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An assay for albumin messenger RNA in an vitro protein synthesizing system from wheat germ.

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ABSTRACT

The synthesis of serum albumin in an *in vitro* protein-synthesizing system from wheat germ stimulated with rat liver polysomal RNA is demonstrated by immunoprecipitation. The newly synthesized albumin has the same electrophoretic mobility as rat serum albumin. There is a linear increase in precursor incorporation into total protein and albumin with increasing RNA concentration. Potassium and magnesium optima for albumin synthesis are different from those for total protein synthesis.

INTRODUCTION

The synthesis of serum albumin is a convenient and appropriate model system for studying the control of specific gene expression in differentiation. Albumin is synthesized uniquely in the liver, representing 3 to 11% of protein synthesis [1 2 3]. It is a single polypeptide chain, a good antigen, and can be easily isolated from serum, of which it comprises about 50% of total circulating protein [4]. Two fundamental aspects of differentiation, the selective synthesis of specific proteins and the intracellular control that maintains a stable phenotype in the differentiated cell, have been explored through the synthesis of serum albumin. The dormant albumin gene in fibroblast cells has been "activated" by hybridization with hepatoma cells [5], and an array of hepatoma clonal variants has been used to study quantitative control of albumin synthesis [6,7].

In order to assess the transcriptional and translational events controlling albumin synthesis in liver and hepatoma cells in culture, a simple quantitative assay of albumin messenger RNA (mRNA) is needed. Albumin mRNA has been translated in heterologous rabbit reticulocytes and homologous liver

cell-free protein-synthesizing systems [8] [9] [10]. However, both systems have high endogenous mRNA activity and are relatively complicated to prepare.

Extracts from wheat germ and wheat embryos have been used to translate mRNA's from a variety of viral [11] [12], plant [13], and animal sources [12] [14] [15]. Wheat germ has minimal or no endogenous mRNA activity, is inexpensive, and extracts are simple to prepare [16]. Since the efficiency of translation and optimal conditions differ for mRNA's from different sources, the present work was undertaken to develop an assay for albumin mRNA in wheat germ extracts.

MATERIALS AND METHODS

Total polysomal RNA was prepared on a discontinuous sucrose gradient from a postmitochondrial supernatant according to the procedure described by Taylor and Schimke [8] from livers of rats, strain AC, that were fasted overnight. The isolated polysome fraction was made 1% sodium dodecyl sulfate (SDS), 50 mM EDTA (pH 7.0), and 100 mM NaCl; and the RNA precipitated overnight with 2.5 volumes of ethanol at -20°C. The precipitated RNA was dissolved in sterile distilled H_2O, then adjusted to 0.1 M NaAc, pH 5.0 and 0.5% SDS and extracted once with phenol-chloroform (1:1) and twice with chloroform [17]. The RNA was precipitated again with ethanol, washed three times with 3 M NaAc, pH 6.0 [17], and reprecipitated with ethanol. All RNA preparations were stored as ethanol precipitates and dissolved in distilled water for each assay.

A few drops of diethyl pyrocarbonate were added to all solutions to remove ribonuclease activity. Diethyl pyrocarbonate was then inactivated by heating the solutions in a boiling water bath for 45 minutes, shaking vigorously at intervals [17].

In Vitro translation

Extracts of wheat germ (General Mills, Vallejo, California) were prepared by the procedure of Marcu and Dudock [16], a modification of those of

Roberts et al [12] and Marcus et al [18], starting with 6 g of wheat germ. The complete protein-synthesizing reaction mixture in a final volume of 50 µl contained: 20 mM HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), pH 7.6, adjusted with 1 N KOH; 2 mM dithiothreitol, 1 mM ATP, 20 µM GTP, 8 mM creatine phosphate, 40 µg/ml of creatine phosphokinase, 100 mM KCl, 3 mM $MgAc_2$, 100 µCi/ml [^{3}H]leucine (Schwarz-Mann; specific activity, 46 Ci/mmole), 19 unlabeled amino acids (25 µM each) and 10 µl of S30 wheat germ extract. Reactions were performed at 25°C and stopped by emersion in an ice-water bath; immediately 10 µl of 0.1 M unlabeled leucine and 5 µl of 0.5 mg/ml rat serum albumin (Pentex, fraction V) were added to each tube. A 10 µl aliquot was precipitated with 5% TCA then incubated at 90°C for 15 minutes, cooled, and the precipitate was collected on glass fiber filters (Whatman, GF/C).

