Some RNases Involved in the Processing of Bacteriophage T4 RNA

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요약. RNase III, RNase E, 및 RNase P가 각각 종주 또는 복합적으로 결합하는 E. coli 돌연변이 종주들 내에서의 박테리오파지 T4 tRNA의 전구 RNA로 부터의 합성을 연구하였다. RNase E 종주에서는 9S RNA로 볼 수 있는 유전인 자.te가 축적되었으며 RNase P 종주에서는 6S 이중띠의 하부띠가 축적되었다. RNase III 종주에서는 T4 tRNA 유전인자 끝(cluster)에 의하여 코드되는(coded) tRNA$^{III}$의 생성이 상하게 억제되며 T4 DNA에 의하여도 코드되지만 T4 tRNA 유전인자에 의하여도 코드되지 않는 species 1 RNA의 양도 단소 억제된다. 그 외 등장하는 T4 tRNA 유전인자 끝에 의하여 코드 되는 6S 이중띠의 상부띠는 RNase III$^+$ 종주의 경우에 비하여 더 크게 축적된다. 그러나 6S 이중띠의 상부띠의 RNA와 tRNA$^{III}$ 사이에는 precursor-product 관계가 없다고 판단되며 RNase III이 precursor RNA를 가수분해 전환한다고 생각하는 개념을 지지할만한 근거가 없음을 지적할 수 있다.

ABSTRACT. Bacteriophage T4 tRNA processing in E. coli mutant strains defective in RNase III, RNase E, and RNase P, respectively, singly or in combinations, was investigated. In RNase E$^-$ strains, a RNA band, which would be referred as 9S RNA, accumulates, while in RNase P$^+$ strains, lower band of 6S double band is accumulated. In RNase III$^+$ strains, the production of tRAN$^{III}$ coded by T4 tRNA gene cluster, is severely depressed and also production of species 1 RNA, which is coded by T4 DNA but not by the tRNA gene cluster, is in somewhat depressed amounts; on the other hand, at the same time, an upper band of 6S double bands, coded by T4 tRNA gene cluster, is accumulated in rather greater amounts as compared to the RNase III$^+$ strain. The upper band RNA of the 6S double band, however, does not appear to be a precursor to the tRAN$^{III}$. The present work points to the lack of evidence for an essential cleavage role of RNase III, although there must be a role for the RNase III in the T4 tRNA processing.
in addition to the mature tRNA sequence, must be processed by various cellular enzymes to yield the mature tRNA. However, the identities and functional activities of RNases involved are not well understood, and only one enzyme, RNase P, has been shown to perform an obligatory function in the endonucleolytic processing of tRNA precursor molecules. The mechanism and rationale of the post-transcriptional processing of tRNA remain one of the least understood area of molecular biology.

In the bacterium E. coli, the processing of E. coli tRNA precursors occurs so fast that it is difficult to get detailed information. In the present work, since the processing of T4 tRNA precursors in cells is known to be dependent upon host cellular RNases, T4 tRNA synthesis in E. coli mutant strains carrying the mutated genes for RNases was investigated. The E. coli mutants employed in the present work were those defective in the enzymes, RNase III (rne); RNase E (rne), and RNase P (rnp), in singly or in combinations. Comparative studies of the cases using a common mutant lesion of an enzyme, e.g., rne, rnp, and rncrp, will not only give some useful insight into the elucidation of the effect of an enzyme on the functional activity of the other, but also can be an effective and convenient approach to the elucidation of the functional activity of the RNases. The bacteriophage T4 have been reported to code for eight unique species of tRNA, and this system has been exploited as a useful system for the study of tRNA synthesis.

While a functional role and mechanisms of RNase III in the T4 tRNA processing remains obscure, the current prevailing concept prescribing an essential cleavage role by this enzyme of a precursor RNA containing tRNA6th lacks supporting evidence. In this paper, a notion that RNase III may participate indirectly in the tRNA processing by affecting the conformation of the RNA molecule to be recognized and cleaved by a secondary RNase species, e.g., RNase P, will be presented.

**MATERIALS AND METHODS**

Organisms: All bacteriophage and E. coli strains were obtained from Dr. D. Apirion (University of Washington, U.S.A.). The characteristics of deletion of tRNA genes in bacteriophage mutant strains used are as follows: Δ27, deletion of all tRNA genes except those encoding tRNA6th and tRNA4th; Δ33, deletion of all tRNA genes; Δ8, deletion of all tRNA genes except those encoding tRNA6th, tRNA3th, tRNA4th, and tRNA5th. E. coli strains used were: N 2097, wild type; N 2097, rnc (RNase III-); N 2021, rnp (RNase P-); N 2018, rncrp (RNase III- and RNase P-); N 3421, rnc (RNase E-); N 3589, rnc-rnc (RNase III- and RNase E-); N 3522, rncrp (RNase E- and RNase P-).

