IDENTIFICATION OF THE TRANSLATION PRODUCTS OF α-1-ANTITRYPSIN mRNA FROM BABOON LIVER POLYSOMES

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1. Introduction

Alpha-1-antitrypsin (A1A), also known as α-1-proteinase inhibitor, is a major protease inhibitor of human plasma [1]. This glycoprotein of M_r 52 000 [2] is present in normal human serum at mean 2.5 mg/ml [3]. Its synthesis in liver is controlled by a system of ⩾40 codominant allelic genes [4]. Individuals homozygous for the *PiZ* allele are strongly deficient in A1A and are predisposed to pulmonary emphysema [5] and juvenile hepatic cirrhosis [6]. The mechanism responsible for this low seric level appears to involve defective export of A1A from the hepatocyte [6]. Human and baboon (*Papio papio*) A1A molecules (52 000 and 60 000 M_r, respectively) behave very similarly when considering protease inhibitory capacities, antigenicity [7] and genetic polymorphism [8]. We found of great interest a study of the molecular mechanisms of regulation and secretion of A1A taking baboon as model.

Here we report the messenger RNA extraction from baboon liver and its translation in a reticulocyte lysate cell free system: The major feature is the observation of 2 protein forms (M_r 48 000 and 43 000), which were specifically immunoprecipitated with purified monospecific antihuman A1A IgGs.

2. Materials and methods

2.1. *Preparation of mRNA*

Baboon livers were rapidly removed, immediately frozen in liquid nitrogen and stored at −80°C. The tissue was first homogeneized in a Potter-Kontes homogeneizer with 3 vol. buffer A (200 mM Tris–HCl (pH 8.6), 25 mM $MgCl_2$, 5 mM β-mercaptoethanol) supplemented with 250 mM sucrose, 500 μg/ml heparin and 4 mg/ml yeast tRNA and then adjusted to 1% sodium deoxycholate and 1% Triton X-100. The homogenate was centrifuged. The supernatant was layered over sucrose cushions made of 4 ml 2.5 M (bottom), 4 ml 1.5 M and 6 ml 1 M sucrose (top) in buffer A containing 100 μg/ml heparine. After centrifugation at 130 000 × *g* for 4 h at 4°C, the polysomes were harvested at the interphase 1.5–2.5 M sucrose. Polysomal RNA was obtained by phenol–chloroform (1/1) extraction. Highly purified poly(A)-RNA was obtained by 2 oligo(dT)-cellulose chromatographies [9].

2.2. *Cell-free translation system*

mRNA was translated in a reticulocyte lysate [10]. Protein synthesis was carried out in a total volume of 25 μl containing 11 μl nuclease-treated reticulocyte lysate [11], 0.4 μg mRNA and 15 μCi [^{35}S]methionine. After 2 h incubation at 30°C, incorporation of [^{35}S]methionine into proteins was measured on 2 μl aliquots precipitated in cold trichloroacetic acid 11%, heated at 100°C for 15 min and filtered on glass fiber filters. The filters were washed in trichloroacetic acid 25% and ethanol successively, then dried and the radioactivity counted in Aquasol (New England Nuclear) with a β-counter (Intertechnique).

2.3. *Anti-A1A antibody preparation*

Anti-human A1A antibodies were raised in goat by immunization with human A1A purified by thiol–Sepharose as in [12]. The antibodies elicited against other serum proteins were discarded by chromatography of the crude goat antiserum onto an immunoadsorbent made of a *Pi null* human serum (i.e., con-

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taining no A1A) [13] immobilized on CNBr-activated Sepharose 6B. The monospecific antiserum thus obtained was further chromatographed on a normal human serum immobilized on CNBr-activated Sepharose 6B [14] and the bound anti-A1A IgGs were recovered by acidic elution at pH 2.2 and immediately buffered at pH 7.0.

