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In vitro synthesis of rat fibrinogen: Identification of preAα, preBβ, and preγ polypeptides*

(fibrinogen/cell-free translation/rat hepatoma cells in culture/tunicamycin)

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ABSTRACT Vertebrate fibrinogen consists of two sets of three nonidentical polypeptides that are synthesized in the liver. The subunits of fibrinogen have been synthesized in a cell-free, membrane-free translation system and compared with (*a*) polypeptides of fibrinogen purified from rat plasma and (*b*) subunits synthesized and secreted by hepatoma cells grown in culture. Rat hepatoma monolayers were grown with or without tunicamycin to prevent or allow glycosylation of the Bβ and γ subunits, respectively. Sodium dodecyl sulfate polyacrylamide gel analysis indicated that each of the polypeptides translated *in vitro* from mRNA is larger than its corresponding nonglycosylated fibrinogen chain. The primary translation Aα, Bβ, and γ chains are larger than their authentic nonglycosylated counterparts by 600, 1100, and 3000 daltons, respectively. Furthermore, the preAα and preBβ translation products are thrombin sensitive. These results strongly imply that signal peptides exist on each of the primary translation products of fibrinogen.

Vertebrate fibrinogen is a plasma glycoprotein that is capable of being activated by thrombin to form the clotting molecule, fibrin (1). Because of its large size and complexity, it may have unique and characteristic steps in its biosynthesis. Rat fibrinogen, like other vertebrate fibrinogens, consists of six polypeptides in two sets of three nonidentical chains, termed Aα, Bβ, and γ. The molecular weights of these chains are about 61,000, 58,000, and 51,000 respectively (2, 3). The rat Aα chain is not glycosylated whereas carbohydrate moieties are found in both Bβ and γ chains (unpublished data). This pattern of glycosylation is the same as in human fibrinogen (4, 5). Fibrinogen is synthesized in the liver, and current evidence suggests that fibrinogen chains are synthesized from three separate mRNAs, and the nascent polypeptide chains start to assemble cotranslationally while bound to the ribosome (6).

This report describes an investigation comparing rat fibrinogen purified from plasma, fibrinogen secreted from a rat hepatoma in monolayer cultures, fibrinogen translated from mRNA in a mRNA-dependent cell-free system, and fibrinogen elongated chains from rat liver polysomes. Evidence is presented showing that each polypeptide in fibrinogen primary translation products appears to be synthesized as a precursor slightly longer than the ultimately secreted polypeptide. The additional amino acids probably are "signal" sequences (7, 8) involved in translocation of the polypeptides from cytosolic to the cisternal side of the rough endoplasmic reticulum (rER).

MATERIALS AND METHODS

Preparation of Polysomes and mRNA. Total polysomes were prepared from rat livers by standard procedures (6, 9). However, cycloheximide and heparin were omitted from all buffers. Total RNA was obtained from polysomes after proteinase K treatment (250 μg/ml, 15 min, 37°C) by phenol/chloroform extraction (10). Total RNA was precipitated by adjusting the solution to 0.3 M in NaCl and adding 2.5 vol of absolute ethanol at −78°C. The RNA precipitate was collected by centrifugation. The precipitate was dissolved in H_2O, and 3 vol of 4 M sodium acetate (pH 5.5) was added to precipitate rRNA and mRNA. mRNA was isolated by oligo(dT)-cellulose chromatography (11). RNA was dissolved in wash buffer (0.5 M NaCl/0.1 M Tris/0.5% sodium lauryl sarcosine/10 mM Na_2EDTA, pH 7.5). The solution was heated to 70°C, cooled quickly, and passed through the oligo(dT)-cellulose. The column was rinsed with wash buffer and a 1:5 dilution of the wash buffer. RNA was eluted with distilled water and stored frozen at −50°C.

