Bevan, M. J., Parkhouse, R. M. E., Williamson, A. R., and Askonas, B. A. (1972), *Prog. Biophys. Mol. Biol. 25,* 131.
Buxbaum, J. N., and Scharff, M. D. (1973), *J. Exp. Med. 138,* 278.
Choi, Y. S., Knopf, P. M., and Lennox, E. S. (1971), *Biochemistry 10,* 659.
Cioli, D., and Lennox, E. L. (1973), *Biochemistry 12,* 3204.
Horwitz, M., and Scharff, M. D. (1969), in Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 253.
Hsu, A., Baynes, J. W., and Heath, E. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2391.
Kiely, M. L., McKnight, G. S., and Schimke, R. T. (1976), *J. Biol. Chem. 251,* 5490.
Knopf, P. M., Sasso, E., Destree, A., and Melchers, F. (1975), *Biochemistry 14,* 4136.
Kuehl, W. M. (1977), *Curr. Top. Microbiol. Immunol.* (in press).
Kuehl, W. M., and Scharff, M. D. (1974), *J. Mol. Biol. 89,* 409.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Laskov, R., and Scharff, M. D. (1970), *J. Exp. Med. 131,* 515.
Maizel, J. V., Jr. (1971), *Methods Virol. 5,* 179.
Melchers, F. (1973), *Biochemistry 12,* 1471.
Melchers, F., and Andersson, J. (1974), *Adv. Cytopharmacol. 2,* 225.
Melchers, F., and Knopf, P. M. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32,* 255.
Milstein, C., Adetugbo, K., Brownlee, G. G., Cowan, N. J., Proudfoot, N. J., Rabbitts, T. H., and Secher, D. S. (1975), *Mol. Approaches Immunol. 9,* 131.
Nisonoff, A., Hopper, J. E., and Spring, S. B. (1975), The Antibody Molecule, New York, N.Y., Academic Press.
O'Brien, P. J. (1977), *Biochemistry 16,* 953.
Parkhouse, R. M. E., and Melchers, F. (1971), *Biochem. J. 125,* 235.
Pless, D. D., and Lennarz, W. J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 134.
Potter, M. (1972), *Physiol. Rev. 52,* 632.
Rhoads, R. E., McKnight, G. S., and Schimke, R. T. (1972), *J. Biol. Chem. 248,* 2031.
Robinson, G. B. (1969), *Biochem. J. 115,* 1077.
Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91,* 653.
Schachter, H. (1974), *Adv. Cytopharmacol. 2,* 207.
Schenkein, I., and Uhr, J. W. (1970), *J. Cell Biol. 46,* 42.
Schubert, D. J. (1970), *J. Mol. Biol. 51,* 287.
Shapiro, A. L., Scharff, M. D., Maizel, J. V., Jr., and Uhr, J. W. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 216.
Sherr, C. J., and Uhr, J. W. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 381.
Uhr, J. W. (1970), *Cell Immunol. 1,* 228.
Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973), *J. Biol. Chem. 248,* 7570.

Synthesis in Vitro of Ribosomal Protein S20 and Its Precursor†

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ABSTRACT: I have purified and characterized two products synthesized in vitro in a system for coupled transcription and translation programmed by DNA from a transducing bacteriophage carrying the gene for ribosomal protein S20. One of these polypeptides appears to be identical with authentic S20 by several criteria, including its electrophoretic and chromatographic mobilities, and its ability to bind to 16S RNA. The second polypeptide is less basic than S20, but exhibits all the structural and functional properties of a precursor to S20, including the presence of an additional methionine residue, apparently as *N*-formylmethionine. Moreover, it is converted, albeit slowly, to S20 in cell-free extracts. The persistence of the precursor form of S20 may be functionally significant as well.

The mechanism of regulation of ribosomal biogenesis remains one of the outstanding unanswered questions of the molecular biology of *Escherichia coli.* I am approaching this problem by exploring the possibility that covalent modification of ribosomal proteins during their assembly into functional ribosomes plays a significant role in governing the rate and/or specificity of this process. I have previously shown that several ribosomal proteins, among them, S20 (Mackie, 1976, 1977), exhibit kinetics of labeling in vivo compatible with the processing of precursors into mature forms of the protein. To confirm this prediction, I have characterized the products synthesized in vitro from the DNA of bacteriophage λddapB2 (Friesen et al., 1976) which carries the gene for ribosomal protein S20 in a system allowing coupled transcription and translation (Zubay, 1973). Among the products are both S20 and, in larger quantities, a protein with structural and functional properties of S20 which has an N-blocked methionine residue, apparently as *N*-formylmethionine. This latter polypeptide is converted to S20 in the crude extracts used for protein synthesis, confirming its likely role as the precursor to S20. L. Reis, L. Lindahl, and M. Nomura (personal communication) have also isolated such a molecule and come to similar conclusions. These findings correct my earlier suggestion that the precursor to S20 might be considerably larger than mature S20 (see the Discussion).

Materials and Methods

Bacterial and Bacteriophage Strains. The following strains were obtained from the named investigators: C600 (P. Leder),

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MRE600 (R. A. Zimmermann), RD100 (M. L. Pearson), JF735, lysogenic for both λc1857S7 (hereafter called λ) and λc1857S7ddapB2 (hereafter, λdapB2) (J. D. Friesen), and λc1857S7plac5 (hereafter, λplac5) (P. Leder).

