucleotides (200 ng), complementary to unique sequences towards the 5′-end of the mouse SAP mRNA, were annealed to 0.5 μg of polyadenylated mRNA for 2 h at 37 °C before extension by using AMV reverse transcriptase in a modified first-strand cDNA synthesis reaction. The same oligonucleotides were used in sequencing reactions with an appropriate SAP genomic M13mp18 template. The products of both procedures were co-run on a standard sequencing gel to allow determination of the SAP mRNA cap site by correlating the position of the primer extension band with the DNA sequence ladder.

Computer analysis

Nucleotide and amino acid sequence analyses were carried out by using the Bionet Resource (funded by National Institutes of Health Grant P41RR01685) accessed via PCGENE software (Intelligenetics, Mountain View, CA, U.S.A.). Sequence comparisons facilitated by the above are by the method of Pearson & Lipman [26].

RESULTS AND DISCUSSION

Different strains of mice display different endogenous serum concentrations of SAP [2,3]. The locus controlling endogenous SAP concentrations [27] maps to the same position on mouse chromosome 1 as the SAP structural gene [10], suggesting that the control of serum SAP concentrations is exercised, at least in part, by elements intrinsic to the gene. CBA/J mice have moderate resting concentrations of circulating SAP (70 μg/ml) that rise

dramatically (to 1350 μg/ml) following an inflammatory stimulus; this increase is subsequent to a marked rise in hepatic SAP mRNA concentrations [28]. In addition, amyloidosis is readily induced in CBA/J mice by daily subcutaneous injection of azo-casein [29], and the susceptibility of the strain to amyloid disease appears to be dependent on a variant at a single, unidentified, locus [30]. Thus CBA/J mice are an ideal strain for the analysis of SAP gene control elements important for induction during the acute phase of inflammation and for determining whether structural or control variants of the SAP gene cause, or contribute to, the genesis of amyloid disease.

Isolation and sequencing of mouse SAP genomic clones

Total CBA/J mouse DNA was digested with the restriction endonuclease *Bam*HI and subjected to Southern-blot analysis with a CBA/J mouse SAP cDNA clone, MSAP5 [10], as a hybridization probe; a single 9 kb band was detected (result not shown). A genomic sub-library was constructed by cloning fractionated *Bam*HI-digested CBA/J strain DNA (8–10 kb) into the EMBL3 vector. This library was screened with MSAP5 to identify genomic clones carrying the SAP gene. One clone, MSAPg1, was plaque-purified, and its insert was excised with *Bam*HI and subcloned into the plasmid vector pUC18 for bulk preparation. Southern-blot analysis of *Pst*I-digested MSAPg1 with oligonucleotides specific for different regions of the published SAP cDNA sequence [10,31] revealed three fragments of 631, 267 and approx. 1500 bases, corresponding respectively to the 5′-end, central portion and 3′-end of the SAP gene. These fragments were subcloned into M13mp18 and sequenced with the use of the M13 universal primer and SAP-specific oligonucleotide primers. In order to establish that the *Pst*I fragments were adjacent, a 2.95 kb *Pvu*II fragment, spanning the *Pst*I-recognition sites flanking the central *Pst*I fragment, was sequenced after being subcloned into M13mp18 between the *Pvu*II sites present in the *laci* and *lacZ* regions. This unconventional strategy yielded a functional bacteriophage construct that could be sequenced with the use of SAP-specific primers; however, as non-recombinant and recombinant bacteriophages both yielded clear plaques, true recombinants containing the desired SAP gene fragment were identified by hybridization of plate lifts with oligonucleotide probes. The analyses outlined above yielded a continuous sequence of 1876 bases: 98% of the sense strand sequence and 100% of the antisense strand sequence were determined (Fig. 1).

