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GENETIC CONTROL OF RIBOSOME ASSEMBLY

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The assembly of E. coli ribosomes has been studied through the analysis of a new group of ribosome maturation mutants. These mutants, all blocked in a late stage in the maturation of 50S ribosomes, map at four different sites on the chromosome. These sites are distant from the known ribosomal protein sites at the str-aro E region of the chromosome. The ribosome precursor particles of the mutants contain precursor-type 23S RNA (p23 RNA) and 5S RNA. 43S particles of one of the mutants contain all but one of the normal complement of proteins. Precursor 43S particles from this mutant can be converted to particles with sedimentation values around 50S by incubation with extracts from either the wild-type organism or from other mutants. This in vitro conversion process differs considerably from the process of ribosome reconstitution and indicates a role for extrinsic factors in the maturation of E. coli ribosomes.

INTRODUCTION

Many of the important physical-chemical problems that are related to ribosome function and assembly are being studied by a wide range of technical approaches. In many of these studies the in vitro reconstitution system of Traub and Nomura (1) is an indispensible component. The in vitro reconstitution of 30S ribosomes of Escherichia coli led to the important notion that ribosomes of bacteria are formed by a self-assembly mechanism. However, this concept may not apply to the processes which govern ribosome assembly inside the cell.

The biosynthesis of ribosomes in vivo requires the participation of a large number of gene products: the genes for structural components (3 different RNA molecules and 55 different proteins) as well as the genes which specify a variety of RNA nucleotide methylases, RNA trimming enzymes and at least one enzyme that acetylates a protein of

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the 50S ribosome. Several years ago we initiated studies on the possible control of ribosome assembly by extrinsic factors. If there are factors which play a role in ribosome maturation in vivo other than ribosomal proteins and RNA, such factors should be revealed by the analysis of ribosome assembly defective mutants. Guthrie et al. (2) and Tai et al. (3) have shown that cold-sensitive mutants of E. coli or Salmonella typhimurium are a rich source of ribosome assembly mutants. Many of these cold-sensitive mutations reported thus far are linked genetically to strA and may involve ribosomal proteins (2–5). Our approach to this problem has been to genetically screen cold-sensitive ribosome assembly mutants for those classes which are not linked to the known structural genes for ribosomal proteins, with the aim of locating genetic loci that control extrinsic factors involved in ribosome assembly. We have described several mutants of E. coli which have these properties (6), and we will summarize our present knowledge of them here.

RESULTS

Mutants Altered in Ribosome Assembly

Over 200 cold-sensitive mutants were screened for defects in ribosome assembly by double-label and zone sedimentation analysis. Ten mutants defective in 50S subunit assembly were found, and these fell into two classes. One class accumulated 43S ribonucleoprotein particles at 20° with little or no 50S subunit accumulation, and the second class accumulated 32S particles at 20°. These mutations were designated rim (ribosome maturation). Figure 1 shows the zone sedimentation profiles of the cells which are typical of each of these classes. The mutants were selected for their inability to grow and form colonies in 48 hr at 20° (6). However, as Table I shows, all of the mutants are capable of exponential growth at slow rates at this low temperature.

Mutant cells that accumulate precursor particles at 20° can convert the particles to normal ribosomes when the cells are put back at 37°. Because the work of Nomura and his coworkers (5) demonstrated that some cold-sensitive ribosome assembly mutants had defects in genes controlling ribosomal proteins, we analyzed the proteins of both 30S and 50S particles of the mutants grown at 37°. Under these conditions there were no differences, as determined by two-dimensional acrylamide gel electrophoresis between the wild-type cells and any of the mutants described here (7).

We concentrated our further studies on four mutants which were characteristic of the four genetic groups. These were designated rim A, rim B, rim C, and rim D. The RNA of the rim mutants and of the parental strain was analyzed by acrylamide gel electrophoresis (8). As Fig. 2 shows, the mutants accumulate precursor ribosomal RNA's when the cells are grown at 20°. In Fig. 2, the slowly migrating RNA species are referred to as P23 and p16 RNA by analogy with the two types of RNA observed in kinetic analysis and by the chemical analysis of pulse-labeled, chloramphenicol-treated, and cold-sensitive ribosome assembly defective cells (9–12). All of these studies have shown that molecules of RNA which migrate more slowly in acrylamide gels are larger than the mature species. In these cases, the processing of the precursor molecules apparently involves the removal