Buffers. Buffer A is 10 mM Tris-acetate,[1] 14 mM magnesium acetate, 60 mM potassium acetate (pH 7.8); buffer B is 50 mM sodium phosphate, 6 M deionized urea, 6 mM β-mercaptoethanol, adjusted to pH 6.5 with a 30% solution of methylamine; buffer C is 50 mM Tris-HCl, 20 mM $MgCl_2$, 350 mM KCl, 6 mM β-mercaptoethanol (pH 7.8).

Preparation of S30 Extracts. Zubay's procedure (Zubay et al., 1970; Zubay, 1973) was followed exactly except for the following slight modifications in the growth and processing of the cells. Cultures of 1.0 L were grown in Zubay's medium (Zubay et al., 1970) in 6-L flasks at 34 °C with vigorous aeration to an A_{600} of 1.5. The cultures were chilled at least 2 min in ice water and then harvested by centrifugation. Rapid chilling, suggested to me by Dr. Jack Greenblatt, was important in obtaining active extracts consistently. The cell pellets were washed twice with buffer A containing 6 mM β-mercaptoethanol. The wet pellet was suspended with 1.5 mL of buffer A containing 1 mM dithiothreitol and 0.5 mM phenylmethanesulfonyl fluoride per g of cells. Subsequently, the cell suspension was lysed in a French Press at 4000–8000 psi, and the S30 extract prepared, with preincubation, as described in Miller (1972). Higher recoveries of protein and greater activity in the cell-free synthesis of β-galactosidase were obtained by this method than by grinding the cell pellet with alumina powder (Miller, 1972). The dialyzed extract was quickly frozen in aliquots and stored at −70 °C until use.

Preparation of Bacteriophage DNA. Lysogenic strains were grown in 1.0-L cultures, induced by heat, and the bacteriophages purified as described by Miller (1972). I was unable to separate λ and λdapB2 bacteriophages with this procedure, although it was successful for several other pairs of transducing phages and their helpers. Presumably, λ and λdapB2 have nearly identical buoyant densities. DNA was prepared by the extraction of the purified bacteriophage(s) with sodium dodecyl sulfate at 60 °C, followed by extensive dialysis (Miller, 1972).

Purification of Ribosomal Components. Purified 30S subunit proteins or 16S ribosomal RNA were prepared by the methods described previously (Muto et al., 1974; Mackie and Zimmermann, 1975). Individual ribosomal proteins were at least 90% pure as judged by polyacrylamide gel electrophoresis.

Cell-Free Protein Synthesis. DNA-directed protein synthesis was performed as described by Zubay (1973) and Miller (1972) with the following modifications. NAD^+ was added to 150 μg/mL on the advice of Dr. Joel Kirschbaum, poly(ethylene glycol) 6000 to 1.5 mg/mL (Pouwels and van Rotterdam, 1975), and the concentrations of Mg^{2+} and DNA reduced to 10.5 mM and 30 μg/mL, respectively (see Results). 3′,5′-Cyclic AMP was included in all incubations at 1 mM and the concentration of S30 protein was 6.2–6.5 mg/mL. Incubations were for 60 min (or as noted in the legends to the figures) at 35–37 °C. Synthesis was terminated by the addition of 0.7 mL of β-galactosidase assay mix (Miller, 1972) or by extraction with 66% acetic acid (Hardy et al., 1969). In the latter case, the incubation mixture was chilled on ice and carrier ribosomes (or subunits) added as required, followed by $MgCl_2$ to 0.1 M and 2 vol of glacial acetic acid. The suspension was stirred for 45 min on ice and then centrifuged to remove precipitated RNA. The extracted proteins were either dialyzed into buffer B for column chromatography, or precipitated several times with acetone (Barriteault et al., 1976) for polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gels were run in slabs 10 × 14 cm (either 1.5 or 0.75 mm thick) using either an 8 M urea (pH 4.5) system (Leboy et al., 1964) or the discontinuous sodium dodecyl sulfate method of Laemmli and Favre (1973). Immediately following electrophoresis, wet gels were processed for fluorographic enhancement (Bonner and Laskey, 1974) and then dried and exposed.

Tryptic Fingerprinting. Material for trypsin digestion was concentrated by precipitation with 12% trichloroacetic acid in the presence of 200 μg of bovine serum albumin in 1–2.5 mL. The precipitate was recovered by centrifugation and washed once with 80% acetone to remove trapped salts and acid. After performic acid oxidation (Weber et al., 1972), the protein was dissolved in 1% NH_4HCO_3 and digested with Tos-PheCH_2Cl-trypsin (Worthington Corp.) at an enzyme to substrate ratio of 1:50 for 16–20 h at 37 °C. Each digest was applied in a 2-cm band to a sheet of Whatman No. 3MM paper and subjected to high-voltage electrophoresis at pH 6.4 for 2 h at 1500 V (approximately 30 V/cm). After the paper was dried, each sample lane was cut into pieces 0.75 cm wide and the pieces were counted by liquid scintillation.

RNA–Protein Binding. The binding of ribosomal proteins to 16S RNA was assayed by the cosedimentation of ^{35}S-labeled proteins synthesized in vitro with 16S RNA essentially as described earlier (Muto et al., 1974; Mackie and Zimmermann, 1975), except that the gradients were fractionated into 30 fractions which were counted directly after mixing with scintillation fluid, rather than after precipitation.

Reagents. Inorganic chemicals were of reagent grade; biochemicals were obtained from Sigma, except for trisodium phosphoenolpyruvate (heptahydrate) which was from Calbiochem. Radiochemicals were products of New England Nuclear and Amersham-Searle.

