Intermediates in the Assembly of Ribosomes by a Mutant of *Escherichia coli*

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Escherichia coli strain 15–28 is a mutant that accumulates ribonucleoprotein ('47S') particles during exponential growth. These particles contain mature 23S rRNA, but lack three of the proteins of the larger ribosomal subunit, to which they are a precursor. In organisms growing at 20°C, assembly of 47S particles involves three intermediates that contain precursor 23S rRNA, one of which has the same sedimentation properties as 47S particles. Assembly of 50S ribosomal subunits in the parent strain is 'normal'. There are three intermediates; each contains precursor 23S rRNA, and one cannot be distinguished from completed subunits by sedimentation. Synthesis of 30S ribosomal subunits in parent and mutant strains is qualitatively similar, but quantitatively different. When growth is at 37°C, assembly in the mutant alters. There are now two sequential precursors to 47S particles. Both contain precursor 23S rRNA; one has the same sedimentation coefficient as 47S particles. In some respects, synthesis in the mutant proceeds as though 47S particles, rather than 50S ribosomal subunits, are the end-product of assembly.

Escherichia coli strain 15–28 is a mutant whose 70S ribosomes are less efficient in the translation of endogenous mRNA than are ribosomes from its parent, strain 15TP (MacDonald *et al.*, 1967). This defect is reflected in a lower peptidyltransferase activity of mutant 70S and 50S ribosomes and their lowered ability to bind an analogue of peptidyl-tRNA (Sims & Wild, 1976). No differences have been detected in the composition of completed ribosomal subunits of parent and mutant strains. We suggested that the biological defects may arise because misassembly in strain 15–28 generates 50S ribosomal subunits that differ in conformation from those of its parent (Butler *et al.*, 1978).

A more dramatic demonstration of altered assembly in the mutant is the finding (MacDonald *et al.*, 1967; Markey & Wild, 1976) that extracts contain large quantities of '47S' ribonucleoprotein particles. The particles contain 23S rRNA that is, by electrophoretic criteria, 'mature' (rather than in precursor form), and lack three of the proteins (L16, L28 and L33) found in completed 50S ribosomal subunits (Markey *et al.*, 1976). Kinetic evidence shows that 50S ribosomal subunits are formed, in

growing mutant bacteria, from 47S particles. These precursor particles are thus unusual. They are present in quantity; their protein complement is different from those of precursors of the larger ribosomal subunit in wild-type bacteria; as a ribosome precursor the particles are unique in containing mature rather than precursor rRNA (Butler *et al.*, 1979).

Previously, we (Butler *et al.*, 1979) used pulse-labelling and sedimentation analyses to compare pathways of ribosome synthesis in parent and mutant organisms. This technique detects precursor particles that differ in sedimentation properties from completed subunits. At both temperatures of growth (20 and 37°C), assembly by the parent was similar to that described for other strains of *E. coli*. Two precursors to the 50S ribosomal subunit were detected and one to 30S ribosomal subunits; these are analogous to the p50S-1, p50S-2 and p30S-1 particles described by Lindahl (1975). Growth and pulse-labelling of the mutant at either temperature revealed a single precursor to the smaller ribosomal subunit. At 37°C there was present a heterogeneous ('p47S') precursor sedimenting more slowly than 47S particles. However, two apparently sequential precursors ('p47S-1' and 'p47S-2' particles) were resolved in organisms grown and labelled at 20°C. The sedimentation properties of the p47S-1 and

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p47S-2 particles were not quite the same as those of p50S-1 and p50S-2 precursors from the parent organism. This suggested that the pathway of assembly of the larger ribosomal subunit in the mutant is altered at an early stage.

The identification of pulse-labelled components as related to either larger or smaller ribosomal subunits is made reasonably secure by their kinetic behaviour when labelling is for different times, by their approximate sedimentation coefficients and by analogies with other work. In the present paper, we use gel electrophoresis of the RNA of the various precursor particles to confirm their identities. This technique also permits identification of additional ribosome precursors that cannot be distinguished by sedimentation and allows examination of the stage in the assembly of 47S particles at which 23S rRNA matures. In addition we present information on the relative compositions of the various ribonucleoproteins and discuss assembly in the mutant strain.

Experimental

Growth of bacteria and preparation of cell-free extracts

The organisms were *E. coli* 15 *thy⁻pro⁻* (15TP) and the mutant (15-28) derived from it (MacDonald *et al.*, 1967). Bacteria were grown, labelled with radioactivity and gently lysed as described previously (Butler *et al.*, 1979). The uracil used for labelling was [5-³H]uracil unless otherwise stated.

Gradient centrifuging

Except for the experiments that also involved isopycnic centrifuging (see below), the buffer used for the preparation of 15–30% (w/w) sucrose gradients contained 10 mM-Tris/HCl/10 mM-magnesium acetate/100 mM-KCl/1 mM-spermidine, pH 7.4. Centrifuging was at 4°C in the Spinco model L2-65B ultracentrifuge. The work used the SW50.1 rotor, operated at 234 000 *g* for 2.75 or 4.5 h as appropriate and the SW27.1 rotor (1.6 cm × 10.2 cm tubes) centrifuged at 75 000 *g* for 16 h or 22 h. Gradients were collected by using an Isco model 185 density-gradient fractionator. For the preparation of ribosomes and their precursors, collection was into tubes; portions (10 µl) of fractions were removed for determination of radioactivity. For analytical purposes, collection was on to strips of glass-fibre paper (Whatman GF82). Measurement of radioactivity was as described previously (Markey & Wild, 1976).

