Translation of Albumin Messenger RNA in a Cell-free Protein-synthesizing System Derived from Wheat Germ*

Purified rat liver albumin mRNA directed the synthesis of albumin in a mRNA-dependent cell-free protein-synthesizing system derived from wheat germ extracts. The \(^{3}H\)leucine-labeled \textit{in vitro} translation product reacted with antibodies specific for albumin and co-migrated with authentic albumin in a gel electrophoresis in the presence or absence of sodium dodecyl sulfate. Higher concentrations of potassium and magnesium ions were required for the translation of albumin mRNA than for total liver mRNAs. These requirements were consistent for the purified albumin as well as when it was a component in the liver mRNA mixture. At the higher potassium or magnesium concentrations, only intact albumin molecules were synthesized, whereas lower concentrations of these ions caused the production of antibody-reactive fragments. These fragments were apparently the result of premature termination of peptide synthesis and not due to endogenous proteolytic activity.

The translation of eukaryotic mRNAs in a cell-free mRNA-dependent protein-synthesizing system could be an important technique for examining certain regulatory events in protein synthesis. The translational control of specific mRNAs and the mechanisms in post-translational processing could be studied in detail. Sensitive assays based on peptide synthesis might also be established to quanitate particular mRNA species. However, the usefulness of these potential applications would depend heavily on the accuracy of translation in this system.

Cell-free protein-synthesizing extracts prepared from un-toasted commercial wheat germ have been developed and examined in several laboratories (1–3). These extracts have been found to be dependent upon the addition of exogenous mRNA for protein synthetic activity and have been applied to a wide variety of translation problems. Among the mRNA species which have been investigated in this system are those coding for globin (2–5), immunoglobulins (6, 7), ovalbumin (8, 9), collagen (10, 11), virus proteins (2, 12–15), and polypeptide hormones (16–22).

We have examined the cell-free translation of purified rat liver albumin mRNA and total rat liver mRNAs in this protein-synthesizing system. Several experimental parameters for albumin synthesis have been investigated. Under optimal conditions for the translation of albumin mRNA, an intact albumin peptide is produced which co-migrates with authentic serum albumin under different electrophoretic conditions. Additional observations, however, indicate that various mRNAs may require different experimental conditions in the same extract for the optimum incorporation of \(^{3}H\)leucine into peptide products. The size distribution of the translation product, as well as the relative efficiencies of translation are markedly altered by variations in the concentrations of potassium and magnesium ions. Suboptimal concentrations of these ions result in premature termination of protein synthesis with the apparent release of peptidyl-tRNA from the polysomes. This technical problem can yield a wide range of estimates in the quantitation of mRNA by translational assays.

**EXPERIMENTAL PROCEDURES**

\textit{Preparation of Antibodies}—Antibodies against homogeneous rat serum albumin were prepared in goats and partially purified by ammonium sulfate fractionation (23). The specificity of the antibodies has been previously verified (22).

\textit{Purification of Albumin mRNA}—Albumin-synthesizing polysomes were separated from total rat liver polysomes through a double antibody technique which allowed isolation of a specific immunoprecipitate (24, 25). The albumin-polysome immunoprecipitate was dissolved in detergent and the polysomal RNA was separated from protein by sucrose gradient centrifugation. Albumin mRNA was then isolated by affinity chromatography on poly(U)-Sepharose (25).

\textit{Preparation of Total Rat Liver mRNA}—Total rat liver RNA was prepared from unfractionated liver homogenates by phenol/chloroform extraction (26), and the poly(A)-containing mRNA was isolated by affinity chromatography on poly(U)-Sepharose. An extinction coefficient of 1 \(A_{260}\) eq/50 \(\mu\)g of RNA was employed, and all mRNA samples had a 260/280 absorbance ratio of approximately 2.2.

\textit{Preparation of Wheat Germ Extracts}—Un-toasted wheat germ was obtained from International Multi Foods Inc., Buffalo, N. Y., and stored at 4°. Extracts were prepared as described by Roberts et al. (2), preincubated for 15 min at 30°, and stored as beads in liquid nitrogen. The final wheat germ extracts routinely had an \(A_{260}/A_{280}\) ratio of 1.6 and a concentration of 125 to 150 \(A_{260}\) ml.