***In Vitro* Translation.** Reticulocyte lysate was prepared by the method of Hunt and Jackson (12). The lysate was treated with micrococcal nuclease and used according to Pelham and Jackson (13). The following were present in the translation system: 50–200 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine (Amersham), 100 mM potassium acetate, 0.63 mM spermidine, 0.2 mM GTP, 1.0 mM ATP, 20 mM creatine phosphate, 1.0 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.35 mg of creatine kinase (Sigma) per ml, and 18 A_{260} units of polysomes or 50 μg of mRNA per ml. Amino acids were present in concentrations as described (12). Usually, protease inhibitors were added to the system: Trayslol (1000 Klett units/ml), ϵ-aminocaproic acid (EACA) (1 mM), and phenylmethysulfonyl fluoride ($PhMeSO_2F$) (90 μM). Translations were done at 25°C, usually for 60 min. For certain experiments, pactamycin at 3 μM was added to the translation system (pactamycin was the kind gift of Upjohn).

Immunoprecipitations. Immunoprecipitations were carried out by the *Staphylococcus aureus* indirect immunoprecipitation procedure (14). Antibodies (20 μg) were added to 0.5-ml samples adjusted to 1% in Triton N-101, 0.5% in sodium deoxycholate, 0.1% in $NaDodSO_4$, 1 mg/ml in ovalbumin, 150 mM in NaCl, 10 mM in $NaPO_4$, 5 mM in methionine, and 1 mM in EDTA at pH 7.4. The samples were incubated overnight at 0°C and then incubated with 5 mg of *S. aureus* for 30 min at 25°C. The antigen–antibody–bacteria complexes were washed four times in 1.0 ml of the above detergent and salt buffer, with a 1-min centrifugation in a Beckman Microfuge. Antigens were recovered by incubation for 4 min in a boiling water bath in 2% $NaDodSO_4$/62.5 mM Tris, pH 6.8 (at 24°C)/10% (vol/vol) glycerol/11 mM dithiothreitol/0.005% phenol red. Bacteria were

Abbreviations: EACA, ϵ-aminocaproic acid; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; rER, rough endoplasmic reticulum; TM fibrinogen, fibrinogen from tunicamycin-treated hepatoma cells.

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removed by a 1-min centrifugation. Samples were alkylated by adding 0.1 vol of 0.5 M iodoacetamide (8).

$NaDodSO_4$ Gel Electrophoresis and Fluorography. The buffer system of Laemmli was used (15). A 4.4% (wt/vol) acrylamide stacking gel and an 11% (wt/vol) acrylamide running gel were used. Samples were always solubilized, reduced, and alkylated as described above for antigens. Both the stacking and running gels contained 0.1% $NaDodSO_4$. The acrylamide-to-methylenebisacrylamide ratio was 20:1 (wt/wt). Fluorography was performed as described by Laskey and coworkers (16, 17).

Purification of Rat Fibrinogen. Turpentine-stimulated rats were exsanguinated individually through the descending aorta into 1-ml of an anticoagulant solution designated buffer A (500 units of heparin and 2000 Klett units of Trayslol per ml, 1 mM *p*-nitrophenylguanidinobenzoate, 50 mM EACA, 1 mM EDTA, 0.11 mM $PhMeSO_2F$, 150 mM NaCl, and 10 mM $NaPO_4$ at pH 7.4). Fibrinogen was isolated by ammonium sulfate precipitation and chromatography on DEAE-cellulose (18).

Hepatoma Cell Cultures. Rat hepatoma cells (Faza) were grown in Williams medium E plus 10% fetal bovine serum in plastic flasks in the presence of insulin at 20 milliunits/ml. For labeling studies the medium was changed to fresh Williams medium E (methionine-free)/10% dialyzed fetal bovine serum/100–200 μCi of [^{35}S]methionine, 20 milliunits of insulin per ml/10 nM dexamethasone. Up to 48 hr later, the medium was harvested. To prepare nonglycosylated fibrinogen, tunicamycin was added at 2.86 μg/ml 12 hr before addition of labeled methionine [tunicamycin (lot 361-26E-239-A) was the kind gift of Eli Lilly Co.]. Medium was harvested and 0.1 vol of buffer A was added to the medium. It was centrifuged to remove cells and debris, extensively dialyzed against 0.1 M ammonium bicarbonate, and lyophilized. The lyophilized material was dissolved in 2 ml of 10 mM $NaPO_4$/150 mM NaCl, pH 7.4, and passed through a Sepharose 6B column. Fibrinogen was immunoprecipitated from appropriate eluted fractions.

