Volume 4 Number 6 June 1977 Nucleic Acids Research

Partial purification of rat α-lactalbumin mRNA

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Received 5 April 1977

ABSTRACT

α-Lactalbumin messenger RNA was partially purified from RNA extracted from 3-5 day lactating rat mammary glands on a poly(U)-sepharose column followed by sucrose gradient centrifugation. α-Lactalbumin mRNA activity was assayed in wheat germ cell-free translational system by immunoprecipitation of the in vitro synthesized protein using specific antiserum prepared against purified rat α-lactalbumin. In the purified mRNA preparation α-lactalbumin mRNA activity comprised approximately 85% of the total mRNA activity.

INTRODUCTION

α-Lactalbumin (α-LA) is the B-protein of lactose synthase which catalyzes the formation of lactose from UDP-galactose and glucose[1]. It is produced by the mammary gland at the time of lactation.

Two species of α-LA which differ in their net negative charge, apparent molecular weights, and in proline content have been separated from rat milk[2]. Both α-LA species are active in the lactose synthase assay and are glycoproteins. By radioimmunoassay α-LA represents about 0.6% of the total protein of 3-5 day lactating rat mammary gland. Several rat mammary tumors contain α-LA in quantities equal to or less than 10% of the amounts found in 3-5 day lactating gland[3].

In order to study the hormonal mechanism(s) which regulates the expression of the α-LA genome in normal and neoplastic tissue we have isolated α-LA mRNA from lactating mammary gland. Here we report the partial purification of this message.

METHODS

[^{3}H]Leucine and RNase free sucrose for density gradients were purchased from Schwarz/Mann. Wheat germ was obtained from General

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Mills, Inc., Minneapolis, Minn. Poly(U)-sepharose 4B was from Pharmacia Fine Chemicals.

Animals. The entire thoracic and abdominal mammary glands were removed from 3-5 day lactating Sprague-Dawley rats, minced, quickly frozen in liquid nitrogen and stored frozen at -70° until RNA extraction.

α-Lactalbumin Isolation. α-Lactalbumin (α-LA) was purified to homogeneity from rat milk obtained from Wistar rats[2]. α-LA isolated from Sprague-Dawley or Wistar strain are identical[2]. Two species of α-LA -- $\alpha\text{-LA}_H$ and $\alpha\text{-LA}_L$ -- were identified with apparent molecular weights of 22,500 and 21,800, respectively. Both are glycoproteins and active in lactose synthetase assay.

A rabbit antiserum was prepared against $\alpha\text{-LA}_L$. With this antiserum the two species of α-LA were found to be immunologically very similar[2]. Immunoprecipitation of casein proteins was not detectable with this antiserum.

Isolation of RNA. Whole frozen mammary glands were pulverized and Dounce homogenized in 7 volumes of a buffer containing 60 mM Tris (pH 7.2), 175 mM NaCl, 35 mM $MgCl_2$, 1% Triton X-100, 1% Na-DOC, 1% SDS, 5 mM Na_2EDTA, 175 mM sucrose, and 0.35 mg/ml heparin. The mixture was shaken for about 10 minutes with equal volumes of a 1:1 mixture of buffer (50 mM Tris, pH 7.1; 25 mM NaCl, 5 mM $MgCl_2$) saturated phenol and chloroform. The emulsion was centrifuged. The aqueous upper phase and the protein interphase were reextracted once more with phenol-chloroform. Extraction was continued three more times with the aqueous phase. Finally, it was made 0.2 M in NaCl, overlayered with an equal volume of cold absolute ethanol. The DNA was then spooled onto a glass rod. The remaining RNA was precipitated by addition of a second volume of cold ethanol, stored at -20° overnight, and finally pelleted by centrifugation. The DNA was dispersed thoroughly overnight in a cold solution of 3 M Na-acetate + 5 mM EDTA (pH 6.0) on a magnetic stirrer. The suspension was centrifuged and the pellet was pooled with the RNA pellet. It was washed five more times with the Na-acetate solution in the cold, each time dispersing the RNA pellet thoroughly with a glass rod. This treatment removed DNA and tRNA (4). A final wash was accomplished with a solution of 70% ethanol containing 0.2 M NaCl. The RNA was dissolved in water, brought to 0.2 M NaCl, and precipitated by addition of 2 volumes of ethanol. The material heretofore will be referred to as "total RNA."

