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THE ISOLATION OF A CLONE FOR HUMAN α 1- ANTITRYPSIN AND THE DETECTION OF α 1- ANTITRYPSIN IN mRNA FROM LIVER AND LEUKOCYTES

J. Rogers", N. Kalsheker*, S. Wallis", A. Speer, C.H. Coutelle', D. Woods^ and S.E. Humphries"

" Biochemistry Department, St.Mary's Hospital Medical School, University of London, London W2 1PG, England

* Clinical Biochemistry Department, Welsh National School of Medicine, Royal Infirmary, Cardiff CF2 1SZ, England

' Cell Differentiation Department, Institute of Molecular Biology, Academy of Sciences GDR, Lindenberger Weg 70, GDR-1115 Berlin, German Democratic Republic

^ Cystic Fibrosis Program, Children's Hospital Medical Center, 300 Longwood Avenue, Boston, Ma 02115, U.S.A.

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A recombinant clone containing an insert complementary to α 1-antitrypsin (α 1-AT) mRNA has been isolated from a human adult liver cDNA library. The clone was selected by direct screening of recombinants with a synthetic oligodeoxynucleotide 17 bases in length corresponding to the known partial DNA sequence of the gene. The insert size of the clone is 250 base pairs. The DNA sequence of the clone has been determined and agrees with the published partial DNA sequence. There is one nucleotide difference from the published sequence, causing a single amino acid change at position 376 where aspartate replaces glutamate. The clone has been used to detect α 1-AT mRNA sequences in human liver and in a mixed leukocyte population containing monocytes and lymphocytes. A single mRNA approximately 1,400 nucleotides in length is observed in both leukocytes and liver. Leukocytes contain only 0.15% as much α 1-AT mRNA as liver.

α 1-AT is a major inhibitor of serine proteinases in human plasma (1,2). The liver is a known site of synthesis, although there are several reports of α 1-AT synthesis in lymphocytes and macrophages (3-7). An important site of action for the inhibitor is in the lung where it prevents the degradation of lung tissue by proteolytic enzymes released from leukocytes, and the secretion of α 1-AT by leukocytes in the lung may be an important local source of the inhibitor. It is thus important to establish whether α 1-AT is synthesised in leukocytes.

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There are approximately 30 α 1-AT (proteinase inhibitor, Pi) variants (1), two of which, S and Z, are particularly associated with disease. About 1/1,000 Northern Europeans are Pi-ZZ or Pi-SZ, both of which are associated with low levels of inhibitor in the plasma. Such individuals are predisposed to chronic pulmonary emphysema and infantile liver cirrhosis (2,8). The site of mutation in the S variant is known; glutamate 264 is replaced by valine (9). For the Z variant, glutamate 342 is replaced by lysine (10). The mutations in both variants cause reduced secretion of the protein into the plasma.

Recently, the entire sequence of the baboon α1-AT cDNA has been reported, as well as the partial cDNA sequence of human α 1-AT (11). We have isolated and identified an α 1-AT cDNA clone from a human cDNA liver library, confirmed the published sequence, and identified one amino acid variant. We have also used this probe to detect α 1-AT mRNA sequences in leukocytes.

MATERIALS AND METHODS

Isolation of adult human liver RNA and construction of the cDNA library Fresh human adult liver was obtained post-mortem and mRNA prepared using oligo(dT)-cellulose (12,13). The cDNA library was constructed by G-C tailing into the PstI site of pAT153, using previously described methods (14). The library contains 2,000 independent recombinants.

Recombinants were plated at low density and individual coloies were picked into mitrotitre wells. Clones were screened with labelled oligonucleotide probes, using a modification of the Grunstein-Hogness procedure (15). After sealing and baking, the filters were washed with 6xSSC/5xDenharts/0.05% sodium pyrophosphate/0.5% sodium dodecyl sulphate/100 µg/ml herring sperm DNA at 37° overnight. Any bacterial protein still adhering to the filters was removed by gentle rubbing with a gloved finger.

The oligonucleotide probe specific for α 1-AT, 17 bases long, was synthesised using the published sequence of 1-AT (11). The probe (GGACCTACTGTAATTTC) is for the last 17 bases of the untranslated region of the mRNA and includes the probable polyadenylation site (Fig 2). The synthetic oligonucleotide (a kind gift from Dr. M. Eaton, Celltech Ltd., Slough) was 5'-labelled with γ -[32P]ATP (5000 Ci/mmole) and T4 polynucleotide kinase (BRL Ltd.) (16). Probes were hybridised to filter-bound DNA at a concentration of 0.5 µg/20 ml, as described in Woods et al (15).

Analysis of DNA Plasmid DNA was isolated from bacteria by a modified cleared lysis method (17). It was digested with restriction enzymes (BRL) and analysed on agarose gels. DNA sequences were determined as described by Maxam and Gilbert (18) or Sanger (19). Appropriate restriction fragments were excised from agarose gels and were cloned in M13 MP8 (20). The recombinant single-stranded phage DNA was used as a template for the sequencing reactions in M13.

