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Evidence for a Precursor–Product Relationship in the Biosynthesis of Ribosomal Protein S20†

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ABSTRACT: The kinetics of labeling ribosomal protein S20 of *Escherichia coli* strains H882 and H882 *groE*$_{44}$ have been examined using partial reconstitution as a means of binding this and some other 30S subunit proteins selectively to 16S RNA from crude extracts prepared by acetic acid extraction of pulse-labeled whole cells. The rate of labeling of S20 during short pulses at 44 °C is less than 20% of that observed at 28 °C. S20 can be recovered from the cells labeled at the higher temperature if they are chased at 28 °C, but not at 44 °C, in the presence of excess sulfate prior to their extraction. These observations suggest that S20 is derived from a precursor whose processing is blocked at 44 °C. Among the proteins extracted from cells labeled at 44 °C capable of binding to 16S RNA is a novel polypeptide, p2, which is not normally present on the 30S subunit. The kinetics of its appearance at 44 °C, and its chasing at 28 °C, suggest a precursor–product relationship with S20. p2 contains a tryptic peptide with the chromatographic properties of the peptide Ser-Met-Met-Arg at position 25–28 in S20. A second methionine-containing peptide at positions 49–59 of S20 is missing from p2. In addition, the apparent molecular weight of p2 (8600) is less than that of S20 (9500). p2 may represent the product of degradation of a precursor to S20, yet retains the ability to bind to 16S RNA. It is much less likely that p2 is a bona fide precursor which is converted into S20 by fusion to some other polypeptide.

It is now well established that ribosomal proteins of the 30S subunit of *Escherichia coli* can self-assemble in the presence of 16S RNA to produce a physically and enzymatically complete 30S subunit in vitro (Nomura, 1973; Nomura and Held, 1974). It is not clear, however, that the same pathway is followed in vivo. In fact, several lines of reasoning argue against such a supposition. First, the conditions under which ribosomal assembly must occur in vivo are somewhat different than those which are optimal in vitro. In particular, ribosomal reconstitution requires a rather high concentration of KCl in the buffers used (Traub and Nomura, 1969), heating to 42 °C (Traub and Nomura, 1969), and mature 16S RNA rather than precursor RNA (Wireman and Sypherd, 1974). Secondly, there are both genetic (Bryant and Sypherd, 1974) and physiological (Mangiarotti et al., 1975) experiments which implicate the participation of nonribosomal morphogenetic factors in ribosomal biogenesis. Third, the slow kinetics of labeling of S18 in 30S subunits in vivo are best rationalized by the existence of a precursor to this protein (Dennis, 1974). Similar precursor–product relationships are quite common in other assembling systems. Proteolytic cleavages, for example, frequently intervene during the assembly of bacteriophages and viruses from their macromolecular precursors (Eiserling and Dickson, 1972; Hershko and Fry, 1975). The additional information present in the putative precursors to some ribosomal proteins might alleviate some of the kinetic or ionic barriers to ribosomal assembly enumerated above.

In the present work, I have examined a temperature-sensitive strain of *E. coli* K12 (Georgopoulos and Eisen, 1974) which is deficient in the processing of some viral precursor polypeptides during infection by T4 or λ bacteriophages in the expectation that this would facilitate the identification of putative precursors to ribosomal proteins. I have found that nonpermissive temperatures and sulfate deprivation block the appearance of ribosomal protein S20 reversibly. Simultaneously, a novel polypeptide not normally associated with the 30S subunit accumulates in extracts of both the mutant and its parent. This protein is capable of binding to 16S RNA under conditions of partial reconstitution, possesses a methionine-containing tryptic peptide chromatographically indistinguishable from one in S20, and behaves kinetically as if it were a precursor to S20, or directly derived from such a precursor.

Materials and Methods

Strains. Strain H882 *groE*$_{44}$ (F$^-$, thi$^-$, *proA*$_{35}$, thr$^-$, leu$^-$, arg$^-$, str^R, ton, tsx, *groE*$_{44}$), and its groE$^+$ parent, H882, are *E. coli* K12 strains and were obtained from C. P. Georgopoulos (Georgopoulos and Eisen, 1974; Georgopoulos et al., 1972). These strains were tested periodically for their growth requirements and for their ability to plate phage T4D. MRE 600 (Cammack and Wade, 1965) was used for the preparation of ribosomal components on a large scale.

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Buffers. Buffer A is 50 mM Tris-HCl,[1] 350 mM KCl, 20 mM $MgCl_2$, 6 mM β-mercaptoethanol, pH 7.7; buffer B is 50 mM sodium phosphate, 6 M deionized urea, 6 mM β-mercaptoethanol adjusted to pH 6.5 with methylamine.

Ribosomal Components. 70S ribosomes, 30S subunits, 16S RNA, and purified ribosomal proteins were prepared essentially as described previously (Muto et al., 1974).

Pulse Labeling. Cultures of H882 *groE*$_{44}$ or H882 were grown at 28 °C in M9 medium (Muto et al., 1974) containing 1 mM $MgCl_2$ and 10 μM Na_2SO_4 in place of $MgSO_4$, supplemented with 0.2% glucose, 25 μg/mL each of arginine, leucine, threonine, and proline, and 10 μg/mL of thiamine hydrochloride. At the onset of starvation for sulfate, the cultures were shifted to 44 °C if so desired. Twenty to thirty minutes later, the cells were pulse labeled with carrier-free $H_2{}^{35}SO_4$ (New England Nuclear Corp.), usually for 4 min. The pulse was terminated rapidly by harvesting 5-mL aliquots by filtration on 0.45-μm nitrocellulose filters (Millipore Corp.), or by adding Na_2SO_4 to a final concentration of 5 mM in the chase experiments. Subsequent samples of 5 mL were also harvested by filtration. The filters were vortexed with 0.6 mL of 20 mM Tris-HCl, 60 mM NH_4Cl, 100 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, pH 7.7, in a 15-mL Corex tube, and the resultant suspension was extracted with 2 volumes of glacial acetic acid with constant stirring on ice. Carrier 70S ribosomes (250 μg) were added at this stage. After 1 h, the filter was removed with a forceps and the precipitated RNA and cellular debris were removed by centrifugation (10 min, 10 000*g*). The clear acid extract was dialyzed against two changes of buffer B, one change of 50 mM Tris-HCl, 1 M KCl, 1 M deionized urea, 6 mM β-mercaptoethanol, pH 8.0, and, finally, two changes of buffer A.

