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Transfer RNA in Posterior Silk Gland of *Bombyx mori*: Polyacrylamide Gel Mapping of Mature Transfer RNA, Identification and Partial Structural Characterization of Major Isoacceptor Species†

Jean-Pierre Garel,‡ Richard L. Garber, and M. A. Q. Siddiqui*

ABSTRACT: Transfer RNAs (tRNAs) from the posterior silk gland and carcass tissues of the silkworm *Bombyx mori* L. were fractionated by high resolution polyacrylamide gel electrophoresis. tRNAs from each source resolved into 50 distinct spots, many of which represented pure tRNA species. Nonlabeled tRNA of the posterior silk gland, purified by benzoylated diethylaminoethyl-cellulose column chromatography and by counter current distribution, were used to aid in identification of $tRNA^{Ala}$, $tRNA^{Gly}$, and $tRNA^{Ser}$ isoacceptor species. These tRNA species constituted about 70% of total tRNA population in the posterior silk gland. The high resolution of tRNA separation on polyacrylamide gels thus provided a quantitative estimate of the posterior silk gland isoacceptor tRNA distribution which is adapted to produce large amounts of the protein, silk fibroin, during the fifth larval instar. Several of the major ^{32}P-labeled tRNA species of the posterior silk gland, isolated by two-dimensional polyacrylamide gel electrophoresis, were subjected to structural analysis by oligonucleotide fingerprinting of RNase T_1 digests and nucleotide separation analysis of RNase T_2 hydrolysates. The results indicated that a specific $tRNA^{Ala}$ species ($tRNA^{Ala}_{2a}$), which is present in the posterior silk gland but absent from the carcass tissue [and in the middle silk gland; see Meza, L., et al. (1977), *FEBS Lett. 77*, 255–260], differs structurally by substitution of a single base in one hexanucleotide indicated in the T_1-oligonucleotide fingerprint map, from the other major $tRNA^{Ala}$ species ($tRNA^{Ala}_{2b}$) found in both tissues. The main $tRNA^{Gly}$ and $tRNA^{Ser}$ species are present in both silk glands and in carcass tissues. The structural properties of these tRNA species are also described.

Significant progress was made during recent years on understanding the mechanism of synthesis and processing of tRNAs encoded by bacteriophage and *Escherichia coli* genes, but little is known about biosynthesis of tRNA in eukaryotes (for reviews, see Burdon, 1971; Schäfer and Söll, 1974; Altman, 1975; Smith, 1976). We have chosen the posterior silk gland of the silkworm, *Bombyx mori* L., as a model eukaryotic system for studies on biosynthesis of tRNA and its regulation. A unique feature of the developing silk gland is the appearance of four preponderant tRNAs specific for glycine, alanine, serine, and tyrosine during the terminal differentiation of the gland in the fifth larval instar (silk fibroin secretion phase). The four tRNAs constitute almost 80% of the total tRNA population of the posterior silk gland (Garel et al., 1970; Chavancy et al., 1971; Delaney and Siddiqui, 1975; Majima et al., 1975). It was proposed (Garel, 1974, 1976) that such an adaptation of tRNA population which results from quantitative changes in specific isoacceptor tRNA species (Garel et al., 1970; Delaney and Siddiqui, 1975; Araya et al., 1975) could ensure rapid and efficient decoding of fibroin mRNA. Silk fibroin is composed mainly of the four amino acids mentioned above and is synthesized exclusively in the posterior part of the silk gland (Tashiro et al., 1968; Daillie et al., 1971). The presence of a relatively small number of predominant tRNA species in the posterior silk gland thus presents an opportunity for the isolation of pure precursor tRNA molecules and for the investigation of the mechanism involved in the accumulation of specific tRNA species.