Media and Cell Growth: The basal medium in Tris buffer (pH 7.4) was adapted from Landy et al. using the guidelines of Neidhardt et al. The medium contains, per liter, 6.05 g of Tris base, 1.50 g of KCl, 1.0 g of ammonium sulfate, 5 ml of a 200X concentrate of trace salts, pH adjusted to 7.4. For labelling with 32Pi, the medium was supplemented with 0.2% glucose and 0.6% peptone (15 µg PO4/ml).

Overnight cultures of E. coli strains were made by inoculating a single colony of each strain to a test tube of rich medium and incubating at 30°C. Experimental cultures were made by transferring the overnight culture to the Tris-glucose medium containing 0.6% peptone and incubating in a water bath at 30°C with shaking for at least 3 doublings.

Bacteriophage Infection: At an A590 of about 0.5 (ca. 3 x 10⁶ cells/ml), cells were infected,
at zero time, by bacteriophage at the MOI (multiplicity of infection) of 10 and at the time of 4 min again infected (superinfected) at the MOI of 10. Temperature sensitive E. coli mutants were transferred to the nonpermissive temperature of 43°C, 90 minutes before the infection with the bacteriophage.

Labeling of RNA and Preparation of Samples for Electrophoresis: Then, eight minutes later, the bacteriophage-infected cells were labeled with 32P by the addition of neutralized 32PO4 (Amersham) to a final concentration of 1.0~2.0 mCi/ml. At 20 minutes after the labeling, was added 4 volumes of ice-cold 90 % ethanol containing 1 % diethylpyrocarbonate (DEP) and was left at -20°C for several hours.

To the precipitate was added 1.0 ml of lysis buffer (0.02 M Tris-HCl, pH 7.4: 0.01 M EDTA: 1 % sodium dodecylsulfate (SDS)), and the precipitate in the lysis buffer was lysed for 2 minutes in boiling water bath at 95°C. After cooling to room temperature, equal volume (1.0 ml) of phenol saturated with the lysis buffer was added, and the mixture was shaken vigorously on a Vortex test tube mixer for 30 minutes at room temperature. The aqueous phase, separated by centrifugation and collected was further phenol-extracted. After the phenol extraction, the nucleic acid was precipitated by the addition of 2 volumes of ethanol containing 0.1 M sodium acetate, pH 5.0. The RNA was allowed to precipitate out of the extract for 15 minutes at -20°C, and then recovered by centrifugation. For the purpose of gel electrophoresis, the RNA was redissolved in boiling sample buffer (0.02 M Tris-HCl, pH 7.6: 0.002 M EDTA: 20 % glycerol: 0.2 % SDS and ca. 0.005 % bromphenol blue dye).

Polyacrylamide Gel Electrophoresis of RNA: 32P-labeled RNA samples in the sample buffer were fractionated on SDS-containing polyacrylamide gel slabs, using the system described by Studier. For analytical purposes, 5~15 %, 5~12 % or 5~10 % tandem polyacrylamide (acrylamide: bisacrylamide=30:0.8) gels were used. Tris-glycine buffer (pH 8.3) with 0.1 % SDS was used as the running buffer. The gels were run at 4°C for 30 minutes at 100 volts, then 4 hours at 150 volts. For autoradiography, the wet gel was covered with plastic wrap and exposed to Kodak XR-5 film.

Two-dimensional gel electrophoresis was carried out according to the technique developed by Ikemura and Dhalberg and modified by Gegenheimer and Apirion, RNA Oligonucleotide Analysis: The minifingerprinting technique of Volkaert el al. was employed. The spots were eluted according to them, and the pancreatic RNase redigestion and DEAE electrophoresis as described by Barrell were followed. Base composition of marker oligonucleotides from DEAE paper was determined after digestion with RNase T2 (Calbiochem) and chromatography on PEI thin-layer plate in 1.0 M LiCl.