Partial Reconstitution. Five-hundred micrograms of 16S RNA was heated 20 min at 37 °C in 0.5 mL of buffer A and then chilled on ice. To this was added the dialyzed acetic acid extract from a 5-mL culture prepared as above, typically 5–7 mL. After being held on ice for 15 min, the mixture was layered on two or three 3–15% sucrose gradients in buffer A and centrifuged 14.5–15.5 h at 22 500 rpm in the Beckman SW27 rotor at 4 °C. The gradients were pumped through a flow cell in a Gilford, Model 2400, recording spectrophotometer and the absorbance at 260 nm was monitored. The portion of the gradients containing the 16S RNA was pooled, and the ribonucleoprotein was concentrated by precipitation with 5% trichloracetic acid and then extracted with 66% acetic acid, 33 mM $MgCl_2$ in the presence of 10–15 mg of carrier 70S ribosomes or 30S subunits (Zimmermann et al., 1974). The extracted proteins were dialyzed against buffer B in preparation for column chromatography.

Column Chromatography. Chromatography of ribosomal proteins on columns of phosphocellulose was performed with slight modifications of techniques described previously (Muto et al., 1974, Zimmermann et al., 1974), using Schwarz/Mann regular high-capacity phosphocellulose in columns 0.9 × 30 cm. A flow rate of 6 mL/h was maintained by pumping and fractions of 1.05 mL were collected.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels (7.5%) containing 8 M urea at pH 4.5 were run as described (Leboy et al., 1964; Muto et al., 1974). Gels containing 0.1% sodium dodecyl sulfate were prepared and run as outlined by Laemmli and Favre (1973).

[1] Abbreviation used: Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

Tryptic Fingerprinting. Attempts to concentrate dilute pools of p2 or S20 by dialysis against 0.1 M NH_4HCO_3 followed by lyophilization consistently resulted in large losses of radioactivity. An alternative procedure which gave 75–90% recoveries involved precipitating the proteins directly with 10% trichloroacetic acid in the presence of 100 μg of bovine serum albumin. The precipitated material was recovered by centrifugation and the pellet was washed with 5% trichloroacetic acid, dissolved in 0.5 mL of 1% NH_4HCO_3, and lyophilized. The proteins so recovered were oxidized with performic acid (Weber et al., 1972). The oxidized proteins were dissolved in 70 μL of 1% NH_4HCO_3 and digested with TPCK-Trypsin (Worthington) at a ratio of 1:60 (w/w) for 16 h at 37 °C. In the fingerprints illustrated in Figure 5a, the samples were applied in bands 2-cm wide, 5 cm on either side of the longitudinal center line of a sheet of Whatman 3MM paper. The paper was subjected to electrophoresis at pH 6.4 or 3.5 for 1 h at 2250 V in a varsol-cooled tank. The paper was dried and cut along the longitudinal center line, and each half subjected to ascending chromatography in 20% acetic acid–1-butanol–pyridine (10:10:17) (Smith, 1965). After drying, the paper was subjected to autoradiography using Dupont Cronex 4 x-ray film. The method used ensures that the mobilities of the peptides in the first dimension can be compared readily.

Preparative-scale fingerprints using 500 μg of unlabeled S20 and 80 000 cpm of [^{35}S]S20 were handled similarly. Sulfur-containing peptides were cut out using an exposed film as a guide. Ninhydrin staining of the paper showed that these three peptides did not overlap other nonradioactive peptides. Each peptide was eluted from the paper with 6 N HCl, hydrolyzed 20 h at 110 °C, taken to dryness, dissolved in pH 2.2 buffer, and applied to the long column of a Beckman, Model 120C, amino acid analyzer, following the manufacturer's instructions. A run was made with standards to facilitate the identification of unknowns. While no information is obtained this way about the C-terminal residue, the composition of the other amino acids in the peptide should be sufficient to permit its identification.

Results

Properties of Strain H882 groE$_{44}$. Strain H882 *groE*$_{44}$ was originally detected by its inability to support the growth of bacteriophages T4 or λ (Georgopoulos et al., 1972, 1973; Sternberg, 1973a,b), ostensibly through the absence of proteolytic cleavages of the products of gene 23 in T4 and gene E in λ (Georgopoulos et al., 1972, 1973). In addition, this strain is temperature sensitive for normal growth (Georgopoulos and Eisen, 1974). In collaboration with C. P. Georgopoulos and K. Reichardt, I found that the labeling of complete ribosomal subunits in this strain is severely blocked at 44 °C, resulting in the accumulation of 22S and 32S particles (our unpublished results). In contrast, labeled amino acids are incorporated into the ribosomal subunits of the parent strain equally well at 28 or 44 °C, with little accumulation of precursor-like particles. These experiments suggested the possibility of proteolytic cleavages of ribosomal protein precursors.

Preliminary attempts to characterize the proteins of the accumulated 22S particles in H882 *groE*$_{44}$ were complicated by contamination of the particles with nonribosomal proteins. Moreover, it seemed quite possible that "processing" or degradation of any precursors could occur during the time necessary for the purification of the 22S particles. Thus, I have employed direct extraction of whole cells with 66% acetic acid (Hardy et al., 1969; Dennis and Nomura, 1975; Hardy, 1975),

which is rapid and which should extract all ribosomal proteins whether bound to RNA or not.