We (Chen and Siddiqui, 1975) and others (Tsutsumi et al., 1974, 1976) have recently described the isolation and characterization of low molecular weight RNA species in the posterior silk gland of *B. mori* that have properties expected of a precursor to tRNA. In continuation of our studies on biosynthesis of tRNA and to elucidate the correlation between the compositions of mature tRNA and the corresponding precursor tRNA population in the silk gland, we looked for a tRNA separation system with high resolution capacity. The polyacrylamide gel electrophoretic procedures described below were designed (i) to establish a gel map that can be used to identify the mature tRNA species or the precursor tRNA species present during distinct physiological stages (growth

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and secretion stages) of the silk gland development, and (ii) to obtain sufficient amounts of labeled tRNA in pure form for structural analysis so that the relationship between mature tRNA species and their counterpart precursor molecules can be established.

In the following report, gel mapping of the posterior silk gland and carcass tRNA by two-dimensional polyacrylamide gel electrophoresis and characterization of the major posterior silk gland tRNA species by ribonuclease T_1 oligonucleotide fingerprinting and by nucleotide composition analysis is described. The tRNA gel patterns reflect the highly characteristic quantitative and qualitative tRNA distributions of the *B. mori* tissues (Matsuzaki, 1966; Garel et al., 1970; Delaney and Siddiqui, 1975). The analysis can also be used for scanning the changes in accumulation of specific tRNA during growth and differentiation of the silk gland and other tissues.

Materials and Methods

Preparation of tRNA. Larvae of Japanese strains of the silkworm *Bombyx mori* L. were grown on mulberry leaves, the silk glands were excised during the fibroin secretion phase, and RNA extracted from the posterior part was fractionated on Sephadex G-100 as described previously (Chen and Siddiqui, 1975). Between 300 and 400 A_{260} units of tRNA was routinely obtained from 100 posterior silk glands of 5-6 day old fifth instar larvae. RNA from the carcass tissue of *B. mori* was extracted using a phenol–isoamyl alcohol–chloroform mixture (Perry et al., 1972) and was subjected to fractionation as above. For preparation of labeled tRNA, 3.0 mCi of neutralized carrier-free [^{32}P]orthophosphate in 100 μL was injected intracoelomically into each larva on the second day of the fifth instar. The glands were excised 48 h later, washed in 1 × SSC[1] (0.15 M NaCl–0.015 M sodium citrate), and were either used immediately or stored at −70 °C. Storage of the glands at −70 °C did not cause any noticeable degradation of RNA. Labeled RNA was extracted and fractionated as above. The specific activity of the RNA thus labeled varied between 3 and 8 × 10^6 cpm per A_{260} unit.

Electrophoretic Separation of tRNA on Polyacrylamide Gels. Electrophoresis of tRNA was done on 9.6% acrylamide–7 M urea or on 20% acrylamide–4 M urea systems using slab gels of 1.5 × 200 × 400 mm for the former and 3 × 120 × 140 mm for the latter. Electrophoretic conditions were similar to those described by Fradin et al. (1975). The acrylamide and bisacrylamide (19:1) were polymerized in 0.089 M Tris base, 0.089 M boric acid, 0.38% (v/v) 3-dimethylaminopropionitrile, and the desired amount of urea. pH was adjusted to 8.2 with HCl and ammonium persulfate was added to a final concentration of 0.19% (w/v). The running buffer consisted of Tris-HCl and boric acid, pH 8.2, in concentrations as above. tRNA (0.1 to 1.0 A_{260} unit) in Tris–borate buffer containing sucrose and xylene cyanol-FF as marker dye were applied directly to gel slot. Electrophoresis was performed in the cold at about 10 mA and 450 V for 45 ± 5 h for the 9.6% gel and at 10 mA and 125 V for the 20% gel for 55 ± 5 h.