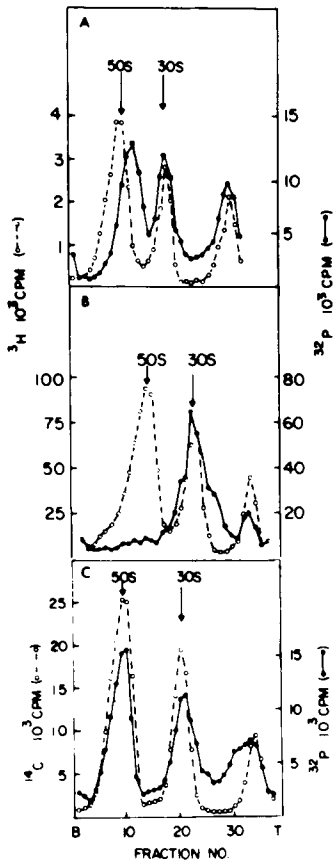

Fig. 1. Zone sedimentation profiles of ribosomal subunits of cold-sensitive mutants and parental strain: A) rim A, B) rim C, C) parental strain, AB1472. Cultures were grown in MOPS medium at 37° C in the presence of H^{3}° - Uracil to a cell density of 4 X 10^{8} to 6 X 10^{8} cells/ml. Cultures were then shifted to 20°C and grown in the presence of $P^{32}O_4^{=}$ until Klett readings had increased by 25 to 50%, and then harvested. Cell extracts were sedimented through a 5–20% (wt/vol) linear sucrose gradient. From Bryant et al. (17).

TABLE I. Doubling Times of Parental and Mutant Strains

Strain	Doubling Time* (37°C)	Minute (20°C)
Parental strain (AB 1472)	40	155
rim A	55	350
rim B	45	170
rim C	75	410
rim D	70	360

*Doubling times were determined in MOPS medium (6) by measuring increases in turbidity on a colorimeter.

