Biosynthesis of Transfer RNA: *In Vitro* Conversion of Transfer RNA Precursors from *Bombyx mori* to 4S RNA by *Escherichia coli* Enzymes

(silkworm/acrylamide gels)

G. S. CHEN AND M. A. Q. SIDDIQUI

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT Evidence for the presence of rapidly labeled short-lived RNA species, presumed to be precursors to transfer RNA (tRNA), in the silk glands of *Bombyx mori* is presented. These precursors, which migrate between 4S and 5S markers on acrylamide gels, can be converted *in vitro* into molecules indistinguishable in size from tRNA. The conversion is also catalyzed by a crude enzyme fraction isolated from ribosomes of *E. coli*. Since a similar enzyme preparation cleaves the suppressor tRNA^{Tyr} sequence of *E. coli* from its precursor molecule, it would appear that the precursor to *E. coli* tRNA^{Tyr} and the precursors to silk-gland tRNAs share common structural features.

There is a growing body of evidence that tRNA in both bacterial and mammalian cells is made through a precursor molecule(s) that is longer than mature tRNA by several nucleotides (1-9). It is believed that during maturation of precursor(s), specific enzymes cause "trimming" of extra length and modification of specific nucleotides (1, 10-15). Although isolation of a precursor of suppressor tRNA^{Tyr} in *Escherichia coli* and its subsequent base-sequence analysis has demonstrated unequivocally the existence of a precursor for tRNA^{Tyr} (14), the overall mechanism of tRNA biosynthesis and the nature of specific structural alterations that lead to formation of functional tRNA is not fully understood, especially in animal systems. The posterior part of the silk gland in the silkworm, *Bombyx mori*, represents a highly specialized system where at one stage of development (during the fifth larval instar) almost 40% of the total tRNA population is tRNA specific for glycine (16, 17; Chen, G. & Siddiqui, M. A. Q., in preparation). This offers potentially a system for isolation of a precursor(s) for a single tRNA species that is amenable to analysis of biosynthetic and maturation processes. Our present studies are directed toward elucidating conditions for isolation of a precursor for tRNA^{Gly} that can produce functional tRNA^{Gly} *in vitro* so that tRNA maturation and modification and their functional significance can be understood. In this communication evidence is presented for the existence in the silk gland of *B. mori* of low-molecular-weight precursor molecules that can be converted *in vitro* into molecules indistinguishable from mature tRNA on acrylamide gels. This specific cleavage is also catalyzed by a crude enzyme fraction isolated from ribosomes of *E. coli*. Since a similar enzyme fraction also converts *E. coli* tRNA^{Tyr} precursor (14), and turnip yellow mosaic virus (TYMV) valyl RNA (18) into tRNA-like molecules, it seems that these molecules share common structural features with the tRNA

precursors of *B. mori*. In the following, evidence for specific conversion of tRNA precursors to tRNA-like molecules by extracts from ribosomes of *E. coli* will be described.

MATERIALS AND METHODS

Larvae of *B. mori* were grown on a synthetic diet in Dr. Patrick Gage's laboratory (Roche Institute of Molecular Biology) (16, 19; Chen, G. & Siddiqui, M. A. Q., in preparation). ³²P-Labeled tRNA and 5S rRNA were made by injecting a total of 2.0 mCi of [³²P]orthophosphate into each larva on second, third, and fourth day of fifth instar. The glands were excised 24 hr later and RNA was isolated and purified as described (16; Chen, G. & Siddiqui, M. A. Q., in preparation).