For two-dimensional separation, electrophoresis was done in the first dimension on 9.6% acrylamide–7 M urea gel as above. RNA bands were located either by autoradiography or by staining of gel strips (see below) containing marker nonlabeled tRNA. Strips of gel containing the sample tRNA were then cut out and embedded in 9.6% acrylamide over prepolymerized 20% acrylamide–4 M urea gel slabs. The conditions for electrophoresis were the same as described above. Staining of RNA was done for 2–3 h with 0.2% (w/v) methylene blue in 2% (w/v) sodium acetate, pH 5.0, and the gels were destained for about 15–30 h in running water. Elution of tRNA from gels was done electrophoretically. Gel pieces containing RNA were placed in a glass tube supported at the bottom by a glass-fiber filter. Electrophoresis was done in the cold in a vertical apparatus for 10 h at 150 V using the standard Tris-HCl–boric acid buffer. The RNA was collected directly in a dialysis bag attached to the tube. The contents of the bag were either dialyzed first against distilled water and then lyophilized to dryness or were ethanol precipitated, dialyzed, and then lyophilized.

Preparation of Purified tRNA Species. Nonlabeled isoacceptor $tRNA^{Gly}$ and $tRNA^{Ala}$ from the posterior silk gland were purified by chromatography on benzoylated DEAE-cellulose after phenoxyacetylation of acylated tRNA according to Gillam et al. (1968) and as described previously (Chen and Siddiqui, 1975). Partially purified $tRNA^{Ser}$ and $tRNA^{Tyr}$ species were obtained by countercurrent distribution (Garel et al., 1976b).

Fingerprinting of [^{32}P]tRNA Species. Fingerprint analysis was done by ionophoresis of the ribonuclease T_1 digestion products of purified [^{32}P]tRNA on cellulose acetate membrane strips (Schleicher-Schuell) at pH 3.5 according to Brownlee and Sanger (1967). Oligonucleotides transferred to polyethylenimine-cellulose (PEI) thin-layer plates (MN-Brinkman) according to Southern (1974) were fractionated in the second dimension by homochromatography using homomixture C (Brownlee, 1972) at 55–60 °C for 5–10 h. The plate was dried and spots were located by autoradiography on x-ray film (Kodak NS-54T).

Nucleotide Composition Analysis. The composition of nucleotides was determined by thin-layer chromatography on cellulose of a ribonuclease T_2 hydrolysate of [^{32}P]tRNA according to Nishimura (1972).

Results

Unfractionated tRNAs from the posterior silk gland of *B. mori,* when subjected to two-dimensional gel electrophoresis, were resolved into about 50 tRNA spots (Figure 1), several of which have been identified (see below). The tRNA separation achieved using only the 9.6% acrylamide–7 M urea system or the 20% acrylamide–4 M urea system is shown on the top and on the left panels of the two-dimensional pattern. Gels of these two types consistently resolved the tRNA into 14–16 and 19–20 bands, respectively. The combination of the two gel systems in a two-dimensional gel electrophoresis, however, produced a high resolution of tRNA separation (see also Figure 2). Many of the major spots, when analyzed by RNase T_1 fingerprinting, showed clean patterns expected of pure tRNA species. The two-dimensional gel map was very characteristic of posterior silk gland tRNA at the end of the fifth larval instar and was reproducible from one tRNA preparation to another. For comparison, the tRNA from the carcass tissue of *B. mori,* separated on a two-dimensional gel under identical conditions, is shown in Figure 2a. While the distribution of the posterior silk gland tRNA clearly showed an enrichment in tRNA specific for a few amino acids (Figure 2b), the distribution of tRNA from carcass, which unlike the posterior silk

[1] Abbreviations used: I, inosine; Ψ, pseudouridine; T, ribothymidine; m^1A, 1-methyladenosine; m^1G, 1-methylguanosine; m^2G, N^2-methylguanosine; $m^2{}_2G$, N^2-dimethylguanosine; m^7G, N^7-methylguanosine; m^3C, N^3-methylcytidine; m^5C, 5-methylcytidine; m^1I, 1-methylinosine; Gm, 2′-*O*-methylguanosine; Um, 2′-*O*-methyluridine; i^6A, N^6-isopentenyladenosine; t^6A, N^6-(*N*-threonylcarbonyl)adenosine; ac^4C, N^4-acetylcytidine; D, 5,6-dihydrouridine; mt^6A, 2-methyl-6-threonyladenosine; U*, unknown modified uridine; SSC, 0.15 M NaCl–0.015 M sodium citrate; Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine-cellulose; BD, benzoylated.