Isopycnic centrifuging

Ribosomal subunits, 47S particles and their precursors were fixed with 7% (w/v) formaldehyde and centrifuged to equilibrium in CsCl gradients as previously described (Markey & Wild, 1976). The two buffers (pH 7.4) used contained triethanolamine

in place of Tris and were (i) 5 mM-triethanolamine/HCl/10 mM-magnesium acetate/100 mM-KCl (EHMK buffer) and (ii) 5 mM-triethanolamine/HCl/2 mM-magnesium acetate/100 mM-KCl (ELMK buffer). In ELMK buffer, 70S ribosomes dissociate into their subunits.

Extraction and electrophoresis of RNA

RNA was extracted from cell lysates of isolated ribonucleoprotein particles by the method of Kurland (1960). To resolve precursor and mature forms of 23S rRNA by electrophoresis, it was found essential to carry out the extractions at low temperature (4°C in the experiments reported). RNA was extracted directly from lysates, but particles isolated from sucrose gradients (where K⁺ ions are present) were first precipitated at –20°C with 2 vol. of ethanol. For the isolation of RNA from pulse-labelled precursor particles about 20 µg of carrier 70S ribosomes from strain 15TP were added to the aqueous phase before the ethanol precipitation. After 1 h, the ribonucleoprotein was collected by centrifuging (5 min at 10 000 *g* in a Beckman Microfuge), resuspended in THM buffer (10 mM-Tris/HCl/10 mM-magnesium acetate, pH 7.4) and the RNA extracted. After three cycles of extraction with phenol, RNA was precipitated from the aqueous phase with 1 vol. of ethanol. After 1 h at –20°C, precipitated RNA was collected by centrifugation (as above), resuspended in 1 vol. of ice-cold ethanol, again collected by centrifuging and dissolved in 50–100 µl of THM buffer.

Electrophoresis of rRNA in 2.5–7.5% (w/v) polyacrylamide gradient gels was essentially as described by Jeppesen (1974). The acrylamide and *N,N'*-methylenebisacrylamide were recrystallized as described by Loening (1967). Gels (82 mm × 82 mm × 3 mm) were made at room temperature by using the Pharmacia GSC-8 gel-slab-casting apparatus and could be stored for up to 2 months at 4°C in sealed plastic bags containing a small volume of electrophoresis buffer (40 mM-Tris/acetate/20 mM-sodium acetate/2 mM-EDTA, pH 8.3).

The Gradipore electrophoresis apparatus (Marquis & Kenrick, 1968) was used. Gels were pre-run for 1–2 h at 4°C and 10 V·cm^{–1}. The samples (1–5 µg of RNA containing about 2 × 10⁴ c.p.m. of ³H and 4 × 10⁴ c.p.m. of ³²P radioactivities in 5–20 µl of THM buffer) were loaded with a micro-syringe. Sucrose (about 12%, w/v) and Bromophenol Blue (0.1%) were also present in the samples. Electrophoresis was for 15–16 h at 4°C and 10 V·cm^{–1}. The buffer was recirculated.

After electrophoresis the gels were fixed, stained and destained (Markey *et al.*, 1976). Tracks were excised, frozen with solid CO₂ and chopped into 0.5 mm slices with a Mickle gel slicer. Slices from the

regions of a gel that contained 16S and 23S rRNA were digested at 42°C for 16 h in a sealed polythene scintillation vial with 0.3 ml of 100-vol. H_2O_2 . Scintillant (10 ml of Aquasol) was added. Samples were stored in the dark at room temperature for at least 12 h before measurement of radioactivity.

Chemicals

Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxyribonuclease I was from Worthington Biochemical Co., Freehold, NJ, U.S.A. Brij 58 was from Honeywell and Stein, Carshalton, Surrey, U.K.

Whatman products were from Whatman, Maidstone, Kent, U.K., and Aquasol was from New England Nuclear, Dreieich, W. Germany.

Results

RNA from pulse-labelled precursor particles

(i) *Strain 15TP*. For the experiment of Fig. 1, strain 15TP was grown at 20°C and labelled for 5 min with [^3H]uracil. A lysate was made and a portion mixed with a second lysate obtained from a culture of strain 15TP that had been grown for three generations with [^{32}P]P_i. Centrifuging gave the

Fig. 1. RNA of the ribosome precursors of strain 15TP: growth at 20°C

A culture (20 ml) of strain 15TP was grown at 20°C and carrier free [^3H]uracil [$30\mu\text{Ci}$ ($0.13\mu\text{g}$)/ml] added for 5 min on reaching A_{450} 0.40. A lysate was then made. A second lysate was obtained from a culture (10 ml) of strain 15TP grown for three generations at 20°C to A_{450} 0.40 with [^{32}P]P_i ($4\mu\text{Ci}$ /ml). A portion ($20\mu\text{l}$) of the ^3H -labelled lysate was mixed with $30\mu\text{l}$ of the ^{32}P -labelled lysate. Centrifuging (SW 50.1 rotor) gave the profiles in panel (a). A further portion (0.5ml) of the ^3H -labelled lysate was centrifuged through a linear sucrose gradient in the SW 27.1 rotor. The precursors (p30S-1, p50S-1 and p50S-2 in panel a) were located and collected. A portion ($50\mu\text{l}$) of each precursor preparation was mixed with $30\mu\text{l}$ of ^{32}P -labelled lysate and centrifuged in the SW 50.1 rotor. RNA made from the remainder of each preparation was mixed with ^{32}P -labelled RNA and electrophoresis carried out (panels b, c and d). The ^{32}P -labelled RNA was prepared from a lysate of a culture (25 ml) of strain 15TP grown at 37°C for three generations to A_{450} 0.40 with [^{32}P]P_i ($4\mu\text{Ci}$ /ml). O, ^{32}P radioactivity; ●, ^3H radioactivity.