Anti-Rat Fibrinogen. Antibodies against rat fibrinogen were prepared as described (3, 6).

RESULTS

Sources of Fibrinogen. The fibrinogens studied in these experiments differed in several properties, depending on their source. The variations among the six types of fibrinogen defined below reflect differences in their cotranslational or posttranslational processing. The first type, plasma fibrinogen, is fibrinogen purified from rat plasma. This represents the authentic molecule in its circulating form. The second type, hepatoma fibrinogen, is fibrinogen secreted from rat hepatoma cells in monolayer cultures. The third type, TM fibrinogen, is fibrinogen secreted from rat hepatoma cells treated with tunicamycin. This type apparently lacks asparagine-linked carbohydrate. The fourth type, mRNA translation products, is *in vitro* synthesized fibrinogen chains derived from mRNA. The fifth type, polysome translation products, is *in vitro* synthesized fibrinogen chains that were translated by using rat liver polysomes added to an *in vitro* system. These products were a mixture of chains that were initiated *in vivo* and *in vitro*. It is likely that this fifth type is a heterogeneous mixture of products in various states of cotranslational processing. Presumably, portions of some nascent fibrinogen polypeptides on the rat liver total polysomes were exposed to enzymes in the lumen of the rER whereas other chains were not exposed to those enzymes prior to polysome isolation, giving rise to the heterogeneity. The sixth type, elongated fibrinogen chains, is chains that were initiated *in vivo* but elongated *in vitro*. To obtain these chains, polysomes were added to a translation system supplemented with 3 μM pactamycin. The pactamycin allows nascent polypeptides to be completed but prevents any more initiations (19). To elucidate the steps in processing of fibrinogen, we have compared these types of fibrinogen by $NaDodSO_4$ gel electrophoresis.

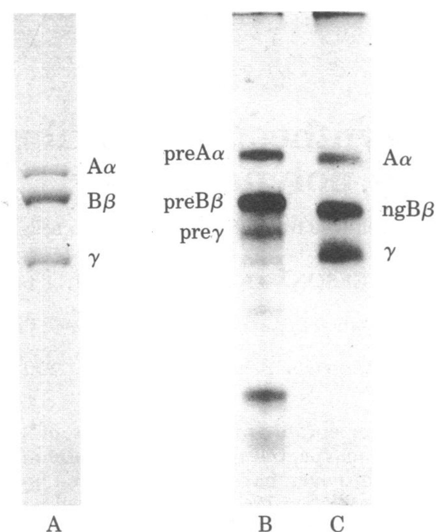

FIG. 1. $NaDodSO_4$ gel of fibrinogen translation products. Lanes: A, purified fibrinogen from rat plasma, stained with Coomassie R-250; B, mRNA translated *in vitro*, fibrinogen immunoprecipitate; C, polysomes translated *in vitro* in the presence of 3 μM pactamycin, fibrinogen immunoprecipitate. The prefix "pre" refers to a precursor form of a chain. The prefix "ng" refers to a chain missing an asparagine-linked carbohydrate moiety.