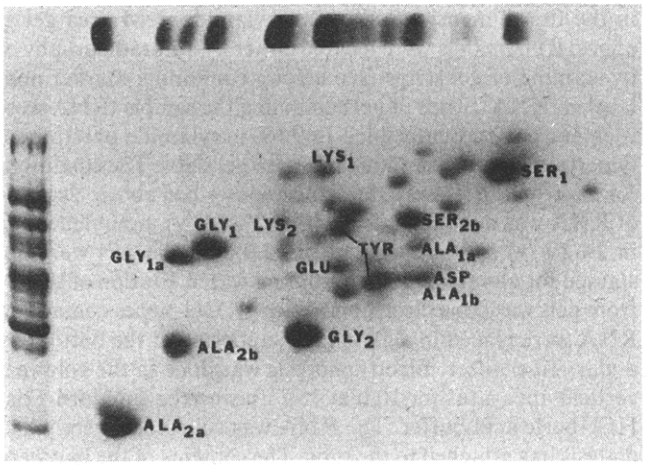

FIGURE 1: Two-dimensional polyacrylamide gel electrophoresis of posterior silk gland tRNA. One A_{260} unit of unfractionated tRNA from the posterior silk gland of 6–7 day old larvae was analyzed on two-dimensional polyacrylamide gel as described in Materials and Methods. Electrophoresis on 9.6% polyacrylamide gel (used as first dimension) and on 20% gel (used as second dimension) alone are shown on the top and left panels of the two-dimensional map, respectively. The direction of migration was from right to left for the first dimension and from top to bottom for the second. Identification of spots was as described in text.

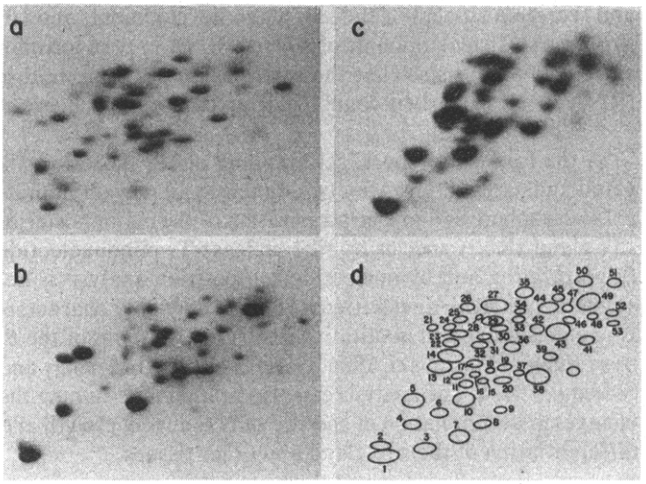

FIGURE 2: Two-dimensional polyacrylamide gel electrophoretic separation of tRNA from posterior silk gland and carcass tissues of *B. mori*. One A_{260} unit each of nonlabeled tRNA from the posterior silk gland and carcass tissue of 6–8 day old larvae and about 200 000 cpm of ^{32}P-labeled tRNA from the posterior silk gland of 3–4 day old larvae were subjected to two-dimensional gel electrophoresis as described in Materials and Methods. (a) Nonlabeled carcass tRNA; (b) nonlabeled posterior silk gland tRNA; (c) [^{32}P]tRNA from posterior silk gland; (d) diagrammatic representation of tRNA separation on two-dimensional gel. Spot 1, $tRNA^{Ala}_{2a1}$; spot 2, $tRNA^{Ala}_{2a2}$; spot 5, $tRNA^{Ala}_{2b}$; spot 10, $tRNA^{Gly}_{2}$; spot 13, $tRNA^{Gly}_{1a}$; spot 14, $tRNA^{Gly}_{1}$; spot 43, $tRNA^{Ser}_{2b}$; spot 49, $tRNA^{Ser}_{1}$; spot 19 (20), $tRNA^{Tyr}$; spot 27, $tRNA^{Lys}_{1}$; spot 22 (and/or 23), $tRNA^{Lys}_{2}$; spot 38, $tRNA^{Asp}$.