2.4. *Immunoprecipitation*

Immunoprecipitation of translation products was performed using a 2 step procedure with solid-phase protein-A. Translation products (25 μl) were immunoprecipitated with 50 μg goat anti-human A1A antibodies and then 30 μl protein-A–Sepharose (Pharmacia) in buffer B (10 mM Na/K phosphate buffer (pH 7.3), 0.14 M NaCl, 2% Triton X-100) (1:1, v/v) were added. The mixture was incubated overnight at 4°C with continuous shaking. The protein-A-bound immunoprecipitates were resuspended in buffer B and collected by centrifugation through a layer of 500 μl buffer B containing 1 M sucrose and 10 mM unlabelled methionine. The immunoprecipitates were further washed several times with buffer B and then dissolved in 50 μl electrophoresis buffer (see below) and heated at 100°C for 5 min before SDS–polyacrylamide gel electrophoresis. For quantitation, immunoprecipitable radioactivity was measured in 5 μl aliquots.

2.5. *SDS–polyacrylamide gel electrophoresis (SDS–PAGE)*

Total translation products and immunoprecipitates were analyzed by electrophoresis in 15% polyacrylamide gel slabs [15]. Molecular mass markers were submitted to electrophoresis in adjacent wells of the gels. Gel slabs were treated for fluorography [16]. Expositions were made on Kodak blue brand Regulix films for 20–48 h at −80°C.

3. Results and discussion

Poly(A)-RNA was extracted by a procedure including the prior isolation of polysomes and subsequent phenol–chloroform extraction. This method gave a better yield than direct isolation by LiCl–urea extraction according to [11]. Table 1 shows the total amounts of mRNAs extracted from baboon liver by the 2 methods. Liver mRNAs are apparently modified by LiCl–urea extraction since they do not bind oligo-(dT)-cellulose.

Addition of baboon liver mRNAs to the reticulocyte lysate cell-free system stimulated incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable proteins 10-fold while purified globin mRNAs stimulated it 20-fold as compared with control translations without addition of any mRNAs. The translation products were then immunoprecipitated with monospecific anti-A1A antibodies in the presence of protein-A–Sepharose (the latter in excess to avoid saturation problems). It was verified by crossed immunoelectrophoresis that these anti-human A1A antibodies cross-reacted well with serum baboon A1A (fig.1). The total and immunoprecipitated products from cell-free translation were analyzed by SDS–PAGE and visualized by fluorography (fig.2). Two polypeptide bands of M_r 48 000 and 43 000 were revealed by specific immunoprecipitation. Furthermore, adding unlabelled purified human A1A abolished the immunoprecipitation of the 2 radioactive translation products (fig.2E, table 2). This points to a close immunochem-

Table 1
Amounts of mRNAs extracted from baboon liver

RNA fraction	Method I[a]		Method II[a]	
	Amount	A_{260}/A_{280}	Amount	A_{260}/A_{280}
Liver	35 g		35 g	
Polysomes	2500 A_{260}	1.55	–	
Total RNA	50 mg: 1250 A_{260}	1.98	48 mg: 1200 A_{260}	1.96
Poly(A)-RNA	500 μg: 12.5 A_{260}	2.00	160 μg: 4 A_{260}	2.00
Yield	1%		0.3%	

[a] Method I, our method (see text); method II, extraction of total RNA by LiCl–urea precipitation as in [11]

