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COMPLEMENTARY DNA CLONING AND NUCLEOTIDE SEQUENCE OF RABBIT SERUM AMYLOID A PROTEIN

Bimal K. Ray† and Alpana Ray†*

Departments of †Veterinary Microbiology and *Veterinary Pathology
College of Veterinary Medicine
University of Missouri
Columbia, Missouri 65211

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A complementary DNA clone encoding serum amyloid A protein has been isolated from an acute rabbit liver cDNA library. Complete nucleotide sequence analysis reveals that the cloned gene contains a 24 bases 5′ untranslated region, 369 bases coding region and a 106 bases 3′ untranslated region. Primer extension analysis indicates that the full-length 5′ untranslated region contains 80 nucleotides. Northern blot analysis of mRNA from normal and acute rabbit livers demonstrates that this gene is expressed constitutively at a low level and undergoes induction of transcription in response to acute inflammation by the administration of turpentine.

Serum amyloid A (SAA) is a high density lipoprotein associated plasma protein which is synthesized by the liver. It is a member of a group of proteins called acute phase reactants that appear in the plasma in response to a variety of stimuli especially related to acute or chronic inflammation, infectious and malignant conditions (1). Gene for this protein codes for a 12 Kd protein found in the serum of men, mice and other mammals. It is well conserved in evolution and antibodies can precipitate SAA across species lines (2). However, sequence heterogeneity was observed with several amino acid substitutions (3, 4). This suggested that multiple chromosomal genes for SAA are likely. Subsequent studies have demonstrated the presence of three SAA structural genes and one pseudogene in mice (5).

In order to investigate whether induction of acute phase is responsible for any change in the expression of this gene in rabbit liver we constructed a cDNA library from an acute phase rabbit liver. Induction of acute phase was achieved by subcutaneous injection of turpentine. In this study, we report cloning of a rabbit SAA cDNA by application of PCR using primers based on homologous sequence in the SAA family.

MATERIALS AND METHODS

mRNA isolation

A New Zealand male rabbit (2-3 kg) was injected subcutaneously with 1.0 ml of concentrated turpentine oil in the dorsal lumbar region (6) to induce acute phase condition. Liver was collected

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24 h after turpentine injection and RNA was isolated by guanidine thiocyanate (7) followed by oligo(dT) cellulose column chromatography (8).

DNA amplification by PCR

Poly(A)$^+$ RNA prepared from acute rabbit liver was used to generate oligo(dT)-primed double stranded cDNA (9, 10). This preparation of cDNA was used as the template in the amplification reaction. Oligonucleotide primers were synthesized on an Applied Biosystems oligonucleotide synthesizer. The sequences of the primers were SAA#1, 5'-AAGTACTTCCATGCTCGG-3' (forward) and SAA#2, 5'-GTCTTTGCCACTGCGGCC-3' (reverse). Choice of these sequences for oligonucleotide primers were made from the conserved region of SAA protein, (3-5). PCR was performed in a Coy Tempcycler with a GeneAmp kit (Cetus) for 30 cycles (1.5 min at 95°C, 2 min at 52°C and 2 min at 72°C). PCR amplified DNA was cloned in pTZ19U plasmid (United States Biochemical) and the sequence was determined by dideoxy nucleotide chain termination method (11) using denatured double-stranded cDNA template. Comparison of this sequence with that of mouse (5) showed more than 75% homology indicating that the insert was indeed a partial cDNA of rabbit SAA protein.

Screening a cDNA library and sequencing

The partial SAA cDNA obtained by PCR amplification was used as a probe to screen a cDNA library of acute liver mRNA (10) by colony hybridization. Several positive clones were obtained. One clone, pSAA55, containing the longest insert was chosen for sequence analysis by dideoxy nucleotide chain termination method (11).

Northern blot analysis

Poly (A)$^+$ RNA from normal and acute rabbit liver was fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred onto a nylon membrane (12). Hybridization with random-primed ^{32}P-labelled pSAA55 cDNA (13) was carried out at 42°C for 15 h in 5X SSC, 50% formamide, 5X Denhardt, 100 μg/ml sonicated salmon sperm DNA, 0.1% SDS followed by washing in 0.2X SSC, 0.1% SDS for 2 h at 45°C. SAA-specific mRNA was visualized by autoradiography.

Primer extension analysis

One microgram of poly (A)$^+$ RNA from acute rabbit liver was hybridized with 5' end-labelled oligonucleotide primer (10^6 cpm) corresponding to positions -21 to -7 with respect to the ATG start codon of the SAA cDNA. The probe and total RNA was heated at 90°C for 15 min in 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA buffer and then hybridized by incubating overnight at 50°C. Annealed mRNA and oligonucleotide was precipitated in ethanol, resuspended in 50 μl of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM spermidine, 1 mM each of four dNTPs, 1000 U/ml RNasin and 1000 U/ml of AMV reverse transcriptase and incubated at 42°C for 90 min. The sample was extracted with phenol/chloroform, and analyzed in a 6% sequencing gel.

