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GENETIC APPROACHES TO THE STUDY OF RIBOSOMES

(E.coli, cooperativity, endoribonucleases, maturation mutants, rRNA)

DAVID APIRION

Department of Microbiology and Immunology
Washington University School of Medicine
St. Louis, Missouri 63110

SUMMARY

Specific mutants of Escherichia coli have been used in order to investigate certain aspects of ribosome function, biosynthesis and decay. These studies show that in the function of the ribosome its various components are very interdependent and cooperative. It was possible to demonstrate that a binding site for erythromycin, which is on the 50S ribosomal subunit, could be affected by alterations in the 30S ribosomal subunit. The alterations are in ribosome proteins as well as in 16S rRNA. On the other hand, a mutational alteration, probably in the 16S rRNA, blocks ribosome maturation but at a relatively late stage. This alteration leads to cell death.

The maturation cleavages of rRNAs were investigated in a strain lacking RNase III, a specific enzyme which cleaves out p16 and p23 from the growing rRNA transcript. These studies suggest that at least three ribonucleases participate in the primary processing (during transcription), while at least three other ribonucleases participate in the secondary processing, which leads to the final mature rRNA molecules.

When cells stop growing exponentially, decay of ribosomes takes place. By analyzing the process in mutants lacking or containing altered ribonucleases, it was possible to elucidate a mechanism for this turnover. The process starts by an endonucleolytic attack on the rRNA in the ribosomal subunits. After such an attack the ribosome disintegrates to fragments of RNA and ribosomal proteins, both of which can be further decayed to soluble material. Two endoribonucleases were identified in cell extracts which could be the enzymes which initiate this important turnover process.

INTRODUCTION

The ribosome is a ubiquitous cellular organelle about which

considerable information has been gathered in the last decade.

Here I would like to summarize studies which were carried out in my laboratory in the last few years which relate to certain areas in which the genetic approach helped toward the understanding of specific facets of the ribosome.

These three areas are: 1) structure and function in the ribosome; 2) turnover of ribosomes; 3) maturation of ribosomal RNA.

In these studies very little information would have been gained without the availability of specific mutants of Escherichia coli.

STRUCTURE AND FUNCTION IN THE RIBOSOME

a) COOPERATION BETWEEN THE RIBOSOMAL SUBUNITS

The ribosome is apparently a self-assembled organelle composed in all organisms studied from two subunits, a large and a small, and the large is about twice the size of the small. Each of these subunits is made from a ribonucleoprotein (RNP) particle composed of RNA and protein. In Escherichia coli the small subunit is composed of 21 proteins and RNA; the 16S RNA molecule contains about 1600 nucleotides. The large subunit is composed of 33 proteins and two RNA molecules, 23S RNA (3100 nucleotides) and 5S RNA (120 nucleotides). The entire particle is composed of about 2/3 RNA and 1/3 protein.

Since the ribosome is the site for protein synthesis, it must contain a number of binding sites for mRNA, tRNA, peptidyl tRNA, etc. In the last decade some detailed description of the complex process of protein synthesis became possible, and the ribosome is the catalyst on the surface of which all those complex reactions are taking place. (NOMURA et al. 1974.)

What could be the relationships among all these many parts of the ribosome in facilitating its working and creating the various binding sites necessary for the functioning of the ribosome? A priori there are two possibilities for the architecture of the ribosome or for any other complex apparatus: a) maximum independence of the various elements in carrying out their function or b) maximum interdependence. The studies which will be described here suggest that the second alternative seems to be the correct one.

Previously, it was shown that the three proteins S4, S5, and S12 are intimately related in the expression of the phenotype of the ribosome with respect to the antibiotic streptomycin (HASENBACK et al. 1973). These three proteins are all in the 30S subunit. I shall describe here cooperation among four mutations that affect the binding of erythromycin to ribosomes.

Erythromycin is a macrolide antibiotic which blocks growth of E. coli cells; it is bacteriostatic. It inhibits growth by blocking protein synthesis by binding specifically to the 50S

ribosomal subunit (for a review see OLEINICK 1975). Such binding is readily demonstrable by incubating ribosomes and labeled erythromycin at room temperature and filtering the mixture through a millipore filter. A mutation altering protein L4 in the 50S ribosomal subunit prevents the occurrence of this binding (WITMANN et al. 1973).

We found that it is possible to restore the binding of erythromycin to the ribosome by altering two proteins in the 30S ribosomal subunit. These are proteins S5 and S12 (APIRION & SALTZMAN 1974). They were altered by introducing to an erythromycin resistant strain mutations for resistance to spectinomycin (S5) and streptomycin (S12). While each of the doubly mutant strains, *ery spc or ery str*, is still resistant to erythromycin and its ribosomes do not bind erythromycin, some of the triple strains (the phenomenon is allele dependent) are sensitive to erythromycin and their ribosomes bind erythromycin (Table 1). By genetic analysis it was shown that the ***ery*** mutation is intact in such phenotypi-

Table 1. Binding of Erythromycin to 70S Ribosomes. Four units of ribosomes were used. Approximately 1 molecule of erythromycin was bound per ribosome of strains AB301 and N54.

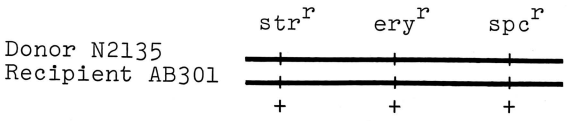
Strain	Genotype	Phenotype	CPM bound to 70S ribosomes
AB301	ery^+ spc^+ str^+ ksg^+	Ery-S Spc-S Str-S Ksg-S	3,785
N54	ery^+ spc_+ str_+ ksg_+	Ery-S Spc-R Str-R Ksg-S	3,284
N282	*ery* spc_+ str^+ ksg_+	Ery-R Spc-S Str-S Ksg-S	31
N214	*ery* spc^+ *str* ksg^+_+	Ery-R Spc-S Str-R Ksg-S	467
N2135	*ery* *spc* *str* ksg^+	Ery-S Spc-R Str-R Ksg-S	2,378
N2183	*ery* *spc* *str* *ksg*	Ery-R Spc-R Str-R Ksg-R	48

cally erythromycin sensitive cells. Such cells can be used as donors in transduction experiments, and they contribute their *ery* gene in normal frequency as compared with erythromycin resistant strains (Table 2). Since such strains are sensitive to erythromycin, we could isolate from them strains in which resistance to erythromycin reappeared. Among such strains some acquired simultaneously resistance to kasugamycin (SALTZMAN, BROWN & APIRION, 1974).

Kasugamycin resistance is known to result from under methylation of the 16S RNA near the 3' end of the molecule (HELSER, DAVIES & DAHLBERG 1971). In such mutants the RNA in the ribosomes can be used as a substrate to be methylated in a cell-free system by an enzyme from the wild type strain. In order for the RNA to be methylated, the ribosome has to be stripped from some of its proteins. This can be achieved by subjecting the ribosome to high salt concentrations of LiCl or CsCl. The *ksg* mutation has been mapped near *leu* (0 min on the E. coli map, TAYLOR & TROTER, 1972), a location which is far from the positions of known struc-

tural ribosomal genes on the map.

Table 2. Genetic analysis of strain N2135 by transduction.
+ represents resistance to an antibiotic.
- represents sensitivity to an antibiotic.



Selection for resistance to	Phenotype with respect to remaining antibiotics		Total number of transductants in each class
Str	Spc	Ery	
	+	+	0
	+	-	6
	-	+	10
	-	-	34
Spc	Str	Ery	
	+	+	0
	+	-	6
	-	+	9
	-	-	64
Ery	Str	Spc	
	+	+	0
	+	-	2
	-	+	4
	-	-	26

It was shown by genetic analysis that the strain in which the erythromycin and kasugamycin resistance reappeared simultaneously contains a *ksg* mutation which maps near to other *ksg* mutations. Moreover, while its supernatant lacked the methylase activity, its ribosomes, after proper stripping of proteins, were acceptors for methyl groups (using s-adenosyl methionine as a methyl donor and an enzyme fraction from a wild type strain, SALTZMAN & APIRION 1976). Thus these experiments showed that the *ksg* mutation is similar in all respects to previously described *ksg* mutations (HELSER et al. 1971) and is caused by under-methylation of the 16S rRNA. Therefore it is clear that we are dealing now with ribosomes which contain a combination of alterations in the 50S and 30S ribosomal subunits: one change in the 50S ribosomal subunit (protein L4), 3 changes in the 30S ribosomal subunit, proteins S5, S12 and in the 16S rRNA.

Since erythromycin binds to 50S ribosomes, (VOGEL et al.

