Similiarity of the Transfer Factors in Novikoff Ascites Tumor and Other Amino Acid-incorporating Systems

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SUMMARY

A highly active in vitro amino acid-polymerizing system has been isolated from Novikoff ascites tumor cells. Considerable difficulty was encountered in the resolution of the transfer enzyme complex. However, purified ribosomes and the transfer components were prepared, and it was possible to demonstrate that 2 factors are involved in the polymerization of amino acids by this tumor cell system. The 2 factors were interchangeable with components of the rabbit reticulocyte system. Other investigators have shown that the 2 transfer factors of the rat liver and reticulocyte systems are interchangeable. These findings suggest that the biosomal mechanism of protein synthesis is quite similar in all mammalian cells and possibly in all cells. However, the ribosomes and Factor T or G of the mammalian and microbial systems are not interchangeable.

INTRODUCTION

An active amino acid-incorporating system obtained from the Novikoff ascites hepatoma has been previously reported (8, 9). During the course of development of this system considerable difficulty was encountered in resolving the transfer factor complex, as has been reported for several other tissues or cells. Allende et al. (2) separated from Escherichia coli 2 complementary factors (T and G) required for polymerization of phenylalanyl-tRNA on E. coli ribosomes. The T factor was divided later into a stable fraction, Ts, and an unstable form, Tu (17). The function of T is presumably in the binding of aminoacyl-tRNA (in the presence of GTP) to the ribosomes and formation of a dipeptide (16). G is needed to complete the cycle of peptide bond formation by catalyzing the translocation of the peptidyl-tRNA to the peptidyl site on the ribosome. Similar or comparable transfer factors have been isolated from other cellular systems. Schweet et al. (3) described procedures for the purification from rabbit reticulocytes of 2 factors, TF-1 and TF-2. Three complementary amino acid-polymerizing factors (S1, S2, and S3) have been isolated from Bacillus stearothermophilus (22); 3 factors (Ts, Tu, and G) were isolated from Pseudomonas fluorescens (17); and 3 fractions (1-P, 2-P, and 3-P) were obtained from yeast (20). Skogerson and Moldave (21), working with rat liver preparations, have demonstrated that transferase I is the aminoacetyl-tRNA binding factor, that transferase II is involved in translocation, and that peptide bond synthetase is an inherent ribosomal enzyme. Two complementary factors (FI, FII) have been separated from calf liver by Klink et al. (12). Recently, Parmeggiani (19) achieved the crystallization of 2 transfer factors from E. coli. From these and other observations, it would appear that the general mechanism of the ribosomal synthesis of protein is similar in all living cells. Species differences in the transfer factors are considered in the "Discussion."

In view of the difficulty encountered in resolving the transfer factor complex of the ascites tumor cells, it appeared that the tumor cells differed from other cells in this respect. With further study it was shown, however, that 2 factors are required for amino acid polymerization in the tumor cell system. This communication describes the procedure for the preparation of ribosomes and partially purified transfer factor fractions from the Novikoff tumor cells and the cross-reactivities with comparable fractions from other cellular in vitro incorporating systems.

MATERIALS AND METHODS

Novikoff ascites tumor cells were collected and processed through the initial high-speed centrifugation stage as described previously by Griffin et al. (9). Tumor 14C-labeled aminoacyl-tRNA's (L-amino acid-14C mixture, New England Nuclear Corporation, Boston, Mass.), prepared by the methods of Canning and Griffin (4) and Yang and Novelli (24), were extracted in 80% phenol, reprecipitated in ethanol, and dialyzed against 1 × 10⁻³ M EDTA to remove bound GTP or other nucleotide triphosphates. Specific activity was approximately 100,000 cpm/mg RNA.

Active incorporating tumor ribosomes, exhibiting a requirement for NH₄⁺, GTP, and transfer factors, were prepared by the following procedures.

Preparation a: Twice DOC-washed Ribosomes. The microsomal pellets were washed twice with a 2.5% DOC buffer, pH 8.0 (9). This ribosomal preparation was stored

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at $-10^\circ$ and contained approximately 10 mg ribosomal protein/ml (absorption at 260 nm assuming that 1 mg ribosomal protein/ml is equivalent to 11.2 A units).

