
An *Escherichia coli* endonuclease responsible for primary cleavage of in vitro transcripts of bacteriophage T4 tRNA gene cluster

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ABSTRACT

An endonuclease activity was isolated from 100,000 g supernatant fraction of *Escherichia coli* using in vitro primary transcripts of T4 tRNA gene cluster as assay substrates. The endonuclease cleaves the polycistronic RNA precursors into fragments containing monomeric and dimeric stable RNA sequences. The results strongly suggest that this enzyme participates in the early steps of T4 tRNA maturation pathway preceding the action of RNase P.

INTRODUCTION

It is now well established that posttranscriptional processing of RNA is a general phenomenon in both prokaryotes and eukaryotes (1). A multitude of enzymes seem to participate in this process but their investigation has been restricted by the requirement for individually purified RNA species which can be used as substrates for in vitro processing. A useful system for the study of RNA processing is provided by the bacteriophage T4 tRNA operon: a cluster of genes coding for ten short phage-specific stable RNAs (2) which are transcribed with the formation of polycistronic RNA precursors (3, 4). Elsewhere (4) we described the isolation of the primary transcripts of the tRNA operon, which were synthesized in vitro on T4 DNA. These polycistronic RNA molecules were processed to mature-size tRNAs by cell extracts of *Escherichia coli*. In the present work we used the transcripts of the T4 tRNA operon as assay substrates for the isolation and preliminary characterisation of a processing endonuclease from *Escherichia coli* extracts.

Fig. 1 presents the map of the T4 tRNA operon and of its major primary transcript A_{wt} as well as the deletion-specific transcripts A_8 , A_{105} and A_{119} which are made on T4 DNA with partial deletions of tRNA genes. The relatively short (about 0.75 kb) deletion-specific transcript A_{119} which contains the sequences of three promoter-distal genes was used as substrate for endonuclease assay during the isolation. The isolated

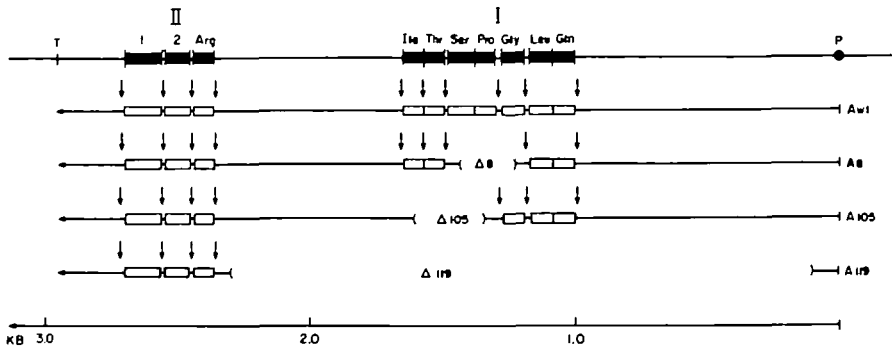


Fig. 1. Bacteriophage T4 tRNA operon and its primary transcripts

The top scheme shows the two subclusters of stable RNA genes (coding for eight tRNAs and Species 1 and Species 2 RNA) which are separated by a spacer of about 0.6 kb (2, 8). Promoter (P) and terminator (T) are located at 1 kb upstream (9) and 0.3 kb downstream (4) from the stable RNA sequences, respectively. The four lines underneath represent the structures of primary transcripts of this region which are synthesized *in vitro* on T4 DNA with intact (A_{wt}) or partially deleted (A_8 , A_{105} , A_{119}) tRNA gene cluster (4). Parentheses represent the deletions $\Delta 8$, $\Delta 105$ and $\Delta 119$ (2). Open boxes represent the stable RNA sequences in the transcript molecules and vertical arrows indicate the sites of cleavages which should occur in the absence of RNase P as predicted on the basis of DNA sequence of the two gene subclusters (1, 10). The lower scale shows the distance from the promoter.

endonuclease activity was then characterised using longer RNA species A_{wt} , A_8 and A_{105} as precursors for the processing reaction.

MATERIALS AND METHODS

Buffers. Buffer A: 50 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 7 mM β -mercaptoethanol.

Buffer B: 20 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 0.1 mM dithiothreitol.

Buffer C: 50 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 0.1 mM dithiothreitol.

TGED: 50 mM Tris-HCl, pH 7.9; 5% (v/v) glycerol; 0.1 mM EDTA; 0.1 mM dithiothreitol.