Isolation of Poly(A)-bearing RNA. Poly(A)-bearing RNA was isolated

by chromatography of total RNA on a column of poly(U)-sepharose 4B essentially as described by Lindberg et al.[5].

Protein Synthesis Assay. Wheat germ cell-free extract was prepared essentially as described by Roberts and Patterson[6], except that the preincubation step was omitted. In the mixture the final concentration of KCl was 100 mM, spermine and S-adenosyl methionine were added to a final concentration of 40 μM and 2 μM, respectively. Two hundred μl assays were routinely employed with a 2 hr incubation at 25°. Twenty μl of [^{3}H]L-leucine (0.5 mCi/ml; spec. activity 61 Ci/mm) was added as the radioactive amino acid in the assay. Following incubation the ribosomes were removed by centrifugation at 105,000 x g for 1 hr. Then a 20 μl aliquot was removed, diluted with 1 ml of 5% TCA, and the suspension was boiled for 10 min. The precipitates were collected on glass fiber filters from chilled samples, dried, and counted in 10 ml of toluene-based fluor mixture for a measure of total mRNA activity. Another 20 μl was used for specific immunoprecipitation as follows: Reaction mixtures were diluted 10-fold in a buffer to a final concentration of 0.05 M Na-PO_4 (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 1 mg/ml of bovine serum albumin, 10 mM leucine, 1% Triton X-100, and 1% Na-DOC. To it 10 μl of rabbit antiserum to rat α-LA or casein was added. Following 1 hr incubation at 37°, the mixture was precipitated by adding 150 μl of sheep antiserum to rabbit IgG. After another hour of incubation at 37° the tubes were left overnight at 4°. The immunoprecipitates were collected as described by Rosen et al.[7]. The precipitates were dissolved in 0.05 M NaOH, neutralized with glacial acetic acid, and counted with 15 ml of toluene-Triton X-100 fluor mixture. TCA-insoluble precipitate and immunoprecipitates of translation products were analyzed by SDS-gel electrophoresis as described by Laemmli[8]. Radioactivity of gel slices was determined by extracting overnight at 37° with 1 ml of NCS solubilizer: H_2O (9:1) and counted.

Analytical Sizing Technique. Analysis of RNA was performed by centrifugation in linear 4 to 20% sucrose gradients containing 10 mM Tris (pH 7.0), 0.1 M NaCl and 1 mM EDTA after heat denaturation. The gradients were centrifuged at 27,000 rpm for 20 hr in a Beckmann SW27.1 rotor at 15°C. Fractions were collected from the bottom of the tubes, made to 0.5 ml by the addition of water, and absorbance at 260 nm was determined. The fractions were brought to 0.25 M NaCl, precipitated with 2 volumes of ethanol. The RNA pellets were dissolved in H_2O and

used in the wheat germ translation system.

α-LA mRNA was characterized by electrophoresis on composite 3% acrylamide-0.5% agarose[9] as well as on 2.5% agarose gels containing 6 M urea at pH 3.5. Extraction of mRNA activity from the composite gel was performed by homogenization of slices in 0.5 ml cold 0.1 M sodium acetate (pH 5.0) + 0.1% SDS. Following centrifugation at 20,000 x g for 15 min, the supernatant was extracted with a 1:1 mixture of phenol-chloroform and finally with ether. Ether was removed under mild vacuum, the solution was brought to 0.5 M NaCl, and RNA was precipitated by addition of 2 volumes of alcohol.

RESULTS

In the wheat germ translation system rat mammary gland poly(A)-bearing RNA caused 20- to 40-fold stimulation in incorporation of [^{3}H]-leucine into TCA-insoluble product after 2 hr of incubation. The stimulation of incorporation was proportional to the amount of RNA and reached a plateau level at a concentration of 200 μg of RNA/ml of reaction mixture. These results are consistent with the other studies[10,11] that wheat germ has a high degree of specificity for translating mRNA.

In initial experiments α-LA mRNA activity was determined in poly(A)-bearing RNA. This RNA directs 40-50% of the total radioactivity incorporation into material specifically immunoprecipitable with anti-α-LA serum. The addition of 50 μg of unlabeled α-LA reduced the radioactivity in the precipitate to 8% (Table 1). Normal serum precipitated about 6% of the total radioactivity. Only 4% of the translation product of rat liver poly(A)-bearing RNA was precipitated with anti-α-LA serum. No background counts were subtracted to compute the results for this small amount of nonspecific precipitation of radioactivity.