Recovery of Ribosomal Proteins from Extracts by Reconstitution. Reconstitution was first employed as a means of purifying ribosomal proteins by Kaltschmidt et al. (1974) in their studies of the synthesis in vitro of ribosomal proteins. These workers performed several steps of purification to eliminate acidic proteins prior to the actual reconstitution with 16S RNA. I avoided these preliminary manipulations and mixed a dialyzed whole cell extract with 16S RNA under conditions conducive to partial or total reconstitution of 30S subunits. Ribonucleoprotein particles, products of the incubation, were isolated from unbound proteins by sucrose-gradient centrifugation. Figure 1 illustrates such an analysis, using as a source of protein a culture which had been labeled with [^{3}H]amino acids for several generations and $^{35}SO_4^{2-}$ for 5 min to distinguish between stable and newly synthesized proteins. In Figure 1a, the components were kept on ice prior to centrifugation, and the 16S RNA added is apparently recovered intact. About 5% of the [^{3}H]protein and 1% of the [^{35}S]protein cosediment with the 16S RNA. Comparable recoveries have been obtained repeatedly. When the added protein is present in excess, the resultant particle sediments at 21 S, the same as intermediates of reconstitution (Nomura and Held, 1974). In the experiments reported here, however, 16S RNA was added in excess in an effort to bind all the available ribosomal protein. In the gradient illustrated in Figure 1b, the recovery of 16S RNA is poorer than at 4 °C, and much of the RNA sediments at less than 16 S, presumably as a consequence of nuclease action during the incubation at 40 °C. Some radioactive proteins still cosediment with the RNA fragments, but the overall yield is reduced. For this reason, heating was avoided in subsequent experiments.

Identification of the Proteins Bound to 16S RNA. The proteins which cosedimented with the 16S RNA after partial reconstitution were extracted from the RNA with acetic acid and resolved preparatively by chromatography on columns of phosphocellulose. A typical separation is illustrated in Figure 2a. In this case, strain H882 *groE*$_{44}$, labeled for three generations at 28 °C with a mixture of [^{3}H]amino acids and for 4 min at 44 °C with $H_2{}^{35}SO_4$, was the source of protein. Qualitatively similar results were obtained when strain H882 was treated similarly.

Stable [^{3}H]proteins S4, S16/17, and S20 can be identified unambiguously from their position on the chromatogram (cf. Figure 1 of Muto et al., 1974). Confirmation was obtained by electrophoretic analysis. The radioactive proteins in the more complex peaks have been identified in two ways. In the first, aliquots of the mixed pools have been subjected to electrophoresis in polyacrylamide slab gels containing urea or sodium dodecyl sulfate. The radioactive components have been visualized by autoradiography and their mobilities were compared to those of stained standards. In the second, an unfractionated mixture of labeled proteins bound to the 16S RNA after partial reconstitution was analyzed by electrophoresis in polyacrylamide gels containing urea at pH 4.5. As a consequence, I have identified both S6 and S8 in the peak labeled S6/8 in Figure 2a, S9 and S18 in peak S9/11/18, and S15 in peak S14/15. Proteins S7, S13, and S19 may also be present in low yields in the mixture of bound proteins in agreement with previous experiments (Zimmermann et al., 1974), as indicated by the presence of small amounts of radioactivity in the appropriate regions of many chromatograms (cf. Figure 2b), and from faint bands in polyacrylamide gels (not shown). The proteins enumerated above comprise over 95% of the radioactivity stably

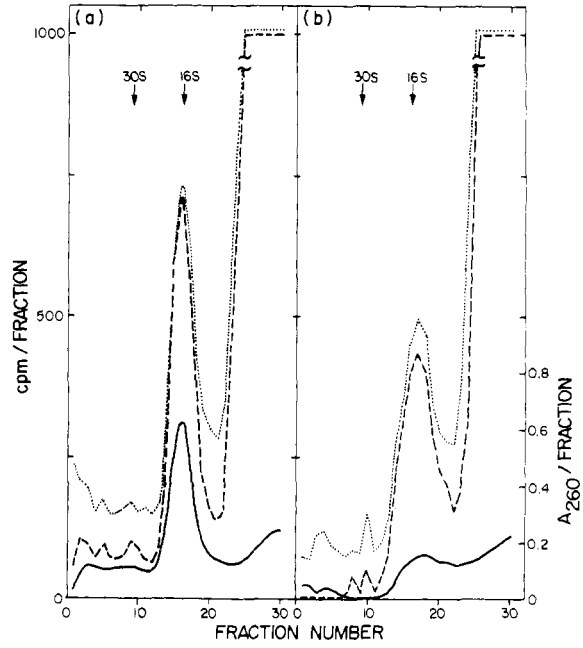

FIGURE 1: Effect of temperature on the binding of acid-extractable proteins to 16S RNA. Strain H882 was grown in 5 mL of M9 medium supplemented with 0.05% casamino acids (Difco), 0.2% glucose, 10 μg/mL thiamine hydrochloride, and 50 μCi of [^{3}H]amino acid mixture (New England Nuclear) at 28 °C. After 5 h (four generations), the medium was supplemented further with casamino acids to 0.25%. Thirty minutes later, the cells were harvested by filtration and the filter was added to the extraction mixture described below. Meanwhile, a parallel culture of H882 was grown to 3 × 10^8 cells/mL in the low-sulfate medium described under Materials and Methods, shifted to 44 °C for 20 min, then pulse labeled with 400 μCi of $H_2{}^{35}SO_4$ for 5 min, harvested by filtration, and then extracted with acetic acid (see Materials and Methods for details). The dialyzed extract contained 7.5 × 10^6 ^{35}S cpm and 5.3 × 10^5 ^{3}H cpm in 7.5 mL of buffer A. Duplicate samples of 25 μg of 16S RNA in 50 μL of buffer A were heated for 20 min at 37 °C and then chilled on ice. 0.35 mL of the extract prepared as described above was mixed with each sample of RNA. One (a) was held on ice, and the other (b) was incubated for 20 min at 40 °C and then chilled. Both were layered on 3–15% sucrose gradients in buffer A and centrifuged for 16 h at 23 500 rpm in the Beckman SW40 rotor. 16S RNA and 30S subunit markers were applied to a third gradient run in parallel. Fractions were collected from the bottom of the gradients and the A_{260} was monitored. Each fraction was mixed with 3 mL of Aquasol (New England Nuclear) and counted. The background and spillover of ^{35}S into ^{3}H have been subtracted. (—) A_{260}; (···) ^{35}S cpm; (- - -) ^{3}H cpm.

bound to the 16S RNA under the conditions used. Identical results have been obtained with either [^{3}H]amino acids or $H_2{}^{35}SO_4$ as long term labels. The composition of the ribonucleoprotein particles obtained here is similar to the composition of the 21S intermediates of reconstitution studied by Nomura and his co-workers (Nomura, 1973; Nomura and Held, 1974).

