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T7 Early RNAs and *Escherichia coli* Ribosomal RNAs are Cut from Large Precursor RNAs *In Vivo* by Ribonuclease III

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ABSTRACT **The early region of T7 DNA is transcribed as a single unit in a Ribonuclease III-deficient *E. coli* strain to produce large molecules essentially identical to those produced *in vitro* by *E. coli* RNA polymerase. As with the *in vitro* RNAs, these molecules are cut by purified RNase III *in vitro* to produce the messenger RNAs normally observed *in vivo*. Thus, the normal pathway for producing the T7 early messenger RNAs *in vivo* appears to involve endonucleolytic cleavage by RNase III. The uninfected RNase III-deficient strain contains several RNAs not observed in the parent strain. Patterns of labeling *in vivo* suggest that the largest of these RNAs, about 1.8 $\times$ 10^6 daltons, may be a precursor to the 16S and 23S ribosomal RNAs. When this large molecule is treated *in vitro* with purified RNase III, molecules the size of precursor 16S and 23S ribosomal RNAs are released; hybridization competition experiments also indicate that the 1.8 $\times$ 10^6 dalton RNA does indeed represent ribosomal RNA. Thus, RNase III cleavage seems to be part of the normal pathway for producing at least the 16S and 23S ribosomal RNAs *in vivo*. Several smaller molecules are also released from the 1.8 $\times$ 10^6 dalton RNA by RNase III, but it is not yet established whether any of these contain 5S RNA sequences.**

The RNAs transcribed from T7 DNA *in vitro* by purified *Escherichia coli* RNA polymerase come from the early region of T7 DNA but are not the same size as the early T7 RNAs observed *in vitro* (1–4). Most of the RNAs synthesized *in vitro* apparently begin at one of three initiators near the left end of T7 DNA and end at a terminator at the right end of the early region, approximately 20% of the way down the T7 DNA molecule. Each of these large *in vitro* RNAs contains the five T7 early messenger RNAs normally observed *in vivo* plus a specific initiator fragment at the left end. An endonuclease that cuts these *in vitro* RNAs at five specific sites to produce RNAs essentially the same as those observed *in vivo* has been purified from uninfected *E. coli* (4). Since virtually all of the RNA synthesized *in vitro* in the presence of the endonuclease corresponds to the major species of RNA observed *in vivo*, it seemed likely that the early T7 RNAs are produced this way *in vivo*.

The endonuclease which sizes the T7 RNAs was found (4) to resemble RNase III (5), an *E. coli* enzyme which selectively degrades double-stranded but not single-stranded RNAs. Experiments done in collaboration with H. D. Robertson (Robertson and Dunn, unpublished) have shown that the sizing activity and the activity against double-stranded RNA are in fact both due to RNase III, the most convincing demonstration being that the sizing activity is competitively inhibited by added double-stranded but not single-stranded RNA. Therefore, we were very interested to learn of an RNase III-deficient mutant of *E. coli* that had been isolated by Hofschneider's group for use in their studies of the replication of bacteriophage M12 RNA (6, 7). Such a strain could provide a critical test of whether RNase III is involved in the processing of T7 RNAs *in vivo*, and in addition provide information on whether RNase III has a role in sizing host RNAs. Cultures of this mutant and of the parental strain from which it was derived were kindly provided by Dr. Hofschneider so that these possibilities could be tested.

MATERIALS AND METHODS

Pancreatic RNase A and electrophoretically purified DNase I were purchased from Worthington Biochemical Corp. DNase was treated with iodoacetate to inactivate residual RNase activity (8). Double-stranded RNA from a virion associated with *Penicillium chrysogenum* (9) and single-stranded F2 RNA (10) were kindly provided by H. D. Robertson. *E. coli* DNA was a gift of S. Lacks. Nitrocellulose membranes (type-B-6) were obtained from Carl Schleicher and Schuell & Co. Carrier-free $^{32}PO_4$ was purchased from New England Nuclear Corp.

