Isolation and Characterization of Large Transfer Ribonucleic Acid Precursors from *Escherichia coli*

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Several transfer RNA precursors which accumulate in a strain of *Escherichia coli* temperature-sensitive for RNase P have been described. These precursors range from 135 to 690 nucleotides in length. Their tRNA content has been determined by digestion of the precursors to 4 S RNA, followed by Sanger fingerprint analysis of the purified 4 S material. Identification of some of these tRNAs, as well as an estimate of the number of copies of tRNA in each precursor has been achieved. Many of these precursor RNA molecules contain multiple copies of the same tRNA sequence, indicating a tandem arrangement of the corresponding tRNA genes in the *E. coli* genome.

Our current concepts of transfer RNA biosynthesis have been derived largely from structural studies of tRNA precursor molecules. To date, tRNA precursors from both prokaryotic and eukaryotic sources have been described, and in all cases the biosynthetic pathway appears to be similar (1). That is, tRNA genes are transcribed into large, unmodified polynucleotide precursors containing complete tRNA sequences plus extra nucleotides on the 5' and 3' ends and in the inter-tRNA spacer regions. Cellular enzymes subsequently process the precursors to mature transfer RNA in reactions involving cleavage of extra segments from the 5' and 3' ends, nucleotide modification, and in some cases, addition of the sequence -C-C-A-G-A to the 3' end of the tRNA. Processing at the 5' end of most precursors is accomplished by the endonuclease RNase P (1-3). Processing at the 3' end seems to require prior 5' end processing, and a role for RNase II in exonucleolytic digestion of extra 3'-terminal sequences has been suggested (1, 3). In some cases, other enzymes such as RNase P₂ (3) or tRNA nucleotidyltransferase (RNase II) (57, 77, 95) may also be required for processing (1, 4). Modification enzymes apparently act on precursors at various stages in the processing sequence to give fully modified, mature tRNA (1). In vitro, processing of precursors can be accomplished through the use of a crude cell-free extract (590) (5).

The study of large precursors of tRNA should provide information about the transcription of tRNA genes. In *Escherichia coli*, for example, tRNA genes occur singly or in clusters located throughout the genome. Clusters of tRNA genes may contain more than one copy of the same tRNA gene, as at the su zone locus specifying tRNA_{Glu}^{57} (6) and the glyV locus specifying tRNA_{Glu}^{57} (7); or, they may be composed of different tRNA genes, as at glyT thrT tyrT which specifies the synthesis of tRNA_{Glu}^{57}, tRNA_{Thr}^{57} and tRNA_{Tyr}^{57} (8, 9). Of particular interest are questions concerning the transcriptional products from clusters of tRNA genes, especially from loci such as su, glyV, and glyT that have been mapped and the number of gene copies estimated. Are the tRNA genes at these loci transcribed singly, is only part of a cluster transcribed, or can an entire gene cluster be transcribed as a multi-tRNA precursor? Conversely, the characterization of large precursors should provide information about loci containing tRNA genes that have not been mapped; for example, an estimate of the number of gene copies can be made, and tRNA genes that are closely linked can be identified. Large tRNA precursors should also be good substrates for defining the mechanism of action of processing or modification enzymes.

In the present investigation, we have isolated and characterized a number of tRNA precursors which accumulate at the nonpermissive temperature in the *E. coli* strain A49. In this strain, RNase P, the endonuclease responsible for processing of many precursors at the 5' end of tRNA sequences, is temperature-sensitive (10). The precursors to be described here range from 135 to 690 nucleotides in length; they are mostly precursors to a single species of tRNA and they contain as many as five complete transfer RNA sequences.

**EXPERIMENTAL PROCEDURE**

*Bacterial Strains—* *Escherichia coli* strain A49 (obtained from P. Schedl, Stanford University) carries the genetic markers *ts_{A}*, *trpA*(−) *argA* *glnA* *Sm* and has a mutation rendering the RNase P activity temperature-sensitive (10). The lysogen A49/pCu*- *glyT* (this laboratory) was constructed by S. Chang (this laboratory) using the transducing phage λCu*- *glyT* which carries the genes for tRNA_{Glu}^{57}, tRNA_{Thr}^{57} and tRNA_{Tyr}^{57} (8). Strain B926 (W3110 rel *trpA*(−) *glnA* *Sm*) has wild type RNase P activity.