Determination of SAP mRNA cap site

To identify the 5′-terminus of the SAP mRNA, primer extension analysis was performed with the use of an oligonucleotide (5′-TTTCAGGTTTTGTATGTG-3′) complementary to nucleotide residues 44–61 of the genomic sequence (Fig. 2). The oligonucleotide was annealed to polyadenylated mRNA from the liver of a CBA/J mouse that had been injected 12 h earlier with thioglycollate; this mRNA contained maximally induced amounts of SAP mRNA [28]. The oligonucleotide primer was extended by using a modified first-strand cDNA synthesis reaction, and the product was co-run on a sequencing gel together with a set of sequencing reactions obtained with the same oligonucleotide (Fig. 3). The primed product yielded a single band that co-migrated with nucleotide residue 1 of the gene sequence (see Fig. 2), indicating that this is the position at which transcription of SAP RNA is initiated. This position was unexpected, as the New Zealand Black SAP cDNA sequence reported by Ishikawa *et al.* [31] and the DBA/2J cDNA sequence subsequently reported by Mole *et al.* [32] contain respectively an additional 15 and four upstream nucleotide residues that correlate with those present in the gene. Solely on the basis of the assumption that the cDNA sequence published by Ishikawa *et al.* [31] represents a full-length clone, Nishiguchi *et al.* [33] recently tentatively assigned the SAP RNA cap site to nucleotide residue −13. To strengthen our experimental data defining the SAP RNA cap site in the CBA/J strain, we performed an additional primer-extension analysis with the use of an oligonucleotide (5′-AACAAAAGTGTGGTGTCT-3′) complementary to nucleotide residues 78–95. In this case the major product was again a discrete band co-migrating with nucleotide residue 1 (result not shown); although several other bands of very high M_r were observed, there was no band in the region of nucleotide residue −13. We therefore consider that the CBA/J strain SAP RNA cap site is at nucleotide residue 1, and differs from the transcription-initiation site used by New Zealand Black and DBA/2J mice to synthesize the SAP RNA that was used by Ishikawa *et al.* [31] and Mole *et al.* [32] to generate cDNA clones. This may reflect the utilization of different start sites in different mouse strains or the

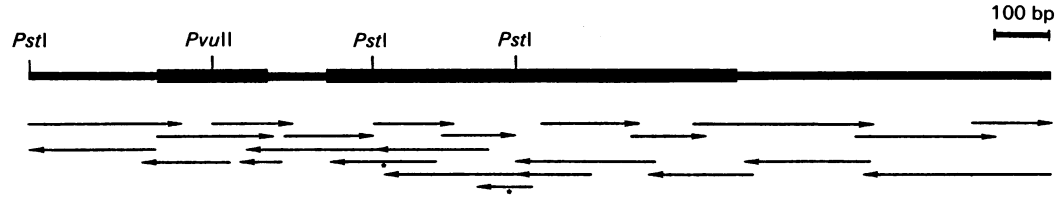

Fig. 1. Strategy for sequencing clone MSAPg1

The Figure is a diagrammatic representation of clone MSAPg1 encompassing exons 1 and 2 (emboldened), the single intron, 234 nucleotide residues of sequence 5′ to the RNA cap site and 577 nucleotide residues downstream from the 3′-terminus of the portion of the gene specifying the mRNA 3′-untranslated region. Sequence was obtained for the entire 631 bp 5′ *Pst*I fragment, the entire 267 bp central *Pst*I fragment and 978 nucleotide residues of the 3′ *Pst*I fragment. Sequence spanning the internal *Pst*I sites was obtained by analysis of the 3′ *Pvu*II fragment as indicated (*). A total of 11 specific oligonucleotides were used to sequence the sense strand and 13 specific oligonucleotides were used to sequence the antisense strand.