The RNAase III-deficient *E. coli* strain was isolated by Kindler and Hofschneider (6) from the RNase I-deficient strain A19 (11) and is referred to by them as AB301/105 or AB105. Both A19 and AB105 seem to be male strains, and as such restrict the growth of T7 (12); however, T7 can abortively infect male strains and produce normal early RNAs (13). Neither strain grows in minimal media but both are able to grow in tryptone broth (1% Bacto tryptone, 0.5% NaCl); cultures grown to an absorbance of approximately 0.2 at 600 nm were used for labeling RNA.

RNA was labeled with $^{32}PO_4$ and analyzed by electrophoresis on slab gels essentially as described previously (4, 14). Details are given in the legends to the figures.

RNase III [specific activity 10 units/μg when assayed using poly(A-U) (5)] was purified as previously described (4), with minor modifications. Treatment with RNase III was carried out in 20 mM Tris·HCl (pH 7.9)–10 mM $MgCl_2$–50 mM KCl–0.1 mM ethylenediamine tetraacetate (EDTA)–0.1 mM dithiothreitol at 35°. The reaction mixture contained 200 μg/ml of bovine-serum albumin and 40 μg/ml of stripped *E. coli* B tRNA. After incubation the RNA products were prepared for electrophoresis as previously described (4).

Hybridization competition experiments were done in 0.2–0.4 ml of two times concentrated standard saline–citrate containing 10 μg/ml of alkali-denatured *E. coli* DNA plus an appropriate mixture of the RNAs to be tested. After incubation for 5 hr at 66° the reaction mixtures were chilled and treated for 45 min at 25° with 10 μg/ml of pancreatic RNase

(which had been heated to 95° in water). Hybrids were collected by filtration through presoaked nitrocellulose filters; the filters were then washed with two times concentrated standard saline–citrate dried, and counted.

Unlabeled ribosomal RNA for hybridization was isolated by phenol extraction of ribosomes purified from an *E. coli* strain that has normal RNase III activity. The 16S and 23S RNAs were then separated by zone centrifugation. ^{32}P-Labeled ribosomal RNA with a specific activity of 175,000 cpm/μg was obtained by preparative gel electrophoresis. The 16S and 23S RNA bands were located by autoradiography of the wet slab, cut out, and eluted from the gel with 300 mM NaCl–50 mM Tris·HCl (pH 6.8)–2 mM EDTA–0.1% sodium dodecyl sulfate. After ethanol precipitation, the RNA was sedimented through a discontinuous CsCl gradient to separate it from what is presumed to be polyacrylamide that elutes from the gel along with the RNA. The RNA was then precipitated twice with ethanol to remove CsCl. Both the labeled and unlabeled ribosomal RNA solutions used in hybridization were adjusted to contain equimolar amounts of the 16S and 23S species. The 1.8×10^6 dalton RNA from AB105 that was used in hybridization experiments was also purified by gel electrophoresis. Two preparations were used, one of specific activity 130,000 cpm/μg, the other 6000 cpm/μg.