**Preparation b: Twice DOC-Low Mg$^{2+}$-0.5 M NH$_4$Cl-washed Ribosomes.** Further purification of the twice DOC-washed ribosomes was attained by modification of the procedures of Takanami (23) and of Gasior and Moldave (7). The twice DOC-washed tumor ribosomes were suspended by diluting 10-fold with buffer of low Mg$^{2+}$ concentration and centrifuged at 150,000 $\times g_{max}$ (50 Ti rotor) for 40 min. The resulting pellets were suspended in standard buffer (0.25 M sucrose-0.05 M Tris-HCl, pH 7.3). This suspension was centrifuged at 40,000 $\times g_{max}$ for 15 min, and the pellets were resuspended in standard buffer to a final concentration of 1 to 2 mg ribosomal protein/ml. The ribosomes purified by this procedure were unstable and were used immediately in the polymerization assays.

**Preparation c: DOC-Sucrose Gradient-0.5 M NH$_4$Cl-washed Ribosomes.** Tumor ribosomes were prepared by the method of Keller and Zamecnik (10) as modified by Felicetti and Lipmann (6). Following the original high-speed centrifugation, the microsomes were suspended by a mechanical homogenization and DOC was added to a final concentration of 0.5%. This tumor mixture was layered on a discontinuous sucrose gradient of 0.5 M sucrose over 2 M sucrose buffers containing 0.004 M MgCl$_2$. The suspension was centrifuged at 150,000 $\times g_{max}$ for 3 hr. Maintaining a concentration of 1 mg ribosomal protein/ml, the DOC-sucrose ribosomes were diluted with a 0.5 M NH$_4$Cl buffer and recentrifuged for 90 min at 150,000 $\times g_{max}$. The washed ribosomes were resuspended in 8 ml of the above buffer without 0.5 M NH$_4$Cl, and retained full activity for 2 weeks when stored at $-10^\circ$.

**Preparation d: DOC-DEAE-cellulose-0.5 M NH$_4$Cl-washed Ribosomes.** The procedure of Kirsch et al. (11) has been adapted for preparation of tumor ribosomes. Following sedimentation of the microsomes, the pellets were homogenized in standard buffer, and the suspension was diluted 5-fold with 0.9 M sucrose-0.004 M MgCl$_2$-0.025 M KCl solution. The suspension was adjusted to 0.009 M by the addition of 0.1 M MgCl$_2$ with constant shaking. A 2.5% DOC solution was pipetted dropwise until a 0.25% DOC concentration was obtained. Following centrifugation at 175,000 $\times g_{max}$ (50.1 Beckman rotor) for 20 min, the precipitate was discarded and the supernatant fraction was recentrifuged at 275,000 $\times g_{max}$ for 60 min. The pellets were resuspended in 3 times with standard buffer, resuspended in 2.0 ml of the same, and passed over a DEAE-cellulose column (0.5 x 16 cm) previously equilibrated with 0.05 M Tris-HCl, pH 7.3-0.004 M MgCl$_2$ buffer.

Approximately 70 to 80 mg tumor ribosomal protein were chromatographed on the DEAE-cellulose column. Fractions were eluted with the 0.05 M Tris-HCl buffer, pH 7.3, containing 0.25 M sucrose and collected in 1-ml volumes at 5-min intervals. Tubes collecting the eluted ribosomes appeared turbid. A yield of 28 to 44 mg ribosomal protein was obtained from the initial 50 ml packed ascites cells. Prior to use, the DOC-DEAE-cellulose ribosomes were washed further with 0.5 M NH$_4$Cl.

One ml tumor ribosomes was diluted 10-fold with the 0.05 M Tris-HCl buffer, pH 7.3, composed of 0.25 M sucrose, 0.004 M MgCl$_2$, and 0.5 M NH$_4$Cl. Following centrifugation at 150,000 $\times g_{max}$ (50 Ti rotor) for 60 min, the pellets were resuspended in 1.2 ml of the same buffer without the 0.5 M NH$_4$Cl. The final ribosomal concentration ranged between 1 and 4 mg ribosomal protein/ml.

**Transfer Factor Preparations.** The pH of the supernatant material from the pH 5 soluble fraction was increased to 7.1 and fractionated with special enzyme grade solid (NH$_4$)$_2$SO$_4$ (Mann Research Laboratories, New York, N. Y.). While a temperature of 4° was maintained, this supernatant material was adjusted slowly to 22% (NH$_4$)$_2$SO$_4$ saturation (12.8 g/100 ml solution). After 45 min of constant stirring, the suspension was centrifuged at 40,000 $\times g_{max}$ for 15 min, dissolved in 0.01 M Tris-HCl buffer, pH 7.6, containing 0.2 M sucrose and 1 X $10^{-3}$ M DTT, and dialyzed overnight against the same buffer to remove excess (NH$_4$)$_2$SO$_4$. This 22 to 60% (NH$_4$)$_2$SO$_4$ fraction served as a partially purified and enriched source of the tumor transfer factors.