1971), it is possible to test the phenotypic behavior of the ribosomes of all those strains by themselves or in combination with a variety of 30S ribosomal subunits. In Table 1 it was shown that 70S ribosomes which contain an altered L4 protein can bind erythromycin. In order to test more directly the influence of the 30S ribosome on the binding of erythromycin by the 50S ribosome, in a second set of experiments the 50S and 30S ribosomal subunits were prepared and tested by themselves or in various combinations for erythromycin binding. Subunits were prepared from the various strains. 30S subunits should not bind the drug, and this is the case in all assays using only the 30S ribosomes (Table 3), while the 50S subunits can bind

Table 3. Binding of erythromycin to 50S and 30S ribosomes. Three units of ribosomes were used.

50S Ribosomal subunits	30S Ribosomal subunits	CPM bound
AB301	——	1,691
——	AB301	90
AB301	AB301	1,926
AB301	N282	1,952
AB301	N2135	2,164
AB301	N2183	1,502
N282	——	10
——	N282	31
N282	N282	72
N282	AB301	59
N282	N2135	1,286
N282	N2183	69
N2135	——	28
——	N2135	14
N2135	N2135	1,584
N2135	AB301	29
N2135	N282	17
N2135	N2183	80

erythromycin if they come from a sensitive strain. It is significant that the separated 50S ribosomes from strain N2135 which bind erythromycin in the 70S form (Table 1, and APIRION & SALTZMAN, 1974) do not bind erythromycin and behave exactly as ribosomes from the original resistant strain N282. Addition of any of the 30S subunits to 50S from the parental sensitive strain AB301 did not affect binding. Notice that the addition of any 30S subunits to the resistant 50S ribosomal subunits from either strain N282 or N2135 did not restore binding, but for the 30S from strain N2135, these 30S caused good binding of erythromycin to resistant ribosomes from either strain N282 or N2135. Notice that the addition of the *ksg* mutation to the doubly altered 30S

ribosomes from strain N2135 counteracted their effect on the restoration of binding.

Thus these data affirm our notion that the ribosome could be looked upon as a single unit where conformational changes in one subunit are affected by alteration in another subunit. The cooperative effect of the four alterations in the ribosome discussed here on the binding of erythromycin to the ribosomes is described in Fig. 1.

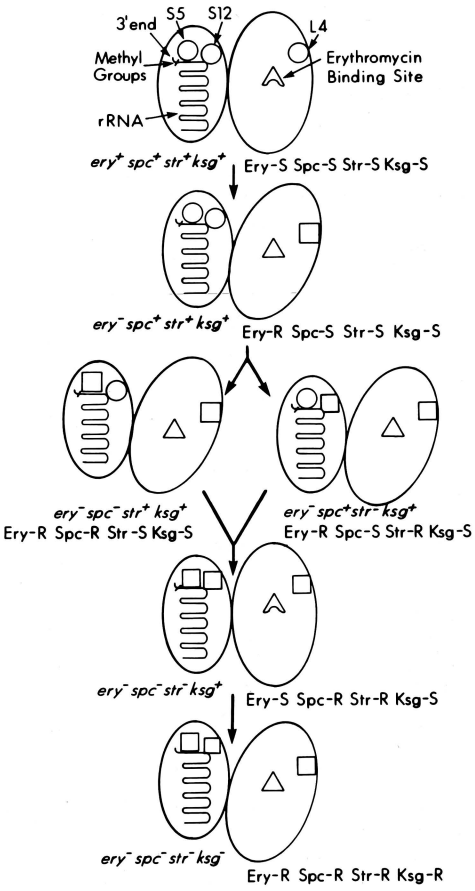


Figure 1. A schematic presentation of the interactions among four ribosomal elements which affect the binding of erythromycin to the ribosome. The 30S ribosomal subunit is on the left and 50S is on the right. The mutations in proteins L4, S5 and S12 are symbolized by alterations from circular to square forms.

The mutation to kasugamycin resistance results in loss of methyl groups at the 3' end of the 16S rRNA, (compare the last two ribosomes in the figure). The erythromycin binding site is on the 50S subunit, depicted with a cavity when the site is open and binding occurs (1st and 4th steps) and as a triangle when erythromycin cannot bind to the ribosomes. The conformation of the ribosome which permits binding is depicted by both subunits being parallel one to the other, while in the non-binding conformation they are in an angle one to the other.

b) A MUTATION WHICH AFFECTS MATURATION OF rRNA AND CAUSES IRREVERSIBLE LETHALITY TO THE CELL

We have seen above how a change in methylation of the 16S rRNA can affect the binding of erythromycin to the 50S ribosomal subunit. The experiments described below suggest that improper synthesis (maturation) of the rRNA plays a role in the synthesis as well as in the function of the ribosome.

Ribosomes exist in large numbers, about 20,000 per cell (WATSON 1974). When the cell starves, ribosomes decay. In order to isolate mutants defective in ribosomes which are not due to resistance or sensitivity to antibiotics, cells were mutagenized and starved at a high temperature, under conditions where wild type cells can recover. Mutants which fail to recover from such a treatment were designated sts (starvation temperature sensitive; APIRION et al. 1969). Among these mutants, some are and some are not temperature-sensitive (fail to grow at elevated temperatures). Among the *Sts*$^-$ mutants which are *Ts*$^-$, most are not killed by the temperature, but some are. From a collection of 150 *Sts*$^-$*Ts*$^-$ mutants we picked up a mutant which is killed very efficiently by being incubated at the non-permissive temperature (43°C., Fig. 2). This killing is rather dramatic and unusual for *Ts*$^-$ mutants, but it can be prevented by adding a number of antibiotics which block protein synthesis when the cells are incubated at the non-permissive temperature (Fig. 2).

This suggested that the mutation might affect the ribosome since it behaves at 43°C like the ribosomal antibiotic streptomycin. Our interest in this mutation was further increased when we found that it maps near the *rna* and *lip* loci (see TAYLOR & TROTER 1972), a region which does not contain structural ribosomal genes. In a series of experiments we looked for altered ribosomal proteins in this strain, but none were found. The defect seems to be in the maturation of rRNA.

While during short pulses at the non-permissive temperature the level of the rRNA is normal, a good part of it consistently disappears differentially. This can be seen in Fig. 3, where the level of newly synthesized rRNA in ribosomes after a pulse chase is greatly diminished as compared to tRNA, while the level of tRNA is similar to that found in the parental cells. Moreover,

the rRNA does not appear immediately as a mature species. It is first cut from the growing transcript into a precursor (PACE 1973; GEGENHEIMER & APIRION 1975), which is subsequently matured to the final rRNA. This process is relatively easy to follow with the 16S RNA, since its immediate precursor is about 17S in size. As can be seen in Fig. 4, the rRNA made at the non-permissive temperature does not mature to 16S RNA, while at the same time the 17S made in a parental strain, or in a tight temperature-sensitive activating enzyme mutant, does mature. Thus it seems that the defect in maturation of 17S and 16S is relevant to the substrate not being properly made in strain N4721.

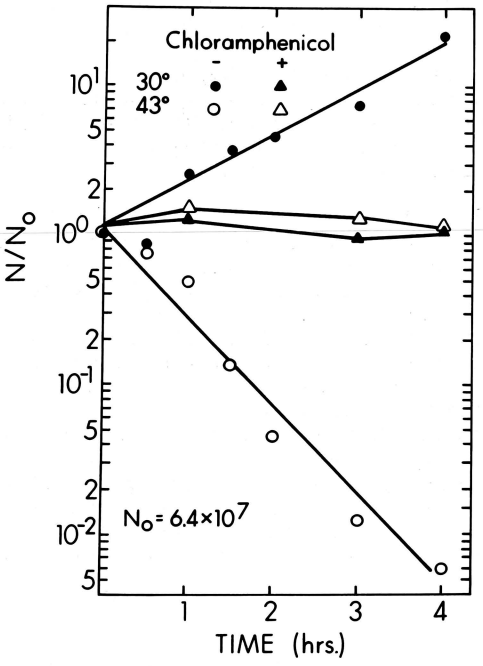


Figure 2. Cell viability. Cultures were grown at 30°C and transferred to 43°C. Cell viability was determined by diluting and plating samples on rich medium at 30°C. Chloramphenicol 300 µg/ml was added at time 0 when cultures were transferred to 43°C.

Some of these immature ribosomes probably enter polysomes and thus lead to an irreversible reaction which results in cell death (as is the case with streptomycin-treated ribosomes (KOGUT & PRIZANT 1975). While other mechanisms are possible, this one can explain all the various observations with the mutant described here.