sedimentation profile in Fig. 1(a), which shows the three putative ribosome-precursor particles previously denoted p30S-1, p50S-1 and p50S-2 (Butler *et al.*, 1979). A second portion of the ^3H -labelled lysate was centrifuged and peak fractions corresponding to the three precursor particles were retained. A part of each fraction was mixed with the ^{32}P -labelled lysate and centrifuged to show that each precursor was homogeneous and sedimented to a position very similar to that occupied in Fig. 1(a). RNA extracted from the remainder of each precursor preparation was mixed with ^{32}P -labelled RNA (from strain 15TP grown for three generations at 37°C with $[^{32}\text{P}]\text{P}_i$) and electrophoresed through polyacrylamide gels. The radioactivities of gel slices are shown in Figs. 1(b), 1(c) and 1(d). The p30S-1 precursor particle contains very largely precursor ('p') rather than mature ('m') 16S RNA. This confirms that the particle is a precursor of the smaller ribosomal subunit. The p50S-1 and p50S-2 particles both contain 23S RNA, the majority of which is in the precursor form.

Ribosome assembly in strain 15TP at 37°C is very similar to that at 20°C , because sedimentation of lysates from pulse-labelled cells shows what are apparently the same three precursor particles (Butler *et al.*, 1979). In an experiment similar to that of Fig. 1, strain 15TP was labelled for 25s with $[5,6\text{-}^3\text{H}]\text{uracil}$ and the precursor particles were isolated. The subsequent electrophoresis of extracted RNA again showed that p30S-1 particles contain almost exclusively p16S RNA, whereas p50S-1 and p50S-2 particles possess 23S rRNA, 60–70% of which is in the precursor form.

Electrophoresis permits the detection of ribosome precursor particles that have the sedimentation properties of completed 'native' subunits, but contain precursor forms of RNA. Strain 15TP was grown at 20°C and labelled with $[^3\text{H}]\text{uracil}$ for 25min. A lysate was mixed with a second lysate from organisms grown for three generations with $[^{32}\text{P}]\text{P}_i$ and centrifuged. Under these conditions, the labelling with $[^3\text{H}]\text{uracil}$ is long enough for pulse-labelled (^3H) and steady-state (^{32}P) profiles virtually to coincide (Fig. 2a). Fractions containing ^3H -labelled material with the sedimentation properties of 30S and 50S ribosomal subunits were isolated from a second gradient. A portion of each fraction was centrifuged to confirm its sedimentation properties and RNA was isolated from the remainder. Electrophoresis (Figs. 2b and 2c) shows the RNA to be almost exclusively in the precursor forms. A similar experiment was carried out for which strain 15TP, grown at 37°C , was labelled for 3min 20s with $[^3\text{H}]\text{uracil}$. The result was the same. Newly made material with the sedimentation properties of 50S or 30S ribosomal subunits contained RNA that was very largely p23S RNA or p16S RNA respectively.

Fig. 2. RNA of newly synthesized subunits of strain 15TP: growth at 20°C

A culture (10ml) of strain 15TP was grown at 20°C to A_{450} 0.36, when it received $[^3\text{H}]\text{uracil}$ [$30\mu\text{Ci}$ ($0.2\mu\text{g}$)/ml] for 25min. A lysate was made. A second lysate was prepared from a culture (10ml) of strain 15TP grown for three generations at 20°C to A_{450} 0.39 with $[^{32}\text{P}]\text{P}_i$ ($4\mu\text{Ci}$ /ml). A portion ($10\mu\text{l}$) of ^3H -labelled lysate was mixed with $70\mu\text{l}$ of ^{32}P -labelled lysate and centrifuged (SW 50.1 rotor; panel a). A further portion ($200\mu\text{l}$) of the ^3H -labelled lysate was centrifuged (SW50.1 rotor) and fractions in the position of 'native' 30S and 50S ribosomal subunits were collected and RNA was made from them. This RNA was mixed with ^{32}P -labelled RNA (made as for the experiment of Fig. 1) and electrophoresis carried out (panels b and c). O, ^{32}P radioactivity; ●, ^3H radioactivity.

These results therefore confirm the status of p30S-1, p50S-1 and p50S-2 particles and show that, as noted for other strains of *E. coli* (Lindahl, 1975), strain 15TP contains additional precursors whose sedimentation properties are indistinguishable from those of the subunits into which they mature. We follow Lindahl's (1975) nomenclature and call these additional and apparently sequential precursors p30S-2 and p50S-3 particles.

