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Molecular cloning and nucleotide sequence of cDNA for murine senile amyloid protein: nucleotide substitutions found in apolipoprotein A-II cDNA of senescence accelerated mouse (SAM)

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ABSTRACT

cDNA clones encoding the murine senile amyliod protein (AS_{SAM}) have been isolated from animal models of accelerated senescence (SAM-P/1) and from normal aging (SAM-R/1). Immunochemical and protein sequence studies revealed that apolipoprotein (apo) A-II is a serum precursor of AS_{SAM}. A 17-base synthetic oligonucleotide based on residues 39-44 of AS_{SAM} was used as a hybridization probe for screening newly constructed SAM-P/1 and SAM-R/1 liver cDNA libraries. The structure of murine apo A-II cDNA is of interest because of the amino acid substitution found in AS_{SAM} and serum apo A-II of SAM-P; in SAM-R or other random bred slc:ICR mice, amino acid residue 5 of mature apo A-II is proline but, in SAM-P, this amino acid is changed to glutamine. This amino acid replacement is caused by two nucleotide substitutions (CCA for proline codon to CAG for glutamine codon). The third base mutation may not be relevant to the substitution of amino acid. Attention is directed to the relation of this amino acid substitution to the specific deposition of apo A-II, as a tissue amyloid fibril.

INTRODUCTION

We continued the sister-brother mating of several pairs of AKR strain mice which had been donated by the Jackson Laboratory (Bar Harbor, ME) in 1968 and became aware of the presence of certain litters in which most of the mice showed a moderate to severe loss of activity, alopecia and lack of hair glossiness, skin coarseness, periophthalmic lesions, increased lordokyphosis of the spine and shortened life span, despite the relatively low incidence of thymic lymphoma. Among them we selected and maintained four substrains with severe exhaustion as "senescence prone" (P-series) and three substrains with a normal aging process as "senescence resistant" (R-series). The former four series are -P/1, -P/2, -P/3 and -P/4, and the latter three series are -R/1, -R/2 and -R/3. Judging from findings in the survivors and Gompertzian function together with the growth pattern in body weight, the aging pattern in this model seems to be due to the senescence accelerated after a normal process of development (accelerated senescence) rather than to the ageing prematurely appearing in early development (premature aging). Thus, this

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P-series was named "Senescence Accelerated Mouse" (SAM) (1). The substrains of P and R series have been designated SAM-P/1, -P/2, -P/3 and -P/4, and SAM-R/1, -R/2 and -R/3, respectively (2). Among pathological findings, spontaneous age-associated amyloidosis is one of the most characteristic findings in these mice (3). An unique senile amyloid fibril protein termed "AS_{SAM}" was isolated from SAM-P/1 liver (4, 5) and AS_{SAM}-related antigenic substance was found in low molecular weight apolipoproteins of High Density Lipoprotein (HDL) (6), which was termed "apo SAS_{SAM}". The biochemical characteristics suggested that apo SAS_{SAM} is apolipoprotein A-II (apo A-II) (7), one of the major protein constituents of plasma HDL. This was confirmed by the complete amino acid sequence of apo SAS_{SAM} and its prominent homology with the human apo A-II (8). In humans, apo A-II mRNA encodes a 100 amino acid long preproapoprotein; an 18 amino acid prepeptide or signal peptide is cleaved co-translationally, a 5 amino acid propeptide is removed post-translationally, then the mature apo A-II in blood plasma is raised as a dimer of 77 amino acid peptide (9, 10, 11 and references therein).

The complete amino acid sequence of mature apo A-II of both SAM-P/1 and SAM-R/1 was determined and the former protein sequence proved to be identical with that of AS_{SAM} (8 and unpublished result). The interesting feature of the primary structure is that the proline at position 5 in SAM-R/1 is changed to glutamine in the SAM-P/1. To certify this amino acid substitution, we isolated and sequenced cDNA clones for apo A-II of SAM-P/1 and SAM-R/1. As a result, the entire amino acid sequence of murine liver preproapo A-II derived from its cDNA sequence was presented. It is of particular interest to determine whether the amino acid substitution of proline for glutamine is the cause of senile amyloidosis in SAM-P. The isolated apo A-II cDNA clones may be useful for related studies.

