Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3392–3396, September 1975
Biochemistry

Nonribosomal proteins associated with eukaryotic native small ribosomal subunits

(initiation factors/gel electrophoresis/globin synthesis)

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Communicated by Severo Ochoa, June 16, 1975

ABSTRACT **The native small ribosomal subunit (S^n) from rabbit reticulocytes which is able to initiate translation of globin mRNA in a cell-free system carries additional protein components. The latter can be separated from the subunit in a high salt sucrose gradient yielding a top fraction (*T*) and a complex fraction (*C*), sedimenting at about 4 and 15 S, respectively. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed that fraction *T* contained four dominant polypeptides, while fraction *C* represents a large protein complex consisting of at least 10 polypeptides. S^n isolated from other sources showed similar patterns of their nonribosomal proteins.**

Reconstitution experiments revealed that fraction *C* is absolutely required for protein synthesis, while fraction *T* enhances protein synthesis only in the presence of *C*.

The adherence of these protein factors to the subunit is not mediated by magnesium ions. Treatment of S^n with EDTA and centrifugation in a magnesium-free sucrose gradient caused unfolding of the subunits and dissociation of several ribosomal proteins, but not of the factors. The unfolded ribosomal subunits sedimented as two distinct peaks. The more slowly sedimenting peak contained proteins of fraction *T* and the faster sedimenting one contained the 15S complex, indicating heterogeneity of the S^n population with respect to the factors attached to them.

Initiation factors for protein synthesis in pro- and eukaryotes are in general found to be bound to ribosomes, from which they can be extracted by high concentrations of monovalent ions (1, 2). In eukaryotes it was recently established that the so-called native small ribosomal subunits (S^n), which comprise only a few percent of the total ribosomes, are able to initiate translation of mRNA in a reconstituted system containing derived large ribosomal subunits and pH 5 enzymes as the only other components (3, 4). Furthermore, it was reported that S^n contained several nonribosomal proteins (5) and that a salt extract from S^n could stimulate globin mRNA translation in a cell-free protein synthesis system (6).

These findings prompted us to identify the nonribosomal proteins found in S^n and to investigate their possible localization in other components of the ribosomal fraction, such as monosomes and polysomes, their mode of linkage to the small ribosomal subunit, their occurrence in a variety of cells, and their role in a reconstituted system.

METHODS

Preparation of Native Small Ribosomal Subunits (S^n). Procedures were essentially as described (3). In brief: blood from anemic rabbits (7) was collected in the presence of cycloheximide. Further operations were performed in the cold (1–4°). Washed reticulocytes were lysed in 2 volumes of a solution of 10 mM Tris·HCl (pH 7.5)–10 mM KCl–1.5 mM $MgCl_2$–2 mM dithiothreitol and homogenized with three strokes in a Potter-Elvehjem homogenizer. The postmitochondrial supernatant (10 min, 20,000 × g_{av}) was centrifuged first for 30 min and the resulting supernatant was subsequently centrifuged again for 5 hr at 105,000 × g_{av} in a Spinco no. 40 rotor, yielding a 30-min and a 5-hr pellet of ribosomes. The 5-hr pellet, containing the bulk of S^n, was resuspended in buffer A [10 mM Tris·HCl (pH 7.5)] at a concentration of 120 A_{260} units/ml; 0.5-ml aliquots were layered on 12.5 ml of a 10–40% sucrose gradient in buffer B [20 mM triethanolamine·HCl (pH 7.5)–100 mM KCl–3 mM MgCl–2 mM dithiothreitol] and centrifuged 5 hr at 190,000 × g_{av} in an SB283 rotor of an IEC centrifuge. The S^n peak was collected using an ISCO gradient analyzer and subsequently was sedimented in a Spinco no. 40 rotor for 10 hr at 105,000 × g_{av}. Pellets were stored at −80°.

S^n from duck reticulocytes (a gift of Dr. R. Ruiz-Carillo), mouse myeloma MOPC 41, Krebs ascites, and rat liver were prepared in an identical manner.

Fractionation of S^n. Amounts of S^n as indicated in figure legends were resuspended in 200 or 300 μl of buffer C [500 mM KCl–50 mM triethanolamine·HCl (pH 7.5)–5 mM $MgCl_2$] and layered on 12.5 ml of a 10–40% or 3.8 ml of a 10–30% sucrose gradient, both in buffer C, and were centrifuged for 5 hr at 40,000 rpm in an SB283 rotor of an IEC or in a SW56 rotor of a Beckman centrifuge. Fractions (*T* = top, *C* = complex, and *S* = small subunit) were collected.

