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A ROLE FOR RIBONUCLEASE III IN SYNTHESIS OF BACTERIOPHAGE T4 TRANSFER RNAs

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SUMMARY

Synthesis of T4 $tRNA^{Gln}$ depends on normal levels of Escherichia coli ribonuclease III. Infection of cell strains carrying a mutation in the gene for this enzyme resulted in severe depression in $tRNA^{Gln}$ production, as revealed by chemical and suppressor tRNA analyses. The remaining seven T4 tRNAs were synthesized in the mutant cells. The requirement of ribonuclease III for synthesis of $tRNA^{Gln}$ points to an essential cleavage by the enzyme of a precursor RNA containing $tRNA^{Gln}$.

Discovery of the involvement of precursor RNAs in the synthesis of mature tRNAs has raised questions about the process by which the large intermediates are converted into finished products. Of immediate interest are the identities of the participating enzymes and the structures of the precursor RNA intermediates. The tRNAs coded by bacteriophage T4 have provided a useful system for the study of tRNA synthesis. Synthesis of T4 tRNAs relies almost entirely on host cell gene products which are also involved in synthesis of cellular tRNAs. For this reason, studies of T4 tRNA biosynthesis are valuable because they provide descriptions of actual substrates and enzymes employed in cellular processes (1).

Genes for the eight T4 tRNAs are grouped together within a small region of the genome, and this segment may be transcribed into one large precursor RNA (1). However, the largest precursor RNA molecule identified thus far contains only two tRNA sequences, while smaller ones contain a single tRNA chain. The observation that these precursor RNAs contain 5'-monophosphate groups indicates that they arise by cleavage of one or more larger molecules (1). Ribonuclease P and ribonuclease III are the two enzymes in Escherichia coli that are known

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to cleave precursor RNAs to liberate products with 5'-monophosphate groups. Ribonuclease P was initially identified by its ability to cleave the precursor RNA to E. coli $tRNA^{Tyr}$ (2). Subsequent work showed that the enzyme performs an analogous cleavage of the small precursor RNAs of T4 (1). Ribonuclease III is responsible for cleavage of E. coli precursor RNAs leading to rRNAs and for cleavage of polycistronic mRNAs of bacteriophage T7 (3). In this report we show that ribonuclease III is also involved in the synthesis of a T4 tRNA.

MATERIALS AND METHODS

Organisms. Bacteriophage T4 strains were from our collection and have been described previously (1, 4). Genes encoding T4 tRNAs are not essential for bacteriophage growth, and four of the eight tRNA genes have been converted into suppressors; Table 1 gives the genetic symbols of the suppressor genes and the associated tRNA species. The suppressible nonsense mutations used in this study are located in the T4 e (lysozyme) gene: eL1 is an ochre mutation, whereas eL1a and eL1G are amber and opal mutations, respectively, at the same codon site as eL1.

Wild type E. coli was strain B/5. Ribonuclease III- mutant strains of E. coli were AB301-105 and BL214 (3) and N2048 (5). Strains BL214 and N2048 contain the ribonuclease III-negative mutant allele of strain AB301-105. Strain A19 is the ribonuclease III-positive parent of strain AB301-105 (3). Strain N2053 is a ribonuclease III-positive transductant of strain N2048 (5).

Efficiency of plating measurements. Bacteriophage were incubated for about 10 m at room temperature with 0.5 ml of a growing culture of E. coli (~$3x10^8$ cells/ml in broth). The suspension was then overlayed onto agar plates with 2 ml of top agar. The plates were incubated overnight at 37°C. Platings were done to obtain about 800 plaques. Media recipes have been described (4).