Northern blots Leukocytes and monocytes were prepared from fresh blood cells by using a Ficoll/Hypaque gradient (21). By this method of preparation, about one-third of cells are monocytes and two-thirds are lymphocytes. Total RNA was isolated from leukocytes (14), and poly(A)+ RNA was prepared using oligo(dT)-cellulose chromatography (13). Total RNA was prepared from frozen human adult liver as described (22). Northern blots were performed as described by Thomas et al (23). cDNA probes were nick translated using α-[32 P]dCTP (400 Ci/mmole) and a kit from Amersham, to a specific activity of 10 cpm/µg. Autoradiographs were scanned using a Helena Quick Scan R&D.

RESULTS

Initially, 350 recombinants were screened with the oligonucleotide probe, and an example of the resulting hybridisation is shown in Fig 1. One positive recombinant, pJNI, was selected for further study.

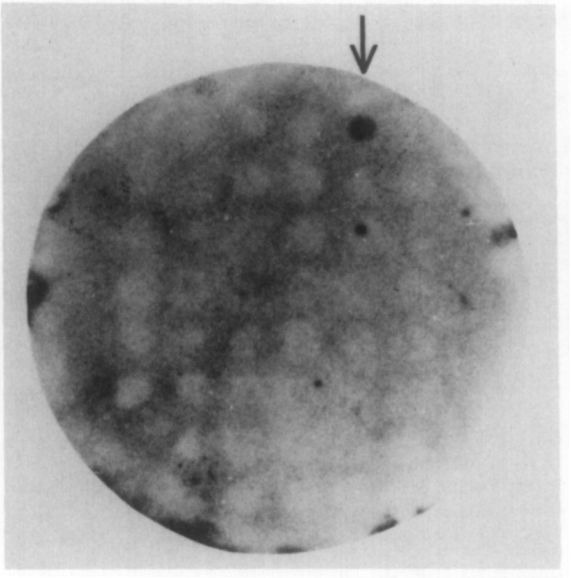

Figure 1: Detection of α1-AT recombinant. 350 recombinants were screened with the oligonucleotide probe. The filter were hybridised with 10^7 cpm in 10 mls at 42° for 24 hours, washed in 6xSSC at 40° and autoradiographed for 16hrs. The positive recombinant is indicated with an arrow.

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A           363                                   370
            Glu Val Lys Phe Asn Lys Pro Phe Val Phe Leu Met
CCCCCCCCCCCCCCGAG GTC AAG TTC AAC AAA CCC TTT GTC TTC TTA ATG


375                                            385
Ile [Asp] Gln Asn Thr Lys Ser Pro Leu Phe Met Gly Lys Val Val
ATT GAC CAA AAT ACC AAG TCT CCC CTC TTC ATG GGA AAA GTG GTG
     ↑

390             394
Asn Pro Thr Gln Lys Stop COOH
AAT CCC ACC CAA AAA TAA CTGCCTCTCGCTCCTCAACCCCTCCCCTCCATCCCT


      Oligonucleotide Probe
           -----------------
GGCCCCCTCCCTGGATGACATTAAAGAAGGGTTGAGCTGGAAAAAAA(50bps)CCCCCC
                       ------            ----------------
                       Poly A            Poly A Tail
                 recognition site
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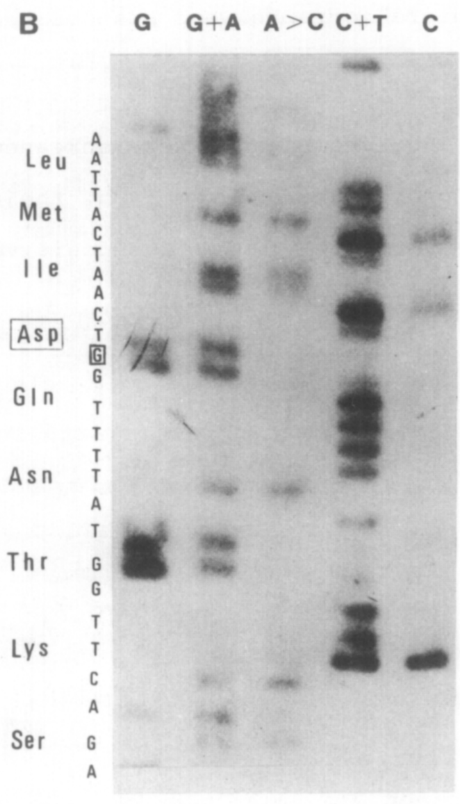

Figure 2:
A. The complete nucleotide sequence of the insert of pJN I. The nucleotide sequence of the coding strand, and the corresponding predicted amino acid sequence are shown. The A to G change resulting in the substitution of glu 376 by asp is indicated. The sequence of the oligonucleotide probe is also shown.

B. Sequence of the cDNA clone pJN I.
The base specific chemical cleavages are indicated over each lane. The sequence obtained from the gel is interpreted with the nucleotides and amino acids as shown. The change from the published sequence is indicated.