Analysis of Translation Products. In Fig. 1, two types of translation products are compared to plasma fibrinogen. All samples were reduced with dithiothreitol and alkylated with iodoacetamide prior to $NaDodSO_4$ gel electrophoresis. Lane A shows the usual types of subunits of plasma fibrinogen. Lane B shows mRNA translation products. These are the primary translation products and presumably contain all the amino acids encoded by the mRNAs for fibrinogen. Furthermore, these products could not have been modified by enzymes found in the lumen of the rER because there were no rER constituents in reticulocyte lysate. In lane C, polysomal elongated chains are shown. These chains were from polysomes translated *in vitro* in the presence of 3 μM pactamycin. Only chains initiated *in vivo* could be elongated in this system because pactamycin at this concentration inhibits initiation but not elongation (19). Many of the nascent fibrinogen chains on the polysomes had presumably already started to translocate through the lumen of the rat liver rER. It is highly probable that the chains with their NH_2 termini translocated could have undergone modifications associated with processing in the rER lumen of the rat liver. For example, a signal peptide could have been removed, intra- or interchain disulfide bonds could have formed, or core oligosaccharides could have been added to certain asparagine residues. Thus, elongated chains cannot be considered to be the primary translation products.

The molecular weight of each *in vitro* translated chain has been estimated. A least-squares fit of a plot of logarithm of molecular weight versus distance migrated for each chain in the $NaDodSO_4$ gel was performed. Protein molecular weight markers were run on the same gel as the translation products but are not shown in Fig. 1. The correlation coefficient of the best fit line was 0.987. The estimated molecular weights calculated from the best-fit line of the mRNA translated fibrinogen chains and the elongated polysome nascent chains (pactamycin treated) are shown in Table 1. The differences in molecular weights be-

Table 1. Molecular weights estimated from translation products

From total mRNA		From polysomes + pactamycin		Differences
Chain	Molecular weight	Chain	Molecular weight	(putative signal peptides)
PreAα	66,000	Aα	65,400	600
PreBβ	57,600	Bβ	56,500	1100
Preγ	53,800	γ	50,800	3000

tween the mRNA-translated and the polysome-elongated chains are attributed to NH_2-terminal signal peptides present on primary translation products that had been removed from nascent polypeptides that had extended through the rER membrane *in vivo* in the rat liver prior to isolation of the polysomes.

Thrombin Treatment of Translation Products. Treatment of rat fibrinogen with thrombin releases fibrinopeptides A and B from fibrinogen, giving rise to fibrin. Thrombin cleaves 17 and 14 residues from the NH_2 termini of authentic rat Aα and Bβ chains, respectively, but does not cleave γ chains. In order to determine whether or not *in vitro* translated rat fibrinogen chains were sensitive to thrombin, 2 μg of thrombin (99% pure human thrombin; 2–4 NIH units/μg) was added to 0.5 ml of previously synthesized translational products. Polysomes had been used as messenger source for this experiment. The *in vitro* synthesized products were treated for 10 min at 25°C with thrombin. The thrombin-treated translation products were then immunoprecipitated with antibodies against native rat fibrinogen, electrophoresed, and subjected to fluorography. These *in vitro* translation products are shown in Fig. 2, lanes D and E (with and without thrombin treatment). They were compared to radioactive authentic rat fibrinogen derived from rat hepatoma cells (Faza).

The carbohydrate present on Bβ and γ chains complicated comparison of these two chains to their *in vitro* translated counterparts. These two chains could be obtained in nonglycosylated forms by treating the hepatoma cells with tunicamycin. This drug prevents asparagine-linked glycosylation by preventing