TURNOVER OF RIBOSOMES DURING STARVATION

Ribosomal RNA and tRNA are stable during exponential growth in bacteria. When bacteria are starved for essential nutrients, however, extensive degradation of these molecules takes place (MANDELSTAM & HALVORSON 1960). Since microorganisms in nature grow exponentially for relatively short periods, and since enteric bacteria do not possess any spores or other durable forms, these processes of degradation of macromolecules are of primary biological importance.

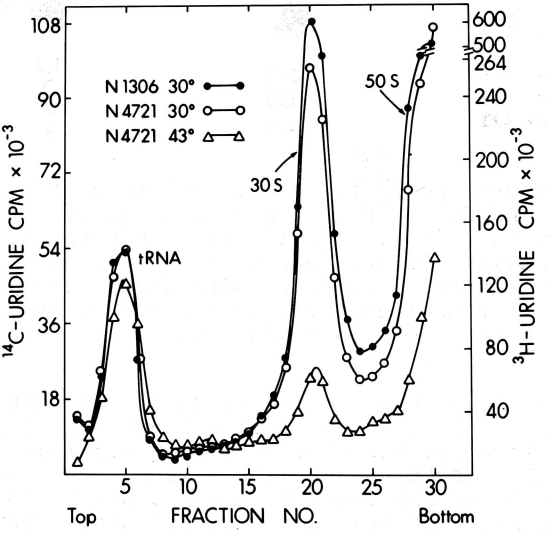


Figure 3. Display of ribosomal subunits on sucrose gradients. Cultures were grown in minimal medium. N1306 at 30° received ^{14}C-uracil (0.2 µCi/ml) during all its growth period. All other cultures were grown at 30° and shifted to a 43° shaking water bath during mid-log phase. After 30 min. at 43°C cultures were labeled for 30 min. with ^{3}H-uracil 2µCi/ml. At that time, unlabeled uracil 50 µg/ml was added, and the cultures were incubated for 1 hour. After opening the cells by a freeze-thaw technique, 13 ml 5-20% sucrose gradients were loaded with 0.5-1.0 mg of material. Gradients were spun for 6.5 hours at 40,000 rpm in an SB-283 rotor in low magnesium (10 mM Tris, pH 7.6, 0.1 mM $MgCl_2$).

Using a series of mutant strains missing or deficient in the enzymes RNase I, RNase II, and RNPase, it became possible to study the turnover of the ribosome in E. coli in a series of deprivations for carbon, nitrogen, amino acids and phosphate. These studies led to the following conclusions: that RNA degradation during starvation is necessary for cell viability; that the degra-

dation of 23S and of 16S RNA starts with an endonucleolytic event which produces relatively small fragments of RNA; that these pieces are digested to nucleotides, at least to some extent by enzymes such as RNase II and PNPase; and that after the endonucleolytic cleavages, which seem to occur only in free 50S and 30S subunits, but not in 70S ribosomes or polysomes, the ribosomes fall apart and their proteins can be degraded.

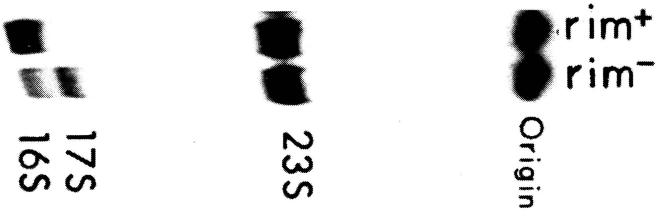


Figure 4. Maturation of 17S rRNA. Cultures of strains N1306 (*rim*$^+$) and N4721 (*rim*$^-$) were grown at 30°C and then labeled with ^{32}Pi (10μ C/ml) for 20 minutes in a low phosphate medium (GEGENHEIMER & APIRION 1975). At that time the concentration of Pi was increased to 0.05 M and after another hour cultures were sampled, cells opened and analyzed on a polyacrylamide gel.

The process of ribosome decay during starvation is adaptive since strains in which it is blocked survive starvation less well than strains in which the process is not blocked. However, it does not require protein synthesis. During the process, one can observe the flow of ribosomes from polysomes to monosomes and to ribosomal subunits and the immediate disintegration of ribosomes to proteins and pieces of RNA upon being subjected to endonucleolytic attack (KAPLAN & APIRION 1975a, b). During the process a ribosome and the rRNA molecules within it are either intact or being degraded rather rapidly; i.e., when 20% of total RNA disappears, about 20% of the total ribosomes and rRNA molecules disintegrated to soluble material. (Fig. 5).

When the decay of stable RNA is compared in all the mutants studied, (Fig. 6) it seems that all the three enzymes, RNase I, RNase II, and PNPase, can contribute to the decay of pre-existing RNA (mainly rRNA) during starvation. Further analysis showed that the involvement of RNase I is probably incidental, for it is observed only at elevated temperatures (45° and 50°C). Since the enzyme is periplasmic, it was suggested that at elevated temperatures RNase I enters the cell through the damaged membrane and then exerts its effect (KAPLAN & APIRION 1975a). This leaves the two enzymes PNPase and RNase II, which are exonucleases (for a brief review on E. coli ribonucleases, see APIRION 1974). Were these the only enzymes involved in the process, then one would expect to see intermediate rRNA molecules of all sizes during the process of rRNA degradation. As seen in Figure 7, however, no intermediate RNA molecules of large size are observed, and the

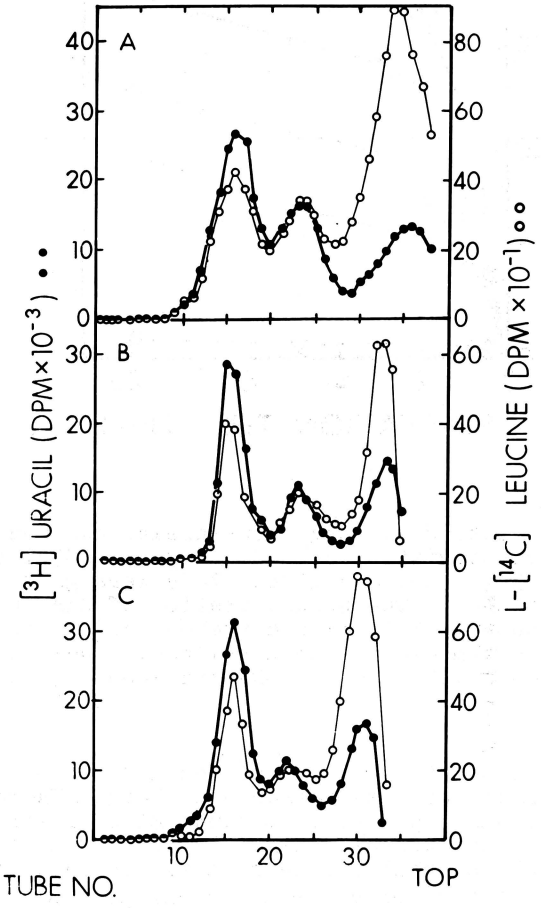


Figure 5. Sedimentation patterns of ribosomal subunits prepared from starved E. coli cells. E. coli A19 was grown in casamino medium supplemented with ^{3}H-uracil and ^{14}C-L-leucine. Extracts prepared from control cultures (A) and from cells after 30 min. (B) or 60 min. (C) of carbon starvation at 50°C were separated on 5 to 20% linear sucrose gradients which contained a low-Mg^{2+} (0.1 mM) Tris buffer.

23S and 16S RNAs disappear to almost the same extent in all four strains studies. The differences among the strains can be obser-

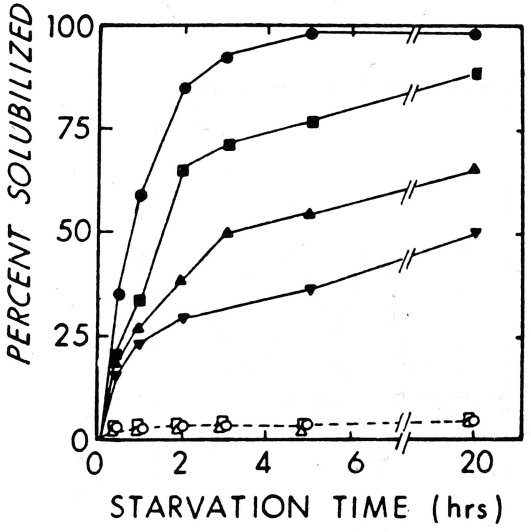


Figure 6. Degradation of long term-labeled RNA and protein during carbon starvation at 50°. Strains AB301 (●, ○), A19 (■,□), Q13 (▲, △), and N7060 (▼,▽) were grown at 37° and starved at 50°. The solubilization of RNA and of protein were measured. RNA, closed symbols and solid lines. Protein, open symbols and broken lines. 100% values represent 20,000 to 25,000 cpm for RNA and about 3,000 cpm for protein.

ved in accumulation of small RNA pieces and can be seen by quantitative analysis of the disappearance of large RNA molecules and accumulation of small pieces in mutants missing exonucleases but not in strains which contain them (Fig. 8). Thus from such studies we concluded that the degradation process starts by an endonucleolytic attack which produces RNA pieces which are degraded to nucleotides by exonucleases.