Many procedures have been reported in detail for the isolation, separation, and purification of the transfer factors from various biological sources. An attempt was made to identify the tumor transfer factors with some of these procedures. The 22 to 60% (NH$_4$)$_2$SO$_4$ fraction from the above paragraph was chromatographed on TEAE-cellulose columns with a K$^+$-free buffer followed by a convex gradient of 0.01 M $\rightarrow$ 0.2 M KCl, pH 7.4, as described by Klink and Richter (13) for yeast transfer factors. When the tumor transfer factors did not appear to be fractionated by the TEAE-cellulose columns, the 22 to 60% (NH$_4$)$_2$SO$_4$ fraction was treated with either alumina C$_7$ gel as reported by Nishizuka and Lipmann (18) or hydroxyapatite (19) for E. coli. Utilizing another approach, the 22 to 60% (NH$_4$)$_2$SO$_4$ material was adsorbed to a 3% Ca$_3$(PO$_4$)$_2$ gel suspension. The fraction eluting from the gel with a 0.25 M potassium phosphate buffer, pH 6.8, was subsequently filtered on Sephadex G-200 columns. However, the tumor transfer factors did not appear to separate into 2 complementary factors as did the factors from normal rat liver (7). Since the tumor transfer factor activity emerged with the lead fractions on Sephadex G-200 columns, Sepharose 4B (fractionation range, M.W., 300,000, 3 X $10^6$, Pharmacia, Uppsala, Sweden) was used for fractionation studies. Approximately 75 to 100 mg sample (0.25 M potassium phosphate eluate) were applied to a column (2.4 x 33 cm) previously equilibrated with a buffer containing 0.25 M sucrose-0.05 M Tris-HCl (pH 7.4)-0.1 M KCl, 1 X
10⁻³ m EDTA, and 1 × 10⁻³ m DTT. Fractions were eluted with the same buffer at a flow rate of 5 ml/10 min.

Preparative polyacrylamide columns were also utilized in order to fractionate the transfer factors. The 22 to 60% (NH₄)₂SO₄ material was concentrated with Aquacide II (Calbiochem, Los Angeles, Calif.) to approximately 60 mg/ml (assuming that 0.6 A unit is equivalent to 1.0 mg protein/ml) and dialyzed overnight against the 0.01 m Tris-HCl buffer, pH 7.6. One ml sample containing 0.1 ml 50% sucrose was applied to a 7-cm preparative polyacrylamide gel column that had been perfused previously with the electrolyte buffer for 20 min (Shandon Scientific Company, Ltd., London, England). The gel medium, Cyanogum 41, was polymerized with the chemical catalysts TMED and AP. Separation was achieved in a continuous electrophoresis system with a modified buffer of Davis et al. (5): 0.035 M asparagine-5 × 10⁻⁴ m DTT-1.0 m Tris to adjust the pH to 7.9. Electrophoresis proceeded at 10 ma for 20 min followed by an increase to 19 ma at constant current until completion of the run (10 to 12 hr). Flow rate of the eluting buffer (asparagine-DTT-Tris, pH 7.9) was 2 ml/10 min, and all fractions were assayed for amino acid incorporation. The area of peak activity was pooled, concentrated to 2 to 3 ml with Aquacide II, and dialyzed overnight against the 0.01 m Tris-HCl buffer, pH 7.6.

Under the same conditions, purity of the preparative gel fractions was checked with an E-C vertical gel system. A 3% gel plug, was prepared by the procedure in Vertical Gel Electrophoresis Manual (E-C Apparatus Corporation, Philadelphia, Pa.). The protein samples (80 to 750 μg), containing sucrose and bromphenol blue, were applied to slots 1 cm wide. Electrophoresis proceeded with 60 ma and the T₁ and T₂ factors were prepared according to the procedure of Arlinghaus et al. (3) as noted under "Materials and Methods." The tumor 40 to 70% (NH₄)₂SO₄ fraction and the T₁ and T₂ factors were prepared according to the procedure of Arlinghaus et al. (3) as noted under "Materials and Methods." The complete amino acid-incorporating system, in a total volume of 0.1 ml, consisted of 0.05 ml ribosomes (450 μg ribosomal protein for DOC-DEAE and 150 μg ribosomal protein for 0.5 NH₄Cl-washed ribosomes), 0.5 μmole PEP, 5 to 10 μg PEP kinase, 0.04 μmole GTP, 0.6 μmole 2-mercaptoethanol, 10 μmole (NH₄)₂SO₄, 0.01 ml amino acid-¹⁴C mixture tRNA (60 μg), 64 μg tumor (NH₄)₂SO₄, 8.0 μg tumor acrylamide fraction, and 15 μg Tumor T₁, 18.5 μg Tumor T₂, 20 μg Reticulocyte T₁, and 35 μg Reticulocyte T₂ when assayed with the DOC-DEAE ribosomes, or 30 μg Tumor T₁, 18.5 μg Tumor T₂, 40 μg Reticulocyte T₁, and 7.0 μg Reticulocyte T₂ in presence of 0.5 M NH₄Cl-washed ribosomes. The mixture was incubated at 37° for 25 min, and Adsorbed on a Whatman No. 3MM paper disc placed directly into the assay tube. The discs were prepared for liquid scintillation counting as described in "Materials and Methods."
somes were treated in the same manner as described for the tumor DOC-DEAE-cellulose ribosomes. Following DEAE-cellulose chromatography, the ribosomal concentration was 4.4 mg ribosomal protein/ml.