Immunoprecipitation

To each reaction tube were added 50 µl of an antibody mixture containing 0.16 A_{280} units of anti-albumin γ-globulin, 1% sodium deoxycholate, 1% Triton X-100, 25 mM leucine, 10 mM Na_2HPO_4, pH 7.2, and 0.15 M NaCl and incubated at 25°C for 1.5 hours and at 4°C overnight. (This amount of antiserum precipitated 3 µg of rat albumin at its equivalence point). The precipitated antigen-antibody complex was sedimented through 200 µl of 1.0 M sucrose containing 1% Triton X-100, 1% sodium deoxycholate and 10 mM leucine. After washing the pellets three times with PBS (10 mM Na_2HPO_4, pH 7.2; 150 mM NaCl) containing 0.01 M leucine, they were dissolved in 0.2 ml of 0.1 N NaOH, neutralized with 1N HCl, then reprecipitated with 10% TCA and the precipitates collected on glass fiber filters. All filters were digested with 0.5 ml of NCS (Amersham-Searle) and radioactivity determined using Omnifluor (New England Nuclear) toluene-based scintillation fluid.

Specific antiserum to rat serum albumin was prepared from rabbits immunized with chromatographically pure rat albumin as described previously [5]. Immune sera were precipitated twice with 40% ammonium sulfate and dissolved

in PBS at one-half the original serum volume (γ-globulin fraction).

Electrophoresis

Immunoprecipitates, prepared and washed as described above, were suspended in PBS, pH 7.5, containing 1% SDS and incubated at 50°C for 3 hours and applied to 7% polyacrylamide gels containing 0.1% SDS, 7 M urea, 0.1 M Na_2HPO_4 (pH 7.2), and 0.02 M EDTA. Electrophoresis was performed at room temperature with a constant current of 3 mA/gel using bromphenol blue as a marker [19]. The protein bands were stained with a solution of 0.05% Coomassie blue, 25% isopropanol and 10% acetic acid in water and the gels destained with the latter solution minus the dye. The gels were sliced into 2.75 mm fractions that were then dried overnight at 60°C, incubated in 0.3 ml 30% H_2O_2 at 50°C until dissolved and radioactivity determined using Bray's scintillation fluid.

RESULTS AND DISCUSSION

Total polysomal RNA from rat liver when added to a protein-synthesizing system from commercial wheat germ stimulated the incorporation of [^{3}H]leucine into protein. Incorporation into total protein increased linearly for about 15 minutes and then leveled off (Figure 1). At an RNA concentration of 305 µg/ml there was a ten-fold stimulation above background. The background counts without added RNA did not increase during the incubation.

The synthesis of rat serum albumin in the wheat germ lysate was detected by immunoprecipitation. The specificity of the antiserum for rat albumin was demonstrated by immunodiffusion in which a single precipitin line formed against either rat serum or liver homogenate, and in both cases an arc with no spur developed when purified albumin was in an adjacent well. The incorporation of [^{3}H]leucine into serum albumin began after a slight delay and then followed an incorporation curve similar to total protein (Figure 1).