Results

Characteristics of the Cell-Free System. The rationale behind this work is to examine the synthesis of ribosomal proteins, S20 in particular, in the absence of ribosomal assembly, in the hope of isolating unprocessed precursor polypeptides. Of the systems available for coupled transcription and translation, that developed by Zubay and his collaborators (Zubay, 1973) entails a minimum of fractionation and produces enzymatically active products. As there is no convenient enzymatic assay for any of the ribosomal proteins of *Escherichia coli*, I chose to optimize the ionic conditions for cell-free synthesis of β-galactosidase directed by λplac5 DNA. This assumes that the synthesis of other proteins directed by other phage templates will occur under these same standard conditions. Some support for this hypothesis stems from Dottin's (1974) finding that the ionic conditions optimal for the synthesis of anthranilate synthetase coincided with those for β-galactosidase.

Accordingly, I have assayed for the synthesis of β-galactosidase as a function of these variables: concentration of Mg^{2+}, concentration of DNA, and concentration of protein in the extract added. Unlike many other investigators, I find that the maximal synthesis of β-galactosidase occurs at 10–11 mM Mg^{2+} for concentrations of $CaCl_2$ and trisodium phosphoenolpyruvate of 7.4 and 22 mM, respectively (Figure 1a). This optimum has been obtained reproducibly from strains RD100

[1] Abbreviations used are: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; NAD^+, oxidized nicotinamide adenine dinucleotide; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DEAE, diethylaminoethyl.

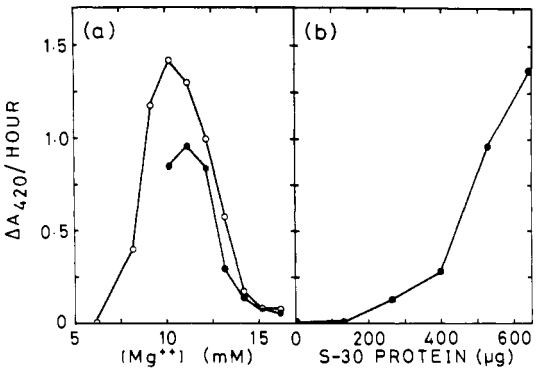

FIGURE 1: Synthesis of β-galactosidase as a function of concentration of Mg^{2+} and S30 protein. The synthesis of β-galactosidase directed by λplac5 DNA was performed as described under Materials and Methods for 60 min at 36 °C in a final volume of 0.1 mL per sample. The enzyme synthesized was detected by further incubation for 30 min using the assay procedure described by Miller (1972). In (a), only the concentration of magnesium acetate was varied. Two S30 extracts were tested: from strain RD100 (●) or from strain C600 (○). The $\Delta A_{420}/h$ at 10.2 mM Mg^{2+} in the absence of added DNA was 0.03 in the former case and 0.42 in the latter, due to endogenous activity (strain C600 is not *lacZ*$^-$). These backgrounds have been subtracted. In (b), the protein concentration in the incubation was varied, at constant ionic strength. The final concentration of Mg^{2+} was 10.5 mM at all points tested.

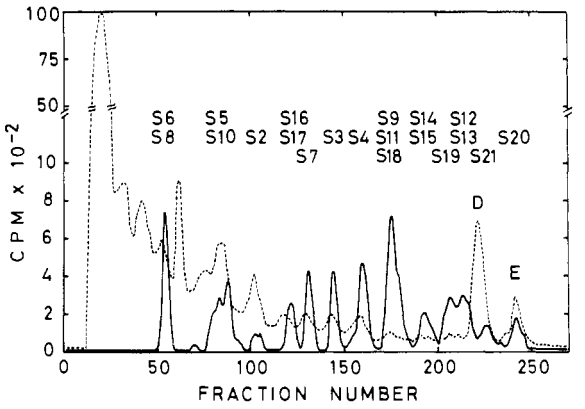

FIGURE 2: Resolution of ^3H-labeled 30S subunit proteins and ^{35}S-labeled proteins synthesized in vitro from λdapB2 DNA. An in vitro synthetic reaction mixture was prepared using 30 μg of a mixture of λ and λdapB2 DNAs, S30 protein from strain RD100, 125 μCi of [^{35}S]methionine (final concentration, 0.15 mM), 10.5 mM Mg^{2+}, and other components as described under Materials and Methods. The final volume was 1.0 mL and the incubation was performed for 60 min at 36 °C. Subsequently, 12 mg of ^3H-labeled 30S subunits (10^6 cpm total) was added to the chilled reaction mixture, and the suspension was extracted with 66% acetic acid in the presence of 33 mM $MgCl_2$ and 2 mM β-mercaptoethanol. The extracted protein was dialyzed extensively against buffer B before being applied to a column (0.9 × 25 cm) of phosphocellulose. Proteins were eluted with a 300-mL gradient from 0 to 0.5 M NaCl in buffer B at a constant flow rate of 6 mL/h (Mackie, 1977). Fractions of 1 mL were collected and aliquots of 80 μL mixed with 3 mL of Biofluor (New England Nuclear Corp.) and counted by liquid scintillation. The ^3H subunit proteins were identified on the basis of their chromatographic mobilities (cf. Figure 1 of Muto et al., 1974): (—) ^3H; (- - -) ^{35}S.

and C600 in extracts prepared either by grinding with alumina powder or by breakage in a French Press. The significance of this difference with previously published reports is unclear, since it could arise from any number of factors not necessarily related to protein synthesis per se. Preliminary experiments indicate that this same optimum holds for the synthesis of ribosomal protein S20 in my hands.

