Protein Synthesis by Hybrid Ribosomes reconstructed from Rabbit Reticulocyte Ribosomal Core-Particles and Amphibian or Fungal Split-Proteins

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It was shown that high-salt (2.75 M-NH₄Cl/69 mM-MgCl₂) shock treatment at 0°C of the larger subparticles (L-subparticles) of rabbit, *Xenopus laevis* and *Neurospora crassa* cytoplasmic ribosomes yielded split-protein fractions that were not only functionally equivalent but also interchangeable. Thus, although the remaining core-particles were inactive in both the puromycin reaction and in poly(U)-directed polyphenylalanine synthesis, activity was restored when they were combined with either homologous or heterologous split-protein fractions. This technique was used to prepare active hybrid L-subparticles, e.g. rabbit cores/*N. crassa* split-proteins, and also active hybrid ribosomes, e.g. rabbit smaller subparticle/*X. laevis* core-particle/rabbit split-proteins. Rabbit and *X. laevis* split-protein fractions labelled with ¹⁴C by reductive methylation with [¹⁴C]formaldehyde and sodium cyanoborohydride were both shown to bind to rabbit core-particles in approximate correlation with the degree of re-activation. The split-protein fractions of rabbit and *X. laevis* L-subparticles were analysed by two-dimensional and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The molecular weights (measured in sodium dodecyl sulphate gels) of the split-proteins of rabbit and *X. laevis* L-subparticles were found to be similar. These results demonstrate that the peptidyltransferase active centre of cytoplasmic ribosomes of eukaryotes has components that are interchangeable over a wide evolutionary range. Evidently the essential molecular architecture of the active centre is highly conserved.

Cytoplasmic ribosomes have diverged during evolution (for review see Bielka & Stahl, 1978). Evolutionary changes are particularly marked in the case of the major RNA component (L-rRNA) of the larger subribosomal particle (L-subparticle); see, for example, Lava-Sanchez *et al.* (1972). The number of proteins reported for a wide range of species was found to be approx. 30 for the smaller subribosomal particle (S-subparticle) and 45–50 for the L-subparticle (Bielka & Stahl, 1978). An underlying pattern was discerned after comparison of patterns obtained by two-dimensional gel electrophoresis of proteins from different species; it was inferred that the size and charge of individual proteins have tended to be conserved during evolution (Delaunay *et al.*, 1973,

1974). An immunological comparison of the proteins of rat and chicken ribosomes indicated that, despite similarities in two-dimensional electrophoretograms, there are extensive differences in the structure of the proteins of the ribosomes from the two species (Fischer *et al.*, 1978). However, eukaryotic acidic proteins homologous to proteins L7/L12 of *Escherichia coli* have antigenic determinants in common with their bacterial counterparts and also seem to be their functional equivalents (Stoffler *et al.*, 1974).

One way of examining the extent to which proteins from different species are functionally equivalent is by means of partial reconstruction systems. The treatment of the L-subparticle of rabbits at 0°C with high salt (2.75 M-NH₄Cl/69 mM-MgCl₂) yields core-particle and split-protein fractions. By themselves the core-particles were found to be inactive in both the puromycin reaction and poly(U)-directed polyphenylalanine synthesis; activity in both reactions was restored when the core-particle and split-protein fractions were recombined (Cox & Greenwell, 1976).

Abbreviations used: S-subparticle, the smaller subribosomal particle; S-rRNA, the RNA component of the S-subparticle; L-subparticle, the larger subribosomal particle; L-rRNA, the major (23–28S) RNA component of L-subparticles.

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The possibility of activating core-particles with a heterologous split-protein fraction was examined; for example, rabbit core-particles were incubated with the split-protein fraction of either *X. laevis* or *N. crassa*. The split-protein fractions were characterized by gel electrophoresis, by measuring their capacity to bind to core-particles, and by measuring the capacity of hybrid L-subparticles to function in poly(U)-directed polyphenylalanine synthesis. The results indicate that the split-protein fraction of one species (e.g. *N. crassa*) can replace the split-proteins of another (e.g. rabbit) in re-activating core-particles, thus allowing the formation of hybrid L-subparticles that are active in protein synthesis.

Methods

General methods

U.v. spectrophotometry. Unicam SP.500 and SP.1800 spectrophotometers were used for routine measurements with 1 cm-light-path cells (3.0 or 0.5 ml capacity). Concentrations of L-subparticle solutions were measured by using the value $A_{260}^{1\%} = 140$ (Cox & Greenwell, 1976).

Ribosome buffers. The pH of buffer solutions was adjusted at 20°C. 'Storage buffer' for stock solutions of L-subparticles was 0.1 M-NH₄Cl/2 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6 (Cox *et al.*, 1976b). When L-subparticles were to be labelled by reductive methylation with [¹⁴C]formaldehyde the primary amino components in this buffer, NH₄Cl and Tris/HCl, were replaced by 0.1 M-KCl and 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (potassium salt), pH 7.6, respectively, to give 'Hepes storage buffer'. 'Sample buffer' for dilution of L-subparticles and core-particles before assay for polyphenylalanine synthesis was 25 mM-KCl/5 mM-MgCl₂/mm-dithiothreitol/0.25 M-sucrose/50 mM-Tris/HCl, pH 7.6. 'Activation buffer' for reconstruction of core-particles (Cox & Greenwell, 1976) was 0.3 M-NH₄Cl/15 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6.

Isolation of ribosome, polyribosome and enzyme fractions

Preparation of rabbit reticulocyte polyribosomes and supernatant enzyme fractions. These were prepared from phenylhydrazine-treated rabbits as described previously (Cox & Hirst, 1976; Arnstein *et al.*, 1964).

Preparation of *N. crassa* ribosomes. Wild-type *N. crassa* (74A) was grown in Vogel's minimal medium for 23 h at 25°C (with shaking), as described by Davis & de Serres (1970). The hyphae from 4 litres of culture were filtered by gentle suction on a Buchner funnel and washed with ice-cold water. The filter

cake was ground in a pestle and mortar with half its own weight of acid-washed sand, and extracted with 5 vol. of sample buffer. The mixture was centrifuged for 15 min at 5°C (15 000 rev./min, 20 000 $g_{av.}$, MSE 8 × 50 ml rotor), and the supernatant fraction was taken for ultracentrifugation (3 h at 5°C, 40 000 rev./min, 105 000 $g_{av.}$, MSE 8 × 25 ml rotor). The resultant pellets of ribosome fraction were stored at -80°C.