Among the proteins labeled during a pulse at 44 °C in the experiment illustrated in Figure 2a, S4, S15, and S16/17 are recovered in yields consistent with their sulfur content and with the recoveries expected by comparison with the long-term labeling. No firm conclusions can be drawn regarding S7, S9, S13, and S19, as they are present in low amounts and/or insufficiently resolved from other components of the mixture. In contrast to the first group of proteins, the recoveries of S6, S8, S18, and S20 are very low. This is especially surprising in the cases of S6 and S8, which are rich in sulfur. Two additional pulse-labeled polypeptides, denoted by p1 and p2 in Figure 2a, do not correspond to any of the stable ribosomal proteins. The former seems to be related to S9 or S18 on the basis of its fingerprint and will be discussed elsewhere. The characterization

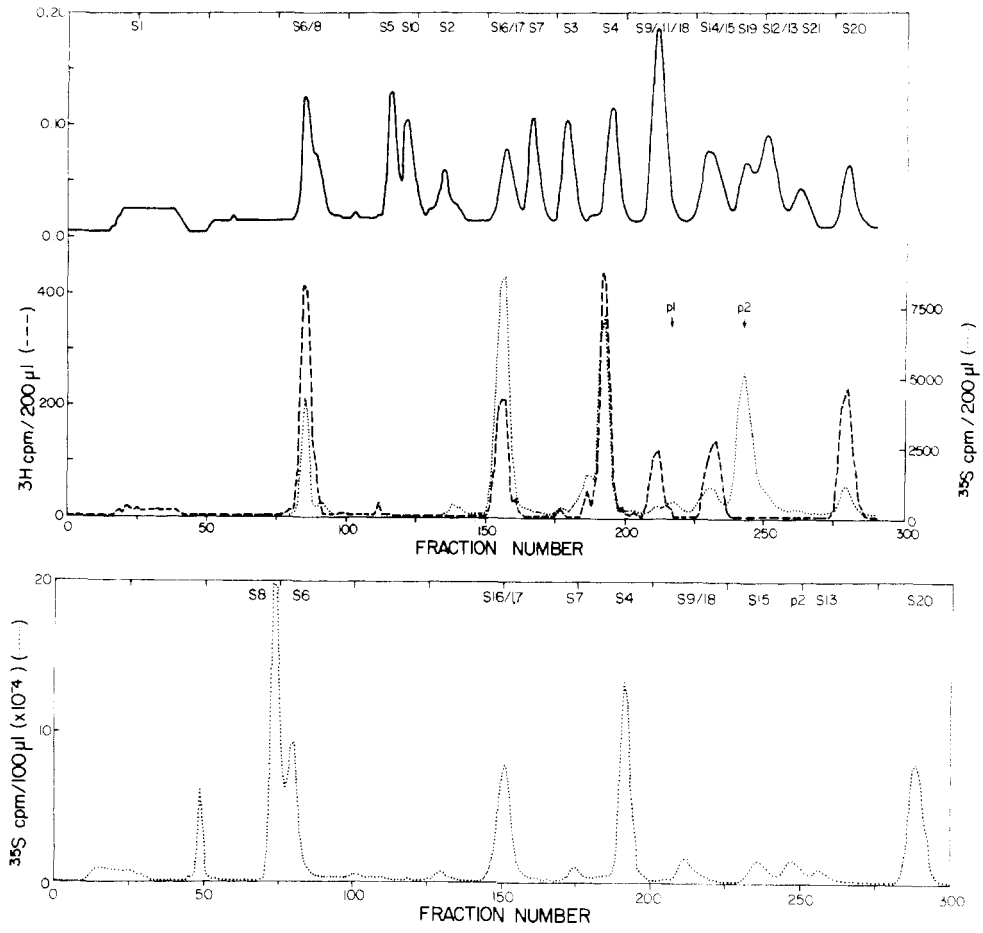

FIGURE 2: Chromatography on phosphocellulose of proteins bound to 16S RNA. (a, top) Five-milliliter cultures of strain H882 *groE*$_{44}$ were labeled for several generations with 100 μCi of a [^{3}H]amino acid mixture, as in the legend to Figure 1, or pulse labeled for 4.0 min at 44 °C with 1.6 mCi of $H_2{}^{35}SO_4$ as under Materials and Methods. Each culture was harvested by filtration and the filters were extracted simultaneously. Processing of the acid extracts, partial reconstitution, sucrose-gradient centrifugation, and recovery of those proteins bound to 16S RNA are outlined under Materials and Methods. The extracted proteins (1.3×10^6 ^{35}S cpm and 6.1×10^4 ^{3}H cpm), including 5 mg of unlabeled 30S subunit proteins added as carrier, were applied to a column of phosphocellulose and eluted with a gradient from 0 to 0.5 M NaCl in buffer B. Aliquots of 200 μL were mixed with 3 mL of Riafluor (New England Nuclear Corp.) and counted. An additional sample of 100 μL from each fraction was used for the determination of protein (Lowry et al., 1951). The curve representing the A_{750} has been displaced upwards in Figure 2a for clarity. (—) A_{750}/100 μL (Lowry protein); (- - -) ^{3}H cpm/200 μL; (···) ^{35}S cpm/200 μL. (b, bottom) In this experiment, the labeling with the [^{3}H]amino acid mixture was omitted and the cells were pulse labeled with 1.0 mCi of $H_2{}^{35}SO_4$ for 4.0 min at 28 °C. Subsequently, the cells were harvested and processed as in Figure 2a. (···) ^{35}S cpm/100 μL.

of p2 is described in detail in the following experiments.