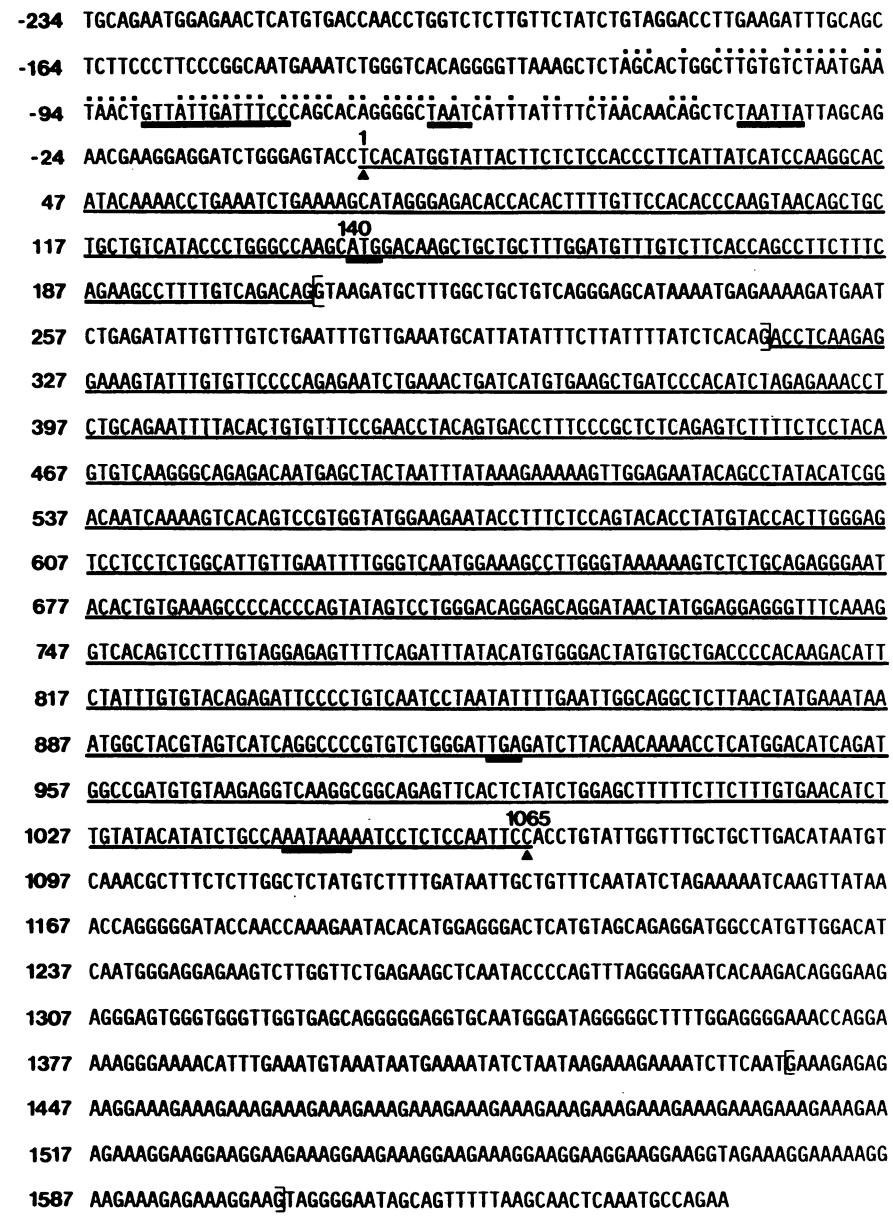

Fig. 2. Sequence of clone MSAPg1

Sequence (1876 nucleotide residues) was obtained as depicted in Fig. 1. Exon 1 and exon 2 are underlined. Between them the single intron is bracketed. The purine-rich GAAA repeat region between nucleotide residues 1438 and 1603 is also bracketed. The boundaries delimiting the SAP primary transcription product are indicated (▲) at positions 1 (RNA cap site) and 1065 (3′-terminus). The following elements are boldly underlined: the putative hepatocyte-nuclear-factor-1-recognition site, modified CAAT box and modified TATA box at nucleotide residues −89, −64 and −37 respectively; the ATG codon specifying the *N*-terminal methionine of pre-SAP at nucleotide residue 140, the TGA stop codon at nucleotide residue 922, and the AATAAA polyadenylation signal sequence at nucleotide residue 1044. The region between nucleotide residues −117 and −41 is highly homologous to the corresponding portion of the 5′-sequence of the human SAP gene: conserved nucleotides are indicated (■). It should be noted that the 5′ mRNA sequence depicted here differs from that derived from the sequencing of New-Zealand-Black-strain and DBA/2J-strain cDNA clones by Ishikawa *et al.* [31] and Mole *et al.* [32] as follows. At nucleotide residue 33 both groups report an A rather than a G; at nucleotide residues 44, 77 and 133 Mole *et al.* [32] report T, C and A respectively rather than A, C and G. In the coding sequence, at position 700 Mole *et al.* [32] report a G rather than an A and at position 709 Ishikawa *et al.* [31] report a C rather than a G; the former difference does not alter the amino acid encoded, but, however, the latter difference specifies an arginine in the derived amino acid sequence instead of the glycine reported in the present paper (see Fig. 5).

utilization of alternative start sites in response to different acute-phase stimuli.

Structure of the CBA/J mouse SAP gene

The sequence presented in Fig. 2 predicts a CBA/J precursor RNA species of 1065 bases, beginning with the transcription-initiation site at nucleotide residue 1 and ending at nucleotide residue 1065, 16 bases downstream from the AATAAA polyadenylation signal. The coding region encompasses nucleotide residues 140–921 and is interrupted by a single 110 bp intron between nucleotide residues 207 and 316. Therefore the mature mRNA is