Preparation of *X. laevis* oocyte ribosomes. The ovaries of about four mature South African toads (gifts from Dr. C. Lane, National Institute for Medical Research, or Dr. J. Gurdon, Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) were homogenized in 2.5 vol. of sample buffer at 0°C with five passes of a loose-fitting (radial clearance approx. 100 μm) Teflon plunger rotating at 1500 rev./min. The homogenate was filtered through glass wool, and the filtrate was centrifuged for 20 min at 5°C (17 000 rev./min, 26 000 $g_{av.}$, MSE 8 × 50 ml rotor). The supernatant was decanted and freed from the lipid pellicles, and was ultracentrifuged for 3 h at 5°C and 105 000 $g_{av.}$ to yield pellets of ribosomes, which were stored at -80°C.

Separation of ribosomal subparticles

The polyribosome (rabbit, *N. crassa*) or ribosome (*X. laevis*; see Pratt & Cox, 1971) preparations were dissociated by incubation at 37°C for 30 min in 0.5 M-KCl/3 mM-MgCl₂/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, together with 0.2 mM-GTP and 0.2 mM-puromycin. The subunits were then separated by zonal centrifugation in a sucrose gradient containing 0.3 M-KCl/3 mM-MgCl₂/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, as described by Cox & Hirst (1976); the profile of the gradient within the MSE B XIV titanium rotor increased parabolically with radius from 15 to 37% (w/v) sucrose, and ended with a cushion of 45% (w/v) sucrose. When subparticles were to be subjected to reductive methylation (see below), the Tris/HCl in the gradient buffers was replaced by 20 mM-Hepes (potassium salt), pH 7.6. The pooled fractions of subparticles were pelleted by centrifuging at 105 000 $g_{av.}$ for 17 h at 5°C (MSE 8 × 25 ml rotor), resuspended in storage buffer or Hepes storage buffer at 250 A_{260} units/ml, rapidly frozen in a solid CO₂/ethanol bath and stored at -80°C.

Poly(U)-directed polyphenylalanine synthesis

The incorporation of [¹⁴C]phenylalanine (sp. radioactivity 50 mCi/mmol) into acid-insoluble material by L-subparticles in the presence of S-subparticles, supernatant factors etc. at 37°C was measured as described by Cox & Hirst (1976). Samples were generally 50 μl of 2.0 A_{260} units/ml in an assay mixture of 0.5 ml. The assay blank, the

incorporated radioactivity equivalent to zero synthesis, was determined by omitting the energy source (pyruvate kinase, phosphoenolpyruvate, ATP and GTP) from a complete control assay mixture. When the samples of L-subparticles were labelled with [^{14}C]methyl groups, it was necessary to run such a blank in parallel to the assay for each sample.

Assay of peptidyltransferase by the puromycin reaction

The transfer of acetyl-[^3H]leucyl groups from acetyl-[^3H]leucyl-tRNA to puromycin, catalysed by L-subparticles at 20°C in 30% (v/v) methanol, was carried out and measured in a small scintillation vial as described by Cox *et al.* (1976b). When the L-subparticles were labelled with [^{14}C]methyl groups, it was necessary first to separate the toluene-based liquid-scintillation phase from the aqueous phase, because ^{14}C , unlike ^3H , registers in the liquid-scintillation counter when it is dissolved in the aqueous phase. Separation was achieved by centrifuging the assay vials (1000 rev./min for 2 min), freezing the lower phase with solid CO_2 /ethanol, and decanting the toluene phase with the acetyl-[^3H]leucyl-puromycin into a fresh vial. All assays were run in duplicate and the results averaged.

Separation of core-particles and split-protein fractions by pelleting

The production of core-particles and split-proteins from rabbit L-subparticles by salt-shock treatment was described in detail previously (Cox & Greenwell, 1976). Effective conditions for producing extensively inactivated core-particles with good potential for reconstruction were taken from the earlier work, and applied to L-subparticles from all three species considered here. Thus L-subparticles at 250 A_{260} units/ml in storage buffer were mixed at 0°C with 4 vol. of salts solution such that the final composition was 2.75 M- NH_4Cl /69 mM- MgCl_2 /3.5 mM-EDTA/1 mM-dithiothreitol/3% (w/v) glycerol/20 mM-Tris/HCl, pH 7.6. After 15 min at 0°C, the reaction mixture was centrifuged for 30 min at approx. 0°C and 40 000 rev./min in the MSE 8 $\times$ 10 ml rotor (1.25 ml portions in 10 ml polycarbonate tubes; 145 000 g_{max}). The supernatants containing the split-proteins were pipetted off, and the pellets were homogenized in a volume of storage buffer equal to the volume of salt-shock mixture, to yield a cloudy suspension of core-particles.

Separation of core-particles from split-protein fractions on short-column preparative sucrose gradients

The preparation of core-particles by ultracentrifugation out of the salt-shock mixture into a pellet (see above, and Cox & Greenwell, 1976) yields material which is often difficult to resolubilize and quantify by absorbance measurements, probably