Both strains H882 *groE*$_{44}$ and H882 have been pulse-labeled at 28 °C, rather than at 44 °C. The chromatographic profile of the pulse-labeled proteins in this situation is identical to that of the stable proteins, with slight variations due to the differences in the relative sulfur content of the proteins, as shown in Figure 2b. The formation of the novel polypeptides p1 and p2 is probably a consequence of heat shock and sulfate starvation, rather than of the presence of a mutation in the *groE* gene (see Discussion).

Kinetics of Labeling of p2 and S20. The relative rates of labeling of p2 and S20 were examined in strain H882 by varying the length of the pulse of $H_2{}^{35}SO_4$. H882 was chosen for this experiment because it exhibits a greater rate of incorporation of radioactivity. Figure 3 summarizes the recoveries of p2 and S20 after pulses of increasing length. At 28 °C, only small quantities of p2 could be detected, even after a 90-s pulse, at most 0.025% of the radioactivity added to the partial reconstitution assay. This is less than one-tenth the recovery of S20 in the same experiment and is a maximum estimate, since the presence of any S19 may inflate this figure. In contrast, p2 comprises up to 0.40% of the extracted radioactivity in cells labeled at 44 °C. There is roughly sixfold more radioactivity in p2 than S20 after 90 s, and threefold more after 240 s. If these recoveries reflect the rates of labeling of p2 and S20, then p2 is labeled much more rapidly than S20 at 44 °C, but barely detectably at 28 °C.

Disappearance of p2. The fate of p2 was examined in pulse-chase experiments in which the pulse-labeling of strain H882 *groE*$_{44}$ was performed at 44 °C, and the chase in the presence of excess unlabeled Na_2SO_4 at either 28 or 44 °C. Samples were taken at various times after the addition of Na_2SO_4, harvested by filtration, and processed as described under Materials and Methods. Figure 4 indicates the recoveries of p2, S20, and, for comparison, S4, at each time. At 44 °C the proportions of p2 and S20 remain relatively uniform, with a slight decrease in that of p2 and a corresponding increase in that of S20 after 10 min. The sum of the recoveries of the two proteins remains essentially constant during the chasing period. At 28 °C, the proportion of S20 increases with time throughout the chase, approaching 0.18% of the radioactivity in the acid extract, which is that found for cells labeled at 28 °C. The proportion of the radioactivity recovered as p2 decreases concomitantly. The recovery of p2, particularly at later times

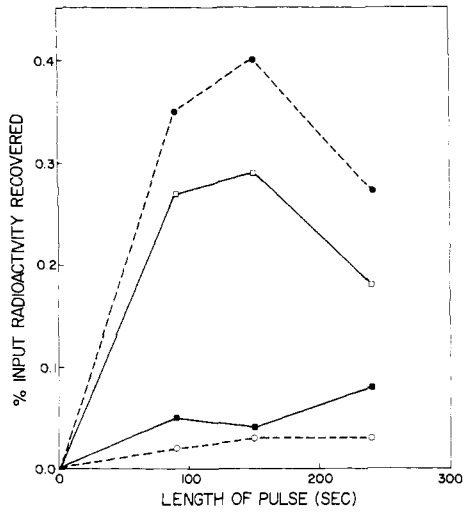

FIGURE 3: Kinetics of labeling of p2 and S20. A culture of H882 was grown in the modified low sulfate M9 medium (Materials and Methods) at 28 °C. At the onset of sulfate exhaustion, 15 mL was shifted to 44 °C, while 15 mL remained at 28 °C. Twenty-five minutes later each sample was pulse labeled with 200 μCi/mL $H_2{}^{35}SO_4$. Aliquots of 5 mL were withdrawn after 90, 150, and 240 s, harvested by filtration, and processed as described under Materials and Methods and in the legend to Figure 2a. Proteins capable of binding to 16S RNA were recovered by partial reconstitution and resolved by chromatography on columns of phosphocellulose (cf. Figure 2). The radioactivity in p2 or S20 is expressed as the percentage of the total input in the reconstitution assay, normalized to a recovery of S4 of 0.19%, the average in the six samples and typical of many determinations. (○) p2 (28 °C); (●) p2 (44 °C); (□), S20 (28 °C); (■), S20 (44 °C).

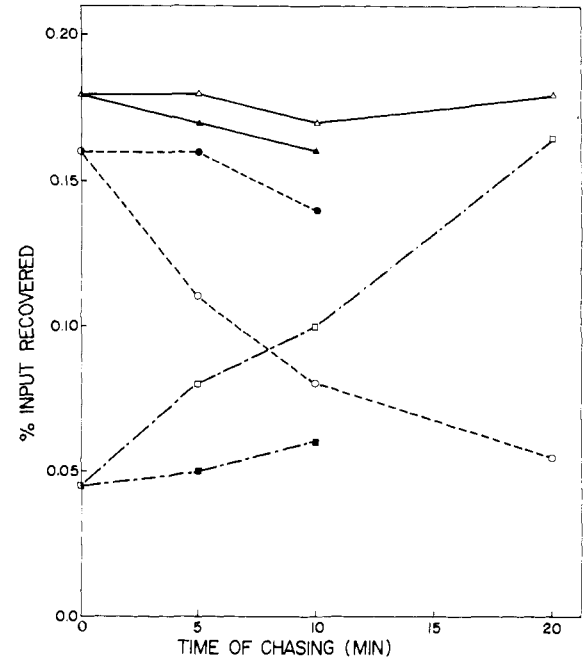

FIGURE 4: Kinetics of chasing of p2, S4, and S20. A culture of H882 *groE*$_{44}$ was grown to sulfate exhaustion at 28 °C, shifted to 44 °C, and pulse labeled 25 min later for 4.0 min with 200 μCi/mL $H_2{}^{35}SO_4$. Portions of the culture were chased at 44 °C as described under Materials and Methods, or at 28 °C by transferring labeled cells to a flask at 28 °C containing sufficient Na_2SO_4 to produce a final concentration of 5 mM. Samples were taken at 0, 5, 10, and 20 min, as indicated under Materials and Methods. The acid-extractable proteins which bound to 16S RNA were separated by column chromatography, as in Figure 2. The radioactivity recovered in a particular protein peak is expressed as a percentage of the total input in the reconstitution assay. The temperature in parentheses below refers to the temperature at which the chase was performed. (△) S4 (28 °C); (▲) S4 (44 °C); (○) p2 (28 °C); (●) p2 (44 °C); (□) S20 (28 °C); (■) S20 (44 °C).