gland makes protein of more uniform amino acid composition, was markedly different. The separation of [^{32}P]tRNA of the posterior silk gland (Figure 2c) was generally similar to that of nonlabeled tRNA. The relative differences in intensity of a few spots were due to differences in the age of larvae used for RNA extraction (labeled tRNA was obtained from 3–5 day old larvae and nonlabeled tRNA was from 6–7 day old fifth instar larvae), and possibly was also due to the shorter accumulation time for the labeled RNA.

The identification of tRNA spots and assessment of their purity was accomplished by parallel electrophoresis of purified isoacceptor tRNA species, coelectrophoresis of purified nonlabeled tRNA with unfractionated ^{32}P-labeled tRNA, and fingerprint analysis of RNase T_1 digests of labeled tRNA spots. A few nonlabeled tRNA species were also eluted from gels and analyzed for amino acid acceptance (Delaney and Siddiqui, 1975). To aid in identification, marker nonlabeled $tRNA^{Ala}$ and $tRNA^{Gly}$ species were prepared by BD-cellulose column chromatography (Delaney and Siddiqui, 1975; Meza et al., 1977) and $tRNA^{Ser}$ and $tRNA^{tyr}$ were obtained by countercurrent distribution (Garel et al., 1976b; Hentzen et al., 1976). Alanyl-tRNA from the posterior silk gland of 5–7 day old larvae separated on BD-cellulose into a minor ($tRNA^{Ala}_1$) and a major ($tRNA^{Ala}_2$) peak in a ratio of about 20:80, the latter eluting at a higher salt concentration. Glycyl-tRNA, under similar conditions, separated into two major peaks, $tRNA^{Gly}_2$ and $tRNA^{Gly}_1$, in a ratio 40:60. $tRNA^{Ser}$ fractionated on countercurrent distribution into two peaks, a polar one ($tRNA^{Ser}_1$), and a lipophilic one ($tRNA^{Ser}_2$) which resolved further into two isoaccepting species on RPC-5, a major $tRNA^{Ser}_{2b}$ and a minor $tRNA^{Ser}_{2a}$ (Hentzen et al., 1976). $tRNA^{Tyr}$ separated as a single peak on countercurrent distribution (Garel et al., 1976b). The electrophoretic separation of the $tRNA^{Ala}$ and $tRNA^{Gly}$ peaks from BD-cellulose column chromatography on 20% polyacrylamide–4 M urea gel is shown in Figure 3. The peak 1 or $tRNA^{Ala}_1$ moved mainly as one band (column 2),[2] but the peak 2 or $tRNA^{Ala}_2$ resolved into two distinct bands. However, upon electrophoresis on two-dimensional gel, $tRNA^{Ala}_2$ resolved into three spots, 1, 2, and 5 (Figure 2d); spots 1 and 2 were similar in structural analysis, but spot 5 was different (see below). These spots, 1, 2, and 5, will be referred from here on as $tRNA^{Ala}_{2a1}$, $tRNA^{Ala}_{2a2}$, and $tRNA^{Ala}_{2b}$, respectively. The probable location of the minor $tRNA^{Ala}_1$ species on the two-dimensional gel is shown in Figure 1 (see footnote 2). $tRNA^{Gly}_2$ from BD-cellulose moved as a single band on 20% gel (column 5) and as a single spot 10 in two-dimensional gel (Figure 2d), but $tRNA^{Gly}_1$ appeared to contain a major (spot 14) and a minor (spot 13) species. These two spots, 14 and 13, will be referred to from here on as $tRNA^{Gly}_1$ and $tRNA^{Gly}_{1a}$, respectively. The partially purified $tRNA^{Ser}$ and $tRNA^{Tyr}$ were also examined on two-dimensional polyacrylamide gel. The tRNAs were also charged with the respective labeled amino acid, stabilized by nitrous acid treatment (Hervé and Chapeville, 1965) and subjected to electrophoresis as above. After staining the spots were cut, digested in 30% H_2O_2, and counted for radioactivity. The gel maps for charged and uncharged tRNA examined were identical. Spots 49 and 43 were thus identified as $tRNA^{Ser}_1$ and $tRNA^{Ser}_{2b}$, respectively, and spot 19 (and/or 20) and spot 32 represented $tRNA^{Tyr}$ species and spot 27 and spot 22 (and/or 23) were identified as $tRNA^{Lys}_1$ and $tRNA^{Lys}_2$, respectively.[2] $tRNA^{Asp}$ was found in spot 38 and radioactivity of the [^{3}H]seryl-tRNA, presumably representing minor species of this tRNA, was also distributed in spots 44, 45, 48, and 50. The precise identity of these spots is under investigation.

