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Characterization of cDNA coding for human factor XIIIa

(DNA sequence analysis/amino acid sequence identity/blood coagulation)

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ABSTRACT **A cDNA library prepared from human placenta has been screened for sequences coding for factor XIIIa, the enzymatically active subunit of the factor XIII complex that stabilizes blood clots through crosslinking of fibrin molecules. Two oligonucleotides, based on the amino acid sequences of tryptic peptides of factor XIIIa, were used as hybridization probes. Of 0.36 × 10^6 independent recombinants, 1 clone was identified that hybridized to both probes. The insert of 1704 base pairs coded for the amino-terminal 541 amino acid residues of the mature factor XIIIa molecule. Blot-hybridization analysis using this cDNA as a probe showed that the factor XIIIa mRNA from placenta has a size of approximately 4000 bases. The insert was used to rescreen cDNA libraries and to identify further factor XIIIa-specific sequences. The total length of the isolated factor XIIIa cDNA is 3905 bases, and it codes for a protein of 732 amino acids. In spite of the presence of factor XIII in blood plasma, we could not identify a leader sequence typical for secreted proteins.**

Factor XIII (FXIII) is a blood coagulation protein that is distributed both extracellularly (in plasma) and intracellularly (in megakaryocytes and platelets) (1, 2). In placenta the exact cellular location of FXIII is not known. Data obtained by indirect immunofluorescence suggest, however, that enzymatically active FXIII is located both intracellulary and extracellulary in the stroma of placental villi (3). FXIII is the last zymogen to become activated in the coagulation cascade during the blood-clotting process of vertebrates (4). FXIII molecules from blood platelets and megakaryocytes and from placental tissue are indistinguishable by several criteria and are each dimers composed of two identical *a* subunits (a_2) with a monomeric molecular weight of about 80,000 (3). Blood plasma FXIII, on the other hand, is a tetramer (a_2b_2) of molecular weight 300,000–350,000. It contains two noncatalytic *b* subunits ($M_r \approx 90,000$) in addition to its two catalytic *a* subunits, which are believed to be identical to those of platelet, megakaryocyte, and placental origin (3). Upon activation by thrombin and in the presence of Ca^{2+}, the plasma-derived FXIII dissociates its noncatalytic *b* subunits and yields the same active enzyme, FXIIIa*, as zymogens derived from megakaryocytes, platelets, or placenta. The enzymatic active form of FXIII, the FXIIIa* [also called activated fibrin-stabilizing factor, fibrinoligase, or plasma transglutaminase, EC 2.3.2.13 (protein-glutamine:amine γ-glutamyltransferase)] contains a cysteine in its active center and acts as a transglutaminase in the blood coagulation process. It catalyzes the formation of a γ-glutamyl–ε-lysyl bond between fibrin molecules (5), fibrin and α_2-plasmin inhibitor (6), fibronectin molecules (7), fibronectin and fibrin (7), and fibronectin and collagen (8) during the formation of an insoluble clot. The formation of covalent crosslinks by FXIII is important not only for normal blood coagulation but also for wound healing and placental retention (3).

Normal concentrations of FXIII in plasma are 10–20 mg/liter (9). Deficiency of FXIII results in inefficient wound and bone healing, and women with FXIII deficiency have a high risk of early abortions. The hereditary deficiency of FXIII is rare, with a frequency of 1 in 2 × 10^6 (10). Hereditary and acquired FXIII deficiencies are treated by substitution therapy.

Inherited deficiency of FXIII usually results from a failure to produce active subunit *a* or from the production of functionally abnormal forms (11, 12). Family studies of genetic variants of both *a* and *b* subunits have established that the structural loci for the two subunits are on different autosomes (13, 14). Moreover, it has been reported recently that the gene for the *a* subunit is distal to *HLA* on chromosome 6 (15).

The activation of FXIII is a multistep process involving (*i*) thrombin cleavage of the bond between Arg-37 and Gly-38 and release of the NH_2-terminal 37-residue peptide (16), (*ii*) a conformational change induced by calcium ions, and (*iii*) in the case of the plasma zymogen, weakening of the strong noncovalent interaction between subunits *a* and *b* (17).

Prior to this study, two groups have reported partial amino acid sequence data for enzymatically active human FXIII: (*i*) the amino acid sequence of the 37-residue peptide released during the activation of FXIII by thrombin (16) and (*ii*) a Gly-Gln-Cys-Trp sequence exposed at the active site of the molecule (18).