Preparation of S-100 extract. *E. coli* MRE-600 cells (50 g) were ground with 50 g glass powder. 75 ml of Buffer A containing 0.05 M KCl were added and the cell debris removed by centrifugation at 8 000 rpm for 20 minutes. The supernatant was treated with 200 μ g of DNase I (RNase-free, Worthington) and centrifuged in a Spinco 60Ti rotor at 17 000 rpm for 25 minutes. The pellet was discarded and the supernatant centrifuged in the same rotor

at 44 000 rpm for 4 hours. The upper two-thirds of the supernatant were recovered, diluted to a protein concentration of 50 µg/ml and stored at -20°C.

Primary transcripts of T4 tRNA gene cluster were synthesized *in vitro*, purified by gel electrophoresis and recovered from the gel as described elsewhere (4). The specific activity of (α -³²P)-labelled RNA was 1-4 Ci/mmol GMP. The specific activity of (β -³²P)-labelled RNA was 75 Ci/mmol ATP. (β -³²P)-ATP was a generous gift of Dr. Y. Groner.

Standard endonuclease assay. The standard 20 µl assay sample contained (³²P)-RNA substrate; 2 µg of rRNA carrier purified by gel-filtration; 10 mM Tris-HCl, pH 7.9; 5 mM MgCl₂; 100 mM KCl; 0.1 mM EDTA; 0.1 mM dithiothreitol and varying amounts of the assayed enzyme fraction. Unless specified otherwise, the samples were incubated for 60 minutes at 37°C, deproteinized with phenol, precipitated with ethanol with 3-4 µg of cold tRNA carrier and analysed by gel electrophoresis.

Electrophoresis in 10% polyacrylamide gel slabs (400 x 200 x 1 mm) containing 6.6 M urea in Tris-Borate buffer system (5) was performed as described elsewhere (4). The gels were autoradiographed at -70°C in Kodak X-ray cassettes with Cronex intensifying screens using preflashed AGFA Curix RP-1 films.

Recovery of cleavage fragments from the gel. Radioactive bands were cut out of gels and transferred into Eppendorf tubes. The gel was crushed with a glass rod and 0.2 - 0.3 ml of the extraction buffer (0.5 M ammonium acetate; 0.01 M magnesium acetate; 0.1 mM EDTA; 0.1% SDS) added. The tubes were incubated for 10-18 hours at 37°C with occasional stirring. The slurry was put on top of 3 ml columns of water-equilibrated Sephadex G-25 (Coarse) in plastic syringes from which the free volume liquid was removed by centrifugation. The syringes were centrifuged and the flowthrough fractions were concentrated to the desired volume by evaporation in Evapomix.

Analysis of proteins by electrophoresis in SDS gel was performed according to Ref. 6. with standard size markers of Pharmacia. Protein concentrations were calculated from UV absorbancy at 260 and 280 nm according to Ref. 7.

RESULTS

Isolation of the processing activity

The 100 000 g supernatant fraction of *E. coli* (S-100) contains all the enzymes nece-

ssary for the formation of mature-size stable RNAs from primary transcripts of the T4 tRNA operon (4). Fig. 2 presents the kinetics of digestion of band A₁₁₉ precursor RNA with S-100. The concentration of S-100 protein in the reaction mixture was about 5 mg/ml and it took about 90 minutes to completely digest the precursor band. The three major products