The values represent the average of 4 separate determinations for each method

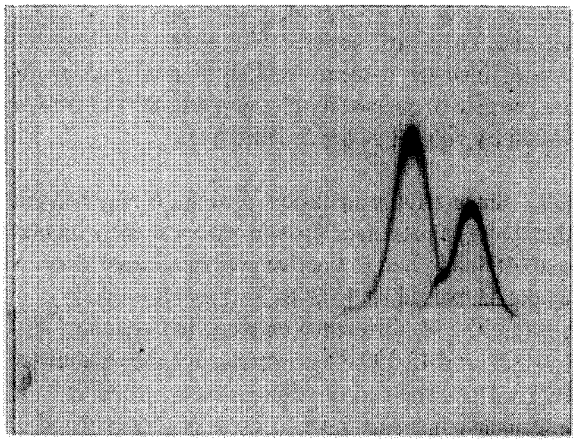

Fig.1. Tandem crossed immuno-electrophoresis of human and baboon normal sera. First and second dimension electrophoreses were performed in 1% agarose in Tris–veronal buffer (pH 8.6) according to general procedures [17]: 1st dimension, right well, 2 μl human serum diluted 1/10; left well, 2 μl baboon serum diluted 1/20; anode at right, 10 V/cm; 2nd dimension, 7.5 μg/cm² purified goat anti-human A1A antibodies; anode at the top, 2 V/cm.

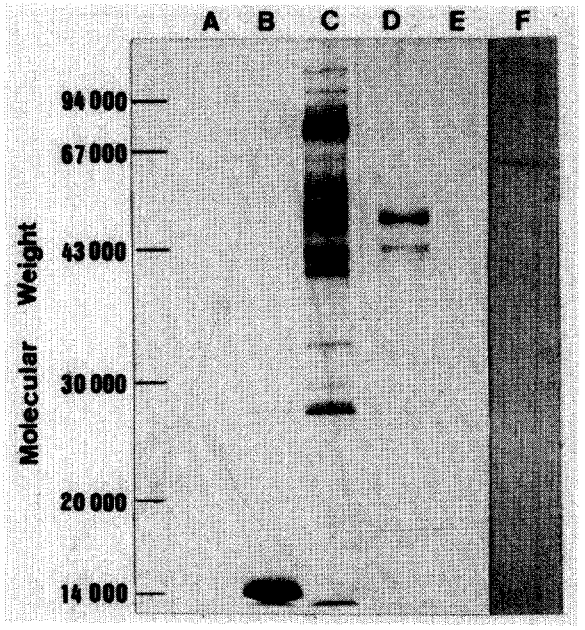

Fig.2. SDS–polyacrylamide gel electrophoresis of translation products synthesized in reticulocyte cell-free system: (A) reticulocyte lysate, no mRNA added; (B) + globin mRNA; (C) + baboon liver mRNA; (D) immunoprecipitation with anti-α-1-antitrypsin IgG; (E) immunoprecipitation after addition of 10 μg purified A1A; (F) purified baboon A1A stained by amido black.

ical relationship between both protein bands and rules out a possible immunoprecipitation of contaminant.

Table 2 shows the total amount of labelled methionine incorporated in the cell-free translation system and the total amount of immunoprecipitable material. The immunoprecipitable products represented 3.9% of the total translation products. The products of M_r 48 000 and 43 000 represented, respectively, 60% and 30% of the immunoprecipitable radioactivity, as revealed by densitometry (not shown). When separating total mRNAs on 5–25% sucrose gradients and translating the fractions, the 2 proteins are found in 4 fractions according to their M_r-value with a progressive shift of their ratio. Strikingly, the protein of lower M_r seems to be coded by the heavier mRNA (fig.3).

According to [18] the M_r of Rhesus monkey A1A is 60 000 and sugar moiety represents 12%. The M_r of human A1A is 52 000 and sugars also represent 12% of the molecule [2]. Furthermore, we verified by SDS–PAGE that baboon A1A is a single polypeptide chain with a M_r 60 000 (fig.2D). From an evolutionary viewpoint, baboon is located between Rhesus mon-

Table 2
Cell-free protein synthesis and specific A1A immunoprecipitation

	[^{35}S]Methionine trichloroacetic acid-precipitable	[^{35}S]Methionine immunoprecipitable	% Immunoprecipitation
Globin mRNA	4.3×10^6 cpm	2.1×10^4 cpm	0.5%
Baboon mRNA	2.1×10^6 cpm	8.3×10^4 cpm	3.9%
Baboon mRNA + unlabelled A1A	2.4×10 cpm	1.1×10^4 cpm	0.4%

