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ANALYSIS OF FIBRINOGEN GENES IN PATIENTS WITH CONGENITAL AFIBRINOGENEMIA

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Several cDNA clones coding for Aα, Bβ and γ chains of fibrinogen have been isolated from a human liver cDNA library. They were selected by differential hybridization with probes raised against fractionated liver mRNA (positive probes) and muscle and albumin mRNA (negative probes), then firmly identified by positive hybridization selection. Three of these clones, encoding Aα, Bβ and γ fibrinogen chain sequences, were further characterized by restriction maping and used as probes to characterize fibrinogen mRNAs from adult and fetal liver and fibrinogen genes in normal individuals and two afibrinogenemic patients.

The results indicate that there is a single copy of the fibrinogen genes which are present and grossly intact in afibrinogenemic DNA

Fibrinogen is a major glycoprotein of the blood which plays a dual role in hemostasis and is involved in many thrombotic disorders. In the primary hemostatic plug formation fibrinogen modulates the aggregation of platelets via its interaction with specific receptors (1). In the coagulation cascade the molecule is transformed into fibrin monomers by thrombin and clots (2). The molecule is composed of three non identical chains : Aα(Mr = 66,000), Bβ(Mr = 54,300) and γ (Mr = 48,500) (3) which are synthesised under the direction of three different and coordinately expressed RNAs(4-7). Recent structural analysis of chromosome from somatic cell hybrids has now established that mamalian fibrinogen is composed of three coding sequences for Aα , Bβ and γ chains linked in a small portion of chromosome 4 (8). Two human inherited disorders involve fibrinogen. Dysfibrinogenemia generally describes families of functionally altered molecules with amino-acid substitution.

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Afibrinogenemia and hypofibrinogenemia represent patients with defective production. Whether these deficiencies result from a gene deletion or insertion, an abnormal RNA processing or metabolism, or a translational defect has yet to be established. With the use of recombinant DNA it is now feasible to analyze the structure and the organization of the fibrinogen genes of these patients. We now report the first restriction endonuclease analysis of the DNA from two patients with congenital afibrinogenemia using specific cDNA probes for each fibrinogen constitutive chain. Results of this analysis indicate that the Aα, Bβ and γ genes are present and grossly intact in these two patients who produce no fibrinogen.

MATERIALS AND METHODS

Materials : Tissue sampling, purification of total human liver mRNA and cell free synthesis were performed according to methods already detailed (7, 9, 10). Human fibrinogen was purified and characterized as previously described, including freedom from contaminants (11). Antibodies were raised against denatured fibrinogen after boiling in the presence of 2 % SDS and 5 % 2-mercaptoethanol and monospecific IgG were purified as described (7). The purified antibodies were used to identify the neosynthesized Aα, Bβ and γ chains from a cell free translation mixture using a microimmunoaffinity chromatography technic as previously described (7, 9, 10). The neosynthesized peptides were analyzed on SDS-polyacrylamide gels according to Laemmli (12) and detected by autoradiography of incorporated ^{35}S-Methionine.

Hybridization probes

Poly A$^+$ mRNA were prepared from total liver mRNA by oligo dT-cellulose chromatography and fractionated by sucrose gradient sedimentation in the presence of 20mM methylmercury hydroxide. Fractions containing Poly A$^+$ mRNA coding for Aα, Bβ and γ chains were selected and reverse transcribted to make ss ^{32}P-cDNA. These probes were used to screen 1000 colonies from a human liver cDNA library (13, 14). A rat albumin ss^{32}P-cDNA and ss^{32}P-cDNA obtained from total human muscle mRNA were used as negative probes. All the clones hybridizing with the rat albumin probe and human muscle probes were eliminated. 40 remaining clones were selected as they hybridized intensly with ^{32}P-cDNA obtained from the fractionated poly A$^+$mRNA

Hybridization selection

The 40 isolated recombinant plasmids were analyzed for their capacity to select fibrinogen mRNA from a poly A$^+$ mRNA preparation, using the hybridization selection method (15-16). Typically the selected recombinant DNA was transfered to a nitrocellulose filter and denatured in situ by boiling for 90 sec. The filters were prehybridized for 2h at 42°C in the presence of 10 ug/ml polyadenylic acid and 10 μg/ml tRNA in 50% formamide, 10 mM piperazine, N, N-bis-(2-ethansulfonic acid) at pH 6.9 containing 0.4M NaCl, 4% SDS and 15 mM iodoacetate. Hybridization was performed with 40 ug poly A$^+$mRNA for 4h at 42°C. The selected mRNAs were eluted with distilled water containing 10 mM methylmercury hydroxyde and 5 μg tRNA, and translated in a cell free rabbit reticulocyte system. Three of the tested clones hybridized with Aα mRNA, 5 with Bβ and 4 with γ. Three of these clones, containing inserts higher than 0.8 kb, were further analysed ans used as probes : 60E1 for Aα 5B8 for Bβ and 61B9 for γ cDNA.