The DNA concentration used throughout this work is 30 μg/mL; beyond this, no increase in the synthesis of β-galactosidase occurs (data not shown). Enzyme synthesis increases in proportion to the amount of extract added at all amounts tested (Figure 1b). This observation suggests that the reason for the plateau in synthesis at 30 μg/mL DNA is that the system is saturated at this concentration of DNA. Alternatively, the DNA preparation could contain an inhibitor of the synthesis of β-galactosidase. If this is the case, it cannot be removed by extensive dialysis.

Separation of the Products Encoded by λdapB2 DNA. Friesen et al. (1976) have previously demonstrated that bacteriophage λdapB2 carries the structural gene for ribosomal protein S20, *rpsT*. Since I was unable to separate this phage from its helper, DNA was extracted from the mixture of phages and used as a template in the in vitro system. The products of the reaction were labeled with [^{35}S]methionine and extracted with 66% acetic acid in the presence of 30S subunits labeled in vivo with a mixture of ^3H-labeled amino acids. Figure 2 illustrates the separation of the extracted proteins by ion exchange chromatography on a column of phosphocellulose. The ^3H-labeled ribosomal proteins serve as markers. Most of the protein synthesized in vitro elutes in the flow-through (fractions 10–25). Further chromatography of this material on DEAE-cellulose at acidic pH (Möller et al., 1972; Zimmermann and Stöffler, 1976) resolves at least six components of this mixture (not shown). Of the discrete peaks which are retained by the ion exchanger, peak E cochromatographs with S20 labeled in vivo. A second rather basic polypeptide, peak D, elutes at a concentration of NaCl 35 mM lower than peak E. Neither peak E nor peak D is found if λ DNA is used as a template (not shown). The polypeptides eluting before peak D have not yet been characterized extensively.

The absolute yields of peaks D and E can be calculated from the specific activity of the added methionine and the knowledge that S20 contains three methionine residues (Wittmann-Liebold et al., 1976) and peak D, four (see below). In a typical preparation, 60 pmol of peak D and 21 pmol of peak E were recovered after a 60-min incubation using 1 pmol of a mixture of λ and λdapB2 DNAs and S30 protein from strain RD100. Under similar conditions, the yield of β-galactosidase is almost two orders of magnitude lower (Dottin, 1974). The much higher efficiency of synthesis of peaks D and E is undoubtedly a function of their smaller size.

I have also tested the effect of the source of the S30 protein on the relative yields of the products synthesized in vitro from λdapB2 DNA. Parallel synthetic reactions were performed using [^3H]methionine with the S30 protein from strain C600 and [^{35}S]methionine of identical specific activity with the S30 protein from strain RD100. After 30 or 75 min of incubation, equal amounts from each reaction were mixed and extracted. The proteins so obtained were separated by column chromatography as in Figure 2. The normalized $^3H/^{35}S$ ratios were identical within 10% across the column, except in the regions of peaks D and E. Here there was up to twofold more peak D and correspondingly less peak E from the incubations with the S30 protein from strain C600, at both time points examined. I conclude that the synthesis of the proteins in peaks D and E is independent of the source of the S30 protein, but that the latter may influence their relative proportions.

Purity of the Products Synthesized in Vitro. The purity of the material in peaks D and E of the separation shown in Figure 2 has been ascertained by subjecting aliquots of each to electrophoresis in two types of polyacrylamide gel. Figure 3a shows the separation obtained on gels containing 8 M urea at pH 4.5. Peak D (lane 2) migrates as a single component slightly less basic than peak E (lane 1) or authentic S20 purified from ^{35}S-labeled 30S ribosomal subunits (lane 3). Peak D cannot

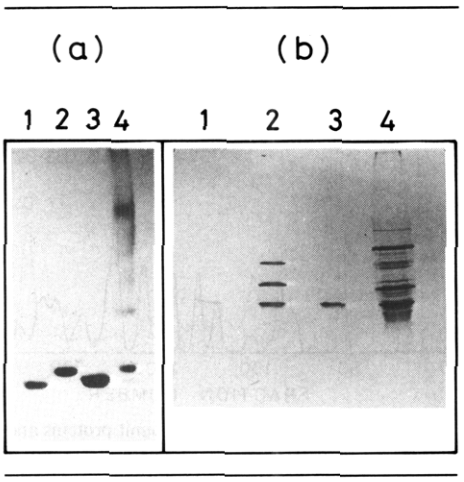

FIGURE 3: Polyacrylamide gel electrophoresis of purified proteins synthesized in vitro. (a) Aliquots of pooled fractions from a column similar to that illustrated in Figure 2 (but without the ^3H-labeled 30S subunit protein markers) were concentrated by precipitation with acetone, dissolved in 8 M urea, and applied to a 7.5% polyacrylamide slab gel containing 8 M urea at pH 4.5 (refer to Materials and Methods for further details). Samples: (1) peak E, (2) peak D, (3) authentic S20 purified from ^{35}S-labeled 30S subunits, (4) a sample of the total protein applied to the column (a better gel is shown in Figure 6). (b) Samples of pooled fractions were precipitated first with 12% trichloroacetic acid in the presence of 0.1 mg of bovine serum albumin, and the pellets obtained by centrifugation were washed once with 80% acetone. This procedure removes salts which otherwise interfere with the stacking of the samples in the discontinuous gel. The precipitated proteins were dissolved in 75 μL of sample buffer and applied to a 10–25% polyacrylamide gradient slab gel containing 0.1% sodium dodecyl sulfate (see Materials and Methods). Samples: (1) peak E, (2) a mixture of authentic S4, S5, and S20 (from top to bottom), (3) peak D, (4) a mixture of all the proteins synthesized in vitro.