RESULTS

The autoradiograms of 32P-labeled RNA extracted from T4-infected cells of E. coli mutant strains defective in the RNases in singly or in combination and fractionated by electrophoresis on a 12 % polyacrylamide gel are shown in Fig. 1. In the autoradiogram, one can see that a RNA band, which would be referred as 9S RNA, appears in the E. coli strains defective in RNase E, i.e., mce, mcne, and mcrep, while in the strains defective in RNase P, i.e., mc, mcrep, and mcrep, double bands corresponding to the size of 6S RNA accumulate. In the counterpart RNase P+ Strain, i.e., mce and wild strain (N2099), the lower band of the 6S double bands occurs in relatively reduced
Fig. 1. Gel-electrophoretic patterns of RNA from T4-infected E. coli mutant cells defective in the enzymes, RNase III (rnc), RNase P (rpm), and RNase E (rne), in singly or in combinations. Each lane is labeled with both the strain number and the symbol of mutant, characteristic for the defective enzyme system. The picture shown here is of an autoradiogram of the 12% portion of the gel. Experimental procedures are described in the text. The band, labeled as "140 bases", is referred as "species 1 RNA" in the text.

amounts or disappears completely, and this observation is reproduced again in the Fig. 2. A comparison of the RNA patterns of rpm vs. rpm rmcp, and rpm vs. the wild strain will show that the upper band of the 6S double bands occurs in large amounts in the RNase III- strains, as compared to RNase III+ strains. The deletion of their characteristic RNA bands in their tRNA regions can also be confirmed in these pictures. Thus, while the final verification remains to be attained by additional studies of the relationship between the precursor RNA and the product tRNA bands in fingerprints and in vitro processing experiments, the results based on the figures of autoradiograms of 32P-labeled RNAs, as summarized, may lead to the speculation that the 9S RNA band is affected by RNase E, the upper band of the 6S double band is affected by RNase III, and that the lower band of the 6S double band is affected by RNase P respectively.

In an effort to improve the knowledge of the possible functional role of RNase III in the processing of the tRNA precursors, A27 and A8 deletion phage mutants were used in the infection of the E. coli cells of the wild strain (N2099) and the rnc strain. Fig. 2 shows the 32P-labeled RNA gel electrophoresis (15% acrylamide) patterns of wild type and rnc cells infected with T4 and deletion mutant phages respectively. The deletion mutant phages carry a deletion in the tRNA operon1. Infection of the
Fig. 3. Two-dimensional gel electrophoresis of tRNA region of RNA from T4-infected wild type and rnc E.coli strains respectively. The technique developed by Ikemura and Dahlberg, and modified by Grora and Apiriom3 was employed in the experimental procedures. The arrow in the N2099 gel indicates the spot of tRNA\(^{Gln}\), and the arrow in the N2097 gel indicates the corresponding position, where the tRNA\(^{Gln}\) spot is missing.

Fig. 4. RNase TI two-dimensional fingerprint of tRNA\(^{Gln}\) spot extracted from the gel of wild type strain (N2099) shown in Fig. 3. The first dimension was electrophoresis, followed by homochromatography in homomixture CJO (10 min hydrolysed homomixture C)\(^{n}\) in the second dimension.