because of extensive aggregation of the particles. It was therefore desirable to prepare soluble monomeric core-particles for use in quantitative reconstruction experiments with ^{14}C -labelled split-proteins. This was achieved by ultracentrifugation of the salt-shock mixture into a short-column sucrose gradient, which effected the separation in about the same time as pelleting and allowed recovery of the separated fractions at concentrations suitable for use in reconstruction experiments. The sucrose gradients were prepared in polyallomer tubes for the Beckman SW50.1 6 $\times$ 5 ml swing-out rotor by layering of the following pre-cooled solutions. First a cushion of 1.0 ml of 60% (w/v) sucrose in reconstruction buffer was pipetted in, followed by 0.3 ml of each of 45, 35, 25 and 20% (w/v) sucrose. This last layer was made up in reconstruction buffer when the sample (0.75 ml) contained control L-subparticles, but in salt-shock buffer (2.75 M- NH_4Cl etc.) when the sample was salt-shocked L-subparticles. The latter precaution was necessary to avoid partial reconstruction of the salt-shock mixture (50 A_{260} units/ml) at the top of the gradient. Centrifugation was at 50 000 rev./min (120 000 g_{av}) and 5°C for 1 h in a Beckman L2-65B ultracentrifuge. This was just sufficient to separate the split proteins from the compact band of core-particles, but not enough to take the latter to the top of the steep concave gradient, where zone destabilization was found to occur with subsequent loss of particles into the sucrose cushion. The gradients were pumped out of the tubes (Pharmacia P3 peristaltic pump) through an LKB Uvicord II instrument to record percentage transmission at 254 nm, and 200 μl fractions were collected manually. Distribution of ^{14}C in the fractions was determined by liquid-scintillation counting of 5 μl portions in 2.5 ml of Tritosol fluid (see under method of reductive methylation below). The split-protein fraction (approx. 1.4 ml) and the core-particle fraction (approx. 1.2 ml) were recovered with less than 2-fold overall dilution by pooling the appropriate fractions.

Reconstruction of active L-subparticles

Core-particles were recombined with homologous or heterologous split-proteins under the conditions found by Cox & Greenwell (1976) to give significant recovery of enzymic activities. The solutions of core-particles and split-proteins (routinely a 1.5-fold excess) were mixed and dialysed for 16 h at 0–4°C against 50 vol. of activation buffer to remove excess salts and sucrose. The reconstruction mixture was then incubated for 1 h at 37°C before portions were taken for duplicate assays of polyphenylalanine synthesis and/or the puromycin reaction. In the latter case it was necessary to concentrate the reconstructed particles approx. 10-fold by pelleting for 30 min at 0°C and 145 000 g (cf. core-particle pre-

paration above) and then resuspending in storage buffer. When ^{14}C -labelled split-proteins were present, portions of the reconstruction mixtures were also analysed on sucrose gradients as described below.

Binding of ^{14}C -labelled split-protein to core-particles measured on analytical sucrose gradients

Portions (0.8 ml, approx. $2.5 A_{260}$ units/ml) of the reconstruction mixtures to be analysed were layered on linear 20–40% (w/v) sucrose gradients (4.2 ml) made up in 0.1 M- NH_4Cl /2 mM- MgCl_2 /1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6. Centrifugation was in a Beckman SW50.1 swing-out rotor at 50000 rev./min and 5°C for 2.5 h. The gradients were pumped out and 0.3 ml fractions collected in plastic scintillation mini-vials. The fractions were diluted with 1.0 ml of water, and their A_{260} values measured. The whole fractions were then homogenized with 4.0 ml of Tritonol scintillation fluid in order to measure their content of ^{14}C -labelled split-protein. Integration of the particle peak in the ^{14}C and A_{260} profiles of the gradient then allowed calculation of the stoichiometry of binding of split-protein.

Reductive methylation of L-subparticles with [^{14}C]formaldehyde and sodium cyanoborohydride

A new method was devised for radioactive methylation of ribosomal proteins, which is more convenient and more efficient than the commonly used procedure of Means & Feeney (1968) (see also Rice & Means, 1971); the radioactivity is introduced as [^{14}C]formaldehyde and the adduct is reduced with cyanoborohydride.

Previously, sodium boro[^3H]hydride has been used successfully to reduce the formaldehyde adduct at around pH 9 with bacterial (Moore & Crichton, 1973) and mammalian (Reboud *et al.*, 1977) ribosomes, but we have found that the use of [^{14}C]formaldehyde with sodium borohydride at pH 9 incorporated radioactivity on to L-subparticles at about 1–3% efficiency. The process was improved to 25–50% efficiency by the use of sodium cyanoborohydride at physiological pH because, as Borch *et al.* (1971) have shown, this reducing agent selectively reduces the amine–formaldehyde adduct rather than the free aldehyde. Routinely, less than one formaldehyde molecule per lysine residue was used, but higher specific radioactivity of the proteins was readily achieved by using more formaldehyde.

L-subparticles (0.5 ml) at $250 A_{260}$ units/ml (approx. 6 mM in lysine residues; Reboud *et al.*, 1977) in Hepes storage buffer, pH 7.6, were made to react with 0°C for 15 min with $50\mu\text{l}$ of 40 mM- ^{14}C]formaldehyde (4 Ci/mol). Then $50\mu\text{l}$ of sodium cyanoborohydride [8.4 mg/ml in 0.2 M-Hepes (potassium salt), pH 7.5] was added and the mixture kept

at 0°C for 8 h before being dialysed against two changes of ribosome storage buffer ($2 \times 500\text{ ml}$).

Radioactive split-proteins were prepared from the [^{14}C]methylated subparticles by the standard salt-shock procedure in 2.75 M- NH_4Cl . Measurement of ^{14}C in salt-shock supernatants, and also in fractions from sucrose gradients, was by liquid-scintillation counting in the Tritonol scintillation fluid of Fricke (1975). At room temperature (20°C) or below, 4.0 ml of this fluid could take up to 1.1 ml of salt-shock mixture, or up to 0.35 ml of 60% (w/v) sucrose when diluted with 1.0 ml of water.

Determination of protein by precipitation as Coomassie Blue complex with trichloroacetic acid

A simple procedure was developed to measure the concentration of ribosomal proteins dissolved in concentrated solutions of NH_4Cl or urea. The sample (0–10 μg of protein) was diluted to $200\mu\text{l}$ in a 1.5 ml polypropylene micro-centrifuge tube, and was then mixed with $500\mu\text{l}$ of a saturated solution of Coomassie Blue R250 in 15% (w/v) trichloroacetic acid (this reagent was made by stirring 50 mg of the dyestuff in 100 ml of the acid solution and filtering out the undissolved solid). The mixture was kept at room temperature for 15 min or more, then centrifuged in a micro-centrifuge (Jobling 320) at approx. 10000 g for 3 min. The supernatant was removed, the pellet surface and tube were rinsed with $3 \times 1.0\text{ ml}$ of 15% (w/v) trichloroacetic acid, and the pellet was dissolved in 1.0 ml of a solution containing 1% sodium dodecyl sulphate and 1% NaHCO_3 in ethanol/water (1:1, v/v). The A_{570} was then measured (generally between 0.05 and 1.20) to estimate the protein content by comparison with standard samples containing 0–10 μg of cytochrome c. The standards were adjusted to approximately the same concentration of NH_4Cl or urea as the sample solutions. The assay is subject to gross interference by detergents, RNA and poly(ethylene glycol) (P. Greenwell, unpublished work).