The major product precipitated by the antiserum was rat albumin, because upon electrophoresis of SDS-dissociated antigen-antibody complex on polyacryl-

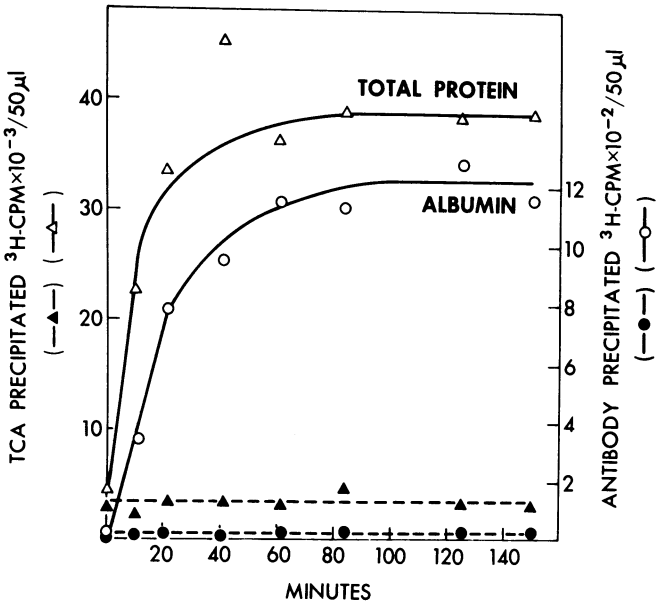

Fig. 1 - Kinetics of *in vitro* [^{3}H]leucine incorporation into albumin and total protein. Reaction mixtures of 750 µl, with (open symbols) and without (closed symbols) 305 µg/ml liver polysomal RNA, were incubated at 25°C and duplicate 50 µl samples taken for each time point (see METHODS).

amide gels containing SDS and urea the major radioactive band comigrated with purified rat serum albumin (Figure 2). The radioactivity that did not enter the gel upon electrophoresis was most likely albumin too tightly bound to the immunoglobulin to be dissociated by the present treatment, since upon electrophoresis on a 5% polyacrylamide gel this slower moving radioactivity comigrated with immunoglobulins (unpublished results). A reducing agent like dithiothreitol was not used to dissociate the antigen-antibody complex as others have done [8], since this treatment broke up the immunoglobulin into subunits that migrated very near the albumin band. Despite the immunoprecipitation reaction being carried out in Triton X-100 and deoxycholate [8], extensive washing of the immunoprecipitate is still necessary to remove non-specifically bound polypeptides. The minor bands seen in polyacrylamide gel electrophoresis (Figure 2) could be some of these polypeptides or they

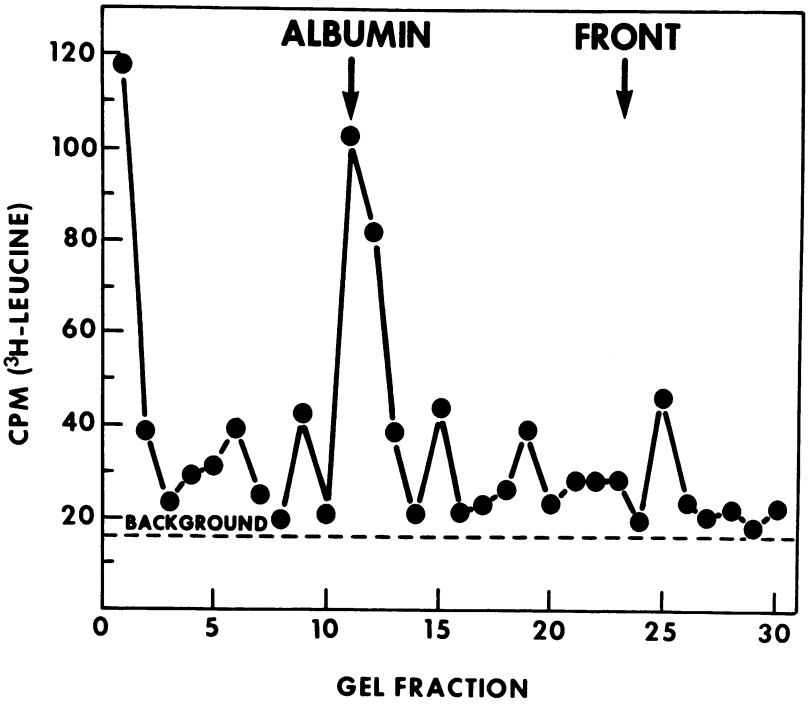

Fig. 2 - SDS-urea-polyacrylamide gel electrophoresis of the immunoprecipitated product from a 100 µl reaction mixture containing 300 µg/ml liver polysomal RNA. Incubation time was 60 minutes. The arrows indicate positions of pure albumin and bromphenol blue.