(ii) *Strain 15-28*. Previous work (Markey & Wild, 1976; Markey *et al.*, 1976) showed that, although 47S particles of strain 15-28 are a precursor to 50S ribosomes, they contain mature ('m') 23S RNA. In addition, RNA from the native 30S ribosomes of the mutant is a mixture of the mature and precursor forms of 16S RNA in a ratio of about 2.3:1 (Markey & Wild, 1975). These observations were made on organisms grown at 37°C. The experiment of Fig. 3 used the electrophoretic techniques of the present paper to confirm and extend these results to growth at 20°C. It shows that at both temperatures the 23S RNA from 47S particles is 'mature'; at both temperatures native 30S ribosomes contain precursor 16S RNA whose proportion falls from 30% (at 37°C) to about 22% (at 20°C).

Strain 15-28 also differs from its parent in that the pathway of synthesis of 47S particles (and therefore 50S ribosomes) alters with the temperature of growth. Fig. 4(a) shows that, as noted previously (Butler *et al.*, 1979), pulse-labelling with [³H]uracil of organisms grown at 20°C (for 30 min in this case) reveals a single precursor (p30S-1) to 30S ribosomal subunits and two precursors (p47S-1 and p47S-2) to 47S particles. These three particles were isolated from a second sucrose gradient and a portion of each preparation was re-centrifuged to confirm their essential purity and retention of sedimentation properties. Electrophoresis of extracted RNA showed (Figs. 4b, 4c and 4d) that p30S-1 particles contain p16S RNA and that the 23S RNA of p47S-1 and p47S-2 particles is predominantly in the precursor form. This confirms previous deductions (Butler *et al.*, 1979) about the particles.

Growth and labelling of the mutant at 37°C (for 2 min 30 s in the experiment of Fig. 5) detects a p30S-1 particle and a single apparently heterogeneous 'p47S' precursor to 47S particles. RNA isolated from the former is precursor 16S RNA (Fig. 5b), whereas p47S particles contain almost exclusively the precursor form of 23S RNA (Fig. 5c). Thus at either temperature the precursors to 47S particles resolved by sedimentation contain largely precursor 23S RNA, whereas the RNA of the 47S particles themselves is mature. The experiment of Fig. 6 examines the possibility that the final stage in assembly of 47S particles may, by analogy

Fig. 3. RNA of 47S particles and native 30S ribosomes of strain 15-28

A culture (20 ml) of strain 15-28 was grown at 37°C from A_{450} 0.06 to 0.42 with [³H]uracil [$15 \mu\text{Ci}$ ($10 \mu\text{g}$)/ml] present. A lysate was made and centrifuged through a sucrose gradient (SW 27.1 rotor) to isolate 47S particles and native 30S ribosomal subunits. From these RNA was extracted. A second lysate was prepared from a culture (20 ml) of strain 15TP grown for three generations with [³²P] P_i ($4 \mu\text{Ci}/\text{ml}$). The ³²P-labelled RNA was mixed with the ³H-labelled RNA from 47S particles (panel a) or 30S subunits (panel b) and electrophoresis performed. For panels (c) and (d) the experiment was repeated with a culture of strain 15-28 grown under the same conditions but at 20°C. O, ³²P radioactivity; ●, ³H radioactivity.

with the synthesis of 50S ribosomes in the parent strain, involve the maturation of p23S RNA contained in material with the sedimentation properties of 'completed' 47S particles.

At 20°C the mean generation time of the mutant strain is about 400 min, and labelling for 2 h with [³H]uracil is required to give a sedimentation profile (Fig. 6a) in which p47S-1 and p47S-2 particles can be scarcely detected in the trough between newly

Fig. 4. RNA of ribosome precursors of strain 15-28: growth at 20°C

A culture (20 ml) of strain 15-28 was grown at 20°C to A_{450} 0.60. [${}^3\text{H}$]Uracil [$30\mu\text{Ci}$ ($0.5\mu\text{g}$)/ml] was then added for 30 min. A lysate was prepared. A second lysate was made from a culture (10 ml) of strain 15-28 grown at 20°C from A_{450} 0.06 to 0.41 with [${}^{32}\text{P}$]P_i ($2\mu\text{Ci}$ /ml). For panel (a), a portion (10 μl) of ${}^3\text{H}$ -labelled lysate was mixed with 25 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged (SW50.1 rotor). A further portion (0.5 ml) of ${}^3\text{H}$ -labelled lysate was centrifuged in the SW27.1 rotor. The precursors (p30S-1, p47S-1 and p47S-2 in panel a) were located and collected. A portion (50 μl) of each precursor preparation was mixed with 25 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged. RNA made from the remainder of each preparation was mixed with ${}^{32}\text{P}$ -labelled RNA (made as for Fig. 1) and electrophoresis carried out (panels b, c and d). O, ${}^{32}\text{P}$ radioactivity; ●, ${}^3\text{H}$ radioactivity.

synthesized components with the sedimentation properties of 30S ribosomes and 47S particles. A fraction with the sedimentation properties of 47S particles was isolated from a similar gradient. This material retained the sedimentation properties of 47S particles when re-centrifuged (Fig. 6b). The RNA from this component was 23S RNA, about half of which was in the precursor form (Fig. 6c). Thus at 20°C there exists a third precursor ('p47S-3') distinguished from 47S particles only by its content of p23S RNA. Growth at 37°C gave a very similar result. Labelling with [${}^3\text{H}$]uracil was for 15 min, and the sedimentation profile of the lysate (Fig. 6d) was then similar to that of the lysate from organisms grown for three generations with [${}^{32}\text{P}$]P_i. When

re-centrifuged, the fraction selected for RNA isolation had the sedimentation properties of 47S particles (Fig. 6e); the RNA from this material was 23S RNA, of which one-third or more was in the precursor form (Fig. 6f). Thus, at 37°C too, synthesis of 47S particles proceeds through a precursor with the sedimentation properties of 47S particles, but which contains precursor 23S RNA.