Here we report the isolation and DNA sequence determination of a cDNA coding for the placental *a* subunit, which we refer to as FXIIIa.

MATERIALS AND METHODS

Reagents. Restriction endonucleases were purchased from New England Biolabs or Boehringer Mannheim. Reverse transcriptase and RNase H were from P. H. Stehelin (Basel, Switzerland). Phage λgt10 DNA and an *in vitro* packaging system were obtained from Vector Cloning Systems (San Diego, CA). *Eco*RI methylase was purchased from Bethesda Research Laboratories.

Isolation of RNA and Preparation of cDNA Libraries. RNA was prepared from a mature human placenta by the standard guanidinium thiocyanate extraction procedure (19). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (20). Poly(A)$^+$ RNA (≈4 μg) was copied into cDNA with reverse transcriptase, and double-stranded cDNA was prepared by the method of Gubler and Hoffman (21). After treatment with T4 polymerase, the cDNA was methylated with *Eco*RI methylase and ligated to *Eco*RI linkers. The products were cleaved with *Eco*RI and separated in a 5–20% KOAc gradient in 1 mM EDTA containing 1 μg of ethidium bromide per ml at 50,000 rpm for 3 hr in a Beckman SW 65 rotor. cDNA larger than 1000 base pairs (bp) was pooled and precipitated by ethanol. An aliquot of 50 ng was ligated to

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Abbreviations: bp, base pair(s); kb, kilobase(s); FXIII, factor XIII; FXIIIa, *a* subunit of FXIII.
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*Eco*RI-cleaved λgt10 DNA (1 μg) and packaged *in vitro*. Phages were plated on *Escherichia coli* C600HFL. About 1 × 10^6 clear plaques were obtained.

Screening of a Human Placenta Library. The cDNA library was screened with two oligonucleotides derived from FXIIIa peptide sequences. Information on different FXIIIa peptides was made available to us by N. Takahashi and F. W. Putnam (personal communication). We chose the sequence Met-Met-Asp-Ile-Thr-Asp-Thr for the synthesis of a "short" 20-mer, degenerated probe with the sequence: ATG ATG GAY ATH ACN GAY AC, in which Y = T or C, H = T or A or C, and N = T or A or C or G. In addition, a "long" 66-mer, undegenerated probe was constructed according to the rules of Lathe (22) by using the following strategy. First, two overlapping oligonucleotides corresponding to the sequences Tyr-Gly-Gln-Phe-Glu-Asp-Gly-Ile-Leu-Asp-Thr-Cys-Leu and Asp-Thr-Cys-Leu-Tyr-Val-Met-Asp-Arg-Ala-Gln-Met-Asp were synthesized. The sequences of the two oligonucleotides A and B are:

(A) 5′ TATGGCCAGTTTGAGGATGGCATCCTGGACA-CCTGCCTG 3′

and

(B) 3′ CTGTGGACGGACATACACTACCTGGCCCGGG-TCTACCTG 5′,

with an overlapping complementary sequence of 12 nucleotides corresponding to the amino acid sequence Asp-Thr-Cys-Leu (underlined).

Next, the two oligonucleotides were separately labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled oligonucleotides were hybridized, and the double-strand was completed by incubation with DNA polymerase I, large fragment, using [α-^{32}P]ATP and three unlabeled deoxynucleotides. The resulting double-stranded probe was purified by electrophoresis on a 15% polyacrylamide gel, the specific activity being 2 × 10^8 Bq/μg. Both the 20-mer and the 66-mer probe were used for screening of the placenta cDNA library. For hybridization, the filters were washed in 0.45 M NaCl/0.045 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at 65°C overnight and hybridized after prehybridization in 0.9 M NaCl/0.006 M EDTA/0.09 M Tris, pH 8.3/0.2% polyvinylpyrrolidone/0.2% Ficoll/0.2% bovine serum albumin at calculated temperatures (e.g., 42°C for the 20-mer and 65°C for the 66-mer or nick-translated cDNA probes). All washes were performed in 0.90 M NaCl/0.09 M sodium citrate, pH 7/0.5% sodium pyrophosphate (23).