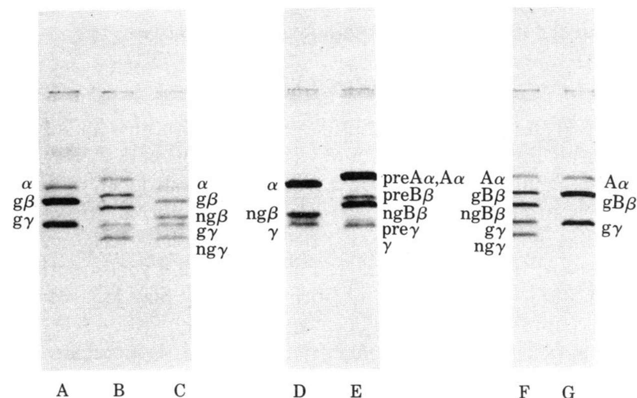

FIG. 2. Comparison of hepatoma cell fibrin and fibrinogen to polysome translation products. Lanes: A, fibrin chains from rat hepatoma cells; B, fibrinogen from tunicamycin-treated hepatoma cells; C, fibrin from tunicamycin-treated hepatoma cells; D, thrombin-treated polysome translation products; E, polysome translation products; F, fibrinogen from tunicamycin-treated hepatoma cells; G, fibrinogen from hepatoma cells. All samples were immunoprecipitated, solubilized, reduced, blocked with iodoacetamide, electrophoresed, and fluorographed. The prefix "g" (glycosylated) refers to chains that contain asparagine-linked carbohydrate; the prefix "ng" (nonglycosylated) refers to chains that lack the asparagine-linked carbohydrate moiety; the prefix "pre" refers to precursor forms of chains; presumably containing signal sequences.

the formation of *N*-acetylglucosaminylpyrophosphoryl dolichol (20). Both glycosylated and nonglycosylated fibrinogen chains can be obtained from a single culture under appropriate conditions, and the nonglycosylated chains can be readily identified. Authentic and nonglycosylated fibrinogens were similarly treated with thrombin and analyzed on the same gel. Lane G in Fig. 2 shows the typical banding pattern for rat fibrinogen from hepatoma cells with Aα, glycosylated Bβ chain (gBβ), and glycosylated γ chain (gγ). These bands comigrate with purified plasma fibrinogen. Lane F shows the banding pattern for fibrinogen from tunicamycin-treated hepatoma cells (TM fibrinogen). Five bands can be seen on the fluorogram; three of these bands comigrated with Aα, Bβ, and γ bands of authentic rat fibrinogen (lane G) and are marked as Aα, gBβ, and gγ. The two additional bands, labeled ngBβ and ngγ, are putative nonglycosylated Bβ and γ chains. These bands migrated distances corresponding to a molecular weight ≈3000 smaller than that of their glycosylated counterparts.

That there are only two forms of the Bβ or γ chains with respect to glycosylation, either normally glycosylated or nonglycosylated, implies that there is only one asparagine-linked carbohydrate moiety per chain. If there were two asparagine-linked carbohydrate moieties per chain, one would expect three bands for Bβ or γ at an intermediate dosage of tunicamycin. One band would have no carbohydrate moieties, one band would have one asparagine-linked derivate with carbohydrate, and a third band would have two asparagine residues derivatized with carbohydrate. Our result implies that each Bβ or γ chain has only one asparagine-linked carbohydrate, a result identical to that with human Bβ and γ chains (5).

Hepatoma fibrinogen and TM fibrinogen were treated with thrombin. The resulting fibrins were compared to the original fibrinogens (Fig. 2). (Lane A of Fig. 2 shows hepatoma fibrin.) After thrombin treatment, the α chains, now missing fibrinopeptide A, were 2000 daltons smaller than hepatoma fibrinogen Aα chains. When one compares fibrin α chains (lane A) to fibrinogen Aα chains (lanes B, F, and G), the difference in mass is readily observed. Glycosylated fibrin β chains, gβ, are smaller by about 1500 daltons than the glycosylated fibrinogen Bβ chain, gBβ (compare fibrin gβ in lane A to fibrinogen gBβ in lanes B, F, and G). The migration of glycosylated γ chains, gγ, is as expected, unaffected by thrombin treatment. The several bands of TM fibrinogen are shown in lanes B and F. The five bands of TM fibrinogen, from largest to smallest, are Aα, gBβ, ngBβ, gγ, and ngγ. Lane C shows the result of treating TM fibrinogen with thrombin. The resulting TM fibrin chains are α, gβ, ngβ, gγ, and ngγ. The top three TM fibrin bands—α, gβ, and ngβ on lane C—are smaller than the corresponding TM fibrinogen Aα, gBβ, and ngBβ chains (lanes B and F). They are smaller by about 2000, 1500, and 1500 daltons, respectively. These changes in size correspond to the losses of fibrinopeptides A and B. The last two bands from TM fibrin, gγ and ngγ, are not affected by thrombin treatment.