Composition of precursor particles: isopycnic centrifuging

The low absolute concentration of ribosome precursor particles necessitates their detection by means such as pulse-labelling. This consideration also applies to the p30S-1 particles and precursors

Fig. 5. RNA of ribosome precursors of strain 15-28: growth at 37°C

A culture (20 ml) of strain 15-28 was grown at 37°C to A_{450} 0.42, when it received [^3H]uracil [$30\mu\text{Ci}$ ($0.5\mu\text{g}$)/ml] for 2.5 min. A lysate was made. A second lysate was made from a culture (20 ml) of strain 15-28 grown at 37°C for three generations to A_{450} 0.42 with [^{32}P]P_i ($2\mu\text{Ci}$ /ml). A portion ($10\mu\text{l}$) of the ^3H -labelled lysate was mixed with $50\mu\text{l}$ of the ^{32}P -labelled lysate and centrifuged (SW50.1 rotor) to give the profiles in panel (a). A further portion (0.5 ml) of the ^3H -labelled lysate was centrifuged in the SW27.1 rotor. Peak fractions corresponding to the p30S-1 and p47S particles of panel (a) were located and collected, and RNA was extracted from them. The RNA was mixed with ^{32}P -labelled RNA (made from strain 15TP as for Fig. 1) and electrophoresis carried out (panels b and c). O, ^{32}P radioactivity; ●, ^3H radioactivity.

to 47S particles in the mutant strain. Although it is therefore difficult to determine directly the composition of the various precursors, isopycnic centrifuging of preparations fixed with formaldehyde can give some information.

Strain 15TP was grown at 20°C (generation time 140 min) and then labelled for 5 min with [^3H]uracil. Portions of a lysate were sedimented with results very similar to Fig. 1(a). Precursor (p30S-1, p50S-1 and p50S-2) particles were isolated and mixed with ^{14}C -labelled 30S or 50S ribosomal subunits, obtained from strain 15TP. The mixtures were fixed with formaldehyde and a portion of each was centrifuged through sucrose gradients to show the essential homogeneity and unchanged sedimentation properties of the precursors. Isopycnic centrifuging (Figs. 7a, 7b and 7c) showed that ^{14}C -labelled 50S ribosomal subunits band at a buoyant density of 1.695 g/ml; the p50S-2 particles also band sharply (Fig. 7c) with a density (calculated from the mid-point of the half-peak heights) of 1.710 g/ml. The p50S-2 particles therefore contain 11% less protein per unit of RNA than do 50S ribosomal subunits. The p50S-1 particles give a more disperse band (Fig. 7b), but the average buoyant density (1.725 g/ml) corresponds to a greater (about 20%) protein deficiency. The smaller ribosomal subunits band less sharply than the larger, and at a lower buoyant density (1.645 g/ml). The p30S-1 particles, although rather disperse, have a mean density of 1.695 g/ml and so contain about 17% less protein, per unit of RNA, than the subunits.

In a similar experiment the mutant strain, 15-28, was grown at 20°C and labelled with [^3H]uracil for 20 min. Fig. 8(a) shows that centrifuging resolved, as expected, p47S-1, p47S-2 and p30S-1 particles. The sedimentation properties of the precursors were maintained when the isolated, fixed particles were re-centrifuged mixed with ^{14}C -labelled 47S particles or native 30S ribosomes from the mutant. Isopycnic centrifuging (Figs. 8b, 8c and 8d) showed that 47S particles have a buoyant density of 1.705 g/ml; because 47S particles lack three proteins they have a higher density than 50S ribosomal subunits (Markey *et al.*, 1976). The p47S-1 particles have an even higher buoyant density (1.725 g/ml) and so on average contain about 11% less protein than do 47S particles. The difference between p47S-2 and 47S particles is slight; the former are marginally the denser and possibly contain about 3% less protein, on average, than 47S particles. As with the parent strain, the 30S ribosomal subunits from the mutant give a broad band, but of the same average buoyant density (1.645 g/ml) as subunits from the parent; the average density of mutant p30S-1 particles (1.695 g/ml) is the same as for the equivalent p30S-1 particles from the parent.