The overall process of ribosome disintegration during starvation, as envisaged now, is detailed in Figure 9. The process begins with polysome changing to monosomes, which separate to ribosomal subunits. The RNA in the ribosomal subunits is attacked endonucleolytically near or at the double stranded regions of the RNA. When this happens, the ribosome falls apart into proteins and pieces of RNA, the latter then being further degraded to nucleotides.

A number of features of the process are worthy of comment. (1) The process is adaptive; thus do E. coli and presumbly other bacteria adapt to starvation. It is not accomplished by the

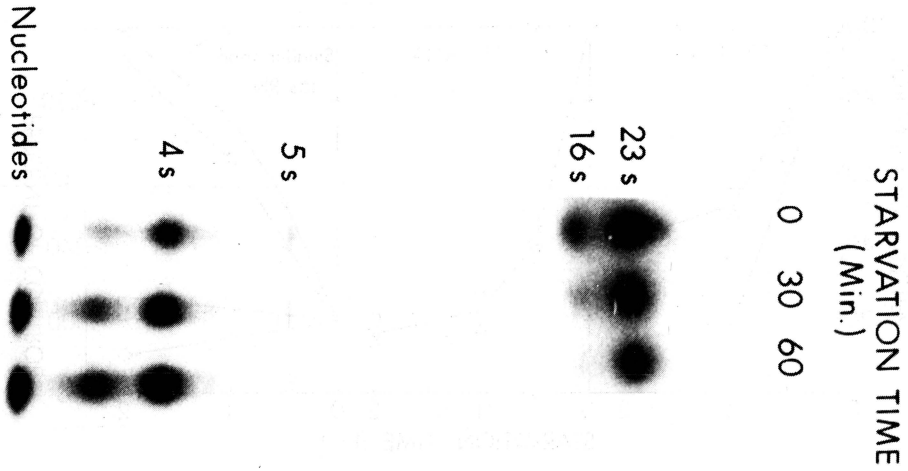


Figure 7. Separation of RNA on a 12% polyacrylamide gel. ^{32}P-RNA prepared from cultures of N7060, 0, 30 and 60 min. after carbon starvation. (For details about gel electrophoresis see KAPLAN & APIRION 1975a).

synthesis of any new protein (since no protein is synthesized at 50°C under carbon starvation) but is brought about by pre-existing enzymes, and apparently the availability of the substrate plays a crucial role in this process. (2) RNA, even rRNA in E. coli, is not necessarily stable. It is stable only when protected in the polysome or in the monosome. Thus, as suggested previously, (APIRION 1973), RNA in E. coli can be degraded by existing enzymes unless it is protected. This leads to the function of the monosome. (3) Here for the first time it can be demonstrated that one of the functions of the monosome is to protect rRNA from degradation, and these findings remove any lingering doubt that this species, the 70S monosome, is a physiological entity. If the rRNA is protected in the monosome but not in the subunits, factors controlling this equilibrium should play an important role in the stability of rRNA. Indeed, preliminary experiments showed that mutations in various ribosomal proteins affect rRNA stability during starvation (unpublished observations). Some mutations increase rRNA stability and others decrease it; protein S8 apparently plays a significant role in this respect. (4) An important feature of the process is that when a given amount of RNA decays, not all the rRNA molecules partially decay, rather a fraction of the molecules disappears completely. This apparently comes about by the endonucleolytic cutting into small pieces of rRNA molecules one at a time. The simplest way to envisage the process is as follows: The riboso-

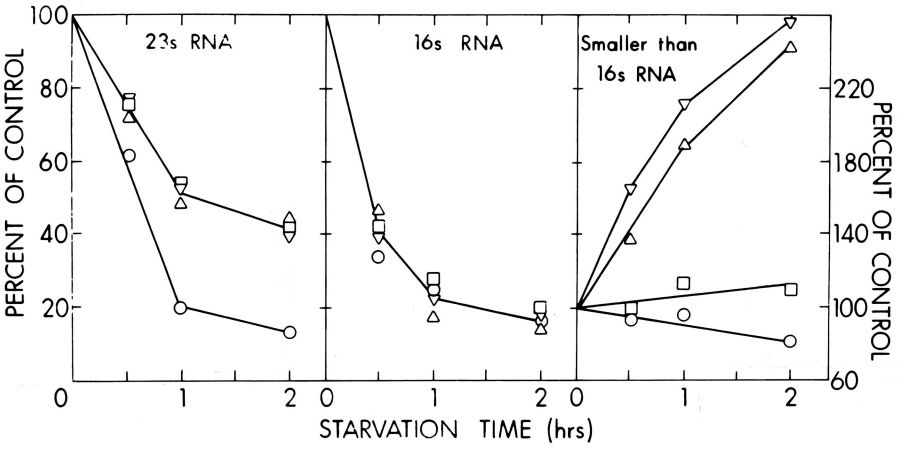


Figure 8. Disappearance of 23S and 16S RNA during carbon starvation at 50°C ^{32}P-RNA, prepared from control cultures and from cells starved for 30 min., 60 min., and 120 min., was separated on 3.2% polyacrylamide gels. The amounts of 23S RNA, 16S RNA, and RNA smaller than 16S were determined and corrected. The data are percentages of RNA species remaining in the starved cells as compared with the control. The left-hand scale applies to 23S and 16S RNA. O, AB301; □, A19; Δ, Q13; ∇, N7060 (for further details see KAPLAN & APIRION 1975a).

mal subunits in their normal state or in a special configuration contain a number of sites that can be attacked by the putative endonuclease; once one such site is attacked and a cut is made in the ribosome, the subunit cannot return to its more protective configuration and further endonucleolytic cleavages follow.

Thus these studies established for the first time a molecular mechanism for a ubiquitous process of turnover which apparently happens in every cell in nature. These studies suggested very strongly the participation of an endonuclease in the process of decay of the ribosome. From the known endoribonucleases of E. coli (APIRION 1974, NIYOGI & DATTA 1975) only RNase III could be a putative candidate to start this reaction.

An E. coli strain was isolated in which the RNase III activity was missing or greatly reduced (KINDLER et al. 1973). This strain contained a large number of additional mutations which were probably introduced in the same step. The mutation was mapped and transferred to a different genetic background. Isogenic strains were prepared, one being RNase III$^+$ (*rnc*$^+$) and the

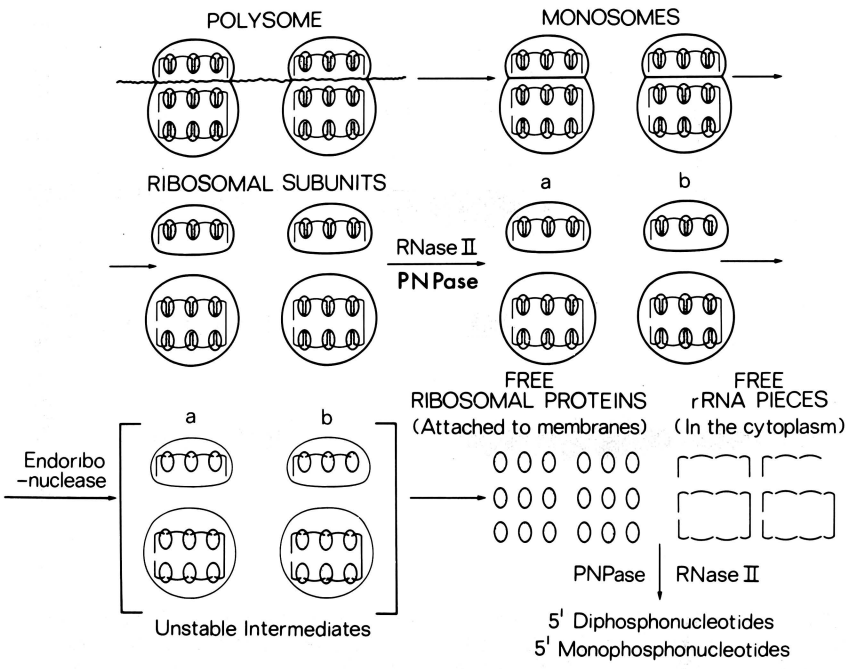


Figure 9. Model for disintegration of ribosomes during carbon starvation. Note that pathways a and b are identical, except that in b the 3' end is missing from the 16S rRNA; this could result from an attack on such ends by either RNase II or PNPase. The unstable intermediates resulting from the endonucleolytic attack on the ribosomal subunits are shown in brackets since they were not observed. The small ellipsoids designate ribosomal proteins bound to double-stranded regions of the rRNA.

other *rnc*$^-$ (APIRION & WATSON 1974, 1975).