Characterization of cDNA Inserts. Positive phages were plaque-purified and grown in liquid culture. After lysis the bacterial debris was sedimented, and bacteriophage DNA was extracted as described (24) but without centrifugation in cesium chloride. cDNA inserts were mapped by suitable restriction endonuclease double digests and limited digests of ^{32}P-end-labeled fragments (25). Overlaps were specified by Southern blot analysis (26) and sequence analysis. Nucleotide acid sequence was determined by the chemical method of Maxam and Gilbert (27) as well as by the dideoxy method of Sanger (28). DNA sequence data were analyzed by University of Minnesota Apple II sequence analysis programs.

RESULTS

Isolation of Human FXIIIa cDNA. The amino acid sequence of human placental FXIIIa has been determined by classical methods involving Edman degradation (29). From the available data (N. Takahashi and F. W. Putnam, personal communication), we selected suitable amino acid sequences to design oligonucleotide probes (see *Materials and Methods* and Fig. 3). A 66-mer unique probe was constructed according to rules published by Lathe (22), and a 20-mer was synthesized that was 48-fold degenerate.

Clear plaques (3.6 × 10^5) of the human placenta cDNA library were screened with either the 20-mer or the 66-mer oligonucleotide as probe. Seven plaques hybridized to either one of the probes, but only one (λgt10-12) hybridized specifically when rescreened with both probes.

Phage DNA from this positive plaque was isolated, digested with *Eco*RI, and analyzed by Southern blotting with the same two probes. The analysis resulted in a large (1161 bp) *Eco*RI fragment hybridizing to the 66-mer probe and a small (543 bp) *Eco*RI fragment hybridizing to the 20-mer probe. As the 66-mer unique probe was constructed from partially complementary 39-mer oligonucleotides A and B, the λgt10-12 insert was also probed with A and B separately. Both 39-mer probes hybridized specifically to the large 1161-bp fragment, which was another strong indication that the cDNA insert indeed carries FXIIIa-specific sequences; this subsequently was confirmed by DNA sequencing.

The 1704-bp insert of λgt10-12 was used to screen the total cDNA library of 1 × 10^6 clear plaques for further FXIIIa-specific sequences. A total of 16 recombinants showed positive signals and were further characterized. Four of the positive signals showed DNA inserts located at the 5′ end of the λgt10-12 insert, but none of them were found to extend more than six bases from the 5′ end of the λgt10-12 insert sequence. Several cDNA inserts were identified from the 3′ end of the FXIIIa coding sequence, of which only the insert of λgt10-11 extends more than 2 kilobases (kb) beyond λgt10-12, the overlap between both inserts being 237 bp. Nucleotide analysis showed that this clone specifies the 3′ end of the FXIIIa mRNA.

Attempts using the primer extension approach to obtain clones from the 5′ end of the mRNA that extend further upstream into the nontranslated region were without success.

Identification of FXIIIa-Specific mRNA. The size of mRNA coding for FXIIIa was determined for poly(A)$^+$ RNA from human placenta and liver by electrophoresis through gels containing formaldehyde and by subsequent blot-hybridization analysis with the 1704-bp insert of λgt10-12 as a hybridization probe. One mRNA species of about 4 kb hybridized to the FXIIIa cDNA probe. This size of the FXIIIa mRNA agrees well with the size of the mRNA deduced from isolated cDNA inserts shown in Fig. 1. Only the placental poly(A)$^+$ RNA showed a signal, while no such signal could be detected in the case of liver poly(A)$^+$ RNA (Fig. 2). This finding agrees with our observation that screening of liver cDNA libraries containing a total of 5 × 10^5 independent recombinants with

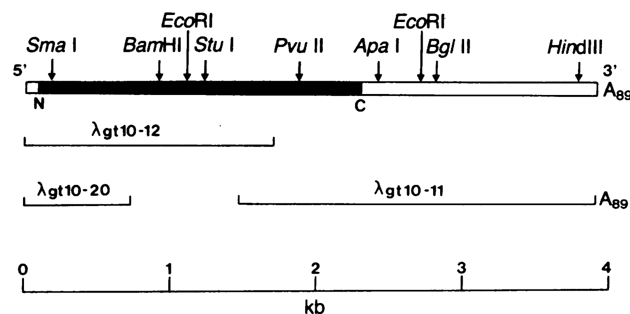


FIG. 1. Restriction map of human placental FXIIIa cDNA and the location of coding sequence. The figure shows the restriction map of FXIIIa cDNA and the location of the coding sequence (heavy bar). N and C indicate the amino and carboxyl terminus of the FXIIIa protein molecule, respectively. The overlapping cDNA inserts of clones λgt10-11, λgt10-12, and λgt10-20 cover the complete FXIIIa cDNA sequence.