RESULTS

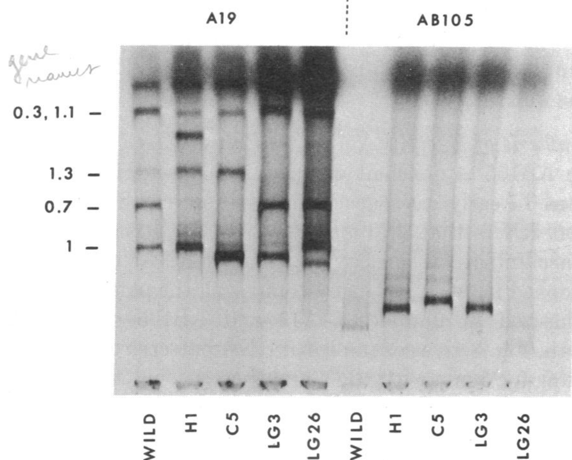

FIG. 1. T7 early RNAs produced after infection of A19 and AB105. Labeling and analysis of the T7 early RNAs were essentially as described previously (4, 14). Cultures of A19 and AB105 growing in tryptone broth were UV irradiated to suppress host RNA synthesis, and 0.1-ml samples were infected with wild-type T7 or with different deletion mutants in the presence of 360 μg/ml of chloramphenicol plus 100 μCi/ml of $^{32}PO_4$. After 10 min at 30°, 1 ml of ice-cold M9 was added and the infected cells were pelleted by centrifugation, suspended in 20 μl of 50 mM Tris·HCl (pH 6.8)–1% sodium dodecyl sulfate–1% 2-mercaptoethanol–10% glycerol, and heated for 2 min in a boiling-water bath. Electrophoresis was for 2.5 hr at 70 V through a slab gel containing 2% acrylamide plus 0.5% agarose, followed by autoradiography. The origin of electrophoresis is at the bottom of the figure. The bacterial strains used are identified above the patterns, the phage strains beneath. The normal T7 early messenger RNA bands are identified at the left. The positions of the different deletions in T7 DNA and their effects on T7 RNAs *in vitro* and *in vivo* have been described (4, 14, 17).

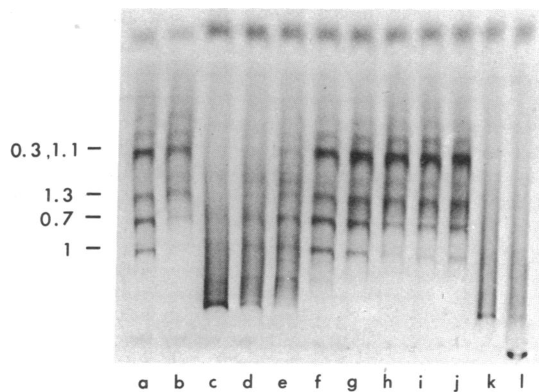

FIG. 2. RNase III treatment of wild-type T7 early RNAs isolated from A19 and AB105. Labeling and electrophoresis of samples was essentially as described in the legend to Fig. 1, with the following exceptions. A 20-ml sample of A19 or AB105 growing in tryptone broth was harvested by centrifugation and suspended in 8 ml of a low-phosphate minimal medium (14) plus 2 ml of tryptone broth before the UV treatment. The entire sample was infected with wild-type T7; $^{32}PO_4$ was present at 20 μCi/ml. After centrifugation, each sample was suspended in 1 ml of the sodium dodecyl sulfate buffer without glycerol and heated for 4 min in a boiling-water bath. The samples were then extracted twice with phenol and the RNA was precipitated twice with ethanol. The RNA was dissolved in a final volume of 2 ml of the buffer used for reaction with RNase III and 50-μl samples were treated with RNase III. The RNA of samples *a* and *b* was from A19, that of samples *c–l* from AB105. Samples *a*, *b*, and *h–l* were incubated for 10 min and *c–g* for 5 min with the following amounts (in units) of RNase III: (*a*) none; (*b*) 0.5; (*c*) none; (*d*) 0.06; (*e*) 0.12; (*f*) 0.25; (*g–l*) 0.5. Samples *i* and *j* received 1 and 10 μg, respectively, of single-stranded RNA, and samples *k* and *l* received 1 and 10μg, respectively, of double-stranded RNA before the RNase III was added.

type T7 or by different deletion mutants of T7 were examined in both the parental strain A19 and in the RNase III-deficient strain AB105 (Fig. 1). The patterns obtained after infection of A19 are identical to those obtained (4, 14) after infection of *E. coli* B, a normal female host for T7. However, the patterns observed after infection of AB105 are entirely different, and are in fact essentially identical to those obtained when the corresponding DNA is transcribed *in vitro* by purified RNA polymerase in the absence of RNase III (4). Most of the label is found in a large RNA which covers the entire early region, and no discrete bands are observed at the positions of the early RNAs observed in A19 or B. As discussed previously (4, 14), the changes in mobility of the RNA bands from the different T7 deletion mutants demonstrate that these RNAs come from the early region of T7 DNA.