Monosomes and Polysomes(from dimers to heptamers) were prepared using sucrose gradients identical to those used for the preparation of S^n except that the time of centrifugation was reduced to 1.5 hr.

Derived Small and Large Ribosomal Subunits (S^0 and L^0) and Globin Messenger Ribonucleoprotein (mRNP) were prepared from reticulocyte ribosomes by the puromycin–KCl method (8, 9).

A pH Enzyme from a 5-hr S100 of Krebs ascites cells was prepared as described (10).

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate ($NaDodSO_4$/Polyacrylamide Gel Electrophoresis) was performed as described (11) in a slab gel apparatus using a 10–15% acrylamide gradient as a resolving gel and a 5% spacer gel (1 mm thick). Samples were prepared as described (3). Gradient fractions were precipitated with one volume of 20% trichloroacetic acid at 0°. The precipitate was collected by centrifugation at 2000 × *g* and was dissolved during a 20-min incubation at 37° in 25 μl of a solution of 30% sucrose–100 mM Tris base–5% $NaDodSO_4$–0.01% bromophenol blue–10 mM dithiothreitol. After boiling for 2 min at 100° and cooling to room temperature, 5 μl of a 1.0 M solution of α-iodoacetamide was added. Rabbit globin (α and β chain), porcine chymotrypsinogen, ovalbu-

Abbreviations: S^n, native small ribosomal subunit; mRNP, messenger ribonucleoprotein; $NaDodSO_4$, sodium dodecyl sulfate; S^0 and L^0, derived small and large ribosomal subunit, respectively.

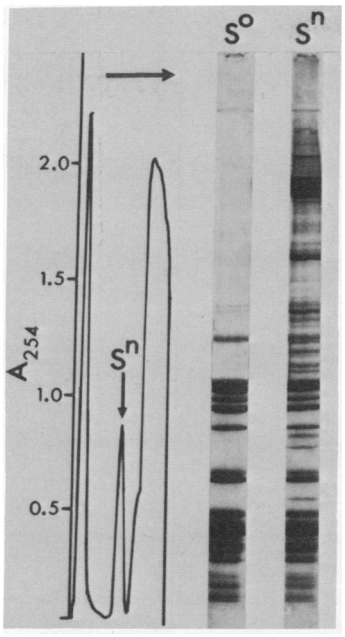

FIG. 1. Presence of nonribosomal proteins on S^n. S^n was obtained from a preparative sucrose gradient (*left*) as described in *Methods*. For comparison three A_{260} units of S^0 prepared by the puromycin–KCl method and two A_{260} units of S^n were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The horizontal arrow indicates the direction of sedimentation.

min, bovine albumin, and β-galactosidase of *Escherichia coli* were used as standards for molecular weight determination.

***In Vitro* Polypeptide Synthesis** was essentially as described (3) except that the volume of the reaction mixture was 0.5 ml instead of 0.25 ml. One-half milliliter contained 50 μmol of KCl, 10 μmol of Tris·HCl (pH 7.5), 1.5 μmol of $MgCl_2$, 1.0 μmol of dithiothreitol, 0.5 μmol of ATP, 0.1 μmol of GTP, 3.0 μmol of creatine phosphate, a few crystals of creatine phosphokinase, 15 nmol of each L-amino acid except for leucine, 1.0 μCi of [^{14}C]leucine (specific activity 312 Ci/mol), 100 μl of pH 5 enzyme, and 0.02 A_{260} unit of globin mRNP. As specified in figure legends, either S^n and L^0 were present in the incorporation system or S^0 and L^0 together with various amounts of fractions T and C derived from S^n. In some assays S^0 and L^0 were replaced by monosomes. Incubation was at 37°. Twenty-five microliter samples were removed at indicated time points and spotted on 3MM Whatman filter paper discs which were processed according to Mans and Novelli (12). Radioactivity was determined in toluene-Liquifluor in a Beckman LS 350 scintillation counter at about 75% efficiency.