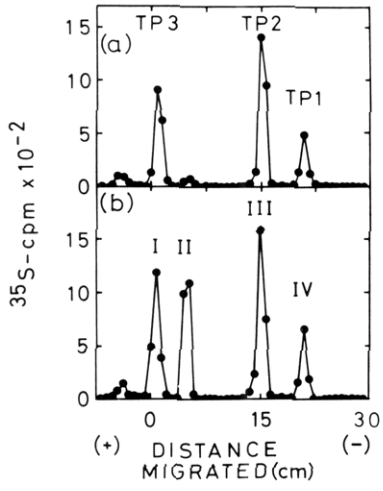

FIGURE 4: Tryptic fingerprints of peak D and S20. Samples of authentic S20 (a) or peak D (b) containing about 10^4 cpm each were concentrated, oxidized with performic acid, and then digested with trypsin, as described under Materials and Methods. The products were separated by electrophoresis at pH 6.4. The anode is to the left, the cathode to the right.

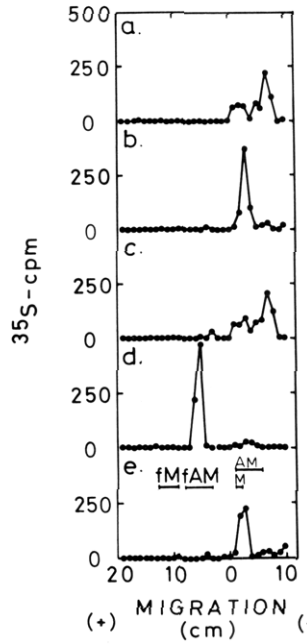

FIGURE 5: Subtilisin digestion of tryptic peptides from S20 and peak D. Pieces of paper corresponding to the major peptides of the separation illustrated in Figure 4 were soaked for 16 h at 37 °C in 2 mL of 1% NH_4HCO_3 containing 0.4 mg/mL subtilisin. The digested material was concentrated by lyophilization, dissolved in 0.1 mL of H_2O, and applied to a sheet of Whatman No. 3MM paper, each sample in a 2-cm wide band. Following electrophoresis at 1500 V for 2 h at pH 3.6, each sample lane was cut into strips 1 cm wide and the radioactivity located by liquid scintillation counting. Nonradioactive standards including methionine (M), alanylmethionine (AM), *N*-formylmethionine (fM), and *N*-formylalanylmethionine (fAM), the latter two synthesized by the procedure of Sheehan and Yang (1958), were run simultaneously on the same paper. These markers were detected by staining with ninhydrin. Samples: (a) TP-3 of S20, (b) TP-2 of S20, (c) peptide I of peak D, (d) peptide II of peak D, and (e) peptide III of peak D.

be S21, with which it coelutes on phosphocellulose (Figure 2), since S21 migrates more rapidly than S20 in this particular gel system (Muto et al., 1974). Coelectrophoresis of peak E with S20 is further evidence for their identity.

On polyacrylamide gels containing sodium dodecyl sulfate illustrated in Figure 3b, peak D (lane 3) comigrates with authentic S20 (in lane 2, together with S4 and S5 as markers). Peak E also coelectrophoreses with authentic S20 but the band is too faint to see in the photograph. On the basis of these analyses, I conclude that peak D is pure as isolated by column chromatography, and that it differs from S20 in charge, but not appreciably in size.

The Structural Relationship between Peak D and S20. Aliquots of pooled fractions containing peak D or authentic S20 were concentrated and digested with trypsin. The products were then separated as described under Materials and Methods and shown in Figure 4. Authentic S20 gives rise to three peptides containing methionine (Figure 4a). Peptides TP-1 and TP-2 have the primary structure Ser-Met-Met-Arg and arise from positions 25–28 in the primary sequence of S20, while peptide TP-3 corresponds to positions 49–59 (Wittmann-Liebold et al., 1976; Mackie, 1977). Figure 4b indicates that peak D contains four labeled tryptic peptides, three of which comigrate with peptides obtained from S20 (I with TP-3, III with TP-2, and IV with TP-1). Moreover, the relative yields of peptides I and III + IV are in direct proportion to those of TP-3 and TP-2 + TP-1 of S20. Since the ratios of the yields of I:II:III + IV are in the order 1:1:2, it is clear that peak D contains one additional methionine relative to S20 in peptide II. S20 of peak E synthesized in vitro has a fingerprint identical with that of purified S20, except for the presence of a small amount of radioactivity in the position of peptide II of peak D (not shown).

