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Structure and Synthesis of the Ribosomal Ribonucleic Acid of Prokaryotes

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INTRODUCTION

Gene expression is generally considered in terms of two separable events, transcription and translation. The process of the transcription of deoxyribonucleic acid (DNA) into informational ribonucleic acid (RNA) molecules is carried out by RNA polymerases and regulated by the various factors governing initiation or termination of RNA chains. The second event, translation of informational RNA into a linear polymer of amino acids, is accomplished by a complex interaction of numerous macromolecular components which are collectively referred to as the translation apparatus. This collection includes the various aminoacyl transfer RNA (tRNA) species; dissociable protein factors involved in polypeptide chain initiation, elongation, and termination; and, finally, the ribosome, which is the hub of the translation apparatus. The ribosome furnishes the surface upon which protein synthesis takes place, and it

probably also coordinates and provides catalytic assistance to the numerous interacting components of the translation apparatus.

Because of its obvious importance and the continuing progress in exploring its extreme complexity, the prokaryotic ribosome, and more particularly that of *Escherichia coli*, has become a perennial favorite for review. This essay also concerns the prokaryotic ribosome, but it will be limited in large part to a consideration of only one of its ingredients, the ribosomal RNA (rRNA). A few review articles have been directed toward ribosomal RNA. Particularly valuable recent efforts are those of Attardi and Amaldi (4), which are concerned with both prokaryotic and eukaryotic rRNA, and of Burdon (33), which consider only eukaryotic rRNA. Most reviewers of protein or RNA synthesis also consider, at least in passing, rRNA. Many of these reviews will be referred to during the course of this essay. For the benefit of those readers who have only passing interest in the rRNA of prokaryotes, I have included a summary of what I consider to be the most noteworthy points (see Summary). The literature survey for this article was completed in the spring of 1973.

GENERAL FEATURES OF PROKARYOTIC RIBOSOMES

Diversity in Bacterial Ribosomes

The detailed structure of bacterial ribosomes (as exemplified by those of *E. coli*) and their function during protein synthesis have been reviewed extensively (60, 146, 166, 208, 215, 264). In brief, active ribosomes at physiological Mg^{2+} concentrations have the sedimentation properties of approximately 70*S* particles (304). The ribosomes of prokaryotes are distinctly smaller than those of eukaryotes, which sediment as 80*S* particles (301). At low Mg^{2+} concentrations (305) or when not engaged in protein synthesis (134, 172, 286), the 70*S* bacterial ribosomes dissociate into two components, one about 50*S* and the second approximately 30*S* in size. The anhydrous molecular weight of the 50*S* component of *E. coli* is 1.55×10^6, and that of the 30*S* particle is 0.9×10^6 (121). These particle weights represent the total of numerous components, both RNA and protein. The 50*S* subunit is constructed of 30 or more proteins (146), which comprise about 25% of its mass; the remainder is RNA. The 30*S* ribosomal component is approximately 60% RNA and 40% protein, including at least 21 identifiable polypeptide chains (146). When mixed, the RNA and protein moieties of the 30*S* ribosomal subunits spontaneously recombine to yield particles which are functional in protein synthesis (208, 209). Functional 50*S* subunits of *Bacillus stearothermophilus* (83, 210) and *E. coli* (179) also have been reconstituted from their component proteins and RNA, but the in vitro assembly process appears rather more complex than that of 30*S* subunits.

Ribosomes from prokaryotes other than *E. coli* are similar in their general properties. Taylor and Storck (301) have carefully examined and compared the sedimentation properties of ribosomes purified from 25 microorganisms, including members of the taxonomic orders *Pseudomonadales*, *Eubacteriales*, and *Actinomycetales* as well as representatives of the blue-green algae. All possess characteristic "70*S*" prokaryotic ribosomes, although the precise sedimentation values vary slightly among the different organisms. Less precise measurements have been made on ribosomes and their subunits from members of other taxonomic orders, including *Myxobacterales* (266), *Mycoplasmatales* (129) and even *Rickettsiales* (255, 302). All possess the 70*S* ribosomes characteristic of prokaryotes, which dissociate at low Mg^{2+} concentrations into 50 and 30*S* subunits. Since the ribosomes of such an evolutionarily diverse collection of organisms exhibit these properties, it seems unlikely that exceptions will be found among the prokaryotes.

Similarities among the ribosomes isolated from prokaryotes of different taxonomic orders and their structural differences from eukaryotic ribosomes are not limited to gross particle size. The RNA components of all prokaryotic ribosomes thus far examined are similar in dimensions, although they differ considerably in detailed structure (see below), and these RNA size classes are quite distinct from those of eukaryotic cells (160, 300). Furthermore, the individual proteins associated with the prokaryotic ribosomes display certain common features. For example, Traut and his colleagues (10, 287) examined the ribosomal proteins from various representatives of the orders *Pseudomonadales*, *Actinomycetales*, and *Eubacteriales* by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and found extensive similarities regarding the quantities and molecular weights of the ribosomal proteins. These features were not common with proteins purified from eukaryotic ribosomes (10). The similar sizes of the classes of prokaryotic ribosomal proteins do not reflect similar primary structures, however, since in the absence of sodium dodecyl sulfate the ribosomal proteins isolated from the various orders do not have similar electrophoretic properties (321), and they generally display little or no serological cross-reac-

tion. As might be anticipated, ribosomal proteins purified from organisms within a single taxonomic family (*Enterobacteriaceae*) show considerable antigenic similarity (236).

It is clear, then, that prokaryotes have diverged greatly in the detailed structures of the components of their ribosomes. Nevertheless, functional homology has been retained at least among diverse members of the order *Eubacteriales*. This is evident in the observation (43, 298) that 50*S* ribosomal subunits from the genus *Bacillus*, including the thermophile *B. stearothermophilus* (2), can combine with *E. coli* 30*S* subunits to form hybrid ribosomes which are active in protein synthesis. Furthermore, Nomura and his colleagues (211) have reconstituted functional, hybrid 30*S* ribosomal subunits from the component proteins and RNA from different eubacteriales, including *E. coli*, *Micrococcus lysodeikticus*, *Azotobacter vinelandii*, and *B. stearothermophilus*. This is possible even though the ribosomal proteins (321) and ribosomal RNA (211, 308) of these organisms have little resemblance in their detailed primary structures. The interaction between the RNA and protein components leading to functional aggregation is not a nonspecific phenomenon, since rRNA isolated from rats or yeasts is incapable even of particle formation with the proteins of 30*S* subunits from *E. coli* (308). Therefore, even though the RNA and protein components of ribosomes from diverse prokaryotes have deviated significantly in their structures during evolution, they have retained the details which permit their functional interaction. This suggests that comparative studies of the components of ribosomes from various taxonomically diverse prokaryotes should be of considerable value in identifying those functional details.

STRUCTURES OF THE PROKARYOTIC rRNA MOLECULES

Prokaryotic rRNA Molecules

Deproteinization of purified, bacterial ribosomes generally yields three distinct RNA components. Two of these, classified by their approximate sedimentation velocities as 23*S* and 5*S* rRNA's, are derived from the 50*S* ribosomal subunit (145, 247). The third RNA component, 16*S* rRNA, originates from the 30*S* ribosomal subunit (145). The molecular weights of 23, 16, and 5*S* rRNA's are, respectively, about 1.1 million, 0.55 million, and 0.04 million (145, 247, 280). These weights correspond to polynucleotide chain lengths of about 3,300 nucleotides for 23*S* rRNA, 1,650 nucleotides for 16*S* rRNA, and 120 for 5*S* rRNA. All of the rRNA components present in actively growing *E. coli* are generally considered to be metabolically stable (91), and density-labeling experiments have shown that there is no exchange of rRNA among the available pool of ribosomes in the cell (133, 181).

Ribosomal RNA molecules of approximately 23, 16, and 5*S* in size have been isolated from all prokaryotes examined, including members of the order *Pseudomonadales* (76, 160, 177, 300), numerous members of *Eubacteriales* (76, 145, 160, 190, 247, 300; S. Sogin, Ph.D Thesis, Univ. of Ill., 1971), *Myxobacteriales* (7), *Actinomycetales* (23), *Rickettsiales* (107), and various representatives of the blue-green algae (123, 160, 300; W. F. Doolittle, personal communication). Detailed comparisons have revealed, however, that 23*S* and 16*S* rRNA molecules isolated from various genera differ slightly in their sedimentation properties (300) and in their electrophoretic mobility through polyacrylamide gels (160). Evidence has even been presented that the 23*S* and 16*S* rRNA's of *E. coli* may each be separated into several mobility classes by polyacrylamide gel electrophoresis (59, 257), but this heterogeneity probably reflects minor diversity in the secondary structures of the rRNA molecules in solution (59). Similarly, variation in the physical properties of the rRNA molecules from diverse genera may not reflect chain length but rather conformational differences. A decision between these alternatives could be achieved by comparing the electrophoretic mobilities of the rRNA components after heating in the presence of formaldehyde so as to irreversibly denature any secondary structure (16). Certainly the chain lengths of 5*S* rRNA from different organisms vary slightly. The 5*S* molecules of *Pseudomonas fluorescens* (76) and *E. coli* (32) are 120 nucleotides in length, whereas those of *Bacillus subtilis* are composed of only about 115 to 116 nucleotides (222; S. Sogin, Ph.D. thesis, Univ. of Ill., Urbana, 1971).

An interesting anomaly regarding the structure of 23*S* rRNA is emerging from studies of certain photosynthetic organisms. Lessie first reported (155) that ribosomes isolated from *Rhodopseudomonas spheroides* and *Rhodopseudomonas capsulata* do not possess a 23*S*-sized rRNA component, whereas other athiorhodaceae, including *Rhodospirillum rubrum* and the *Rhodopseudomonas* species *palustris* and *gelatinosa*, contain normal complements of 23 and 16*S* rRNA's. Instead of 23*S* rRNA, *Rhodopseudomonas spheroides* 50*S* ribosomal subunits contain two RNA molecules, one about 16*S* in size (0.53×10^6 daltons) and a secor

approximately 15S in size (0.42×10^6 daltons) (177, 244). It has been claimed that these molecules might be fragments of 23S rRNA generated during purification, and indeed 23S-sized molecules may be isolated from *R. spheroides* (19, 294), but these are dissociable by heat or formamide treatment into the 16 and 15S "halves" (177, 244). Moreover, Marrs and Kaplan (177) have shown that an RNA molecule of intact, 23S size in fact transiently exists in *R. spheroides* 50S ribosome subunits, but that it is cleaved during growth to the smaller components. Lack of an intact 23S rRNA molecule does not affect the function of the *R. spheroides* ribosome (177), so it has been suggested that the cleavage products remain noncovalently associated.

The blue-green alga *Anacystis nidulans* also cleaves its 23S rRNA into two fragments (70, 293). The larger of these is about 0.88×10^6 daltons, and the smaller is 0.17×10^6 daltons. It is quite clear in this case that the fragments are not an artifact of isolation. The cleavage process is very slow relative to ribosome maturation times; the half-life of intact 23S rRNA is several hours (70). Furthermore, scission of the 23S rRNA is retarded by inhibitors of energy metabolism, which implies that it is closely coupled to growth rather than being the result of random nuclease action (70). Ribosomal RNA lability also has been reported for species of *Agrobacterium* (103) and for numerous eukaryotes (119, 143, 283). The utility to these organisms of cleaving the rRNA remains obscure.

rRNA Integrity and Ribosome Function

The existence of organisms which cleave their 23S rRNA molecules, for whatever reason, suggests that rRNA intactness is not necessary for ribosome function. Apparently it is not. Several investigators (34, 61, 92) have examined the effects of ribonuclease treatment on the capacity of ribosomes from *E. coli* to carry out polypeptide synthesis, with the conclusion that as long as Mg^{2+} concentrations are sufficiently high to stabilize the digested ribosomes, they remain functional (34). Probably the divalent cations are chelated by adjacent polynucleotide fragments, and hence the structural integrity of the rRNA-protein aggregates is maintained (34). Unfortunately, all of these investigations examined only the capacity of digested ribosomes to participate in polyuridylate-directed polyphenylalanine synthesis. It would be of interest to test their ability to translate faithfully a heteropolymer.

Eukaryotic Analogy

It is instructive while considering the prokaryotic rRNA components to consider briefly their evolutionary homologues among the eukaryotes.

80S ribosomes from animal cells are composed of two subunits, one about 60S and a second about 40S in size (301). Deproteinization of the smaller, 40S subunit yields one RNA molecule, about 18S in size and 0.7×10^6 in molecular weight (160, 300). The 60S subunit yields three distinct RNA molecules. The largest of these is about 28S (300). This corresponds to a molecular weight of about 1.7×10^6, although the exact molecular weight may vary somewhat according to the animal source (160). The larger rRNA component of lower animals and plants may be substantially smaller than that of mammals, with molecular weights as low as 1.4×10^6 recorded (160). The 60S ribosomal subunits, like the prokaryotic 50S subunits, also contain 5S rRNA (0.04×10^6 daltons) as well as another low-molecular-weight RNA component which originally was termed "7S" rRNA (228). "7S" rRNA, which is actually 5.5S and about 0.05×10^6 daltons, is intimately associated with 28S rRNA; it is released by treatments with disrupt hydrogen bonding, for example by high concentrations of urea or by heating in solutions of low ionic strength (228). Consequently, this RNA species has been referred to as "28S-associated" (28S-A) rRNA (317).

The ribosomes of eukaryotes, therefore, have certain fundamental physical properties which distinguish them from the prokaryotic ribosomes. The ribosomes derived from eukaryotes are significantly larger than those of prokaryotes; the same is true of the two high-molecular-weight rRNA components, 28 and 18S rRNA's. Finally, there are two low-molecular-weight rRNA components of eukaryotic ribosomes, as contrasted with only one in prokaryotes. It is tempting because of their equivalent sizes to assert that the eukaryotic 5S rRNA is the functional homologue of the prokaryotic 5S rRNA, and that 28S-A rRNA is a relative newcomer on the evolutionary scene, but this may be incorrect (see below). When knowledge of the detailed function of the rRNA components of both prokaryotes and eukaryotes becomes available, it will be possible to decide just how profound the differences are between their ribosomes.

Base Compositions of rRNA from Prokaryotes

The nucleotide compositions of the high-molecular-weight rRNA components isolated

from a wide variety of prokaryotes have been determined. For comparative purposes, some of these are listed in Table 1, along with the DNA guanosine plus cytidine (G+C) content of the organism indicated. The listed values are those of the combined 16 and 23S rRNA components; the nucleotide compositions of the individual molecules are nearly the same, but careful analyses have revealed minor differences (119, 280, 322). The noteworthy feature of the available rRNA base compositions is their remarkable similarity, even though the genome base compositions, as reflected in the G+C content of the DNAs, are extremely variable. Of course, the rRNA molecules are defined by a very small fraction of the cellular DNA, and so their genes do not significantly contribute to the observed G+C values of the DNA. The implication of the disparate G+C values for the genome DNA, as compared with the rather constant G+C content of rRNA, is that the requirements of the translation apparatus for a particular rRNA structure are rather fastidious. That is, whatever selective pressures have driven the genomes of the organisms toward their present G+C compositions have affected very little the nucleotide composition of the rRNA cistrons; the rRNA molecules are evolutionarily highly conservative in that regard. Nevertheless, the nucleotide sequences of the rRNA molecules from diverse microorganisms differ extremely (see below). Therefore, the conservative features of the rRNA molecules which are reflected in their base compositions are not chiefly the primary structures, but rather their secondary or tertiary structures. Evidence that these latter features probably largely define the specificity of the interactions of the rRNA molecules with the ribosomal proteins is provided by the findings of Nomura et al. (211) that functional, hybrid 30S ribosomes may be reconstituted from the 16S rRNA and the 30S subunit proteins of organisms whose rRNA components possess relatively little nucleotide sequence homology. The possible importance of RNA sequences not associated with helical regions must not be ignored, however. Modification of bases not involved in stable base pairing in purified 16S rRNA in solution abolishes the ability of the molecule to combine functionally with ribosomal proteins (208).

The 16 and 23S rRNA components each contain, in addition to the four "natural" bases, minor quantities of a variety of modified nucleosides. All of the modified nucleosides known to be associated with the bacterial rRNA molecules, except one (5-ribosyluridine), are methyl-substituted. This distinguishes the minor components of rRNA from those to tRNA, which also include hypermodified and thiol-substituted nucleosides. Ribosomal RNA from *E. coli* also has been reported to contain thiol-substituted nucleosides (51), but this observation proved to be a consequence of contaminating tRNA (42, 81). The chemistry and biology of the modified nucleosides, with particular reference to tRNA, have been reviewed recently and concisely by Hall (108).