Translation products from rat liver polysomes are shown in lane E of Fig. 2. These polysomes were added to the *in vitro* translation system in the absence of pactamycin so that some new rounds of initiation could occur in addition to the elongation of nascent polypeptides still attached to the polyribosomal complex. Polysomes translated under these conditions yielded two sets of identifiable products. Some of the immunoprecipitated polypeptides contained signal peptides; however, presumably many of the elongated nascent chains already had had their signal peptides removed by signal peptidase *in vivo* prior to polysome isolation. Due to the small apparent difference in molecular weights of preAα and Aα, these two bands appear to comigrate in this fluorogram. However, preBβ and its pro-

cessed form, likely not glycosylated and identified as ngBβ, are clearly resolved. Likewise, preγ and γ, although faint, are also clearly resolved. When these translation products were treated with thrombin (lane D), the thrombin-sensitive Aα and Bβ chains were affected, yielding α chains and nonglycosylated β chains of expected mobilities. The translation product α chain comigrated with authentic α chain, as can be seen from the gel. After thrombin clipped the translation product preBβ and ngBβ chains, the resulting fibrin chain comigrated with nonglycosylated β fibrin chain from tunicamycin-treated hepatoma cells, identified as ngβ on lane C. The mobilities of translation products preγ and γ chain remained unaffected by thrombin treatment.

It is interesting that the *in vitro* elongated γ chain (lane E in Fig. 2) migrated close to the mobility of the glycosylated γ chain from authentic rat fibrinogen (lane G). We suggest that the elongated γ chain has already had carbohydrate attached to it *in vivo*. If the carbohydrate on the γ chain of the rat were near the NH_2 terminus of the molecule, as it is in the human (5), then one could expect this modification to occur as a cotranslational event. It has been shown that core oligosaccharide addition can occur as soon as an appropriate asparagine residue has translocated to the lumenal side of the rER membrane (21). If the rat γ chain asparagine residue is near the NH_2 terminus, it is likely that most of nascent γ polypeptides have been derivatized *in vivo* with the core oligosaccharide as a cotranslation event. When these modified nascent chains, still ribosome bound, are elongated *in vitro*, they would migrate with a mobility close to that of normally glycosylated authentic γ chain, gγ.

The opposite result probably occurs with rat Bβ chain. Human Bβ chain is glycosylated near the COOH terminus (5). If rat Bβ is glycosylated near the COOH terminus, then few of the appropriate asparagine residues would reach the lumenal side of the rER membrane while the polypeptide is still ribosome-bound. The consequence is that nascent Bβ chains elongated *in vitro* would be expected to migrate with the same mobility as nonglycosylated Bβ chains. The elongated Bβ chains had the same mobility in this gel system as nonglycosylated Bβ chains.

Signal Sequences Are at the NH_2 Termini of the Chains. To demonstrate that signal peptides are on the NH_2 termini of Aα and Bβ, a total mRNA fraction and a polyribosome fraction to which pactamycin was added were translated, and both sets of translation products were then treated with thrombin. Fig. 3, lane D, shows mRNA translation products. Lane C shows polysome-plus-pactamycin translation products. Each mRNA translation product appeared to be slightly larger than the analogous polysome elongated chain, strongly suggesting the presence of signal peptides. Lane B shows mRNA translation products treated with thrombin. Lane A shows the polysome elongated chains treated with thrombin. The α chains on lanes A and B are the same size. Also, the β chains on lanes A and B are the same size. The γ chains remained unaffected. Because the α chains, the large COOH-terminal fragments resulting from thrombin digestion of preAα and Aα chains, are the same size and because preAα is larger than Aα, it follows that the NH_2-terminal thrombic fragments of preAα and Aα are different sizes. The extra mass present in preAα must be on the NH_2 terminus of the preAα chain or bound to one of the first 17 amino acids at its NH_2 terminus. Similarly, the extra mass of the preBβ chain not in ngBβ elongated chain must precede the NH_2 terminus of the authentic Bβ chain or be covalently bound to one of the first 14 amino acids in the Bβ chain. These extra masses, 600 and 1100 daltons on the NH_2 termini of Aα and Bβ chains in all likelihood are signal peptides and must precede the authentic NH_2 termini of Aα and Bβ chains.