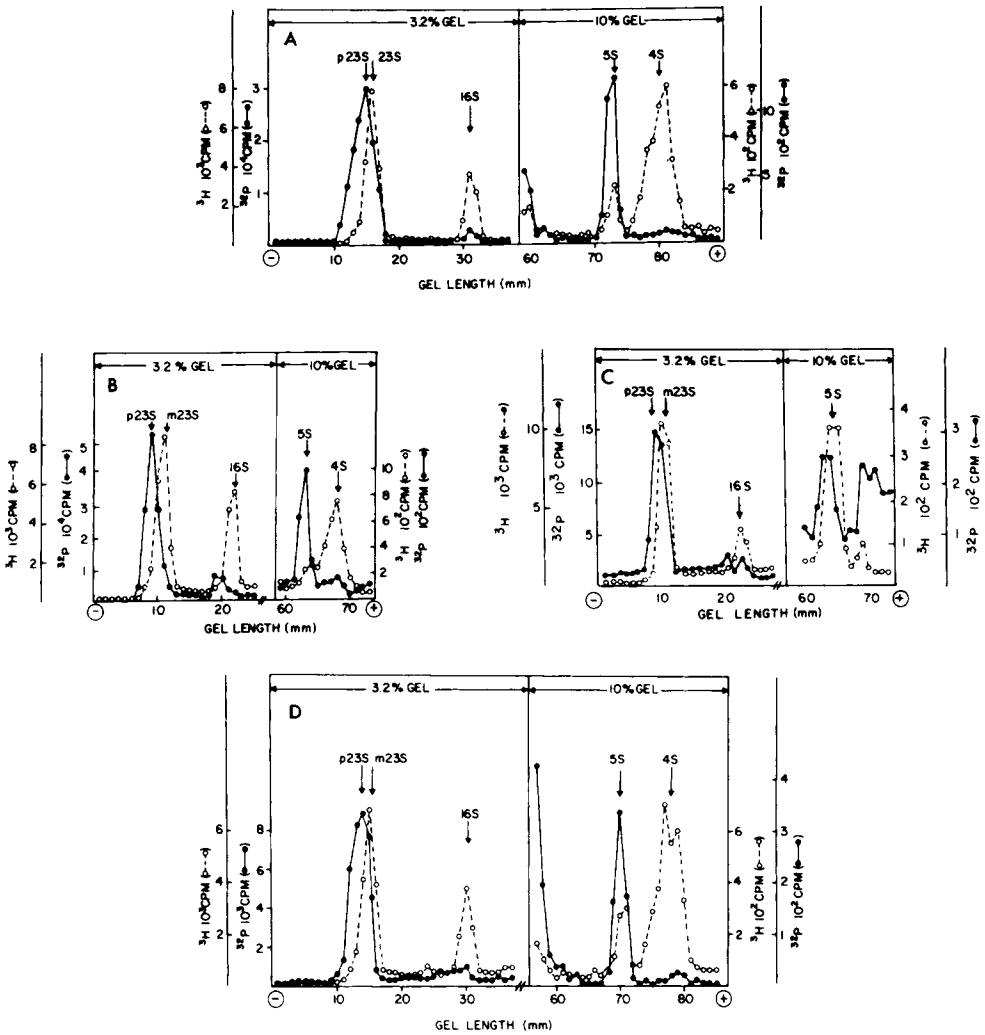

Fig. 2. Polyacrylamide gel patterns of RNA extracted from the particles of cold-sensitive rim mutants. Gels consist of composite 3.2%/10% acrylamide. H^3-labeled RNA was added as a marker- A) rim A, B) rim B, C) rim C, D) rim D. From Bryant et al. (7).

of oligonucleotides from the 3′ and 5′ ends of the p16 RNA and at least the 5′ end of the p23 RNA (13). The available evidence shows that the slower migrating species of rRNA behave kinetically as precursors to the faster, or mature, forms. The data in Fig. 3 show that RNA from 30S ribosomes of the parental strain grown at 20° consists of both the p16 and m16 RNA species. This seems to indicate that even in the wild-type cells, the processing of RNA is considerably slower at 20°. But more importantly, it also indicates that the trimming of the excess oligonucleotides is a very late step in ribosome maturation,

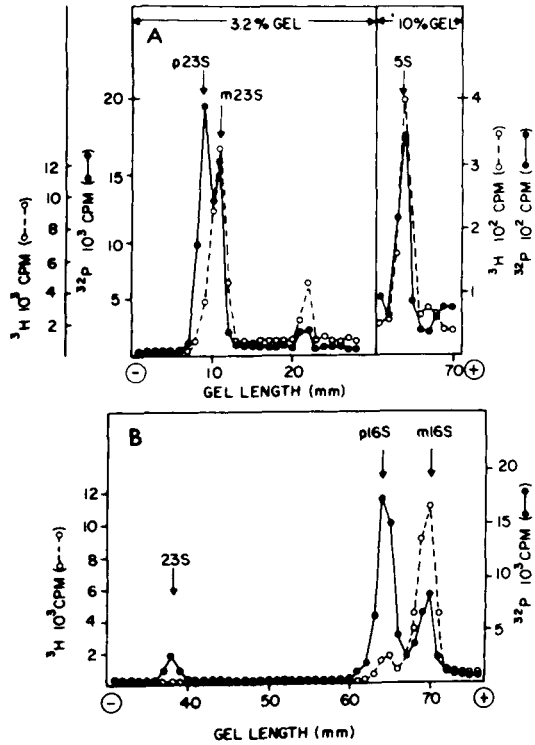

Fig. 3. Polyacrylamide gel patterns of RNA extracted from ribosomes of AB1472, the parental strain of the rim mutants: A) composite 3.2%/10% acrylamide gel of RNA extracted from 50S subunit. B) 2.8% acrylamide gel of RNA extracted from the 30S subunit. From Bryant et al. (7).

since 30S ribosomes contain the precursor molecules. The accumulation of p16S and p23S RNA at 20° was also found to occur in other E. coli strains.

The data in Fig. 2 show that 5S RNA is present in both the 43S and 32S particles of the mutant bacteria. Quantitative estimates of the 5S RNA content of these particles gave variable results. However, we could conclude that the precursor particles contained from 50 to 100% of the normal 5S RNA content. The lower figure is probably due to a loss of 5S RNA from the precursors during particle isolation (14).