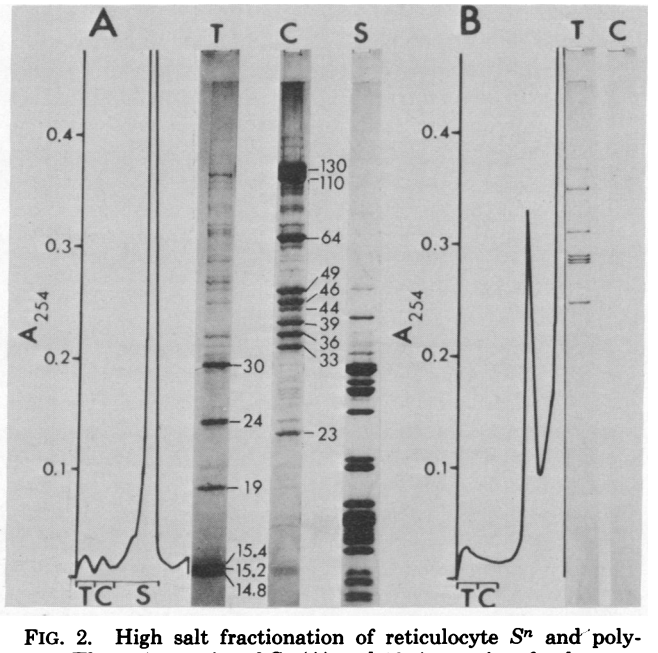

FIG. 2. High salt fractionation of reticulocyte S^n and polysomes. Three A_{260} units of S^n (A) and 10 A_{260} units of polysomes (dimers to heptamers, B) were resuspended each in 0.3 ml of buffer C and centrifuged in 10–40% sucrose in buffer C as described in *Methods*. Indicated on the abscissa of the sedimentation profiles (left side of A and B) are those fractions (T = top fraction, C = complex fraction, S = small subunit fraction) which were subsequently analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in slots marked correspondingly (right side of A and B). Numbers at the right of the columns × 1000 indicate the molecular weights of the polypeptides.

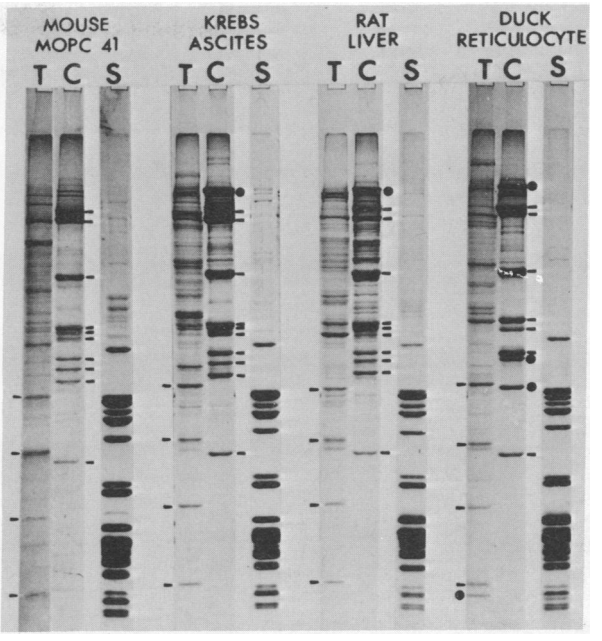

FIG. 3. Fractions of S^n from various sources. S^n were isolated from mouse myeloma MOPC 41, Krebs ascites, rat liver, and duck reticulocytes. S^n in amounts of 3.0, 2.5, 2.3, and 2.5 A_{260} units, respectively, were resuspended and centrifuged. Gradient fractions were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described in legend of Fig. 2. *Bars* indicate corresponding, and *dots*, differing polypeptides.

RESULTS

A comparison (Fig. 1) of the polypeptide banding pattern in $NaDodSO_4$/polyacrylamide gel electrophoresis between native small ribosomal subunits (S^n) of reticulocytes and the derived small ribosomal subunits (S^0) obtained from reticulocyte polysomes by the puromycin–KCl procedure revealed the presence of additional distinct, nonribosomal polypeptides in the S^n fraction. Similar additional polypeptides were previously described in the S^n fraction isolated from ascites ribosomes (5).