\textit{Standard Translation Assays}—A reaction mixture of 100 \(\mu\)l with a final pH of 7.0 contained: 20 \(\mu\)l of preincubated wheat germ extract, \(28 \text{ mm Hapes}^{1}, 2 \text{ mm dithiothreitol}, 1 \text{ mm ATP}, 25 \mu\text{M GTP}, 8 \text{ mm creatine phosphate}, 4 \mu\text{M of creatine phosphokinase (Sigma), 40 \muM spermine, 25 \muM concentrations of each of 19 L-amino acids required for protein synthesis, 10 \muCi of \(^{3}H\)leucine (New England Nuclear, 40 to 60 Ci/mmol). 2.5 \text{ mm magnesium acetate}, 75 \text{ mm KCl}, and 0.1 to 1.0 \mu\text{g of mRNA. The reaction mixtures were incubated at 25° for 120 min.}
remaining reaction material was used immediately or stored at -20°C until further analysis. The data presented in Fig. 2 showed that these three polyamines stimulated translational activity, with the most highly charged molecule, spermine, being the most effective, and at the lowest concentrations. No synergistic effects of polyamine addition on peptide synthesis were observed when 60 μm spermine was added to 0.3 mm spermidine (data not shown). In this latter case, both polyamines were at their optimum concentrations. These observations on the effect of polyamines on translation in wheat germ extracts correlated quite well with the results reported by Atkins et al. (28) for a fractionated protein-synthesizing system derived from rabbit reticulocytes and Krebs ascites cells in which several different mRNA species were translated.

An investigation of several different preparations of wheat germ extracts revealed a relatively low level of amino acid incorporation activity in the absence of exogenous mRNA. In an attempt to further reduce this background activity, the effect of varying the preincubation time of the freshly prepared extracts was examined. Fig. 3B shows that the background activity was lowered about 25% by a 15- to 20-min preincubation while having a relatively minor effect on the translational capacity for exogenous total liver mRNA (Fig. 3A). As a result of this preincubation, the relative stimulation of peptide synthesis by exogenous mRNA appeared to be enhanced significantly (Fig. 3C). With various extracts and mRNA preparations, 10 μg/ml of added mRNA usually resulted in a stimulation of protein synthesis of 25- to 50-fold over background activity.

**RESULTS**

**Characterization of Wheat Germ Extracts** — General characteristics of the wheat germ protein-synthesizing system were examined. As a starting point, the initial experimental conditions were similar to those of Roberts and Paterson (2) and a total liver mRNA preparation was employed. Fig. 1A shows that the addition of exogenous liver mRNA to the assay mixture resulted in a linear incorporation of [3H]leucine into trichloroacetic acid-precipitable peptide material for about 30 min, with the activity gradually diminishing over the following 90-min period. Increasing the amount of added mRNA to 10 μg/ml resulted in a linear increase in amino acid incorporation into protein, but amounts greater than 20 μg/ml caused significant inhibition of protein synthesis (Fig. 1B). The optimum pH for protein synthesis with our wheat germ extracts ranged from pH 6.7 to pH 7.0 in the final complete reaction mixture (Fig. 1C). The optimum concentrations of potassium and magnesium ions for amino acid incorporation into peptide material (as codified for by total liver mRNA) were also determined and these results are discussed in detail in a subsequent section.

Spermine had been included in all of the characterization studies since polyamines have been implicated as being involved in protein synthesis (27). The effect of spermine, as well as spermidine and putrescine, on amino acid incorporation into protein was investigated. The data presented in Fig. 2 showed that these three polyamines stimulated translational activity, with the most highly charged molecule, spermine, being the most effective, and at the lowest concentrations. No synergistic effects of polyamine addition on peptide synthesis were observed when 60 μm spermine was added to 0.3 mm spermidine (data not shown). In this latter case, both polyamines were at their optimum concentrations. These observations on the effect of polyamines on translation in wheat germ extracts correlated quite well with the results reported by Atkins et al. (28) for a fractionated protein-synthesizing system derived from rabbit reticulocytes and Krebs ascites cells in which several different mRNA species were translated.