Genetics of Rim Mutants

All but one of the ribosomal proteins that have been mapped have been shown to be specified by loci near strA at minute 64 on the E. coli chromosome (15). The one exception is protein S18 which maps between 76 and 88 min (16) on the chromosome. The demonstration that a ribosome mutation is at strA constitutes strong genetic evidence that the mutation resides in a gene coding for a ribosomal protein. An important question

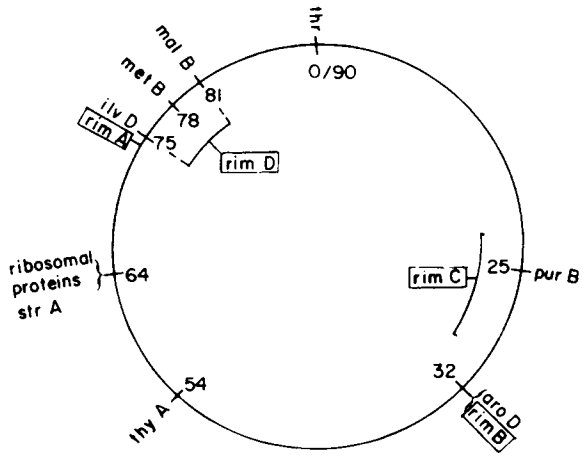

Fig. 4. Genetic map of E. coli indicating the map positions of rim loci. The positions of rim C and rim D are based on conjugation data and are approximate. The positions of rim A and rim D were determined by contransduction with ilv and aro D, respectively. After Bryant and Sypherd (6).

regarding the molecular nature of the assembly defects in the rim mutants was whether the mutations involved ribosomal proteins. Mutants representing 3 of the 4 rim loci were tested for co-transduction of the cold-sensitive marker with aroE, which serves as a nutritional marker for the strA region. Lysates of transducing phage P1 were prepared on mutants rim A, rim B, and rim C. An aroE strain was used as the recipient in transduction experiments; $aroE^+$ recombinants were selected and scored for cold sensitivity. None of the $aroE^+$ transductants was cold sensitive. In addition, several $aroE^+$ recombinants from each cross were tested for defects in ribosome assembly and all were found to be normal. These data show that the ribosome assembly defects do not map close to strA and suggest that they do not involve ribosomal proteins.

Positive genetic map positions were determined by a combination of conjugation and transduction. Figure 4 summarizes the results of these experiments by showing the map positions determined for the rim mutations (6). As the figure shows, mutations involving ribosome assembly map at four different loci well away from the strA region and separate from each other.

Mutations in ribosomal proteins that lead to resistance to streptomycin and resistance to spectinomycin are recessive to the wild-type sensitive allele. As another test of the possibility that the rim mutations involve structural ribosomal proteins, we performed dominance tests with three of the rim mutants. The dominance tests were carried out by forming stable partial diploid strains, with the wild-type allele on an episome covering each rim mutation. A recA derivative was formed for each of the rim mutants tested. The partial diploid strains were analyzed extensively for the presence of the episome to show that integration of the wild-type allele had not taken place and that the episome had not been lost. In each case we could show that the episomes were transferable and that segrega-

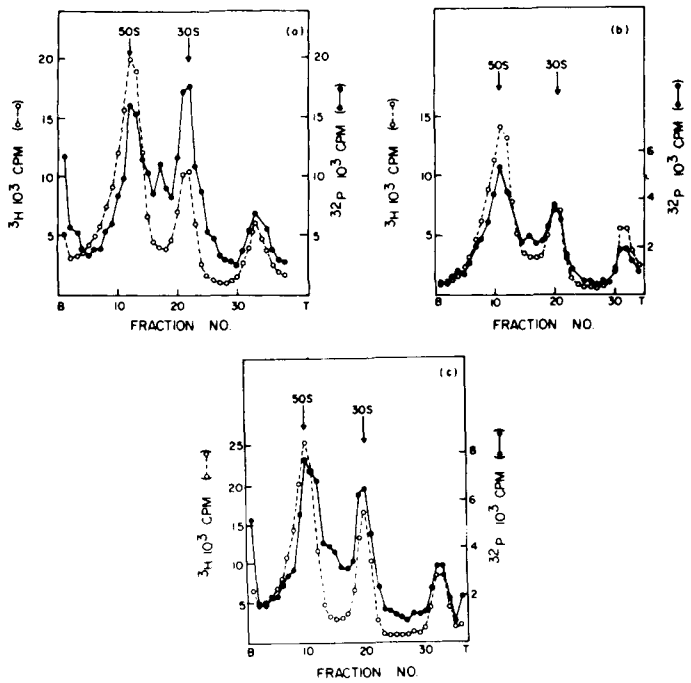

Fig. 5. Zone sedimentation profiles of ribosomes isolated from merodiploids heterozygous for the wild-type allele and A) rim A, B) rim B, and C) rim D. Merodiploids were prepared by transferring an episome into F^- recA strains bearing the respective rim allele as described by Bryant and Sypherd (6). Conditions of culture growth and sedimentation analysis are the same as for Fig. 1.