Preparation and analysis of ^{32}P-labeled RNA. Detailed procedures for cell growth, T4 infection, ^{32}P labeling and RNA analysis are described elsewhere (4). A 5 ml culture of infected cells was incubated with 2 mCi ^{32}P from 4 to 15 m after infection. Three methods were used to extract RNA from infected cells. (i) The standard method involved adding liquid phenol directly to the culture medium at the end of the labeling period (4). (ii) The hot SDS method involved adding an equal volume of boiling sodium dodecyl sulfate buffer (1% sodium dodecyl sulfate; 0.2M NaCl; 0.04M ethylenediaminetetraacetic acid; 0.02M tris (hydroxymethyl) aminomethane, pH 7.5) to the culture medium at the end of the labeling period. The sample was mixed and then placed in a bath of boiling water for 90 s. Liquid phenol was then added to the sample. (iii) The proteinase K method proceeded as in (ii), but following the 90-s incubation in the boiling water bath, the sample was cooled to 37°C and proteinase K was added to a final concentration of 2.0 mg/ml. The sample was incubated at 37°C for 1 h, at which time phenol was added.

After phenol extraction, nucleic acid was precipitated by the addition of ethanol. The sample was then treated with DNase and fractionated by DEAE-cellulose column chromatography. The RNA was electrophoretically fractionated on a slab gel containing 10% polyacrylamide.

RNA fingerprint analysis. Two-dimensional fingerprints of ribonuclease T_1 digestion products were prepared as described previously (4). The identity of RNA species was ascertained by comparing its fingerprint to those of known species (1, 4).

Table 1. Effect of Host Ribonuclease III Mutation on Suppressor Functions of T4 tRNAs

T4 strain	Suppressor tRNA	Plating efficiency (%)
psu^+_{2oc}-eL1	$tRNA^{Gln}$	<1
psu^+_{1am}-eL1a	$tRNA^{Ser}$	101
psu^+_{3am}-eL1a	$tRNA^{Leu}$	95
psu^+_{4op}-eL1G	$tRNA^{Arg}$	114
psu^+_{2oc}	$tRNA^{Gln}$	94
psu^+_{1am}	$tRNA^{Ser}$	100
psu^+_{3am}	$tRNA^{Leu}$	96
psu^+_{4op}	$tRNA^{Arg}$	104

Plating efficiency is the percentage of bacteriophage that grew on the ribonuclease III-mutant strain, AB301-105, compared to the wild type strain, B/5. Similar plating efficiencies were obtained on strains B/5 and A19. Strain A19 is the parent of strain AB301-105 (3).

RESULTS AND DISCUSSION

To determine whether ribonuclease III participates in T4 tRNA synthesis, we examined the ability of T4 suppressor tRNAs to function in a ribonuclease III-mutant strain. T4 strains carrying a suppressor form of a tRNA gene together with a suppressible nonsense mutation were tested for growth on ribonuclease III-mutant cells and wild type cells, both of which are nonpermissive for nonsense mutant strains. If formation of the suppressor tRNA is blocked in ribonuclease III-mutant cells the bacteriophage will not grow. Bacteriophage growth was determined by plating efficiency.

Table 1 shows that the psu^+_2 function of T4 $tRNA^{Gln}$ was reduced in the ribonuclease III-mutant strain, although the plating efficiency of the single T4 mutant, psu^+_2, carrying only the suppressor tRNA was unaffected. The remaining data in Table 1 show that the suppressor functions of T4 $tRNA^{Ser}$, $tRNA^{Leu}$, and

$tRNA^{Arg}$ were unaffected by the ribonuclease III mutation. These results demonstrate that a normal level of functional $tRNA^{Gln}$ is not made in cells mutant for ribonuclease III.

We analyzed RNA from infected cells to determine if loss of functional $tRNA^{Gln}$ in the mutant cells was accompanied by decreased levels of this tRNA. Fig. 1 shows polyacrylamide gel profiles of ^{32}P-labeled, T4-coded $tRNA^{Gln}$ and $tRNA^{Leu}$ synthesized in mutant and wild type cells. The T4 strain used in this experiment carries a deletion mutation spanning six T4 tRNA genes, leaving only $tRNA^{Gln}$ and $tRNA^{Leu}$ genes intact (1, 4). (Cellular tRNAs were not labeled in this experiment because host RNA synthesis terminates after T4 infection.) To insure complete recovery of RNA, three methods of RNA extraction were used.