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**Fig. 1.** Characterization of the protein synthesis reaction. A, time course of protein synthesis. Standard translation assays were performed with (- - - -) or without (O O O) 15 μg/ml of total rat liver mRNA for varying lengths of time. The trichloroacetic acid-precipitable, radioactive material in 5-μl aliquots was measured. B, the effect of mRNA concentration on protein synthesis. Standard translation assays were performed for 2 h with varying amounts of total rat liver mRNA, and the peptides in 5-μl aliquots were measured. C, the effect of pH on protein synthesis. The final reaction mixtures were adjusted to different pH values. Standard translation assays were then performed with 5 μg/ml of total rat liver mRNA and radioactivity in 10-μl aliquots was measured.

**Fig. 2.** The effect of polyamines on protein synthesis. Standard translation assays were performed with 10 μg/ml of total liver mRNA and varying concentrations of A, spermine; B, spermidine; and C, putrescine. Peptide material in 5-μl aliquots was measured.
Translation of Albumin mRNA

Purified albumin mRNA (25) was translated in the cell-free wheat germ system employing the optimum conditions as determined above, for total liver mRNA. Following the incubation period, the ribosomes were removed by centrifugation, the released peptide material was treated with RNase, and the unincorporated [3H]leucine was removed by dialysis. The translation product was denatured with sodium dodecyl sulfate in the presence of dithiothreitol, then examined by electrophoresis on polyacrylamide gels. Analysis of the radioactive material in the gels (Fig. 4A) showed an unexpected heterogeneous size distribution. In a separate experiment, the albumin mRNA translation product was mixed with albumin antibodies in the presence of unlabelled albumin carrier, and the immunoprecipitate was collected, denatured, and examined by gel electrophoresis (Fig. 4B). About 90% of the radioactive peptide material had reacted with the antibody, and only a portion of the lowest molecular weight material was not in the immunoprecipitate. Additional experiments showed that the discrete peptide at Fraction 10 co-migrated with authentic [3H]leucine-labeled serum albumin. Examination of the low endogenous background activity, which was present in the extracts in the absence of added mRNA showed no radioactive peaks, and no material which adsorbed to the immunoprecipitates (Fig. 4, A and B). Reducing the protein synthesis incubation time to 60 min, or varying the mRNA concentration over a 5-fold range did not significantly alter the peptide pattern.

Approximately 40% of the radioactive trichloroacetic acid-soluble material had sedimented with the ribosomes. Analysis of this material by gel electrophoresis (Fig. 4C) showed no unusual peaks of peptide material, whereas the released translation product showed discrete major peptides. The size distribution and relative amounts of these peptides were reproducible for three different preparations of albumin mRNA and two different wheat germ extracts.

The nature and cause of these albumin fragments were investigated further. Since all of the larger fragments reacted specifically with the albumin antibody, they probably contained the normal peptide sequences from the NH2-terminal portion of the protein; and they were not likely to be the result of random peptide initiation towards the COOH terminus. If the fragments were the result of improper initiation in the middle of the mRNA, then this initiation would be required to occur in the correct codon phase in order to account for the antibody reactivity of 90% of the fragments. If initiation occurred randomly out of phase, two-thirds of the fragments (3 nucleotides/codon) would not be expected to correspond to albumin peptides and would not react with the specific antibody. Prolonging the protein synthesis incubation time to 7 h did not affect the peptide pattern or the material at Fraction 10, suggesting that the fragments did not arise as a result of endogenous proteolytic activity. Incubation of the wheat germ extract with [3H]labeled poly(U) suggested a moderate amount of RNase activity. Up to 40% of the poly(U) was hydrolyzed to acid-soluble material. Preincubation of total liver mRNA with fractionated wheat germ extracts, followed by supplementation to permit protein synthesis, also resulted in up to a 40% loss of mRNA activity. Translation of an albumin mRNA preparation which contained additional partially degraded albumin mRNA pieces2 derived from the 3' terminus did not result in the appearance of new peptide peaks in the translation product. The mixture of intact and degraded mRNA had a greatly reduced protein synthetic activity, but no significant change in the relative size distribution of the peptide pattern was observed. These observations have not strictly eliminated the possibility of improper initiation, premature peptide