Restriction endonuclease analysis

DNA was prepared from peripheral white blood cell, by lysis in 0.1% SDS and proteinase K (17). Typically 300 ug of genomic DNA were recovered from 20 ml of citrated blood. Restriction enzymes were obtained from BRL and Amersham. Restriction digestions of human DNA were performed on 10-15 μg DNA with 2U enzyme/mg for 4h at 37°C. Complete digestion was monitored by electrophoresis of the endonuclease digests in 0.8% agarose gels. The digests were subsequently transfered on nitrocellulose filters by the method of Southern (18) and hybridized with ^{32}P-labeled inserts from 60 E1, 5B8 and 61B9 clones.

Northern blots hybridization

RNAs were separated by electrophoresis on 1.5 % (w/v) agarose gels in 10 mM phosphate buffer (pH 7), 2.2 M formaldehyde (19). After blotting on nitrocellulose sheet (20), hybridization was performed as in (21), using purified, nick-translated inserts as probes (10^6 CPM/ml).

RESULTS

Positive hybridization selection

SDS-polyacrylamide gel electrophoresis analysis of the polypeptides neosynthesized under the direction of mRNAs hybridizing with the three selected recombinant plasmids is shown in Fig. 1A. Purification of the synthesized material by immunoaffinity microchromatography indicated that 60E1, 5B8 and 61B9 inserts contained sequences hybridizing with mRNA coding for the preforms of Aα, Bβ and γ chain of fibrinogen (7).

Characterization of Aα, Bβ and γ messenger RNAs in human adult and fetal liver

Fig. 1B shows that 60E1 (Aα) plasmid probe hybridized with a single major species of mRNA, of 2400 bases. Further exposure of the filters for a long time revealed the presence of two additional heavy bands of 3700 and 4200 bases (not showns).

With 5 B8 (Bβ) probe, two mRNA species were observed, the more intense was 1850 bases long and the second 2120 bases long. With longer exposure two heavier supplementary species could also be detected, of 2400 and 3600 bases. (not showns).

61B9 insert hybridized with two discrete species of mRNA of about 2000 and 1800 bases respectively.

Characterization of the ds cDNA recombinant plasmids

The length of the inserts isolated by hydrolysis with PstI restriction enzyme were 920 bp for 60E1, 670 bp for 5B8 and 1470 bp for 61B9. Partial restriction

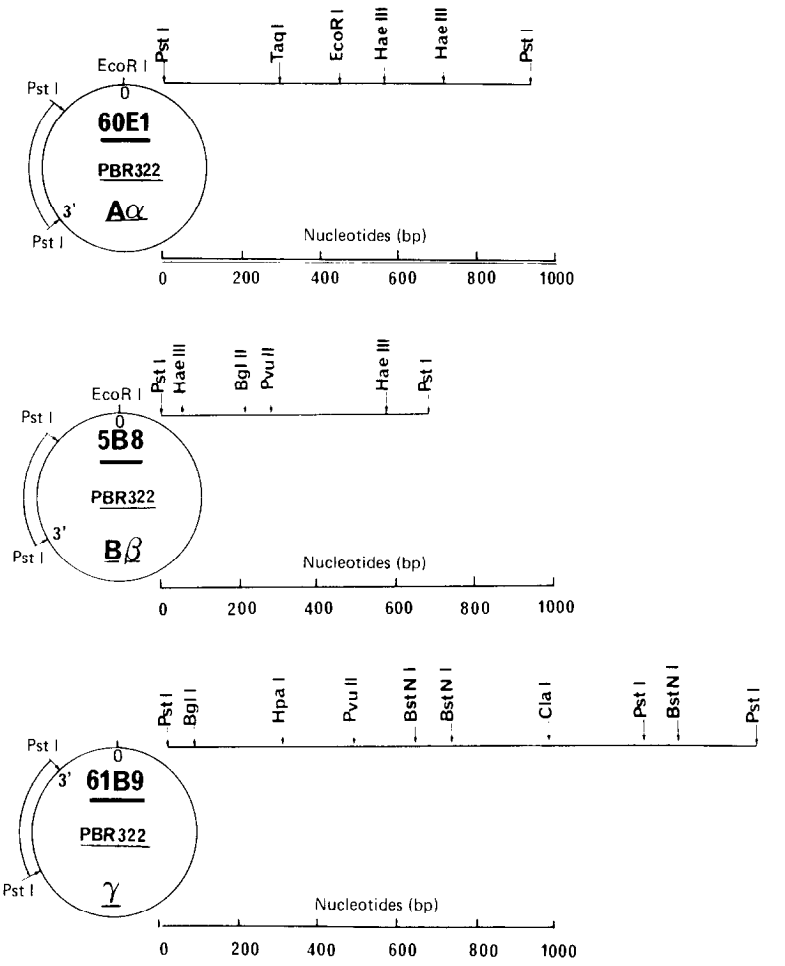

Fig. 1. Restriction maps of plasmids 60E1, 5B8 and 61B9 The 3' ends were localized by correct fitting of the restriction sites with published sequences (20, 22)