The majority of detailed studies of methylated nucleosides in rRNA from prokaryotes have focused on *E. coli*. The methyl-substituted nucleosides in *E. coli* rRNA fall into two classes, the first containing methylated bases and the second possessing a methyl substitution at the 2'-O-position on the ribose moiety of the nucleoside. About 1% of the total nucleotides of *E. coli* rRNA are methylated (73, 86). It has been estimated (73, 86) that the 16S and 23S rRNA molecules contain approximately 22 and 23 methyl groups, respectively, although somewhat lower values have been reported (113). About 90% of the methylated nucleosides in the

TABLE 1. *Base compositions of rRNA and total DNA from various bacteria*[a]

Organism	A	G	C	U	rRNA G+C	Reference	DNA G+C (%)	Reference
Mycoplasma hominis	28.8	26.9	19.5	24.7	46.4	129	27-29	129
Proteus vulgaris	26.2	31.4	21.7	20.7	53.1	183	~38	120
Bacillus megaterium	25.2	31.1	22.0	21.6	53.1	217	38	120
Streptococcus pneumoniae	25.4	31.0	19.1	24.2	50.1	322	39	120
Vibrio marinus 15381	26.9	30.9	21.2	20.9	51.1	217	40	49
Bacillus subtilis	25.9	31.0	22.3	20.8	53.3	66, 183	43	120
Escherichia coli	25.1	32.3	21.4	21.2	53.7	280	52	120
Bacillus stearothermophilus	22.7	35.5	23.7	18.0	59.2	217	52	217
Spirillum intersonii	23.4	33.2	23.8	19.5	57.0	217	55	120
Aerobacter aerogenes	25.5	31.5	21.9	21.1	53.4	183	~56	120
Pseudomonas aeruginosa	25.7	31.6	21.7	21.0	53.3	114, 183	~65	120
Micrococcus lysodeikticus	22.7	32.7	21.2	23.1	53.9	322	72	120

[a] Abbreviations: A, arginine; G, guanosine; C, cytidine; U, uridine.

rRNA of *E. coli* are base substituted; the remainder are 2'-O methylated (73, 86, 203). This contrasts with the rRNA of the higher eukaryotes, in which most of the methyl groups are contained on the ribose moiety of the nucleosides (28, 269). The 5*S* rRNA molecules of both prokaryotes (31, 32, 76) and eukaryotes (6, 30, 89, 223, 315) lack modified nucleosides.

The structures of the minor nucleosides of *E. coli* rRNA, excepting those substituted only on the ribose, and their distributions between the 16 and 23*S* molecules are shown in Fig. 1. No detailed cataloging of the modified nucleosides present in the rRNA components of prokaryotes other than *E. coli* has been carried out, although methylation of the high-molecular-weight rRNA components has been reported for a variety of diverse organisms (129, 273). The fraction of modified nucleosides does not appear to be constant, however. For example, the rRNA of *Mycoplasma hominis* contains only one-half as many methyl-substituted nucleosides as that of *E. coli* (129).

The methylated nucleosides of the prokaryotic rRNA molecules are not randomly distributed, but rather are associated with particular nucleotide sequences (86, 273); the methylation events are highly specific and, therefore, presumably have some function. Moreover, Sogin et al. (273) have found that most of the methylated nucleosides and the nucleotide sequences in their immediate vicinity are identifiable in the 16*S* rRNA components of bacterial species which otherwise have relatively little similarity in primary structure. The occurrence of these features within the 16*S* rRNA molecules from phylogenetically diverse bacterial species implies that not only the methylated nucleosides but also the nucleotide sequences surrounding them are somehow important in the construction or the function of ribosomes; they otherwise would not have been conserved evolutionarily.

Although some of the methylated nucleosides appear to be important in rRNA function, the nature of the involvement is not known. Some methylated components may be indispensable; others may merely refine properties already inherent in unmodified nucleotide sequences (273). Still other modifications appear superficially to be inconsequential. For example, the mutation of *E. coli* to resistance to the antibiotic kasugamycin is attributable (117, 118) to the loss of a methylase specific for 16*S* rRNA. 16*S* rRNA from kasugamycin-resistant cells consequently lack the methylated, T1 ribonuclease (RNase)-generated oligonucleotide me_2-Ame_2ACCUG, and yet the cells appear to grow normally; this methylated sequence plays little,

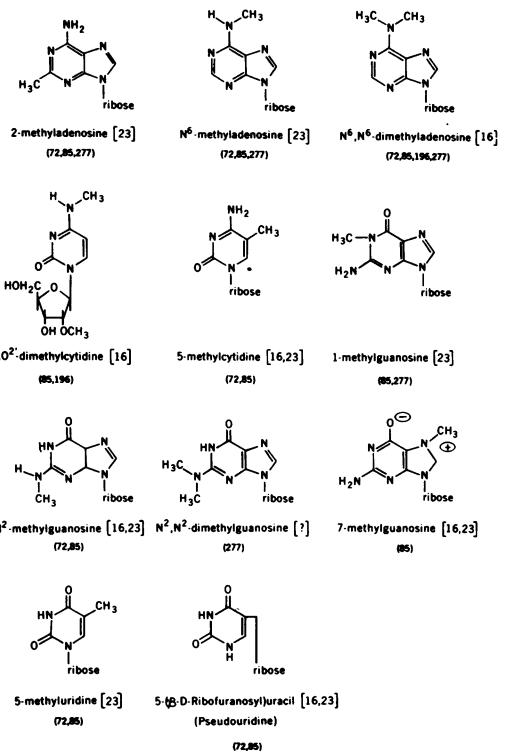

FIG. 1. *Base-methylated nucleosides in the rRNA of Escherichia coli. Numbers in brackets after the names of the compounds are the rRNA components in which the modified nucleosides have been positively identified. References are listed parenthetically below the names of the compounds.*

if any, role in ribosome formation or function. Nevertheless, these methyl substituents obviously influence the properties of the kasugamycin-resistant ribosomes; they lose the capacity to interact with the drug.

Another indication that the methylation of the rRNA components may subtly influence the function of ribosomes comes from studies of induced drug resistance. Certain strains of *Staphylococcus aureus* are physiologically induced by erythromycin to resistance to this and some other drugs which interact with the 50*S* ribosome subunit. Lai and Weisblum (149) have observed that the induction to drug resistance is accompanied by the appearance of N^6-dimethyladenosine in the *S. aureus* 23*S* rRNA; this modified nucleoside is not present in 23*S* rRNA of uninduced cultures. It has not yet been proven that the presence of the N^6-dimethyladenosine indeed confers resistance to the antibiotic, but certainly the rRNA is involved. 23*S* rRNA purified from induced drug-resistant cells, but not that from sensitive cells, confers

drug resistance upon reconstituted 50S ribosomal subunits (150). Still another example of an apparently unimportant modified nucleoside is 1-methylguanosine. Björk and Isaksson (12) have isolated a mutant of *E. coli* which lacks this component in its rRNA, and yet the mutant grows normally, at least in the laboratory.

It should be obvious that our understanding of the role of the modified nucleosides in rRNA is limited at best. One hopes that as investigators continue to chip away at the intricacies of the ribosome, this understanding will broaden. It is probable that studies of modified nucleosides in tRNA will also provide information on the mechanisms by which methylation may affect polynucleotide structure and function.

Primary and Secondary Structures of the rRNA

Many questions regarding the structures of the rRNA molecules, their interactions with the ribosomal proteins, and their function during protein synthesis will only be understood when the complete nucleotide sequences of the molecules are available. This is truly a formidable task because of the large sizes of 16 and 23S rRNA's and the tedious, albeit elegant, nature of the experimental techniques involved. This technology has been reviewed by Sanger and Brownlee (252) and by Gilham (97). Nevertheless, several laboratories have undertaken the determination of the primary structures of the rRNA molecules, and a limited amount of information has become available.

At this time, the only completely known nucleotide sequences of prokaryotic rRNA are those of the 5S rRNA molecules of *E. coli* (31, 32) and of *Pseudomonas fluorescens* (76). The structures of the 5S rRNA components from these two ostensibly phylogenically distant bacteria differ substantially, but certain common features are present. Most notably, the middle two-thirds of the molecules show a high degree of similarity in nucleotide sequence, and approximately 75% of the identical bases in the two molecules can be aligned (76). Also, the 5'- and 3'-terminal eight or nine nucleotides of both 5S rRNA species are structurally capable of forming antiparallel, hydrogen-bonded "stalks", which confer amphora configurations on the molecules (31, 76). This same feature is inherent in the primary structures of 5S rRNA from eukaryotes (30, 89, 223, 315). The 5S rRNA molecules of both the prokaryotes and eukaryotes also contain the sequence -G-A-A-C-, which at least in principle could interact by hydrogen bonding with the -G-T-Ψ-C- sequence of the common arm of the known tRNA molecules (32). It is premature to assert that these common features are somehow involved in 5S rRNA function, but as the structures of 5S rRNA from other prokaryotes become available, comparative examinations should prove useful in defining the functional regions of the molecule.

The 5S rRNA populations of *E. coli* and *P. fluorescens* are largely homogeneous with regard to their primary structures, but minor heterogeneity is evident. For example, *E. coli* strain MRE600 produces two forms of 5S rRNA (32), differing only at position number 13 (nucleotide positions are conventionally numbered from the 5'-terminus of the polynucleotide); one form contains a G at that position and the second contains a U. Another *E. coli* strain, CA265 (32), has two 5S rRNA forms which differ at position 12. Similarly, the 5S rRNA population of *P. fluorescens* is composed of two classes, differing at two positions (76). It is unlikely that these minor structural variations within the 5S rRNA populations of a given organism have any functional significance. All of the rRNA molecules are defined by multiple genes (see below), which presumably are evolving more or less independently. Point mutations in any one of the multiple cistrons therefore would lead to a mixed population of rRNA structures.

Unlike the example presented by studies of transfer RNA, the availability of the primary structures of some 5S rRNA molecules has not led to precise information regarding their secondary structures. After the determination of the exact nucleotide sequences of 5S rRNA molecules from *E. coli* and humans and the presentation of physical data indicating that about 70 to 80% of the nucleotides in 5S rRNA are hydrogen bonded (17, 36, 53, 156), several possible models for the secondary and tertiary structures of the 5S molecules were conceived (35, 156, 168, 237). When the nucleotide sequence of 5S rRNA from *P. fluorescens* then became available, attempts to fit it to the proposed models were more or less unsuccessful (76). Jordan (131) and Mirzabekov and Griffin (187) have evaluated experimentally these various models by subjecting *E. coli* 5S rRNA to very gentle digestions with ribonuclease and examining the products of hydrolysis. Since RNase cleaves only at nucleotides which are not involved in hydrogen bonding, the regions of the molecule which are relatively resistant to digestion should be those which are involved in some sort of stable secondary structure. The results were not completely compatible with any of the proposed models. Therefore, the secondary structure of 5S rRNA remains elusive, and in

fact Richards has concluded (242) from studies of molecular models that there may be no unique secondary structure for 5S rRNA in solution. Also, it must be appreciated that the secondary and tertiary structures of any of the rRNA molecules in solution are probably significantly different from their conformations when packaged within the ribosome in juxtaposition with potentially interacting proteins. The numerous physical studies of the rRNA molecules in solution and in the ribosome have been reviewed recently by Spirin and Gavrilova (277). The 16 and 23S rRNA molecules have about the same degree of secondary structure in solution and in the ribosome, but the association with the ribosomal proteins imparts to the duplex regions of the RNA considerably more stability to thermal denaturation than observed with RNA in solution (96, 182). Furthermore, 23S rRNA in solution occupies about twice the effective volume that it assumes in the ribosome (182); the tertiary structures of the molecule in solution and in the particle are certainly quite different. Evidence also has been presented (291) that the binding of ribosomal proteins to 16S rRNA may markedly influence its secondary structure.

The nucleotide sequences of the 16 and 23S rRNA molecules are very difficult to evaluate because of their large sizes. However, through the efforts of several investigators (23, 80, 84, 253, 254), the sequence of 16S rRNA from *E. coli* should be available soon. The nucleotide sequence of about 70% of the molecule, distributed over several large fragments, is more or less completely known (80, 84, 85). The determined sequence is compatible with physical measurements (52, 111) indicating that about 60 to 70% of bases in 16 and 23S rRNA's are involved in hydrogen bonding. Fellner and his colleagues have suggested that the consequent secondary structure is likely predominantly "local" (85). That is, adjacent sequences of 10 to 30 nucleotides tend to be approximately complementary, so that the molecule in solution, and presumably in the ribosome, might be envisioned as a bewildering cluster of hairpin loops.

The partial sequence of the 16S rRNA of *E. coli*, as that of the prokaryotic 5S rRNA molecules, contains several positions which are variable (85, 192, 329). These, too, probably are a consequence of the multiple genes specifying rRNA. It is noteworthy that most if not all of the variable positions are clustered. This implies either that the variable regions are functionally unimportant and therefore structural drift is permissible, or that these areas of the 16S molecule require very precise primary structures and that any successful variation must be accompanied by compensatory changes in the immediate vicinity. Fellner and his associates (85) also have pointed out that the primary structure of 16S rRNA, as that of 5S (32) and possibly 23S rRNA's (86), appears to contain a significant amount of repetitive sequences. The meaning of these sequences is not known, but probably the repetitive sequences have equivalent functions, for example as common portions of binding sites for the ribosomal proteins.

Two approaches toward defining the regions within the 16S rRNA sequence which interact specifically with particular ribosomal proteins are being made by a number of investigators. One of these experimental approaches involves the binding of purified ribosomal proteins to purified 16S rRNA and then treating the complex with ribonuclease. By virtue of its association with the protein, the specific, interacting region of the rRNA is protected from digestion. The aggregate of the rRNA fragment and the ribosomal protein then may be isolated, and the involved RNA sequence may be determined (260, 261). In a second type of approach, 16S rRNA fragments of known sequence are tested for their capacity to bind particular ribosomal proteins (333). This type of analysis, coupled with continued evaluation of the 16S rRNA primary structure, should provide within the next few years a reasonably complete picture of the smaller ribosomal subunit of *E. coli*.

Question of Functional Heterogeneity in the rRNA Molecules

The finding that certain of the proteins associated with the 30S ribosomal subunit of bacteria are present in less than unimolar concentrations led to some consideration that individual ribosomes might be differentiated in their function (209). If extensive heterogeneity in rRNA primary structure exists, then this also would suggest that there is functional differentiation of ribosomes. However, there is no evidence at this time for such heterogeneity within the rRNA population of a given prokaryote. It was noted above that minor variability exists in the rRNA primary structures, but that this probably reflects the independent evolutionary drift of the multiple rRNA genes. There is one frequently cited report (3) that nutritional conditions may influence rRNA structure, but this finding is almost certainly incorrect for technical reasons.

As an assay for rRNA heterogeneity, Aronson and Holowczyk (3) examined the relative quantities of particular oligonucleotides released by pancreatic RNase digestion of ^{32}P-labeled 16S

rRNA purified from ribosomal subunits of both *E. coli* and *Pseudomonas aeruginosa*. No significant differences were found in the oligonucleotide contents of 16*S* rRNA from cells grown in "poor" versus "rich" media when the isotopic labeling times were lengthy. When, however, cultures were shifted from a low growth rate to a higher one by addition of a better carbon source and the cultures were labeled with [^{32}P]orthophosphate for only 5 to 6 min, then the quantities of certain labeled oligonucleotides recovered from 16*S* rRNA varied significantly from those labeled for lengthy time periods during steady-state growth. It was cautiously concluded that perturbation of balanced growth results in a temporary change in the pattern of rRNA synthesis.

In retrospect, the observed differences in oligonucleotide recoveries almost certainly were not a consequence of differences in the structure of the 16*S* molecules. Instead, the brief labeling period, coupled with the isolation of labeled RNA from 30*S* ribosomes, would have resulted in the purification of 16*S* rRNA which was not uniformly labeled along its molecular length. The time required for synthesis of a 16*S* rRNA molecule is about 30 s, and an additional 4 to 5 min are required for the completed rRNA chain to appear in mature 30*S* ribosomes. Therefore, in a pulse labeling experiment, the synthesis of most radioactive RNA molecules isolated from mature ribosomal particles would have been initiated before addition of the isotope. Consequently, a steep gradient would exist in the specific radioactivity (^{32}P/mole of nucleotide) of individual nucleotides along the molecular length of the 16*S* rRNA, with the gradient increasing toward the 3′-terminus (the terminal point of transcription) of the molecule. This means that Aronson and Holowczyk were effectively comparing radioactive oligonucleotides from the (pulse-labeled) 3′-proximal regions of the 16*S* molecule to those of the uniformly labeled (steady-state) molecule. They of course differed. This conclusion is borne out by the observation of these investigators that with progressively increasing labeling times, the oligonucleotide content of pulse-labeled 16*S* rRNA progressively approached that of the uniformly labeled molecule.