2 Rat liver polysomes which contained slightly degraded starting material were immunoprecipitated to isolate albumin-synthesizing polysomes (25). The polysomal RNA was extracted, and albumin mRNA purified by poly(U)-Sepharose affinity chromatography (25). This mRNA preparation, which contained a small amount of intact mRNA, consisted primarily of partially degraded pieces derived from the poly(A)-containing 3' terminus. Partially degraded mRNA pieces showed a size distribution of about 8 to 17 S as compared to intact 17 S albumin mRNA (25) when examined by sucrose gradient sedimentation. The degraded mRNA pieces had essentially no translational activity.
release from a degraded mRNA, or ribosome-associated proteolyis during translation. However, studies in subsequent sections suggest that fragment production is the result of other reaction parameters.

The size distribution of the polysomes formed in the protein synthesis reaction mixture was examined at various incubation times (Fig. 5). The average size of the polysomes at 60 min was quite similar to that of the 30-min profile, whereas some peptide-bearing monosomes had accumulated by 120 min. The finding that the size of the polysomes does not change in the first 60 min of incubation while there is nearly a linear increase in released peptide material (with no accumulation of radioactive monosomes), suggests that the albumin mRNA molecules are reinitiated. Supplementing the protein synthesis reaction mixture with either a ribosome-free wheat germ extract, or a pH 5.0 fraction from a rat liver homogenate (29), and using low amounts of mRNA did not affect the peptide pattern, indicating that the various translational components were probably not limiting.

The various experiments, discussed above, suggested that the wheat germ extract was capable of translating albumin mRNA but that a moderate amount of endogenous RNase activity appeared to be a technical problem. Despite this problem, however, some full length peptides continued to be synthesized. At this point, it seemed possible that the optimum conditions established for the translation of the total liver mRNAs might be different for the translation of albumin mRNA.

**Effect of Potassium Ion Concentration on Albumin mRNA Translation**—The optimum potassium ion concentration for protein synthesis was re-examined (Fig. 6) and purified albumin mRNA was found to have a higher optimum (by 15 mM) than total liver mRNA. Immunoprecipitation analysis of the albumin translation product from the total liver mRNA also showed a higher optimum, which was identical to that of the isolated albumin mRNA. These results were consistently found with three different wheat germ extracts and three different mRNA preparations examined over a 10-fold concentration range. Further analysis of the data indicated that the per cent of the total liver mRNA translation product which was immunologically identified as albumin could vary over a 6-fold range, depending on the potassium ion concentration (Table I). This finding indicates that serious problems may exist in the interpretation of mRNA quantitation data based on general translational assays in wheat germ protein-synthesizing systems.

The peptide material synthesized by the purified albumin mRNA at different potassium ion concentrations was examined by gel electrophoresis (Fig. 7). The radioactivity profiles showed that at lower ion concentrations, only incomplete fragments were produced. Intact albumin became the dominant product only at higher potassium levels. Above the optimum potassium concentration, only complete albumin molecules were detected, but the yield of the peptide material was reduced.

**Effect of Magnesium Ion Concentration on Albumin mRNA Translation**—The optimum magnesium ion concentration for protein synthesis was also re-examined (Fig. 8) and purified albumin mRNA was found to have a higher optimum (by 0.5 mM) than total liver mRNA. In these experiments, the optimum potassium ion concentration (90 mM) for albumin mRNA was employed. Immunoprecipitation analysis further showed different KCl concentrations with 10 μg/ml of total liver mRNA (---). The ribosome-free supernatant fluids were then collected and treated as described under "Experimental Procedures." The trichloroacetic acid-precipitable, radioactive material in 10-μl aliquots was measured and the albumin immunoprecipitation assays (---) were performed on 70-μl aliquots. The data in both determinations were normalized to the total supernatant fluid volume recovered. In separate protein synthesis experiments, 2.5 μg/ml of purified albumin mRNA (---) was assayed and the total peptide material in 5-μl aliquots was measured.