The TCA-insoluble and immunoprecipitated translation products were further characterized by SDS-polyacrylamide gel electrophoresis after mixing with purified unlabeled α-LA protein marker. TCA-insoluble product showed a major peak of radioactivity comigrating with purified α-LA markers along with a heterogeneous mixture of smaller peptides. Immunoprecipitates showed only one component comigrating with the major TCA-insoluble peak.

Having established the specificity of immunoprecipitation, we employed the procedure to quantitate the amount of immunoprecipitable α-LA radioactivity in the total RNA stimulated translation products. We observed from several experiments that 20-30% of the total incorporated

Table 1. Immunoprecipitation Specificity of In Vitro Synthesized Protein

Source of Poly(A)-RNA for protein biosynthesis	Antiserum	Competitor	% of TCA insoluble counts immuno-precipitated[1]
Lactating rat	Anti-α-LA	--	47
Mammary gland	Normal serum	--	6
	Anti-α-LA	Rat α-LA (50 μg)	8
	Anti-α-LA	Rat casein (50 μg)	41
Rat liver	Anti-α-LA	--	6
	Anti-casein	--	4

[1] 82,000 cpm (TCA insoluble counts) were used for immunoprecipitation.

radioactivity precipitated with anti-α-LA serum and the remainder with anti-casein serum.

Rat casein was prepared according to Rosen et al.[7] and rabbit anti-casein serum was obtained. Poly(U)-sepharose 4B chromatography resulted in selection of poly(A)-bearing RNA, while most of the rRNAs were removed. This treatment caused 29-fold enrichment of α-LA mRNA specific activity (Table 2). Analysis of poly(A)-containing RNA on a linear 4-20% sucrose gradient after heat denaturation is shown in Fig. 1. A peak of absorbance sedimenting at about 8.3S contains mostly α-LA mRNA activity, and a shoulder of RNA absorbance includes the peaks of casein mRNA activity ranging from 11S to 16S. A pool of material containing α-

Table 2. Partial Purification of α-LA mRNA

	RNA (mg)	Total mRNA activity X 10^{-8} cpm	α-LA mRNA activity X 10^{-8} cpm	α-LA mRNA/ Total mRNA* (%)	Fold
Total RNA+	190	34.80	7.30	21	
Poly(A)-bearing	1.086	2.28	1.20	53	29x
"Pooled material" 1st sucrose gradient	0.227	0.87	0.63	72	72x
"Pooled material" 2nd sucrose gradient	0.114	0.57	0.48	84	110x

*No correction has been made for 15% loss during immunoprecipitation assayed using ^{125}I-labeled α-LA internal standard.
+Thirty grams of lactating gland were used for the extraction of total RNA.

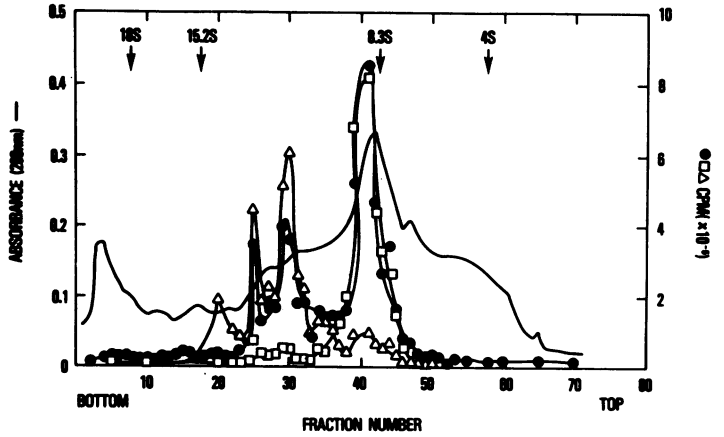

Figure 1. Fractionation of Poly(A)-containing RNA on a linear 4-20% sucrose gradient. Total mRNA activity (●——●); immunoprecipitated radioactivity with anti-α-LA (□——□) and anti-casein (△——△) serum.