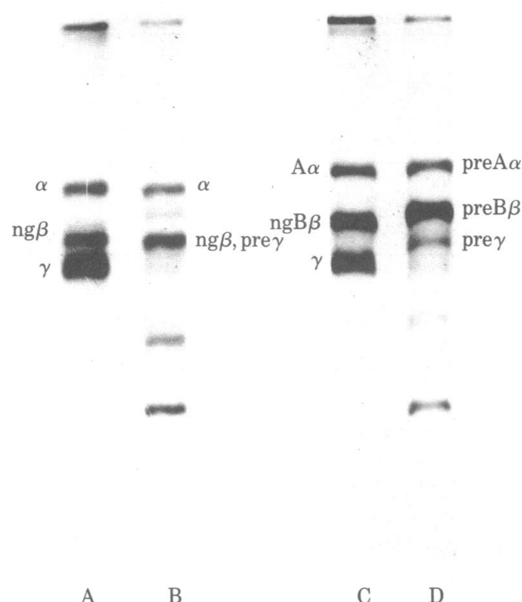

FIG. 3. Comparison of mRNA primary translation products to elongated chains from polysomes: effect of thrombin. Lanes: A, elongated chains from polysomes treated with thrombin; B, mRNA translation products treated with thrombin; C, elongated chains from polysomes (no thrombin treatment); D, mRNA translation products (no thrombin treatment). Polysomes were added to an *in vitro* translation system containing 3 μM pactamycin to block any new initiations. The products from this translation were only elongated chains. "Pre" refers to precursor forms of the chains; "ng" refers to nonglycosylated forms of chains (i.e., lacking asparagine-linked carbohydrate moieties).

The two minor bands of low molecular weight present on lanes B and D probably are incompleted mRNA translation products. One of the polypeptides was sensitive to thrombin and probably represents an incomplete preAα or preBβ chain. The other band was not sensitive to thrombin and likely represents an incomplete preγ chain. The small amounts of incomplete translation products may have arisen as a result of slightly nonideal conditions for protein synthesis *in vitro*.

DISCUSSION

The NH_2 termini of polypeptide precursor proteins have been intensely investigated in recent years. The notion that NH_2-terminal signal peptides are involved in the translocation of secretory proteins from the cytosolic to the cisternal side of the rER has been of particular significance (7, 8). The NH_2 termini of *in vitro* translated fibrinogen chains contain such peptides; in addition, these termini of fibrinogen may play key roles in the assembly of fibrinogen because all six polypeptide NH_2 termini appear to be tightly clustered in the native molecule. We have presented evidence that fibrinogen Aα chain is translated as a precursor slightly larger than the authentic Aα chain, that fibrinogen Bβ chain is synthesized as a precursor slightly larger than nonglycosylated Bβ chain, and that fibrinogen γ chain is translated as a precursor slightly larger than nonglycosylated γ chain. The data are consistent with the idea that these precursors contain NH_2-terminal signal sequences. The additional amino acids present in the *in vitro* translated chains are positioned to the NH_2-terminal side of the thrombin cleavage site on the preAα and preBβ chains. *In vitro* translated thrombin-treated α and β chains are the same molecular weight as the authentic fibrin α chain and the nonglycosylated fibrin β chain, respectively. If the extra amino acids had been on the COOH-