Analysis of ribosomal proteins by two-dimensional polyacrylamide-gel electrophoresis

Ribosomal proteins were recovered from subparticles and split-protein fractions by acetic acid extraction and trichloroacetic acid precipitation respectively, as described by Cox & Greenwell (1976). The samples, dissolved in 8 M-urea/10 mM-2-mercaptoethanol, were then analysed in the gel system used by Cox & Greenwell (1976), in which the first dimension was a 4% (w/v) polyacrylamide tube gel, pH 5.5 (Knopf *et al.*, 1975), and the second dimension was an 18% (w/v) polyacrylamide slab, pH 4.5 (Kaltschmidt & Wittmann, 1970). The slabs were stained overnight in 500 ml of 0.0125% Coomassie Blue R250 in methanol/acetic acid/water

(81:15:104, by vol.), and excess stain was allowed to diffuse into 500 ml of the same solvent.

The molecular weights of the polypeptides in some of the spots on the slab gels were determined by excising and extracting the spots, and running the extracts in the sodium dodecyl sulphate gel system (see below) as a third dimension in the manner described by Howard *et al.* (1975).

Polyacrylamide-gel electrophoresis of ribosomal proteins in the presence of sodium dodecyl sulphate

Ribosomal proteins were separated according to molecular size in the discontinuous-buffer system of Laemmli (1970). The separating gel contained 12.5% (w/v) acrylamide and 0.1% sodium dodecyl sulphate in a vertical slab 10 cm high. The stacking gel (1 cm high) contained 5% (w/v) acrylamide. Ribosomal proteins isolated as precipitates or urea solutions (see above) were dissolved or diluted 5-fold in the sample buffer, which was 0.001% (w/v) Bromophenol Blue/2.0% (w/v) sodium dodecyl sulphate/10.0% (v/v) glycerol/0.1 M-dithiothreitol/80 mM-Tris/HCl, pH 6.8. The sample mixtures (50–100 μ l) were heated at 100°C for 2 min, cooled, and then electrophoresed into the gel at 50V for the first 1 h, and at 100V until the tracking dye reached the bottom (approx. 3.5 h). The electrode buffer was 0.1% (w/v) sodium dodecyl sulphate/192 mM-glycine/25 mM-Tris, pH 8.3. Each gel slab was stained overnight in 250 ml of methanol/acetic acid/water (81:15:104, by vol.) with 0.0125% (w/v) Coomassie Blue R250, and destained by diffusion in 250 ml of the same solvent. The apparent molecular weights of the ribosomal proteins were estimated by comparison of the mobilities with those of a mixture of standard marker proteins. The calibration curve obtained by plotting the logarithm of the molecular weights of the standards against their migration distances was drawn as a shallow sigmoid curve, not a straight line (cf. Neville, 1971).

Materials

Materials were as described by Cox & Greenwell (1976). In addition, [14 C]formaldehyde (4 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and Hepes, sodium cyanoborohydride, and most molecular-weight marker proteins were from Sigma Chemical Co., Poole, Dorset, U.K. Antibody light-chain and pig smooth-muscle myosin were gifts from Dr. M. J. Crumpton.

Results

Activity of hybrid ribosomes in poly(U)-directed polyphenylalanine synthesis

The activities of preparations of the three species of ribosomes studied are given in Table 1, together with the activities of hybrid ribosomes formed from the S-subparticle of one species and the L-subparticle of another. Relative to the homologous system of the L-subparticle, the hybrids were fully functional, in accord with earlier studies (see, for example, Martin *et al.*, 1970; Cammarano *et al.*, 1972). The practical advantage of this result was that single preparations of one species of S-subparticle and supernatant enzymes could be used for assaying the activity of a range of L-subparticles and their derivatives under identical conditions.

Reconstruction of active L-subparticles from core-particle and split-protein fractions

The production of core-particles and split-proteins by high-salt shock was described in detail previously (Cox & Greenwell, 1976). The activity of the reconstructed L-subparticles in polyphenylalanine synthesis ranged from 15 to 50% of the activity of control non-treated L-subparticles; the activity of the core-particles alone was usually 1–10% of the control.

Attempts to improve the recovery of activity by

Table 1. Activity in polyphenylalanine synthesis of hybrid ribosomes formed from smaller and larger subparticles of different species

Activity in polyphenylalanine synthesis was measured by means of the standard assay (see the Methods section). The radioactivity of the control (100%) amounted to 2688 c.p.m./assay (8.4 nCi/assay). These results suggest that the *N. crassa* S-subparticle fraction was partly inactivated during the isolation procedure, whereas the L-subparticle fraction was not impaired.

S-subparticle	L-subparticle	Activity in polyphenylalanine synthesis (% of rabbit control)
Rabbit	—	3
—	Rabbit	6
Rabbit	Rabbit	100
Rabbit	<i>X. laevis</i>	75
Rabbit	<i>N. crassa</i>	116
<i>X. laevis</i>	<i>X. laevis</i>	88
<i>N. crassa</i>	<i>N. crassa</i>	57

using a range of proteinase inhibitors such as phenylmethanesulphonyl fluoride (1 mM), aprolinin (0.1%), soya-bean trypsin inhibitor, or the addition of polyamines (1–10 mM) such as spermine, spermidine and putrescine, raised the activity of both the control and reconstructed samples by 10–20%, but led to no improvement in the fraction of activity recovered on reconstruction (results not shown). Similarly, no improvement was obtained by using precautions to minimize degradation of subparticles by ribonuclease activity. Indeed, L-subparticles deliberately treated with either ribonuclease 1 or T_1 , such that the rRNA was degraded to small fragments (approx. 5S), could not be distinguished from control particles either in the stability of their polyphenylalanine-synthetic activity on treatment with high salt or in recovery of the activity on reconstruction of the salt-shock mixtures (results not shown).