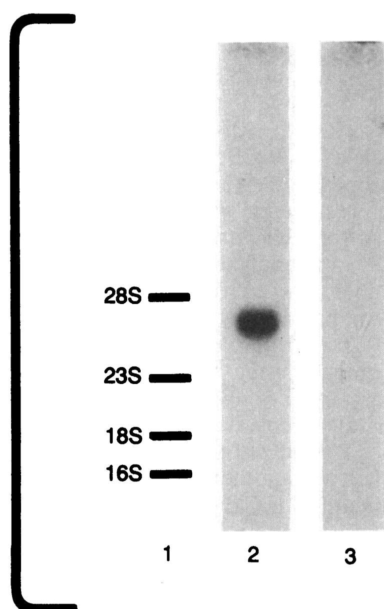
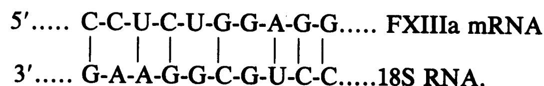


FIG. 2. Blot-hybridization analysis of mRNA. Poly(A)$^+$ RNA (4 μg) of human placenta (lane 2) and liver (lane 3) were transferred after electrophoresis through a denaturing agarose gel containing formaldehyde to a nitrocellulose filter. The filter was hybridized with the nick-translated cDNA insert of λgt10-12 at 65°C, washed as described, and exposed to Kodak XAR-5 films in DuPont cassettes with intensifying screens "Quanta Fast Detail." Lane 1 shows the positions of human and bacterial (*Escherichia coli*) ribosomal RNAs.

the oligonucleotide probes and even with the λgt10-12 insert did not result in isolation of positive clones.

Analysis of cDNA and Deduced Amino Acid Sequence. Nucleic acid sequencing was carried out by the chemical method of Maxam and Gilbert as well as by the dideoxy method of Sanger. This way 90% of the total cDNA was sequenced by two independent methods. The entire nucleotide sequence of the FXIIIa cDNA as determined from clones λgt10-11, λgt10-12, and λgt10-20 is displayed in Fig. 3. The deduced amino acid sequence is also shown. The cDNA shows an open reading frame up to nucleotide 2280 that carries the total information for 732 amino acids, including the initiator methionine for FXIIIa. The 5′ nontranslated sequence extends 84 bases upstream from the first AUG codon. Nucleotides 44 to 53 in the 5′ nontranslated region are partially complementary to the 3′-terminal region of the eukaryotic 18S RNA (30) and thus could represent a ribosome binding site:



The stop codon UGA is followed by about 1.6 kb of noncoding region, which ends in a poly(A) tail of 89 residues. At the 3′ end of the mRNA, the putative poly(A) addition signal AATAAA is located 15 nucleotides upstream of the beginning of the poly(A) tail. The codon usage for the FXIIIa mRNA is in good agreement with the data for eukaryotic proteins presented by Lathe (22). In fact, the 66-mer that was used as a hybridization probe shows differences in only seven positions to the actual sequence (*Materials and Methods* and Fig. 3).

From the deduced amino acid sequence, the mature FXIIIa, after removal of the initiator methionine, is a protein of 731 amino acids and has a molecular weight of 82,996. It has six potential glycosylation sites (asparagine in positions 17, 46, 541, 556, 613, and 686) located at the NH_2-terminal third and the COOH-terminal third of the molecule and nine cysteine residues clustered with one exception (Cys-695) at the middle third of the molecule. Through activation by thrombin, the Arg-Gly bond at amino acid positions 37–38 is cleaved. The sequence of the 37 residues released in the activation step agrees with data published by Nakamura *et al.* (31) and Takahashi *et al.* (29) but shows an additional valine in position 35 when compared to the data of Takagi and Doolittle (16). The sequence Gly-Gln-Cys-Trp at the active site was located at positions 312–315 of the FXIIIa molecule.

DISCUSSION

The amino acid sequence predicted here from the cDNA sequence is in agreement with the sequence of FXIIIa completed by classical protein sequence analysis (29). Minor changes ($Leu^{88}\rightarrow Phe^{88}$, $Gln^{651}\rightarrow Glu^{651}$), which have been verified after exchange of data prior to publication, might be due to a polymorphism of the FXIIIa gene within the population. While the purified protein represents a pool collected from many individuals, the mRNA was isolated from an individual mature placenta. A heterogeneity also was observed at positions 77 and 78, where the residues Gly-Lys could be detected in addition to the residues Arg-Arg that are specified by the cDNA-derived sequence. The fact that the extra methionine at the 3′ end was not found by protein sequencing might be due to protease activity and heterogeneity at the 3′ end of the isolated FXIIIa protein.

