IN VITRO SYNTHESIS OF RAT PRE-PROALBUMIN

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SUMMARY: An mRNA fraction prepared from rat liver was translated using radioactive amino acids and a cell-free protein synthesizing system derived from wheat germ. The radioactive albumin produced was isolated by immunoprecipitation with rabbit antiserum to rat serum albumin and analyzed on slab gel SDS-polyacrylamide (5-30%) gel electrophoresis. The main radioactive protein obtained migrated on electrophoresis slightly behind authentic rat serum albumin indicating a larger molecular size. Radioactive amino acid sequence analysis of the amino terminal portion of the protein indicated that the nascent protein contained an octadecapeptide extension coupled to proalbumin. The peptide extension contains methionine at its amino terminus and many hydrophobic amino acid residues.

Several proteins, when their mRNAs are translated by cell-free protein synthesizing systems, appear as larger molecules and some of these have been shown to contain an additional hydrophobic peptide extension at the amino-terminus (1-8). It has been postulated that this peptide extension occurs in secretory proteins and may be involved in the attachment of polysomes to the endoplasmic reticulum (ER) membrane or that it is also present in certain non-secretory proteins, such as membrane proteins, and may be involved in directing these proteins to their correct cellular destinations (1,9,10).

Rat albumin is a secretory protein which, in normal adult rat liver, is predominantly synthesized on membrane-attached polysomes (11-14), is vectorially discharged into the rough ER and is then transported stepwise to the smooth ER and to the Golgi prior to its secretion into the Space of Disse (15,16). During its passage within the rough and smooth ER, nascent albumin exists as a precursor protein (proalbumin) which contains a hexapeptide extension at its amino terminus (17-19). This precursor protein is converted into serum albumin just prior to its secretion into the blood (20,21).

In this report we show that the translation of mRNA from rat liver by

Abbreviation: SDS, sodium dodecyl sulfate

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a cell-free protein synthesizing system derived from wheat germ, yields an albumin-like product (pre-proalbumin) which appears to be larger in size than serum albumin and which, on partial analysis of its amino acid sequence, is shown to contain an octadecapeptide extension coupled to proalbumin.

MATERIALS AND METHODS

Young male rats were starved overnight and RNA was extracted from the livers by phenol-chloroform as described by Killewich et al. (22). A poly-A-rich messenger RNA fraction was then obtained by chromatography on oligo(dT)-cellulose columns (23).

A wheat germ cell-free protein-synthesizing system was prepared and used with slight modifications of the methods described by Roberts and Paterson (24). Wheat germ was obtained from Niblack Food, Inc., Rochester, N.Y. and several batches were tested for the ability to complete translation of the albumin mRNA. The following incubation conditions were found to yield optimal albumin synthesis. mRNA (50 µg/ml) was incubated at 25° for 2 hours together with the wheat germ extract (6 mg protein/ml) in the following reaction mixture: 20 mM HEPES buffer at pH 7.6, 2 mM dithiothreitol, 1 mM ATP 20 µM GTP, 8 mM creatine phosphate, 50 µg/ml creatine phosphokinase, 74 mM KCl, 3 mM Mg acetate, 10 µg/ml spermine, 40 µM of 19 non-radioactive amino acids and the radioactive amino acid to be used. The following were used: L-[^{35}S]methionine (555 Ci/mmole), L-[2,3-^{3}H]leucine (55 Ci/mmole), L-[5- ^{3}H]arginine (11 Ci/mmole), L-[2,3-^{3}H]valine (17 Ci/mmole), and L-[4-^{3}H]phenylalanine (12 Ci/mmole). In the experiment shown in Figure 1, a mixture of L-[U ^{14}C]amino acids was used. All of the radioactive amino acids were purchased from Amersham-Searle.