The decay of total RNA, 23S and 16S RNA was compared in a number of pairs of *rnc*$^+$, *rnc*$^-$ strains, and in all cases the decay was not decreased in the *rnc*$^-$ strains (APIRION & WATSON 1974, APIRION, NEIL & WATSON 1976). Thus is became clear that the endonuclease which starts the decay of rRNA during starvation is a thus far unrecognized enzyme.

Therefore we started to search for an activity that could degrade rRNA in ribosomes. Two activities were found in extracts of E. coli which might just do this. One of the experiments is

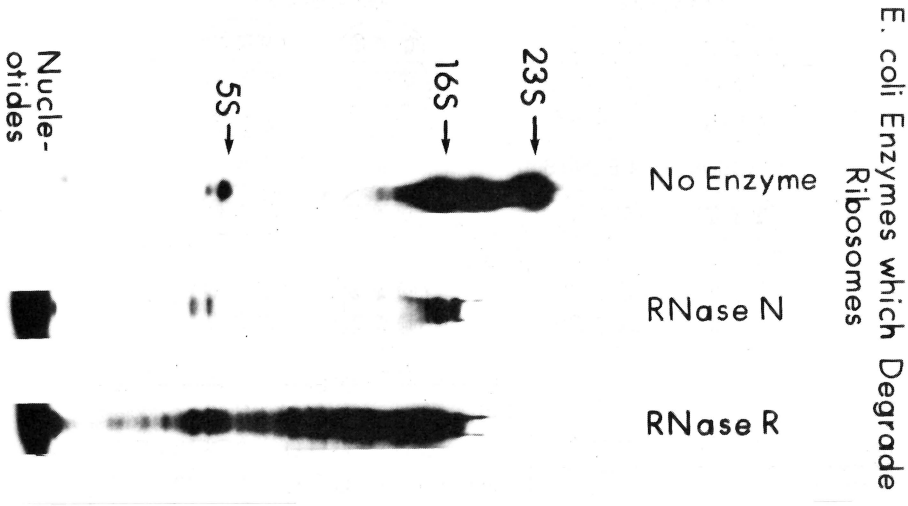


Figure 10. ^{32}Pi labeled ribosomes were incubated by themselves (top) or with protein fractions isolated from a ribosome wash for 2 hrs. Sodium dodecyl sulfate was added to the mixture and aliquots were electrophorized on a sandwich 5% top, 12% bottom gel. The gel was dried and autoradiographed, and the photograph of the autoradiograph is presented.

shown in Fig. 10. In this experiment rRNA from ribosomes incubated without the enzyme is shown on the left and in the third lane are the pieces of the rRNA found after two hours' incubation with this activity (RNase R). It is clear from such experiments that we are dealing with an endonuclease since most of the material which disappears from the 23S and 16S regions appears as oligoribonucleotides. Not enough nucleotides were accumulated to be able to explain the reaction by an exonucleolytic activity. In the second lane of Fig. 10, we see an activity which we call RNase N since it is able to degrade rRNA in ribosomes to nucleotides and very short oligonucleotides. During the last year we carried out some studies with these activities which seem to be two new endoribonucleases. These activities could be found either in a high salt ribosome wash or in the post ribosomal supernatant of the cell.

We carried out some purification of these two enzymes. First, we carried an ammonium sulfate fractionation of the post-ribosomal supernatant. A fraction which contains both activities was further purified on a DEAE sephadex column, followed by

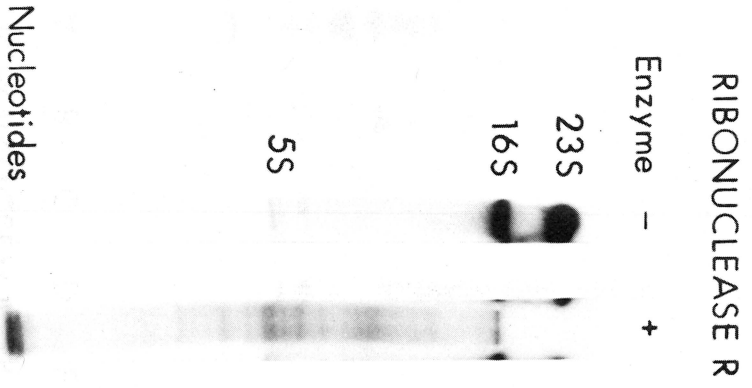


Figure 11. ^{32}Pi labeled ribosomes were incubated by themselves (bottom) or with a protein fraction, purified by DEAE Sephadex chromatography, for 45 minutes at 40°C. Sodium sulfate was added to the mixture and samples were radiographed. Photograph of the autoradiograph is presented.

phosphocellulose and sephadex columns. Only the RNase N activity was purified throughout all these steps, while RNase R was purified only through the first column. In Fig. 11, a result of a reaction with RNase R, purified through the DEAE sephadex column, is shown. As in Fig. 10, very distinct fragments of rRNA can be observed. An analysis of material from the various purification steps revealed that our most purified preparation of RNase N contains only two major and one minor protein (Fig. 12E).

The limited analysis that we carried out up to now clearly indicates that both these enzymes are new endonucleases. Purified RNase N when used in limiting amounts starts to decay the rRNA by producing RNA fragments which eventually are converted to nucleotides and short oligonucleotides, while even a very prolonged activity of RNase R does not convert all the rRNA fragments to small oligonucleotides.

Since RNase I is missing completely from the strains used for the isolation of these enzymes and since none of the known endoribonucleases of E. coli (RNase III, RNase P or RNase H) can attack rRNA, it is clear that these are new activities. Moreover, RNase N is much more heat-stable than any of the other known ribonucleases. From a given protein fraction we can inactivate RNase II and RNase III but still retain RNase N activity. We found that the optimal temperature for RNase N activity is about 45°C (Fig. 13), which makes it a very reasonable candidate for an endoribonuclease that attacks ribosomes *in vivo*, since it has been shown that *in vivo* decay of ribosomes is differentially increased at higher temperatures (KAPLAN & APIRION 1974, 1975a). The decay of free rRNA and rRNA in ribosomes by RNase N were compared, and both are degraded almost to the same extent.

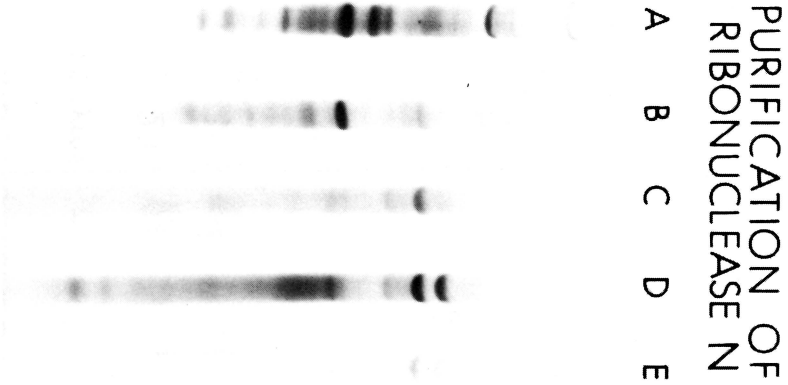


Figure 12. Polyacrylamide gel electrophoresis of protein fractions obtained through different steps of purification. Electrophoresis was carried out for 4 hrs at 2 ma per gel at 4°. Gels were stained overnight with Coomassie Brilliant Blue G 250 and destained by diffusion. A: Cell-extract, B: Postribosomal supernatant fraction, C: DEAE Sephadex A_{50} fraction, D: Phosphocellulose fraction, E: Sephadex G 150 fraction.

These two enzymes are excellent candidates for starting the process of ribosome decay in E. coli . At the higher temperatures where most of the detailed studies of ribosome decay were carried out, one does not observe large rRNA fragments, which might suggest that the enzyme RNase N might be the major endonuclease which attacks rRNA under these conditions. It is still possible that RNase R participates in the process but that the pieces produced are degraded very efficiently by exonucleases. At present there are three enzymes in E. coli which could degrade pieces of single stranded RNA; they are RNase II and PNPase for larger fragments and oligoribonuclease (NIYOGI & DATTA 1975) for very small fragments. It is interesting that these three enzymes produce 5' mono- or di-nucleotides, since these nucleotides could be easily reutilized by the cell.