MATERIALS AND METHODS

Preparation of RNA

Total RNA was isolated from tissues of mice by the guanidine thiocyanate method (12). About 3 to 5-month old SAM-P/1 and SAM-R/1 were used as tissue donors.

Isolation of poly A^+ RNA and construction of cDNA libraries

Total RNA from liver was used for further purification of poly A^+ RNA by oligo(dT)-cellulose (13) or messenger activated paper (mAP) (14). cDNA was prepared by using avian myeloblastosis virus reverse transcriptase, under the conditions specified by the supplier. cDNA was made double-stranded, tailed with dCTP and annealed to dG-tailed PstI-cleaved pUC9 vector as described by

Gubler and Hoffman (15). For construction of the SAM-P/1 cDNA library, the recombinant plasmid DNA was transformed to strain *E. coli* DH1, as described (16). For the SAM-R/1 cDNA library, *E. coli* RR1 was transformed according to the high frequency procedure of Hanahan (17).

Screening of the cDNA libraries

A 17-base synthetic oligonucleotide mixture complementary to the mRNA region encoding for the known amino acid residues 39-44 of SAM-P/1 apo A-II was from Toyobo Co., Ltd. This oligonucleotide probe was used for the isolation of apo A-II cDNA clones from the SAM-P/1 cDNA library. The method for screening of the cDNA library with the oligonucleotide probe was essentially as described (18). About twenty positive recombinant clones were identified after 3×10^3 colonies had been screened. One of these SAM-P/1 apo A-II cDNA clones, pSPA2-16 was used for the screening of the SAM-R/1 cDNA library. A PstI 500-bp insert of pSPA2-16 was purified by LMP agarose and labeled with α-^{32}P-dCTP by nick translation. 5×10^3 colonies were screened as described (19) and about thirty positive recombinant clones were obtained.

RNA and DNA blotting analysis

Northern transfer of glyoxylated RNA from agarose gels to a nitrocellulose filter and subsequent hybridization were carried out as described by Thomas (20). RNA dot blot hybridization was carried out as describred (19). Southern transfer of DNA to nitrocellulose filter was performed as described (21), except that soaking the gels in 0.25 M HCl was reduced to 5 min. The filter was prehybridized in 5 x SSPE (1 x SSPE is 180mM NaCl, 10mM NaH_2PO_4, pH 7.4 and 1mM EDTA, pH 7.4), 5 x Denhardt's solution (1 x Denhardt's is 0.02% BSA, 0.02% Ficol 400 and 0.02% polyvinylpyrrolidone), 0.5% SDS, 50% (v/v) formamide and 250 μg/ml sheared heat-denatured salmon sperm DNA at 42°C for 12 h in sealed bag. Hybridization was performed for 24 h at 42°C in 5 x SSPE, 5 x Denhardt's, 0.5% SDS, 50% formamide, 10% dextran sulfate, 250 μg/ml sheared heat-denatured salmon sperm DNA and nick-translated apo A-II insert DNA of pSPA2-16. Washing of the filter after hybridization was performed as described (19).

DNA sequence analysis and other methods

Restriction endonuclease fragments of the cDNA was subcloned into pUC19 at appropriate restriction sites. Sequencing by the dideoxy nucleotide procedure was performed as reported (22, 23). Plasmid DNA isolation, isolation of genomic DNA from tissues, radio-labeling of DNA by nick-translation and other conventional methods were as described and referenced therein (19).

Materials and protein sequence data used

Restriction enzymes were purchased from Toyobo Co., Ltd. or Takara Shuzo Co., Ltd. (Japan). mAP paper, dideoxy sequence kit, Klenow fragment of DNA polymerase I, T4 ligase, T4 kinase, reverse transcriptase and terminal deoxynucleotidyl transferase were purchased from Takara Shuzo Co., Ltd. *E. coli* ligase and Ribonuclease H were from New England Biolabs. Oligo(dT_{12-18}) and oligo(dT)-cellulose (type 7) were from PL-Biochemicals. Nick translation kit and radioisotopes were from Amersham.