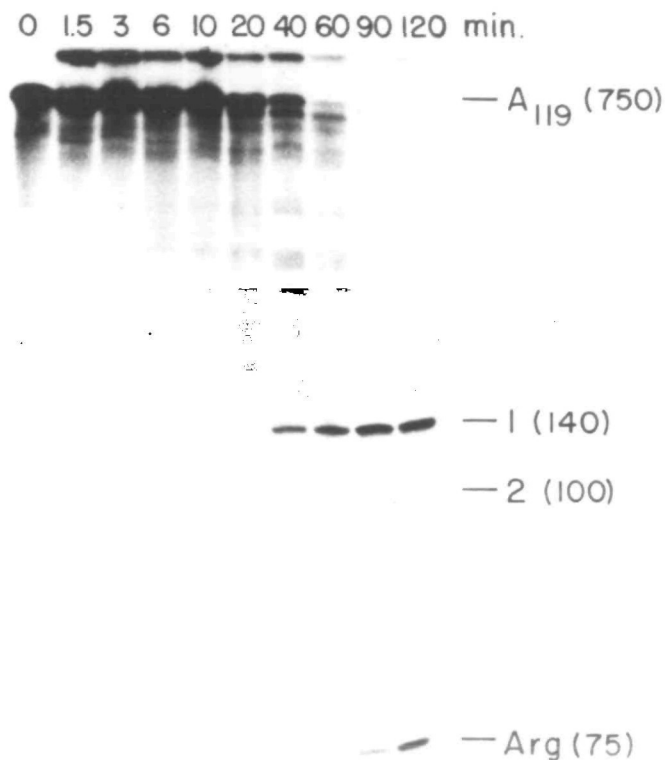


Fig. 2. Kinetics of digestion of band A₁₁₉ RNA with S-100.

Band A₁₁₉ RNA was incubated with S-100 (5 mg/ml) for different periods of time under standard assay conditions and the products were analysed in a 10% acrylamide gel. The identity of the stable products was established as described elsewhere (4). The lengths of RNA (nucleotides) are given in parentheses.

have been previously identified as Species 1 RNA (140 nucleotides), Species 2 RNA (100 nucleotides) and tRNA^{Arg} (75 nucleotides) (4). The S-100 fraction was used as the starting material for the isolation of the processing activity using band A₁₁₉ RNA as substrate for assay.

DEAE-cellulose chromatography. S-100 obtained from 50 g of cells was diluted to 100 ml with Buffer B containing 0.05 M KCl and loaded onto a 100 ml DEAE-cellulose column equilibrated with the same buffer. After loading, the column was washed with 150 ml of the same buffer and the bulk protein eluted with Buffer B containing 0.33 M KCl and precipitated with (NH₄)₂SO₄ (0.5 g per ml solution). The precipitate was collected by centrifugation, dissolved in TGED and dialysed extensively against TGED containing 0.03 M KCl. This fraction (DE-I, final volume 100 ml) had approximately the same level of processing activity as S-100 (Fig. 3, lanes 1 and 2) and was used for further purification.

Phosphocellulose chromatography. DE-I fraction was loaded onto a 12 x 1 cm phosphocellulose column equilibrated with TGED containing 0.03 M KCl. The column was washed with the same buffer until OD₂₈₀ in the eluate was less than 0.05 and the adsorbed material was eluted stepwise with 0.32 M, 0.50 M and 1.0 M KCl in TGED. Most of the protein was recovered in the flowthrough fraction (PC-F) and the amount of protein eluted at the three salt concentrations (fractions PC-0.3, PC-0.5 and PC-1.0) was 2 mg, 3 mg and 1.4 mg, respectively. As one can see from Fig. 3 (lanes 3 to 6) most of the processing activity was recovered in the PC-1.0 fraction.

DEAE-Sephadex chromatography. PC-1.0 fraction was extensively dialysed against Buffer C containing 0.05 M NH₄Cl and loaded onto a 5 x 1 cm column of DEAE-Sephadex (A-50, Pharmacia) equilibrated with the same buffer. About half of the protein was recovered in the flowthrough (DES-F) fraction and the absorbed material was eluted as a single peak (DES-P fraction) with a 0.05 - 0.5 M gradient of NH₄Cl in buffer C. As one can see from Fig. 3 (lanes 7 to 12), all of the processing activity was recovered in the DES-F fraction. The material of DES-F was transferred into a dialysis bag, concentrated by imbedding the bag into dry Sephadex G-100 for several hours and dialysed against several changes of TGED. This preparation (0.5 mg protein in 1 ml) was used in the subsequent experiments and will be hereafter referred to as precursor cleaving nuclease (PC nuclease).

The protein fractions obtained at different stages of endonuclease isolation were