MATURATION OF rRNA IN THE E. COLI CELL

Ribosomal RNA cistrons in bacteria, coding for 16S, 23S and 5S rRNA, are organized into single transcriptional units with the cistrons transcribed from a single promoter in the order given (PACE 1973). This transcriptional unit can be compared to that which in mammalian cells codes for different rRNA molecules and is transcribed as a single 45S molecule which is processed by a series of steps (for a review, see ATTARDI & AMALDI 1970). In all cells the original rRNA transcript is larger (contains more sequences) than the final rRNAs found in ribosomes; moreover, the final molecules in prokaryotic, as well as in eukaryo-

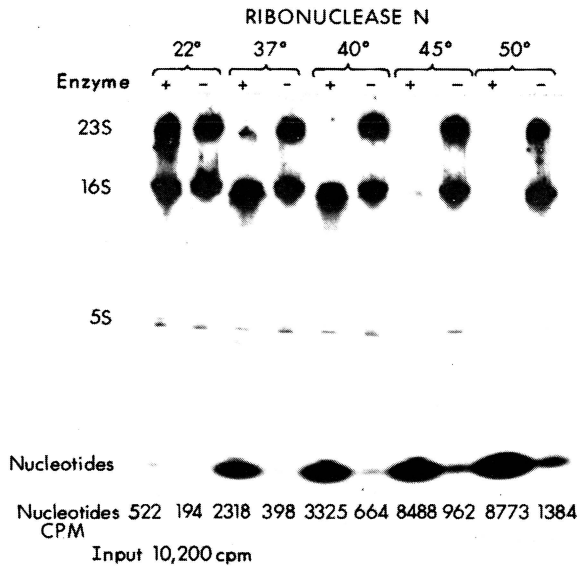


Figure 13. ^{32}Pi labeled ribosomes were incubated by themselves or with RNase N preparation as described in the text. Sodium dodecyl sulfate (0.2%) was added to the mixture and samples were electrophorized on a sandwich 5% top, 15% bottom gel. The gel was dried, autoradiographed and quantitated. A photograph of the autoradiograph is presented. For quantitation, the autoradiogram was superimposed on the gel and the position of the nucleotides, as visualized on the film, was marked. A strip corresponding to each slot was cut, and the areas containing nucleotides, as well as the rest of the area in each lane, were cut and counted in toluene-based scintillation fluid. Background values, i. e. counts, in similar gel areas in which no radioactive compounds have been separated, were subtracted.

tic, cells contain a variety of bases such as methylated adenine which are not transcribed as such from the DNA template but are modified later by a variety of enzymes. Thus the rRNA goes through two types of maturation steps, one which involves a series of cuttings and trimmings and in the other secondary base modifications occur (not necessarily in this order).

In E. coli until recently only a limited amount of trimming was recognized, especially that of 17S to 16S or p16 (precursor) to m16 (mature 16S), and p5 to m5. The difference between the first two molecules is about 100 to 200 bases, while between the last two is only three bases (PACE 1973, MONIER 1974). Since there is only a single promoter for the three species they can arise either by termination events which would necessitate termination with a new initiation of RNA synthesis (a phenomenon not yet recognized, but which cannot be excluded) or by enzymatic cutting events. Recent studies from a number of labs and those

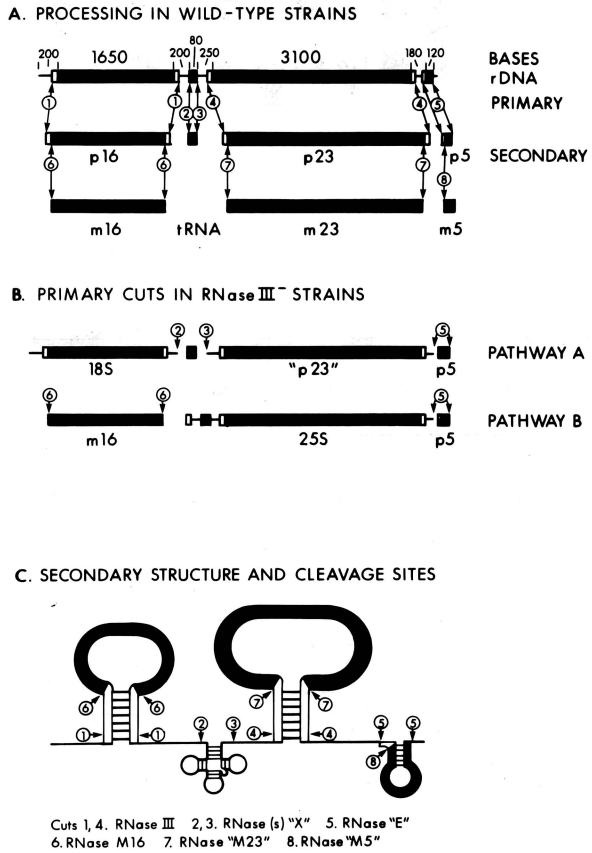


Figure 14. Proposed processing map for ribosomal RNA. A. Processing in wild-type strains: the first line shows the transcriptional sequence of the rRNA genes and the length in bases of important sections (distances are between vertical bars above the map). Thick solid bars represent mature RNAs, thick open bars are precursor sequences removed in secondary processing steps, and the thin lines are non-conserved sections of the primary transcript, discarded during primary processing. The precursor sequences of p16 and p23 are presumed to be about 50-100b long, as judged from examination of the electron micrograph of WU & DAVIDSON (1975) and other data (see text). Primary cuts are

those which release sequences directly from the nascent transcript, and secondary cuts are those which act on products of primary processing, and which are inhibited by chloramphenicol treatment. Each cut or cutting event is given a separate number, as discussed in the text.
B. Primary cuts in RNase III$^-$ strains. The primary and secondary cuts of wild-type strains, which act in the primary processing of the nascent rRNA transcript of RNase III$^-$ mutants, are shown above the products they release. Numbers are those of panel A.
C. Secondary structure and cleavage sites. Using the evidence of WU & DAVIDSON (1975) for loop formation by p16 and (p)23 sequences, the known structure of the su_3^+ $tRNA^{tyr}$ precursor (as a general model for tRNA precursors), and the specificities of ribonucleases mentioned in the text, the secondary structure of regions involved in primary and secondary processing can be drawn. Arrows indicate cleavage sites; numbering is the same as in panel A. The existence of an RNase "E", different from RNase III, is discussed in the text. RNase M5 is presumed to exist in E. coli by analogy with B. subtilis; RNase "M23" is a generic term for the enzymatic system which matures p23 RNA; and RNases P, P2, and O are mentioned as examples of endonucleases which can separate tRNA or pre-tRNA sequences from larger transcripts.

that will be discussed below lead to the conclusion that cutting events are occurring during transcription of the nascent rRNA transcript. From the studies that will be summarized here, together with some studies from other laboratories, the following picture emerges.

A ribosomal RNA transcription contains four regions which give rise to stable RNA, 16S, a tRNA molecule, 23S and 5S, in this order. These regions are not contiguous; they are sandwiched on both sides with sequences which are cleaved and trimmed. Those sequences contain recognition signals for specific maturation enzymes.

The process and the enzymes which participate in it can be divided into two classes: primary events and secondary events (Fig. 14). The primary cutting events occur while the molecule is being synthesized, and the secondary processing events occur after the various pieces are released from the growing transcript. There are probably three enzymes which cut the primary transcript; one is RNase III which cuts out from the growing transcript the p16 and p23 molecules, thus leaving 3 other pieces, a relatively small piece at the 5' end, a middle piece (a ptRNA) and the 3' end piece, which is being further cut, again probably during transcription to a p5 RNA and an extra small RNA. This cut is made, not by RNase III (see below), but by some other enzyme which could be a thus far unidentified enzyme. The third enzyme probably cuts a ptRNA from the spacer region between p16 and p23.

The first piece near the 5' end probably gets degraded. The second piece is the well-recognized species 17S or p16 RNA which is being matured to 16S RNA by at least one complex endonucleolytic cut made by RNase M (MEYHACK, MEYHACK & APIRION 1974). The cut, if it is a single one, involves both ends of the molecule since the p16 contains sequences on both ends of the molecule which are not found in the m16 RNA (PACE 1973).

The knowledge about the p23 is more rudimentary, but the same general features probably hold true for the fourth piece of the RNA. The cut by RNase III produces a p23 molecule which is further cut by some maturation enzyme that will be referred to here as RNase M23. (One problem is that the p23 and m23 are not separable on the regular gels used, while p16 and m16 are easily separable (Fig. 16).