LA mRNA activity represents a 43-fold increase over total Poly(A)-bearing RNA activity. α-LA mRNA activity represents at least 72% of the total activity at this stage of purification. Sedimentation of this material on a similar second sucrose gradient (Fig. 2) completely removes any contaminating RNA. A single peak of absorbance coincides with the peak of activity profile. Analysis of TCA-insoluble and immunoprecipitated translation products of peak fraction RNA by SDS-acrylamide gel electrophoresis (Fig. 3) shows comigration of major radioactivity peak with α-

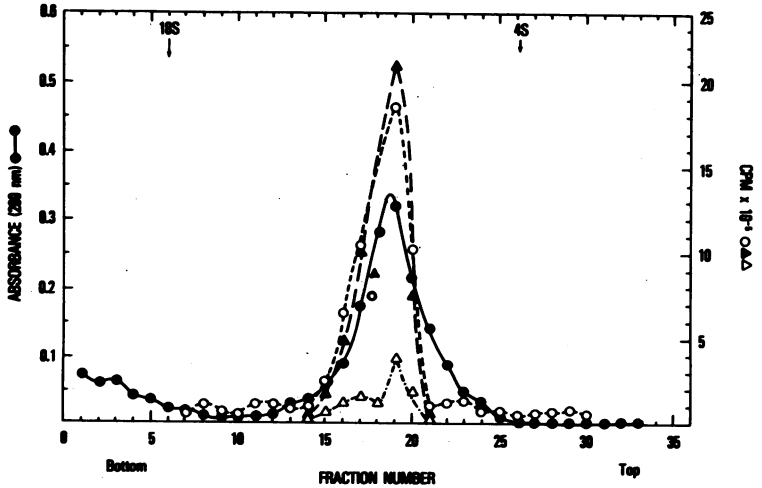

Figure 2. Purification of α-LA message on a second sucrose gradient. Total mRNA activity (o——o); immunoprecipitated radioactivity with anti-α-LA (▲——▲) and anti-casein (△——△).

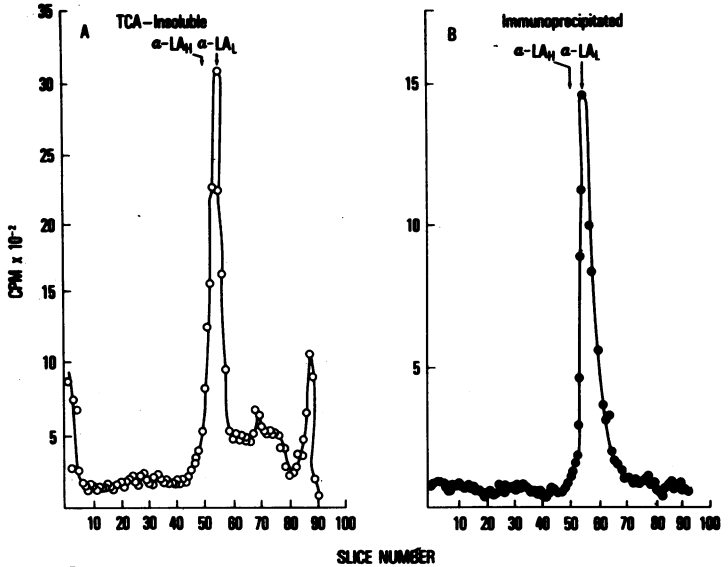

Figure 3. SDS-Polyacrylamide gel analysis of TCA-insoluble (A) and immunoprecipitated (B) translation products of peak fraction from 2nd sucrose gradient.

LA markers. Analysis of RNA from the pooled second gradient peak fractions on a composite 3% acrylamide-0.5% agarose gels[9] reveals the presence of two major bands with a shoulder (Fig. 4). When the activity

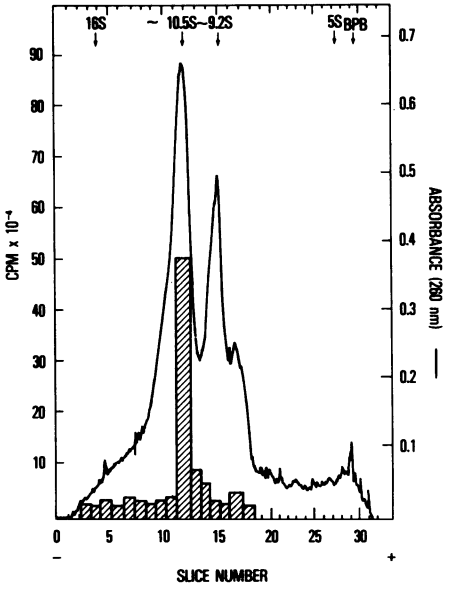

Figure 4. Analysis of RNA from pooled second sucrose gradient peak fractions on 3% polyacrylamide-0.5% agarose gels. Before slicing the gels they were scanned at 260 nm. Total mRNA activity from RNA extracted from gel slices (%).