Keil and Hofschneider (7) showed that extracts of AB105 have lost most of the ability to degrade double-stranded RNA when compared with extracts of the parental strain. To confirm that the abnormal RNA synthesis observed after infection of AB105 by T7 is due to a deficiency of RNase III activity *in vivo*, we have purified the labeled T7 early RNA from AB105-infected cells and treated it with highly purified RNase III (Fig. 2). As with RNA produced *in vitro* (4), this *in vivo* RNA is cut by purified RNase III at specific sites to produce an RNA pattern essentially indistinguishable from that observed in normal hosts *in vivo*. This cleavage is in-

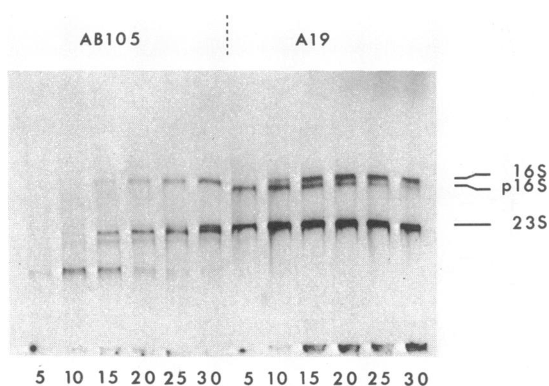

FIG. 3. Labeling of A19 and AB105 RNA. Cultures of A19 and AB105 growing at 30° in tryptone broth were labeled with 20 μCi/ml of $^{32}PO_4$. After 5 min in the presence of the label, rifampicin was added to give a concentration of 100 μg/ml. Immediately before the addition of the rifampicin and at 5-min intervals thereafter, 0.1-ml samples were removed and added to 1 ml of ice-cold 0.02 M KCN, 0.03 M sodium phosphate buffer (pH 7). Samples were analyzed as described in the legend to Fig. 1. The time after addition of label at which each sample was collected is indicated beneath the patterns. The 23S, precursor 16S (p16S), and mature 16S ribosomal RNA bands (15, 16) are identified at the right.

hibited by small amounts of double-stranded RNA but not by single-stranded RNA (Fig. 2*h–l*). High levels of RNase III cause some degradation of the larger RNA products (compare Fig. 2*a* with *b* and *f–h*), but it is not clear whether this degradation is due to the RNase III itself or to a minor contaminant. The relative amounts of label in the bands produced by cleavage *in vitro* are strikingly similar to what is observed *in vivo* in A19 (compare Fig. 2*g* and *a*).

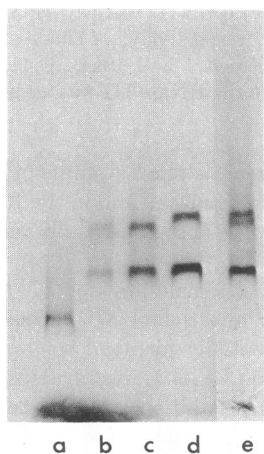

FIG. 4. RNase III cleavage of the 1.8×10^6 dalton RNA from AB105. A 20-ml sample of AB105 growing in tryptone broth was harvested by centrifugation and suspended in 8 ml of a low-phosphate minimal medium (14) plus 2 ml of tryptone broth. The cells were labeled for 5 min at 30° in the presence of 20 μCi/ml of $^{32}PO_4$ and harvested by centrifugation. The RNA was extracted as described in the legend to Fig. 2. The 1.8×10^6 dalton RNA was further purified by zone centrifugation and treated with RNase III. The products were subjected to electrophoresis alongside or in combination with marker RNAs: (*a*) purified 1.8×10^6 dalton RNA incubated 10 min without RNase III; (*b*) same as sample *a*, except incubated with 0.5 unit RNase III; (*c*) A19 cells labeled with $^{32}PO_4$ for 5 min to label precursor 16S and 23S RNAs; (*d*) ribosomes purified from A19 cells that had been labeled and then chased for 2 hr to label mature 16S and 23S RNAs; (*e*) mixture of samples *b* and *d*. Samples were analyzed as described in the legends to previous figures.