Assay for Amino Acid Incorporation. The ability of the transfer factors to catalyze the incorporation of amino acids into polypeptides was measured in the following assay system: 0.05 ml ribosomes (9 mg ribosomal protein/ml for crude, 3 mg/ml for purified ribosomes), transfer factors (as indicated in tables), 0.5 μmole PEP, 5 to 10 μg PEP kinase, 0.04 μmole GTP, 0.6 μmole 2-mercaptoethanol, 10 μmoles (NH₄)₂SO₄, 60 μg aminoacyl-tRNA-¹⁴C mixture in a total volume of 0.1 ml. The mixture was incubated at 37° for 25 min and adsorbed on a Whatman No. 3MM paper disc placed directly into the assay tube. Following the usual treatment with hot TCA and alcohol washing, the discs were counted in a liquid scintillation spectrometer with a 60% efficiency (9). The cpm were based on the total 0.05 ml ribosomes present in the assay mixture and are expressed as cpm/mg ribosomal protein.

RESULTS

A stable ribosomal preparation was obtained from the Novikoff tumor cells by DOC washing of the microsomal fraction. This preparation, designated twice DOC-washed, had a low background when tested in the standard incorporation assay and transfer factors, GTP, monovalent cation, and sulfhydryl source were essential for optimal amino acid incorporation. Approximately 200 μmoles labeled amino acid were incorporated per mg ribosomal protein. With this in vitro ribosomal system, it was not possible to demonstrate the involvement of 2 transfer factors as has been observed in microbial and certain mammalian systems.

Ribosomal preparations were obtained by several different procedures as described in "Materials and Methods." Concurrently, attempts were made to fractionate the transfer factor complex by various chromatographic and other procedures. Considerable difficulty was encountered in obtaining a system wherein a requirement for 2 transfer factors for amino acid incorporation could be demonstrated. Most of the ribosomal preparations described in "Materials and Methods" (Preparations a, b, and c as well as Preparation d) exhibited low background incorporation without added transfer factors and good incorporation in the presence of most of the transfer factor fractions. However, no complementation or additive response was obtained by any combination of the transfer factor preparations. It was assumed incor-

Table 2

<table>
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<th>Reticulocyte ribosomes</th>
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Chart 1. The incubation mixture included the tumor DOC-DEAE-cellulose-0.5 M NH₄Cl-washed ribosomes (3 mg/ml ribosomal protein), Tumor T₁ or T₂ as designated in the chart, and other components as described in "Materials and Methods." Incubation, sampling, and counting were performed as specified in "Materials and Methods." Tumor transfer Factors T₁ and T₂ were prepared according to the procedure of Arlinghaus et al. (3) as noted in "Materials and Methods" and the legend to Table 1.
Transfer Factors in Novikoff Ascites Tumor

Correctly for a long time that the tumor transfer factors existed in a complex not resolvable by the procedures that were used. In retrospect, the major obstacle in these studies was the inability to remove all traces of T2 from the ribosomes. This was finally accomplished by extraction of ribosomes prepared by DOC treatment and passage over DEAE-cellulose, with 0.5 M NH4Cl. Only at this stage was it possible to demonstrate a 2-transfer factor requirement by the tumor system. In fairness to those authors whose methods were initially used to fractionate the tumor transfer factors with no apparent success, the difficulty was inherent in the ribosomes and not in the fractionation procedures. If factor-free ribosomal preparations had been employed in the assay procedure, complementation very probably would have been observed. The objective of this study was to demonstrate a 2-factor requirement for amino acid incorporation by the tumor in vitro system, and, once achieved, no further attempt was made to repeat all of the transfer factor fractionation procedures mentioned earlier in the text.