in the chase, may be overstated due to contamination of p2 with S19. Fingerprinting and gel electrophoresis (see Figures 5 and 7) show that p2 is at least 80% pure after a 4-min pulse. If the binding of S19 to 16S RNA is equimolar with that of S7 (see Zimmermann, 1974), and if it contains a single sulfur atom (Wittmann-Liebold, 1973), then after a chase of 20 min the p2 peak could contain as much as 40% S19. No correction has been made in the data plotted in Figure 4 to account for this. In contrast to the behavior of p2 and S20, the recovery of S4 was virtually constant with time in chases at both 28 and 44 °C.

Essentially the same experiment was repeated in strain H882. The rate of chasing of p2 at 28 °C was indistinguishable from that found in the mutant. As an additional check of the stability of S20 during the chase, a culture of strain H882 was pulse labeled at 28 °C for 4 min and then chased for 10 min at 44 °C. The recovery of S20, processed as above, was 0.20%, practically the same as from cells pulse labeled at 28 °C and extracted without chasing. The behavior of p2 and S20 in this and the preceding experiment implies that S20 is derived from a precursor whose processing is retarded at the higher temperature.

Kinetics of Labeling of S20 in 30S Subunits. Independent confirmation of some of the preceding results was sought by examining the proportion of S20 in newly synthesized 30S subunits by column chromatography of the constituent proteins. Cultures of strain H882 *groE*$_{44}$ were pulse labeled at 43 °C and then chased at 28 °C as described above. Samples were collected by filtration and the 30S subunits were isolated from them by zonal sedimentation of sonic extracts. After a pulse of 3 min, virtually no S20 could be detected in the total 30S protein. However, the proportion of S20, relative to S4, increased gradually to 60–80% of its level in continuously labeled cells after 15 min of chasing of the pulse labeled culture at 28 °C. This lag in the appearance of S20 on 30S subunits is consistent with the apparent precursor–product relationship described above. The complicated chromatographic profile in the region where p2 is eluted (cf. Figure 2a, upper panel), exacerbated in these experiments by the presence of contaminating 32S particles in the 30S subunits, precluded its identification among the proteins on the newly synthesized 30S subunits.

The Structural Relationship between p2 and S20. Tryptic fingerprints of performic acid oxidized ^{35}S-labeled p2 and S20 are illustrated in Figure 5a. S20 reproducibly gives rise to three labeled peptides, TP-1, TP-2, and TP-3, although the proportions of TP-1 and TP-2 vary from digest to digest. p2 always contains a peptide TX-1, which runs identically with TP-2 in both dimensions, and, occasionally, a faint spot which comigrates with TP-1. A light spot in the digest of p2 which runs ahead of TP-3 in the first dimension corresponds to a mixture of the major sulfur-containing peptide of S19 and a minor product also found in S20. Admixtures of digests of p2 and S20 separated in a pattern identical to that of S20 alone in both the pH 3.5 and 6.4 systems, as illustrated in Figure 5. Both TP-2 and TX-1 are resistant to digestion with chymotrypsin. Interestingly, when each of these peptides is eluted and electrophoresed again at pH 6.4, it partitions into two components with mobilities identical to TP-1 and TP-2 (see below). This further strengthens the apparent identity of TP-2 and TX-1.

The composition of the three radioactive peptides in S20 was determined in order to place them in the primary sequence of S20. Peptides TP-1 and TP-2 gave similar amino acid analyses,

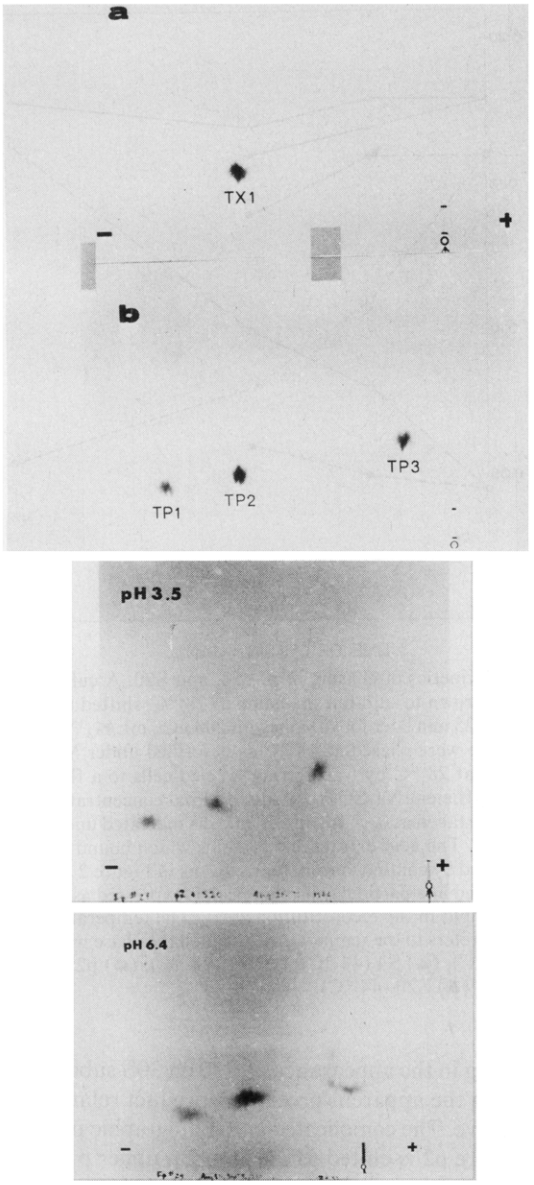

FIGURE 5: Tryptic fingerprints of p2 and S20. (a, b). Tryptic digests of p2 (a) or S20 (b) were separated by electrophoresis at pH 6.4 from left to right with the indicated polarity on the same sheet of paper, and subsequently by chromatography from bottom to top as described under Materials and Methods. About 12 000 cpm of each sample was applied to the paper. The resultant spots were visualized by autoradiography. (pH 3.5 and 6.4) Admixtures of tryptic digests of p2 and S20 were applied to the same paper and separated by electrophoresis at pH 3.5 or pH 6.4. The papers were then subjected to chromatography as described above. In c, 8400 cpm of p2 and 8100 cpm of S20 were mixed; for d, 9500 cpm of p2 and 12 000 cpm of S20 were used.