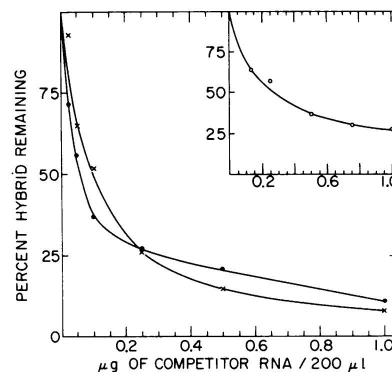

FIG. 5. Hybridization competititon between the 1.8×10^6 dalton RNA and normal ribosomal RNA. Constant amounts of labeled 1.8×10^6 dalton RNA (●——●, 60 ng, 8000 cpm) or ribosomal RNA (×——×, 63 ng, 11,000 cpm) were annealed with denatured *E. coli* DNA in the presence of increasing amounts of unlabeled ribosomal RNA. In the *insert*, a constant amount of ribosomal RNA (○——○, 63 ng, 11,000 cpm) was annealed with DNA in the presence of increasing amounts of low specific activity (6000 cpm/μg) 1.8×10^6 dalton RNA. Approximately 10% of the input RNA was found in hybrids when no competing RNA had been added.

These results indicate that processing by RNase III is part of the normal pathway for the production of T7 early RNAs *in vivo* and that AB105 is deficient in RNase III activity *in vivo* as well as *in vitro*.

Synthesis of E. coli RNA in the RNase III-Deficient Strain. Since AB105 is deficient enough in RNase III so that uncleaved T7 early RNA accumulates, it seemed likely that any *E. coli* RNAs that are normally sized by RNase III would also accumulate as uncleaved precursor RNAs in this strain. We looked for such RNAs by comparing the patterns of RNA synthesized in uninfected A19 and AB105 during active growth. Fig. 3 gives examples of RNA patterns obtained after labeling for 5 min with $^{32}PO_4$ and then chasing in the presence of rifampicin (which was added to prevent further initiation of RNA chains). The labeling pattern of the A19 ribosomal RNAs is similar to that normally observed in *E. coli* (15, 16). The pattern of incorporation into AB105 RNA is strikingly different. In a short pulse label is found in an RNA that is considerably larger than 23S RNA, at a position corresponding to approximately 1.8×10^6 daltons [relative to T7 early RNAs as standards (17)]. During the chase, label is lost from this large band and accumulates in RNAs of approximately the same size as the normal ribosomal RNAs. The patterns are further complicated by the presence of other distinct bands in this region of the gel.

The 1.8×10^6 dalton RNA observed in AB105 is large enough to contain both the 16S and 23S ribosomal RNAs, and from the labeling patterns, as well as by analogy with the T7 early RNAs, it seemed likely that this large RNA could represent an uncleaved precursor to the ribosomal RNAs. To test this idea, labeled 1.8×10^6 dalton RNA was purified from AB105 and treated with RNase III. As shown in Fig. 4, this RNA is cut by RNase III to produce molecules the size

of 16S and 23S ribosomal RNAs: the smaller RNA migrates at the position of the precursor to 16S RNA that is normally observed *in vivo* (15, 16) and resolves from mature 16S RNA when coelectrophoresed with RNA from purified ribosomes; the larger RNA comigrates with mature 23S RNA. [Since the RNA samples were heated before electrophoresis, precursor and mature forms of 23S RNA would not be expected to resolve (16).] As was found for the large precursor to T7 early RNAs, cleavage of the 1.8×10^6 dalton RNA is inhibited by small amounts of double-stranded but not single-stranded RNA (data not shown).