The amino acid sequences referred to in this report are as follows: deposited amyloid protein purified from SAM-P/1 liver (AS_{SAM}) (8); serum apo A-II (apo SAS_{SAM})of SAM-R/1; apo SAS_{SAM} of random bred slc:ICR mice; apo SAS_{SAM} of SAM-P/1. The complete sequence data of these proteins, except AS_{SAM}, are being prepared for publication.

RESULTS

Isolation of apo A-II cDNA clones from SAM-P/1 and SAM-R/1 mice

The complete amino acid sequence of senile amyloid protein (AS_{SAM}), that is deposited fibrils of serum apo A-II (7) was determined by Yonezu et al. (8). A 17-base synthetic oligonucleotide corresponding to residues 39-44 of the amino acid sequence was prepared as an apo A-II specific probe (Fig. 1).

About twenty out of 3 x 10^3 independent clones were hybridized with the apo A-II specific probe. Five randomly selected clones were further analyzed by restriction endonuclease mapping and four of these clones had common restriction sites. Clone pSPA2-16, which contained the longest insert of 0.5 kb was used as a probe for Northern analysis. As shown in Fig. 2, apo A-II

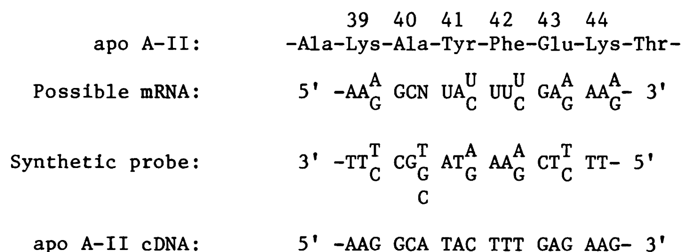

Fig. 1 Design of the synthetic oligonucleotide probe used to screen recombinant plasmids containing sequences for mouse apolipoprotein A-II. The top line indicates the amino acid sequence of mouse apo A-II. The mRNA sequences that could code for this segment are given on the second line (N refers to any of the four nucleotides). The third line gives the synthetic oligonucleotide mixture used as a probe. The bottom line is the actual sequence of the cDNA.

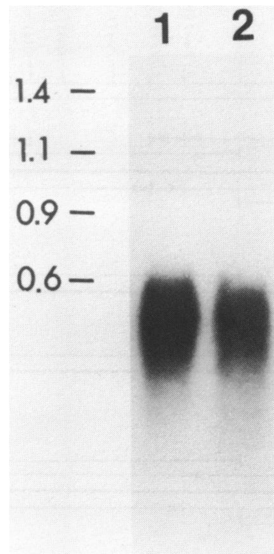

Fig. 2 Northern blot analysis of RNA from mouse liver. RNA was separated by 1.4% glyoxal-agarose gel, transferred to a nitrocellulose filter and probed with nick-translated clone pSPA2-16. Lanes 1 and 2 are 30 μg of total RNA from SAM-P/1 and SAM-R/1 liver, respectively. Mr. markers (shown in kb) were run in a parallel lane and visualized by ethidium bromide staining. The filter was exposed to X-ray film for 12 h.

mRNA is about 0.5 kb in both SAM-P/1 and SAM-R/1, and this value corresponds to pSPA2-16 insert.

Screening of SAM-R/1 apo A-II cDNA was performed after having completed the DNA sequence analysis of pSPA2-16. About thirty out of 5 x 10^3 independent clones were hybridized with the nick-translated pSPA2-16 insert. We randomly selected five clones and four had common restriction sites.

Apo A-II cDNA sequences of SAM-P/1 and SAM-R/1 mice

A physical map of the cloned murine apo A-II cDNA inserts and the sequencing strategy used in the study are shown in Fig. 3. The complete nucleotide sequences of the apo A-II cDNA inserts of SAM-P/1 and SAM-R/1 are shown in Fig. 4.