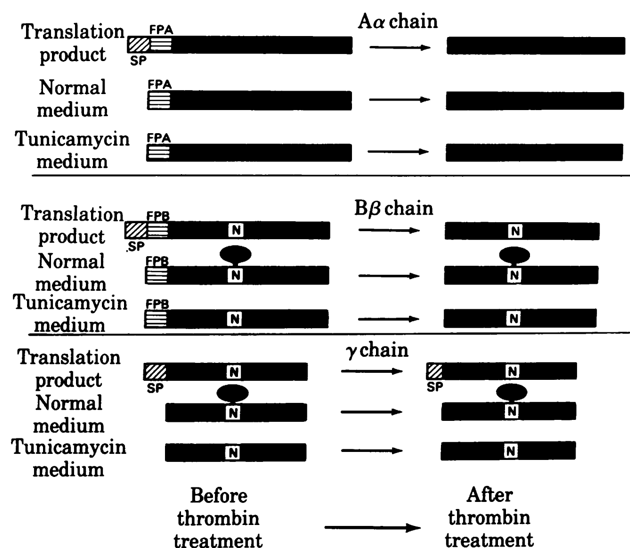

FIG. 4. Proposed model of the effect of thrombin cleavage on fibrinogen chains. Thrombin treatment of Aα chain translated from mRNA *in vitro* yields an α chain and a single fragment containing both the signal sequence and fibrinopeptide A. Thrombin treatment of authentic Aα chain yields the same α chain and fibrinopeptide A. Thrombin treatment of Aα chain from tunicamycin-treated hepatocytes gives the identical result as thrombin treatment of authentic Aα chain. Thrombin treatment of mRNA translated Bβ chain yields a nonglycosylated β chain and a single fragment containing a signal sequence and fibrinopeptide B. Thrombin treatment of authentic Bβ chain, normally glycosylated from hepatocyte medium, yields fibrinopeptide B and a glycosylated β chain slightly larger than the nonglycosylated β chain. Thrombin treatment of Bβ chain from tunicamycin-treated hepatocyte medium yields fibrinopeptide B and a nonglycosylated β chain. Thrombin has no effect on γ chains regardless of the origin of the chain from media or from *in vitro* translation of mRNA. FPA, fibrinopeptide A; FPB, fibrinopeptide B; SP, signal peptide; N, asparagine. The black ovals represent carbohydrate attached to asparagine residues.

terminal side of the thrombin cleavage site they would have made the *in vitro* translated α and β chains longer than the authentic fibrin α chain or nonglycosylated fibrin β chain. Although we have not examined for the presence of a single peptide containing both a signal sequence and a fibrinopeptide, the difference in molecular weight, 2600, between mRNA translated Aα chain and its thrombin-treated counterpart is the estimated sum of a signal sequence (600) and fibrinopeptide A (2000). Similarly, the difference in molecular weight, 2600, between mRNA translated Bβ chain is the estimated sum of a signal sequence (1100) and fibrinopeptide B (1500) (Fig. 4).

The idea of three separate NH_2 terminal signal sequences is consistent with the notion that there are three separate mRNAs coding independently for the three individual Aα, Bβ, and γ chains. Had fibrinogen Aα, Bβ, and γ chains been synthesized as a polyprotein Aα-Bβ-γ promolecule or as Aα-Bβ or Aα-γ promolecules, these pro forms might have been detected by using an *in vitro* translation system. Such as Aα-Bβ-γ polyprotein would need only one initiator methionine, and, if it were to follow the conventional version of the signal hypothesis (7, 8), such a polyprotein would need only one NH_2-terminal signal peptide. No large polyprotein precursors were detected in mRNA translation experiments. Furthermore, evidence was shown here that there is not just one signal peptide, but there are three signal peptides, one on each of the three chains. Consequently, this evidence weighs against the idea of one mRNA coding for a polyprotein fibrinogen precursor.

In summary, we have demonstrated the *in vitro* synthesis of the three different chains in fibrinogen. The chains are released from polysomes as individual chains. The Aα and Bβ *in vitro* translated chains are sensitive to thrombin. All three chains are synthesized as precursor molecules slightly larger (600–3000 daltons) than the nonglycosylated polypeptides of the authentic secreted fibrinogen molecule. Rat Bβ and γ chains are glycosylated, via asparagine-linked moieties; Aα is not glycosylated.

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