of RNA extracted from gel slices is determined in the wheat germ assay, only the major band migrating about 10.5S is found to be active. At least 85% of the incorporated radioactivity in the synthesized product is immunoprecipitable with anti-α-LA serum, and the major peak of radioactivity of TCA-insoluble product comigrates with α-LA marker proteins (data not shown). However, when the same RNA material is analyzed on 2.5% agarose gels in the presence of 6 M urea at pH 3.5, a closely spaced doublet is observed corresponding to the active major peak of composite gel, along with another major band and small fragments. The 9S globin mRNA also splits into two main components when run on a parallel gel. This observation is consistent with the observation of Beckman and Lebleu[12]. At present, whether the two major bands of α-LA mRNA correspond to $\alpha\text{-LA}_H$ and $\alpha\text{-LA}_L$ proteins is not known.

DISCUSSION

Alpha-lactalbumin is a specifier of the galactosyl transferase enzyme. It is synthesized in the mammary gland at the time of lactation and makes up about 0.6% of the total protein of 3-5 day lactating rat mammary gland as measured by radioimmunoassay[3]. Two species of α-LA were isolated from rat milk, having molecular weights of 22,500 and 21,800 daltons, respectively, for $\alpha\text{-LA}_H$ and $\alpha\text{-LA}_L$, and both species are glycoproteins[2]. The carbohydrate moiety slightly affects their mobility on SDS-acrylamide gel. Wheat germ assay synthesized proteins comigrate with the purified α-LA markers. It is unlikely that the wheat germ S-30 fraction glycosylates the nascent chains.

By selection of poly(A)-bearing material and by sizing on sucrose gradients, it has been possible to purify α-LA mRNA activity from contaminating rRNA and other RNAs starting from total RNA extracts. Of the total RNA α-LA mRNA activity comprised about 20-30% and the remainder was casein. Chromatography of the total RNA on poly(U)-sepharose 4B results in 6% recovery of the total mRNA activity of which α-LA activity consisted of about 40-50% and the remainder was casein.

The poly(A)-bearing RNA was further discriminated on the basis of their size by centrifugation in 4-20% linear sucrose gradient after heat denaturation. In the first sucrose gradient the bulk of the α-LA mRNA activity was separated from the casein mRNA activity as well as some inactive RNAs. Wheat germ assay of each fraction of the gradient and immunoprecipitation of the synthesized product, either with anti-α-LA serum or with anticasein serum, revealed the distribution of total, α-LA

and casein mRNA activities. The peak of α-LA mRNA activity was found to sediment at about 8.3S. At this step α-LA mRNA activity consisted of at least 70% of the total mRNA activity. A second centrifugation of the RNA from active peak fractions resulted in almost complete purification of α-LA mRNA activity. Anti-α-LA serum precipitated 84% of the radioactivity in the synthesized product. However, this is an underestimation since no correction has been made for 15% loss during immunoprecipitation assayed using an ^{125}I-labeled α-LA internal standard. Analysis of synthesized product on SDS-acrylamide also did not assess any discernible biological impurity.

RNA, from the activity peak fractions of the second sucrose gradient, was analyzed by composite acrylamide-agarose gel electrophoresis. Upon electrophoresis the RNA separates into two major bands migrating about 10.5S, 9.2S with a shoulder of lower S value. The proportion of the second band varied from experiment to experiment with the same batch of RNA suggesting breakdown of mRNA during electrophoresis. The band migrating about 10.5S was active in stimulating the protein biosynthesis in wheat germ cell-free translational system. The in vitro synthesized protein comigrates with purified α-LA markers. Thus no precursor product relationship was observed between the synthesized protein and the finished α-LA protein obtained in milk. Eighty-five percent immunoprecipitation of the synthesized protein with anti-α-LA serum also describes the absence of measurable impurity with biological activity. Upon characterization of the same RNA on 2.5% agarose-urea gel, the major band resolved as a closely spaced doublet. It has not yet been determined whether each of these bands corresponds to the mRNA for individual α-LA species. Work is in progress to get sufficient amounts of this RNA extracted from the gels to allow its further characterization.

ACKNOWLEDGMENTS

We thank Dr. P.M. Gullino for constant support for this work, and Miss C. Gerken and Mr. R. Adler for their help.

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