A SAM-P/1 apo A-II cDNA clone, pSPA2-16 had the longest insert and contained an open reading frame corresponding to preproapo A-II and 5' and 3' noncoding regions of its mRNA. Although the primary translation product of murine apo A-II was not characterized, a peptide from residues -23 to -1 was in good agreement with the human and rat prepro sequences (24), as shown in

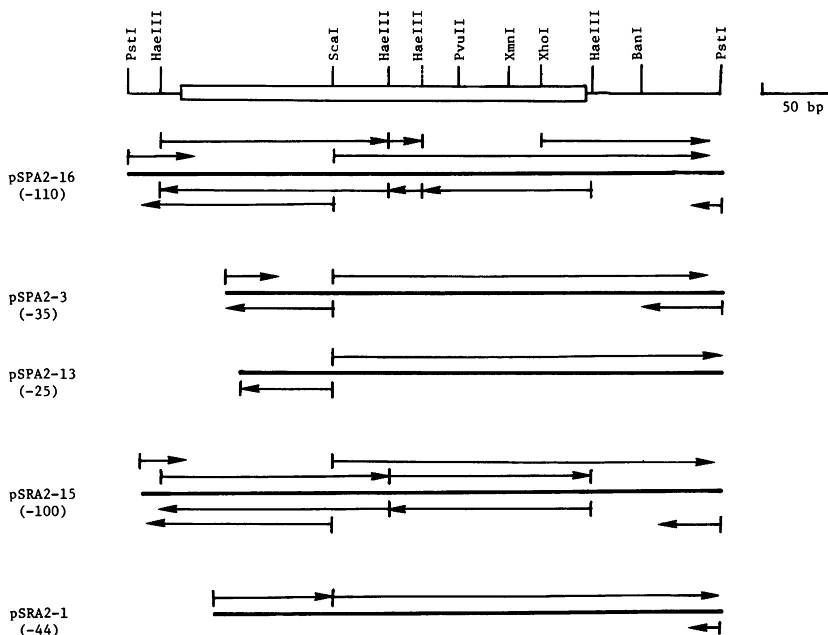

Fig. 3 Physical map and sequencing strategy of the mouse apo A-II cDNA clones. An open box represents the open reading frame. Hae III restriction site indicated by the broken vertical line is lost in SAM-R/1 cDNA clone. The number in parenthesis shows the left end of the fragment (solid line) by nucleotide numbers in Fig. 4. Arrows indicate the direction of sequencing.

Fig. 5. The remaining peptide from residues 1 to 78 was identical to the amino acid sequences of AS_{SAM} and mature serum apo A-II of SAM-P/1.

From the cDNA sequences, there was an additional COOH-terminal amino acid, lysine. All five cDNA clones sequenced had this amino acid. However, in the course of amino acid sequence analysis, this COOH-terminal lysine was not detected. The result is quite unexpected and post-translational processing seems to be a most plausible explanation. Rat apo A-II deduced from the cDNA sequence also have this COOH-terminal lysine (24). In humans, mature apo A-II consists of 77 amino acids in length measured from both the peptide and cDNA sequencing data (9, 10, 11). Two amino acids at the COOH-terminus of murine apo A-II, alanine and lysine were not found in human apo A-II.

While 5' and 3' untranslated regions and prepropeptide coding region of SAM-P/1 cDNA clones are identical to those of SAM-R/1 cDNA clones, nucleotide substitutions were found in the apo A-II coding region of SAM-P/1 compared

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-110                          CATAGAATCGCAGCACTGTTCCTAGGCCATAGTCTGCCATC ATG AAG CTG   -61
(-23)                                                                   Met Lys Leu  (-21)

-60   CTC GCA ATG GTC GCA CTG CTG GTC ACC ATC TGT AGC CTG GAA GGA GCT TTG GTT AAG AGA   -1
(-20) Leu Ala Met Val Ala Leu Leu Val Thr Ile Cys Ser Leu Glu Gly Ala Leu Val Lys Arg  (-1)