955 bases long (not including polyadenylation), a size that is in good agreement with that observed experimentally [28,32]. The first exon contains the mRNA 5′-untranslated region, sequence encoding the entire pre-SAP leader peptide, and six nucleotide residues encoding the first two amino acid residues and the first base of the triplet encoding the third amino acid residue of mature SAP. The 110-base intron is followed by 605 nucleotide residues encoding the bulk of the mature SAP protein and sequence specifying the TGA stop codon and the 141-base SAP mRNA 3′-untranslated region. The 139-base 5′-untranslated region specified is somewhat larger than that of the human SAP mRNA (96 nucleotide residues) and those of the human and rabbit CRP mRNAs (104 and approx. 110 nucleotide residues respectively). Both the mouse and human SAP mRNA 5′-untranslated regions lack the heat-shock consensus (Pelham) sequences [19] evident in the 5′-untranslated regions of the human and rabbit CRP mRNAs. The 141-nucleotide residue 3′-untranslated region of the mouse SAP mRNA is 14 nucleotide residues shorter than that of the human SAP mRNA; both SAP mRNA 3′-untranslated regions are an order of magnitude shorter than those of the human and rabbit CRP mRNA 3′-untranslated regions. The position of the single intron in the mouse SAP gene with respect to coding regions is identical with that of the single introns of the human SAP, human CRP and rabbit CRP genes. At 110 bases it is similar in size to the 115-base intron of the human SAP gene; in addition, both lack the extensive purine–pyrimidine repeat region that is present in the introns of the larger human CRP [17,18] and rabbit CRP [20] introns. The mouse and human SAP introns are highly homologous, with 78 conserved nucleotide residues (Fig. 4). This high degree of evolutionary stability contrasts with the less uniform and less marked sequence similarity between the respective 5′- and 3′-untranslated regions of the mouse and human SAP mRNAs (results not shown) and may indicate an important role for the intron in SAP expression.