**Fig. 5 (left).** Polysome formation during translation in wheat germ extracts. Standard protein synthesis assays were incubated for 30 min (●-●), 60 min (○---○), and 120 min (▲-▲) with 6 μg/ml of purified albumin mRNA. Translation was stepped by the addition of 1 μM cycloheximide, and the reaction mixtures were incubated at 0° and adjusted to 10 mM leucine and 0.5 μg/ml of sodium heparin. The mixtures were then layered on linear gradients of 15 to 50% sucrose containing 5 mM MgCl₂, 25 mM NaCl, 50 mM Tris at pH 7.4, and 0.50 mg/ml of sodium heparin, and centrifuged at 280,000 × g for 120 min at 5°. Gradient fractions of 0.6 ml were collected and adjusted to 50 mM leucine and 250 μg/ml of rat serum albumin, mixed, then adjusted to 10% in trichloroacetic acid. The protein precipitates were collected on glass fiber filters (GF/C, Whatman), dried, and digested with 0.75 ml of Protosol, and radioactivity was measured. Sedimentation is from left to right, and 80 S indicates the position of ribosome monomers. The inset shows the time course of this assay and a control reaction without added mRNA (---).

**Fig. 6 (center).** Effect of potassium ion concentration on the translation of albumin and total liver mRNA. Standard protein synthesis assays (in 2.5 mM magnesium acetate) were performed at different KCl concentrations with 10 μg/ml of total liver mRNA (○-○). The ribosome-free supernatant fluids were then collected and treated as described under "Experimental Procedures." The trichloroacetic acid-precipitable, radioactive material in 10-μl aliquots was measured and the albumin immunoprecipitation assays (---) were performed on 70-μl aliquots. The data in both determinations were normalized to the total supernatant fluid volume recovered. In separate protein synthesis experiments, 2.5 μg/ml of purified albumin mRNA (---) was assayed and the total peptide material in 5-μl aliquots was measured.

**Fig. 7 (right).** Gel electrophoresis of albumin mRNA translation products at various potassium ion concentrations. Standard translation assays (in 2.5 mM magnesium acetate) were performed as described in Fig. 6, with 5 μg/ml of purified albumin mRNA at different KCl concentrations: A, 45 mM; B, 60 mM; C, 75 mM; D, 90 mM; and E, 105 mM. Gel electrophoresis and analysis of the translation products were carried out as described under "Experimental Procedures." Direction of migration is from left to right. Bromphenol blue dye migrated to Fraction 38. Under these electrophoretic conditions, "C-labeled serum albumin migrated to Fraction 10. Note that Panel E is illustrated at an enlarged scale.
that the albumin mRNA preferred the higher magnesium ion levels when it was a component in the total liver mRNA mixture. Once again, calculations showed that the per cent of the total liver mRNA translation product which was immunologically detectable as albumin varied over a wide range, depending on the magnesium ion concentration. Examination of the peptide material translated by the purified albumin mRNA at different magnesium levels (Fig. 9) by gel electrophoresis revealed that incomplete fragments were produced at low magnesium concentrations and that raising the concentration of this ion resulted in the synthesis of intact albumin molecules.

**Nature of Intact Albumin mRNA Translation Product**—Purified albumin mRNA was translated under its optimum conditions, and the intact protein product was analyzed by gel electrophoresis. In the presence of sodium dodecyl sulfate (Fig. 10A), at neutral pH, and long electrophoresis times, the translation product migrated one gel fraction slower than serum [14C]albumin. Electrophoresis in the absence of detergent (Fig. 10B), at pH 9, showed that the translation product co-migrated exactly with serum [14C]albumin, even with respect to the albumin dimer (Fractions 15 and 16) which appeared to form spontaneously under these conditions. In previous work (25) we have shown that the albumin translation product in wheat germ extracts was identical to serum [14C]albumin with respect to tryptic peptide analysis, except that the in vitro product contained an extra leucine-rich peptide. These data suggest that the apparent albumin precursor is not much larger than the native secreted protein and are in agreement with the finding of only a short precursor segment associated with albumin in rat liver homogenates (30, 31).