Hybridization competition experiments (Fig. 5) also indicate that the 1.8×10^6 dalton RNA does indeed represent ribosomal RNA. Unlabeled ribosomal RNA competes with labeled 1.8×10^6 dalton RNA for sites on *E. coli* DNA with about the same efficiency as it competes with labeled ribosomal RNA; 1.8×10^6 dalton RNA of low specific activity effectively competes against ribosomal RNA of high specific activity. Thus, most of the RNA in the 1.8×10^6 dalton piece represents ribosomal RNA, and most (if not all) of the ribosomal RNA sequences are represented in this piece.

These results indicate that 16S and 23S ribosomal RNAs are normally transcribed as a unit *in vivo* and that the primary transcripts are normally cut by RNase III. Whether the products of RNase III cleavage are in fact identical to the precursor 16S and 23S RNAs observed *in vivo* will have to be determined by sequence analysis.

DISCUSSION

Role of RNase III In Vivo. RNase III appears to participate in the processing of both the T7 early messenger RNAs and the *E. coli* ribosomal RNAs *in vivo* by cutting the primary transcripts at a few specific sites. The large precursor RNAs have not previously been observed *in vivo*, an indication that cleavage by RNase III is normally so efficient that susceptible sites are cut almost as soon as they are synthesized, but in the RNase III-deficient strain the uncleaved precursors do accumulate. It is clear that these RNAs are normally cut by RNase III, because the primary transcripts isolated from the RNase III-deficient host are cut by highly purified RNase III to produce RNAs that are indistinguishable from those normally found *in vivo*.

The RNase III-deficient host was isolated after heavy mutagenesis of the parental strain (6), and it is likely that secondary mutations are also present. Therefore, it is not certain that the RNase III deficiency alone is responsible for all of the effects on RNA synthesis observed in this strain. For a thorough analysis of the effects of RNase III deficiency *in vivo* it will be necessary to have RNase III-deficient mutations in a defined genetic background.

T7 RNA Synthesis In Vivo. The combined results of these and previous (1–4) studies demonstrate that the synthesis of T7 early messenger RNAs normally proceeds in at least two steps: (*1*) the primary transcripts are synthesized by the *E. coli* RNA polymerase, starting at any one of three initiators near the left end of T7 DNA and terminating specifically at the terminator at the end of the early region; and (*2*) the primary transcripts are cut at five specific sites by RNase III to produce the messenger RNAs normally observed *in vivo*. Our results do not exclude the possibility that the products of RNase III cleavage might be further processed *in vivo*, perhaps, for example, by slight modification of the ends, but this can be determined by appropriate sequence analysis.

This pathway would be expected to generate equal numbers of RNA molecules from each of the T7 early genes. However, labeling patterns have generally seemed to indicate that the closer a gene is to the promoter region the more copies of RNA are transcribed from it (Figs. 1 and 2, refs. 14 and 18). Such a labeling pattern could be produced if an appreciable fraction of the RNA chains which were started during the labeling period did not reach the end of the early region, either because they terminated prematurely or because they were still in the process of synthesis at the time the cells were harvested. If chains do terminate prematurely, such terminations do not seem to occur at specific points, since few if any discrete RNAs smaller than the RNA which covers the entire early region are observed in the RNase III-deficient host. Of course, other factors, such as different rates of degradation for the various early RNAs, could also influence the molar ratios observed in labeling experiments.

Other pathways for the production of T7 early messenger RNAs have been proposed, involving specific rho-mediated terminations within the early region (19) or specific initiations and terminations within the early region (3). Our results indicate that such pathways, if they exist *in vivo*, probably produce no more than a minor fraction of the T7 early messenger RNAs; and any such transcripts which contain a cleavage site would also be processed by RNase III. It is not yet known whether the RNase III-deficient strain has a normal amount of rho, but little if any of the early T7 RNA produced in this strain is the size of the rho-terminated fragments observed *in vitro*.