*Low Phosphate Medium—* Low phosphate peptone and low phosphate casamino acid media were prepared as described by Chang and Carbon (11).
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Isolation of tRNA Precursors—Escherichia coli strain A49 is temperature-sensitive for RNAase P activity and has been shown to accumulate tRNA precursors at the nonpermissive temperature (43°C) (3, 10, 16). In the present investigation, strain A49 was incubated in the presence of [32P]phosphate for 45 min at 43°C. Total cellular [32P]RNA was isolated and subjected to gel electrophoresis on a 5% polyacrylamide gel in formamide, according to the method of Staynov et al. (13). The advantages of this gel system for isolating tRNA precursors are 2-fold: (a) since aggregation is minimized, the precursor bands are sufficiently well resolved that further purification is usually not required, and (b) in the range from 4 S to 16 S, a linear relationship exists such that the size of precursor bands can be determined directly from a plot of migration in the gel vs. molecular weight or length in nucleotides expressed on a log scale.

Fractionation of total cellular [32P]RNA from strain A49 labeled at the nonpermissive temperature consistently gave the gel pattern shown in Fig. 1, in which a number of RNA bands are clearly resolved. The mobility and size of each of the bands was plotted as shown in Fig. 2, using 4 S RNA, tRNA \( ^* \), 5 S RNA, the tRNA \( _{a} \)-tRNA \( _{a} \)-c precursor, and 16 S RNA as markers. The data from Fig. 2 are also summarized in Fig. 1. The numerous bands migrating on the gel slower than 4 S RNA were considered as possible precursors of tRNA. No attempt was made to identify tRNA precursors in the range from 4 S to 5 S, since bands in this region were likely to be contaminated with the larger tRNAs, 4.5 S RNA or 5 S RNA. Bands moving slower than 5 S RNA, labeled A through M, were eluted electrophoretically from the gel and were analyzed and characterized as markers. The data from Fig. 2 are also summarized in Fig. 1.

In Vitro Processing of Precursors—Isolated precursors were processed in vitro by incubation at 37°C in 200 μl of a mixture containing 0.01 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.065 M MgCl₂, 10-3 M EDTA, 10-3 M 3-mercaptoethanol, and 350 μg of protein (26). After incubation for 60 min at 37°C, an additional 130 μg of S30 protein was added and incubation at 37°C was continued for 30 min. The reaction was terminated by the addition of 0.5 ml of buffer and 1 ml of water-saturated, distilled phenol. The RNA in the aqueous phase was precipitated with ethanol and analyzed on a 4.5% polyacrylamide gel.

The yield of 4 S RNA product following S30 digestion was used to calculate the number of copies of tRNA present in precursor bands. An equal amount of radioactivity in paired samples was incubated at 37°C in buffer with or without S30, then extracted with phenol, ethanol-precipitated, and analyzed on a 4.5% polyacrylamide gel. Recovery of radioactivity was monitored at each step, and bands corresponding to the precursor control, uncleaned precursor, and 4 S RNA products were cut from the gel. The yield of 4 S RNA was expressed as a percentage of counts recovered in the precursor control (correcting if necessary for counts in uncleaned precursor). From the yield of 4 S RNA, the number of tRNA-specific nucleotides in each precursor was calculated and the number of copies of tRNA determined. T1-RNAse fingerprints were used to verify the identities of the 4 S RNA products.

CLEAVER OF Precursors with RNase P, RNase Pr, or RNase II was carried out according to the procedure described by Robertson et al. (5) with digestion with RNase P (2). Cleavage products were analyzed in the same way as products from S30 digestion.

Materials—Materials were obtained from the following sources: T, ribonuclease from Calbiochem; RNase P and RNase II from P. Schled, Stanford University; RNase P by purification from E. coli through the DEAE-Sephadex chromatography step (2); acrylamide from J. T. Baker Chemical Co.; N,N'-methylenebisacrylamide, X,N,N'-tetramethylethenediamine, and medical x-ray film (RP14 and “no screen”) from Eastman Kodak Co.; ammonium persulfate (electrophoresis grade) and analytical grade mixed bed resin, AG 501-X8 (20 to 50 mesh), from Bio-Rad Laboratories; DEAE-cellulose paper, DE81, from Whatman; cellulose acetate strips, No. 2900 (30 x 570 mm), from Schleicher and Schuell, Inc.; formamide (99% pure) from Matheson, Coleman and Bell; carrier-free H,PO, from International Chemical and Nuclear Corp.; and NCS tissue solubilizer from Amersham/Searle. Toluen-based scintillation fluid contained 0.4% Omnifluor (New England Nuclear Corp.). Bacteriological media were purchased from Difco. Sterile buffers were used and were prepared with analytical grade chemicals. All glassware utilized in biochemical analyses was heat treated and siliconized prior to use.
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FIG. 1. Autoradiogram of an electrophoretic fractionation in a formamide-5% polyacrylamide gel of \(^{32}P\)-labeled RNA from strain A49. The procedures for isolation of \(^{32}P\)RNA and for preparation of the formamide-acrylamide gel were as described under “Experimental Procedure.” Electrophoresis was carried out at 400 V for 30 hours at 4°C. The values for the lengths of Bands A through M were determined from the plot shown in Fig. 2.