tion could occur (6). Figure 5 shows the zone sedimentation profiles of extracts of the differentially labeled partial diploids. The profiles show that the wild-type allele is dominant over the rim mutation, since the $P^{32}O_4^=$ incorporated at 20° appears primarily in mature particles. However, the dominance is incomplete because a small but significant amount of material still accumulates in the 43S region at 20°. This lack of complete dominance of the wild-type allele can be explained if catalytic assembly factors were transiently bound to the precursor particles in stoichiometric amounts. This might slow down the maturation seen in the diploids.

Genetic analysis of the ribosome assembly mutants shows that all four loci lie outside the strA region and that all these mutations are recessive to the wild-type allele. These data, together with the two-dimensional acrylamide gel electrophoretic analyses, suggest very strongly that these mutations do not involve structural components.

In Vitro Evidence for Extrinsic Factors

The strong possibility that the mutants analyzed here are altered in factors that control ribosomal assembly led us to develop an in vitro assay for such factors. The approach

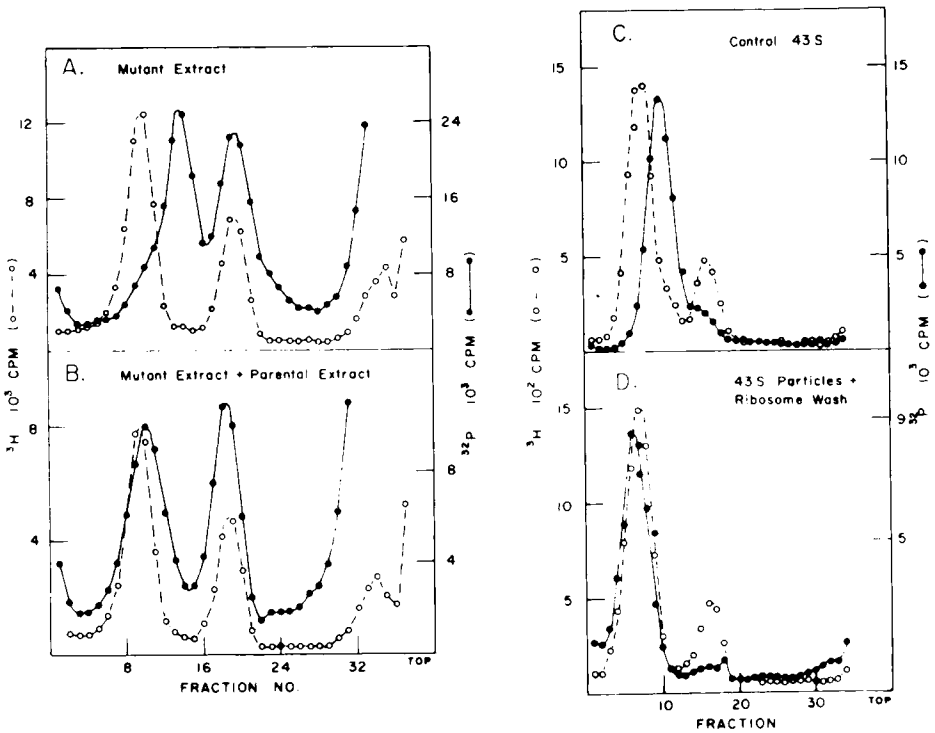

Fig. 6. The in vitro conversion of precursor 43S particles from rim A to "50S" particles in the presence of a crude extract from the parental strain. The rim A mutant was grown in H^3-Uracil at 37° and precursor particles were labeled at 20° in $P^{32}O_4^{=}$. The extract of the parental strain was unlabeled. Extracts from the two organisms were mixed, held at 30° for 30 min in a buffer containing 2 mM Mg^{2+} and 100 mM KCl. After the incubation period, the mixtures were layered onto sucrose gradients prepared in 0.1 mM Mg^{2+}. A) rim A extract alone; B) rim A extract + wild-type extract; C) isolated 43S particles of rim A labeled with $P^{32}O_4^{=}$; D) isolated 43S particles of rim A incubated with a 0.5 M ammonium chloride wash of parental ribosomes.