Phylogenetic Differences in the rRNA of Prokaryotes

The rRNA molecules from taxonomically diverse prokaryotes are superficially very similar in their properties. Equivalent rRNA components are similar in base composition and size (see above), and, more important, ribosomes reconstituted from RNA and proteins from different prokaryotes are more or less functional (211). These facts imply that the evolution of the rRNA molecules has been rather conservative. Nevertheless, it is known that extensive departures in the primary structures of the rRNA components of diverse prokaryotes have occurred.

Determinations of the primary structures of the rRNA components from diverse organisms will be invaluable in achieving an understanding of rRNA function. Those portions of the rRNA molecules which are required for function, whether they are primary or secondary structural features, should be ubiquitous, whereas less important regions will appear highly diverse in structure. It is desirable for this sort of comparison to have detailed, primary structures available, but for the time being this is experimentally feasible only with 5*S* rRNA. In principle, considerable useful information could be obtained from phylogenetically diverse 16*S* and 23*S* rRNA molecules by comparison of large (and therefore probably unique) oligonucleotides released from the molecules by nuclease digestions and by comparison of the regions of the rRNA molecules which interact with specific ribosomal proteins. These approaches are currently being undertaken by Woese and his colleagues (273, 274).

Although it is not yet possible to compare the detailed structures of the high-molecular-weight rRNA molecules from diverse prokaryotes, the extent of their similarities and dissimilarities can be evaluated approximately by DNA-RNA hybridization. The methodology and interpretation of nucleic acid hybridization experiments have been reviewed recently by Gillespie (98) and Kennell (136). Two types of DNA-RNA hybridization experiments, which provide comparable results (296), have been used most frequently in evaluating the relatedness of rRNA from various prokaryotes. The first of these is hybridization saturation, in which constant amounts of DNA are annealed with increasing quantities of radioactive rRNA. When no more radioactivity enters a "true" (RNase-resistant) hybrid, then the complementary sequences in the DNA are saturated and the fraction of DNA complementary to the rRNA may be calculated. When DNA and rRNA from different organisms are annealed, then the amount of RNA hybridizing with the heterologous DNA provides some measure of the relatedness of the primary structures of the rRNA molecules from the two organisms. The second approach, that of hybridization competition, is to anneal DNA of a given organism

with radioactive, homologous rRNA in the presence of a large molar excess of unlabeled, heterologous rRNA. If the rRNA's from the two organisms have any sequence similarities, then the unlabeled rRNA competes with the labeled rRNA for the complementary DNA sequences. The difference in the amounts of labeled, homologous RNA hybridizing in the absence and in the presence of the competing, heterologous rRNA defines the extent of similarity between the two rRNA populations.

Several groups of investigators have used these methods to delineate the extent of diversity of prokaryotic rRNA primary structures (67, 75, 219, 296). Perhaps surprisingly, in view of their superficial similarities, the primary structures of the prokaryotic rRNA molecules may vary considerably. For example, *E. coli* and *B. subtilis* 16 and 23*S* rRNA's have about 20 to 30% of homology (75, 219, 296); *Streptomyces griseus* and *B. subtilis* have only about 2% common sequences in their rRNA's. Bacteria from the same genera possess extensive rRNA homology, but often the results indicate, once again, the arbitrary nature of bacterial classification. For example, Marmur and his colleagues (74) and Doi and Igarashi (67) have considered rRNA homologies within the genus *Bacillus*, which is, to be sure, a catch-all genus. The observed rRNA homologies ranged from 50 to 100%, whereas total DNA (as represented by the RNA products of in vitro transcription) from the various species displayed far less similarity in primary structure (75); rRNA structure indeed is more conservative, evolutionarily, than the bulk of the information residing in the DNA. Quantitatively the rRNA of *B. polymyxa* (75) is about as closely related to that of *B. subtilis* as is the RNA of *Alcaligenes faecalis* or of *Staphylococcus epidermidis* (296). Therefore, by the criterion of DNA-RNA hybridization, the genus *Bacillus* should be broken into several other genera. On the other hand, certain organisms bearing little superficial resemblance may have considerable homology in their rRNA components. *E. coli* rRNA saturates *Vibrio metschnicovii* DNA to the same extent as the homologous RNA, although the two organisms have little structural similarity in their messenger RNA (mRNA) (pulse-labeled RNA) populations (227). Pace and Campbell (219) have compared by hybridization competition the rRNA homologies of numerous organisms to two little-related members of the eubacteriales, *E. coli* and *Bacillus stearothermophilus*, and they found that rRNA populations more closely related to the rRNA of *B. stearothermophilus* were less related to that of *E. coli*, and vice versa. This reciprocal relationship implies that there exists a spectrum of relatedness among the rRNA molecules of the prokaryotes examined and that these organisms may have a common ancestor. Therefore, the phylogenetic approach to evaluating structure-function relationships in prokaryotic rRNA is a valid one.

The outcome of heterologous DNA-rRNA hybridization competition or saturation experiments is generally expressed as "percent relatedness," and it measures the degree to which heterologous rRNA is capable of competitively preventing a certain fraction of homologous rRNA from annealing to complementary DNA sequences, or alternatively, the extent to which heterologous RNA is capable of interacting with the complementary DNA sequences in a sufficiently precise fashion to confer RNase resistance on portions of the RNA molecule. It is not yet clear whether exact base pairing of a heteropolymer DNA-RNA hybrid is necessary for RNase resistance of the hybrid (98, 136), so the "homologous" regions of the rRNA molecules from different organisms in fact may not be identical. Also, it is effectively impossible to decide whether or not the "homologous" regions of the rRNA of different organisms have the same linear organization in the molecules. The difficulties of interpreting DNA-RNA hybridization results in terms of primary structural homologies between organisms is exemplified by two sorts of experiments. The first of these is the observation by Pederson and Kjeldgaard (227) that rRNA from *E. coli* will form RNase-resistant hybrids with the entire rDNA of *Vibrio metschnicovii*. However, the rate of formation of the heterologous DNA-RNA hybrid is only about one-fourth that of the homologous pair. This means that more DNA-RNA collisions are required to produce a stable heterologous hybrid than with either homologous pair. The homologous regions are scattered throughout the rDNA (227), or, alternatively, the heterologous DNA-RNA hybrids in fact are not composed of perfect complements, even though they are resistant to ribonuclease digestion. Therefore, each collision between *E. coli* rRNA and *V. metschnicovii* DNA would have a lower probability of forming a hybrid than would the homologous pair.

A second example of experimental evidence for the ambiguity of heterologous DNA-RNA hybridization experiments is the apparent homology between 16 and 23*S* rRNA's in some organisms. It is fortunate that the first DNA-RNA hybridization tests for the uniqueness of the 16 and 23*S* genes were performed with *Bacillus megaterium* (327). With the rRNA of

this organism (327) and other members of the genus *Bacillus* (75; B. Pace, Ph.D. thesis, Univ. of Ill., Urbana, 1968), the 16 and 23*S* rRNA molecules form hybrids with only their respective genes, and neither molecule competes with the other in hybridization tests. On the other hand, 16 and 23*S* rRNA from *E. coli* (5, 171; B. Pace, Ph.D. thesis, Univ. of Ill., Urbana, 1968) or *Alicaligenes faecalis* (B. Pace, Ph.D. thesis, Univ. of Ill., Urbana, 1968) do display considerable homology in hybridization saturation or competition experiments. This result is not attributable to cross-contamination of the rRNA preparations, but careful hybridization competition experiments do show that the affinities of 16*S* rRNA for the 23*S* genes and 23*S* rRNA for the 16*S* genes are only a fraction of the homologous affinities (171, 231; B. Pace, Ph.D. thesis, Univ. of Ill., Urbana, 1968) and that heterologous hybrids are substantially more unstable than the homologous ones. One is left with the feeling that primary structural homologies determined by DNA-RNA hybridization do not necessarily mean that the "homologous" sequences in fact are identical, but rather that they are only similar at best.

McCarthy and his colleagues (9, 189) realized the difficulties of evaluating the structural relatedness of rRNA molecules from different organisms by DNA-RNA hybridization, and they have proposed a different approach, one which involves annealing rRNA from a given organism to DNA from other organisms and then testing the stability of any resulting hybrids to dissociation by heat. The temperatures required to release RNA from hybrids is some function of the number and type of interacting, complementary base pairs; DNA-RNA pairs which conform more perfectly to one another are considerably more stable to thermal denaturation than if the complementary fit is a poor one. Therefore, a hierarchy of relatedness of any rRNA to that of other organisms may be established on the basis of the temperatures at which the rRNA is released from hybrids formed with the DNA of those organisms. Unfortunately, this method does not lend itself well to quantitative evaluation because the thermal transitions are not sharp. This implies that the sequences distributed along a heterologous rRNA-DNA hybrid have a wide spectrum of relatedness.

To summarize, it appears that although the rRNA molecules of prokaryotes have many superficial resemblances, they in fact may differ considerably in their detailed, primary structures. Nevertheless, the rRNA molecules from diverse organisms do possess more or less extensive similarities in their nucleotide sequences, as revealed by DNA-RNA hybridization. At first glance it might appear that rRNA structural similarity would provide a useful index for classification purposes, but the available means for determining sequence homologies are not very precise. However, DNA-RNA hybridization competition is sufficiently straightforward that it can be used as a taxonomic tool, particularly at the family level. The method, in fact, has been used to bolster a reclassification scheme for the genus *Desulfovibrio* (218).

Prokaryotic Origin for the Eukaryotic Organelles?

In passing, I would like to consider briefly the general features of the ribosomes and rRNA from mitochondria and chloroplasts, for the reason that both of these organelles often are considered to be derived evolutionarily from prokaryotic "parasites" (45, 175, 196, 241). This supposition is based in large part upon ostensible similarities between the translation apparatus of the organelles and that of prokaryotes. The case for the prokaryotic origin of chloroplasts is a reasonably sound one. The ribosomes of chloroplasts are clearly prokaryotic in character; the 70*S* chloroplast ribosomes (285) dissociate into 50 and 30*S* subunits which contain, respectively, bacterial-like 23*S* (153, 161) and 5*S* rRNA's (285) and 16*S* rRNA (153, 161). Similar to some of the photosynthetic prokaryotes, the 23*S* chloroplast rRNA is subject to post-transcriptional scission (153). Probably most important is the fact that the ribosomal subunits of chloroplasts from both lower (154) and higher (104) plants are capable of forming active hybrid ribosomes with the ribosomal subunits of *E. coli*. Furthermore, the rRNA components of *Euglena* chloroplasts display a relatively high degree of structural similarity to those of certain blue-green algae, as assayed in DNA-RNA hybridization tests (234).

The ribosomes of mitochondria, on the other hand, are quite unlike those of the prokaryotes; their physical properties have been reviewed recently by Borst and Grivell (22). The mitoribosomes of the ascomycetes are the most similar to the ribosomes of prokaryotes, but they are structurally distinct. The mitoribosomes, their subunits, and their high-molecular-weight rRNA components are 10 to 20% larger in molecular size than those of *E. coli* (22), they lack an identifiable 5*S* rRNA molecule (159), and, unlike the ribosomal subunits of chloroplasts, those of the ascomycetes cannot form functional hybrid ribosomes with the subunits of *E. coli* ribosomes (104). The mitoribosomes of

the vertebrates are even more unlike the prokaryotic ribosomes. The unit ribosomes are 50 to 60S in size, depending upon the animal source. They dissociate into subunits of about 40 and 30S which contain, respectively, rRNA molecules only about 16 to 18S and 12 to 14S in particle size (22). In brief, the properties of the mitochondrial ribosomes and their rRNA components do not support the notion that mitochondria are derived from a prokaryotic symbiote. This thesis has been critically reviewed recently by Raff and Mahler (238). An alternative explanation is, of course, that the mitochondrial ribosomes have evolved in the host to a sufficient extent that they no longer are recognizable as prokaryotic.

GENES SPECIFYING rRNA

Gene Dosage for rRNA

When purified ribosomal RNA molecules from several bacterial species initially were hybridized with their homologous DNA by Yankofsky and Spiegelman (325, 326), it was noted that considerably more rRNA specifically annealed than would be anticipated if only one gene were specifying each rRNA component. In all prokaryotes examined, the fraction of the genome which is complementary to rRNA is remarkably constant, comprising about 0.3 to 0.4% of the total DNA (135, 213, 220, 251), or about 0.6 to 0.8% of the potentially available genetic information. In *E. coli*, in which the genome size is known with some precision, the amount of DNA hybridizing with rRNA corresponds to about six copies of each of the rRNA genes. For *Mycoplasma*, whose genome is only about 20 to 25% the size of that of *E. coli*, the amount of rDNA present is equivalent to only one gene for each of the rRNA components (251). Furthermore, DNA-RNA hybridization saturation experiments performed with the individual rRNA components, 16 and 23S (5, 213, 271; B. Pace, Ph.D. thesis, Univ. of Ill., Urbana, 1968) and 5S (220) rRNA's, have shown that the number of genes specifying each is the same. These observations satisfactorily account for the equimolar accumulation of the three rRNA molecules in the cell without requiring the preferential production of any of them.

Mapping the rRNA Genes in E. coli

Determination of the locations of the rRNA genes within the bacterial chromosome has proved to be technically difficult. There are no known bacterial mutants which are ascribable to the rRNA genes, and any search for such mutants is a rather speculative undertaking because of the multiplicity of the rRNA genes; most deleterious mutations would be recessive and therefore undetectable. Even in the absence of appropriate mutants, however, considerable experimental athletics have provided a relatively good map position for at least part of the rDNA of *E. coli*.

Initial attempts to define the location of the rDNA within the genome of *E. coli* were made by Chargaff and his colleagues (248, 249), who supposed that as the rDNA is replicated more genes specifying rRNA would be available, and that an increase in rRNA output would immediately result. Consequently, synchronous cultures of *E. coli* were pulse-labeled with [^{32}P]-orthophosphate at different intervals during a cell division cycle, and the base compositions of the radioactive RNA synthesized during the pulse periods were evaluated. Two waves of synthesis of RNA with base compositions biased toward rRNA were observed. After examining two Hfr strains, which were thought to have different initiation points for DNA replication, map positions were suggested (249), assuming unidirectional DNA synthesis at a uniform rate (but see below). Re-evaluation (11) of the origins of DNA replication in the Hfr strains employed in the experiments places one of these clusters in the 70- to 80-min region of the Taylor and Trotter map of the *E. coli* chromosome (299) and the second cluster at 35 to 50 min. In retrospect, these experiments of Chargaff and his collaborators yielded approximately the correct answer, at least with regard to the major rDNA cluster in the 70- to 80-min region, but it is not clear why. Doubling the amount of rDNA in cells apparently does not double the continued output of rRNA (56, 62, 64), although the rate of rRNA production may be enhanced temporarily as the rDNA is duplicated (56).

Cutler and Evans (56) also have used synchronously dividing populations of *E. coli* to evaluate the map position(s) of the rDNA. Unique portions of *E. coli* DNA were pulse-labeled with 5-bromouracil at intervals during synchronous division and then separated, by CsCl buoyant density centrifugation, from the less dense portions of the DNA which were not being replicated during the pulse interval. Ten unique parts of the *E. coli* DNA, comprising the entire genome (55), were isolated, and their abilities to form hybrids with purified rRNA were tested. Two regions were found to do so; it was proposed that these are located in the 60- to 75- and 10- to 25-min regions of the linkage map.

The use of synchronously replicating bacterial populations to define the map position of

the genes specifying rRNA is, at best, an imprecise experimental probe. More importantly, the basic assumptions of this approach, that the genome DNA replicates at a constant rate and in a unidirectional fashion, are probably incorrect (224). An alternative avenue toward the determination of the map positions of the rRNA genes became available with the construction, by several investigators, of a large collection of F-merogenotes. The basic idea here, which first was exploited by Yu et al. (330), is that any strain containing an F-merogenote is partially diploid for a given region, and if the episome contains the rDNA, then the rRNA gene dosage, as measured by DNA-RNA hybridization saturation experiments, is increased over the haploid value by a quantity corresponding to the number of rRNA genes present in the episome. Yu et al. (330) examined the rRNA gene dosage for strains of *E. coli* carrying a variety of F-merogenotes, which collectively spanned the entire chromosome. Contrary to the results of the indirect experiments suggesting two locales for the rDNA, it was concluded that only one region of the chromosome was complementary to rRNA. By comparison of the amounts of rRNA hybridizing to the DNA of strains carrying various episomes, the genes defining rRNA could be localized in the 74- to 77-min region of the *E. coli* map. However, Yu et al. examined only two F-merogenotes which span the 10- to 70-min region of the linkage map. Both of these episomes were about 30 min in length, corresponding to approximately 30% of the total genome. If these episomes did not contain rDNA, then the rRNA/DNA ratios observed in hybridization saturation experiments should have been lower in the diploids than in the corresponding haploid strains; the episome ostensibly contributes no rDNA, but it does contribute to the total mass of DNA in the cell. And yet, one of the diploids (15 to 40 min) contained normal proportions of rDNA, and the second (40 to 74 min) had only slightly reduced levels of rDNA. It therefore might appear that both of these episomes in fact contain rDNA. Unfortunately, the communication presented does not contain the detailed hybridization saturation data, and so it is difficult to judge the seriousness of the discrepancy between expected and observed results.