T4 $tRNA^{Gln}$ and $tRNA^{Leu}$ are derived from a common precursor RNA molecule (1), and as expected, approximately equal yields of these two tRNAs were obtained in wild type cells (Fig. 1). Infection of cells deficient in ribonuclease III under the same conditions resulted in production of $tRNA^{Leu}$, but little or no $tRNA^{Gln}$. (The precursor RNA containing $tRNA^{Gln}$ and $tRNA^{Leu}$ is unstable, and does not accumulate in either wild type or mutant cells in sufficient amounts to be observed in polyacrylamide gels.) Severe reduction of $tRNA^{Gln}$ in ribonuclease III-deficient cells points to an involvement of this enzyme in the synthesis of $tRNA^{Gln}$.

An experiment analogous to the one shown in Fig. 1 has been performed with wild type T4. Again, the gel profile of ribonuclease III-deficient cells showed a dramatic reduction in $tRNA^{Gln}$. Bands corresponding to the remaining seven T4 tRNAs were present. The identities of the tRNAs in the bands were confirmed by two-dimensional fingerprint analysis.

Ribonuclease III mutant strain AB301-105 was used for the experiments reported in Table 1 and Fig. 1. This strain was derived from strain A19 by mutagenesis and may contain several mutations in addition to the ribonuclease III lesion (3). We tested two other ribonuclease III-mutant strains and obtained results similar to those reported in Table 1 and Fig. 1 (results not

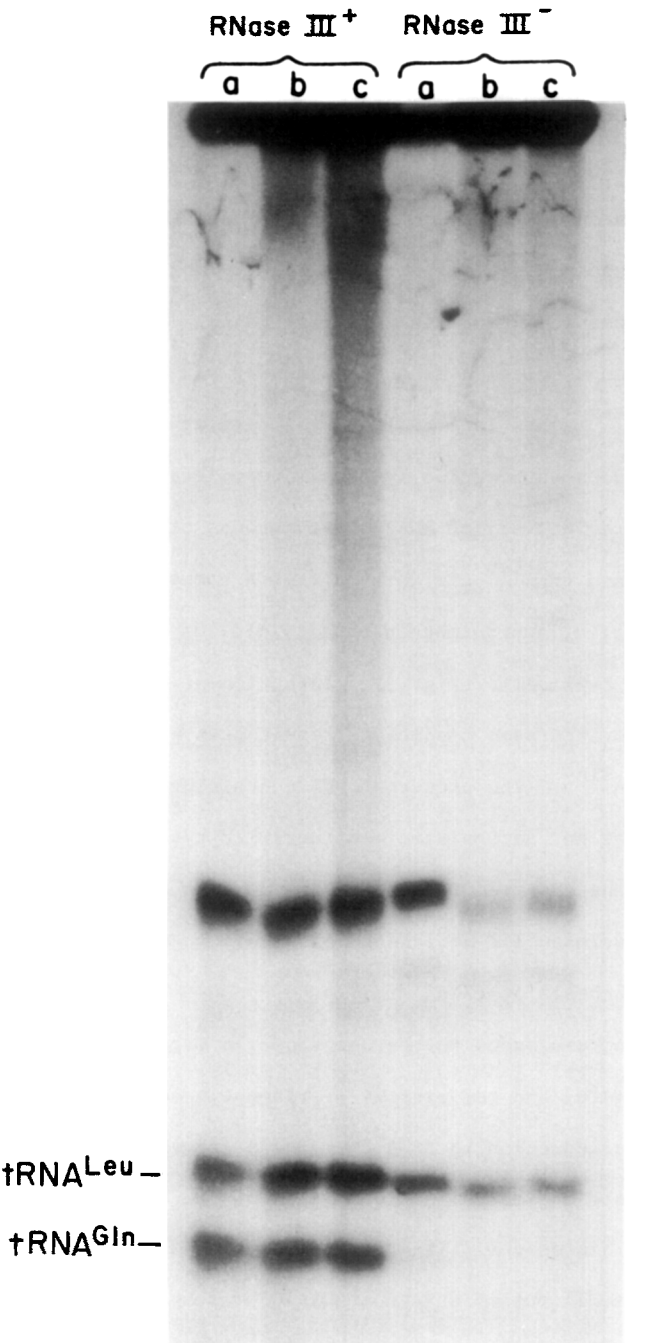

Fig. 1. Gel of ^{32}P-labeled RNA from T4-infected cells. Wild type E. coli strain, B/5, is designated RNase III$^+$, and the mutant E. coli strain, AB301-105, RNase III$^-$. The bacteriophage T4 strain, Δ27, bears a deletion of all tRNA genes except those encoding tRNA^{Gln} and tRNA^{Leu} (1, 4). Three methods were used to extract RNA from infected cells: a, the standard method; b, the hot SDS method; and c, the proteinase K method. The predominant RNA migrating