followed the analogy of phage T_4 assembly in extracts. We selected rim A, labeled its precursor 43S particles at 20°C with radioactive phosphorous, and mixed an extract of these cells with an extract of the parental strain. The mixture was held at 30°C for 30 min and then layered onto sucrose gradients and centrifuged. The results, shown in Fig. 6A and B, show that the wild-type extract had the effect of converting the 43S particles to particles that sediment around 50S. The exact conditions for this conversion reaction were explored further. Figure 7 shows the magnesium requirement for this in vitro conversion. The reaction did not require a high ionic strength, since conversion took place equally well from 1.4 mM to 100 mM KCl. It should be noted from these data that the conditions for this in vitro conversion of 43S particles are quite different from the conditions for in vitro reconstitution of 50S ribosomes of Bacillus stearothermophilis and the conditions for 30S ribosome assembly in E. coli. In these cases, 20 mM Mg^{2+} and 0.4

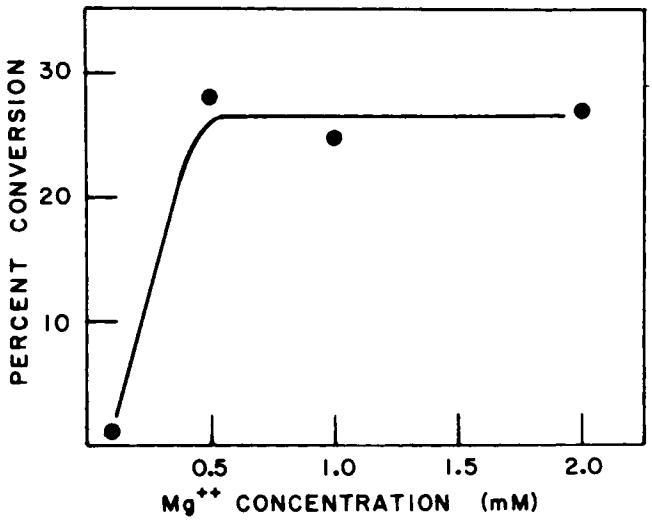

Fig. 7. The magnesium dependence of the 43S particle conversion to "50S." The rim A mutant was grown and labeled as in Fig. 1. An extract of rim A was mixed with a 0.5 M ammonium chloride wash of wild-type ribosomes and held at 30°C for 30 min in a buffer containing the indicated level of magnesium and 10 mM KCl. After the incubation period, the mixtures were layered onto sucrose gradients prepared in 2 mM Mg and 100 mM KCl.

M KCl are required for optimal reassembly. We could also show that extracts from other rim mutants would cause the conversion of 43S rim A particles to "50S" particles. However, extracts prepared from rim A were inactive in converting particles from rim A. Thus, the mutant was not self-complementary.

We attempted to fractionate the wild-type extract to purify the activity responsible for the apparent in vitro maturation of 43S particles. We discovered that the activity could be washed from ribosomes of wild-type cells with 0.5 M ammonium chloride (Fig. 6C, D). The activity could also be adsorbed to DEAE cellulose and recovered in 0.2 M NaCl. The ribosome wash fraction was used for the remainder of our studies. Two-dimensional gel electrophoresis analysis showed that there were no 50S ribosomal proteins in the fraction used for in vitro conversion. The material eluted from DEAE, which was responsible for the apparent in vitro maturation of precursor particles, was referred to as maturation activity (MA). MA is heat labile, being completely inactivated at 50° in 5 min. The interaction between MA and 43S particles occurs over a wide temperature range with rates at 0°C being nearly as great as those at 30°C. The reaction which we observed in vitro with MA and 43S particles is apparently not a catalytic one. This is shown by the data in Fig. 8 where MA appears to react stoichiometrically with the particles. It appears, therefore, that MA binds to the particle and is removed from solution. The apparent stoichiometric interaction of MA with the particles could be explained if MA is released only after a subsequent action on the particles, e.g., by the trimming of oligonucleotides.