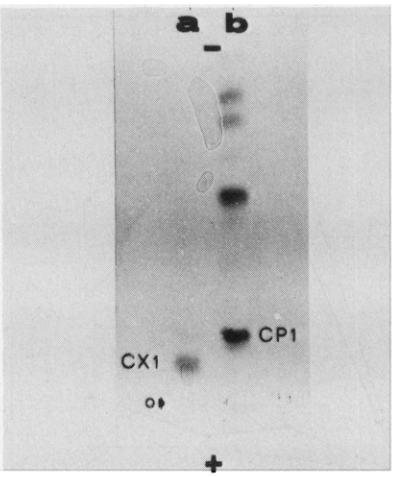

FIGURE 6: Chymotryptic fingerprints of p2 and S20. Samples (about 10 000 cpm each) of p2 (a) or S20 (b) were digested with chymotrypsin at an enzyme to substrate ratio of 1:50, essentially in the same manner employed for tryptic digestion. The digests were separated by electrophoresis for 2 h at 1500 V on Whatman 3 MM paper at pH 6.4, and the products were visualized by autoradiography.

containing serine and methionine in the ratio 1:2. Why this peptide should migrate heterogeneously is not clear; it is probably a consequence of incomplete oxidation of the vicinal methionines. Its amino acid composition is consistent with the sequence of the tryptic peptide Ser-Met-Met-Arg, which occurs at positions 25–28 of S20 (Wittmann-Liebold et al., 1976). Peptide TP-3 has the composition Ala, Asx_2, Glx_2, Ile, Met, Phe, Pro, and presumably Arg or Lys, corresponding to the peptide T3 of Wittmann-Liebold (1971) which occurs at positions 49–59 in the amino acid sequence of S20 (Wittman-Liebold, et al., 1976). The clear absence of TP-3 from the tryptic fingerprint of p2 indicates that, if p2 is homologous to S20, this homology encompasses sequences in the N-terminal portion of S20 not extending beyond the methionine at position 52 (Wittmann-Liebold et al., 1976).

Since the first aromatic residue of S20 is a phenylalanine at position 30 (Wittmann-Liebold et al., 1976), I have attempted to characterize the chymotryptic peptides of p2 and S20 as a means of identifying the N terminus of p2. As shown in Figure 6, S20 yields four or more peptides containing methionine, presumably as a result of oversplitting. It has not yet been possible to determine the sequences of the peptides CP-1 and CX-1. Nonetheless, it is noteworthy that CX-1 obtained from p2 is more acidic than CP-1 or any other peptide from S20. This suggests, but does not prove, a modification in the N-terminal sequence of p2 relative to S20.

Electrophoretic Mobilities of p2 and S20. Samples of pooled fractions containing p2 or S20 were concentrated by precipitation with trichloroacetic acid (see Materials and Methods) and then subjected to electrophoresis in 10–22.5% polyacrylamide gradient slab gels containing 0.1% sodium dodecyl sulfate. Figure 7 illustrates that p2 runs slightly ahead of S20, and is just resolved from it in mixtures. For comparison, the whole cell extract from which p2 was purified and a mixture of S4 and S16/17 from the same purification were run in the same gel in adjacent slots. In other gels, including those containing 15% acrylamide throughout, p2 has reproducibly migrated ahead of S20 and S16/17. Molecular weights calculated from electrophoretic mobilities in a number of experiments using purified ribosomal proteins, myoglobin, lysozyme, and cytochrome *c* as markers are 12 700 for S20 and 11 500 for p2. However, the molecular weight of S20 calculated from its sequence is 9554 (Wittmann-Liebold et al., 1976); accordingly, that of p2 ought to be approximately 8600.

Discussion

The method exploited in this work for the isolation of precursors to ribosomal proteins relies on the high affinity of many ribosomal proteins for ribosomal RNA (Zimmermann, 1974), and affords both great specificity and convenience. Implicit in this approach is the assumption that any such precursors bind to 16S RNA in vitro as well as their ultimate products. It is entirely conceivable that a precursor to a ribosomal protein might need to be processed in order to bind to ribosomal RNA; in such a case, the technique used here would fail. The other

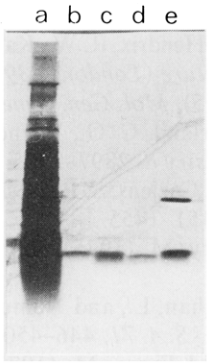

FIGURE 7: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of p2 and S20. Samples of (a) a total acid extract of pulse labeled strain H882 *groE*$_{44}$; (b) pooled fractions of S20; (c) equal amounts of p2 and S20; (d) pooled fractions of p2; and (e) a mixture of S4 and S16/17 were applied to a 10–22.5% gradient slab gel and subjected to electrophoresis, as described under Materials and Methods. The ^{35}S-labeled proteins were identified by autoradiography of the dried gel. Migration is from top to bottom.

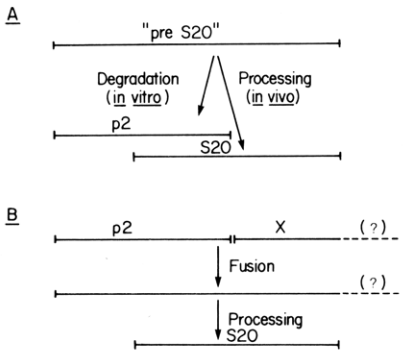

FIGURE 8: Alternative models to account for the relationship between p2 and S20. For details see text.

limitation to the method in its present form is the necessity of performing the reconstitution at 4 °C, allowing the fractionation of only a subset of the 30S subunit proteins.