Fig. 6. RNA of newly made 47S particles

A culture (10 ml) of strain 15-28 was grown at 20°C to A_{450} 0.41, when it received [${}^3\text{H}$]uracil [$15 \mu\text{Ci}$ ($2 \mu\text{g}$)/ml] for 2 h. A lysate was made. A second lysate was obtained from a culture (20 ml) of strain 15-28 grown at 20°C for three generations to A_{450} 0.41 with [${}^{32}\text{P}$]P_i ($2 \mu\text{Ci}$ /ml). A portion (10 μl) of the ${}^3\text{H}$ -labelled lysate was mixed with 70 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged (SW50.1 rotor) to give the profiles in panel (a). A further portion (200 μl) of the ${}^3\text{H}$ -labelled lysate was centrifuged (SW50.1 rotor), the peak of 'newly-made' 47S particles located, collected, and a portion (5 μl) mixed with 25 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged (SW50.1 rotor; panel b). RNA was extracted from the remainder of the preparation of 47S particles, mixed with ${}^{32}\text{P}$ -labelled RNA (made as for the experiment of Fig. 1) and electrophoresed (panel c). The experiment was repeated but with strain 15-28 grown at 37°C. A culture (10 ml) of strain 15-28 at A_{450} 0.42 received [${}^3\text{H}$]uracil [$12 \mu\text{Ci}$ ($1 \mu\text{g}$)/ml] for 15 min. A second culture (20 ml) was grown for three generations to A_{450} 0.40 with [${}^{32}\text{P}$]P_i ($2 \mu\text{Ci}$ /ml) present. Lysates were made. A portion (10 μl) of ${}^3\text{H}$ -labelled lysate was mixed with 70 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged (SW50.1 rotor; panel d). A further 200 μl of ${}^3\text{H}$ -labelled lysate was centrifuged (SW50.1 rotor) and newly made 47S particles were located and isolated. A portion (5 μl) of this preparation was mixed with 25 μl of ${}^{32}\text{P}$ -labelled lysate and centrifuged (SW50.1 rotor; panel e). RNA extracted from the remainder of the 47S particles was mixed with ${}^{32}\text{P}$ -labelled RNA (made as above) and electrophoresis carried out (panel f). O, ${}^{32}\text{P}$ radioactivity; ●, ${}^3\text{H}$ radioactivity.

Pulse-labelling of strain 15-28 during growth at 37°C (e.g. Fig. 5) shows a single presumed heterogeneous precursor (p47S) to 47S particles. The heterogeneity might result from continuous variation in composition across the p47S fractions of a gradient; the buoyant density of these fractions should then differ. The spread might result from variable conformation but constant composition; the buoyant densities of the fractions should then be the same. A third explanation could be that the heterogeneity of p47S particles is an artefact of centrifugation; in this case, material isolated from the different fractions should behave identically when re-centrifuged through sucrose gradients. These possibilities were tested in the experiment of Fig. 9. A culture of strain 15-28 growing at 37°C was labelled for 2 min with [³H]uracil. A lysate was made and centrifuged. Three fractions (1, 2 and 3) from different regions of the p47S particles (Fig. 9a) were collected, separately mixed with ¹⁴C-labelled 47S particles and re-centrifuged through sucrose gradients. Figs. 9(b), 9(c) and 9(d) show that the fractions retain their individual sedimentation properties. Thus the heterogeneity is 'real'. Isopycnic centrifuging (Figs. 9e, 9f and 9g) shows that there are some differences in composition. All three fractions are more dense than 47S particles; the average density of fraction 1 (1.740 g/ml, equivalent to 17% less protein than in 47S particles) is appreciably greater than that of fractions 2 or 3. The latter fractions are similar in density, but differ appreciably

Fig. 7. Isopycnic centrifuging of ribosome precursors from strain 15TP; growth at 20°C

A culture (10 ml) of strain 15TP grown at 20°C to A_{450} 0.40 received [³H]uracil [75 μ Ci (0.2 μ g)/ml] for 5 min. A lysate was made and a portion (200 μ l) centrifuged (SW50.1 rotor) through a sucrose gradient in EHMK buffer. The sedimentation profile was very similar to that in Fig. 1(a); fractions containing p30S-1, p50S-1 and p50S-2 particles were collected. A second culture (25 ml) of strain 15TP received [¹⁴C]uracil (1 μ Ci/ml with no added carrier) and was grown for two generations to A_{450} 0.40. A lysate was made and a portion (0.25 ml) centrifuged (SW50.1 rotor) through a sucrose gradient made in ELMK buffer. Fractions containing 30S and 50S ribosomal subunits were collected. Mixtures (110 μ l of precursor particle plus 30 μ l of ribosomal subunit plus 80 μ l of EHMK buffer) were fixed with formaldehyde. Portions (25 μ l) of each mixture were centrifuged (SW50.1 rotor) through sucrose gradients in EHMK buffer and further portions (150 μ l) centrifuged in CsCl. Precursor particles p30S-1 plus 30S ribosomal subunits gave panel (a); p50S-1 particles plus 50S ribosomal subunits gave panel (b); p50S-2 particles plus 50S ribosomal subunits gave panel (c). O, ¹⁴C radioactivity; ●, ³H radioactivity; ■, density (g/ml).

Fig. 8. *Isopycnic centrifuging of ribosome precursors of strain 15-28: growth at 20°C*

A culture (10 ml) of strain 15-28 grown at 20°C to A_{450} 0.40 received [${}^3\text{H}$]uracil [$50 \mu\text{Ci}$ ($1 \mu\text{g}$)/ml] for 20 min. A lysate was made. Another culture (25 ml) was grown at 20°C for three generations to A_{450} 0.40 with [${}^{14}\text{C}$]uracil [$1.5 \mu\text{Ci}$ ($10 \mu\text{g}$)/ml] present and a second lysate made. Samples ($150 \mu\text{l}$) of each lysate were centrifuged (SW50.1 rotor) through sucrose gradients in EHMK buffer. The ${}^3\text{H}$ -labelled p30S-1, p47S-1 and p47S-2 particles were isolated from one gradient and ${}^{14}\text{C}$ -labelled 47S particles and 30S subunits from the other. A mixture ($30 \mu\text{l}$ of ${}^3\text{H}$ - and $10 \mu\text{l}$ of ${}^{14}\text{C}$ -labelled lysate) was also centrifuged (panel a). Mixtures [$110 \mu\text{l}$ of precursor particle plus $40 \mu\text{l}$ of 30S ribosomes (or 47S particles) plus $140 \mu\text{l}$ of EHMK buffer] were fixed with formaldehyde. Portions ($50 \mu\text{l}$) of each mixture were centrifuged (SW50.1 rotor) through sucrose gradients in EHMK buffer and further portions ($105 \mu\text{l}$) centrifuged in CsCl. Precursor particle p30S-1 plus 30S ribosomal subunits gave panel (b); p47S-1 particles plus 47S particles gave panel (c); p47S-2 particles plus 47S particles gave panel (d). O, ${}^{14}\text{C}$ radioactivity; ●, ${}^3\text{H}$ radioactivity; ■, density (g/ml).