Logically, the additional peptide of peak D, peptide II, ought to contain *N*-formylmethionine. To examine this possibility, the pieces of paper containing peptides I, II, and III of peak D and peptides TP-2 and TP-3 of S20 were eluted and digested with subtilisin. The products were separated by electrophoresis at pH 3.6, along with standards, as illustrated in Figure 5. Only peptide II contains an acidic product which coelectrophoreses

TABLE I: Binding of Proteins Synthesized in Vitro to 16S RNA in the Presence of Unlabeled Ribosomal Proteins.[a]

Expt	^{35}S-Labeled protein tested	Competitor (pmol)	Relative binding,[b] %
1	Peak D		75
		S20 (30)	62
		S20 (60)	7
		S20 (150)	2
		S20 (300)	1
2	Peak D		72
		S4 (200)	70
		S12 (100), S13 (100), S19 (100)	78
		S21 (150)	80
3	Peak E		61
		S20 (30)	36
		S20 (60)	26
		S20 (90)	21

[a] Samples of pools of peak D or peak E, prepared essentially as described in the legend to Figure 2, except that ^{3}H-labeled 30S subunit proteins were omitted, were precipitated with 5 vol of acetone in the presence of 0.2 mg of bovine serum albumin and 10 μmol of β-mercaptoethanol per mL at −20 °C. The precipitated proteins were recovered by centrifugation and dissolved in 0.15 mL of 4 M urea containing 0.5 M KCl and 5 mM β-mercaptoethanol. Portions of 12 μL were diluted into 0.20 mL of buffer C containing 0.10 mg of bovine serum albumin, 100 pmol of a mixture of ribosomal proteins S16 and S17,[2] 25 μg of 16S RNA, and the unlabeled competitor protein(s) as indicated in the table. The mixture was incubated for 30 min at 37 °C and then chilled and applied to a 3–15% sucrose gradient in buffer C. Centrifugation, fractionation, and counting methods are described under Materials and Methods. [b] Relative binding is the percentage of the radioactivity added to the incubation which cosediments with the 16S RNA. For peak D, 100% is 1200 cpm and 0.3 pmol; for peak E, 100% is 690 cpm and 0.2 pmol, ignoring the nonradioactive (carrier) S20 in peak E.

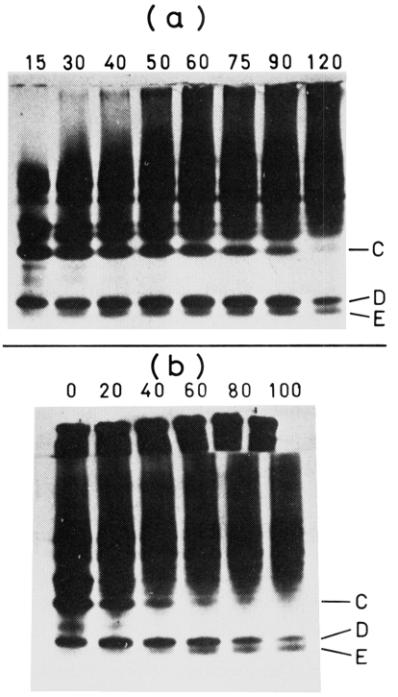

FIGURE 6: The kinetics of synthesis of peak D and S20. (a) Time course of synthesis. An in vitro synthetic reaction programmed by a mixture of λ and λdapB2 DNAs identical in composition with that described in the legend to Figure 2 was incubated at 37 °C. Samples were withdrawn from it at various times (in minutes) indicated on the figure and extracted with 66% acetic acid–33 mM $MgCl_2$. The extracted proteins were precipitated several times with acetone (Barriteault et al., 1976) and then dissolved in 8 M urea. Samples containing about 8×10^4 cpm each were resolved by polyacrylamide gel electrophoresis at pH 4.5. Peaks D and E (S20) were identified by comparison with purified standards (not shown on this gel). Other experiments demonstrate that band C is encoded by λ DNA alone. (b) Chasing of peak D in vitro. An in vitro synthetic reaction identical with the one described above was prepared and incubated for 20 min at 36 °C. At this time, methionine and rifampicin were added to final concentrations of 1 mM and 50 μg/mL respectively. This completely halts further incorporation into acid-insoluble material. Aliquots of 0.1 mL were withdrawn at the times indicated (in minutes) and extracted, and the labeled proteins analyzed by polyacrylamide gel electrophoresis as noted above.

with formylalanylmethionine (Figure 5d). All the others give rise to products which migrate in the position of methionine or alanylmethionine. It is significant that the profile of radioactivity obtained from peptide I (Figure 5c) resembles that of peptide TP-3 (Figure 5a). Likewise, peptide III (Figure 5e) and peptide TP-2 are digested to apparently identical products. This is further evidence for homology between peak D and S20.

Comparable results have been obtained from the analysis of total subtilisin digests of peak D or S20. Only the former contains an acidic peptide which coelectrophoreses with formylalanylmethionine (not shown).

Binding of Peak D Protein to 16S RNA. Ribosomal protein S20 is one of six 30S subunit proteins capable of binding independently to 16S RNA prepared by phenol extraction (Zimmermann, 1974). Indeed, this is essentially the only known property of S20. The ability of peak D to bind to 16S RNA at the binding site for S20 was tested in competition experiments. Although the concentration of peak D in the incubations was rather low (approximately 1.5 nM), it nonetheless bound quite well to 16S RNA in the presence of nonradioactive proteins S16 and S17,[2] as an average of 75% of the added radioactivity cosediments with the 16S RNA (Table I). Only S20 is effective in competing with peak D for binding (experiment I, lines 2–5). Proteins S4, S21, and a mixture of proteins S12, S13, and S19 (proteins of comparable basicity) were without effect (experiment 2, lines 2–4). S20 synthesized in vitro (peak E) also binds to 16S RNA, and nonradioactive purified S20 competes with its binding too (experiment 3). It is clear from these experiments that peak D shares the same RNA binding site as S20, and is thus functionally indistinguishable from S20.