It also was proposed that the rDNA might even be confined to the rbs-ilv span (74 to 74.7 min), but this almost certainly is not the case. Purdom et al. (235) have presented reasonably convincing evidence that individual clusters of rRNA genes, each probably comprised of one 16, 23, and 5*S* gene (148, 149), are separated by stretches of DNA at least 10×10^6 daltons. This means that the total length of the rDNA map region, in order to contain five to six genes for each of the rRNA molecules, would span at least two map minutes.

Birnbaum and Kaplan (11) also used F-merogenotes in DNA-RNA hybridization experiments, and they, too, have determined that part of the rRNA genes are scattered throughout the 74- to 77-min region, excluding the rbs-ilv area. This conclusion was drawn by hybridizing rRNA with the DNA of several *E. coli* F-merogenotes purified from *Proteus mirabilis* carrying the episomes. The G+C content of the *P. mirabilis* DNA is significantly lower than that of *E. coli* F-merogenote DNA, and so the episomes may be more or less purified from the genome DNA. These investigators noted, however, that only about half of the total rRNA cistrons could be accounted for within the 74- to 77-min region, an observation which also probably is compatible with the data of Yu et al., so more rRNA gene clusters remained to be added to the map. Kaplan and his colleagues (312), through further experiments with *E. coli* F-merogenotes isolated from *P. mirabilis*, have proposed that two doses of each of the rRNA genes are located somewhere in the 54- to 59-min span of the chromosome. The finding supports the earlier suggestion of Jarry and Rosset (128) that some of the genes specifying 5*S* rRNA might lie in the 40- to 66-min region of the linkage map, in addition to the 74- to 77-min area, and equivalent numbers of 16 and 23*S* genes should accompany the 5*S* rDNA (see below). Also, Gorelic (102) has provided evidence for increased rDNA levels in an *E. coli* strain which is diploid for the 60- to 66-min region, but this result could not be confirmed by Unger et al. (312).

It is not completely certain whether copies of the rDNA exist outside the 74- to 77- and 54- to 59-min regions of the linkage map; they conceivably all are accounted for. However, episomes containing DNA outside these spans should be examined more closely for any rDNA content. This would be done best by partially purifying the episomal DNA, perhaps as described by Birnbaum and Kaplan (11), before hybridizing with rRNA. If the episome is isolated, its presence in the hybridization assays is guaranteed. Bacterial strains which are diploid for rDNA conceivably are unstable with regard to at least those genes and possibly the entire episome (330); the multiple rRNA genes in the chromosome probably are stabilized against recombinational deletion by the presence of

essential genes within the non-rDNA stretches which separate the individual rDNA clusters. Further, the determination of the rDNA content of an episome by hybridization saturation experiments with the entire diploid genome is probably not a very reliable method. It is our experience that rRNA gene dosage values, determined through hybridization saturation experiments with different preparations of rRNA and DNA from even the same strains of bacteria, may vary by 10 to 20% or even more. Isolation of the episomal DNA before the hybridization tests minimizes quantitative uncertainties implicit in the technique.

Mapping the rRNA Genes in B. subtilis

The applicability of DNA transformation in *B. subtilis* has made the location of its rDNA, relative to other markers, rather more simple than with *E. coli*, but the answer is less precise than information gained from F-merogenotes of *E. coli*. Oishi and Sueoka (213) and Marmur and his collaborators (74, 271) have used density labeling of synchronously replicating *B. subtilis* DNA to locate at least most of the rRNA genes. These experiments were performed by inducing synchronous DNA replication through germination of spores (213, 271) in 5-bromouracil or D_2O-containing medium, or by diluting stationary cultures pregrown in D_2O-containing medium into water-containing medium (74). DNA of density indicative of newly synthesized material could then be purified pycnographically from the remainder of the genome, and its content of rDNA could be determined by DNA-RNA hybridization. The time of appearance of the rRNA genes in the newly synthesized DNA then was correlated, by genetic transformation, to the replication times of various nutritional and antibiotic resistance markers. About 60 to 80% (271) of the rDNA was located within the 25% of the genome replicating initially; the remaining 20 to 40% of the rRNA genes appeared to be replicated with the terminal 25% of the genome. The positioning of the major rDNA cluster possibly could be even more narrowly located within the DNA region lying 15 to 20% of the replication distance from the origin (74), but it certainly is distinct from the str locus (267). Therefore, in *B. subtilis* as in *E. coli*, there probably are at least two regions of the chromosome containing the rDNA clusters.

All of these mapping experiments with the *B. subtilis* genome are compatible with the intermingling or close association of the individual genes specifying 16 and 23*S* (74, 213) as well as 5*S* (271) rRNA's. Colli and Oishi (46) and Colli et al. (48) have refined these observations by shearing *B. subtilis* DNA to small size (about 3×10^5 daltons), isolating the fragments capable of annealing with either 16*S* or 23*S* rRNA's, and then testing their ability also to hybridize with the other high-molecular-weight rRNA component or 5*S* rRNA. It was determined that 16*S*-specific DNA fragments are also capable of hybridizing with 23*S*, but not 5*S*, rRNA. On the other hand, 23*S*-specific DNA could be annealed with both 16 and 5*S* rRNA's. This demonstrates that the three rRNA genes are closely adjacent to one another and linked in the order 16*S*-23*S*-5*S*. Presumably these individual clusters in *B. subtilis*, as in the gram-negative bacteria examined (235), are separated by lengthy stretches of non-ribosomal DNA, which may contain various other genes (99) not necessarily related to the ribosome (11, 299).

Isolation of the rDNA

The ready availability of quantities of the rRNA molecules makes possible the isolation of the DNA regions complementary to them, and, indeed, the rDNA has been substantially purified from several organisms (47, 140, 275, 311). Isolation procedures generally begin by shearing the cellular DNA to fragments of sizes not too much larger than the combined molecular weights of the rRNA molecules. The fragments of DNA are then annealed with rRNA, and the hybrids are isolated from the remainder of the DNA by various means, including CsCl (275) or Cs_2SO_4 (containing Hg^{2+} ions; 47) buoyant density centrifugation or chromatography on columns of hydroxylapatite (140) or deoxycholate-saturated benzoylated diethylaminoethyl cellulose (311). All of these procedures more or less cleanly resolve the various products of the hybridization reactions, which include single-strand DNA and RNA, duplex DNA, and the coveted DNA-RNA hybrid. Also, the hybridization step may be prefaced by enrichment for the rDNA by chromatographic means (47) or by preferentially denaturing the majority of the genome DNA, if its G+C content is sufficiently less than that of the rDNA (295). This latter enrichment scheme is based on the fact that the temperature at which duplex DNA denatures increases as a function of increasing G+C content (176). Therefore, if the genome DNA is of lower average G+C content than the rDNA, then the bulk DNA may be heated to a temperature sufficiently high to denature a large portion of the non-ribosomal DNA. The remaining duplex DNA, now considerably enriched for rDNA, may easily be separated from the single-strand DNA.

As their rationale for seeking to purify DNA

complements to the rRNA molecules, some investigators have offered the possibility of using these as templates for in vitro studies of rRNA synthesis. It is unlikely that the products of the rDNA purification schemes devised thus far will be useful for such experiments. Only the DNA complements of the rRNA can be isolated in quantity, and these are of variable length and completeness as a consequence of the necessary shearing procedures involved in the purification schedules. A more fruitful avenue toward in vitro studies of rDNA transcription appears to be the construction of the appropriate rDNA-containing F-merogenotes, even though substantial quantities of non-ribosomal DNA must be tolerated.

TRANSCRIPTION OF THE rRNA GENES

rRNA Transcriptional Unit

In eukaryotic cells, coordinate synthesis of the 28, 18, and probably 28S-A rRNA molecules is guaranteed by the fact that the genes specifying each of these rRNA components are all part of the same "transcriptional unit". A transcriptional unit is a segment of DNA bounded by transcription initiation and termination sites, which is read without interruption by any RNA polymerase molecule effectively beginning RNA synthesis at the initiation site. A transcriptional unit may be comprised of one or many genes, and if it contains a demonstrable operator locus, then it appropriately is termed an operon. In the mammalian cell, the initial product of the rRNA transcriptional unit is about 4×10^6 daltons and 45S in molecular size, and it is cleaved in a series of endonucleolytic steps to yield the individual, mature rRNA molecules (4, 33). This compound precursor of the rRNA molecules is readily demonstrable in pulse-labeling experiments (33).

Such an elaborate scheme for production of the rRNA molecules in prokaryotes was not suspected until rather recently, since the precursors of the mature prokaryotic rRNA components, as identified by pulse-labeling experiments, are only slightly larger than the mature molecules (see below). This was generally considered as weakly implying that the genes defining the rRNA molecules were independent transcriptional units. The first solid indication that this probably was not the case was provided by the "transcriptional mapping" experiments of Woese and his collaborators (13, 14), which used the drug actinomycin D (AMD) to measure the sizes of transcriptional units generating rRNA and tRNA in *Bacillus subtilis*. AMD binds at random to the DNA, interrupting the progress of RNA polymerase molecules during transcription, and so the sensitivity of the synthesis of any given RNA molecule to AMD should be a function of the size of the DNA segment preceding and including the gene defining that RNA molecule in its transcriptional unit. If cells are exposed to AMD concentrations sufficient to inhibit RNA synthesis only partially, then the larger transcriptional units should be preferentially inactivated by virtue of the larger target size which they present to the drug. When the quantities of the individual rRNA molecules accumulating in the presence of low concentrations of AMD were tested (14), it was found that the synthesis of 5S rRNA was inhibited concomitantly with 23S rRNA and that both of these were more sensitive than 16S rRNA to the effects of the drug. In view of the very small size of the gene for 5S rRNA, if it in fact were an independent transcriptional unit, then the synthesis of 5S rRNA should have been refractory to AMD concentrations sufficient to retard 90% of the 23S rRNA synthesis. Bleyman et al. consequently proposed (13) that the 5S genes in *B. subtilis* are promoter distal in transcriptional units containing at least the 23S genes as well.

Armed with this information and the observations of Colli and Oishi (46) that the genes for 16 and 23S rRNA's in *B. subtilis* are physically linked, other investigators used rifampin to provide evidence that in *E. coli* the rRNA molecules are derived from transcriptional units consisting each of one 16S, one 23S, and one 5S gene, which are read by the RNA polymerase in that order (71, 72, 225). The drug rifampin (316) inhibits the initiation of RNA synthesis by the RNA polymerase, but it does not affect completion of nascent chains. When rifampin and a radioactive precursor of RNA are simultaneously added to growing bacterial populations, the amount of radioactivity appearing in any given RNA molecule is a consequence of the size of that molecule and the number of RNA polymerase molecules which transcribe its gene after addition of the drug. In analyzing the transcriptional organization of the rRNA genes, the necessary considerations are that equal numbers of the genes defining 16, 23, and 5S rRNA's serve as templates in producing equal numbers of the rRNA molecules. Therefore, the frequencies at which the three genes are transcribed are the same, and the numbers of RNA polymerase molecules associated with each of the rRNA genes at any instant are proportional to the sizes of the genes. To an acceptable approximation, the genes specifying the rRNA molecules are of the same length as the mature

rRNA species. Therefore, the amounts of isotopic label appearing in any of the rRNA components after residual RNA synthesis in the presence of rifampin are predictable for any conceivable transcriptional organization. The outcome of such experiments with *E. coli* is that, whereas the 16*S* gene apparently is read only by those RNA polymerase molecules residing upon it at the time of addition of the drug, the amount of label appearing in 23*S* rRNA, relative to the amount in 16*S* RNA, could only be accounted for if the 23*S* gene is preceded in its transcriptional unit by a segment of DNA corresponding in size to the 16*S* gene; not only do polymerase molecules associated with the 23*S* rDNA deposit label in 23*S* rRNA, but they also associate with polymerase molecules read into the 23*S* rDNA from the DNA segment preceding it (72, 225). Furthermore, considerably more label accumulates in 5*S* rRNA than would be expected if it comprised an independent transcriptional unit; the amount of label deposited in 5*S* rRNA in the presence of rifampin is compatible only with the 5*S* gene being preceded in its transcriptional unit by a DNA segment about the size of the combined 16 and 23*S* genes (71, 225). Equivalent experiments with *B. subtilis* (N. R. Pace and M. L. Pato, unpublished observations) have the same outcome, in support of the findings of Bleyman et al. (13). In this organism, too, the rRNA molecules are derived from transcriptional units containing genes for 16, 23, and 5*S* rRNA's, which are read by the RNA polymerase in that order.

The experiments with rifampin are corroborated by other results. When rRNA synthesis is induced by restoration of an essential amino acid to a starved amino acid auxotroph of *E. coli*, the synthesis of 16*S* rRNA, as identified by DNA-RNA hybridization tests, commences before that of 23*S* rRNA (144). Also, the time duration of 16 and 23*S* rRNA synthesis in *E. coli* after addition of rifampin is compatible with their genes being arranged in compound transcriptional units (24, 127, 186). Therefore, the organization of the prokaryotic rRNA genes in polycistronic transcriptional units is quite analogous to the situation in eukaryotes, except that the prokaryotic 5*S* gene is included in the transcriptional unit, whereas in eukaryotes a gene defining the 28*S*-A ("7*S*") rRNA molecule is included. The genes responsible for eukaryotic 5*S* rRNA production are not even associated with the nucleolus, which is the site of synthesis of the 18, 28, and 28*S*-A rRNA molecules (4, 33). If this complex transcriptional organization for the rRNA genes has any functional basis other than guaranteeing coordinate synthesis of all of the rRNA molecules, then it is conceivable that the eukaryotic 28*S*-A rRNA, and not the 5*S* rRNA, is the evolutionary homologue of the bacterial 5*S* rRNA (72).

Interaction of the RNA Polymerase with the rDNA

About 40 to 50% of the RNA being synthesized at any instant by rapidly, exponentially growing cultures of *E. coli* is identifiable as rRNA in DNA-RNA hybridization competition experiments (135, 231). This implies that the six or so transcriptional units specifying the rRNA components in *E. coli* are read by the RNA polymerase with a frequency which is, on the average, 50 to 100 times greater than the frequency of transcription of genes specifying the several hundred (135) mRNA species required for cell growth. Since one knows the number and size of the rRNA transscriptional units, the rate at which transcription occurs (about 50 nucleotides/s; 26, 173), and the number of ribosomes (10^4 to 2×10^4) which must be synthesized during a division cycle of 30 to 40 min, then the approximate number of RNA polymerase molecules associated with the rDNA at any time may be calculated. This exercise yields a value of about 100 to 150 RNA polymerase molecules per rRNA transcriptional unit. Since RNA polymerase molecules are about 7.5 nm in diameter (185) and the rRNA transcriptional unit is about 1.7 μm in length (5,000 base pairs $\times$ 0.34 nm per base pair), about 70% of the absolute maximal number of polymerase molecules which can be accommodated within the transcriptional unit in fact are loaded on during rapid growth. The distance between adjacent polymerase molecules therefore must be only about 10 to 12 base pairs, whereas the polymerase itself occupies in the order of 20 to 25 base pairs.