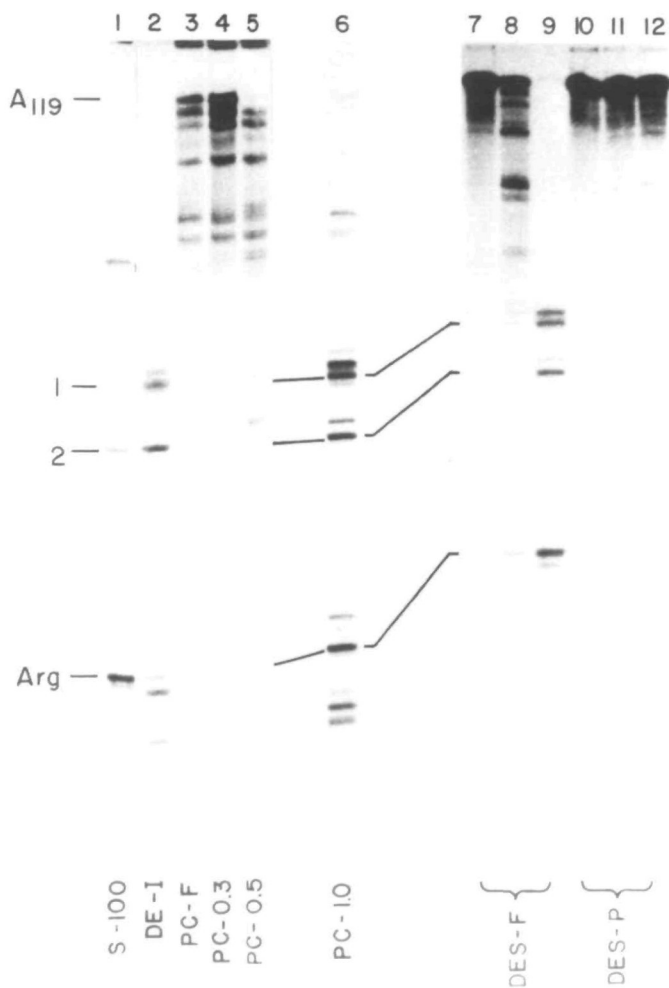


Fig. 3. Isolation of processing activity from S-100

Gel separation of the products obtained upon incubation of band A₁₁₉ RNA with different protein fractions described in the text. Protein concentrations and incubation times were as follows:

- S-100, DE-1 and PC-F..... 3 mg/ml, 2 hours;
- PC-0.3 and PC-0.5.....150 µg/ml, 1 hour;
- PC-1.0.....30 µg/ml, 1 hour;
- DES-F and DES-P..... 5 µg/ml (lanes 7,10), 1 hour,
15 µg/ml (lanes 8,11), 1 hour,
50 µg/ml (lanes 9,12), 1 hour.

analysed by electrophoresis in SDS-polyacrylamide gel (Fig. 4). One can see that the final active preparation (DES-F) separates into several protein bands of relatively low molecular weight. Obviously, the endonuclease contained in this preparation is not yet purified to homogeneity.

In order to obtain a semi-quantitative estimation of the degree of purification, the kinetics of cleavage of band A₁₁₉ precursor RNA with the PC nuclease were determined (Fig. 5A). The concentration of protein in the reaction mixture was about 50 µg/ml and it took about 20 minutes to completely cleave the precursor band. Since the same effect could be obtained by digestion with S-100 for 90 minutes at a concentration of 5 mg/ml protein (Fig. 2) one can conclude that the processing activity of isolated endonuclease is about 400 times higher than that of S-100. This value, of course, is an overestimation of the degree of enzyme purification since a large amount of nucleic acid material present in S-100 might have inhibited the processing.

The PC nuclease is quite stable: it can be stored frozen at -20°C for at least two months or at +4°C for as long as two weeks without apparent loss of activity.

Analysis of the products of band A₁₁₉ cleavage with PC nuclease

The PC nuclease cleaves band A₁₁₉ RNA (about 750 nucleotides) into six major short RNA fragments (shown in Fig. 5A). The cleavage products migrate in the gel in