could be unfinished polypeptides of albumin. However, it is doubtful that these bands are significant since none of them were more than 2-fold above background and they were not reproducible. Also, the background radioactivity in the immunoprecipitate that was analyzed by electrophoresis was higher than that in the routine assay since the former was not dissolved in NaOH and reprecipitated with acid. Notwithstanding, the assay appears to be quantitative since there is a linear RNA dose dependence for both total protein and albumin synthesis up to a liver polysomal RNA concentration of 400 µg/ml (Figure 3). Also, the curve for albumin does extrapolate to zero. In addition, rat hepatoma cell lines that produce serum albumin at different rates have been shown by the present assay method to have pro-

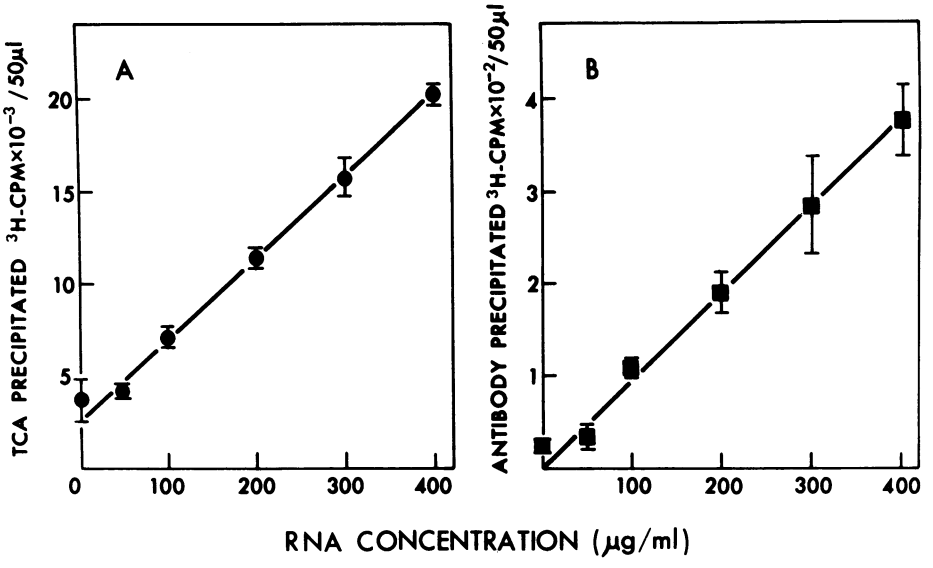

Fig. 3 - Effect of liver polysomal RNA concentration on *in vitro* incorporation of [^{3}H]leucine into total protein (A) and albumin (B). Triplicate reaction mixtures of 50 µl were incubated for 60 minutes.

portionate amounts of albumin mRNA activity [7]. Therefore, relative levels of translatable albumin mRNA can be estimated with this method.

Albumin synthesis represents 3% of total in this *in vitro* system, a percentage that is within the range observed in fasted animals *in vivo* [1,2]. However, the proportion of total *in vitro* protein synthesis that albumin represents may not be an accurate estimate of the *in vivo* value, since the reaction optima and efficiency of translation may not be the same for each mRNA.