An example of the recovery of activity after the individual steps in the reconstruction process is illustrated in Fig. 1. The activity of the homologous reconstructed L-subparticles in any given experiment establishes the criterion by which the activity of hybrid L-subparticles should be judged.

Formation and activity of hybrid L-subparticles

L-subparticles from rabbits and *X. laevis* were subjected to high-salt shock and the core-particles and split-protein fractions were isolated by the standard procedure. Examples of the activities of L-subparticles reconstructed with either homologous or heterologous split-protein fractions are given in Table 2. The results show that the hybrid L-subparticles had gained approximately as much activity in polyphenylalanine synthesis as had the L-subparticles reconstructed by using the homologous split-

Fig. 1. Activity in polyphenylalanine synthesis of the rabbit L-subparticle fraction at different stages in the reconstruction procedure

Activity in poly(U)-directed polyphenylalanine synthesis was measured by the standard method (see the Methods section). The high-salt-shock treatment (2.75 M-NH₄Cl/69 mM-MgCl₂) and the separation of core-particle and split-protein fractions by pelleting are described in the Methods section. Where indicated, dialysis of reconstruction mixtures was carried out at 0°C against activation buffer for 16 h. Some samples were then heated at 37°C for 1 h in activation buffer. (a), Control L-subparticles after dialysis; (b), control L-subparticles after dialysis and heating; (c), salt-shock mixture diluted with sample buffer and assayed without further treatment; (d), a sample from (c) was first dialysed before assay; (e), a sample from (c) but dialysed and heated; (f), core-particle resuspended in activation buffer and dialysed; (g), core-particle fraction dialysed and heated; (h), core-particle fraction was mixed with 1.5 equivalents of split-protein fraction and dialysed; (i), core-particle fraction plus 1.5 equivalents of split-protein fraction dialysed and heated.

Table 2. Activity in polyphenylalanine synthesis of L-subparticles reassembled from core-particles and either homologous or heterologous split-protein fractions

Activity in polyphenylalanine synthesis was measured by means of the standard assay (see the Methods section); the control (100%) was 1472 c.p.m./assay for rabbit and 1024 c.p.m./assay for *X. laevis*.

S-subparticle	L-subparticle	L-subparticle components		Activity in polyphenylalanine synthesis (% of control)	
		Core-particle	Split-proteins	Rabbit	<i>X. laevis</i>
Rabbit	Rabbit	—	—	100	—
Rabbit	—	Rabbit	—	3	—
Rabbit	—	—	—	3	—
Rabbit	—	Rabbit	Rabbit	18	—
Rabbit	—	Rabbit	<i>X. laevis</i>	15	—
<i>X. laevis</i>	<i>X. laevis</i>	—	—	—	100
<i>X. laevis</i>	—	<i>X. laevis</i>	—	—	5
<i>X. laevis</i>	—	—	—	—	4
<i>X. laevis</i>	—	<i>X. laevis</i>	<i>X. laevis</i>	—	21
<i>X. laevis</i>	—	<i>X. laevis</i>	Rabbit	—	16
Rabbit	—	<i>X. laevis</i>	Rabbit	—	32
Rabbit	—	<i>X. laevis</i>	<i>X. laevis</i>	—	22

protein fractions. Functional ribosomes were obtained from a variety of combinations such as rabbit S-subparticle/*X. laevis* core-particle/rabbit split-proteins, or rabbit S-subparticle/rabbit core-particle/*X. laevis* split-proteins.

Core-particles are inactive in both the puromycin reaction and in polyphenylalanine synthesis, but activity in both assays is recovered, in parallel, on reconstruction (Cox & Greenwell, 1976). The split-protein fraction therefore contains not only the acidic proteins that are implicated in translocation (Moller, 1974), but also proteins that affect the peptidyltransferase centre of the L-subparticle, i.e. protein(s) that effect one or more of aminoacyl-tRNA binding, peptidyl-tRNA binding or peptidyltransferase catalysis. Whatever the detailed composition of the split-protein fraction may be, it appears that rabbit and *X. laevis* split-proteins are both functionally equivalent and interchangeable.

A similar result was obtained when comparable experiments were carried out with rabbit and *N. crassa* L-subparticles, namely that the *N. crassa* split-protein fraction was interchangeable with rabbit

split-proteins in re-activating rabbit core-particles (see Table 3). Evidently, the structure of functionally equivalent proteins within the split-protein fraction is sufficiently conserved to permit them to be interchangeable even for species as diverse as moulds and mammals.

Reconstruction of monomeric core-particles with homologous and heterologous ¹⁴C-labelled split-proteins

Information on the functional roles of ribosomal proteins should be derived more readily from experimental reconstruction of core-particles if the quantity and identity of the re-attached components could be detected by physical means as well as by enzymic assays. The split-protein fraction was labelled by reductive [¹⁴C]methylation of the lysine residues and then recombined with core-particles. Reductive methylation was used to introduce radioactivity, because this modification is known not to impair ribosomal functions (Moore & Crichton, 1973; Reboud *et al.*, 1977). Radioactivity was introduced in the form of [¹⁴C]methyl rather than [³H]methyl

Table 3. Activity in the puromycin reaction and in [¹⁴C]polyphenylalanine synthesis of hybrid L-subparticles assembled from rabbit core-particles and an *N. crassa* split-protein fraction

Activity in polyphenylalanine synthesis was measured by means of the standard assay (see the Methods section); the control (100%) was 1568 c.p.m./assay (4.9 nCi/assay). Activity in the puromycin reaction was measured by means of the standard assay (see the Methods section); the control (100%) was 1352 c.p.m./assay.