Our goal for the subfractionation of S^n was to dissociate only the nonribosomal polypeptides, leaving the small ribosomal subunit intact; the latter requires that extraction as well as subsequent centrifugation in a sucrose gradient is performed in the presence of magnesium ions. It can be seen from the A_{254} recording in Fig. 2A that sedimentation of the

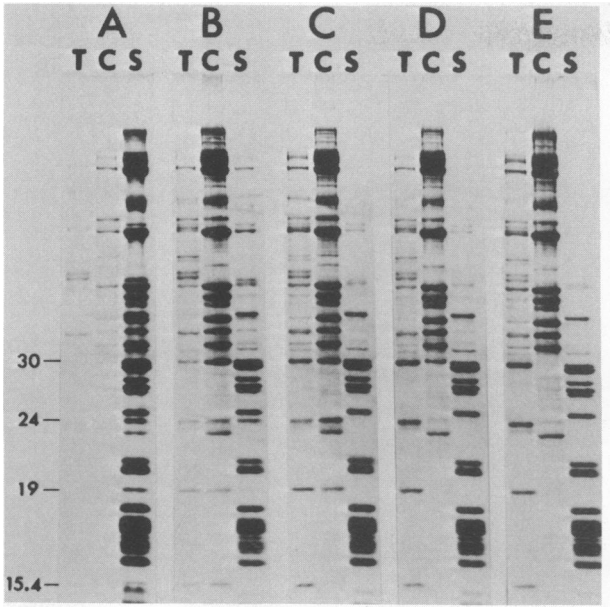

FIG. 4. Effect of increasing KCl concentrations on S^n. 2.5 A_{260} units of reticulocyte S^n were resuspended in buffer A and centrifuged in 10–40% sucrose gradients containing 20 mM triethanolamine·HCl (pH 7.5) and 100 mM KCl–3 mM $MgCl_2$ (A); or 150 mM KCl–3 mM $MgCl_2$ (B); or 200 mM KCl–3.5 mM $MgCl_2$ (C); or 250 mM KCl–3.5 mM $MgCl_2$ (D); or 500 mM KCl–5 mM $MgCl_2$ (E). Centrifugation and gradient fractionation was performed as described in legend of Fig. 2. Top fraction = T, complex fraction = C, and small subunit fraction = S were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Numbers at the left × 1000 indicate the molecular weights of the polypeptides characteristic for fraction T.

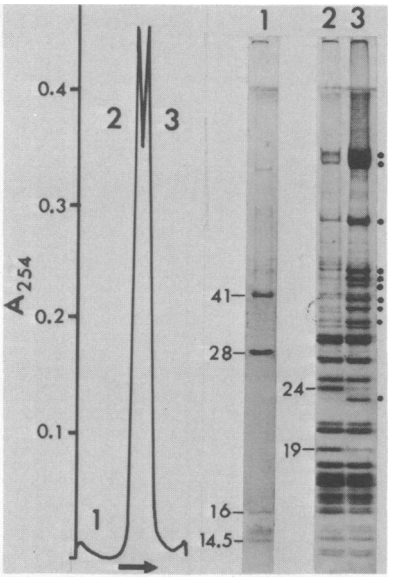

FIG. 5. Effect of EDTA on S^n. 2.5 A_{260} units of reticulocyte S^n were resuspended in 0.3 ml of a solution of 20 mM triethanolamine·HCl (pH 7.5)–50 mM KCl containing 0.75 μmol of EDTA and layered on 12.5 ml of a 10–40% sucrose gradient in 20 mM triethanolamine·HCl (pH 7.5)–50 mM KCl. Centrifugation was at 4° for 5 hr at 40,000 rpm in a SB283 rotor (IEC). Gradient fractions 1, 2, and 3, as indicated in the sedimentation profile (left), were collected and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (right). Numbers to the left of columns 1 and 2 indicate molecular weights in thousands. Dots on the right of column 3 indicate polypeptides identical to those found in fraction C (see Fig. 2A).