Therefore, the reaction optima for Mg^{++} and K^{+} for albumin mRNA translation were compared to those for total [^{3}H]leucine incorporation. As shown in Figures 4 and 5, the optimal Mg^{++} and K^{+} concentrations for albumin synthesis are in the same range as those observed for other mRNA's [12,14,16], but differences are observed. The Mg^{++} optimum for albumin synthesis is 2.5 mM and that for total protein is 3.0 mM. Optimal concentrations for K^{+} are the same for albumin and total protein synthesis. However, the lat-

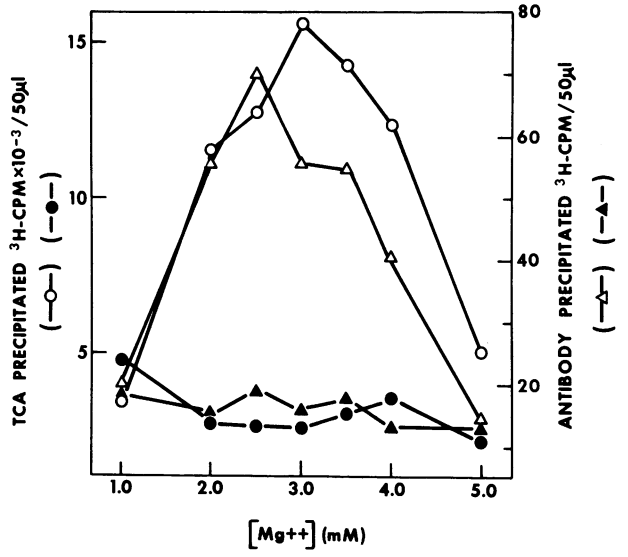

Fig. 4 - Effect of Mg++ concentration on *in vitro* [^{3}H]leucine incorporation into protein (circles) and albumin (triangles). Duplicate reaction mixtures were incubated at 25°C for 55 minutes with (open symbols) and without (closed symbols) 260 µg/ml liver polysomal RNA.

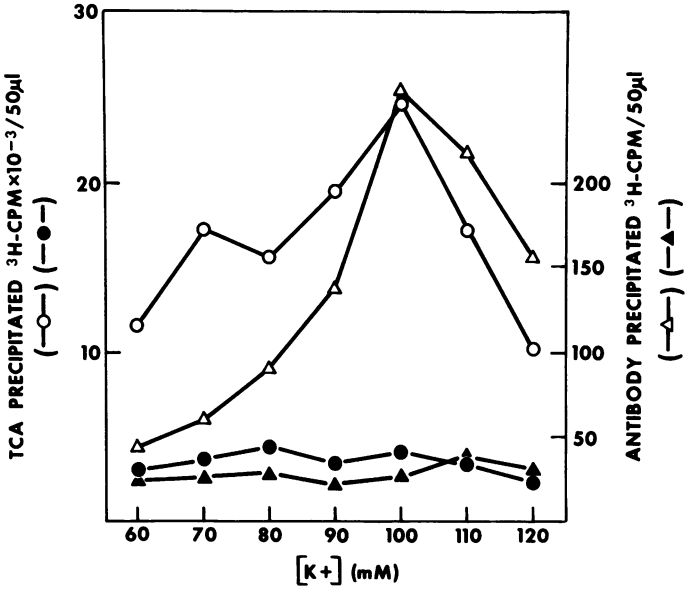

Fig. 5 - Effect of K+ concentration on *in vitro* [^{3}H]leucine incorporation into total protein (circles) and albumin (triangles). Duplicate reaction mixtures were incubated at 25°C for 55 minutes with (open symbols) and without (closed symbols) 250 µg/ml liver polysomal RNA.

ter decreases more rapidly than albumin synthesis at higher than optimal $[K^+]$, therefore causing albumin synthesis to represent a greater proportion of total protein synthesis at these higher K^+ concentrations. Potassium and magnesium concentrations were found to have a dramatic effect on the ratio of α and β chains of hemoglobin synthesized *in vitro*[20], and collagen synthesis requires a K^+ concentration of 180 mM in the wheat germ system[21,22].

In conclusion, crude lysates of commercial wheat germ can be used to compare quantitatively the levels of translatable albumin mRNA in different preparations of polysomal RNA. However, an estimation of albumin mRNA levels relative to those of other mRNA's may not be accurate due in part to differences in Mg^{++} and K^+ optima.

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