Rates of incorporation of [^{35}S]methionine in proteins are expressed in cpm/μg total mRNA. The blank values have been subtracted for computative purposes

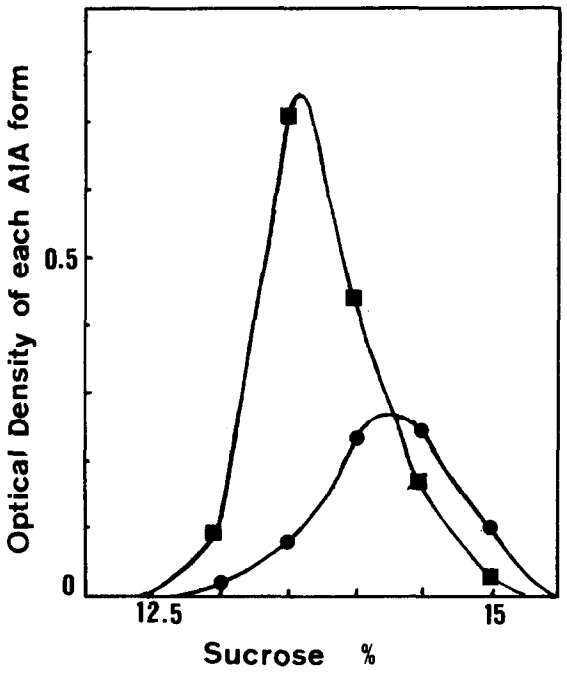

Fig.3. Densitometric analysis of the A1A forms translated from 2 A1A mRNAs separated on a 5–20% sucrose gradient: centrifugation, 40 000 rev./min, for 16 h, in a SW41 rotor; (■——■) protein of M_r 48 000; (●——●) protein of M_r 43 000.

key and human. We thus assumed the sugar moiety of baboon A1A to also represent 12%. The polypeptides synthesized in vitro in reticulocyte lysate are not glycosylated as protein glycosylation is a post-translational step occurring in the endoplasmic reticulum. Consequently, the 2 immunoprecipitable bands likely correspond to unglycosylated forms of A1A.

It should be outlined that, so far, the only indication of A1A synthesis in liver was the immunofluorescent detection of this protein in liver biopsies [19]. This work gives a distinct proof of the A1A synthesis by hepatocyte.

Various explanations can account for these results which exhibit a heterogeneity of the immunoprecipitable translation products:

(1) The protein of M_r 48 000 may be the native A1A without its carbohydrate moiety. In this case, M_r 43 000 could be a product of incomplete translation. This remains unlikely unless a breakage easily occurs at a sensitive place in the A1A mRNA. We could think as well that production of the 43 000 M_r protein from the 48 000 M_r protein occurs via an intrinsic enzymatic activity within the reticulocyte lysate; however, it seems unlikely that an endoprotease capable of accurately cleaving 48 000–43 000 M_r would be present in the reticulocyte.

(2) Since A1A is an export protein, it may be synthesized as a precursor protein with a slightly higher size (48 000) than the native unglycosylated chain whose M_r would be 43 000. This seems unlikely because the M_r difference between native serum A1A (60 000) and 43 000 is too important to be accounted for by M_r of the carbohydrate moiety, which is assumed to be ~7200, i.e., 12% of M_r as discussed above.

(3) The liver mRNA would direct the synthesis of 2 separate A1A precursors, as suggested by sucrose gradient centrifugation. Such a possible occurrence of 2 mRNA translation products would require different glycosylated chains to display an analogous final M_r. This A1A heterogeneity would be responsible for the A1A polymorphism observed by isoelectric focusing [8].

All of the above possibilities are being investigated in our laboratory. Improvements in purification of A1A mRNA by additional size preparation procedures are in progress. When purified A1A mRNA is obtained, synthesis of complementary DNA and clonage will allow one to reach a new field of investigations relevant to A1A synthesis and regulation, in health and disease.

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