It was surprising, however, that the deduced amino acid sequence of the cloned FXIIIa mRNA does not contain a leader peptide, which is common in secreted proteins. The translational initiation codon was assigned to the first inframe AUG, which is located 84 bases downstream from the 5′ end of the isolated cDNA sequence.

As the open reading frame extends to the 5′ very end of the sequence, in theory one could assign another 28 amino acids 5′ to the methionine. There are, however, several reasons that make this methionine very likely to be the starting point of translation of the FXIIIa molecule. First, of four cDNA inserts that carry the 5′ end of the mRNA molecule, all stopped within six bases at the same place. This was taken as an indication that the 5′ end of the mRNA had been reached. Second, the preceding 28 amino acids that could be assigned show no resemblance to a typical leader peptide—e.g., have no stretches of hydrophobic amino acids. Third, the first amino acid of the mature FXIIIa molecule is a serine, which is acetylated (16, 29, 31). This is in agreement with an analysis of 1764 eukaryotic protein sequences that showed that serine, alanine, or glycine in the second position favor removal of the initiator methionine and N-acetylation of the NH_2-terminal amino acid (32). Fourth, the third base preceding the codon for the first methionine is an adenine, which is typical for the initiation of translation in eukaryotes (33, 34). Fifth, 41 nucleotides upstream from the initiation codon appears a putative ribosome-binding consensus sequence CCTCTG, which is frequently found in eukaryotic mRNAs (35). The above points indicate that FXIIIa does not have an NH_2-terminal signal sequence, which is typical for an intracellular protein. However, FXIIIa is obtained as an extracellular protein from plasma in association with the *b* subunit. The mechanism that promotes the release from the corresponding tissue has yet to be established.

FXIIIa contains six potential glycosylation sites (Fig. 3). Therefore, it was surprising that recent protein sequence analysis did not detect carbohydrates (29) in placental FXIIIa. In the literature, glycosylation of placental FXIIIa has been reported to be 1.5% (3).

The deduced amino acid sequence shows nine cysteine residues. However, from protein analysis there is evidence for only one of the cysteines being involved in a disulfide