RESULTS AND DISCUSSION

PCR amplification of a partial cDNA of rabbit SAA protein

Two regions containing conserved amino acid sequences of SAA protein (5) were chosen for designing primers for PCR analysis. Poly (A)$^+$ RNA was isolated from acute rabbit liver and first strand cDNA was synthesized by oligo(dT)-primed reverse transcription and second strand by using RNase H, E. coli DNA polymerase I and Klenow fragment of DNA polymerase I. Such ds cDNA was used as a template for DNA amplification by PCR using SAA#1 and SAA#2 primers.

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                                M  K  L  L  S  G  L  L  L  C  S  L  V  L    14
GCTG CTCCACCAAA ACCCAGCACC ATG AAG CTC CTC TCA GGC CTG CTT CTC TGC TCC TTG GTC CTG   66

 G  V  S  G  Q  G  W  F  S  F  I  G  E  A  V  R  G  A  G  D                34
GGA GTC AGC GGC CAA GGC TGG TTC TCC TTC ATC GGT GAG GCT GTG AGA GGG GCA GGG GAC   126

 M  W  R  A  Y  T  D  M  R  E  A  N  Y  I  N  A  D  K  Y  F                54
ATG TGG AGA GCC TAC ACT GAC ATG AGA GAA GCC AAT TAC ATA AAT GCA GAC AAG TAC TTC   186

 H  A  R  G  N  Y  D  A  A  Q  R  G  P  G  G  V  W  A  A  K                74
CAC GCT CGG GGC AAC TAT GAC GCT GCC CAA AGG GGC CCT GGG GGT GTC TGG GCT GCC AAG   246

 V  I  S  D  V  R  E  D  V  Q  R  L  M  G  H  G  A  E  D  S                94
GTC ATC AGT GAC GTC AGA GAG GAC CTT CAG AGA CTC ATG GGC CAC GGA GCA GAG GAC TCG   306

 M  D  D  Q  A  A  N  E  W  G  R  S  G  K  D  P  N  H  F  R               114
ATG GAC GAC CAG GCT GCC AAC GAA TGG GGC CGC AGT GGC AAA GAC CCC AAC CAC TTT CGA   366

 P  K  G  L  P  D  K  Y  *                                                 122
CCC AAG GGC CTA CCC GAC AAA TAC TGA GCTCCCTACT. CTGCTGCCCC TGAGGACTGA CCCAGAGTAC   433

TGAGTTCCGT GTGCGTGAGC TGGTGGCGGG CATACAACTG GTGTCTAATA AATGCGGTATA AGACTGAAAAAA   503
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Figure 1. Nucleotide sequence of cDNA for rabbit liver serum amyloid A protein (pSAA55). In addition to the putative coding DNA sequence of 369 nucleotides, a 24 nucleotide 5′ untranslated region and a 106 nucleotide 3′ untranslated region have been sequenced. The putative amino acid signal sequence containing leucine-rich hydrophobic amino acid domain and the consensus sequence for polyadenylation signal are underlined. Sequences corresponding to the PCR amplified cDNA and PCR primers (SAA#1 and SAA#2) are underlined with solid and dashed line, respectively.

An amplified product of about 174 bp size was detected by agarose gel electrophoresis (data not shown). The PCR product was subcloned in pTZ19U vector and the sequence was determined from a purified clone (pSAA4). The nucleotide sequence of the insert was found to be 75% homologous to mouse SAA indicating that the insert was a partial cDNA of SAA protein and represented as a rabbit SAA probe.

Isolation of SAA-specific cDNA clones and sequence analysis of pSAA55

The cDNA library of acute rabbit liver was screened using the insert of pSAA4 as a probe and 50 positive clones were obtained from about 5000 independent recombinants. The largest clone (pSAA55) had an insert approximately 500 base pairs in length and was selected for further study. The nucleotide sequence of this cDNA is shown in Fig. 1. Comparison of the deduced amino acid sequence of rabbit SAA with that of mouse SAA shows 76% homology (5). The clone pSAA55 contains a putative leader sequence of 57 bases (underlined). As is commonly found, this leader peptide of 19 amino acids is rich in hydrophobic amino acids. Other features of the cDNA include 24 nucleotides of 5′ untranslated sequence, 312 nucleotides including the termination codon coding for the mature protein, 106 nucleotides of 3′ untranslated region (3′ UTR). The 3′ UTR contains usual sequence for polyadenylation (namely, AATAAAT in Fig. 1).