Disappearance of Peak D. If peak D is in fact a precursor to S20, it ought to be convertible into S20. Two experiments have been performed to examine this possibility. In the first, the synthesis of various proteins directed by a mixture of λ and λdapB2 phage DNAs was investigated as a function of time. Aliquots were removed from an incubation at various times and the proteins extracted. Incorporation of methionine into acid-precipitable material was linear for 75 min, and then declined gradually. Samples containing the same amount of radioactivity were analyzed by electrophoresis in polyacrylamide gels containing 8 M urea at pH 4.5, conditions which permit the resolution of peak D and S20 (cf. Figure 3a). Figure 6a illustrates that at 15 and 30 min, virtually no S20 is detectable, whereas peak D is quite prominent. Both appear to be synthesized continuously for up to 120 min. In contrast, at least one other major band at 15 min (band C) is either unstable, or its synthesis ceases after a brief period. This protein

[2] The partial requirement for ribosomal proteins S16 and S17 in the binding of protein S20 to 16S RNA is documented by G. A. Mackie and R. A. Zimmermann, manuscript submitted for publication.

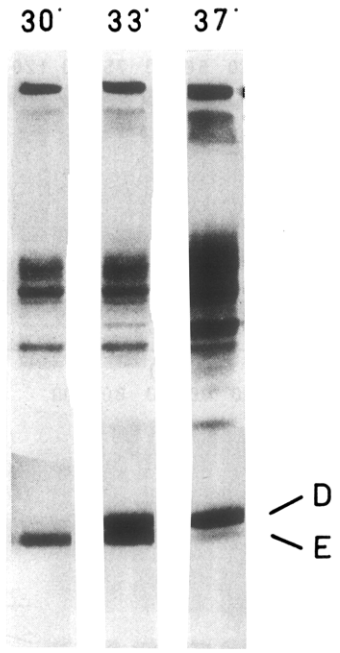

FIGURE 7: The effect of temperature on the synthesis of peak D and S20. Parallel synthetic reactions similar in composition to that of Figure 2 but in volumes of 50 μL were prepared and incubated 75 min at 30, 33, or 37 °C. Subsequently the samples were chilled, made 7 M in urea, and adjusted to pH 4.5, and the radioactive proteins resolved by polyacrylamide gel electrophoresis as in Figure 3a.

is encoded by λ DNA alone (data not shown) and is not related to peak D or S20.

In a second type of experiment, incorporation in the in vitro synthetic reaction was allowed to proceed for 20 min. Rifampicin was added to 50 μg/mL and unlabeled methionine to 1 mM, a seven-fold excess. The incubation was continued for 100 min, while aliquots were removed at 20-min intervals and extracted. Figure 6b illustrates the resolution of the products at each time point by polyacrylamide gel electrophoresis. After 20 min of synthesis (time 0), virtually no S20 is present. S20 does gradually appear with time. However, even after 100 min, there is no more than 50% apparent conversion of peak D into S20. Identical results have been obtained repeatedly. While this experiment is consistent with the idea that peak D can be converted into S20, it suggests that the rate of this process at 37 °C is quite slow, particularly since some other ribosomal proteins synthesized in vitro from λfus2 DNA (Lindahl et al., 1976; Nomura, 1976; Mackie, unpublished results) are rapidly converted into their mature forms.

In view of the dramatic effect of temperature on the rate of appearance of mature S20 in vivo (Mackie, 1977), I have investigated the dependence of the synthesis in vitro of peaks D and E (S20) on temperature. In one experiment the temperature of incubation was varied and the products of the reaction resolved by polyacrylamide gel electrophoresis as illustrated in Figure 7. Clearly, at 30 °C, only band E (S20) is found. Increasing the temperature favors the accumulation of peak D (compare the lane at 33 °C with that for 37 °C), either by altering the enzyme(s) necessary for its conversion into mature S20 or by rendering peak D itself an unfavorable substrate.

In a second experiment, the chase in the presence of rifampicin described above was performed at 30 °C (after synthesis at 37 °C) with the addition of fresh portions of the S30 extract to compensate for any inactivation during the incubation at 37 °C. Even in this case, only 40–50% of the material in peak D was converted into S20 over an 80-min period. I interpret this and the preceding experiments as follows: processing (i.e., deformylation and removal of the terminal methionine) of peak D is thermolabile and, even at permissive temperatures, is efficient only when coupled to translation.

Discussion

The present experiments were undertaken to clarify my earlier observation (Mackie, 1977) that the kinetics of labeling of S20 in vivo suggested the existence of a precursor to this protein of more than ephemeral lifespan. I have found that the products of transcription and translation in vitro of bacteriophage DNA carrying the gene for ribosomal protein S20, *rpsT*, include a molecule (peak D of Figure 2) very similar, but not identical with S20. The most important properties of this polypeptide defining a precursor–product relationship with S20 are its tryptic fingerprint (Figure 4) which includes both methionine-containing tryptic peptides of S20, its ability to interact with the binding site for S20 on 16S RNA (Table I), and its apparent conversion, in vitro, into a product which coelectrophoreses with mature S20 (Figure 6). I propose that this molecule be called pre-S20.

Pre-S20 possesses several noteworthy features. First, it appears to differ from mature S20 only by the presence of *N*-formylmethionine at its N terminus as evidenced by its comigration with mature S20 in polyacrylamide gels containing sodium dodecyl sulfate (Figure 3b). I cannot exclude the possibility that one or a few additional amino acids are situated between *N*-formylmethionine and the N-terminal alanine of mature S20. If this is the case, these residues must be uncharged at pH 6.4, since the electrophoretic mobility of the "extra" peptide (peptide II) of pre-S20 is consistent with the sequence fMet-Ala-Asn-Ile-Lys (cf. Wittmann-Liebold et al., 1976). Nonetheless, it is remarkable that the presence of the blocked methionine in pre-S20 dramatically alters its mobility relative to S20 on both phosphocellulose and on polyacrylamide gels containing urea at pH 4.5.