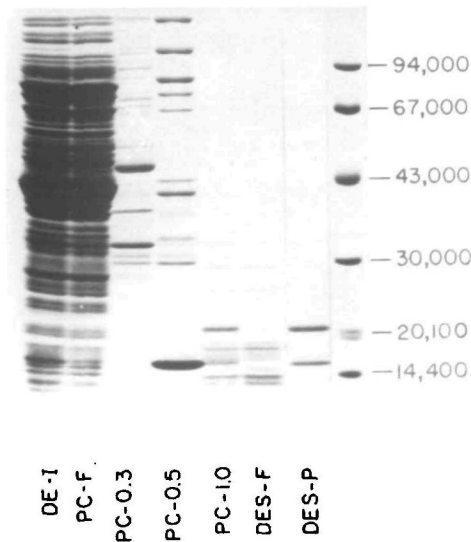


Fig. 4. Analysis of protein fractions by SDS gel electrophoresis

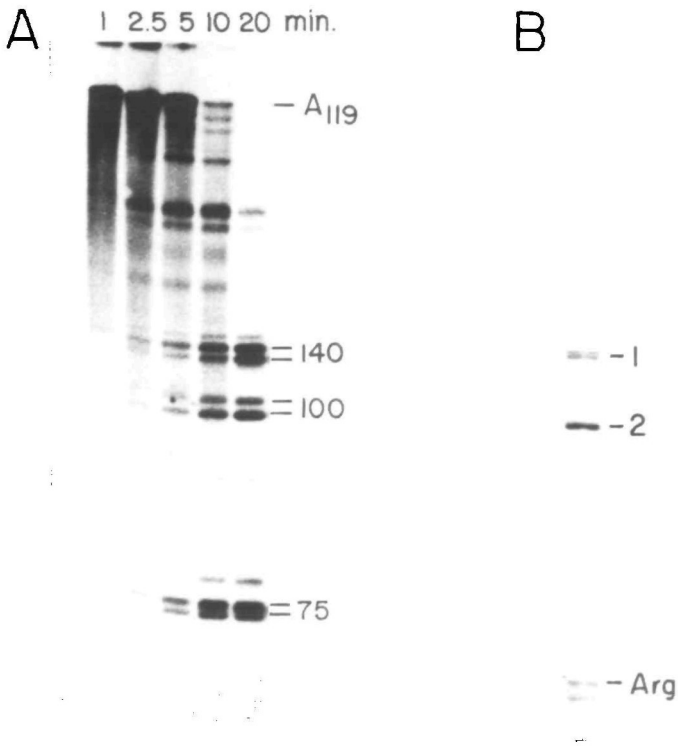


Fig. 5: The products of PC nuclease cleavage of band A₁₁₉ RNA

- A: band A₁₁₉ RNA was incubated with PC nuclease (50 µg/ml) for different periods of time and the products were analyzed in a 10% gel. Major cleavage products and their approximate length (in nucleotides) are indicated
- B: Band A₁₁₉ RNA was cleaved with PC nuclease, the products were deproteinized with phenol, precipitated with ethanol, dissolved and digested with S-100.

three pairs with electrophoretic mobilities close to those of stable Species 1 RNA (140 nucleotides), Species 2 RNA (100 nucleotides) and tRNA^{Arg} (75 nucleotides). Subsequent digestion of these cleavage products with S-100 yields the three mature-size stable RNA

species (Fig. 5B). Although the exact relationship of each of the six PC nuclease cleavage products with the final S-100 resistant species is not clear, there is no doubt that the PC nuclease cleaves band A₁₁₉ precursor either to mature-size stable RNAs or to their monomeric precursors; indeed, none of the six PC nuclease products is long enough to include the sequences of two stable RNAs.

The kinetics of cleavage with PC nuclease of band A₁₁₉ RNA labelled with [β -³²P]-ATP at its 5' end is presented in Fig. 6. The end-labelled precursor RNA was incubated with PC nuclease for different time periods and the products were analyzed by electrophoresis followed by autoradiography. Only the cleavage fragments containing the intact 5'-terminus of the precursor are seen on the autoradiogram. The leftmost lane of Fig. 6 presents the products of partial PC nuclease cleavage of the same precursor internally labelled with [α -³²P]-GTP which were separated on the same gel. Among the cleavage products of 5' end-labelled band A₁₁₉ RNA one can observe several fragments migrating in pairs. The fragments comprising each pair differ in length by no more than ten nucleotides. This surprising phenomenon is further addressed in the Discussion. The approximate sizes of the major end-labelled cleavage products can be estimated from their mobility relative to that of the fragments of known length (75, 100 and 140 nucleotides) and band A₁₁₉ (750 nucleotides). These sizes are about 370 nucleotides (**a**-products), 230 nucleotides (**b**-products), 120 nucleotides (**c**-products) and 40 nucleotides (**d**-products). Thus, the cleavage resulting in the formation of **a**-products occurs approximately in the middle of A₁₁₉ RNA molecule while shorter end-labelled fragments seen on the gel represent cleavage sites situated in its 5' half. One can also distinguish several longer end-labelled fragments just below band A₁₁₉. These products indicate that there are several cleavage sites clustered in the 3' end of the A₁₁₉ RNA molecule.