For pulse-labeling RNA, 50 μ Ci of [^{5-³H]uridine was injected intracoelomically into each larva during the second or third day of fifth instar (the stage of maximum growth of the gland). The glands were removed, washed once, and suspended in 0.5 ml of extraction buffer containing 5 μ mol each of Tris·HCl (pH 7.4), magnesium sulfate, and potassium chloride. The suspension was immediately homogenized in the presence of 0.5% sodium dodecyl sulfate and an equal volume of freshly redistilled phenol saturated with the buffer above. The aqueous phase was recovered by centrifugation and RNA was precipitated with 2.5 volumes of 95% ethanol in the presence of 2% potassium acetate (pH 5.5).}

Polyacrylamide disc-gel electrophoresis was performed in a vertical electrophoresis apparatus (Shandon Southern) essentially as described by Peacock and Dingman (20). Gels consisted of 7.5% acrylamide crosslinked with 0.2% bis-acrylamide in 0.89 M Tris·HCl (pH 8.3)-56 mM boric acid-2.4 mM disodium EDTA-1.75 mM ammonium persulfate-0.02% (v/v) *N,N,N',N'*-tetramethylethylenediamine. The reservoir buffer consisted of Tris·HCl-boric acid-EDTA in concentrations as above. Samples were applied to gels with bromophenol blue added as marker as described in legend to Fig. 2. Electrophoresis was performed at 5 mA per tube for about 3 hr at 4°. Gels were cut in a Gilson gel fractionator, digested overnight at 60° in the presence of 30% H₂O₂, and counted in 5 ml of Insta-gel emulsifier (Packard Instrument Co., Inc.) in a Beckman model LS-100 spectrometer.

Crude enzyme fraction from *E. coli* K12 ribosomes, a gift from Dr. Kung of our Institute, was prepared by washing ribosomes for 3 hr at 0° in 1 M ammonium chloride-10 mM Tris-acetate (pH 8.0)-14 mM magnesium acetate-60 mM potassium chloride-1 mM dithiothreitol. The ribosomes were pelleted by centrifugation at 50,000 rpm for 90 min in a Spinco rotor 65. The supernatant was dialyzed against the

Abbreviation: TYMV, turnip yellow mosaic virus.

buffer without ammonium chloride and stored frozen at -170° .

RESULTS AND DISCUSSION

The kinetics of [^3H]uridine incorporation into Cl_3CCOOH -insoluble material, presented in Fig. 1, shows that there was a rapid uptake of the label which reached a maximum in about 2 hr. After 4.5 hr of continuous labeling, almost 85% of the total uridine entering the silk glands appeared as acid-precipitable RNA material. When RNA pulse-labeled with [^3H]uridine for 20 min, 1 hr, and 4 hr was examined on 7.5% acrylamide gels, distinct RNA species migrating between 4S and 5S markers were observed (Fig. 2). Their presence was demonstrable as late as 4 hr in continuous labeling. At least two peaks were obvious, one (P-1) migrating close to tRNA, and the other (P-3) moving with 5S rRNA. An additional fraction was a shoulder (P-2) that moved between these two peaks and appeared consistently up to 30 min of pulse-labeling. Prolonging the labeling time resulted in reduction of ^3H counts in these slow-moving fractions with a concomitant increase in the fraction migrating as tRNA. The peak (P-3), which should also contain 5S rRNA, remained unchanged after 1 hr of pulse-labeling with the counts remaining in the peak in an amount expected of 5S rRNA (the ratio between 5S rRNA and tRNA was about 1:8; measurements were based on separation of these two RNA species on G-100 Sephadex columns and on acrylamide gels). When appropriate fractions from these intermediate peaks (P-1 and P-3) and from the tRNA region were pooled and subjected to electrophoresis again on 7.5% gels, each peak moved as a single band exactly at the same position relative to 4S and 5S markers (Fig. 3). The rapid accumulation of label in these fractions and its transfer into the tRNA region both *in vivo* and *in vitro* suggested that these intermediate peaks are precursor(s) to tRNA. The consistency of their appearance and the conversion into tRNA-like molecules suggested

Fig. 1. Rate of incorporation of labeled uridine. 50 μCi of [^3H]uridine (specific activity 24.7 Ci/mmol) at a concentration of 10 mCi/ml in H_2O was injected intracoelomically into each larva during the second day of the fifth instar. At intervals the glands were excised and homogenized immediately. Duplicate samples of 10 μl each from the pooled aqueous layer after phenol extraction were assayed for acid-insoluble and -soluble counts. Radioactivity plotted represents total amount per animal. $\circ$, acid-insoluble; $\bullet$, total.