ciably in sedimentation properties. It is therefore likely that both composition and conformation changes contribute to the heterogeneity of p47S particles.

Discussion

Our experiments detect two precursors to 30S ribosomal subunits in the parent organism. The

Fig. 9. Isopycnic centrifuging of ribosome precursors from strain 15-28; growth at 37°C. A culture (10 ml) of strain 15-28 was grown at 37°C to A_{450} 0.40, when it received [^3H]uracil [$100\mu\text{Ci}$ ($0.5\mu\text{g}$)/ml] for 2 min. A lysate was then made. A second culture (25 ml) was grown at 37°C from A_{450} 0.075 to 0.40 with [^{14}C]uracil [$1\mu\text{Ci}$ ($10\mu\text{g}$)/ml] present and a lysate made. A mixture of lysates was centrifuged (SW50.1 rotor) to give the profiles in panel (a). Further samples ($160\mu\text{l}$) of each lysate were centrifuged separately through sucrose gradients in EHMK buffer. The ^{14}C -labelled 47S particles were isolated from one gradient and three fractions from the ^3H -labelled p47S particles were, as illustrated in panel (a), retained from the other. Each particle fraction was diluted to $250\mu\text{l}$ with EHMK buffer and ^{14}C -labelled 47S particles ($50\mu\text{l}$) were fixed with formaldehyde. A portion ($50\mu\text{l}$) of each mixture was centrifuged (SW50.1 rotor) through sucrose gradients in EHMK buffer (panels b, c, d) and further portions ($220\mu\text{l}$) in CsCl (panels e, f, g; density (g/ml)). Fraction 1 (plus 47S particles) gave panels (b) and (e); fraction 2, (c) and (f); and fraction 3, (d) and (g). O, ^{14}C radioactivity; $\bullet$, ^3H radioactivity.

p30S-1 particles have a sedimentation coefficient of about 27S; the results of isopycnic centrifuging suggest that they contain about 20% less protein than the completed subunits. The p30S-2 particles contain precursor 16S RNA, but cannot be resolved from native 30S ribosomal subunits by sedimentation. The absolute concentration of these precursor particles in exponentially growing organisms cannot be judged from the present experiments. Lindahl (1975) estimated that, in *E. coli* strain NF523, the 16S RNA of the p30S-2 particle (the major precursor in assembly of 30S ribosomes) amounts to only 2% of the total 16S RNA that the bacteria contain. Because there are only traces of p16S RNA in RNA isolated from native 30S ribosomal subunits of strain 15TP, a similar value may apply here.

The mutant strain, 15-28, assembles the smaller ribosomal subunit similarly to its parent; p30S-1 and p30S-2 particles are again detected. The sedimentation coefficients of 'mutant' and 'parent' p30S-1 particles are similar, as are their protein contents. The p30S-2 particle of the mutant contains p16S RNA, but has the sedimentation properties of completed subunits. It is possible that these resemblances are superficial. Precursor particles with similar sedimentation properties and overall compositions could contain different subsets of ribosomal proteins; relative compositions obtained by isopycnic centrifuging are crude average properties; during the preparation and centrifuging of lysates to obtain ribosomes and precursor particles, proteins are lost to an unquantifiable extent. The spread band given by 30S ribosomal subunits after isopycnic centrifuging suggests that the smaller subunit is more prone than the larger to protein loss. The precursor particles to either subunit may even more easily lose proteins, although this could be combined with 'genuine' heterogeneity among precursor particles of a given category, for which there is some evidence (Blundell & Wild, 1973).

Although assembly of the smaller subunit is qualitatively similar in parent and mutant strains, the p16S RNA of the mutant p30S-2 particles accounts for 20% (at 20°C) to 30% (at 37°C) of the 16S RNA of 30S material. This delay in the assembly of 30S ribosomal subunits in the mutant is more probably a consequence of the obvious alterations in the pathway of synthesis of the larger subunit than vice versa. Although mutations have been described that affect the assembly of only one of the ribosomal subunits (e.g. Rosset *et al.*, 1971), many mutants have a single genetic lesion that under restrictive conditions affects assembly of both subunits (Guthrie *et al.*, 1969; Cabezon *et al.*, 1977).