The end-labelled fragment **a** has a strong counterpart among the products of partial cleavage of internally labelled band A₁₁₉ RNA (Fig. 6, see also Fig. 5A). On the other hand, none of the shorter end-labelled fragments has corresponding bands among the internally labelled products. Among the latter, fragments longer than **a** do appear; however, since the intensity of internal labelling is proportional to the molecular length, these longer fragments are considerably less abundant. Thus fragment **a**, i.e. the intact 5' end half of A₁₁₉ RNA molecule appears to be the major intermediate of the processing reaction. This conclusion is substantiated by the fact that **a**-product is more abundant than other end-labelled fragments during the first minutes of the processing reaction when less than one cleavage per A₁₁₉ molecule has occurred. Since under the conditions of limited

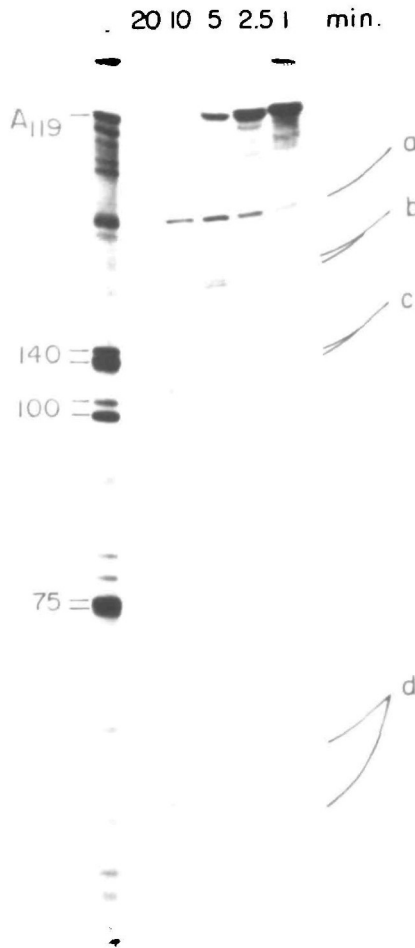


Fig. 6: Kinetics of the cleavage with PC nuclease of band A₁₁₉ labelled at its 5' end

Band A₁₁₉ RNA labelled during its synthesis with $[\beta\text{-}^{32}\text{P}]\text{-ATP}$ was incubated with PC nuclease for different periods of time. The leftmost lane shows the products of partial cleavage with PC nuclease of band A₁₁₉ RNA internally labelled with $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$.

reaction, random cleavage at several sites would produce more short nested fragments than long ones, one can conclude that the precursor cleaving nuclease prefers α -sites over at least all other cleavage sites more proximal to the 5' terminus.

PC nuclease cleavage of tRNA precursors with different composition of stable RNA sequences

The four primary transcripts of the T4 tRNA operon shown in Fig. 1 have different composition of stable RNA sequences. A comparison of the products of their cleavage with PC nuclease can therefore be expected to yield important information about the role of this enzyme in T4 maturation. The primary transcript of the intact tRNA operon, band A_{wt} containing the sequences of ten stable RNAs was digested with S-100 (Fig. 7A, lane 1) or cleaved with PC nuclease (Fig. 7A, lane 2). Among the cleavage products one can see the six bands (indicated by dots) which have been observed on the cleavage pattern of band A₁₁₉ RNA (see Fig. 5A) and are, therefore, derived from subcluster II. In addition, there are several new bands, none of which has the electrophoretic mobility of mature-size products of S-100 digestion. Our preliminary results (data not shown) demonstrate that at least some of the PC nuclease products of band A_{wt} migrating in the 4S region are monomeric precursors of stable RNAs. Two of the cleavage products, bands α and β , have a relatively large size suggesting that they may be dimeric precursors of tRNAs. These two bands were extracted from the gel and subjected to digestion with S-100. As is evident from Fig. 7B, both band α RNA and band β RNA yield upon digestion two stable products migrating in the tRNA region of the gel. These products migrate similarly to tRNA^{Ser}, tRNA^{Pro} and tRNA^{Leu}, tRNA^{Gln} suggesting that the respective bands α and β are dimeric precursors of these tRNAs.

Since RNA bands α and β are not present among the cleavage products of band A₁₁₉ RNA, they must be transcribed from the part of the operon which is removed by the deletion Δ 119. In order to map these two dimeric tRNA precursors more precisely, we checked their occurrence among the PC nuclease cleavage of deletion-specific transcripts A₈ and A₁₀₅ which lack shorter segments of the tRNA operon. As is evident from Fig. 8, band β is present among the cleavage products of both A₈ and A₁₀₅, while band α is missing in both cases. This result together with the data of Fig. 1 and Fig. 7B strongly indicate that band α RNA is a dimeric precursor of tRNA^{Ser} and tRNA^{Pro} and band β RNA is a dimeric precursor of tRNA^{Leu} and tRNA^{Gln}.