Fig. 2. Acrylamide gel pattern of rapidly labeled, low-molecular-weight RNA. Animals were labeled. RNA was extracted by deproteinization with phenol and ethanol precipitation and stored in H_2O at -20° . 10 μl of each sample containing about 60,000 trichloroacetic acid-precipitable total cpm (about 10–12% of which represents low-molecular-weight RNAs) were placed on 7.5% gels in 25% sucrose along with [^{32}P]tRNA (9150 cpm) as marker for electrophoresis. The position of 5S [^{32}P]rRNA run simultaneously on a separate gel is indicated by an arrow. Gels were fractionated into 2-mm slices and counted. Fraction no. 1 represents the bottom of gel. $\circ$, ^3H ; $\bullet$, ^{32}P .

strongly that they were not derived as nonspecific degradation products of high-molecular-weight ribosomal RNA. Further, the relative amount of each fraction did not change regardless of the procedure used for RNA extraction.

An endonuclease from *E. coli* described by Smith and co-workers (14, 21) that specifically cleaves 41 nucleotides from

Fig. 3. Electrophoresis of material eluted from gel fractions in Fig. 2. Three fractions representing (1) tRNA, (2) precursor tRNA (P-1) migrating close to tRNA, and (3) precursor tRNA (P-3) migrating with 5S rRNA from appropriate slices from the gel containing 20-min pulse-labeled RNA were eluted with Tris-borate-EDTA buffer used for electrophoresis. The gel pieces were removed by filtration on a Millipore membrane. The solution was concentrated by lyophilization and dialyzed overnight against the buffer above. Each fraction (fraction 1, 7200 cpm; fraction 2, 6000 cpm; fraction 3, 8700 cpm) was analyzed separately on gels along with the 4S and 5S markers indicated by arrows. Δ , tRNA; $\circ$, P-1; $\bullet$, P-3.

FIG. 4. Analysis, after incubation with *E. coli* enzyme, of low-molecular-weight RNA pulse-labeled for 20 min. 20 μ l of RNA pulse-labeled for 20 min, representing about 120,000 cpm of total RNA, were incubated with *E. coli* ribosomal extract (200 μ g of protein) for 20 min at 37° in a total volume of 40 μ l containing 3.3 mM Tris-OAc (pH 8.2)–5 mM Mg(OAc)₂–20 mM KCl–0.3 mM dithiothreitol. The mixture was chilled in ice for 2 min and applied to a gel for electrophoresis as above, along with the markers [³²P] tRNA and 5S [³²P]rRNA. □, before incubation; ●, after incubation; ○, 5S [³²P]rRNA and [³²P]tRNA (fractions 37–43 represent 5S rRNA and fractions 11–30 are tRNA).

the precursor of suppressor tRNA^{Tyr} was recently shown to hydrolyze TYMV valyl RNA, releasing a tRNA-like fragment that can be acylated with valine (18). In an attempt to test the possibility that the enzyme might recognize other molecules resembling tRNA, the RNA fraction from the silk glands labeled for 20 min, which contained the largest amount of precursor fractions, was incubated with the ribosomal extracts of *E. coli* as described in Fig. 4 and analyzed on gels as before. Most of the RNA moving slower than tRNA before incubation now moved faster, in fact precisely at the same position as tRNA. The peaks (P-1 and P-3) were significantly reduced, whereas the shoulder fraction, P-2, seemed unaffected. To ascertain that the peaks that appeared after digestion were derived from precursor tRNAs and not from ribosomal RNAs, we digested and examined purified 18S and 28S [³²P]rRNA (a gift from Dr. R. Green of our Institute)

FIG. 5. Effect of enzyme on 5S [³²P]rRNA. 5S [³²P]rRNA (4500 cpm) purified from silk-gland RNA on a Sephadex G-100 column (16; Chen, G. & Siddiqui, M. A. Q., in preparation) was incubated with *E. coli* enzyme and analyzed on gel as in Fig. 4. ●, before incubation; ○, after incubation; Δ, [³²P]tRNA.