                      Pro
                      CA
1     CAG GCG GAC GGA CAG GAT ATG CAG AGC CTG TTC ACT CAG TAC TTT CAG AGC ATG ACT GAA   60
(1)   Gln Ala Asp Gly Gln Asp Met Gln Ser Leu Phe Thr Gln Tyr Phe Gln Ser Met Thr Glu  (20)

                                                                      Val
                                                                       T
61    TAT GGC AAA GAT TTG GTG GAG AAG GCC AAG ACC TCA GAG ATT CAG AGC CAG GCC AAG GCA  120
(21)  Tyr Gly Lys Asp Leu Val Glu Lys Ala Lys Thr Ser Glu Ile Gln Ser Gln Ala Lys Ala  (40)

121   TAC TTT GAG AAG ACA CAC GAG CAG CTG ACA CCC CTT GTC AGG TCA GCA GGA ACT AGT CTG  180
(41)  Tyr Phe Glu Lys Thr His Glu Gln Leu Thr Pro Leu Val Arg Ser Ala Gly Thr Ser Leu  (60)

181   GTG AAC TTC TTC AGC AGT TTA ATG AAC CTC GAG GAG AAA CCG GCT CCT GCG GCT AAG TGA  240
(61)  Val Asn Phe Phe Ser Ser Leu Met Asn Leu Glu Glu Lys Pro Ala Pro Ala Ala Lys ***  (79)

241   GATGTGCCAGGCCAGTCTTCCCACCCCAGCTGCTCCACTGGCCACCGCTAGAGCCCCTCTCCCTACCTTCTGCCTGTTT  319

320   TCTCCAATAAATGCGGAAGGAGTT-poly(A)                                                 343
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Fig. 4 Nucleotide sequence of mouse apo A-II cDNA. The sequence is compiled from the sequences of the clones in Fig. 3. Amino acids encoded in the open reading frame are shown below the DNA sequence. Nucleotide substitutions found in SAM-P/1 are indicated by corresponding SAM-R/1 nucleotides above them (boxed) and altered amino acids are written above boxes. Prepeptide and propeptide are shown by solid and broken underlines, respectively. Amino Acid residues 1 to 79 correspond to AS_{SAM} or mature serum apo A-II. Asterisks show the termination codon. A putative poly A signal is indicated by a wavy line.

with that of SAM-R/1. In the SAM-R/1 clone, pSRA2-15, the residue 5 deduced from the cDNA sequence is proline specified by CCA codon. This is also the case with another SAM-R/1 clone, pSRA2-1. But, the codon sequence is changed to CAG, glutamine, in SAM-P/1 clone, pSPA2-16. This is unusual because the second and the third letters of the codon are simultaneously changed. This mutation is considered not to be a cloning artifact in that other cDNA clones of SAM-P/1; pSPA2-3 and pSPA2-13 have the same nucleotide substitutions. This observation is consistent with the protein sequence data, that the residue 5 of serum apo A-II of SAM-R/1 and random bred slc:ICR mice is proline and that

```
rat     M K L L A M V A L L V T I C S L E G | A L V R R
        * * * * * * * * * * * * * * * * * * | * * * o *
mouse   M K L L A M V A L L V T I C S L E G | A L V K R
        * * * * *         * * o * * * * * * * | * * * o *
human   M K L L A A T V L L L T I C S L E G | A L V R R

                  prepeptide                  propeptide
```

Fig. 5 Comparison of the prepeptide and propeptide of mouse, rat and human apo A-II. Sequences of human and rat were predicted from the nucleotide sequence of the cDNA (24). Asterisks and open circles denote the identical amino acid and amino acid representing conservative substitutions, respectively.