Upstream from the cap site 234 bp of clone MSAPg1 was sequenced. There is a modified TATA box, TAATTA, and a modified CAAT box, TAAT, 37 bp and 64 bp 5′ to the cap site respectively; these are possible initiation and attachment sites for RNA polymerase II. In addition, there is a sequence (GTTATTGATTTCC) beginning 89 nucleotide residues upstream from the cap site that is similar to the consensus site GTTAATNATTAAC defined by Courtois *et al.* [34] as being the target for hepatocyte nuclear factor 1 in genes expressed in liver. The position of this sequence in the mouse SAP gene, which is expressed principally in liver, suggests a probable involvement in promoter activity. Furthermore, the putative hepatocyte-nuclear-factor-1-recognition site is in a region of the mouse SAP gene that is highly homologous to the corresponding region of the human SAP gene (Fig. 2).

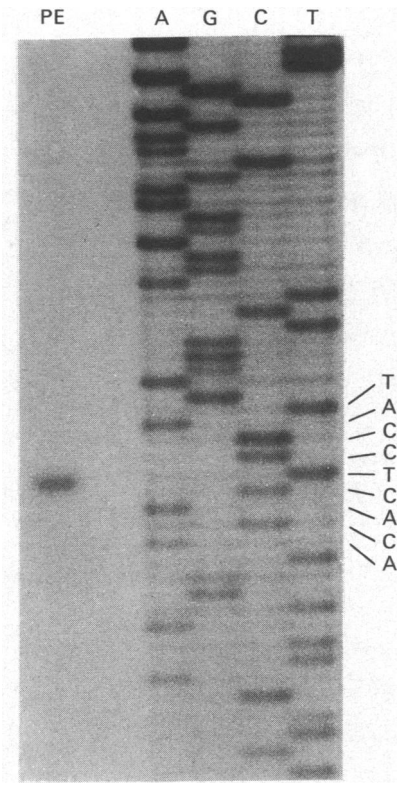

Fig. 3. Determination of SAP RNA cap site

PE indicates the product of primer extension analysis with the use of an oligonucleotide (5′-TTTCAGG-TTTTGTATGTG-3′) complementary to nucleotide residues 44–61 of the SAP gene sequence and acute-phase liver polyadenylated RNA as a template; the single band corresponds to the product obtained. AGCT indicates the sequence obtained from the 5′ *Pst*I M13 template of clone MSAPg1 with the same oligonucleotide co-run to facilitate sizing of the primed product, and positioning of the RNA cap site relative to the SAP gene sequence.

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Mouse     GTAAGatGcTttgGctgctGTCAgGgAgCATAAAaTGAGAAAA   GaTGAAtCTGAG
          |||||  | |   |     |||| | | |||||| ||||||||   | |||| |||||
Human     GTAAGgaGgTgaaGgaatgGTCAaGaAtCATAAAgTGAGAAAAtagGtTGAAgCTGAG

Conserved GTAAG--G-T---G-----GTCA-G-A-CATAAA-TGAGAAAA---G-TGAA-CTGAG


Mouse     ATATtgTTTgtCTGaATTT  gtTGAAatgCATTATaTTTCTTatTTTATCtCaCAG
          ||||  |||  ||| ||||    ||||   |||||| ||||||  |||||| | |||
Human     ATATctTTTccCTGcATTTatacTGAAggtCATTATcTTTCTTtcTTTATCcCgCAG

Conserved ATAT--TTT--CTG-ATTT----TGAA---CATTAT-TTTCTT--TTTATC-C-CAG
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Fig. 4. Alignment of the introns of the mouse and human SAP genes

The mouse and human SAP intron sequences are indicated with the conserved nucleotide residues depicted below. Alignment was made by using the Genalign program of the PCGENE suite of software programs (Intelligenetics) in combination with the Bionet database resource.