**DISCUSSION**

The experimental results here, while confirming a previous observation\(^6,17\) that the level of tRNA\(^{Gln}\) and species 1 RNA is reduced in RNase III\(^{+}\) strains, are extended to show that also the level of the upper band of the 6S double band is increased in RNase III\(^{+}\) strains. They also demonstrate the accumulation of a 9S RNA band in RNase E\(^{-}\) strain and the lower band of 6S double band in RNase P\(^{-}\) strain respectively. Whereas the 9S band, the 6S double band, and the species 1 RNA band are all coded by T4 DNA, the 9S and the 6S RNase III\(^{-}\) strain (rnc) with he \(\triangle 27\), the production of the tRNA\(^{Gln}\) can not occur and, if any, only in minute amounts. By the comparison of the two-dimensional gel electrophoresis patterns of the \(^{32}\)P-labeled RNA from T4-infected wild type and rnc cells in Fig. 3, the depressed production of tRNA\(^{Gln}\) in rnc can be further confirmed. The \(^{32}\)P-labeled RNA spot of N2099 (wild type) corresponding to tRNA\(^{Gln}\) was extracted from the Fig. 3 and then its structure was analyzed by two-dimensional fingerprint after digestion with Ti, pancreatic and T2 RNase. The result of the fingerprint analysis shown in Fig. 4 identifies the RNA deleted to be the tRNA\(^{Gln}\) (see also References 6 and 17). Further studies will be required for the elucidation of a specific role of the RNase III in the process of the tRNA\(^{Gln}\) production.
bands only are coded by the tRNA gene cluster and not the species 1 RNA band, since the former two band species do not occur, while the latter band does, when the phage J33, which contains a deletion of all the tRNA gene cluster\(^{10,19}\), is used (see Fig. 1 and Fig. 2.) Thus, the results obtained in the present work show that the role of RNase III is not limited only to the processing of tRNA\(^{\text{Gln}}\) but also is implicated in the level of upper 6S band and species 1 RNA. These results prompted the author to look for any precursor-product relationship between the upper 6S band and the tRNA\(^{\text{Gln}}\), employing the techniques of in vitro processing of the 6S RNA with an extract from an RNase III\(^+\) strain and also with an RNase III enzyme preparation, but the experimental data obtained did not indicate any of such a relationship between the two RNA species. A separate experiment in the structural analysis of the 6S RNA, using digestions with T1 RNase followed by digestion with pancreatic or T2 RNase, again showed a negative result as to the precursor-product relationship between the 6S RNA and the tRNA\(^{\text{Gln}}\). However, the structural analysis indicated that the 6S RNA contains tRNA\(^{\text{Pro}}\) and tRNA\(^{\text{Asx}}\). The effect of the RNase III on the 6S RNA and the species 1 RNA is not so striking, though as obvious, as on the tRNA\(^{\text{Gln}}\) synthesis, and the previous reports\(^6,17\) might have missed to recognize the RNase III effect on these RNA species.

We can notice in Fig. 1 rather little difference in their RNA patterns in rnc vs. rncup and vs. rncrne respectively. Thus one can speculate some delicate functional roles of RNase III other than drastic hydrolytic cleavage functions, and that RNase III may affect the conformational feature of the nucleic acid, instead of a hydrolytic cleavage role, thereby rendering the nucleic acid molecule to be recognized by proper enzymes, e.g., RNase P, or other factors. Indeed, Guthrie et al.\(^{26}\) have shown that RNase P recognizes a conformational feature shared by all precursor molecules, and that loci far removed in space from the cleavage site can alter the enzyme-substrate recognition process. Pragai et al.\(^{17}\) reported that a T4 tRNA precursor species (10S band), accumulated in RNase III\(^-\) strain, could be processed in vitro to tRNA\(^{\text{Gln}}\) and tRNA\(^{\text{Asx}}\) by an extract from RNase III\(^+\) strain. However, as they did not carry out additional studies using purified RNase III and RNase P preparations separately and also together in combinations, the result they obtained can not be a supporting evidence

\(\text{Fig. 1. Electrophoretic analysis (15% acrylamide) of RNAs of wild type, rnc, and rncp, infected with bacteriophage T4 wild and J33 respectively. Experimental procedures are the same as those described in the legend of Fig. 2.}\)
that T\(N\)ase III has an essential cleavage role in the T\(4\) t\(\text{RNA}^{\text{m}}\) processing.

As to the question how R\(N\)ase III may function in the synthesis of only one T\(4\) t\(\text{RNA}^{\text{m}}\), t\(\text{RNA}^{\text{a}}\), without significant effect on t\(\text{RNA}^{\text{b}}\), it should be mentioned that all of the T\(4\) t\(\text{RNAs}\) are synthesized in a single transcript having t\(\text{RNA}^{\text{a}}\) nearest the 5' end of the molecule and yet the relative amounts of different mature t\(\text{RNA}\) species can vary extensively, depending on the precise arrangement of sequences surrounding the T\(4\) t\(\text{RNA}\) genes\(^6\).

Verification of the possibilities presented here (1) that a role of R\(N\)ase III is not limited only to the t\(\text{RNA}^{\text{a}}\) metabolism but is extended over to the metabolism of more such RNA species as 6\(S\) upper band RNA and species 1 RNA, (2) that R\(N\)ase E and R\(N\)ase P are involved in the accumulation of 9\(S\) and 6\(S\) lower band RNA respectively, and (3) the evaluation of the postulation outlined above regarding to the plausible functional role of R\(N\)ase III in the T\(4\) t\(\text{RNA}\) processing, will require further extensive work, including isolation of precursor RNA species that accumulate in an R\(N\)ase-defective strain, in vitro processing studies of the isolated precursors with appropriate R\(N\)ase systems, structural analysis to establish precursor-product relationships, and physicochemical studies to test the plausible effect of R\(N\)ase III on the conformational transition of nucleic acids.

REFERENCES