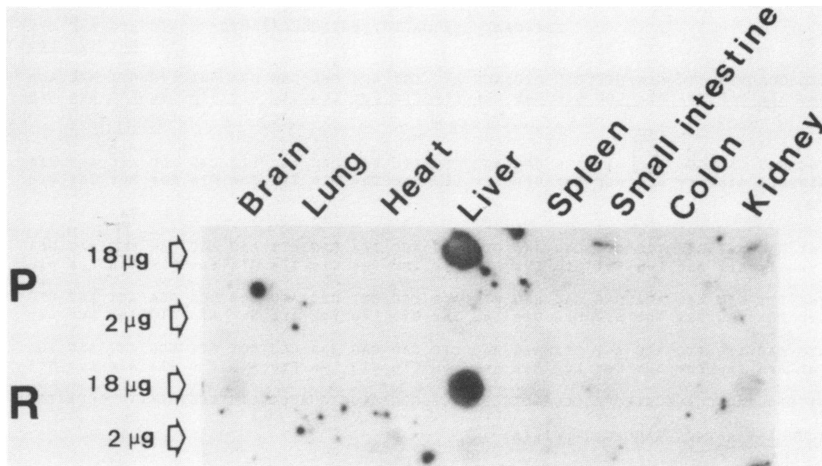

Fig. 6 Tissue distribution of apo A-II mRNAs. Total RNA isolated from tissues from SAM-P/1 and SAM-R/1 was dot blotted onto nitrocellulose filter and the filter hybridized with nick-translated pSPA2-16 probe and exposed for 24 h.

of both the AS_{SAM} and serum apo A-II of SAM-P/1 is glutamine.

Additional nucleotide substitution can be seen at residue 38 (Fig. 4). In SAM-R/1 clones, the residue 38 is valine, specified by GTC codon. In SAM-P/1 clones this residue is alanine by GCC codon. These amino acid substitutions agree well with the protein sequence analysis of serum apo A-II of SAM-R/1 and SAM-P/1.

RNA dot blot analysis

The insert of pSPA2-16 was used as a hybridization probe in dot blot analysis of total RNA prepared from the tissues of SAM-P/1 and SAM-R/1 (Fig. 6). Apo A-II gene was predominantly expressed in the liver and its expression was much the same, in both strains. No significant amount of apo A-II mRNA was detected in other tissues; brain, lung, heart, spleen, small intestine, colon, kidney.

Southern blot analysis

The pSPA2-16 probe was used in a Southern blot analysis of the genomic DNA from SAM-P/1 and SAM-R/1 (Fig. 7). Each DNA sample was digested with XhoI, HindIII, or AvaII. The results showed that the pSPA2-16 probe hybridizes to a single 10 kb XhoI fragment, a single 4 kb HindIII fragment and three fragments of 1.5 kb, 0.45 kb and 0.35 kb in the AvaII digest. It should be noted that the size of each fragment is indistinguishable between SAM-P/1

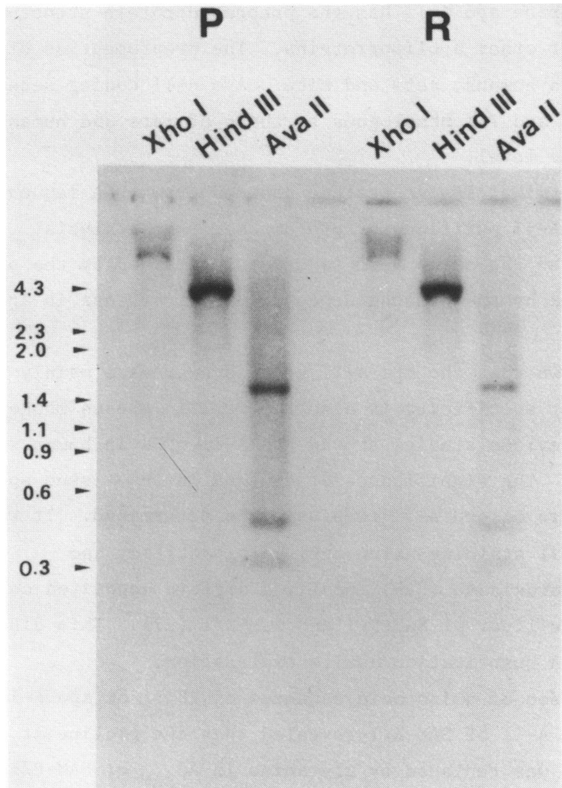

Fig. 7 Southern blot analysis of the apo A-II gene of SAM-P/1 and SAM-R/1 mice. 10 μg of DNA was digested by the restriction enzyme indicated above each lane, fractionated by 1.2% agarose gel, transferred to nitrocellulose filter, hybridized with nick-translated pSPA2-16 probe, and exposed for 24 h.

and SAM-R/1. This suggests that the apo A-II gene of SAM-P/1 suffers no gross change such as a large deletion or insertion of DNA, compared to that of SAM-R/1.