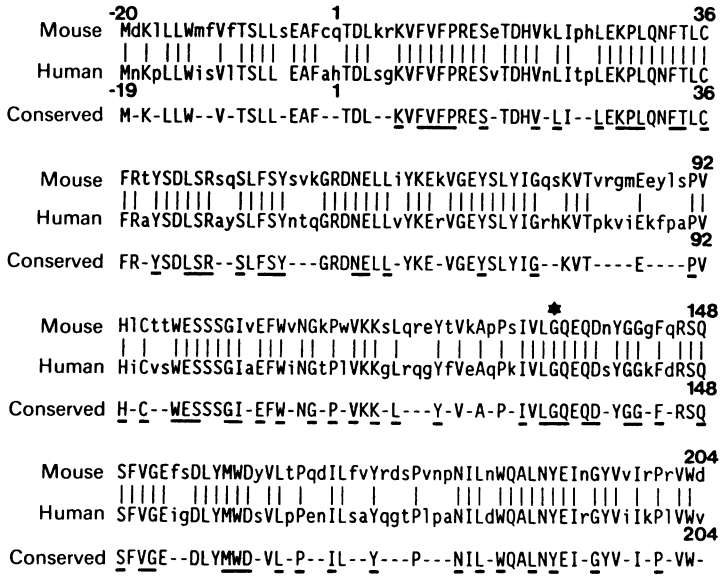

Fig. 5. Alignment of the derived mouse and human pre-SAP amino acid sequences

The mouse and human pre-SAP sequences are indicated, with the conserved residues depicted below. ✱, The glycine residue at position 134 is in agreement with the amino acid sequence derived from the SAP cDNA clone reported by Mole *et al.* [32] rather than the arginine residue reported by Ishikawa *et al.* [31]. Residues conserved between all of the mammalian pentraxins reported thus far, i.e. human CRP [17,18], rabbit CRP [20], Syrian-hamster SAP [35], human SAP [16] and mouse SAP [31,32], are underlined. The alignment of mouse SAP and human SAP, and the determination of residues conserved in all mammalian pentraxins, was obtained by using the Genalign program of the PCGENE suite of programs (Intelligenetics) in combination with the Bionet databank resource.

Downstream from the sequence delimiting the 3′-terminus of the SAP RNA, between nucleotide residues 1438 and 1603 is an extensive region of repeats comprising GAAA motifs and variants thereof. Database searches revealed that such repeat elements are present in a wide range of sequences, including spacer DNA within ribosomal DNA clusters. We currently have no information as to whether this region plays any role in the expression of the mouse SAP gene.

Derived amino acid sequence of mouse SAP

Clone MSAPg1 contains sequence encoding a 20-amino-acid-residue leader peptide preceding the mature 204-residue mouse SAP protein. The derived amino acid sequence of mouse pre-SAP is identical with that derived recently by Mole *et al.* [32] from analysis of a DBA/2J-strain mouse cDNA clone and is aligned with the human pre-SAP sequence in Fig. 5. There is a very high degree of amino acid identity between the mature SAP molecules in each species, 141 residues (69%) being conserved as shown. When compared with the other mammalian pentraxins for which complete amino acid sequences are known, i.e. human [16] and Syrian-hamster [35] SAP and human [17,18] and rabbit [20] CRP, several regions are shown to be invariant. These are underlined in the consensus sequence depicted in Fig. 5. Of the 71 residues conserved in all pentraxins, 35 are hydrophobic whereas the proportion of hydrophobic residues in the individual pentraxins ranges from 37.4% to 39.8% [12]. It is likely that the highly conserved regions are involved in specifying the folding of the pentraxin monomer and in forming the interactive surfaces that are involved in the non-covalent assembly of native pentraxin pentamers.

CONCLUSION

The determination of the structure of the CBA/J mouse SAP gene provides the basis for future studies of intrinsic SAP gene elements that respond to external stimuli (such as cytokines and the elevated temperatures associated with fever) present during the acute phase of inflammation. Analysis of the control regions of the SAP genes of CBA/J mice, which readily develop secondary amyloidosis, and comparison with those of the SAP genes of other strains, such as A/J, that are resistant to amyloidosis will allow the role of SAP expression in the genesis of amyloid disease to be determined. The possible role of SAP structural variants in amyloid development may also be determined via comparison of derived SAP amino acid sequences.

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