By direct measurements, the radioactivity eluted from each of the major spots, 1, 2, 5, 10, 13, 14, 43, and 49, representing the majority of the $tRNA^{Ala}$, $tRNA^{Gly}$, and $tRNA^{Ser}$ species constituted about 70% of the total activity representing all

[2] The locations of $tRNA^{Ala}_1$, $tRNA^{Tyr}$, and $tRNA^{Asp}$ species on the two-dimensional gel map (Figure 1) are tentative, since they were ascertained only by the separation of labeled tRNAs after aminoacylation with the respective radioactive amino acids. However, the noncharged $tRNA^{Ala}_1$ appeared to move at an alternate position (see Figure 3), the reason for which is not clear.

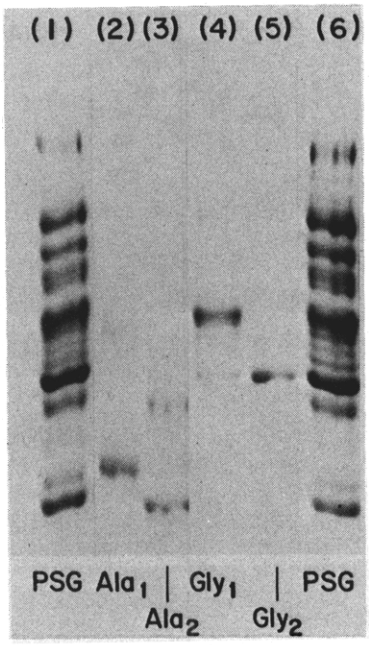

FIGURE 3: Electrophoretic separation of unfractionated tRNA and purified $tRNA^{Ala}$ and $tRNA^{Gly}$ species of posterior silk gland on 20% polyacrylamide–4 M urea gel. One A_{260} of unfractionated nonlabeled tRNA and 0.1 A_{260} each of $tRNA^{Ala}_1$ and $tRNA^{Ala}_2$ peaks (see text and Meza et al., 1977) and of $tRNA^{Gly}_1$ and $tRNA^{Gly}_2$ peaks (see text and Delaney and Siddiqui, 1975) obtained by BD-cellulose column chromatography of tRNA from the posterior silk gland of 6–8 day old larvae were run on 20% acrylamide–4 M urea gel as described in Materials and Methods. PSG, posterior silk gland tRNA; Ala_1, Ala_2, Gly_1, and Gly_2 are $tRNA^{Ala}_1$, $tRNA^{Ala}_2$, $tRNA^{Gly}_1$, $tRNA^{Gly}_2$ peaks of the BD-cellulose column, respectively (see footnote 2).