DISCUSSION

SAM-P series is a murine model for accelerated senescence. Spontaneous age-associated systemic amyloidosis occurs in a high frequency in SAM-P. Immunochemical analysis of this unique senile amyloid protein (AS_{SAM}) purified from SAM-P mice liver revealed that AS_{SAM} is a fibril protein consisting of apo A-II, one of the plasma apolipoproteins (7).

From SAM-P/1 and SAM-R/1 mice, we have identified cDNA clones corresponding to murine apo A-II. Nucleotide sequences of these clones

revealed that murine apo A-II has the preproapoprotein structure, as noted in human apo A-II or other apolipoproteins. The prepropeptide of apo A-II is well conserved in humans, rats and mice. Apo A-II coding sequence of mouse (SAM-P/1) is 84% and 71% homologous to those of rats and humans, respectively, at the nucleotide level.

The COOH-terminal lysine residue deduced from cDNA sequences is not found in AS_{SAM} or apo A-II purified for protein sequence analysis. This type of difference between the amino acid sequence determined by the protein sequence and that of coded by the cDNA sequence is the first case in apo A-II or other apolipoproteins.

We have shown that the apo A-II gene is expressed mainly in the liver in mice. This liver specificity is also reported in Rhesus monkey and humans (25) although previous studies showed apo A-II mRNA in human small intestinal epithelium (26). The significance of changes in the tissue specific expression pattern of apo A-II remains to be determined. It was reported that immunohistological staining using anti-AS_{SAM} antibody and preoxidase-antiperoxidase (PAP) localized amyloid deposited cells in the small intestine and the liver of SAM-P/1 and SAM-R/1 (27). This discrepancy with the apo A-II mRNA quantitation awaits explanation.

The comparison of amino acid sequence of AS_{SAM} or apo A-II of SAM-P/1 with that of apo A-II of SAM-R/1 revealed that the proline at position 5 in SAM-R/1 apo A-II was replaced by glutamine in AS_{SAM} or SAM-P/1 apoA-II. The nucleotide sequence analysis of cDNA clones for apo A-II of SAM-P and SAM-R confirmed that this amino acid substitution is caused by two nucleotide substitutions of a CAG codon for the CCA codon although the third base mutation may be due to polymorphism not associated with the substitution from proline to glutamine. Replacement of proline for glutamine at position 5 may cause conformational alteration in the protein molecule. Replacement of valine for alanine was also found at position 38. However, position 38 seems to be merely a polymorphic amino acid and is probably non-essential for the deposition of apoA-II because the valine/alanine amino acid polymorphism at this position was observed in random bred slc:ICR mice.

To investigate the relation of the position 5 amino acid substitution and SAM amyloidosis, we intended to use the restriction fragment length polymorphism (RFLP) of the apo A-II gene deduced from amino acid substitution at this position. The sequence from nucleotide 9 to 15, CGGACCA of SAM-R/1 apo A-II cDNA clones is cleaved by AvaII (the nucleotide recognition site is GGACC) but not for the corresponding sequence of SAM-P/1, CGGACAG. However, Southern hybridization pattern in Fig. 7 did not meet with our expectation.

Three common bands were detected in both SAM-P/1 and SAM-R/1. In humans and rats, the second intron is located at the 29 base upstream from the first nucleotide coding for the residue 5 of mature apo A-II (24, 28). If the insertion site of the intron is conserved in mice, the nearby AvaII site in the second intron would make it difficult to distinguish the restriction fragment length of SAM-P/1 from that of SAM-R/1.

While there is no evidence to show the significance of position 5 amino acid substitution for the apo A-II deposition as an amyloid fibril, cDNA clones described in this report may be useful for investigations on the molecular mechanisms and genetics of amyloidosis.

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