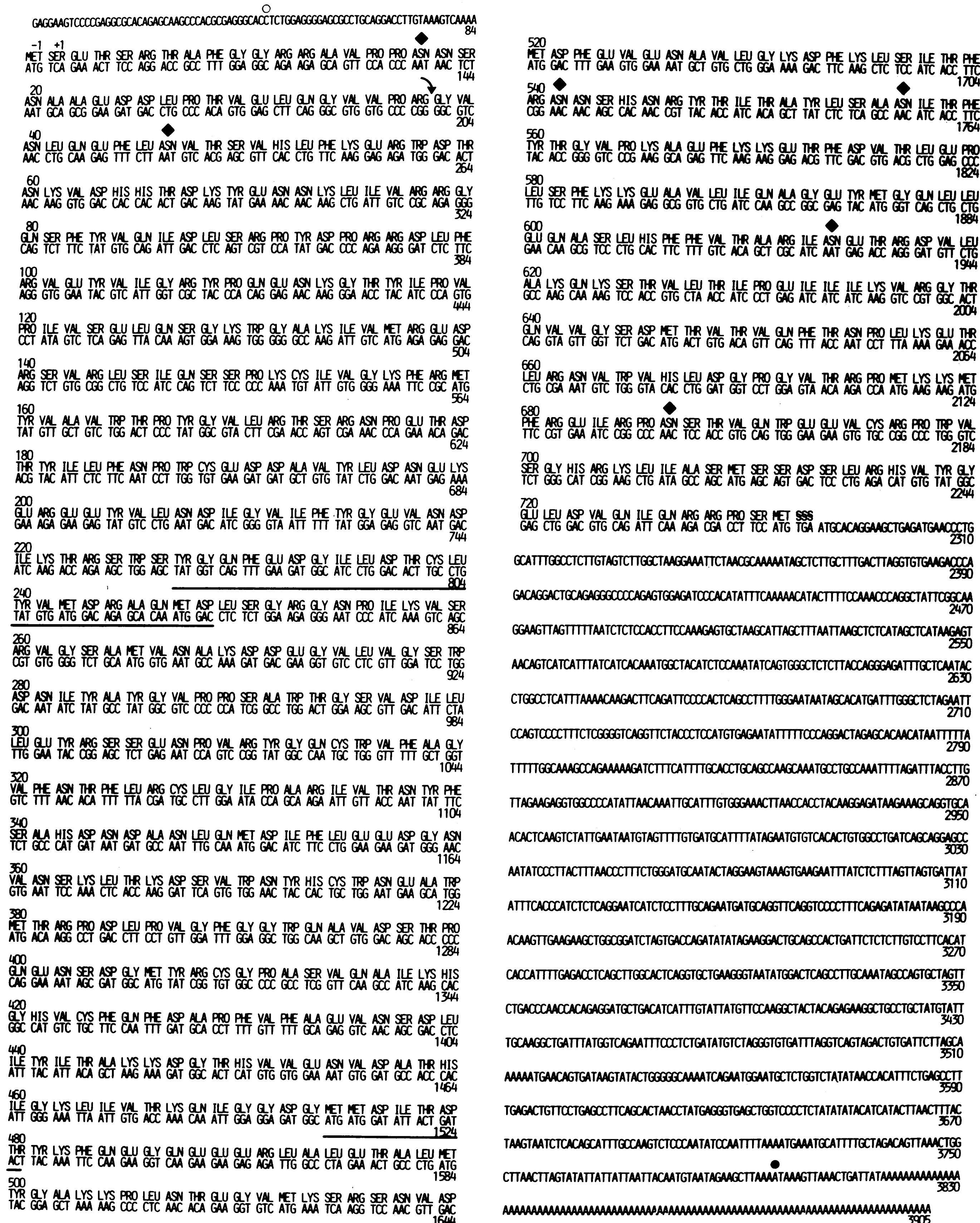


FIG. 3. Nucleotide sequence of human placental FXIIIa cDNA. The nucleotide sequence was determined by analysis of the overlapping clones shown in Fig. 1. The deduced amino acid sequence is displayed above the DNA sequence. Numbering of amino acids is relative to the amino-terminal serine (position 1). The solid arrow indicates the thrombin-activation cleavage site of FXIIIa. Potential attachment sites for carbohydrates are shown by solid diamonds. A putative ribosome binding site 41 bp upstream of the initiation codon AUG is marked by an open circle, and the poly(A) addition site 15 bp upstream of the poly(A) sequence is marked by a closed circle. Positions of the 20-mer and 66-mer oligonucleotides are underlined.

bridge, with four of the cysteines being in the free SH form (29). The fact that most cysteine residues are in a reduced form can also be taken as an indication for the intracellular biological role of FXIIIa.

Blot-hybridization analysis of poly(A)$^+$ RNA from placental tissue showed a mRNA for FXIIIa that is about 4000 bases in length. This is in good agreement with the length of the cloned cDNA sequence of 3905 bases. Thus, the FXIIIa mRNA carries 2196 bases of protein coding sequence, 1625 bases of 3′ noncoding sequence, and at least 84 bases of 5′ noncoding sequence. The unusually long 3′ noncoding sequence carries several open reading frames. Three of them, starting with a methionine, have potential coding capacities for >50 amino acids. The longest could code for a peptide of 67 amino acids (nucleotides 2691–2891), and the others, for peptides of 55 (nucleotides 3348–3512) and 54 (nucleotides 3317–3478) residues, respectively. Whether these open reading frames have any biological significance remains to be shown.

Comparison of blot-hybridization analysis of poly(A)$^+$ RNA from mature placenta and from liver showed that FXIIIa mRNA concentration is at least 20 times higher in placenta compared to liver. Up to now the liver was considered to be the major place for FXIIIa synthesis. The normal concentration of FXIIIa in plasma is about 10–20 mg/liter, while about 30 mg are extracted from a mature placenta (36). The concentrations of FXIIIa during placental development have not yet been reported in the literature, but FXIIIa might be specifically induced at the late stage of placental development where stabilization of blood clots is of high importance. In that respect it is noteworthy that the other examples of secreted proteins that do not carry a leader peptide are induced by hormones: ovalbumin and lipocortin (37, 38). In both cases the NH_2-terminal amino acid of the mature protein molecules (glycine and alanine, respectively) is acetylated.

The cloning of FXIIIa cDNA now enables questions to be answered regarding the glycosylation pattern and the mechanism of secretion by introducing the coding sequence into different host cells.

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