maps of the 60E1, 5B8 and 61B9 plasmids are shown in Fig. 2. It fitted exactly with the partial sequences of human Aα, Bβ and γ mRNAs recently published (22-24). From comparison of the published sequences of fibrinogen chain cDNAs (22-24), it appeared that insert 60E1 included a coding sequence near GLY 404 to PRO 625 in the C-terminal part of the A α chain, insert 5B8 a coding sequence near the ALA 272 to GLU 461 COOH terminal portion of the Bβ chain while the insert 61B9 corresponded to the complete chain mRNA coding sequence.

Analysis of fibrinogen genes in afibrinogenemic patients

Two individuals with congenital afibrinogenemia were studied. Subject 1 was a 20-year-old female and subject 2 a 27-year-old male. The absence of plasma

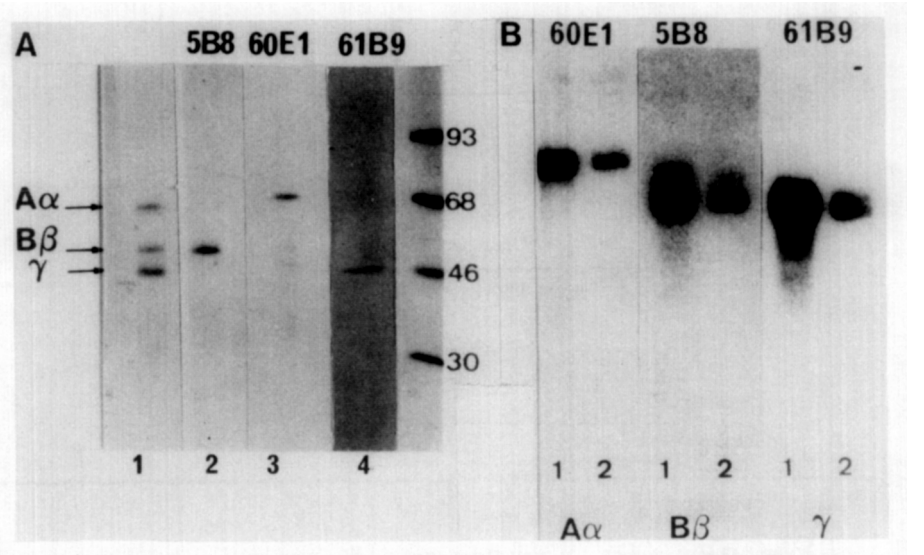

Fig. 2. Panel A - Positive hybridization selection and immunoaffinity microchromatography of the neosynthesized peptides. The mRNA were eluted from the different plasmid DNA-bound filters and translated in a cell free system. The translation mixture was passed over an antifibrinogen column and the adsorbed material was analyzed on SDS polyacrylamide gels. Lane 1 represents standard ^{14}C-fibrinogen. Lane 2 to 4 represent the material synthesized from RNA selected by clones 5B8, 60E1 and 61B9 respectively. Lane 5 are the molecular weights markers.
Panel B - Northern Blot analysis of mRNA from adult and fetal human liver. Two ug of poly A$^+$ mRNA were layered on each plot. Hybridization was performed with three clones selected from a human liver cDNA library.

fibrinogen in both patients have been documented for many years. The plasma and platelet fibrinogen levels of these patients were determined in several occasions by radioimmunoassay. Plasma fibrinogen represented less than 0.05 % (<1 ug/ml) and 0.4 % (<8 ug/ml) of a normal value for subject 1 and 2 respectively. Thus trace amounts of antigen were detectable with a sensitivity of 50 ng/ml. Although bleeding of the patients was performed at least three months after the last fibrinogen injection this minimal plasma content may represent residual fibrinogen from injection. Platelet fibrinogen was undetectable with a high sensitivity of 0.5 $\mu g/10^{11}$ platelets. Restriction endonuclease analysis was performed on the DNA from these two afibrinogenemic patients and compared with normal individuals. Six different enzymes were used and the digestion fragments were analyzed by Southern blotting. The electrophoretic patterns of the products and the intensity of