posterior silk gland tRNAs of the 6–7 day old 5th instar larvae. This ratio was in fair agreement with that derived by in vitro acylation assay for these tRNA (Garel et al., 1970; Chavancy et al., 1971) and that expected from the composition of the cognate amino acids in silk fibroin (see Discussion). Suzuki and Ikemura (1974) have previously separated the ^{32}P-labeled posterior silk gland tRNA into about 20 spots using two-dimensional gel electrophoresis. They observed an accumulation of label in a few spots, which appeared to be similar in mobility to $tRNA^{Ala}$ and $tRNA^{Gly}$, during the early stages of gland development in the fifth instar larvae. The resolution achieved by our electrophoretic conditions would allow a quantitation of specific isoacceptor tRNAs (see Table I) and can be used to monitor changes in tRNA distribution during growth and development of the silk gland.

Another application of the two-dimensional gel system was to obtain labeled tRNA species of high purity in sufficient amounts for structural analysis. The separation of ^{32}P-labeled tRNA was similar to that obtained with nonlabeled tRNA (Figure 2). The spots representing $tRNA^{Ala}$, $tRNA^{Gly}$, and $tRNA^{Ser}$ species, the major constituents of the posterior silkgland tRNA, were eluted from the gel and subjected to oligonucleotide fingerprint analysis after RNase T_1 digestion. The fingerprints for spots 1 and 2 ($tRNA^{Ala}_{2a1}$ and $tRNA^{Ala}_{2a2}$) appeared identical (Figure 4, left panel); on the other hand, the fingerprint map of spot 5 ($tRNA^{Ala}_{2b}$) differed by a shift in the position of one hexanucleotide (indicated by arrow). The T_2 nucleotide separation analyses (Figure 4, right panel) were also quantitatively identical for $tRNA^{Ala}_{2a2}$ and $tRNA^{Ala}_{2a1}$. There was, however, a clear difference between $tRNA^{Ala}_{2a}$ species and $tRNA^{Ala}_{2b}$ with respect to a RNase T_2 stable dinucleotide. With the help of the composite T_2

TABLE I: Quantitative Distribution of Preponderant Isoaccepting tRNA Species in the Posterior Silk Gland of *Bombyx mori* L.[a]

tRNA	Spot No.[b]	% distribution	Anticodon[c]	Major codons in fibroin mRNA[d]
Ala_{2a1}	1	11		
Ala_{2a2}	2	2	IGC	GCU
Ala_{2b}	5	6		
Gly_{1a}	13	7		
Gly_1	14	16	GCC	GGU
Gly_2	10	18	UCC or U*CC	GGA
Ser_1	49	3	GCU	
Ser_{2b}	43	7	IGA	UCA

[a] Long-labeled [^{32}P]tRNAs from the posterior silk gland of 6–7 day old larvae of *B. mori* were purified by two-dimensional gel electrophoresis as described in Materials and Methods. Radioactivity from each spot was measured after dissolving gel pieces for 12 h at 60 °C in H_2O_2 and counted in 10 mL of Bray's solution. [b] See Figure 2. [c] Assignments of anticodons were based on previous experiments on codon responses and partial sequences of isoacceptor species purified by BD-cellulose chromatography and countercurrent distribution and their identification by two-dimensional acrylamide gels (see text). For further details, see Garel, 1976; Garel et al., 1974, 1976a,b; Hentzen et al., 1976. [d] From the sequence analysis of purified fibroin mRNA (Suzuki and Brown, 1972).