Why are T7 early messenger RNAs cut by RNase III? One possibility is that cleavage is needed for or facilitates translation of these messenger RNAs. This possibility can be tested *in vivo* when an RNase III-deficient female strain that can grow on defined medium becomes available. It will be interesting to see whether uncleaved messengers are translated in an RNase III deficient *in vitro* protein synthesizing system.

Ribosomal RNA Synthesis. Previous work on kinetics of labeling and hybridization of the individual *E. coli* ribosomal RNAs (20–22), as well as electron microscopic visualization of ribosomal RNA transcription (23), has suggested that the three ribosomal RNAs are transcribed in tandem in the order 16S, 23S, and 5S. The work reported here shows that at least the 16S and 23S RNAs are transcribed *in vivo* as a single unit of about 1.8×10^6 daltons and are normally cut from the primary transcript by the action of RNase III. It is not yet known whether the 5S ribosomal RNA is also a part of this primary transcript; cleavage by RNase III *in vitro* produces a family of low molecular weight RNAs, most of which seem to be somewhat larger than 5S marker RNA (data not shown). Perhaps some of these bands represent precursors that are further processed *in vivo* to give normal 5S RNA. Others could represent fragments released from the ends or from spacer regions within the 1.8×10^6 dalton RNA.

Label chases out of the 1.8×10^6 dalton band and accumulates in bands the size of normal ribosomal RNAs in the RNase III-deficient strain. In fact, analysis of total RNA in this strain reveals that the 1.8×10^6 dalton RNA is a minor component relative to the RNAs which correspond to normal ribosomal RNAs. Presumably, the ribosomal RNA is slowly processed in this strain either by residual RNase III activity

or by a second enzyme system. It will be interesting to see whether the 1.8×10^6 dalton RNA assembles into a ribosome-like structure, and if so, whether such a structure carries out any ribosomal functions *in vitro*.

Several other RNAs that migrate in the ribosomal region of the gel are made in the RNase III-deficient host. On the basis of *in vivo* labeling patterns, we would guess that at least some of these RNAs are not related to ribosomal RNAs and may represent precursors to other types of RNA, for example transfer RNAs or perhaps a class of rather stable messenger RNAs. It should be possible to determine which RNAs are related to ribosomal RNA and which are not by hybridization competition experiments and by determining what products are released after treatment with RNase III.

Our results are in excellent agreement with those of Pettijohn, *et al.* (21), who have described a system in which ribosomal RNA chains that had been initiated *in vivo* are extended *in vitro*. They observed two RNA species, about 1.7 and 1.25×10^6 daltons, which contain ribosomal RNA sequences; these RNAs are essentially the same size as the two largest RNAs we observe *in vivo* in the RNase III-deficient host. Pettijohn *et al.* suggested two possible explanations for the appearance of these large RNAs *in vitro:* (*1*) the failure of a normal termination mechanism; or (*2*) the 16S and 23S RNAs were transcribed as a unit that would normally have been processed *in vivo*. Our results support the latter interpretation.

Since the processing of T7 messenger RNAs and *E. coli* ribosomal RNA are both competitively inhibited by added double-stranded RNA, it seems likely that the cleavage sites in these single-stranded RNAs have at least some double-stranded character. It will be interesting to see whether enzymes similar to RNase III have a role in processing RNAs in the cells of higher organisms, where both ribosomal and messenger RNAs seem to be processed (24), and if so, to what extent the cleavage specificity might be conserved throughout evolution. In this regard, it is intriguing that the processing of the 45S ribosomal precursor RNA of mammalian cells is inhibited by intercalating agents, a possible indication that the processing sites in this RNA might have some double-stranded character (25), and double-stranded sequences seem to be found in mammalian heterogeneous nuclear RNA but not in messenger RNA (26).

After this work had been completed, we learned that Nikolaev, Silengo, and Schlessinger (27) had independently discovered the high molecular weight precursor to ribosomal RNA in the same RNase-III deficient host.

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