Species of S-subparticle	Species of L-subparticle	L-subparticle components		Activity in puromycin assay (% of control)	Activity in polyphenylalanine synthesis (% of control)
		Core-particle	Split-protein fraction		
Rabbit	Rabbit	—	—	100	100
Rabbit	—	Rabbit	—	12	4.5
Rabbit	—	Rabbit	Rabbit	20	9
Rabbit	—	Rabbit	<i>N. crassa</i>	30	14
<i>N. crassa</i>	<i>N. crassa</i>	—	—	104	120

Table 4. Activity in polyphenylalanine synthesis of rabbit L-subparticles, core-particles and reconstructed core-particles after ¹⁴C labelling by reductive methylation

Activity in polyphenylalanine synthesis was measured in the standard assay (see the Methods section) by using rabbit S-subparticles. The radioactivity of acid-insoluble protein synthesized by the non-labelled control was 1680 c.p.m./assay. When ¹⁴C-labelled components were assayed, the incorporation was measured with and without energy; the difference yielded the incorporation of label into polyphenylalanine. The core-protein and split-protein fractions were separated on sucrose gradients, and reconstructed, as described in the Methods section.

L-subparticle	L-subparticle components		Activity in polyphenylalanine synthesis (% of control)
	Core particle	Split-protein fraction	
Non-labelled	—	—	100
¹⁴ C-labelled	—	—	95
—	Non-labelled	—	15
—	¹⁴ C-labelled	—	19
—	Non-labelled	¹⁴ C-labelled	54
—	Non-labelled	Non-labelled	36

because it is more easily detected by liquid-scintillation counting and by radioautography. Active L-subparticles were labelled by reductive methylation under physiological conditions, and the labelled split-protein fraction was then isolated. This procedure avoided the difficulties encountered in labelling isolated split-proteins arising from their strong tendency to precipitate during manipulations unless denaturants such as urea were present. Labelled L-subparticles were active in polyphenylalanine synthesis, and the labelled split-protein fraction was able to effect reconstruction (see Table 4).

The extent of binding of labelled split-proteins by reconstructed core-particles was measured by analy-

sis of reconstruction mixtures on sucrose gradients. Monodisperse preparations of core-particles were recovered from sucrose gradients (see the Methods section). The yield of control monomeric L-subparticles was approx. 70% of the sample loaded on the gradient. The yield of monomeric core-particles was 35% and a pellet of aggregated core-particles was also recovered. When subjected to the standard reconstruction procedure, the aggregates recovered activity to the same extent as the monomer core-particles (results not shown).

The results for the reconstruction of rabbit monomer core-particles with rabbit or *X. laevis* ^{14}C -labelled split-proteins are shown in Fig. 2. Compari-

Fig. 2. Reconstruction titration of rabbit core-particles with ^{14}C -labelled split-proteins from rabbit (a) and *X. laevis* (b) (a) Monomer core-particles and split-proteins were prepared from unlabelled and ^{14}C -labelled rabbit L-subparticles by treatment with $2.75\text{ M-NH}_4\text{Cl}/69\text{ mM-MgCl}_2$ and separation on preparative sucrose gradients as described in the Methods section. For $x A_{260}$ units of core-particles, one equivalent of the split-protein fraction is the amount of protein (see below) split off from $x A_{260}$ units by salt-shock treatment. In this case, for $1 A_{260}$ unit of core-particles, one equivalent of split-proteins was contained in $42\text{ }\mu\text{l}$ of the salt-shock mixture containing 5250 c.p.m. (approx. $2\text{ }\mu\text{g}$ of protein measured by the dye-binding assay). Reconstruction mixtures, containing $4 A_{260}$ units of unlabelled core-particles and up to 4.4 equivalents of ^{14}C -labelled split fraction per A_{260} unit, were treated according to the standard procedure. Samples of each mixture were then taken for measurement of A_{260} activity in polyphenylalanine synthesis and binding of ^{14}C label to core-particles in analytical sucrose gradients, as described in the Methods section. (b) In a separate but very similar experiment, monomer core-particles were prepared from unlabelled rabbit L-subparticles and ^{14}C -labelled split-proteins from ^{14}C -labelled *X. laevis* L-subparticles. Each equivalent of ^{14}C -labelled split-protein fraction was $44\text{ }\mu\text{l}$ of solution containing 8430 c.p.m. (approx. $3\text{ }\mu\text{g}$ of protein by dye-binding assay). Reconstruction mixtures were prepared and analysed as described above. ▲, Activity of rabbit core-particles in polyphenylalanine synthesis after reconstruction with rabbit split-proteins. △, Activity of rabbit core-particles in polyphenylalanine synthesis after reconstruction with *X. laevis* split-proteins. ●, Binding of rabbit split-proteins to rabbit core-particles. ○, Binding of *X. laevis* split-proteins to rabbit core-particles. ■, Binding of rabbit split-proteins to rabbit control L-subparticles. □, Binding of *X. laevis* split-proteins to rabbit control L-subparticles.

son of the upper panels with the lower panels of Fig. 2 demonstrates that re-activation of core-particles is accompanied by stable binding of split-proteins in both homologous and heterologous systems. The results are in agreement with our earlier finding (Cox & Greenwell, 1976) that recovery of activity in polyphenylalanine synthesis approached maximum when 1.5–2 equivalents of split-proteins were present in reconstruction mixtures (see Fig. 2). [For $x A_{260}$ units of core particles, one equivalent of split-proteins is defined as the amount (e.g. μg) of protein split off from $x A_{260}$ units of L-subparticles by salt-shock treatment.] When 2 equivalents of labelled rabbit split-proteins were present during reconstruction, the re-activated rabbit core-particles bound 0.44 equivalent of split-proteins, compared with an estimated 0.16 equivalent bound to L-subparticles in control experiments; activity in polyphenylalanine synthesis was 25% of control compared with 10% for the cores alone. When 2 equivalents of *X. laevis* proteins were incubated with rabbit core-particles, 0.42 equivalent of split-proteins was bound and activity in polyphenylalanine synthesis increased from 14% for cores alone to 22% of control. Thus both homologous and heterologous split-protein fractions yielded similar results.