shown). The finding that three otherwise unrelated strains carrying the same ribonuclease III mutation behaved similarly indicates, but does not prove, that the effect on $tRNA^{Gln}$ synthesis is a consequence of the ribonuclease III deficiency.

The reduced amount of $tRNA^{Gln}$ observed in Fig. 1 may be correlated with accumulation of a larger precursor RNA that is a substrate for ribonuclease III. We have looked for, but have not found, such a precursor RNA. This effort involved analyzing all major and minor gel bands migrating slower than $tRNA^{Gln}$ by preparing two-dimensional fingerprints of ribonuclease digestion products and examining these for $tRNA^{Gln}$-specific oligonucleotides. We do not know why the putative precursor RNA containing $tRNA^{Gln}$ has not been detected in cells mutant for ribonuclease III. Perhaps this precursor RNA is polydisperse in length and does not migrate in gels as a single molecular species. Such heterogeneity could result from non-specific attacks by ribonucleases on precursor RNA-specific residues. Less likely, though not precluded by the results, is the possibility of degradation of this precursor RNA. Production of $tRNA^{Leu}$ in the mutant cells indicates that the residues of $tRNA^{Gln}$ are synthesized, because these two tRNAs are cleaved from a common precursor RNA chain. Degradation of the $tRNA^{Gln}$ chain seems unlikely given the reputed stability of tRNAs to cellular ribonucleases. This stability is known to depend on the native conformation of the tRNA chain, and the residues comprising $tRNA^{Gln}$ apparently adopt a native, tRNA-like conformation while contained within the precursor RNA chain (1).

Chemical and functional analyses revealed that seven T4 tRNAs were synthesized in cells deficient in ribonuclease III. This observation suggests to us that ribonuclease III does not participate in synthesis of these tRNAs. Two other interpretations of this finding can be offered: (i) residual enzyme

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slower than $tRNA^{Leu}$ is a stable, low-molecular-weight RNA coded by T4 (1). RNA from infected cells of strain A19, the parent of AB301-105 (3), was not analyzed. However, near isogenic strains known to differ only in ribonuclease III locus, strains N2053 and N2048 (5), were analyzed yielding results concordant with those shown in this figure.

activity persists in the mutant cells and is sufficient to support synthesis of some tRNAs; and (ii) ribonuclease III activity lost by mutation can be replaced by an auxiliary enzyme.

How might ribonuclease III function in the synthesis of only one T4 tRNA? As mentioned in the Introduction, the eight tRNA sequences might be contained within a single precursor RNA. Though this putative precursor RNA has yet to be detected in infected cells, we know from the DNA sequence of the T4 tRNA genes that $tRNA^{Gln}$ would occur nearest the 5' end of the molecule (Fukada and Abelson, personal communication). One cleavage of this precursor RNA molecule by ribonuclease III could remove most 5' precursor RNA-specific residues. This cleavage would not generate the mature 5' terminus of $tRNA^{Gln}$, however, because ribonuclease P is known to function in this capacity (1). The absence of $tRNA^{Gln}$ in ribonuclease III-deficient cells indicates a requirement for the ribonuclease III cleavage of the precursor RNA prior to the ribonuclease P cleavage. Perhaps this sequential order operates only with $tRNA^{Gln}$ because of its 5' position within the precursor RNA.

Demonstration that ribonuclease III actually participates in the synthesis of T4 $tRNA^{Gln}$ and evaluation of the hypothesis outlined in the previous paragraph will require further work, including isolation of the precursor RNA that accumulates in the absence of ribonuclease III.

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