Results from representative yield experiments are shown in Fig. 3 and summarized in Table I.

Identification of tRNA Precursors—All except one of the 13 bands were digested to 4 S RNA products following incubation with S30. A single 4 S RNA product was obtained from 10 of the precursor bands, whereas multiple digestion products were obtained from the other two.

Of the smaller precursor bands, Band A (135 nucleotides) is apparently a mixture of precursors, since two bands of 4 S product were obtained following digestion of Band A with S30 (Fig. 3, Lane A (+)). A fingerprint of the faster moving of the two products is shown in Fig. 4a; it contains most of the spots characteristic of tRNA\(_{\text{AUC}}\) that is found in other precursors, but spots corresponding in position to A-A-U-A-G- and U-C-U-C-G- of tRNA\(_{\text{AUC}}\) are missing from this map (7). A fingerprint of the larger 4 S product is shown in Fig. 4b. It is apparently a single species of tRNA but it has not been identified.

Band C is not a tRNA precursor. The size (185 nucleotides), T\(_1\) fingerprint (not shown), and stability of this band to cellular enzymes (see Fig. 3, Lane C (+)) all indicate that it is either 6 S RNA or 6 S precursor (17, 18).

Equal yields of two tRNAs were recovered from Band D (Fig. 3, Lane D (+)). The smaller tRNA gave the fingerprint shown in Fig. 4c; this fingerprint has not been identified. The larger tRNA was identified by fingerprint (shown in Fig. 4d) and base composition analysis as tRNA\(_{\text{AUC}}\) (14). Precursor Band D is approximately 205 nucleotides long and could conceivably contain two tRNA sequences. Alternatively, it could be composed of a mixture of two precursors, each about 205 nucleotides in length. Yield experiments trend to support the latter interpretation, since only 40% of the material in Band D (equivalent to 84 nucleotides) was recovered as 4 S product. The physical separation of untreated Band D into four bands following electrophoresis on a neutral polyacrylamide gel (Fig. 3, Lane D (−)) was unique to this experiment and most likely reflects breakdown of Band D upon storage.

The same T\(_1\) fingerprint (shown in Fig. 4e) was obtained from the single 4 S product of Bands B, F, I, J, and K. This...
FIG. 3. Electrophoresis on a 10% neutral polyacrylamide gel of Bands A through M, following incubation at 37° for 90 min without (–) or with (+) crude cell extract (S30). Conditions for digestion of the bands with crude cell extract and for analysis of the digestion products by polyacrylamide gel electrophoresis were as described under “Experimental Procedure.” Electrophoresis was from top to bottom.

TABLE I
Size, tRNA content, and yield of tRNA in precursor bands

The length of precursor bands, isolated as shown in Fig. 1, was determined as shown in Fig. 2. In vitro processing of precursors and determination of yields of 4 S RNA product were performed as described under “Experimental Procedure.” Standard Sanger fingerprints of T, RNase digests were used to identify the various tRNAs.

<table>
<thead>
<tr>
<th>Band</th>
<th>Length, nucleotides</th>
<th>tRNA from precursor</th>
<th>Yield of tRNA from in vitro processing (No. copies/chain)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>135</td>
<td>Not identified</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>155</td>
<td>tRNA</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>185</td>
<td>tRNA</td>
<td>1.8</td>
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<td>D</td>
<td>205</td>
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<td>2.0</td>
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<tr>
<td>M</td>
<td>690</td>
<td>tRNA</td>
<td>0.6</td>
</tr>
</tbody>
</table>

fingerprint and the base compositions of the T, oligonucleotides correspond to tRNA^\text{Val}^\text{1}_{\text{Glu}} (19). A variable yield of three of the oligonucleotides on the fingerprints (indicated by arrows, Fig. 4e) was the only obvious difference among the 4 S products from each of the tRNA^\text{Val}^\text{1}_{\text{Glu}} precursors. The two upper spots indicated by the arrows are not present on the T, map of mature tRNA^\text{Val}^\text{1}_{\text{Glu}} (19); they may reflect incomplete modification of tRNA segments or incomplete processing of extra 5' or 3' end sequences. Band F and probably Band J are apparently derived from larger sequences, since they accumulate only with prolonged labeling. If a progression in processing exists among all of these valine tRNA precursors, it is not apparent from yield experiments, which indicate that there are 1.4 copies of tRNA in Band B, 3.2 copies in Band F, 2 copies in Band I, 2.7 in Band J, and 1.8 in Band K.