DISCUSSION

The processing endonuclease that we have isolated has a very strong affinity to phosphocellulose which makes it possible to remove most of the proteins of the crude extract in a single purification step. The phosphocellulose-purified enzyme passes through

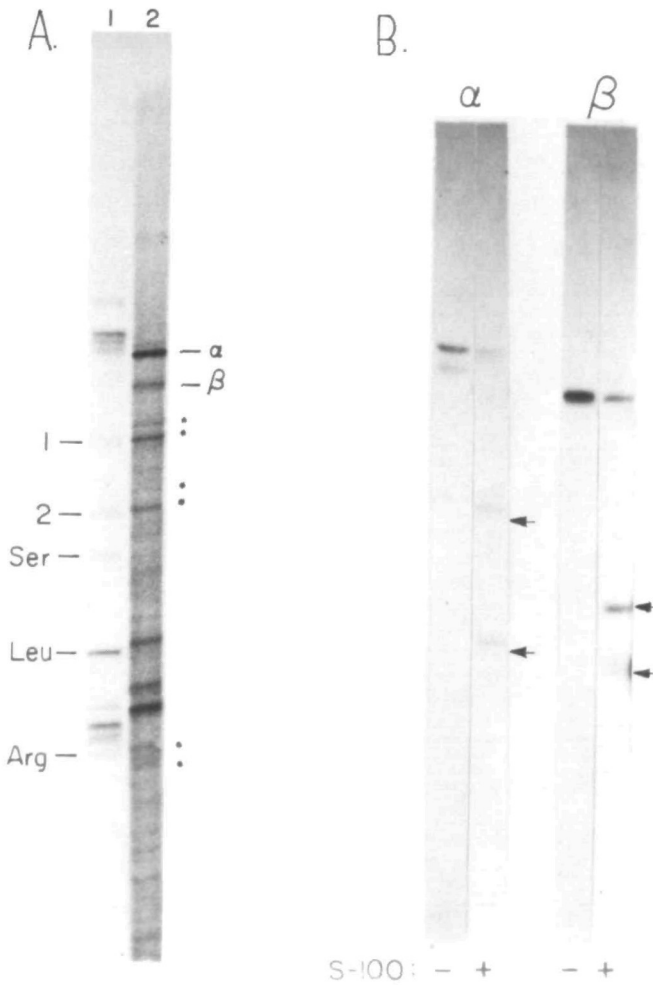


Fig. 7. The products of PC nuclease cleavage of band A_{wt} RNA

A: band A_{wt} RNA was digested with S-100 (lane 1) or incubated with PC nuclease (lane 2) and the products were analysed in a 10% polyacrylamide gel. The identification of S-100 resistant stable RNA species is described elsewhere (4). Dots indicate PC nuclease cleavage products common to band A_{wt} and A_{119} (see Fig. 5A).

B: cleavage products α and β of lane 2 of Fig. 7A were extracted from the gel and digested with S-100. Arrows indicate the migration distances of T4-specific tRNAs for Ser, Pro (α) and Leu, Gln (β) which were determined on the same gel as described in Ref. 4.

Fig. 8. Comparison of PC nuclease cleavage products of bands A₁₁₉, A_{wt}, A_B and A₁₀₅



DEAE-Sephadex. However, in crude extracts, it apparently adsorbs to DEAE-cellulose at low ionic strength. This may be due to the retardation of the enzyme on the column by the phosphates of nucleic acids present in the extract. This phenomenon is useful for purification since a nuclease degrading tRNA is removed in the flowthrough fraction during chromatography of the crude extract on DEAE-cellulose (data not shown). The interaction with traces of nucleic acids can also explain why some of the processing activity was recovered in the flowthrough fraction (PC-F) and in low salt eluates (PC-0.3, PC-0.5) of the phosphocellulose column (Fig. 3); this residual processing activity pro-

duces the same series of RNA fragments that are observed after short-time incubation with isolated PC nuclease (Fig. 5A) implying that the discarded phosphocellulose fractions did not contain any other major endonuclease active on band A₁₁₉. We cannot, however, state that the final PC nuclease preparation contains only one endonuclease activity. Moreover, since no large cleavage products were observed and the long intercistronic RNA sequences of larger precursors were apparently cleaved at random (the background "ladder" in the gel of Fig. 8) we can suspect that our preparations have some non-specific endonuclease activity. Yet, the isolated endonuclease must be relatively pure of exonuclease contamination as is evident from the relative stability of 5' end-labelled cleavage products in the experiment of Fig. 6.