Raising the amount of split-proteins to 4.4 equivalents during the reconstruction led to further binding of split-proteins (0.68 equivalent of rabbit and 0.62 equivalent of *X. laevis* split-proteins) by rabbit core-particles, with little further increase in activity in polyphenylalanine synthesis. A similar effect was noticed when control L-subparticles were

subjected to the reconstruction process, since split-proteins were bound without there being a concomitant increase in polyphenylalanine synthesis; indeed, up to 10% inhibition was often observed. If, whatever the mechanism, the binding of split-proteins to L-subparticles is regarded as 'background', the additional split-proteins bound by rabbit core-particles approached a maximum of 0.34 equivalent of rabbit and a maximum of 0.28 equivalent of *X. laevis* split-proteins. The binding of split-proteins by rabbit core-particles is roughly in proportion with the recovery of activity in polyphenylalanine synthesis.

Comparison of rabbit, N. crassa and X. laevis split-protein fractions by gel electrophoresis

The two-dimensional electrophoretic patterns of the split-protein fractions of *N. crassa* and *X. laevis* are given in Fig. 3 and are to be compared with patterns obtained previously for rabbit L-subparticles (Cox & Greenwell, 1976). Qualitative comparison of electrophoretograms of rabbit, *N. crassa* and *X. laevis* split-protein fractions revealed slight similarities between the patterns, but no obvious general analogies. As the two-dimensional patterns are very sensitive to alterations in the electric charge of the proteins, the rabbit and *X. laevis* split-proteins were compared on the basis of molecular size alone by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; Fig. 4 shows the patterns obtained with preparations that gave good reconstruction of core-particles. The apparent molecular weights estimated for the bands consistently seen in several preparations of split-proteins are given in Table 5. The

Fig. 3. Two-dimensional electrophoretograms of split-proteins from *X. laevis* and *N. crassa* L-subparticles. Split-protein fractions were prepared from L-subparticles by the standard salt-shock with 2.75 M-NH₄Cl/69 mM-MgCl₂ as described in the Methods section, and resolved in two-dimensional electrophoresis as described by Cox & Greenwell (1976). First dimension, migration left to right in 4% (w/v) acrylamide at pH 5.5; second dimension, migration from top to bottom in 18% (w/v) acrylamide at pH 4.5. (a), *X. laevis* split-proteins. (b), *N. crassa* split-proteins.

Fig. 4. Analysis of rabbit and *X. laevis* proteins from intact L-subparticles, core-particles and split-protein fractions by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was used with a 5% (w/v) stacking gel and 12.5% (w/v) separating gel. The proteins were stained with Coomassie Brilliant Blue R250. Gels: (a), (b) and (c), rabbit L-subparticle, core-particle and split-proteins respectively; (d), (e) and (f), *X. laevis* split, core-particle and L-subparticle proteins respectively; (g), molecular-weight marker proteins: myosin (200 000); β -galactosidase (130 000); phosphorylase (97 400); transferrin (78 000); bovine serum albumin (66 000); ovalbumin (45 000); antibody L-chain (30 000); soya-bean trypsin inhibitor (21 500); cytochrome c (12 500).

values obtained for *X. laevis* proteins were similar to those for rabbit proteins. Table 5 also includes the results of a single experiment to correlate the two types of gel pattern by extracting the rabbit polypeptides from the major spots of a two-dimensional pattern and running them in the sodium dodecyl sulphate system as a third dimension (Howard *et al.*, 1975).

Discussion

There are good grounds for believing that bacterial ribosomes are all closely related and that the *E. coli* ribosome is representative of bacterial ribosomes in general. For example, for the S-subparticle this was shown by the formation of functional chimera by utilizing S-proteins of one species and the S-rRNA component of another. The species studied were *Azotobacter vinelandii*, *Bacillus stearothermophilus* and *E. coli* (Nomura, 1973). The existence of sequence homologies among a wide range of bac-

Table 5. Molecular weights of split-protein components estimated from their mobility on electrophoresis in sodium dodecyl sulphate/polyacrylamide gels

The procedure of Laemmli (1970) was followed with 12.5% (w/v) gels (see also Fig. 4). The estimated accuracy of the molecular-weight values is ± 1000 . The values refer to protein bands that are not necessarily pure polypeptides.

10 ⁻³ × Molecular weight	
Rabbit split-proteins*	<i>X. laevis</i> split-proteins
40.5	40
37.5	35.5
36	34
34	32
29	29.5
25.5†	25
22.5†	22.5
20.5‡ (L7)	
19.0 (L28)	
17.5‡ (L7)	17.5
14.5‡ (L7)	
9.0 (L46)	

* The numbers in parentheses refer to spots on two-dimensional gels (nomenclature of Cox & Greenwell, 1976) which were found to contribute to the designated bands on three-dimensional electrophoresis (Howard *et al.*, 1975).

† These bands varied appreciably in intensity, and were scarcely visible in some split-protein preparations capable of re-activating core-particles.

‡ Acidic proteins analogous to L7/L12 of *E. coli* are probably present in these bands, as indicated by chromatographic behaviour on DEAE- and CM-cellulose columns (R. A. Cox & P. Greenwell, unpublished work).

terial S-rRNA species was demonstrated by Pace & Campbell (1971) on the basis of hybridization, and by Woese *et al.* (1975) using a method based on cataloguing the oligonucleotides produced by ribonuclease-T₂ digestion of S-rRNA.

An evolutionary discontinuity separates bacterial and eukaryotic cytoplasmic ribosomes, as shown, for example, by the greater size and number of eukaryotic proteins, the increased sizes of both S-rRNA and L-rRNA (the major rRNA species of smaller and larger subparticle respectively), and by the emergence of 5.8S rRNA in eukaryotic ribosomes (Erdmann, 1976). Despite the evolutionary trends, many features of the active centre of ribosomes must be common to all species, because the substrates are the same for all, namely mRNA, aminoacyl-tRNA and peptidyl-tRNA. It is to be expected that eukaryotic ribosomes should have features in common, and reconstruction systems provide the means to measure their extent.