Three other large precursors, Bands E, G, and H, each contained multiple copies of a single tRNA. Band E, 255 nucleotides in length, contains two copies of a tRNA which has not been identified. The fingerprint of the tRNA is shown in Fig. 4f. Band G is identical with the tRNA^\text{Glu}^\text{1}_{\text{Glu}} precursor described briefly in a previous publication (21). It is 400 nucleotides long and contains three copies of tRNA^\text{Glu}^\text{1}_{\text{Glu}} (fingerprint shown in Fig. 4g). Band H is the precursor band obtained in the highest yield under our labeling conditions and is composed almost entirely of sequences of a tRNA identified by fingerprint and base composition analysis as tRNA^\text{Glu}^\text{1}_{\text{Glu}} (21) (see Fig. 4h). Five copies of tRNA^\text{Glu}^\text{1}_{\text{Glu}} are present in Band H.

From the two largest precursor bands, Bands L and M, very low yields of tRNA were obtained. The 4 S RNA product recovered from Band L represented only about 15% of the precursor (one copy of tRNA per total 620 nucleotide precursor sequence); from Band M, about 7% was recovered in 4 S RNA product (less than one copy of tRNA in a total of 690 nucleotides). The remainder of each of these bands was completely degraded by the S30. The T, fingerprint of the 4 S RNA product from Band L (Fig. 4i) matches that of tRNA^\text{Glu}^\text{1}_{\text{Glu}} (22), while tRNA^\text{Glu}^\text{1}_{\text{Glu}} was recovered from an S30 digestion of Band M (Fig. 4j).

The results from in vitro processing of precursors, fingerprint analyses, and yield determinations are summarized in Table I.

Processing of Precursors with Purified Enzymes—The precursor to tRNA^\text{Val}^\text{1}_{\text{Glu}} was originally isolated on a 5% neutral polyacrylamide gel, according to the procedure described by Chang and Carbon (11). On this gel, the tRNA^\text{Val}^\text{1}_{\text{Glu}} precursor migrates in an aggregate with at least one of the precursors to tRNA^\text{Val}^\text{1}_{\text{Glu}}. This aggregate was treated with RNase P, as described under “Experimental Procedure,” which resulted in
the formation of two 4 S products, one of which gave the characteristic T₁ fingerprint of tRNA\(^{\text{Gly}}\) (plus one additional spot which failed to move from the origin in the second dimension). The other 4 S product gave a T₁ fingerprint characteristic of tRNA\(^{\text{Glu}}\), identical to the fingerprint shown in Fig. 4e. This precursor aggregate was also assayed for its sensitivity to RNase P₂ and to RNase II (supplied by Paul Schedl), with the results shown in Fig. 5. Digestion to 4 S products with RNase P was enhanced if this enzyme was used in combination with RNase P₂ (2) or RNase II. In fact, the combination of RNase P plus RNase II appeared to be as effective as digestion with S30 (data not shown).

The precursor to tRNA\(^{\text{Gly}}\) was a substrate for RNase P, although this precursor was somewhat more resistant to RNase P digestion than were the precursors to tRNA\(^{\text{Glu}}\) and tRNA\(^{\text{Glu}}\). The 4 S product from RNase P digestion of Band G gave a fingerprint corresponding to tRNA\(^{\text{Glu}}\) but it was not characterized further.

The sensitivity of other precursors to RNase P, RNase P₂, or RNase II was not examined.
Transfer RNA precursor molecules ranging from 135 to 690 nucleotides in length and containing from one to five copies of tRNA have been isolated from E. coli strain A49. These precursors fall into discrete size classes, based on their mobility in polyacrylamide gels prepared in formamide. Most of the precursors appear to contain one or more copies of only a single species of tRNA, including tRNA$\alpha$, tRNA$\gamma$, tRNA$\xi$, tRNA$\kappa$, tRNA$\beta$, tRNA$\gamma$, and tRNA$\delta$, and other tRNAs which have not yet been identified.

The accumulation of tRNA precursors in E. coli strains which contain a temperature-sensitive RNase P has been described previously by other groups. Sakano et al. (24) have reported the appearance of new RNA species at the nonpermissive temperature, which have been tentatively identified as precursors to tRNA$\kappa$, tRNA$\gamma$, tRNA$\xi$, and tRNA$\beta$. Schedl et al. (3, 10, 16) have identified a number of precursors which accumulate in strain A49 at the nonpermissive temperature. Among those larger than 5 S were precursors to tRNA$\alpha$, tRNA$\gamma$, tRNA$\xi$, tRNA$\kappa$, tRNA$\beta$, and tRNA$\delta$.