The in vivo rate at which the RNA polymerase synthesizes RNA in *E. coli* is generally accepted to be about 50 nucleotides/s (26, 173) at 37 C, so the times required for the construction of the 16 and 23*S* rRNA chains would be, respectively, about 30 and 60 s. In contrast to these expected times for the synthesis of the rRNA components, Mangiarotti et al. (171) and Adesnik and Levinthal (1) have presented data regarding the rates of appearance of exogenously supplied isotopic label in 16 and 23*S* rRNA's which, in their simplest interpretation, indicate that the times required for construction of the two molecules in *E. coli* might be the same. This conclusion is almost certainly incorrect, however, and the reasons underlying the

observations are not yet completely clear. As Mangiarotti et al. (171) have discussed, there are two possible models whereby the times required for the synthesis of 16 and 23S rRNA's could be the same. The first of these is that the procession rate of the RNA polymerase on the 16S genes is only half that of polymerase molecules associated with the 23S genes. Since equimolar quantities of 16 and 23S rRNA's are produced, equal numbers of polymerase molecules must then be associated with the two classes of genes, and the packing density of polymerase molecules on the 16S gene would be twice that of the 23S gene. Alternatively, equal transcription times for 16 and 23S rRNA's could be accounted for if 23S rRNA were assembled from two, 16S-sized molecules which originate from different DNA segments. This sort of scheme for the synthesis of 23S rRNA has also been considered by Midgley (184). Therefore, two polymerase molecules would be active in production of the 23S rRNA halves for each polymerase reading the 16S gene. Both of these models have definite predictions regarding the amounts of radioactivity deposited in 16 and 23S rRNA's during residual synthesis in the presence of rifampin, and neither model is indicated by such experiments (72, 225), which were described above. Furthermore, Bremer and Berry (24) have found that the persistence of 23S rRNA synthesis after rifampin addition to cultures of *E. coli* is three times that of 16S rRNA, an observation compatible only with the 23S rRNA synthesis time being twice that of the 16S molecules and with the arrangement of the 16 and 23S genes in a tandem transcriptional unit. These results also suggested that only about 20 s is required for the synthesis of a 16S rRNA molecule in *E. coli*. This is about the same length of time (18 s) proposed by Zimmermann and Levinthal (332) for the synthesis of 16S rRNA in *B. subtilis*. Both estimates yield a chain elongation rate for the RNA polymerase of about 75 to 85 nucleotides/s, which is somewhat more rapid than the generally accepted velocity of 50 nucleotides/s. This latter value was obtained by measuring the rate of synthesis of the total cellular RNA, however, and it therefore essentially averages the chain elongation rates of mRNA and rRNA. Therefore, the rate of nucleotide addition to growing mRNA chains indeed may be substantially slower than 50/s, whereas the rate of chain elongation of rRNA could be in the order of 75 to 85 nucleotides/s during rapid growth. Diffusion of ribosomes associated with the nascent mRNA conceivably slows the RNA polymerase in its procession, whereas the nascent rRNA chains, which are not associated with ribosomes, would minimally fetter the polymerase.

Initiation of rDNA Transcription

The RNA polymerase holoenzyme is composed of a "core" enzyme, which is constructed of four subunits and is responsible for RNA synthesis, and a dissociable protein factor, σ, which is required for meaningful initiation of transcription. The chemistry of the RNA polymerase and the process of RNA synthesis has been reviewed recently by several authors (8, 40, 95, 162). One facet of RNA synthesis which remains to be defined is that which determines the distribution of the RNA polymerase among different genes. This question is particularly pertinent to the synthesis of rRNA, since the rRNA transcriptional units can be read with frequencies 50 to 100 times greater than those of most genes.

There are two obvious mechanisms which might govern the very high relative rate at which the rRNA genes are read by the RNA polymerase. The first of these supposes that the affinity of the RNA polymerase for the promoter of the rRNA transcriptional unit is 50 to 100 times greater than its affinity for promoters of DNA segments which define mRNA, and therefore the frequency at which the rDNA is transcribed is correspondingly higher than the rate of production of any given mRNA molecule. The second possible scheme requires the existence of a protein factor which would bind either to the RNA polymerase, activating it so that the rDNA promoter may be recognized, or to the rDNA promoter, to form a complex for which the RNA polymerase would have an exceptionally high affinity.

At least in principle, a decision between these two alternative models could be made by confronting purified *E. coli* DNA with purified RNA polymerase and examining the quantity of rRNA which is produced relative to total RNA. When this sort of experiment was performed, it was observed initially (231, 310) that little if any of the in vitro product could be prevented from hybridizing with DNA by the presence of competing, authentic rRNA. This indicated that little rRNA was produced and, therefore, that some sort of auxiliary factor probably was required in order to enhance the affinity of the RNA polymerase for the rDNA. Travers and his colleagues (310) then reported the isolation of a protein, termed ψr, which could stimulate purified RNA polymerase to preferentially produce rRNA in vitro. This observation did not prove reproducible (112), however, and ψr is now considered to be a nonspecific stimulator of in

vitro RNA synthesis (162) whose exact function in normal cells is unknown.

More recently, several investigators have determined that, contrary to the earlier reports, the purified RNA polymerases from *E. coli* (112), 230) and *B. subtilis* (125) are, in fact, capable of preferentially transcribing the rDNA. With reasonably intact DNA, about 10% of the in vitro RNA transcripts are homologous with authentic rRNA in DNA-RNA hybridization competition tests. It is not clear why rRNA synthesis was not observed in previous experiments, but there were probably at least two contributing factors (112). The first of these is that the purified DNA originally used as the template for in vitro RNA synthesis might have been partially single-stranded and sufficiently nicked by deoxyribonuclease so that only a very small fraction of the total RNA chains produced was "physiologically meaningful". Disruption of the DNA duplex causes aberrant transcription with regard to initiation and probably termination. Consequently, any rRNA produced in vitro would have been diluted by the aberrant transcripts beyond the sensitivity of the hybridization assays. The second factor in the inability to detect in vitro rRNA synthesis might have been the nature of the DNA-RNA hybridization tests used in searching for the rRNA. Experimenters who failed to detect any rRNA among the in vitro transcripts (231, 310) used unlabeled rRNA in hybridization competition with the RNA which was isotopically labeled in vitro. Even the presence of 10% rRNA sequences among the in vitro RNA might not have been detected since this value lies within the expected error of the method. Investigators who subsequently detected the synthesis of rRNA in vitro used much more sensitive tests. One of these measured the ability of the in vitro RNA to compete with isotopically labeled, authentic rRNA in DNA-RNA hybridization competition tests (112, 125). In this protocol, only the quantity of rRNA among the in vitro products was measured in terms of the amount of in vitro product required to prevent a known amount of labeled rRNA from hybridizing. Another method used purified rDNA in searching for rRNA among the in vitro products (230). This, too, minimized the quantitative uncertainties of discriminating between the amounts of rRNA produced in vitro and the non-ribosomal RNA sequences, which are the majority.

We are left with the conclusion that highly purified RNA polymerase is capable of preferentially transcribing the rDNA without additional cellular components. It should be realized that the observed 10% rRNA among the total in vitro transcripts probably represents much more than 10% of the physiologically significant RNA products present, since it is likely that many, if not most, RNA polymerase molecules utilize incorrect initiation sites and ignore proper termination sites in vitro with the DNA templates used. Consequently, a minority of the in vitro RNA products may be equivalent to those produced by the cell, and it is this minority which the quantity of rRNA synthesized must be related to. There is no way of judging what fraction of the total in vitro product is physiologically relevant, so it can be asserted only that the high frequency of rDNA transcription is governed largely by the affinity of the RNA polymerase (holoenzyme) for the promoter of the rRNA transcriptional unit. The existence of modulating devices similar to Ψr, which could enhance the affinity of the polymerase for the rDNA promoter, is not excluded, however.

Regulation of rRNA Synthesis During Balanced Growth

The availability of nutrients determines the growth potential of any population of organisms. As a result of their evolution in an environment which presents, alternately, feast and famine, the prokaryotes have devised means for modulating macromolecular synthesis according to the suitability of the medium for proliferation. If the growth rate of a cell population is limited by the availability of nutrients rather than the limitations imposed by the potential of the biosynthetic machinery, then it behooves the cells to restrict their production of the components of this machinery. The stable RNA molecules of the prokaryotic cell are components of the protein synthesizing apparatus, and as such their rates of synthesis are governed by the growth rate of the cell population.

Early studies of variations in the RNA content of bacterial cells as a function of growth rate have been reviewed by Maaløe and Kjeldgaard (167), and more recent efforts have been considered in detail by Koch (138). The fundamental observation regarding variation in cellular RNA content, derived from the work of Schaechter et al. (77, 256), is that, to a good approximation, the RNA content and the number of ribosomes per cell are proportional to the rate of cellular protein synthesis and the rate of growth of the population. For example, in *Salmonella typhimurium* (77) or *E. coli* (138), the amount of RNA accumulating per cell mass increases two- to threefold over a fourfold increase in growth rate. Total cellular RNA of course includes mRNA, rRNA, and tRNA, so

the question we must here address is: are the rates of synthesis of these RNA species coordinately or independently controlled by the rate of growth?

Coordinate control of RNA synthesis could be achieved by cellular manipulation of the availability of nucleoside triphosphates or of active RNA polymerase molecules. Probably intracellular levels of nucleoside triphosphates do not determine the RNA synthetic capacity of the cell (139, 197-9), but the role of active versus inactive RNA polymerase molecules is difficult to assess. Some evidence suggests (225) that slowly growing cells possess a pool of polymerase molecules which are not engaged in RNA synthesis but which become active upon shift to a more rapid growth rate. It is not known whether the pool of unengaged polymerase is inherently inactive or if it is a consequence of restricted availability of promoter sites in the DNA template. Independent regulation of the synthesis of rRNA, mRNA, and tRNA of course would require regulatory devices capable of interacting directly with the transcriptional units involved, and their effects would be superimposed upon any restraints which affect all RNA synthesis during balanced growth.

Early, rather indirect measurements of variation in the rates of synthesis of mRNA, rRNA, and tRNA at varying rates of balanced growth provided suggestive evidence for the independent control of these classes of RNA (137, 167, 246), but this conclusion was not unanimous (90). More recent measurements, largely by rather convincing DNA-RNA hybridization competition techniques, indicate that the rates of synthesis of at least mRNA and rRNA may differ during rapid and slow cell growth, but that the differences in the synthetic rates of the two classes of RNA are insufficient to account for differences in their observed quantities during growth.

Norris and Koch (212) have adjusted by glucose limitation the balanced growth rates of *E. coli* cultures in chemostats and have measured the instantaneous rates of synthesis of mRNA, rRNA, and tRNA at varying growth rates by DNA-RNA hybridization competition of pulse-labeled RNA with unlabeled rRNA, tRNA, or a mixture of the two. The conclusion was reached that the newly synthesized mRNA:rRNA ratio in a population doubling each 10 h is about twice that of cultures with generation times of 55 min. The composition of the medium was the same at both growth rates, and so presumably the mRNA complement required for growth was the same; the difference in mRNA:rRNA ratios, therefore, suggests that independent controls of mRNA and rRNA synthesis exist. This same general conclusion was drawn by Lazzarini and Winslow (152), who employed similar analytical techniques but different carbon sources (glucose versus lactate) to achieve a four- to fivefold difference in the generation times of cultures. The observed, pulse-labeled, total RNA:rRNA ratio at the lower growth rate was about twice that at the high rate, again indicating a preferential restriction of rRNA output at reduced growth rates.

The twofold difference in the rates of synthesis of mRNA and rRNA at widely different growth rates apparently is not the only factor influencing rRNA accumulation in the cell, however. Julien et al. (132) and Norris and Koch (212) have pointed out that a significant fraction of the rRNA synthesized at low growth rates does not eventually appear among the stable RNA components of the cell; the fraction apparently is degraded soon after its synthesis. This observation is strengthened by the findings of Pederson (personal communication), who has compared by DNA-RNA hybridization the amounts of rRNA synthesized residually in the presence of rifampin to the amounts which eventually are stabilized. During rapid culture growth, all of the rRNA produced is stable, whereas at very much lower growth rates, on poor carbon sources, only about half of the rRNA is stabilized.

The suggestions that part of the rRNA produced during slow growth is unstable conceivably could cast doubt on the measurements of the relative rates of synthesis of mRNA and rRNA at varying growth rates. The elevated mRNA:rRNA ratios at low compared with high growth rates could be a consequence of rRNA degradation if the half-life of rRNA destined to be destroyed is significantly shorter than that of mRNA. However, Pato and von Meyenburg (225) found no obvious differences in the half-life of unstable RNA over a wide range of growth rates. The lower growth rates examined fell within the range where appreciable rRNA degradation should occur, and so if rRNA breakdown is rapid then some perturbation of the decay kinetics might be expected. Instead, the data suggest that any unstable rRNA decays at about the same rate as mRNA, and so the conclusion seems correct that the rate of synthesis of rRNA, relative to that of mRNA, is restricted by some controlling factor other than those which limit total RNA synthesis at reduced growth rates. The accumulation of ribosomes in the cell, therefore, appears to be governed at several levels.

One control mechanism affects overall cellu-

lar RNA synthesis and might involve the availability of *active* RNA polymerase molecules. A second control seems to restrict rRNA synthesis relative to that of mRNA and, at its simplest, would preferentially restrict utilization of the rRNA transcriptional units by the RNA polymerase. It has been proposed that uncharged tRNA (147, 282) or disengaged ribosomes (191, 245) might mediate in suppression of rDNA transcription, but no substantiation for either of these proposals has been offered. Still a third means for regulating the accumulation of ribosomes appears to function subsequent to transcription: rRNA is degraded. Conservation of rRNA conceivably could be determined by the availability of ribosomal proteins. At relatively rapid growth rates, ribosomal protein and rRNA synthesis may be coordinate (109), and ribosomal protein synthesis is reduced relative to total protein synthesis as the rate of balanced growth slows (262), but the relative rates of rRNA and ribosomal protein synthesis have not been examined during slow but balanced growth. If the availability of the ribosomal proteins were to become limiting at reduced growth rates, then a fraction of the newly synthesized rRNA would not be incorporated into rRNA-protein aggregates, and therefore it would be susceptible to enzymatic degradation.

It is not clear at this time whether the synthetic rates of rRNA and tRNA are independently controlled. Judging the relative, instantaneous rates of rRNA and tRNA synthesis by the available DNA-RNA hybridization competition experiments (212) is difficult. Measured variations in the tRNA content of pulse-labeled RNA at different cell growth rates probably fall within the inaccuracies of the methodology, since tRNA comprises such a minor component of the RNA being synthesized at any instant. The cellular tRNA:rRNA ratio during very slow, balanced growth may be 1.5 to 2.0 times that during rapid cell growth (63, 137, 246), but these measurements reflect only the amounts of tRNA and rRNA which accumulate. Therefore, the disparity in the tRNA:rRNA ratios may simply be a consequence of the preferential degradation of rRNA.

Regulation of rRNA Synthesis During Perturbation of Balanced Growth

The evidence for noncoordinate control of the synthesis of rRNA and mRNA is rather clear when studies are performed with cultures whose balanced growth rate is abruptly reduced by a change in available nutrients or whose growth is halted by removal of an essential amino acid. The regulatory mechanisms invoked by these unbalancing events differ at least in part.

The influence of growth rate transitions upon bacterial RNA synthesis has been the subject of numerous investigations. The basic facts (167, 201) are that, if a culture is removed from a medium permitting rapid proliferation and is placed in one supporting only poor growth ("step-down"), then rRNA synthesis is temporarily reduced until the ribosome:cell mass ratio characteristic of the slower growth rate is achieved. For example, in one study (152), a four-to fivefold reduction in the growth rate of *E. coli* (achieved by changing the carbon and energy sources from glucose to lactate) immediately resulted in a 75% decrease in the instantaneous rate of rRNA synthesis but only a 25% reduction in mRNA production, both as measured in DNA-RNA hybridization competition experiments. This is strong and convincing evidence for regulatory mechanisms which are capable of independently influencing rRNA and mRNA production. Conversely, during "shift-up", from relatively poor to rich media, the stable RNA (rRNA plus tRNA):unstable RNA (mRNA) ratio rapidly becomes characteristic of the faster growth rate (225), and there is evidence for the transient, preferential synthesis of rRNA (205, 206).

A second type of growth perturbation leading to noncoordinate reduction of rRNA and mRNA synthesis results from the removal of an essential amino acid from auxotrophic cultures. This has immediate and profound consequences for macromolecular synthesis; the phenomenology has been reviewed recently by Edlin and Broda (78) and Ryan and Borek (250). Most important in the present context is that net RNA accumulation abruptly slows upon removal of the required amino acid. Restriction of rRNA synthesis is more profound than the decrease in the rate of mRNA production; Lazzarini and Winslow (152), for example, find by DNA-RNA hybridization competition experiments that the rates of synthesis of rRNA and mRNA are reduced upon amino acid deprivation to 10 and 40%, respectively, of the rates observed in growing cells. The disproportionate inhibitions of rRNA and mRNA synthesis imply, again, that cells possess the capacity to regulate independently the transcription of genes specifying the two classes of RNA. The synthesis of rRNA, at least, is retarded under these conditions by prevention of the initiation of transcription of the rDNA by the RNA polymerase (279). This specific reduction in rRNA production is magnified by other consequences of amino acid deprivation which might affect RNA synthesis. These include, for example, a decrease in the

purine nucleoside triphosphate pools (79, 94) and a reduction in the nucleotide step time of the RNA polymerase (320). Moreover, Donini has pointed out (68) that the reductions in the rates of synthesis of rRNA and mRNA during starvation for an essential amino acid are probably insufficient to account for the reduction in the rate of RNA accumulation. This suggests that much, if not all, of the rRNA which is produced during starvation is rapidly degraded. Presumably the degradation is a consequence of the lack of synthesis of the ribosomal proteins; therefore, the rRNA molecules are not packaged into nuclease-resistant particles.