The validity of the present method as a semiquantitative assay has been tested in two ways. In the first, the recovery of labeled S4 bound to 16S RNA typically represents from 0.17 to 0.19% of the radioactive input, even in independent preparations. The recoveries of S8 and S20 from extracts of either strain used labeled at 28 °C have been equally reproducible. Moreover, these recoveries are not significantly altered if the cells are subsequently chased at 44 °C prior to their extraction. Thus, there is apparently little degradation in vivo at the higher temperature. In a second test, I have measured the binding of labeled S4 or S20 to 16S RNA in the presence of an unlabeled, dialyzed, acetic acid extract of H882 *groE*$_{44}$. There was no interference with the binding of S4 or S20 by other proteins in the extract, aside from a small amount of isotope dilution due to unlabeled S4 or S20 in the extract.

After cultures have been pulse labeled at 44 °C, the recoveries of S6/8, S9/18, and S20 are much lower than at 28 °C. These proteins are all recoverable after a chase at 28 °C. Thus, at 44 °C these proteins are apparently synthesized in forms (i.e., as "precursors") that are either unextractable or are incapable of binding to 16S RNA in the reconstitution assay. The kinetic data presented here firmly support the notion that S20 is derived from a precursor molecule.

Besides 12 mature ribosomal proteins in varying yields, at least two hitherto uncharacterized polypeptides labeled at 44 °C can bind to 16S RNA under the conditions used. One of these, p1, has yet to be characterized completely. The other, p2, seems to contain a sequence of up to 50 amino acid residues identical to that of the N-terminal portion of S20, based on the apparent identity of peptides TX-1 and TP-2. p2 may also contain additional sequences at its N-terminal extremities. While the chymotryptic fingerprint of p2 is consistent with this suggestion, it has not yet been possible to purify sufficient p2 to determine its N-terminal amino acid sequence directly.

Two models which can account for the results presented in this paper are summarized diagrammatically in Figure 8. In model A, the product of the structural gene for S20, *rpsT*, is a polypeptide, "pre-S20", larger than S20. In vitro, it appears to be degraded to p2 prior to partial reconstitution by enzymes extracted with it by the acetic acid treatment. In contrast, in vivo, "pre-S20" is converted to S20 by a highly specific protease. The chasing experiments clearly eliminate the possibility of degradation occurring before the extraction of the cells. Attempts to isolate the postulated "pre-S20" are underway.

Alternatively, as shown in model B of Figure 8, p2 could be a real precursor to S20. The most attractive mechanism for the conversion of p2 into S20 would be protein–protein fusion, which has already been invoked to explain the origin of the minor capsid proteins X1 and X2 of bacteriophage λ (Hendrix and Casjens, 1974). Two arguments can be advanced against this model. First, the series of reactions involved in the postulated conversion of p2 into S20 is highly complex, compared to the one-step maturation scheme of model A. It would seem more logical for fusion to occur at the level of the genes for p2 and "X", rather than at the level of the gene products. Secondly, there is strong genetic evidence (Böck et al., 1974; Wittmann and Wittmann-Liebold, 1974; Friesen et al., 1976) for a single structural gene for S20.

Other models to account for the formation of p2 include translational blockage of the messenger for S20 or "pre-S20" due to starvation of the cultures and consequent deprivation of a limiting aminoacyl-tRNA under the conditions used for labeling. Codons for cysteine and methionine are the most obvious candidates for the sites of such an effect, yet translation proceeds past the peptide Ser-Met-Met-Arg of S20 and p2. Moreover, there is no apparent effect of the growth conditions on the recoveries of S4 and of S16/17 which contain both cysteine and methionine.

The effect of temperature on the relative yields of p2 and S20 is quite striking. Twenty minutes exposure of the cells to 44 °C almost completely arrests the appearance of S20 in a reconstitutable form. This could be a consequence of the inactivation of processing enzymes, of the thermal alteration of the substrate(s), or of the activation of a protease which degrades the putative "pre-S20" molecule. Whatever the case, the result is entirely independent of the state of the *groE* allele in the strains used. It is conceivable that the role of the *groE* gene product is manifested in a later step of ribosomal assembly than was investigated here.

The fact that the cultures used in these experiments are effectively starved for cysteine and methionine prior to their labeling suggests that a stringent response may contribute to the accumulation of p2 at 44 °C, inasmuch as the genes for some ribosomal proteins are known to be regulated, in part, by the intracellular concentration of "magic spot" nucleotides (Dennis and Nomura, 1974, 1975). I have never succeeded in obtaining p2 in high yields from cultures from which the sulfate had not been completely exhausted, suggesting a role for guanosine polyphosphates in the regulation of the biosynthesis of p2 and S20. In addition, there is a strong interplay with the effect of temperature, since cells labeled at 28 °C are starved

to the same extent, yet show minimal accumulation of p2, even after a 90-s pulse. It may be worthwhile to investigate the accumulation of p2 in isogenic *rel*$^+$ and *rel*$^-$ strains to clarify these effects.

The significance of the processing of S20 to the overall pathway of ribosomal assembly and to ribosomal function is not obvious. Nonetheless, the existence of a precursor to S20 adds a new complication to current thinking about ribosomal biogenesis. Previously, the only modifications to ribosomal proteins thought to occur after their biosynthesis were the removal of the terminal *N*-formylmethionyl residue and perhaps some adjacent residues, and the addition of acetyl or methyl groups, principally, to proteins of the 50S subunit (Schlessinger, 1974; Chang and Chang, 1975). Work in progress supports the existence of precursors to S5, S6, S8, and S18 (my unpublished results). Dennis (1974) has also obtained kinetic evidence for the processing of the latter protein. His approach differs substantially from mine in the protocol for labeling, and in the method of detection of ribosomal proteins. This probably explains his failure to detect processing of S20. The occurrence of precursor polypeptides, and by necessity of the enzymes involved in their processing, demonstrates that the assembly of ribosomes in vivo is likely considerably more complex than reconstitution in vitro where the mature components of the 30S subunit self-assemble quite efficiently.

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