The events which take place with the third piece, which eventually will give rise to a tRNA molecule, are even more obscure, but by analogy with other tRNA there will be a series of cuttings and trimmings which would lead to a mature tRNA molecule (ALTMAN 1975). (I use the word cutting here to denote an endonucleolytic event and the word trimming to denote an exonucleolytic event.) The last piece of the RNA molecule is cut during transcription by another enzyme than RNase III to a molecule (p5) which is only slightly larger (3 nucleotides) than the mature 5S RNA (m5). This molecule p5 will be matured again by an endonucleolytic cleavage to give rise to the 5S molecule (MONIER 1974). While many of the details of the processing of the rRNA still await further analysis, the general features of the process are probably correct, i.e., a series of endonucleolytic cleavages during transcription (primary processing), followed by a series of specific cuts, by specific enzyme secondary processing.

The secondary processing, at least of the 16S, 23S and 5S, is blocked by a number of agents which block protein synthesis, while the primary processing is not. There could be a number of interpretations for these results, but they all have to involve deficiency of enzyme or substrate; we prefer the latter. Thus, while in the primary processing the signal for the cutting is in the RNA itself, it is possible that in the case of the secondary processing the signal for the cutting is in the RNA-protein complex. In absence of protein synthesis the substrate would not be properly synthesized and maturation will not take place. This view of the process is illustrated in Fig. 15. It is very unlikely that the enzyme RNase M responsible for the p16 to m16 maturation is very labile and requires continuous synthesis, since maturation occurs after protein synthesis is blocked by rifampicin or by shifting a temperature-sensitive phenylalanine activating enzyme to the non-permissive temperature.

The primary cuts apparently must take place in a certain order: the last cuts cannot take place if any one of the previous cuts did not take place. With respect to the secondary processing events, it seems that these cuts, or at least some of them, can take place on the primary transcript when the pri-

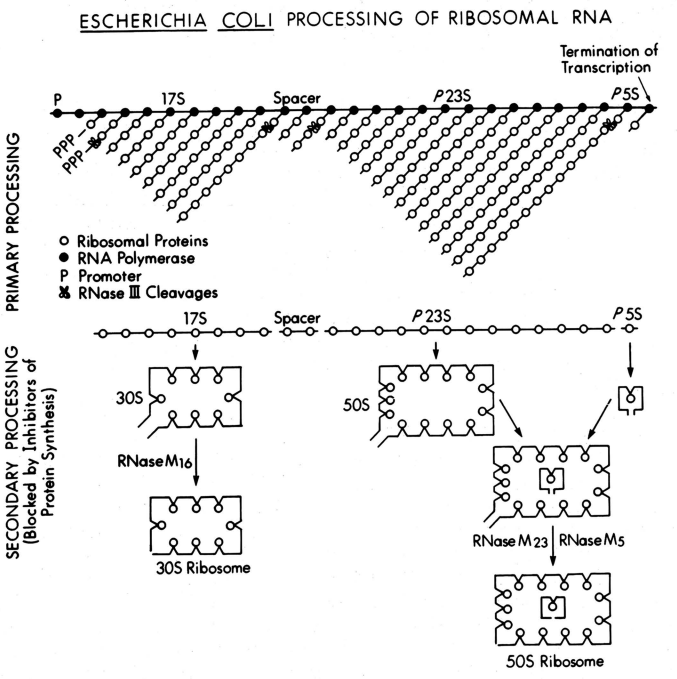


Figure 15. Schematic presentation of maturation of ribosomes. Only the RNase III cleavages are depicted here. The cuts which produce p16 and p23 are each depicted as two independent events. The growing rRNA transcripts, on the DNA, are presented in straight lines to emphasize the fact that the cuts by RNase III do not depend on the proteins (ribosomal) associated with the rRNA. In the secondary processing steps RNP particles are shown, to emphasize the possibility that the substrate for the secondary processing ribonucleases are RNP particles rather than the RNA itself.

mary cuts performed by RNase III cannot take place. This happens in a mutant which is lacking the enzyme RNase III. In such a mutant the m16, but not the m23, can be cut out directly from the growing transcript. Also, the enzyme(s) which cuts out the ptRNA from the growing transcript, which usually acts after RNase III cuts out the p16 molecule, can still introduce its cut even though RNase III did not perform its function. Thus in the absence of RNase III, processing of rRNA still can take place. Some of the studies which led to these conclusions will be described.

The studies were carried out with a strain missing RNase

III. This strain does not show any RNase III activity in extracts (APIRION, NEIL & WATSON 1976), and is completely lacking RNase III in the cell or the level is greatly reduced (GEGENHEIMER, WATSON & APIRION 1976).

In such a strain, when RNA synthesis is followed, rather than the customary species p23 m23 p16 and m16 rRNA, one observes 30S, 25S: p23, 23S, 18S, 17S and m16 species (Fig. 16). All these new species, but for m23 and m16, are transitory, and when cells are labeled with ^{32}Pi and chased, one finds the customary 23S and 16S RNAs (see last lanes in Fig. 16). All these changes are caused by the *rnc*105 allele which is in all likelihood a single point mutation in the structural gene for RNase III, since revertants were isolated which reacquired the full complement of the enzyme and the enzyme behaves in heat inactivation tests as a normal enzyme. Such revertants show normal RNA metabolism and do not harbor the *rnc*105 allele (APIRION, NEIL & WATSON 1976).

The precursor product relationships among all the large molecules were investigated in two ways, first by continuous labeling and quantitative analysis of the various species before they are saturated with label, and by blocking further RNA synthesis by rifampicin prior to saturation labeling and following the fate of the RNA synthesized previously. From all these studies it was concluded that 30S is not a major precursor for the smaller molecules (GEGENHEIMER & APIRION 1975) and that 25S is not the precursor for 23S RNA, while the 18S is the precursor for 17S and 16S RNA. Since, as can be seen in Fig. 16, the "p23" in the *rnc*$^-$ strain is different (somewhat larger) than the p23 in the *rnc*$^+$ strain, and since 17S is formed from 18S in the *rnc*$^-$ strain (in a strain which is in addition *rnb*$^-$ *pnp*$^-$ the 17S is formed only to a small extent), the primary processing in the *rnc*$^-$ strain is abnormal. However, not all the species which appear in the *rnc*$^-$ strain are cut from the growing transcript by the same enzyme.

It is possible to account for the production of the various species by at least two pathways. These two pathways can be dissected apart. First, the relative levels of the various RNA species can be varied with accordance to the growth rate. When the cells grow fast the relative amounts of the 30S and 25S RNA are increased, while when they grow slow their relative amounts are decreased, as compared to the 23S and 16S RNA (Fig. 17). The rationale for this experiment is the following: when cells are growing fast, initiation of rRNA synthesis is relatively fast and the rDNA is packed with RNA polymerase molecules, back to back. When the cells grow slower, the number of growing chains is smaller. Thus, in the absence of RNase III, when the primary processing is impaired, the relative number of misprocessed to processed molecules is increased when more rRNA chains are growing per unit of rDNA.

Another way to separate the two pathways is to add chloramphenicol to the culture. Since it is known from the studies with *rnc*$^+$ cells that chloramphenicol impairs the secondary

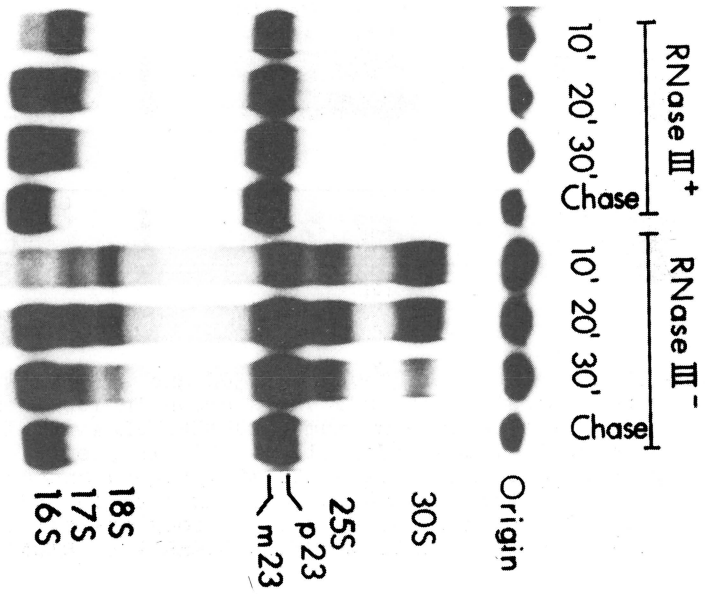


Figure 16. Large RNA species in RNase III $^{+}$ and RNase III $^{-}$ strains. Cultures were labeled with 5 µCi ^{32}Pi/ml (strain N2076, *rnc*$^{+}$) or 10 µCi/ml (strain N2077, *rnc*$^{-}$) and samples taken at the times indicated. The "chase" samples were from cultures labeled with 5 µCi/ml for 2.5 doublings and chased with 20 mM unlabeled Pi for 1.5 doublings. Electrophoresis is from top to bottom on a 3% gel.

processing of all the three mature RNA species, m16, m23 and m5 (PACE 1976), if any of the enzymes, which are involved in these secondary processings, are recognizing their sequences on the nascent transcript and thus play a role in primary processing in an *rnc*$^{-}$ strain, their activity should be inhibited. Indeed, as can be seen in Fig. 18, when chloramphenicol is added to an *rnc*$^{-}$ culture and the label is added after that (it can be as early as 0 or as late as 90 minutes afterwards) no 16S and 25S are formed, while 18S and "p30" continue to be formed as well as 30S. Thus it is clear that the pathway which leads to the formation of 25S is inhibited by chloramphenicol. (It can be seen in Fig. 18 and also from quantitative analysis of 18S, 17S and 16S relationship that some of the 16S is produced in *rnc*$^{-}$ strains directly from the nascent transcript without going through a precursor.)