The ability of cells to respond to amino acid deprivation is governed at least in part by the gene *rel* (250). Wild-type strains are designated as "stringent" with regard to the control of RNA synthesis, whereas *rel*$^-$ strains, in which RNA synthesis continues unabated after removal of a required amino acid, are termed "relaxed". To date, mutants at the *rel* locus are known for *E. coli* (20, 282), *Salmonella typhimurium* (178), and *Bacillus subtilis* (288). One striking metabolic difference between *rel*$^+$ and *rel*$^-$ strains is that immediately after amino acid deprivation the *rel*$^+$, but not the *rel*$^-$, strain accumulates a significant quantity of an unusual nucleotide (38), which has been identified as guanosine, 5′-diphosphate, 2′-(or 3′)-diphosphate (ppGpp; 39). The function of the *rel* gene product is probably limited to conditions of growth which bring about starvation for an amino acid, but the enhanced production of ppGpp is observed concomitantly with any perturbation of growth which results in the restriction of rRNA synthesis. Both *rel*$^+$ and *rel*$^-$ strains of *E. coli* restrict their output of rRNA during step-down imbalance of growth (200) and during starvation for a carbon source (110, 151) or an inorganic nitrogen source (126). These same conditions promote the accumulation of ppGpp in both the *rel*$^+$ and the *rel*$^-$ genotypes (110, 126, 151).

At concentrations observed within *rel*$^+$ cells during amino acid deprivation, ppGpp inhibits in vitro RNA synthesis by purified RNA polymerase with regard to both chain elongation and initiation. The inhibition of chain initiation apparently is directed largely toward RNA chains with guanosine triphosphate (GTP) at their 5′-terminal (37). Cashel has suggested (37), on the basis of this latter observation, that the presence of ppGpp in starved, stringent cells could markedly alter the specificity of the RNA polymerase. If the transcript of the rDNA contains 5′-terminal GTP and if a portion of the mRNA population does not, then the preferential restriction of rRNA production during amino acid starvation might be accounted for. However, this suggestion is not substantiated by the findings of Ikemura and Dahlberg (J. E. Dahlberg, personal communication), who have observed that *rel*$^+$ strains of *E. coli*, starved for an essential amino acid, preferentially accumulate at least one RNA molecule with GTP at its 5′-terminus.

Regulation of rRNA Synthesis During Morphogenesis

Certain prokaryotes have the capacity to alter significantly their morphology and physiology in response to environmental effects and to enter dormant states. Examples of this simple form of morphogenesis include sporulation by the genus *Bacillus* and microcyst formation in *Myxococcus* species. Both of these genera restrict the net formation of ribosomes during the differentiation events, but they probably achieve this through different mechanisms.

The regulation of rRNA synthesis in sporulating *B. subtilis* has been reviewed briefly by Losick (162). During sporulation the synthesis of certain classes of mRNA required for vegetative growth is halted (65, 324), and the production of rRNA is dramatically reduced (124). Losick and his collaborators have provided evidence that the restriction of rRNA synthesis is not a consequence of some regulatory mechanism directed specifically toward the rDNA, but rather the template specificity of the RNA polymerase itself is modified. RNA polymerase purified from vegetative *B. subtilis* can utilize as template both phage ϕe DNA and poly(dA-dT), whereas the enzyme from sporulating cells is capable of actively transcribing only the synthetic DNA (164). The mechanisms responsible for altering the template specificity of the RNA polymerase from sporulating cells are not yet understood (158, 163, 169), but the alteration is also reflected in the inability of the polymerase to synthesize rRNA in vitro (125) and, presumably, in sporulating cells. This very neat scheme for regulating the expression of the rDNA in sporulating *B. subtilis* remains to be confirmed, however. Szulmajster and his colleagues have reported (18) that the basic premise of the scheme, that rRNA production halts in sporulating cells, may be incorrect. They find that although net RNA synthesis indeed is drastically reduced in populations of sporulating cells, the proportion which is attributable to rRNA is only about 30% lower than in cultures growing exponentially. Unfortunately they did not offer compelling evidence that all members of the cell populations studied in fact were undergoing sporulation. Since net RNA synthe-

sis is reduced in the sporulating cultures, if only a few percent of the cells escaped the commitment to sporulation and continued to grow exponentially, then these could account for the observed quantities of residual rRNA synthesis.

In contrast to *B. subtilis*, populations of *Myxococcus xanthus* which are induced to form microcysts continue to synthesize RNA, including ribosomal RNA, at almost the rate observed in vegetative cells (214, 239). This synthesis results in little net accumulation of RNA, however (7, 239). Therefore, either the ribosomes which existed before induction are degraded during cyst formation, or the newly synthesized rRNA is degraded soon after its synthesis. Probably both of these events occur, but degradation of rRNA synthesized during microcyst formation apparently is more important in balancing RNA synthesis with the absence of net accumulation. The immediate precursor of 16*S* rRNA, which is somewhat larger than the mature molecule (see below), is identifiable by sedimentation analysis in cells induced to form microcysts. However, mature 16*S* rRNA is not evident in induced cells after istopic labeling periods sufficiently lengthy to demonstrate its presence in vegetative cells (7). This implies that most (but possibly not all; 7) of the rRNA synthesized during cyst formation does not enter ribosomes, but rather it is degraded, probably at the precursor stage.

POST-TRANSCRIPTIONAL PROCESSING OF rRNA

Hypothetical Tandem Transcript of the rDNA

In mammalian cells, the transcriptional units specifying the rRNA molecules are composed of one gene each for 18, 28, and 28*S*-A rRNA's. The immediate product of transcription is a 45*S* molecule containing the sequences of these three rRNA components plus considerable excess material which is discarded during the post-transcriptional maturation events (4, 33). As described above, the rDNA of bacteria also is arranged in compound transcriptional units consisting of one gene each for 16, 23, and 5*S* rRNA's; any RNA polymerase molecule which reads the 16*S* DNA continues on to produce one 23*S* and one 5*S* rRNA sequence. However, no precursor of bacterial rRNA analogous to the mammalian 45*S* RNA has been isolated from normally growing cells. Instead, the identifiable precursors of 16 and 23*S* rRNA's are only slightly larger than the mature molecules. Following the nomenclature introduced by Hecht and Woese (116), I henceforth shall refer to the mature rRNA components as m16, m23, and m5 rRNA's, and to their precursors as p16, p23, and p5 rRNA's. These will be discussed at some length below.

There are two conceivable explanations for the absence of a compound rRNA precursor in bacteria. The first possibility is that, as the RNA polymerase molecules pass from, for example, the 16*S* gene into the 23*S* gene, they encounter some sort of signal to release the newly completed p16 molecule before commencing the synthesis of p23 rRNA. Alternatively, the cell might possess a specific and very active endonuclease which clips the completed p16 molecule from the nascent RNA chain very soon after the polymerase reads into the 23*S* gene. The available evidence strongly supports the latter possibility.

Pettijohn and his collaborators (231) have isolated the intact genetic apparatus from *E. coli;* the "nucleoids" retain the RNA polymerase molecules and nascent RNA strands which are associated with the DNA at the time of disruption of the cells. When the four nucleoside triphosphates then are added to preparations of the nucleoids, the resident RNA polymerase molecules complete the reading of the transcriptional unit with which they are associated, and they release identifiable classes of RNA molecules (233). The released RNA polymerase molecules are incapable of reinitiating synthesis, however, since the initiation factor σ is not present in the nucleoids. The two major RNA classes completed in vitro include one which apparently is slightly larger than p23 rRNA in size and the second, which has been termed p30, has the electrophoretic mobility in polyacrylamide gels expected of a molecule with the combined molecular lengths of 16 and 23*S* rRNA's. The in vitro reaction yields virtually no RNA that is the size of p16 rRNA. DNA-RNA hybridization competition experiments have shown that the p23-sized material indeed contains m23 rRNA sequences, but the p30 RNA includes both m16 and m23 rRNA; p30 apparently is a transcript of the entire rDNA (232; D. E. Pettijohn and C. R. Kossman, personal communication). If cells are radioisotopically pulse-labeled immediately before preparation of nucleoids, then both the p30 and p23 products completed in vitro contain the pulse label, but the pulse-labeled sequences associated with the p30 product are predominantly 16*S* rRNA (232). Since the 16*S* sequences are associated only with p30, which is completed in vitro, and these 16*S* sequences are initiated in vivo, then the 16*S* sequences must be in the 5′ portion of the p30 molecule. Little

p30-associated 23S RNA is labeled in vivo, so most of the p30 RNA must be the product of polymerase molecules associated with the 16S gene at the time of nucleoid isolation, which in vitro continue on to read through the 23S gene and, presumably, the 5S gene. The association of 5S sequences with p30 has not yet been demonstrated.

The production of a tandem transcript of the rDNA in vitro suggests strongly that the mechanism for the release of p16 from the RNA polymerase as it moves into the 23S gene is not encoded into the DNA in some fashion, but rather the release is effected by a specific endonuclease which is soluble and is removed from the nucleoids during purification. Thus, all of the 16S sequences completed in vitro are associated with p30; no cleavage enzyme is present to dissociate the p16 from the nascent RNA chain. The absence of the requisite, specific cleavage enzyme also may explain the observation (232) that the p23-sized material generated by the nucleoids is somewhat larger in size than the p23 rRNA appearing in vivo. The in vitro p23 (and p30 as well) probably contains the 5S rRNA in addition to the p23 sequence. The linked, p23-p5 RNA species has only a very brief lifetime in growing cells before it is cleaved to the longer-lived p23 and p5 molecules, and probably only part of the completed p5 population passes through the linked state. Isotopic label added to growing cultures of *E. coli* appears in p5 rRNA with higher order kinetics than the labeling of total RNA (1, 221), implying the existence of a rate-limiting step between the transcription of the rDNA and the release of p5 rRNA (27). The rate-limiting step presumably is the cleavage of the linked, p23-p5 rRNA. However, the difference in the rates of labeling of total RNA and p5 rRNA probably is not sufficiently great that all of the p5 sequences pass through the p23-p5 intermediate (1, 221), so it seems likely that many of the p23 rRNA molecules are clipped from the growing RNA chain soon after the polymerase migrates into the 5S gene.

The existence of endonucleases which separate p16 and p23 rRNA from the growing rDNA transcript also is implicit in the results of Chantrenne (44) and Grünberger et al. (105), who observed that *Bacillus cereus* cultures treated with 8-azaguanine accumulate a stable RNA component which is about the size of the tandem transcript of the rDNA. It has not been proven by the appropriate DNA-RNA hybridization experiments that the observed RNA molecule in fact contains both the 16 and 23S sequences, but in light of the data of Pettijohn and his colleagues it probably does. Presumably the 8-azaguanine is incorporated into the RNA, and its presence perturbs the polynucleotide structure such that the substrate sequence can no longer be recognized by the specific cleavage enzyme(s). The presence of the analogue would not be expected to interfere with p16 and p23 rRNA release if the RNA polymerase itself were somehow involved in the release of the completed chains.

Therefore, there are probably at least three endonuclease cuts made in the RNA product during the course of transcription of the rDNA or very soon thereafter. One of these is the removal of the hypothetical 5'-terminus of the rDNA transcript; the event is evidenced only by the observation discussed below that the 5'-terminus of p16 rRNA does not have the properties expected of an immediate product of the transcription reaction. A second scission event releases the p16 sequence from the growing 23S molecule, and a third frees the p23 molecule from the nascent or just-completed 5S chain. The enzymes involved in severing the growing rDNA transcript are highly sophisticated in their action; they must scan polynucleotides, pick a sequence, and cleave it precisely. It is not yet known whether only one enzyme catalyzes these three scission events or if each requires a specific enzyme. In either case, the nucleases effecting scission during transcription are probably distinct from those participating in post-transcriptional cleavage. The latter appear to require ribonucleoprotein aggregates as their substrates (see below), whereas the former are capable of functioning normally with more or less naked polynucleotide chains; inhibitors of protein synthesis do not retard the accumulation of p16, p23, and p5 rRNA's, but they do prohibit the subsequent cleavage events which generate the mature rRNA molecules.

Immediate Precursors of the rRNA Molecules

Soon after the advent of studies of ribosome biosynthesis, it was determined that the pulse-labeled RNA components of the ribonucleoprotein precursors of ribosomes differ somewhat from mature 16 and 23S rRNA's in their sedimentation velocities (141, 180) and in their behavior during chromatography on columns of methylated albumin-kieselguhr (157). In particular, the "immature" 16 and 23S rRNA molecules were observed to sediment slightly more rapidly than their mature counterparts, implying either that p16 and p23 are larger in molecular weight than m16 and m23, or that the precursors possess a more compact secondary

structure. When the precursor and mature forms of the rRNA molecules of *B. subtilis* (116) and *E. coli* (1, 221) were examined by polyacrylamide gel electrophoresis, it was apparent that the maturation of both rRNA components involved a reduction in size. Both p23 and p16 migrate more slowly in the gels than do the mature molecules; they therefore have either a higher molecular weight or a larger molecular cross section than do the mature rRNA components. However, if the precursors were equal to the mature forms in molecular weight but of greater cross section, then they would sediment more slowly than the mature molecules. The converse is the observation, and so p16 and p23 in principle must be of greater molecular length. The electrophoretic mobilities of the p16 and p23 products of both *B. subtilis* (116) and *E. coli* (1,221) suggest that they are, respectively, about 10 and 5% longer than the mature molecules in chain length. A minor portion (10% or so) of the p16 of *E. coli* is of slightly more rapid electrophoretic mobility than the majority (1, 59). This difference may not reflect size differences, but rather conformational alternatives of the p16 molecules (29, 59). Pulse-labeled molecules similar in size to p16 and p23, and therefore presumably occupying precursor relationships to m16 and m23 rRNA's, have been identified in several other diverse prokaryotes, including, for example, *Salmonella typhimurium* (306), *Myxococcus xanthus* (7), the blue-green alga *Anacystis nidulans* (69, 293), and even the parasitic trachoma agent (107). The widespread occurrence of transient p23- and p16-like molecules suggests that similar precursors are involved in rRNA formation in all prokaryotes. A search for exceptions would be of some interest.

The precursor forms of the rRNA molecules are identifiable not only in pulse-labeling experiments, but they also accumulate under any conditions which prohibit protein synthesis while permitting continued RNA production. Antibiotic inhibitors of protein synthesis such as chloramphenicol (122, 148) or puromycin (58, 122, 259) prevent rRNA maturation, as does removal of an essential amino acid from auxotrophic strains carrying the *rel*$^-$ lesion, so that rRNA synthesis is not halted (57, 194, 202). Other conditions which retard the formation of functional proteins and lead to the accumulation of p16 and p23 in *E. coli* include fluorouracil addition to cultures (142), toluene treatment of cells (229), probably potassium depletion (82), and treatment of cells with cobalt chloride (15). The precursor rRNA molecules also accumulate under restrictive growth conditions in conditionally lethal mutants of *E. coli* which are defective in the assembly of ribosomes (*sad* mutants; 106, 195). The rRNA precursors produced by cells treated with chloramphenicol or starved for an essential amino acid have received considerable attention because they are recoverable as ribonucleoprotein aggregates which at one time were thought to be intermediates in ribosome assembly (148, 194). Osawa (215) and Nomura (208) have reviewed these studies in some detail. The only relationship that these particles have with the precursors of ribosomes is their rRNA content, however. The associated proteins are extremely heterogeneous in their electrophoretic properties, and they bear little resemblance to the true ribosomal proteins (263, 328). Presumably the particles are random aggregates of basic proteins and the polyanionic rRNA molecules. The important point is that inhibition of protein production prevents rRNA maturation. This implies either that the enzymes involved in the post-transcriptional cleavage of p16 and p23 recognize as their substrates not the free RNA, but rather a specific rRNA-ribosomal protein aggregate, or that the cleavage enzymes turn over rapidly. The inability of the cold-sensitive *sad* mutants to carry out the maturation of rRNA (195) suggests that the former possibility is correct. These mutants are incapable of assembling ribosomal subunits at restrictive temperatures, but protein synthesis is not immediately affected.