The establishment of the requirement for 2 transfer factors by the tumor cell amino acid-incorporating system is presented in Table 1. With the use of ribosomes obtained by DOC treatment and DEAE-cellulose chromatography it was possible to stimulate considerable incorporation by the addition of the (NH4)2SO4-precipitated fraction or the acrylamide fraction. Addition of T1 from reticulocytes or a T1 fraction from tumor cells also catalyzed considerable incorporation in this system. However, the addition of T2 fractions prepared from the reticulocytes or tumor cells was ineffective. Addition of T1 and T2 did not enhance the incorporation observed by the addition of T1 alone. These findings indicated that the tumor ribosomes still retained sufficient T2 for near optimal incorporation.

Difficulty was encountered in removing the last traces of T2 from ribosomal preparations. Careful washing with 0.5 M NH4Cl finally resulted in preparations that exhibited dependence upon exogenous T2 (Table 1). These ribosomes, active in the presence of the (NH4)2SO4 precipitate, had minimal incorporation in the presence of the acrylamide fraction or the tumor or reticulocyte T1 or T2 fractions separately. In contrast, considerable incorporation was observed when combinations of T1 and T2 from reticulocytes or tumor cells were added to these ribosomes in the standard assay procedure (Table 1). It would appear that the homogenous acrylamide fraction described in "Materials and Methods" represents a good source of the tumor T1.

Further indication of the involvement of 2 transfer enzymes in the tumor cell in vitro system is illustrated in Chart 1. Incorporation in the presence of either factor occurred at a low level and increased with the addition of the complementary factor. Approximately 15 µg T1 and 10 µg T2 appeared to be optimal in the standard assay procedure.

The interchangeability of the tumor and reticulocyte transfer factors is shown in Tables 1 and 2. Incorporation by the purified tumor ribosomes was enhanced by the addition of T1 and T2 from either the tumor or reticulocyte or by combinations of the factors from these cells. Also, good incorporation was observed with reticulocyte ribosomes in the presence of the 2 factors from reticulocytes or tumor cells or combinations of enzymes from these 2 sources.

DISCUSSION

A major objective of this study was to ascertain whether the polypeptide elongation steps of protein synthesis in tumor cells proceeded according to the mechanism that has been established for microbial and for several mammalian cells (15). This generally accepted mechanism has emerged from the in vitro recombination of purified ribosomes or polysomes, accessory transfer factors, aminoacyl-tRNA's, GTP, etc. While these amino acid-incorporating systems are artifactual in many aspects, they have been useful in establishing the essential reactions and components involved in the elongation of polypeptides. Considerable difficulty has been encountered in the preparation of ribosomes and in the resolution of the transfer complex from the tumor cells. However, the findings reported in this communication indicate that at least 2 transfer factors, T1 and T2, are required for polypeptide chain elongation in the tumor cells. These enzymes correspond to the T and G factors of E. coli, the TF-1 and TF-2 of reticulocytes, and the transferases I and II of liver. No attempt was made in the current study to fractionate Tumor T1 into the stable and unstable forms that have been found by other investigators for other cells.

The interchangeability of ribosomes, Factors T1 and T2, from Novikoff ascites tumor cells and from reticulocytes in the incorporation of aminoacyl-tRNA's into acid-insoluble forms was demonstrated in this study. Recently, Krisko et al. (14) reported also that the binding of phenylalanyl-tRNA to poly U-charged Novikoff tumor cell and reticulocyte ribosomes was stimulated similarly by E. coli Factor T and by a tumor supernatant fraction. The bacterial T catalyzed amino acid polymerization in both of these mammalian systems in the presence of mammalian T2. Bacterial G would not replace mammalian T2. In contrast, mammalian T1 could not be substituted for bacterial T in an E. coli ribosomal incorporating system. These findings suggest that there are species differences in certain of the major components of protein synthesis. However, the same general mechanism appears to be operative in the biosynthesis of proteins by the polysomal systems of all cell types.

Some consideration should be given to the artifactual nature of the incorporating systems. Mammalian systems polymerize less than 1% of the amino acids that may be incorporated into polypeptides by comparable intact cellular systems. The in vitro systems are not subjected to the organizational and regulatory elements present in the living cell. It is at this higher level of control that differences may exist between tumor cells and their normal...
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counterparts. However, subtle structural alterations in the ribosomes, transfer factors, aminoacyl synthetases, tRNA's, etc., could also affect their response to the regulating elements within the cell or the body. Such alterations in these major components of protein biosynthesis would not be apparent in the in vitro system used in studies of this type. The present findings provide additional indication that the mechanism of polypeptide chain elongation reviewed recently by Lipmann (15) may be similar in all cells.

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