Most likely the enzyme RNase M recognizes its region in

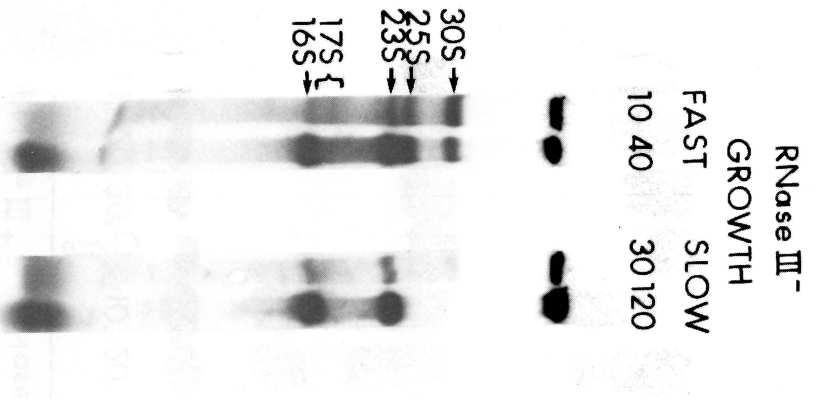


Figure 17. Variation of levels of 30S and 25S RNA with growth rate. Strain N974 (*rnc*⁻) was grown in low phosphate-casamino acids-peptone medium (GEGENHEIMER & APIRION 1975) with 0.2% glucose (doubling = 100 min., left) or without glucose (doubling time = 300 min., right) and labeled with 10 µCi ^{32}Pi/ml; samples were taken at 10 and 40, or 30 and 120 min. and analyzed on 2% polyacrylamide - 0.5% agarose gels as described (GEGENHEIMER & APIRION 1975).

the growing transcript and cuts the molecule, the cut leads to m16, a small molecule from the 5' end, and the rest of the molecule becomes the 25S RNA. However, the formation of the "p23" and 18S are not inhibited because they are formed by a cut which is probably the first step in the maturation of the tRNA which resides in the spacer region between the 16S and the 23S genes (see Fig. 14).

Since 5S RNA exists in the 30S RNA (GINSBURG & ARGETSINGER-STEITZ 1975) but it is also being cut out from the transcript even in the presence of chloramphenicol, we conclude that the first cut which leads to p5 is not made by RNase III and is not inhibited by chloramphenicol.

The usefulness of the *rnc* mutation is that it permits analysis of steady state rather than a transitory condition such as has to be analyzed in conditional mutants. While RNase III is used in the cell to cut out p16 and p23 RNA, the larger molecules "p23" and 18S produced in an *rnc*⁻ strain are also capable of being processed normally, most likely by the secondary processing enzymes, and thus the *rnc*⁻ cell can survive.

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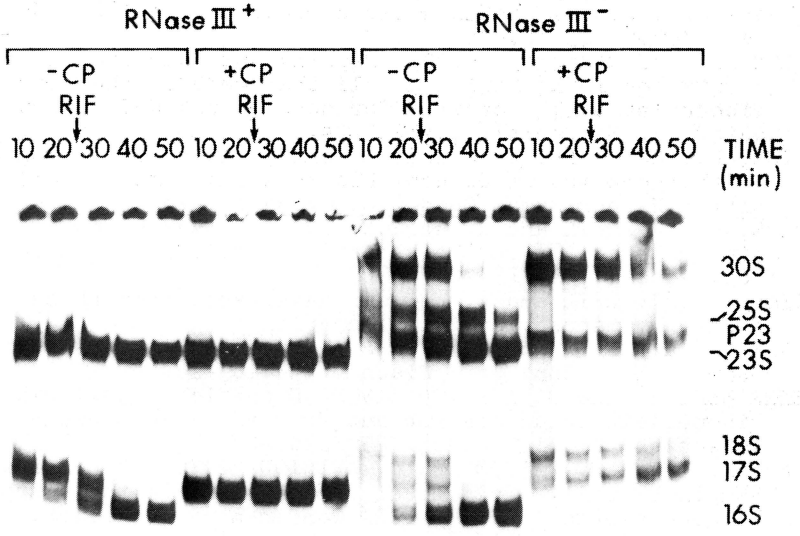

Figure 18. Effect of chlormaphenicol treatment on RNA synthesis in *RNase III*$^+$ and *RNase III*$^-$ strains. Left: Strain N2076 was labeled with 5 μCi ^{32}Pi/ml, either directly (first series of 5 lanes) or 1.0 min. after the addition of chloramphenicol to 400 μg/ml (second series). Samples were taken at the times shown. At 21 min. after labeling, 400 μg rifampicin/ml was added. Right: an identical protocol was followed for strain N2077, except that 10 μCi ^{32}Pi/ml was used. Analysis was on a 3% gel.

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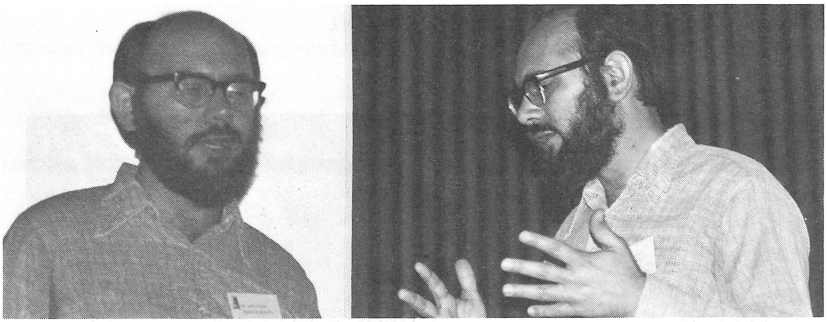

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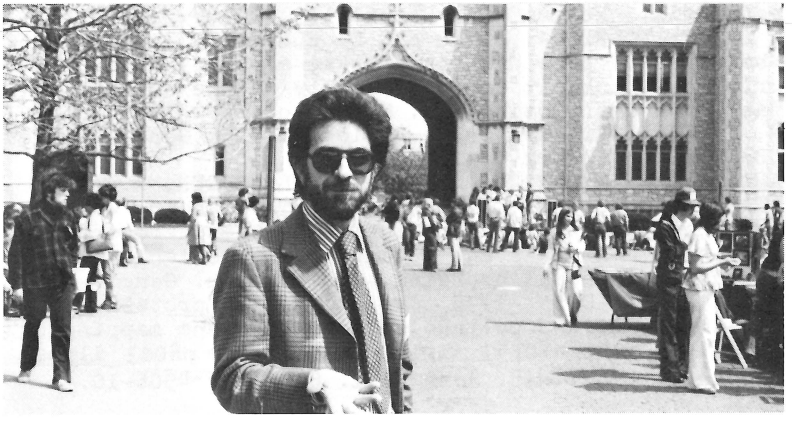

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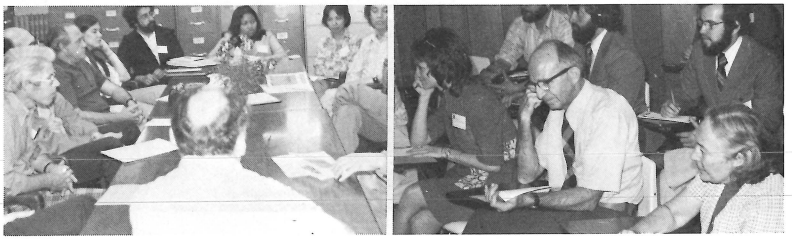

Discussion Groups