The comparisons of the sedimentation and electrophoretic properties of the precursor and the mature rRNA molecules is strong evidence that they differ in their molecular weights, but the data do not prove such differences. In fact, it has been reported that heat denaturation of p16 and m16 renders them indistinguishable by methylated albumin-Kieselguhr chromatography (292) and that denaturation of p23 and m23 converts them to forms which can no longer be separated by polyacrylamide gel electrophoresis (59). These observations would seem to imply that the precursor and mature rRNA molecules are merely conformational isomers of one another, but this conclusion is certainly incorrect, and the basis for the experimental data is not clear. Recent detailed comparisons of oligonucleotides released by T1 RNase digestion of the precursor and mature rRNA molecules of *E. coli* (29, 115, 165, 272), *Bacillus megaterium*, and *B. subtilis* (M. L. Sogin, Ph.D. thesis, Univ. of Ill., Urbana, 1972) have proven beyond doubt that rRNA maturation involves size transitions. T1 RNase releases from any given RNA molecule a specific array of

oligonucleotides, which is characteristic of the primary structure of that RNA molecule; the quantitative yield of the various oligonucleotides is diagnostic of the size of the molecule. The p16 and p23 products of both genera proved to contain nucleotide sequences not present in the mature molecules, and the observed sizes of the precursor and mature molecules confirm the results suggested by gel electrophoresis. The p16 rRNA molecule is about 10% longer than m16 in all organisms examined, whereas p23 is about 5% longer than the mature molecule in *E. coli* and about 7 to 8% longer in the *Bacillus* species. Therefore, during maturation about 150 to 200 nucleotides are cleaved from each of the precursor rRNA chains.

The p16 and m16 rRNA molecules of *E. coli* have received the most detailed attention with regard to their structures (29, 115, 165, 272). The precursor contains sequences at both the 3'- and 5'-termini which are not present in the mature molecule, so the conversion of p16 to m16 rRNA involves at least two cleavage events. However, the distribution of the excess materials at either end of the p16 molecule is not yet known. The sequences associated specifically with the p16 rRNA of *E. coli*, at least, are not particularly distinguishing except that their pyrimidine content is rather higher than that of the mature rRNA molecule as a whole (M. L. Sogin, Ph.D. thesis, Univ. of Ill., Urbana, 1972). It is also noteworthy that the 5'-terminal nucleotide of p16 rRNA is 5'-monophosphorylated uridylic acid (29, 115, 165, 272); it is not a 5'-triphosphorylated purine, as might be expected if it were the initial nucleotide deposited by the RNA polymerase during transcription (25, 170). This suggests that a segment may be removed from the 5'-terminal portion of the RNA transcript of the 16*S* gene before or very soon after the release of p16 from the polymerase-DNA complex.

Rather less detail is available regarding the conversion of p23 to m23 rRNA. It is only known with certainty that the p23 molecules of the organisms examined are larger than their mature counterparts and that the 5'-termini of the precursor and mature components differ; sequences must be removed from at least the 5'-termini during maturation (M. L. Sogin, Ph.D. thesis, Univ. of Ill., Urbana, 1972). The difficulty in precisely defining the p23-specific sequences is that the large sizes of the precursor and mature molecules severely complicate their analysis with the available technology. Structural changes occurring during the maturation of p16 rRNA are more easily monitored only by virtue of the smaller sizes of the molecules involved, but even 16*S* rRNA is sufficiently large that it is not an ideal system for exploring the mechanics and the rationale of the rRNA maturation process.

The kinetics of the maturation of p16 and p23 have been monitored in *B. subtilis* (116) and *E. coli* (1, 221) by polyacrylamide gel electrophoresis. Both p23 and p16 are abruptly reduced to the sizes of the mature rRNA components; transition products intermediate in size between the precursor and mature forms which are sufficiently long-lived to be detected do not appear to exist within the limits of resolution of polyacrylamide gel electrophoresis. In *E. coli* growing at 37 C, the average p16 molecule remains in the precursor form for about 4 to 5 minutes before it is converted to m16 rRNA, whereas the lifetime of p23 is only about 2 to 3 min (1, 221). It is not yet possible to decide conclusively whether the enzymes effecting maturational cleavage draw upon the pools of the precursors at random, or if each precursor molecule must retain the precursor-specific nucleotide sequences for a definite length of time, while certain operations are performed upon the rRNA. Probably the latter possibility is correct; the precursor rRNA components at least must interact with numerous ribosomal proteins before they become suitable substrates for the cleavage enzymes.

Relatively little effort has been expended on the identification of the enzyme or enzymes involved in the cleavage events accompanying rRNA maturation. RNase II, a processive 3'-exonuclease (268, 276), has been implicated in the cleavage of p16 to m16 rRNA in *E. coli*, but recent evidence does not support its involvement. Corte et al. (50) and Yuki (331) first noted that *E. coli* N464, which produces a temperature-sensitive (*ts*) RNase II, is incapable of producing mature ribosomes or mature rRNA at restrictive temperatures. Moreover, purified p16 rRNA was converted in vitro by ostensibly highly purified RNase II to materials migrating in polyacrylamide gels coincidently with m16 rRNA, with the release of a fragment at least 100 nucleotides in length (50).

The simplest interpretation of these results would be that RNase II mediates the conversion of p16 to m16, although the action of the enzyme would seem to be more complex than expected of a simple exonuclease; it also must be able to serve as a highly specific endonuclease. However, two observations invalidate this conclusion. First, Weatherford and his colleagues (314) have examined the RNase II of 40 independent, ts^+ transductants of a temperature-sensitive strain of *E. coli*, which initially produced a thermolabile RNase II. All isolates retained the temperature-sensitive RNase II but were capable of growing normally at elevated temperatures and, therefore, of carrying out

normal rRNA metabolism. 16S rRNA purified from the ts^+ transductants grown at elevated temperatures was definitely m16 in size (314; D. Apirion, personal communication), and so the observed accumulation of p16 rRNA in *E. coli* N464 at restrictive temperatures certainly was not a consequence of the defective RNase II. Furthermore, Weatherford et al. (314) recovered essentially normal levels of RNase II from the ts^+ transductants grown at elevated temperatures; the RNase II^{ts} mutation apparently is not manifested in vivo.

Since RNase II apparently is not responsible for the cleavage of p16 rRNA, then the mutation of rRNA maturation to temperature sensitivity fortuitously accompanied the mutation to RNase II thermolability, and the rRNA cleavage enzyme more or less co-purified with RNase II. These unlikely accidents seem to have occurred. Schlessinger and his co-workers have noted (313) that fractions from diethylaminoethyl-cellulose chromatography of "purified" RNase II do not have constant proportions of endonuclease and exonuclease activities, suggesting that the two reactions are effected by different proteins. The endonuclease activity which ostensibly is capable of converting p16 to m16 rRNA (50) has not yet been characterized in detail. If this endonuclease in fact is responsible for 16S rRNA maturation, then it is curious that it is capable in vitro of utilizing purified p16 rRNA as a substrate, whereas in the cell p16-ribosomal protein aggregates are required.

The involvement of post-transcriptional cleavage steps in the maturation of rRNA is not limited to 16 and 23S rRNA's. The mature 5S rRNA components of all prokaryotes so far examined also are fragments of larger precursors. Since 5S rRNA is small in size (120 nucleotides in *E. coli*), the details of its maturation are more or less amenable to experimental definition. Monier and his colleagues (87, 88, 188) have focused considerable attention on the p5 rRNA of *E. coli*, which is identifiable in pulse-labeled cells and which accumulates in the absence of protein synthesis. As isolated, this precursor is a mixed population of RNA molecules which are only one, two, or maximally three nucleotides larger than the mature 5S rRNA. All of the additional nucleotides are associated with the 5'-terminus (87, 88), and kinetics experiments (87, 93) have demonstrated that during maturation the additional nucleotides are removed in a stepwise fashion until the mature 5S length is achieved. 5S rRNA precursors similar in size to those of *E. coli* have been detected in *Salmonella typhimurium* (240) and the blue-green alga *Anacystis nidulans* (W. F. Doolittle, personal communication), so this scheme for 5S maturation may be rather widespread among the prokaryotes. Unfortunately, the scheme is so simple that it probably cannot provide a useful model system for understanding the tailoring events involved in the production of the mature, high-molecular-weight rRNA components.

The maturation of 5S rRNA in the genus *Bacillus* (222) is very much more complex than that described for *E. coli*, and it resembles to a considerable degree the maturation of p16 rRNA. In the absence of protein synthesis or in pulse-labeling experiments, two precursors of 5S rRNA, present in about equimolar quantities, are evident in *B. subtilis* (222). One of these, $p5_A$, is about 180 nucleotides in length, and the second, $p5_B$, is composed of approximately 150 nucleotides. As with the p16 rRNA of *E. coli*, the non-5S sequences of $p5_A$ and $p5_B$ are associated with the 5'- (216) as well as the 3'- (M. L. Sogin and N. R. Pace, unpublished observations) termini of the 5S sequence. Both $p5_A$ and $p5_B$ are rapidly and apparently independently converted to the mature 5S rRNA, so $p5_B$ does not appear to be an intermediate on the $p5_A$ maturation pathway. There seem to be two possible explanations for the existence of these independent 5S precursors: either they are the products of independent 5S genes, or they are the result of two, alternate substrate sites for the cleavage enzyme that releases p23 rRNA from the rDNA transcript as the RNA polymerase passes from the 23S into the 5S gene during rRNA synthesis. Whether $p5_A$ and $p5_B$ have independent functions during their integration into the immature ribosomes remains to be discovered.

Function of the Precursor-Specific Sequences

Each precursor rRNA species thus far studied has been found to be largely homogeneous with regard to the T1 oligonucleotides attributable to its precursor-specific regions. This homogeneity implies that the sequences cleaved from the precursors during maturation are functional; since multiple genes are involved as templates for the rRNA molecules, the precursor-specific sequences would be expected to diverge in their detailed structures unless held more or less constant by functional constraints. Two sorts of roles might be envisaged. The first of these is that the precursor-specific sequences might facilitate the coalescing of the ribosomal proteins with the rRNA to form the ribosomal subunits. For example, the termini of the precursors might interact in some fashion with the mature rRNA sequences, permitting them to assume required conformations which otherwise would be thermodynamically unfavorable. After removal of

the excess sequences, the conformation of the mature rRNA chain would be stabilized by interaction with the ribosomal proteins. The incorporation of the rRNA molecules into the ribosomal subunits certainly does not require the presence of the precursor-specific termini, however. The purified, mature rRNA molecules and ribosomal proteins spontaneously unite to form 50 and 30S ribosomal subunits under the appropriate in vitro conditions (146, 209). Nevertheless, the assembly rate apparently is more efficient in the cell. Traub and Nomura (309) have investigated the temperature dependence of the in vitro assembly of the 30S subunit from mature 16S rRNA and the 30S subunit proteins from *E. coli*, and they find that above 30 C or so, assembly occurs at about the same rate as in vivo, but below that temperature the in vitro assembly is extremely slow. This suggests that cells possess some mechanisms for facilitating ribosome construction which are not inherent in the structures of the mature rRNA molecules and the ribosomal proteins. The excess sequences associated with the precursor rRNA molecules conceivably participate in this process.

The second possibility for the role of the precursor-specific sequences is that they merely are part of the substrate sites recognized by the enzymes which cleave the tandem transcript of the rDNA into the component rRNA molecules. With either of these two conjectures, removal of the excess sequences would be required if the termini of the mature rRNA molecules were in some manner required for ribosome function.

Regardless of the function of the precursor-specific sequences, at least the p16 rRNA which accumulates in *met*$^-$, *rel*$^-$ *E. coli* starved for methionine is incapable of fulfilling the role of m16 rRNA. Wireman and Sypherd (personal communication) have reconstituted 30S particles from the requisite proteins and relaxed p16 rRNA. The particles formed have certain of the attributes of normal 30S subunits, including the ability to bind polyuridylic acid, but they lack the capacity to bind protein S3, and consequently they cannot bind $tRNA_{Phe}$ (146) and associate with 50S subunits to form the functional 70S complex. However, whether the inability of p16 to bind all of the ribosomal proteins is due to the presence of the precursor-specific sequences or to the lack of methylation in the relaxed RNA is not yet known.

Post-Transcriptional Modification of Nucleosides

The post-transcriptional metabolism of the rRNA components includes not only the cleavage events, but also the methylation of select nucleosides and 5-ribosyluracil formation. Borek and Srinivasan (21, 278) and Hall (108) have discussed these events at length. The basic fact is that nucleosides are not methylated before incorporation into RNA; methyl groups are transferred from *S*-adenosylmethionine to a polynucleotide acceptor. Most studies of the methylation of RNA have been directed toward tRNA, but some information is available regarding the rRNA of *E. coli*. The deposition of methyl groups in the 16S rRNA of *E. coli* probably occurs in two stages. The first stage is during the formation and lifetime of p16 rRNA, and the second either immediately precedes or immediately follows the size transition from p16 to m16 rRNA. The evidence for the first stage of methylation is that the p16 rRNA which accumulates in cells treated with chloramphenicol (73) or in *rel*$^-$ strains starved for an essential amino acid (other than methionine, the methyl donor; 290) contains only about 30 to 50% of the normal complement of methyl groups. The methylated nucleosides present in the p16 rRNA are not representative of the total present in m16 rRNA, however; only specific sequences are methylated (115, 272, 273). These methylation events are effected in the absence of protein synthesis, and so the responsible methylating enzymes must be capable of recognizing the free polynucleotide chain, even if the rRNA is somewhat cluttered by association with the nonribosomal, basic proteins which are constituents of the "relaxed" or "chloramphenicol" particles. It is not yet certain whether methylation is initiated during completion of the nascent rRNA chains or if the completed p16 or p23 molecules are required as substrates, but probably methylation begins before the completion of the transcription process; at least one of the specific methylating enzymes has no requirement for intact rRNA (see below). The second stage of methylation, during which the remainder of the methylated nucleosides characteristic of mature 16S rRNA are formed, occurs only during active protein synthesis; restoration of a required amino acid to starved *rel*$^-$ cells apparently permits completion of methylation of at least some of the submethylated rRNA which accumulates during starvation (290). So, the enzymes effecting these final methyl substitutions presumably require as their substrates not the free polynucleotide chains, but rather the ribonucleoprotein precursors of the ribosomal subunits.

Some attention has been devoted to the isolation and characterization of the specific methylases involved in rRNA metabolism, but most studies have pursued the tRNA methyl-

ases (108). Gordon and Boman (101) first reported that submethylated rRNA, from *rel*⁻ cells starved for methionine (the ultimate methyl donor), is capable of accepting methyl groups in vitro, but no attempts were made to isolate the specific methylases involved. Recently, two rRNA methylases have been partially characterized in vitro. One of these is an example of the enzymes which are capable of methylating free polynucleotides, and the second functions only with rRNA-ribosomal protein aggregates.

Sipe et al. (270) have extensively purified from *E. coli* strain B an adenine (N^6-) methyltransferase, which is responsible for the formation of N^6-methyladenine in at least m23 rRNA (73, 86, 281). The enzyme utilizes *S*-adenosylmethionine as a donor, and it deposits a limited number of methyl groups in purified, methyl-deficient (isolated from *rel*⁻, *met*⁻, methionine-starved cells) rRNA from *E. coli* B, but not in mature rRNA from the same strain of *E. coli*. This methylase, therefore, appears to be quite specific in its action with homologous rRNA, although it has not been demonstrated rigorously that the adenine residues methylated in vitro are associated with the same sequences as in mature rRNA. However, the specificity of the in vitro reaction apparently is reduced if the methyl acceptor is rRNA from *Micrococcus lysodeikticus* or *B. subtilis;* the mature rRNA molecules from these organisms are effective acceptors, and so either spurious methylation is occurring or nonmethylated sequences are present, which in the rRNA of *E. coli* would be methylated.

A second type of methylase, that responsible for dimethyladenosine formation in *E. coli*, has been partially purified by Helser et al. (118). As discussed above, the lack of this enzyme in certain mutants confers resistance to the antibiotic kasugamycin; the me_2Ame_2ACCUG sequence in 16*S* rRNA, which somehow participates in binding the drug, is not produced in the resistant strains (117). Protein preparations containing this methylase use *S*-adenosylmethionine as a methyl donor to specifically dimethylate the appropriate adenine residues in 16*S* rRNA from kasugamycin-resistant strains of *E. coli*, as demonstrated by oligonucleotide fingerprint analysis, but the 16*S* rRNA from normal cells, which already possesses the dimethylated sequence, is not an acceptor. This methylase is distinct from that described by Sipe et al. (270) not only in its reaction products, but also in its substrate requirements. The dimethylase functions only with rRNA-ribosomal protein aggregates; purified rRNA is not an effective substrate. This finding is in concert with observations that the methyl substitutions in the me_2Ame_2ACCUG sequence are not present in p16 rRNA prepared by any of various procedures (115, 165, 273); they have been observed only in 16*S* rRNA from mature 30*S* particles. Therefore, this dimethylase must function either immediately before or immediately after the cleavage of p16. In their studies, Helser et al. used as substrates for the dimethylase the "core" ribosomal particles obtained by buoying mature ribosomal subunits in CsCl gradients (307). This procedure strips many of the ribosomal proteins from the subunits. These core particles may be useful as assay substrates for many of the modifying enzymes, if suitable mutants lacking the modification of interest may be isolated or if enzymes and ribosomal core substrates from different organisms do not possess the same specificities as the homologous pairs (cf. 270).