Primer extension analysis

To determine if the clone pRSAA55 contains a full length 5′ untranslated region we performed a primer extension analysis. A 15 base long antisense oligonucleotide (5′-

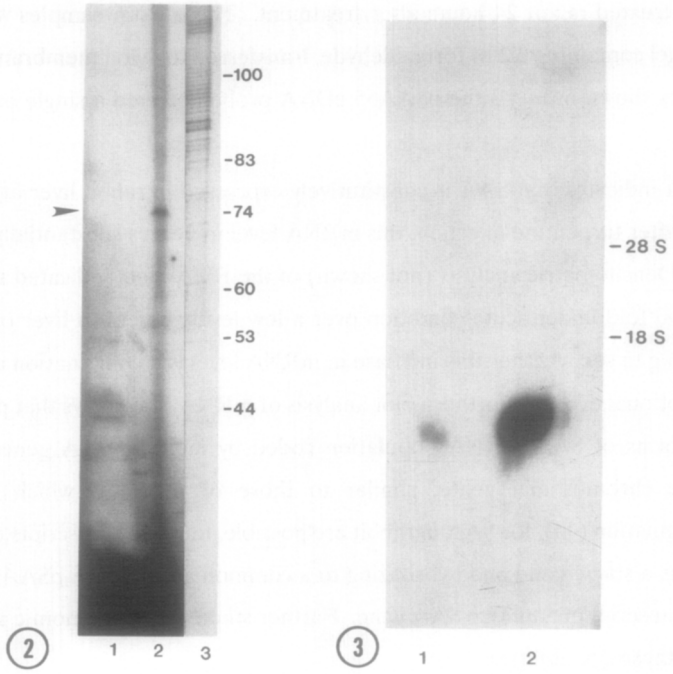

Figure 2. Primer extension analysis of SAA mRNA. A 5′ end-labelled oligonucleotide complementary to a sequence near the 5′ end of sequenced cDNA (-7 to -21 with respect to translation initiator ATG codon) was used to prime cDNA synthesis by reverse transcriptase using poly $(A)^+$ RNA as a template (lane 2). As a negative control, yeast tRNA was also used as template in separate extension reaction (lane 1). The products of primer extension reaction were fractionated in a 6% sequencing gel. Lane 3 contains a G-specific reaction of an unrelated sequence which was used as size marker to determine the length of the primer extended product.

Figure 3. Northern blot analysis of RNA from normal rabbit liver (lane 1) and acute phase rabbit liver (lane 2). One microgram of poly $(A)^+$ RNA was size fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane. Filter was hybridized with radiolabelled SAA cDNA following the procedure described in Materials and Methods. Ribosomal RNA (28S and 18S) and tRNA (4S), not seen here, were used as markers for size estimation and detected by ethidium bromide staining of the gel prior to transfer onto nylon membrane. The autoradiogram presented here was obtained following 24 h exposure of nylon membrane after hybridization.

GGGTTTTGGTGGAGC-3′, sequence present at the 5′ untranslated region of pSAA55) was synthesized. This oligonucleotide was ^{32}P-labelled at the 5′ end, hybridized with poly $(A)^+$ rabbit acute liver RNA and used to extend the cDNA further by using AMV reverse transcriptase. The primer-extended cDNA was fractionated in 6% sequence gel. As shown in Fig. 2 primer extended cDNA is of size 74 bases, which indicates that the clone pSAA55 is not full length and approximately 56 nucleotides shorter at the 5′ end.

Northern blot analysis

Prevalence of positive clones (50 out of 5000 colonies) indicated that SAA messenger RNA might be present at a high copy number in acute rabbit liver. Higher level of this mRNA has earlier been shown to be present in mouse (5). In order to test whether SAA mRNA population is induced in acute rabbit liver we used poly $(A)^+$ RNA isolated from the livers of a control (saline injected)

and a turpentine-treated rabbit 24 hours after treatment. These RNA samples were fractionated on a 1% agarose gel containing 2.2 M formaldehyde, transferred to nylon membrane and hybridized to SAA probe. As shown in Fig. 3 the pSAA55 cDNA probe detected a single band of about 550 bases long.

This result indicates that SAA is constitutively expressed in rabbit liver at a very low level. Following a 24 h after turpentine injection, this mRNA level increases substantially (compare lanes 1 and 2, Fig. 3). Densitometric analysis (not shown) of the RNA-blots indicated that SAA mRNA increases nearly 90 fold under acute condition over a low level present in liver of control animal. It will be interesting to see whether this increase in mRNA level is a combination of transcriptional and post-transcriptional events. Northern blot analysis of mRNA also shows that pSAA55 does not detect multiple forms of SAA mRNA population coded by multiple SAA genes in rabbit liver. Thus, if multiple chromosomal genes, similar to those of mice (5) which are involved in transcriptional regulation (14), for SAA in rabbit are possible, then the transcripts of such genes are either migrating as a single band and hybridizing to a common sequence in pSAA55 or pSAA55 is hybridizing to a transcript of a unique SAA gene. Further studies on the genomic DNA from rabbit liver will explore these possibilities.

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