After incubation the ribosomes were removed by centrifugation at 105,000g for 1 hour and 10 mM of the amino acid was added, to dilute the radioactivity, the solution was made 1% with sodium deoxycholate and Triton - X-100, and the solution was again centrifuged at 10,000g for 15 minutes. To this supernate was added a small amount of rat serum and a slight excess of rabbit antiserum to rat serum albumin. An immunoprecipitate was formed on incubation at 4° overnight. The immunoprecipitate was layered over 1 M sucrose containing 10 mM Tris-HCl buffer pH 7.4, 154 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 10 mM of the amino acid used to label the nascent proteins, and was centrifuged at 10,000g for 15 minutes (25). The resulting pellet was resuspended and washed three times in 0.154 M NaCl. This washed immunoprecipitate was then used for radioactivity determination by liquid scintillation spectrometry, for analysis on SDS-polyacrylamide gel electrophoresis and for amino acid sequence determination. SDS-polyacrylamide gel electrophoresis was performed by treating the immunoprecipitate with 0.1 M sodium phosphate, pH 7.2, 1% SDS, 6 M urea and 1% 2-mercaptoethanol, heating at 45° for 1 hour and subjecting it to SDS slab gel gradient polyacrylamide (5-30%) electrophoresis. The protein bands were identified by Coomassie blue staining and the radioactivity was localized by autoradiography using Kodak x-ray film.

Amino acid sequencing was performed using the Beckman Model 890C automatic sequencer. To each radioactive immunoprecipitate was added 5 mg of sperm whale apomyoglobin as a standard to determine the fidelity and the yields of the automatic sequencer. The method used is essentially that described by Burstein and Schechter (2). The dried anilinothiazolinone amino acid deriva-

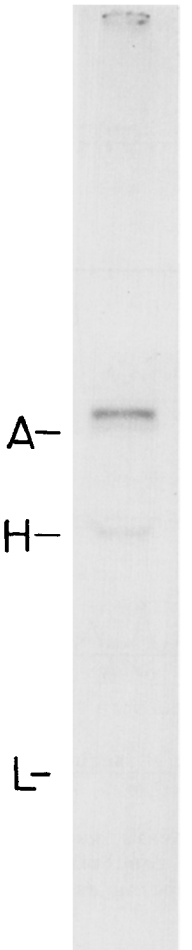

Figure 1. Autoradiogram of SDS-polyacrylamide gradient gel.

A radioactive anti-albumin immunoprecipitate was electrophoresed as described in MATERIALS AND METHODS. An autoradiogram is shown above. The positions where serum albumin (A), heavy (H) and light (L) immunoglobulin chains migrated are marked.

tive from each cycle of the sequencer was dissolved in 0.1 ml of ethyl acetate and 10 mls of Aquasol scintillation fluid was added prior to determination of radioactivity.

RESULTS AND DISCUSSION

The poly-A-rich RNA fraction isolated from rat liver directs the synthesis of proteins which precipitate with rabbit antiserum to rat albumin and which, on co-electrophoresis with authentic rat serum albumin on a 7.5% SDS-urea polyacrylamide gel yields a major radioactive protein which appears to

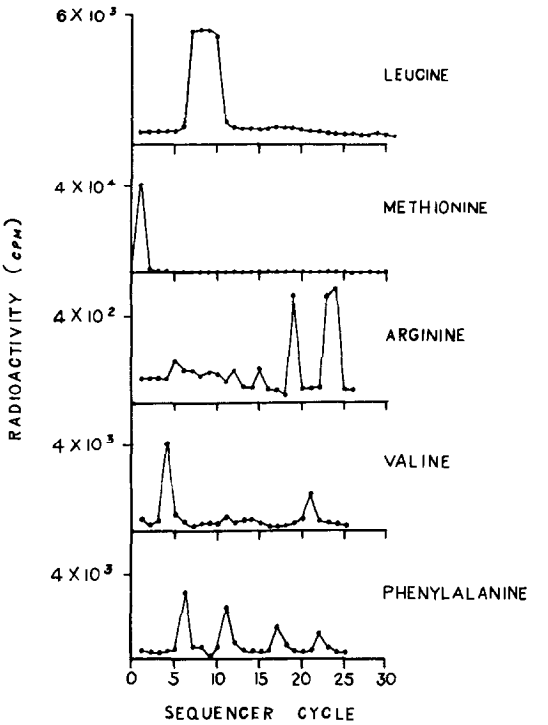

Figure 2. Radioactive amino acid sequences from anti-albumin immunoprecipitates.