S^n fraction into a sucrose gradient containing 500 mM KCl, buffer, and 5 mM $MgCl_2$ resulted in a dominant peak sedimenting at 40S (labeled S in Fig. 2A), and in two small additional peaks sedimenting at about 4 S and about 15 S (labeled T and C, respectively, in Fig. 2A). The corresponding polypeptide patterns of these three peaks in $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2A) showed that the derived small subunit (S) had lost the additional S^n associated polypeptides, retaining only the characteristic ribosomal polypeptides (see Fig. 1), whereas all of the S^n associated polypeptides were found in the two more slowly sedimenting top fraction (T) and complex fraction (C) (Fig. 2A). Fraction T contains four dominant polypeptides. According to their mobilities in $NaDodSO_4$/polyacrylamide gel electrophoresis, the estimated molecular weights are 15,400, 19,000, 24,000, and 30,000 (the 14,800 and 15,200 molecular weight bands are the α and β chains of globin, respectively, and their intensity differs in various preparations; see also Fig. 4). Fraction C consists of ten major polypeptides. It appears therefore that fraction C is the equivalent of the large complex of polypeptides isolated by others from the salt wash of crude ribosomes (13). The sum of the molecular weights of the 10 polypeptides is about 570,000. The exclusive association of these polypeptides with the small ribosomal subunit is demonstrated in Fig. 2B. Sedimentation of polysomes under identical conditions as in Fig. 2A does not result in the release of any of the polypeptides found in fractions T and C of Fig. 2A. Instead, a characteristic set of six polypeptides was released (Fig. 2B, slot T).

The occurrence of specific polypeptides attached to S^n is not restricted to reticulocytes. S^n fractions were also isolated from mouse myeloma MOPC 41, Krebs ascites cells, rat liver, and duck reticulocytes. As in the case of reticulocyte S^n, two groups of polypeptides sedimenting as two peaks at about 4 S and about 15 S can be dissociated by salt. Furthermore, as can be seen from Fig. 3, there is a striking similarity in the polypeptide patterns in $NaDodSO_4$/gel electrophoresis of these two fractions. Thus, at least the molecular weight of most of the polypeptides (see Fig. 3, bands labeled with bars) in the S^n fraction from various sources is identical, although there were some distinct differences (labeled with dots).

So far we have established that there is a specific interaction of the two groups of distinct polypeptides apparently in a salt linkage with the small ribosomal subunit. The following experiments were designed to examine this linkage in more detail. Isolated S^n were sedimented into sucrose gradients containing increasing concentrations of KCl. Fractions were collected as indicated and analyzed by $NaDodSO_4$/gel electrophoresis. Fig. 4 shows that the ten polypeptides that make up the 15S complex were released from the small ribosomal subunit as a group, at 150 mM KCl. In contrast, the four polypeptides characteristic of fraction T were dissociated individually, suggesting that these four polypeptides exist as four single proteins.

Magnesium ions play an important role not only in maintaining the structure of the ribosomal subunits, but also in the interaction of other components with ribosomal subunits. It was therefore of interest to investigate whether chelation of magnesium ions by EDTA would cause dissociation of the T and C fraction polypeptides from the S^n. Fig. 5 (left side) shows the sedimentation profile of S^n that was treated with EDTA and subsequently sedimented into a sucrose gradient containing no magnesium and low concentrations of mono-