FIG. 6. Analysis of tRNA and precursor tRNA (P-1) fractions after incubation with *E. coli* enzyme. The fractions representing tRNA and precursor tRNA (P-1) eluted from the gel containing RNA pulse-labeled for 20 min, as in Fig. 3, were digested with the *E. coli* enzymes and analyzed. A total of 7200 cpm for tRNA (bottom) and 6000 cpm of P-1 fraction (top) were used for digestion, and the same amounts were run on gels as control undigested material. ○, before incubation; ●, after incubation.

and 5S [³²P]rRNA as above. None of these three [³²P]RNA species yielded a low-molecular-weight product that could be detected as a tRNA-like fragment on 7.5% gels. 5S RNA was digested readily, whereas 18S and 28S rRNAs yielded fragments that remained close to the top of the gels (data for 5S rRNA alone is shown in Fig. 5).

An additional demonstration for the specific digestion of precursor molecules comes from the experiment where P-1 and tRNA fractions were eluted from the same gel, incubated with the enzymes, and run again on acrylamide gels as before. While most of the label (60%) from P-1 shifted to the 4S position, tRNA remained resistant to digestion under the experimental conditions (Fig. 6). Altman and Smith (14) have noted similar results with the suppressor tRNA^{Tyr} of *E. coli*. These observations, and the fact that prolonged incubations did not cause any further change in the position of these two fractions (tRNA and P-1) after digestion, support the argument for the specific nature of this conversion, although the extent of degradation varied somewhat with our enzyme preparations. RNA eluted from the peak, P-3, was also digested by the enzymes under identical conditions but to a different extent. Similar conversion of the precursor molecules was also seen with crude enzyme preparations from silk-gland ribosomes, as would be expected, but the level of other contaminant nucleases seemed to be high, since the degradation to smaller fragments was much more rapid than it was under conditions used with *E. coli* enzymes.

Hence, it appears that in accordance with the findings in other systems, precursor molecules for tRNA exist in the silk glands of *B. mori*. Although no compelling experimental evidence is given in this paper to demonstrate the precursor role of these molecules, we believe that the quantitative conversion *in vivo* and *in vitro* and work in progress in our laboratory will provide evidence for the nature and origin of these RNA species. It seems certain, however, that, from the gel

patterns, the silk-gland tRNA precursors, as in other animal systems, are heterogeneous and smaller in size compared with the *E. coli* suppressor tRNA^{Tyr} precursor. In accordance also with the hypothesized processing of precursor tRNA, the precursors in our system are susceptible to enzymes that can "trim" the molecule into tRNA-like fragments. The nuclease(s) from *E. coli* ribosomes not only cleaves the suppressor tRNA^{Tyr} from *E. coli* but also hydrolyzes TYMV valyl RNA releasing a 3'-fragment resembling tRNA. The recognition mechanism of this enzyme for specific cleavages in these molecules must be of interest. Our results indicate that the enzyme is not only specific for *E. coli* tRNA precursors but can recognize other molecules with structure related to tRNA regardless of the species origin. However, 5S RNA, which possesses a structure similar to tRNA (22), was readily degraded. The specificity must be primarily for precursor tRNAs since both mature tRNA and the cleaved products of precursor tRNA were resistant to the hydrolysis. Although very impure at this stage, the enzyme fraction does display a great deal of specificity. The specific conversion of the precursor tRNA in the silk glands may be the net result of activities of more than one ribonuclease, including RNase P shown recently to be present in a 0.2 M NH₄Cl wash of *E. coli* ribosomes (21). The significance of this novel specificity and the mechanism of enzymic action must await further data in terms of structural analysis of the precursor molecules and purification of the maturation enzymes.

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