The complete sequence of pJN1 is presented in Fig 2. The insert size is 250 bp and includes 50 bp poly (A). The PstI site at the poly A end of the sequence has not been reconstituted. The sequence starts at amino acid 363 and agrees exactly with the published sequence of α 1-AT, except at amino acid 376 where an A to G change occurs. This would cause the substitution of glutamic acid 376 by aspartic acid. This change has been confirmed by M13 sequencing of the cDNA recombinant.

Detection of 1-antitrypsin mRNA The probe was used to investigate the size and amount of α 1-AT mRNA in adult liver and leukocytes. Total RNA or poly(A)+ RNA from these tissues was denatured, run on a 1.5% formaldehyde-agarose gel, and blotted. For an internal marker of RNA size, the filter was first probed with a mouse muscle actin cDNA recombinant, pAM91 (Fig 3). This probe contains about 90% of the actin coding region and will hybridise to human non-muscle actin sequences (24,25). As expected from previous reports,the actin probe hybridises to an mRNA of about 2,000 bp in both tissues (26). The α 1-AT probe detects an mRNA species of about 1,400 bases in both liver and leukocytes (Fig 3).

The relative abundance in the two tissues was estimated by densitometric scanning of the autoradiograph (Inset Fig 3). We calculate that leukocytes contain only about 0.15% of the α1-AT mRNA of the liver. Similar results have been obtained from two independent leukocyte RNA preparations.

DISCUSSION

There have been several reports that leukocytes and macrophages synthesise α 1-AT (3-7). For example, α 1-AT has been detected on the surface of macrophages by immunohistochemical methods, and newly synthesised

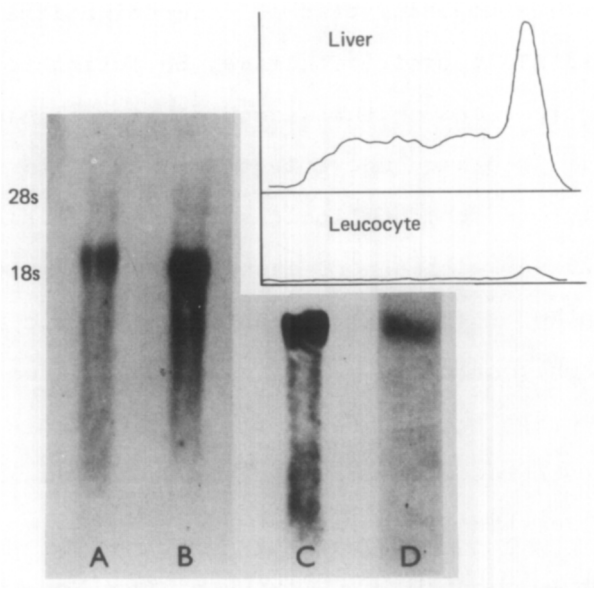

Figure 3: Northern Blot analysis of RNA from human liver and leukocytes. Track A and C, 10 µg of total RNA from human liver; Track B, 10 µg of total RNA and Track C 15 µg of poly A+ RNA from human leukocytes. Filters were hybridised to pAM 91 (A & B), 10^7 cpm in 10 mls, for 16hrs; or with pJN I (C & D), 10^7 cpm in 10 mls, for 16hrs. Filters were washed as described and exposed for 5 days. Tracks A + B and C + D were from separate gels. The size of ∝ 1-AT mRNA in liver was estimated by comparison with 28S, 18S, 23S and 16S ribosomal RNA markers.

From the densitometric scan of the autoradiograph (Inset), the leukocyte RNA sample contains about 1/40th of the ∝ 1-AT mRNA of the liver. 3% of leukocyte total RNA was retained by oligo dT cellulose, thus 10 µg of poly A+ RNA is equivalent to 300 µg of total RNA loaded. It can therefore be calculated that the relative abundance in leukocytes is only 0.15% of that in the liver.

[3H]-labelled protein released from macrophages into culture medium is precipitated by antibodies to ∝ 1-AT. We have used Northern blotting to analyse RNA from leukocytes for sequences that hybridise to an ∝ 1-AT cDNA probe. Hybridisation occurs to RNA of about 1400 bases in adult liver and leukocytes, which is similar in size to that reported by others (11). The level of mRNA in a mixed population of leukocytes (one-third monocytes, two-thirds lymphocytes) is 0.15% of that for liver. While it is possible that these mRNAs are coded by different genes, this seems unlikely as Southern blot analysis demonstrates only a single copy of the gene in the human genome

(27; our unpublished observations). The significance of macrophage α-1 AT is unclear. It may be acting to neutralise intracellular proteinases. However, it is possible that α1-AT synthesised by localised macrophages in an inflamatory lesion might allow a more rapid biological reaction in situ (8).

By comparison with the published DNA sequence of α1-AT, we have detected a G to A change in our cDNA clone. It is possible but very unlikely that such a sequence change could have been produced artefactually during the cloning procedure (28,29). The change we observe results in a Glu to Asp change at position 376. The consequence of this would be a variant protein showing no change in overall charge. The medical record of the individual from whom the liver sample was obtained gives no indication of a decreased plasma concentration of α1-AT, nor of diseases due to α1-AT deficiency. It is in all probability a new neutral mutation occuring in the α1-AT protein.

ACKNOWLEDGEMENTS

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