**DISCUSSION**

Several aspects of the assay conditions in the wheat germ protein-synthesizing system were investigated with a total liver mRNA preparation. As others have observed (1), we found considerable variation in activity with different batches of wheat germ. We also noted some variability in the level of endogenous background activity, the degree of stimulation by exogenous mRNA, and the optimum potassium ion concentration between different wheat germ extracts. Our initial approach was to prepare an extract and establish protein synthesis reaction conditions that would yield a maximum incorporation of amino acids into a peptide product. The data presented here, however, indicate that all mRNA species may not have the same assay requirements, and that minor changes in assay conditions can have dramatic effects on translational efficiencies in this system. Depending on individual needs, it may be necessary to evaluate translational requirements for individual mRNA species in each wheat germ extract employed.

The translation of purified albumin mRNA, under conditions which were optimal for total liver mRNAs, yielded discreet fragments of a lower molecular size than native albumin. Since preliminary experiments indicated that the essential components required for protein synthesis were apparently not limiting in concentration, the possibility of degradation artifacts was considered. No evidence of proteolytic degradation of the translation product was found. A moderate amount of RNase activity was observed, and mRNA degradation which leads to the synthesis of peptide fragments could not be ruled out. However, subsequent studies indicated that certain parameters of the assay conditions were a more likely cause of fragment production.

**Table I**

| KCl (mM) | Albumin synthesis (in % of total trichloroacetic acid precipitable material) |
|----------|--------------------------------------------------------------------------------|
| 45       | 1.6                                                                           |
| 60       | 2.0                                                                           |
| 75       | 3.8                                                                           |
| 90       | 6.0                                                                           |
| 105      | 9.6                                                                           |

**Fig. 8 (left).** Effect of magnesium ion concentration on the translation of albumin and total liver mRNA. Standard protein synthesis assays (in 90 mM KCl) were performed at different magnesium concentrations with 4 µg/ml of purified albumin mRNA (○—○) or 10 µg/ml of total liver mRNA (●—●). The experimental protocols and immunoprecipitation assays (○—○) for albumin synthesized by the total liver mRNA were carried out essentially as described in Fig. 6 with the total reaction mixture.

**Fig. 9 (right).** Gel electrophoresis of albumin mRNA translation products at various magnesium ion concentrations. Standard translation assays (in 90 mM KCl) were performed as described in Fig. 8 with purified albumin mRNA at different magnesium acetate concentrations: A, 1.5 mM; B, 2.0 mM; C, 2.5 mM; D, 3.0 mM; E, 3.5 mM; and F, 4.0 mM. Gel electrophoresis and analysis of the translation products were carried out as described previously. Direction of migration is from left to right. Bromphenol blue dye migrated to Fraction 38. Note that Panel D is illustrated at a reduced scale.
Various reports by the other laboratories indicated that the concentrations of potassium and magnesium ions could affect the length of the translation product. Collagen mRNA required a potassium concentration higher than that for maximum amino acid incorporation to support the synthesis of large peptides (10, 11). Several virus mRNAs required different potassium and magnesium optima in wheat germ extracts for efficient translation (13, 15). Increasing the potassium concentration stimulated the protein synthetic rate and increased the protein-synthesizing system to quantitate mRNA levels by a translation assay is risky. Because the relative amount of albumin synthesized with a liver mRNA mixture can vary (up to 6-fold) with respect to total peptides synthesized under different assay conditions, it may be difficult to employ rates of protein synthesis to quantitate the absolute amount of albumin mRNA in a total liver mRNA preparation. This problem is further complicated if translation yields incomplete peptide products.

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![Figure 10: Gel electrophoresis of albumin synthesized in wheat germ extracts. Standard protein synthesis assays with 90 mM KCl and 3.0 mM magnesium acetate were performed with 3 µg/ml of purified albumin mRNA. Following translation, ribosomes were removed by sedimentation, free [3H]leucine was removed by dialysis, and [3H]-labeled rat serum albumin (16 cpm/µg) was added to each sample. A, analysis of the translation product on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate was performed as described under "Experimental Procedures," except that electrophoresis was for 6 h, and bromphenol blue dye migrated out of the gel. B, electrophoresis was performed in 7.5% polyacrylamide gels in the presence of 25 mM Tris and 190 mM glycine buffer at pH 9.0 without detergent. Direction of migration is from left to right. □□□□ translation product; □□□□□, [3H]albumin.](http://www.jbc.org/)
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