In the parent strain, sedimentation and kinetic analyses detect two precursors to the larger subunit,

the p50S-1 and p50S-2 particles with sedimentation coefficients of about 37S and 44S respectively (Butler *et al.*, 1979). These particles contain, as expected, the precursor form of 23S RNA. The resolution of p23S RNA by electrophoresis enables the detection of a third (p50S-3) precursor particle, with the sedimentation properties of the subunit. In these respects strain 15TP resembles other strains of *E. coli* (Lindahl, 1975). The isopycnic centrifuging suggests that p50S-1 particles contain about 20% less protein than the completed subunits and p50S-2 particles about 10% less. This accords with the isolation of protein-deficient precursors from growing organisms (Nierhaus *et al.*, 1973) and as intermediates in reconstitution *in vitro* (Spillmann *et al.*, 1977). A 10% protein deficiency (as in p50S-2 particles) corresponds to the absence of three to four proteins of average size. The p50S-2 particles isolated by Nierhaus *et al.* (1973) had seven proteins missing and four more present in lower amounts; about 14 proteins were missing from p50S-1 particles. Better agreement can possibly not be expected in view of uncertainties over protein losses during isolations.

Pulse-labelling of the mutant 15-28 at 37°C detects p47S particles, which contain p23S RNA. The p47S particles are heterogeneous, and it is likely that both composition and conformation are elements in their heterogeneity. In organisms grown at 37°C, there is a further precursor particle with the sedimentation properties of 47S particles but distinguished by containing p23S RNA. A p47S-3 particle with these same properties is also present during growth at 20°C, but at this lower temperature gradient centrifuging resolves p47S-1 and p47S-2 particles, both of which also contain p23S RNA. The isopycnic centrifuging suggests that p47S-1 particles have about 11% less protein than 47S particles, and p47S-2 particles are scarcely (perhaps about 3%) protein-deficient. The 47S particles themselves lack about 8% of the protein of completed subunits (Markey *et al.*, 1976); thus, relative to 50S ribosomal subunits, p47S-1 and p47S-2 particles are about 19% and 11% protein-deficient.

Scheme 1, which compares synthesis of 50S ribosomes in parent and mutant strains, shows that synthesis in the mutant proceeds in some respects as though 47S particles, rather than 50S ribosomal subunits, are the end product of the pathway. This is not unexpected, because during exponential growth about two-thirds of the 23S RNA of mutant organisms is in 47S particles and native 50S ribosomal subunits cannot be detected (Markey *et al.*, 1976). Conversion of p23S RNA into m23S RNA without change in sedimentation properties involves the p47S-3 → 47S transition (rather than a precursor sedimenting with 50S ribosomal subunits); there are three sequential precursors to 47S

Mutant strain					
Particle	→ p47S-1	→ p47S-2	→ p47S-3	→ 47S	→ ^{slow} 50S
p or m 23S RNA	p	p	p	m	m
Sedimentation coefficient (S)	37	43	47	47	50
Protein deficiency (%)	19	11	?	8	0
Parent strain					
Particle	→ p50S-1	→ p50S-2	→ p50S-3	→ 50S	
p or m 23S RNA	p	p	p	m	
Sedimentation coefficient (S)	37	44	50	50	
Protein deficiency (%)	20	10	?	0	

Scheme 1. *Synthesis of 50S ribosomal subunits by mutant and parent strains*

particles in the mutant and to 50S ribosomal subunits in the parent. The similarity in sedimentation coefficients and protein contents between p50S-1 and p47S-1 particles, and between p47S-2 and p50S-2 particles, suggest that in the mutant the earlier stages in assembly are as 'normal' as possible in the circumstances of the lesion. However, sedimentation of these pairs of precursors through the same sucrose gradients shows that similar particles are not identical (Butler *et al.*, 1979) and that the pathways must differ at an early stage. The reasons for this divergence require further study.

Gel electrophoresis permits the separation of precursor forms of 16S and 23S rRNA from their 'mature' counterparts. Under the conditions we have used, resolution of p16S RNA from m16S RNA is always more clear-cut than that between the 23S RNA species, and a change in conditions leads to failure to resolve the latter pair: this and similar problems have been noted by others (Dahlberg & Peacock, 1971). Precursor 16S RNA extracted from pulse-labelled material or 'chloramphenicol particles' has a chain some 70 nucleotides longer than mature 16S RNA (Hayes & Vasseur, 1976). This difference in length together with conformation differences are responsible for the separations obtained in non-denaturing gels. We (P. D. Butler, P. F. G. Sims & D. G. Wild, unpublished work) have separated p16S RNA from m16S RNA in gels containing formamide (Pinder *et al.*, 1974), but under these conditions are unable to resolve p23S RNA from m23S RNA. Indeed, there is little evidence that p23S RNA and m23S RNA are other than conformational isomers to which the undermethylation of p23S RNA may contribute (Dahlberg & Peacock, 1971).

These considerations are relevant to one feature of assembly in parent and mutant strains: particles (p50S-1 and p47S-1 in particular) with p23S RNA also contain more m23S RNA than can be accounted for by contamination with subunits (or 47S particles). This observation is also noticeable, for example, in Lindahl's (1975) work. Two explana-

tions are possible. If the transition from precursor to mature 23S RNA is largely conformational, it may well occur, and to a rather variable extent, during extractions. Alternatively RNA maturation may proceed somewhat independently of the rest of the assembly process. If this were so, it would be an interesting example of the limited flexibility that cells retain in ribosome synthesis. This latter possibility is the more attractive, but not necessarily for that reason correct. It was emphasized above that 47S particles are a unique ribosome precursor because, among other things, they contain m23S RNA. This distinction remains valid: the precursors to 50S ribosomal subunits detected in the parent strain and those to 47S particles in the mutant have 50% or more of their RNA as p23S RNA. This species has never been detected on the many occasions on which electrophoresis of RNA from 47S particles has been carried out.

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