The precursor cleaving endonuclease cleaves band A₁₁₉ RNA into six major products. None of them is long enough to be a dimeric precursor of stable RNAs. Since subsequent incubation with S-100 yields the three mature-size stable RNAs, we conclude that at least three of the cleavage products of band A₁₁₉ contain monocistronic sequences of three different stable RNA genes.

The cleavage pattern of 5' terminally labelled band A₁₁₉ suggests that the initial cleavage of the precursor molecule occurs preferentially at its approximate middle and the resulting 5' half (**a**-fragment in Fig. 6) is the major intermediate of the processing reaction. Unless the 3' end fragment generated by the cleavage at the **a**-site has exactly the same length as the 5' **a**-fragment, one can conclude that the 3' half is cleaved much faster than the 5' half of the molecule. These observations underline the question of where in the A₁₁₉ molecule are the three stable RNA sequences situated. From our estimation of the lengths of the primary transcripts (4) combined with the restriction mapping data and the location of the promoter (8, 9) it follows that the termination site is located from 250 to 400 base-pairs downstream from the end of Subcluster II (see Fig. 1). The length of A₁₁₉ RNA molecule is about 750 nucleotides of which about 320 constitute the sequence of Subcluster II. Thus, the three stable RNA sequences seem to be situated close to the 5' end of A₁₁₉ RNA molecule. It is tempting to assume that the 5' end-labelled fragments **d** represent the 5' leader sequence of the A₁₁₉ transcript and fragments **c**, **b** and **a** are mono- di- and trimeric precursors of the three stable RNAs, respectively (see Fig. 5). The size differences between fragments **a**, **b**, **c** and **d** are consistent with this scheme.

Another question posed by our results is why PC nuclease cleavages of band A₁₁₉ RNA yield equimolar amounts of two 5' end-labelled fragments differing in length only

by few nucleotides (Fig. 6). This can be explained in two alternative ways: (a) PC nuclease sites on the precursor molecule contain two close cleavage points which are attacked with equal probability, and (b) band A₁₁₉ RNA is in fact an equimolar mixture of two transcripts one of which has a few extra nucleotides at the 5' end. Clearly, the nucleotide sequence analysis of the cleavage products of band A₁₁₉ RNA would answer the above questions. Such experiments are currently in progress.

An endonuclease, RNase P, which cleaves tRNA precursors at the 5' ends of tRNA sequences (11, 12) has been implicated in T4 tRNA maturation (13, 14). This enzyme contains an RNA moiety which is probably the reason of its very strong binding to DEAE groups and inability to adsorb to a phosphocellulose column (15). The processing endonuclease that we have isolated has exactly opposite chromatographic properties and we believe that it is completely devoid of RNase P contamination.

When a mutant of RNase P is infected with bacteriophage T4 under non-permissive conditions, dimeric precursors tRNA^{Gln}-tRNA^{Leu} and tRNA^{Pro}-tRNA^{Ser} and monomeric precursors of other T4 stable RNAs accumulate (13, 14). Hence, enzymes other than RNase P must be responsible for the generation of these precursors. From the nucleotide sequences of the two subclusters of stable RNA genes (1, 10) it follows that single endonucleolytic cleavages are responsible for the formation of the aforementioned precursors for RNase P. Our results demonstrate that the PC nuclease cleaves the primary transcript of the T4 tRNA operon into several monocistronic RNA fragments and two dimeric tRNA precursors which are likely to be the tRNA^{Gln}-tRNA^{Leu} and tRNA^{Pro}-tRNA^{Ser}. We, therefore, assume that the enzyme that we have isolated is the one that acts in T4 tRNA processing pathway together with RNase P.

The relation of PC nuclease to other partially purified (16, 17) or postulated (18) RNA processing enzymes is not clear. We can only say that RNase III which is known to degrade Species 1 RNA (14) seems not to be active in our preparations. We suggest to name the precursor cleaving endonuclease described in this paper RNase PC if further purification and characterization proves it to be a single enzyme.

ABBREVIATIONS: kb, kilobase-pairs or kilobases; S-100, 100,000 g supernatant fraction of *E. coli*.

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