A second feature of pre-S20 is the persistence of the blocked N terminus. The experiment illustrated by Figure 6b demonstrates that even in a crude extract, the half-life at 37 °C of pre-S20 is 90–120 min. It is conceivable that this apparent stability is an artefact of the cell-free system, a consequence of inactivation of the necessary processing enzymes. Other workers have in fact described the lability of the deformylase activity (Adams, 1968; Livingston and Leder, 1969). An alternative explanation is that pre-S20 is a poor substrate for the terminal processing enzymes, particularly at 37 °C. First, I have interpreted my previous experiments in vivo (Mackie, 1977) to indicate the stabilization of a precursor to S20 in the absence of ribosomal assembly, whereas other proteins such as S4 are synthesized and processed to their mature forms under the same conditions. Secondly, some ribosomal proteins, exemplified again by S4, are recovered in their mature forms after synthesis in vitro from λfus2 DNA (Lindahl et al., 1976; Nomura, 1976; G. A. Mackie, unpublished results), while others such as S11 and S13 retain their formylmethionine blocking group (Lindahl et al., 1976). A common feature of S11, S13, and S20 is their basicity. This may explain why these proteins are poor substrates for the terminal processing enzyme(s).

The slow rate of removal of the *N*-formylmethionine residue from pre-S20 would effectively maintain the latter in a more acidic form, perhaps less prone to aggregate with polyanions (like RNA) than mature S20. This could be important in enhancing the specificity of its interaction with ribosomal RNA precursors in the cell. Presumably, pre-S20 and the immature forms of other proteins such as S11 and S13 would be processed on nascent or newly synthesized 30S subunits rather than on

the ribosomes where they were made. Labeling experiments in vivo (Mackie, 1977) are consistent with, but do not prove, this hypothesis.

I was previously unable to recover pre-S20 from pulse-labeled cells (Mackie, 1977). I did succeed in purifying a polypeptide, p2, whose properties suggested that it was derived from a precursor to S20 by degradation. The electrophoretic mobility of p2 in polyacrylamide gels together with its tryptic fingerprint led me to the erroneous conclusion that the putative precursor to S20 would be considerably larger than S20 itself. I have since determined that p2 (like many ribosomal proteins) migrates anomalously in gels containing sodium dodecyl sulfate and that its actual molecular weight could be as low as 5000. Thus, it is not necessary to postulate the presence of "extra" amino acids at the N terminus of the precursor to S20. Presumably then, the precursor hypothesized to accumulate in vivo during a temperature shock is identical with pre-S20 synthesized in vitro at 37 °C.

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References

Adams, J. M. (1968), *J. Mol. Biol. 33,* 571–589.
Barriteault, D., Expert-Bezançon, A., Guérin, M.-F., and Hayes, D. (1976), *Eur. J. Biochem. 63,* 131–135.
Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83–88.
Dottin, R. P. (1974), Ph.D. Thesis, University of Toronto.
Friesen, J. D., Parker, J., Watson, R. F., Fiil, N. P., and Pedersen, S. (1976), *Mol. Gen. Genet. 144,* 115–118.
Hardy, S. J. S., Kurland, C. G., Voynow, P., and Mora, G. (1969), *Biochemistry 8,* 2897–2904.
Laemmli, U. K., and Favre, M. (1973), *J. Mol. Biol. 80,* 575–599.
Leboy, P. S., Cox, E. C., and Flaks, J. G. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1367–1374.
Lindahl, L., Zengel, J., and Nomura, M. (1976), *J. Mol. Biol. 106,* 837–855.
Livingston, D. M., and Leder, P. (1969), *Biochemistry 8,* 435–443.
Mackie, G. A. (1976), *FEBS Lett. 71,* 178–180.
Mackie, G. A. (1977), *Biochemistry 16,* 1391–1398.
Mackie, G. A., and Zimmermann, R. A. (1975), *J. Biol. Chem. 250,* 4100–4112.
Miller, J. H. (1972), Experiments in Molecular Genetics, Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 419–424.
Möller, W., Groene, A., Terhorst, C., and Amons, R. (1972) *Eur. J. Biochem. 25,* 5–15.
Muto, A., Ehresmann, C., Fellner, P., and Zimmermann, R. A. (1974), *J. Mol. Biol. 86,* 411–432.
Nomura, M. (1976), *Cell 9,* 633–644.
Pouwels, P. H., and van Rotterdam, J. (1975), *Mol. Gen. Genet. 136,* 215–226.
Sheehan, J. C., and Yang, D.-D. H. (1958), *J. Am. Chem. Soc. 80,* 1154–1158.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26,* 3–27.
Wittmann-Liebold, B., Marzinzig, E., and Lehmann, A. (1976), *FEBS Lett. 68,* 110–114.
Zimmermann, R. A. (1974), in Ribosomes, Nomura, M., Tissières, A., and Lengyel, P., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 225–269.
Zimmermann, R. A., and Stöffler, G. (1976), *Biochemistry 15,* 2007–2017.
Zubay, G. (1973), *Annu. Rev. Genet. 7,* 267–287.
Zubay, G., Chambers, D., and Cheong, C. (1970), in The Lac Operon, Zipser, D., and Beckwith, J. R., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Press, pp 375–391.