Cell-free protein synthesis was carried out with the radioactive amino acids listed above and the resulting washed anti-albumin precipitates were subjected to amino acid sequencing as described in MATERIALS AND METHODS.

be similar in molecular size to serum albumin. Other radioactive bands of smaller molecular size are also observed after polyacrylamide electrophoresis but these contain much less radioactivity and may be prematurely terminated albumin molecules. The reason for postulating that these smaller size proteins are incomplete albumin molecules is that the antiserum to rabbit albumin appears to be mono-specific for albumin as shown by a single precipitin band on immunoelectrophoresis against rat plasma and also that when the entire immunoprecipitate obtained from the cell-free system was used for amino acid sequencing, each sequential removal of a terminal amino acid produced homogenous radioactive residues (Fig. 2), thus indicating that the smaller size radioactive bands are not contaminating proteins, but may have common amino terminal

sequences. In contrast to electrophoresis on 7.5% polyacrylamide gels, electrophoresis on SDS-slab gels (5-30% polyacrylamide) showed that serum albumin consistently migrated slightly ahead, but clearly separable, from the major radioactive band. Other radioactive bands, of lower autoradiographic intensity, could also be seen. One of these bands migrated to the same extent as serum albumin and others appeared to be of smaller molecular size (Fig. 1). These experiments demonstrate that translation of liver mRNA by wheat germ extracts yields a radioactive product which immunoprecipitates with anti-albumin and which appears to be larger than serum albumin.

To determine whether the radioactive proteins obtained from this system are serum albumin with peptide extensions at the amino terminus, we partially sequenced the amino terminal portion of the radioactive proteins contained in the immunoprecipitate. It is to be expected that if an extra peptide exists in the in vitro translation products, it would be coupled to proalbumin and the amino terminal end of this polypeptide would be recognized by the following sequence, Arg-Gly-Val-Phe-Arg-Arg- (18). Hence, incubation of liver mRNA with wheat germ extracts under conditions of protein synthesis together with radioactive arginine, valine and phenylalanine should indicate when this proalbumin portion is reached. Since other studies have shown that the extra peptide portion seen on cell-free translation products is usually rich in hydrophobic amino acids and since several of these proteins have amino terminal methionine, we also incubated the in vitro protein synthesizing system in separate experiments with the following amino acids: L-[^{35}S]methionine, L-[4,5-^{3}H]leucine, L-[2,3-^{3}H]valine, L-[5-^{3}H]arginine and L-[4-^{3}H]phenylalanine. The putative pre-proalbumin was then isolated by immunoprecipitation using antiserum to rat albumin and was subjected to automated sequential amino acid analysis. The radioactivity obtained in each of the cycle runs for the various radioactive amino acids used are shown in Figure 2.

These data indicate that a radioactive protein was translated in vitro which contains amino terminal methionine. Since arginine was located in

Table I

Amino Terminal Sequence of Amino Acids in Pre-proalbumin

* Met	--	--	* Val	--	* Phe	* Leu	* Leu
1	2	3	4	5	6	7	8
* Leu	* Leu	* Phe	--	--	--	--	--
9	10	11	12	13	14	15	16
* Phe	--	* Arg	Gly	* Val	* Phe	* Arg	* Arg
17	18	19	20	21	22	23	24

The amino acid residues marked with asterisks are those determined by sequencing the radioactive antialbumin immunoprecipitates as described in MATERIALS AND METHODS. The residues in positions 19 through 24 are those which have been shown to occur at the amino terminus of proalbumin (18).

positions 19, 23 and 24, valine in position 21, and phenylalanine in position 22, the following internal sequence is obtained: Arg-X-Val-Phe-Arg-Arg- which strongly suggests that the radioactive protein is proalbumin coupled to a peptide containing 18 amino acid residues. L-leucine was identified at positions 7,8,9 and 10, valine was also seen in the 4th cycle and phenylalanine was noted in positions 6,11 and 17.

These analyses together with the known amino terminal sequence of intracellular proalbumin suggest that the in vitro translation product which precipitates with antialbumin, is pre-proalbumin and that it contains the amino terminal sequence shown in Table I.

During this study an abstract appeared which shows that albumin mRNA is translated by wheat germ extracts to form a protein which has an extension

at the amino terminal end. The amino acid sequence analysis reported is similar to that shown in Table I (26).

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