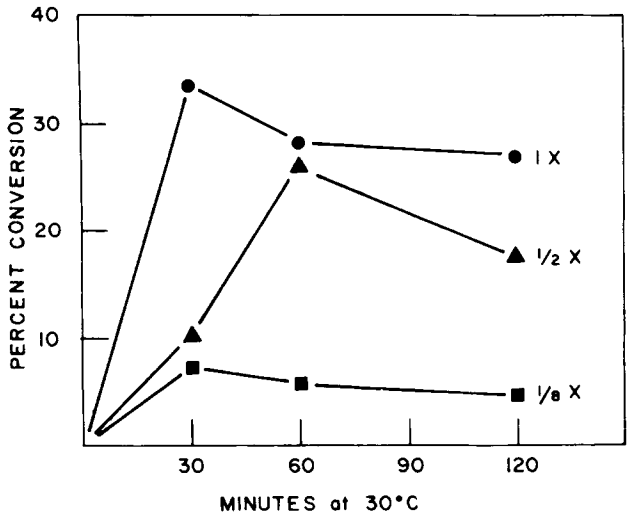

Fig. 8. The extract dependence of the 43S particle conversion to "50S." The rim A mutant was grown and labeled as in Fig. 1. The parental strain was unlabeled. Extracts of the two organisms were mixed in the ratio of one part (—●—), one-half part (—▲—), and one-eighth part (—■—) parental strain to one-half part rim A mutant; all ratios were based on spectrophotometric determinations of total RNA. The mixtures were held at 30°C in a buffer containing 2 mM Mg, 100 mM KCl. Samples were removed at 30 min, 60 min, and 120 min, and layered onto sucrose gradients containing 0.1 mM Mg. (TM4)

DISCUSSION

The rim mutants were isolated much in the same way as the sad mutants described by Guthrie et al. (2). The genetics reported for sad mutants place them close to strA, and sad mutants, and other mutants with defects in ribosome assembly (4, 17, 18, 19) be- protein, S5 (5). Since the genetics for rim and sad mutants are apparently different, it is premature to regard the two phenotypes as equivalent. It is possible that rim mutants, said mutants, and other mutants with defects in ribosome assembly (4, 17, 18, 19) belong to a heterogeneous group with alterations in the assembly and maturation processes.

We have found four classes of ribosome maturation mutants based on map positions. Since each of the four mutational classes is represented by a single mutant, it is quite possible that additional non-strA loci exist. The fact that all four mutations we report here lie outside the strA region suggests that the loci do not code for ribosomal proteins. This suggestion is supported by our failure to find differences in the proteins of either 50S or 30S ribosomes by two-dimensional gel electrophoresis.

The recessive character of the ribosome maturation defects is most simply explained as a defective catalytic activity. The wild-type alleles are incompletely dominant as evidenced by the occurrence of detectable amounts of 43S particles in the merodiploids. These particles will "chase" into 50S particles when growth is continued at 20°.

If the rim mutations do not affect structural components of ribosomes, where

could their defects lie? We consider it likely that these mutants suffer lesions in enzymes that catalyze a modification reaction necessary for proper assembly (e.g., RNA methylation) or in a factor that promotes assembly but does not remain in the mature subunit. In the latter case, a mutation would alter such a factor so that it could bind to the particle. We turned to the in vitro system to gain some evidence on this question. The approach was based on the studies of phage T_4 morphogenesis. The results of mixing the mutant and wild-type extracts clearly showed that wild-type extracts are able to alter the precursor 43S particle of rim A, producing a particle with a sedimentation coefficient around 50S. This 50S particle is not a mature ribosome since it retains p23S RNA after the conversion. Moreover, we have found that high salt treatment of these converted 50S particles will return them to their 43S state, apparently by washing the factor off of the particles. The in vitro reaction which we have followed is an incomplete one since the factor appears to be used stoichiometrically, yet mature ribosomes do not contain this factor as a structural component. Since the 43S particles of rim A lack only protein L20, subsequent steps in the maturation must involve the addition of protein L20 and the trimming of the excess oligonucleotides of p23 RNA. We have not yet been able to carry out these additional steps in the in vitro system.

The in vitro system for converting precursor 43S particles to 50S particles is quite different from the high temperature, Mg^{2+} and KCl required to reconstitute ribosomes from RNA and proteins (1). The studies reported here are the first to suggest that extrinsic factors may control ribosome assembly on maturation. Our additional studies will center on the interaction between the maturation factor and the precursor particles, the nature of the change in the sedimentation coefficient of the particles, and the precise sequence of the subsequent maturation steps. It is anticipated that the other mutants in the collection will yield additional steps involved in ribosome maturation.

ACKNOWLEDGMENTS

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