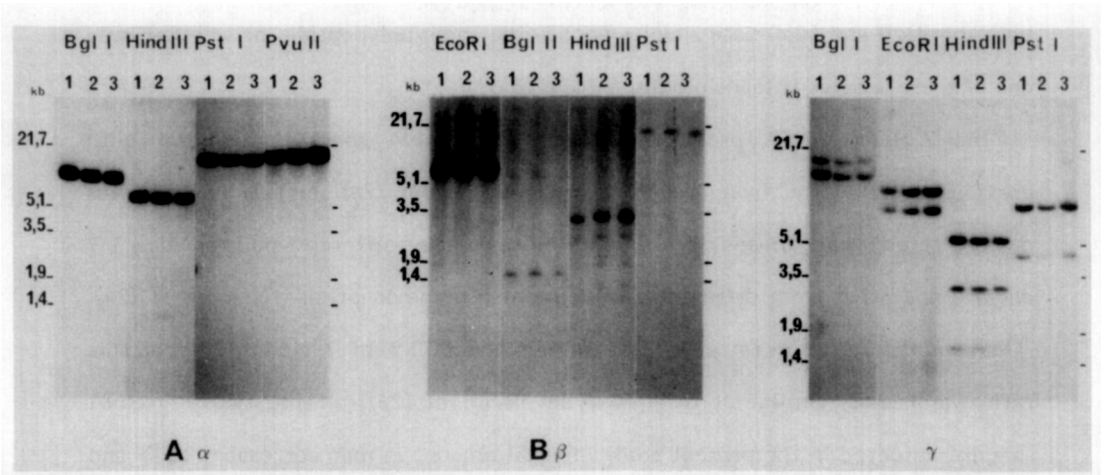

Fig. 3. Southern blot analysis of genomic DNA digested with various restriction enzymes. Hybridization were performed with clones 6OE1, for the A α chain, 5B8 for the B β chain and 61 B9 for the γ chain.
Lane 1 : normal DNA, lane 2-3 : afibrinogenemic DNA.

the bands were identical for the normal and afibrinogenemic individuals for the three chains. Results obtained with only four enzymes are illustrated in Fig. 3.

DISCUSSION

Fibrinogen is an abondant secreted protein, its mRNA represent 1 to 3 % of the total adult liver messenger (25). This proportion fits well with the frequency of fibrinogen clones in the total human liver cDNA library analyzed. A total of twelve clones containing coding sequences for either one of the chains (3 for Aα, 5 for Bβ and 4 for γ) were identified among 1000 colonies.

The lengh of the Aα mRNA (2400 bases) is similar to that previously reported for rat A α mRNA. In contrast, Crabtree and Kant reported that rat cDNA Bβ clone hybridized with a single mRNA species of 2120 bp, which corresponds to the heavier abundant species detected in man (5,6). In man, however, a second more intense mRNA band of 1850 bases exists, in adult as well as in fetal liver. The fact that the hybridization patterns are absolutely identical in several different liver RNA preparations, especially in adult and fetal preparations, seems to exclude the possibility that this heterogeneity is due to purification artefacts. More probably, B β messenger RNA exists in man under several forms of different lenghts, as it is found for various types of RNA. At the present time we cannot determine whether

both the 2120 and 1850 base species encode the same polypeptide, or whether only one of these species is translatable.

The γ chain cDNA probe recognized two mRNA species consistent with previous observations from Crabtree and Kant in rat (26). In this case it was demonstrated that these different mRNAs encode different polypeptides (γ and γ') and arize from differential splicing of a common primary transcript (24). γ' chains represents in rat as well as in man, about 10 % of γ chains (27), and this ratio was grossly similar at the mRNA levels in rat (26). If the two γ mRNA species, detected in the present study in equal amount in man correspond to γ and γ', this would imply that either the heavy species encoding the γ' chains is less translated than the γ mRNA or that the γ' chain is less stable than the γ chain.

It has been shown that the fibrinogen chain genes exist in man as well as in rat as single copy forms. The three genes are closely related on the same region of the chromosome 4 (8). Our results of Southern blotting of genomic DNA from two unrelated afibrinogenemic patients indicate that the absence of circulating fibrinogen in these patients is not due to a major deletion or a gross rearrangement of one or several of these genes, as in the case of α thalassemia (for review, see ref 28). Identification oF mRNA specific for the different fibrinogen chains, in the liver of these patients is impossible since needle or surgical biopsy are forbiden because of bleeding disorders. We don't know, therefore, if the disease is associated with the defect of one or several chains, and if this defect is related to absence or decrease of the specific mRNA. Cloning of the fibrinogen genes from such patients and study of their expression after transfection into cultured cells could will solve this problem in the next future.

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