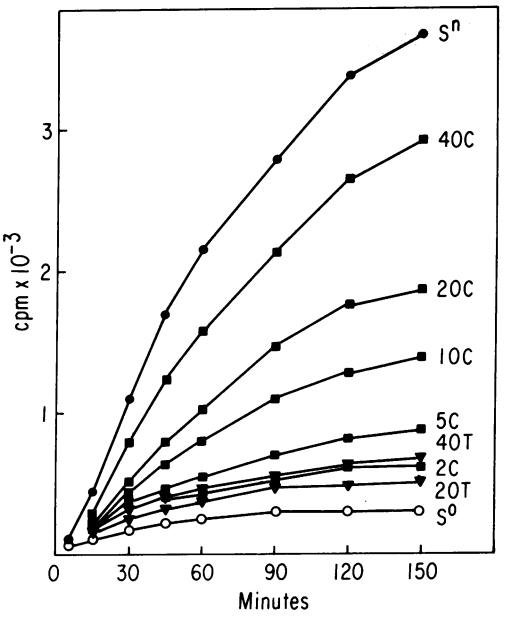

FIG. 6. Time course of polypeptide synthesis in a reconstituted system containing either S^n or S^0, or S^0 and various concentrations of fractions T and C. Fractions T and C were collected each in 0.6-ml aliquots from a sucrose gradient in buffer C (3.8-ml variety, see *Methods*) containing 12.0 A_{260} units of reticulocyte S^n. Various aliquots of fractions T and C were assayed directly, i.e., without removal of sucrose or salt in an amino-acid incorporation system as described in *Methods*. Compensation of the high salt concentration was accomplished through dilution afforded by the relatively large volume (0.5 ml) used in the *in vitro* polypeptide synthesis system. The latter contained in addition to globin mRNP (0.02 A_{260} unit), L^0 (1.0 A_{260} unit), and pH 5 enzyme (100 μl), either S^n (0.4 A_{260} unit) or S^0 (0.4 A_{260} unit) alone (curves marked S^n and S^0, respectively) or S^0 (0.4 A_{260} unit) in combination with various amounts of fractions T and C (numbers next to curves refer to amounts in μl). 20 μl (see curve 20C and 20T) contain an amount of either fraction carried by 0.4 A_{260} unit of S^n.

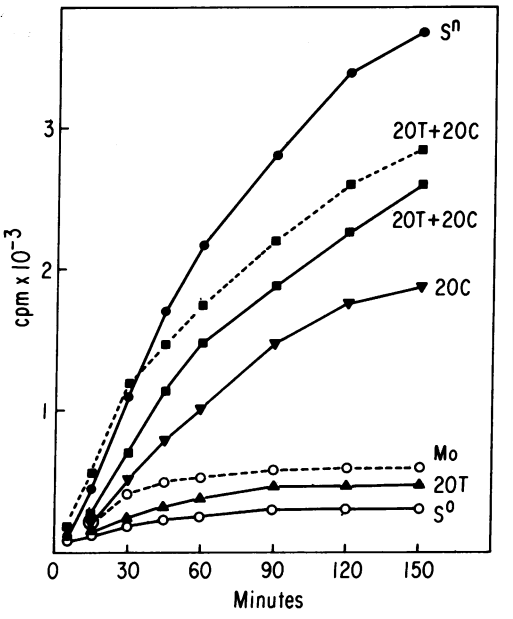

FIG. 7. As Fig. 6, except that fractions T and C were also used in combination and that, in two cases, S^0 and L^0 were replaced by 1.4 A_{260} units of monosomes (dotted lines).

valent ions. Surprisingly, two peaks could be resolved. Na-DodSO_4 gel analysis (Fig. 5, right side) showed that both peaks contained the typical ribosomal polypeptides; however, the more slowly sedimenting peak (Fig. 5, slot 2) contained polypeptides characteristic for fraction T (molecular weight 19,000 and 24,000), while the faster sedimenting peak contained polypeptides typical for fraction C. None of the T or C fraction polypeptides was dissociated from the small ribosomal subunit by EDTA (Fig. 5, slot 1). However, chelation of magnesium led to either nearly complete (slot 1, marked 28) or partial (slot 1, marked 41, 16, and 14.5) removal of typical ribosomal proteins. The dissociation of ribosomal proteins from the small ribosomal subunit by EDTA is not restricted to S^n; identical proteins were dissociated from derived small subunits upon EDTA treatment (data not shown). Thus, it is clear that magnesium chelation induces unfolding of the small subunit (14) with the concomitant loss, more or less complete, of several ribosomal proteins but does not cause the detachment of the characteristic S^n associated polypeptides. Moreover, this result demonstrates that the S^n fraction is heterogeneous, in that approximately half of the S^n contain polypeptides of fraction T (molecular weight, sum of 90,000), and sediment more slowly than the other half, which is associated with fraction C polypeptides (molecular weight, sum of 570,000).

Finally, the proteins dissociated from S^n and separated into two fractions (T and C) on sucrose gradients were tested as to whether they are required for the translation of globin mRNP. Various amounts of fractions T or C, either alone (Fig. 6) or combined (Fig. 7), were added to a system containing derived small (S^0) and large (L^0) ribosomal subunits of reticulocyte ribosomes, pH 5 enzyme, and globin mRNP. For quantitative interpretation of these results, the amount of fraction T and C was related to the amount of S^n from which they were derived by salt extraction; e.g., shown in both Figs. 6 and 7 are the activities of equivalent amounts of both S^0 and S^n and the activities obtained by adding one (20), two (40), or fractional (2, 5, 10) equivalents of fraction T or C to the reconstituted system containing S^0 instead of S^n. It can be seen in Fig. 6 that the addition of fraction C in amounts of either one (curve 20C) or two equivalents (curve 40C) restored about 50 or 78%, respectively, of the activity of the original S^n, whereas one or two equivalents (20T and 40T) of fraction T restored very little activity. However, a combination of both, fraction T and C, in equivalent amounts (Fig. 7, curve 20T and 20C) restored about 71% of the activity of the original S^n fraction; thus the observed stimulation of the combined fractions is more than additive, indicating that both fractions are required in the reconstituted system. The incomplete restoration of the S^n activity by equivalent amounts of the disassembled fractions (T, C, and S^0) may have been the result of partial inactivation of these components during fractionation. The result in Fig. 7 (dotted curve 20T and 20C) show that replacement in the assay of S^0 and L^0 by an equivalent amount of monosomes resulted in a somewhat higher activity.