The majority of methyl substitutions in the rRNA of prokaryotes is on the purine or pyrimidine bases. Only a few 2′-*O*-positions on the ribose moiety of the polynucleotides are methylated. Nichols and Lane (204) have determined that the enzymes involved in these methylations also are amenable to in vitro study. Crude extracts of *E. coli* transfer methyl groups from *S*-adenosylmethionine to the precursor rRNA components from methionine-starved *rel*⁻ cells to form $O^{2'}$-methylguanosine and $O^{2'}$-methylcytidine. This system cannot generate N^4,$O^{2'}$-dimethylcytidine or $O^{2'}$-methyluridine, however Possibly the enzymes responsible for these latter modifications cannot function with the free polynucleotides, but rather require the rRNA-ribosomal protein aggregates.

Even less information is available regarding the formation of 5-ribosyluracil (pseudouridine) in rRNA. The chemistry (41) and biochemistry (100) of this modified nucleoside have been reviewed. Dubin and Günalp (73) have determined that chloramphenicol inhibits the formation of pseudouridine in the rRNA of *E. coli*, so it must be formed post-transcriptionally, and presumably the responsible enzymes require ribonucleoprotein precursors of ribosomes as substrates. There have been no reports of in vitro studies of pseudouridine formation in rRNA, but Johnson and Söll (130) have defined an in vitro system for its formation in unmodified tRNA.

Interaction of the rRNA with the Ribosomal Proteins

During their maturation the rRNA molecules must aggregate with the ribosomal proteins to form the functional ribosomes. This aggregation

process is nonrandom; specific proteins are added to the rRNA-protein complex at specific stages in the formation of the mature ribosomal subunits. The sequence of addition of the ribosomal proteins to the maturing 30*S* subunit of *E. coli* has been more or less completely delineated by a number of investigators through the stepwise in vitro reconstitution of the subunit from its isolated components. These results have been reviewed recently (146, 209). Only six of the 20 to 21 ribosomal proteins from 30*S* subunits are capable of binding to 16*S* rRNA (258, 259, 284); the remainder bind only to rRNA-ribosomal protein aggregates. Similarly, only a fraction of the proteins of the 50*S* subunit will bind in a specific fashion to 23 or 5*S* rRNA's (146, 284). Four, and possibly five, of the RNA-binding proteins from the 30*S* subunit interact with the 5'-terminal half of the 16*S* molecule, and they have no requirement for intact rRNA as a binding substrate (333). Therefore, in the growing cell the ribosomal proteins could begin to aggregate with the rRNA chains even before their synthesis is complete. Protein aggregation with the nascent rRNA probably has no influence upon the transcription process or upon the specific scission of the growing tandem transcript of the rRNA transcriptional unit; these events appear to occur normally in the absence of protein synthesis.

Early investigators of the formation of ribosomes suggested that the rRNA molecules might serve as mRNA in defining some or all of the ribosomal proteins (215, 243). Although the mature rRNA components are incapable of serving in vitro as mRNA for the synthesis of proteins (207, 303, 319), there were some indications that the precursor rRNA molecules, which accumulate during treatment of cells with chloramphenicol (216) or during starvation for an essential amino acid (193), indeed might be able to do so. However, Manor and Haselkorn (174) and Sypherd (289) subsequently found that the template activity attributed to the rRNA precursors probably was due to contaminating, bona fide mRNA. When sufficiently highly purified, the immature rRNA species were ineffective as templates for polypeptide synthesis. More recently, Miller and his colleagues (185) have examined, by electron microscopy, complexes of rDNA and nascent rRNA, which are identifiable in lysates of *E. coli* on the basis of the size of the rRNA transcriptional units and the relatively high packing density of the nascent RNA strands. In contrast to the nascent mRNA molecules, none of the growing RNA chains associated with the putative rDNA has ribosomes attached to it. This observation provides visual assurance that rRNA is never translated.

CONCLUDING COMMENTS

It should by now be apparent that a considerable body of descriptive information regarding the rRNA molecules of prokaryotes is available. However, this information is superficial, and few fundamental questions have been answered. For example, at this time we do not know the function (if any) of the rRNA molecules during protein synthesis beyond their role as skeletal structures for the ribosome. The evolutionarily conservative character of certain features of rRNA implies that the rRNA molecules enjoy rather more importance than that of a mere framework. It is my feeling that the roles of the rRNA molecules probably as well include dynamic participation in the exquisitely complex operation of the ribosome. A few other facets of the rRNA molecules whose functions remain to be discovered are the modified nucleosides and the "excess" nucleotide sequences associated specifically with the precursors of the rRNA molecules. Conceivably these latter features are trivial in function, but I suspect not. In animal cells, about one-half of the rRNA transcript is discarded during maturation of the rRNA-containing segments. The fact that these precursor-specific segments are so extensive implies their utility to the animal cell and, by extension, the utility of their prokaryotic counterparts.

As more information becomes available regarding the interactions of the ribosome and other elements of the translation apparatus, we can look forward to the challenge of intellectually reducing this apparatus to its essential components and speculating on the mechanics of its origin and function in the pre- or protobiotic milieu. The central importance of the protein-synthesizing machinery must have dictated its very early appearance in rudimentary form. Crick has suggested (54) that the requisite, individual RNA components of the primitive translation machinery arose independently in the prebiotic sea. This is not unreasonable, except for the choice of polymers. Divalent cation-catalyzed hydrolysis of polyribonucleotides (via a 2',3'-cyclic phosphate intermediate) probably precluded the random abiotic construction and survival of a sufficiently complex variety of RNA chains. Therefore, the original elements of the pre- or protobiotic translation apparatus likely were DNA, not RNA.

The information now available represents only a first step, but the prospect of achieving an understanding of the detailed mechanics of the translation apparatus appears bright.

SUMMARY

General Features of Prokaryotic Ribosomes

The ribosomes of phylogenetically diverse prokaryotes are superficially similar in their properties. The active ribosomes at physiological Mg^{2+} concentrations sediment as particles which are about 70*S* in size. At low Mg^{2+} concentrations or when not engaged in protein synthesis, each 70*S* particle dissociates into one 30*S* and one 50*S* particle, which are composed of, respectively, about 60 and 75% RNA. The remaining mass in the ribosomal subunits is contributed by numerous ribosomal proteins. The collections of ribosomal proteins from diverse prokaryotes are similar in the numbers and sizes of their components, but the corresponding proteins from different organisms do not necessarily display close antigenic relationships. Therefore, the prokaryotes have diverged considerably in the detailed structures of the components of their ribosomes. Nevertheless, functional homology has been retained; hybrid ribosomal subunits which are active in protein synthesis may be reconstituted from the component proteins and rRNA from little-related organisms.

Structures of the Prokaryotic rRNA Molecules

The ribosomes of prokaryotes generally contain three metabolically stable rRNA components. Two of these, 23 and 5*S* rRNA's, are derived from 50*S* ribosomal subunits. The third, 16*S* rRNA, originates from the 30*S* subunit. The molecular weights of 23, 16, and 5*S* rRNA's are, respectively, about 1.1 million, 0.55 million, and 0.04 million; these weights correspond to chain lengths of about 3,300, 1,650, and 120 nucleotides, respectively. A few photosynthetic prokaryotes, for example, *Rhodopseudomonas spheroides* or *Anacystis nidulans*, possess 23*S* rRNA only transiently. The molecule is specifically cleaved into two fragments, apparently without affecting the capacity of the ribosomes to carry out protein synthesis. The utility of the cleavage of 23*S* rRNA to the organisms remains obscure.

The nucleotide compositions of the total rRNA from diverse prokaryotes are remarkably constant, even though the base compositions of their genomes vary widely. The similar base compositions do not necessarily reflect similar primary structure, however. The rRNA components of phylogenetically dissimilar organisms frequently possess little homology in nucleotide sequence, as defined in DNA-RNA hybridization experiments. Therefore, the evolutionarily conservative features of the rRNA molecules which are reflected in their base compositions are probably their secondary or tertiary structures.

The 16 and 23*S* (but not 5*S*) rRNA components of all prokaryotes thus far examined each contain minor quantities (about 1%) of modified nucleosides, including methylated nucleosides and 5-ribosyluridine. About 90% of the methylated nucleosides are base substituted; the remainder are 2′-*O*-methylated. The function of the modified nucleosides remains to be determined, but their utility is implicit in the fact that many of the methylated nucleosides and the nucleotide sequences in their immediate vicinities are identifiable in the rRNA components of bacterial species which otherwise have little similarity in their primary structures. The functions of certain of the methyl substitutions are sufficiently subtle that they are superficially dispensable; mutants have been isolated which lack some individual modifications but which are capable of apparently normal growth.

Evaluation of the detailed primary, secondary, and tertiary structures of the rRNA molecules is only beginning. The nucleotide sequences of the 5*S* rRNA molecules of *E. coli* and *Pseudomonas fluorescens* are known, and about 70% of the sequence of the 16*S* rRNA of *E. coli* is known to an approximation. The determined structures of the rRNA molecules are compatible with physical measurements indicating that 60 to 70% of the bases are involved in hydrogen binding. In the case of the 16*S* rRNA of *E. coli*, the consequent secondary structure apparently is predominantly local; adjacent, short nucleotide sequences tend to be complementary, so that the molecule in solution and presumably in the ribosome might be viewed as a complex series of hairpin loops. The individual rRNA classes of a given strain of bacteria are largely homogeneous in their structures, but minor differences, in only a few nucleotides, are evident. These presumably occur because each organism possesses multiple copies of the rRNA genes, and their individual structures are evolving more or less independently. There is no evidence for functional heterogeneity within the rRNA population of individual prokaryotic organisms.

Genes Specifying rRNA

DNA-RNA hybridization saturation experiments have revealed that most prokaryotes contain multiple genes specifying the rRNA components. Generally, about 0.3 to 0.4% of the

total genome DNA is complementary to rRNA. For example, in *E. coli* this corresponds to about six copies of each of the genes specifying 16, 23, and 5*S* rRNA's; *Mycoplasma* species possess only one copy of each of the rRNA genes.

The lack of appropriate mutants in the rRNA genes has retarded locating them within the linkage map of *E. coli*, but at least most of the rDNA loci now have been mapped by DNA-rRNA hybridization experiments using episomes which span defined regions of the chromosome. One cluster of rDNA, comprising about three genes for each of the rRNA components, is located at about 74 to 77 min on the linkage map of *E. coli*. A second cluster, containing probably two genes for each of the rRNA species, is somewhere in the 54- to 59-min span. It is not yet certain whether all of the rDNA of *E. coli* is accounted for.

The rDNA in the *Bacillus subtilis* genome also is distributed between at least two regions, but the location of these has not been determined accurately. About 70% of the rDNA is located in the first 25% of the genome to replicate during spore germination; the remainder of the rDNA appears to be replicated with the terminal 25% of the genome. In both *E. coli* and *B. subtilis*, the individual rRNA genes are arranged in clusters containing one gene each for 16, 23, and 5*S* rRNA's, linked in that order. These clusters are separated by lengthy (at least 6 to 10 times the length of the 16-23-5*S* unit) stretches of non-ribosomal DNA.

Transcription of the rRNA Genes

A major fraction of the RNA being synthesized at any instant by *E. coli* is identifiable as rRNA. The rDNA is read by the RNA polymerase with a frequency 50 to 100 times that at which the average mRNA gene is read. The relatively high rate at which the rDNA is read apparently is determined by the affinity of the RNA polymerase for the promoter of the rDNA; no auxiliary protein factors are required for the preferential in vitro synthesis of rRNA by purified RNA polymerase holoenzyme in vitro.

To a good approximation, the RNA content and the number of ribosomes per cell are proportional to the rate of cellular protein synthesis and the rate of growth of the population. The accumulation of rRNA in growing *E. coli* is governed at several levels. The first control mechanism affects overall cellular RNA synthesis and might involve the availability of active RNA polymerase molecules. A second control seems to noncoordinately modulate rRNA synthesis relative to that of mRNA; the rRNA:mRNA ratio among pulse-labeled RNA may vary two- to threefold, depending upon the growth rate of cultures. The mechanism by which the frequency of transcription of the rDNA is specifically modulated is not known. Still a third means for regulating the accumulation of rRNA functions post-transcriptionally: rRNA is degraded. This process conceivably is controlled by the availability of ribosomal proteins.

Certain prokaryotes reduce their rates of accumulation of ribosomes during "differentiation" events. During endospore formation, *Bacillus subtilis* halts rRNA synthesis, apparently because the RNA polymerase is proteolytically modified so that it no longer is capable of recognizing the rDNA promoter. *Myxococcus xanthus* undergoing microcyst formation also restricts the net accumulation of ribosomes, but in this case there apparently is little or no retardation of the relative rate of rRNA synthesis. Instead, the newly synthesized rRNA, as well as probably some pre-existing rRNA, is degraded.

Post-Transcriptional Processing of rRNA

The genes specifying the rRNA molecules comprise transcriptional units; any RNA polymerase molecule which begins to read the 16*S* gene continues on to read the 23*S* gene and then the 5*S* gene before terminating transcription. This transcriptional organization guarantees production of equimolar quantities of the rRNA components. However, a tandem transcript of the rDNA does not normally appear in growing cells. Probably a specific endonuclease cleaves a completed rRNA chain from the nascent RNA soon after the RNA polymerase moves into the adjacent, "downstream" gene in the transcriptional unit.

The immediate products of transcription of the rDNA are not structurally identical to the mature rRNA components. In general, the precursor (p16) of mature 16*S* rRNA (m16) is about 10% larger than the mature molecule; p23 is 5 to 8% greater in molecular length than m23. During the maturation of p16 rRNA in *E. coli*, excess sequences are removed from both the 3'- and the 5'-termini. Sequences are removed from at least the 5'-terminus of p23 during its conversion to m23. The mature 5*S* rRNA molecules also are not the immediate products of the transcription. The p5 rRNA molecule of *E. coli* is maximally only three nucleotides larger than m5, but the maturation of 5*S* rRNA in *Bacillus subtilis* bears resemblance to events affecting the high-molecular-weight rRNA components; extensive, precursor-specific sequences are re-

moved from both termini. The function (if any) of the sequences associated specifically with the rRNA precursors is not known. The character of the enzyme(s) which affects the maturational cleavages also is unknown. The substrates for this cleavage enzyme(s) probably are not the free, precursor rRNA molecules, but rather aggregates of the precursors and specific ribosomal proteins; inhibitors of protein synthesis concomitantly prohibit rRNA maturation.

Post-transcriptional modifications of the rRNA molecules also include methylation of select nucleosides and the formation of 5-ribosyluracil. In *E. coli*, certain of the methylation events may occur in the absence of cellular protein synthesis, and therefore the enzymes catalyzing them are capable of utilizing as substrates polynucleotide chains which are not complexed with ribosomal proteins. Other methylation reactions, as well as the formation of 5-ribosyluracil, do not occur in the absence of protein synthesis; rRNA-ribosomal protein aggregates are required substrates for the responsible enzymes. Examples of methyl transferases, which employ as substrates either free rRNA or rRNA complexed with some ribosomal proteins, have been partially purified and characterized. All use *S*-adenosylmethionine as a methyl donor and specific nucleosides within the polynucleotide chains as acceptors.

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ADDENDUM IN PROOF

Subsequent to the assembly of the above information, several pertinent papers have been published or submitted to press. I would like to call attention to two noteworthy items. First, Travers (A. Travers. 1973. Control of ribosomal RNA synthesis *in vitro*. Nature (London) **244**:15-18) has again implicated the factor ψr in control of rRNA production. He finds that there exist two states for the rRNA promoter, "open" or "closed," depending upon the conformation of the DNA template. If in vitro RNA synthesis is carried out at low salt concentrations (0.01 M) or at elevated temperatures (38 C), then the rRNA promoters are "open" and the RNA polymerase holoenzyme is capable of preferentially transcribing the rRNA genes. At higher salt concentrations (0.1 M) and at a lower temperature (34 C), the rRNA promoters are "closed," and rRNA synthesis by purified RNA polymerase is substantially enhanced by the presence of ψr. Moreover, Travers has presented evidence that the ψr-stimulated rRNA synthesis is inhibited by physiological concentrations of ppGpp.

The second finding that I would like to point out is that of Schlessinger and his colleagues (N. Nikolaev, L. Silengo, and D. Schlessinger, 1973. Synthesis of a large precursor to ribosomal RNA in a mutant of *E. coli*. Proc. Nat. Acad. Sci. U.S.A., in press), who have presented evidence that RNase III is responsible for cleaving the tandem transcript of the rDNA into the immediate precursors of the mature rRNA molecules.

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