Precursors to tRNA$\alpha$, tRNA$\gamma$, tRNA$\xi$, tRNA$\kappa$, and tRNA$\beta$, which we have isolated from strain A49, were not identified in their study. Similarly, we have not recovered precursors to the serine, arginine, or glutamic tRNAs. These results may reflect differences in the gel systems used to isolate precursors from total cellular RNA. On neutral polyacrylamide gels, some tRNA precursors are recovered as large aggregates. If some of the precursors isolated by Schedl et al. (3, 10, 16) contained individual tRNA precursors actually smaller than 5 S, they would not have been identified in the present investigation.

The length of precursors might not necessarily reflect the number of tRNA sequences contained within them. Among the 12 precursors described here, the tRNA content ranged from less than 10% of Band M (less than one copy of tRNA per 690 nucleotide precursor sequence) to 93% of Band H (five copies of tRNA in a total precursor sequence of 440 nucleotides). For most of the precursors, 40 to 70% of the total sequence was tRNA.

The yield experiments used to determine the number of
FIG. 5. Polyacrylamide gel electrophoresis following in vitro cleavage of mixed tRNA precursors. Enzyme reactions and electrophoresis on a 10% neutral polyacrylamide gel were carried out as described under “Experimental Procedure.” Lane 1, digestion with RNase P; lane 2, RNase P + RNase P; lane 3, RNase P + RNase P; lane 4, RNase P + RNase P; lane 5, RNase P + RNase P; lane 6, RNase P; lane 7, RNase P; lane 8, S30. The position of uncleaved precursor on this gel is indicated (P).

copies of tRNA in a given precursor should be interpreted with some caution. They assume: (a) quantitative recovery of radioactivity at each step; (b) equal susceptibility of all precursors to processing; and (c) no degradation of tRNA sequences during the processing reaction. In these experiments, radioactivity in experimental and control samples was monitored at each step and corrections for uncleaved precursor included in the calculations of yields. In addition, the reaction conditions for S30 processing were adjusted to maximize the yield of 4 S RNA product. However, if during processing certain partially digested precursors assumed conformations favoring total degradation, a possibility suggested by Altman et al. (25), some tRNA segments present in precursors may not have been recovered as 4 S product. The low yields of tRNA from Bands L and M and the fractional yields from the tRNA loops in precursors may indicate that these precursors are particularly unstable.

The precursors to tRNA (Band G) and tRNA (Band D) represent transcripts from regions of the E. coli chromosome that have been mapped and are known to contain multiple copies of tRNA genes. Synthesis of tRNA is specified by genes at two locations on the chromosome. A single copy of tRNA occurs at glyW (37), while an estimated three to four copies are located at glyV (86) (7, 26). The tRNA precursor (Band G) contains three copies of tRNA and is probably a transcript from glyV, although this remains to be established.

A gene (tyrT) specifying tRNA (Band G) and tRNA (Band D) represents transcripts from regions of the E. coli chromosome that have been mapped and are known to contain multiple copies of tRNA genes. Synthesis of tRNA is specified by genes at two locations on the chromosome. A single copy of tRNA occurs at glyW (37), while an estimated three to four copies are located at glyV (86) (7, 26). The tRNA (Band G) contains three copies of tRNA and is probably a transcript from glyV, although this remains to be established.

The existence of multiple copies of tRNA sequences in precursor Bands E and H, and in the many bands containing tRNA, leads to the prediction that there are corresponding regions of the genome containing multiple tandem copies of these tRNA genes. There must be at least five copies of the gene specifying tRNA, arranged in tandem at some location on the E. coli chromosome. In addition, there should be a region containing at least two or three copies of the genes for tRNA, as well as a region specifying two copies of tRNA in Band E.

We do not know if any of these precursors are primary transcriptional products with a purine triphosphate at the 5' end. Moreover, nucleotide sequencing studies will be required to determine the nature of the non-tRNA segments, the degree of nucleotide modification, and the presence or absence of mature tRNA 3' ends within the precursor sequences.

Processing of these precursors is also of interest. Since they were isolated in strain A49, they are all necessarily substrates for RNase P. Further processing by other enzymes most likely requires the coordinate activity of RNase P, as was recently proposed by Schedl et al. (16) in a model for processing of large tRNA precursors. Particularly interesting substrates for processing studies include the multi-tRNA precursors to tRNA (Band G) and tRNA (Band H), as well as the numerous precursors to tRNA.

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