DISCUSSION

Our results show that the initiation factors for protein synthesis contained in crude ribosomes are localized exclusively in the native small ribosomal subunit (S^n) which comprises only a few percent of the mass of crude ribosomes. Initiation factors were completely dissociated from S^n without significant loss of activity either of the factors or the derived small ribosomal subunits. This was achieved by extraction of S^n with 500 mM KCl, buffer, and 5 mM $MgCl_2$. Subsequent su-

crose gradient centrifugation resulted in the separation of the initiation factors into two fractions: a top fraction (referred to as fraction *T*) sedimenting at about 4 S and a fraction containing a protein complex, sedimenting at 15 S (referred to as fraction *C*), showing in $NaDodSO_4$/polyacrylamide gels four and 10 polypeptides, respectively. The four polypeptides contained in fraction *T* most likely represent four single proteins since they could be removed individually by increasing concentrations of monovalent ions, whereas the 10 polypeptides contained in fraction *C* could only be dissociated as a complex.

Although salt dissociation could be anticipated from previous work on the isolation of initiation factors, it was surprising to find that magnesium ions apparently play no role in the linkage of the initiation factors to the small ribosomal subunit. Furthermore, magnesium ion chelation by EDTA revealed that the S^n fraction is heterogeneous; approximately one-half of S^n contained fraction *T* polypeptides and the other half contained fraction *C* polypeptides. It is clear that EDTA treatment caused unfolding of the small ribosomal subunit (14) with a concomitant complete or partial dissociation of a few ribosomal proteins. However, the adherence of largely differing amounts of protein to either S^n (molecular weight: fraction *T* about 90,000, fraction *C* about 570,000) produced unfolded S^n particles sedimenting with different S values. Heterogeneity in the S^n fraction has also been detected in other laboratories (5, 15–17). It was found that after aldehyde fixation, the S^n fraction could be separated into several peaks by isopycnic centrifugation in CsCl gradients. However, aldehyde fixation precluded analysis of the polypeptides contained in these peaks. We would like to propose that the two S^n populations detected by EDTA treatment represent physiological intermediates in the initiation process.

Finally, the results of our reconstitution experiments demonstrated that the disassembly of S^n into subfractions did not result in appreciable losses of activity. Although fraction *T* showed little activity by itself, it stimulated protein systhesis considerably in the presence of fraction *C*. Thus it appears that the proteins contained in both fractions are required for the initiation of protein synthesis. The presence of the four and 10 polypeptides in fractions *T* and *C* in such predominant amounts in reticulocyte S^n and the occurrence of polypeptides of similar or identical molecular weight in the fractions derived from S^n of four other sources suggest that all of them are required for initiation. However, it should be emphasized that some of the minor, not designated polypeptides in fraction *T* and *C* may not be merely contaminants, due to unspecific absorption of proteins to S^n during cell fractionation, but may represent initiation factors occurring in fractional amounts. It is conceivable, e.g., that some of these minor bands are indeed initiation factors which during cell fractionation are isolated predominantly in the supernatant rather than in the ribosome fraction. If this were the case, these factors could have been recovered subsequently in the pH 5 fraction. Their essential role in initiation may have escaped detection since our procedures were designed to assay for polypeptide synthesis rather than only for initiation of polypeptide synthesis. Evidence for the existence of soluble, i.e., not ribosome-bound, initiation factors has been reported (18–22).

We acknowledge support by Grant CA 12413 from the National Institutes of Health. C.F. is a recipient of a Deutsche Forschungsgemeinschaft fellowship.

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