Significance of reconstruction of functional ribosomes with heterologous components

An important result of this study is that the split-protein fraction of a mould (*N. crassa*) can replace the split-protein fraction of a mammal (rabbit), giving rise to functional hybrid L-subparticles. Although the functional role of the individual split-proteins is not known, it is clear that the split-protein fractions for rabbit, *X. laevis* and *N. crassa* can substitute for one another. The analysis of the split-proteins of rabbit and *X. laevis* by electrophoresis reveals that there are proteins present that have similar properties (see Figs. 3 and 4 and Table 5), as would be expected if the split-proteins of one species had their structural counterparts in the split-protein fraction of another. This does not necessarily mean that the amino acid sequences of analogous proteins are highly conserved. For example, an immunological comparison of proteins of chicken and rat ribosomes suggests that there are extensive sequence differences in the protein moieties of the two species (Fischer *et al.*, 1978; cf. Noll & Bielka, 1970). We conclude that the split-protein fraction functions in heterologous systems because not only do the split-proteins of one species have their functional analogues in the split-protein fraction of another species, but also the structures of the analogues are sufficiently similar for them to be capable of replacing one another. The replacement of rabbit split-proteins by, for example, *N. crassa* proteins could be useful for structural studies of mammalian ribosomes by utilizing antibodies to the heterologous proteins.

The capacity of *N. crassa* L-subparticles to function with rabbit S-subparticles is also informative about evolutionarily conserved features of eukaryotic ribosomes. The significance of this property is obscured by our ignorance of the mechanism of interaction of subparticles within ribosomes. We have found (R. A. Cox & S. Kotecha, unpublished work) that very mild trypsin treatment (40 ng of trypsin/ A_{260} unit of L-subparticle for 15 min at 30°C) of the rabbit L-subparticle destroys its capacity to synthesize polyphenylalanine because it can no longer bind to S-subparticles, although its catalytic activity in the puromycin reaction is enhanced. We infer that trypsin-sensitive protein(s) contribute to the highly conserved features necessary for the interaction between heterologous subparticles to take place.

Nature of the split-protein fraction

The present reconstruction experiments have shown that split-protein fractions prepared from three eukaryotes under the same conditions of salt-shock are functionally equivalent, but the functions of individual proteins remain unknown. In view of

the partial interchangeability of some acidic ribosomal proteins (Stoffler *et al.*, 1974) and protein-synthesis elongation factors (Grasmuk *et al.*, 1977) across the prokaryote/eukaryote evolutionary discontinuity, a preliminary attempt was made to prepare an analogous split-protein fraction from *E. coli* ribosomes which would contain proteins with known functions. This direct comparative approach by using 2.75 M-NH₄Cl/69 mM-MgCl₂ was not successful, because the *E. coli* L-subparticles (prepared from *E. coli* MRE600, by the method of Cox & Hirst, 1976) were resistant to these standard salt-shock conditions. In order to obtain core-particles almost completely inactive in the puromycin reaction at the standard Mg²⁺:NH₄⁺ ratio of 1:40 it was necessary to use approx. 4 M-NH₄Cl. Alternatively, with the standard 2.75 M-NH₄Cl, the Mg²⁺ concentration had to be lowered from 69 mM to approx. 5 mM (P. Greenwell, unpublished work).

Further information on the individual functions of the eukaryotic split-proteins could be obtained by means of the methods developed in the present paper if the proteins were available in a pure non-denatured form. In practice, numerous attempts to chromatograph ¹⁴C-labelled rabbit split-proteins by methods used in the past for *E. coli* ribosomal proteins (Wittmann, 1974) have led to practically irreversible binding of the proteins to the columns. Newer methods have been developed to maintain split-proteins in less 'sticky' conformations, by avoiding solvents with urea or lower ionic strength [e.g. ion-exchange chromatography as described by Littlechild & Malcolm (1978), or gel filtration on Sephadex G-100 in reconstruction buffer]. These methods have allowed partial purification of the rabbit split-proteins, but not on a sufficient scale to use the resolved fractions in reconstruction experiments (P. Greenwell, unpublished work). The problem of scale may be overcome by utilizing the equivalence of the split-protein fractions of lower eukaryotes and mammals. For example, *N. crassa* ribosomes are more readily available, may more readily be labelled *in vivo* and could therefore provide an abundant source of split-proteins for fractionation; the individual proteins could then be used to examine the re-activation of both *N. crassa* and rabbit cores.

Comparison of features in L-subparticles from X. laevis and N. crassa

It has been shown elsewhere (Cox & Thompson, 1980) that 28% of the nucleotide sequence of L-rRNA from *N. crassa* is homologous (with fewer than 5% mis-matches) with the sequence from *X. laevis*. The homologous regions (approx. 1000 nucleotides) are located within 3000 nucleotides of the 3'-OH terminus of *X. laevis* L-rRNA. This observation is in agreement with the idea that cytoplasmic L-rRNA comprises a core that has tended to be

conserved during evolution, together with characteristic non-conserved sequences that vary from class to class (Cox *et al.*, 1973, 1976a). There is evidence that the L-rRNA is closely involved in the peptidyltransferase active centre of the ribosome (see, for example, Greenwell *et al.*, 1974; Cox *et al.*, 1976b), and the necessity of conserving this function could be an explanation for the conservation of sequences near the 3'-OH terminus of L-rRNA. The present study shows that a subset of ribosomal proteins, the split-protein fraction, from *N. crassa* or *X. laevis* or rabbit is both highly conserved and involved in the peptidyltransferase active centre. We infer that the active centre of cytoplasmic ribosomes in general is highly conserved, and suggest as a working model that it comprises the conserved 3'-OH-terminal sequences of L-rRNA in association with the interchangeable split-proteins. The significance of those features of ribosome structure that have diverged, such as the variations in the properties of L-rRNA at the 5'-terminus (see, for example, Cox & Thompson, 1980), remains unknown.

The peptidyltransferase centre of eukaryotic ribosomes may be studied advantageously by using ribosomes of lower eukaryotes, such as fungi, because both larger quantities of material and well-characterized mutants are available. The present work suggests that such studies can be relevant to and therefore useful for investigations of mammalian ribosomes, where the practical difficulties are greater.

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