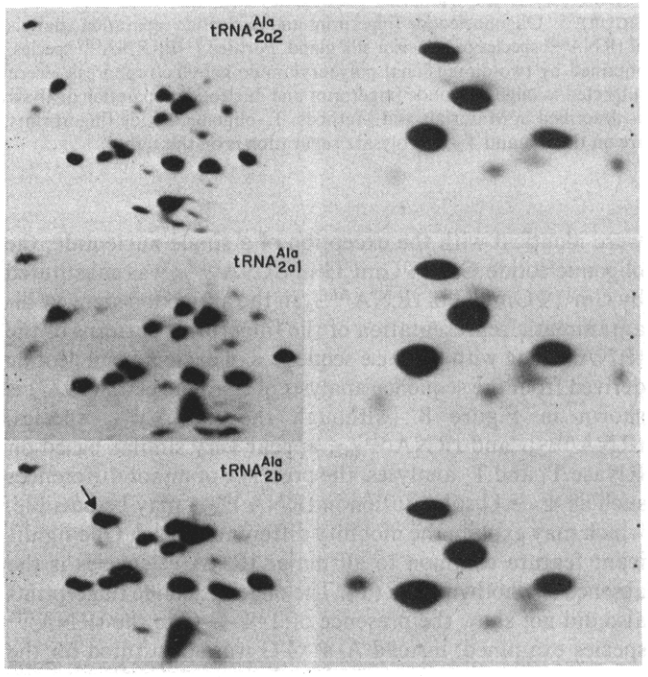

FIGURE 4: Oligonucleotide fingerprints and nucleotide separation analysis isoacceptor $tRNA^{Ala}$ species of posterior silk gland. Purified [^{32}P]$tRNA^{Ala}$ species obtained by two-dimensional polyacrylamide gel electrophoresis (see Figure 2) were digested with RNase T_1 and T_2 separately, and the hydrolysates were subjected to two-dimensional separation as described in Materials and Methods. The RNase T_1 oligonucleotide fingerprint profiles are shown on the left and the RNase T_2 hydrolysate separation patterns are on the right. The arrow indicates the shifted position of an oligonucleotide in $tRNA^{Ala}_{2b}$ (see text).

profile (see Figure 7), it can be deduced that GmpUp present in $tRNA^{Ala}_{2a}$ species is replaced by GmpCp in $tRNA^{Ala}_{2b}$. This would also account for the shift in the first dimension of the hexanucleotide mentioned above. Sprague et al. (1977) have recently completed the base sequence analysis of two major $tRNA^{Ala}$ species of the posterior silk gland of *B. mori*. These tRNAs correspond to isoacceptor $tRNA^{Ala}_{2a}$ and $tRNA^{Ala}_{2b}$ of our studies. The base sequences of the tRNAs

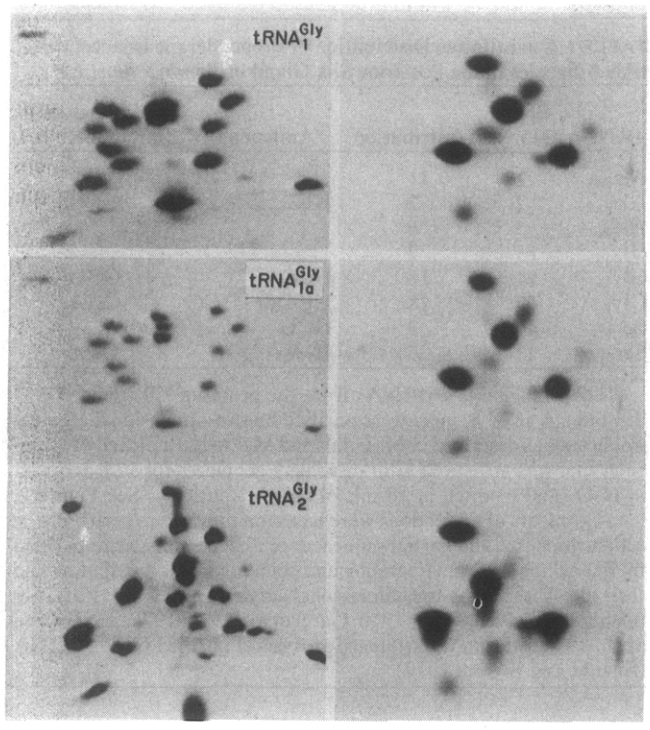

FIGURE 5: Oligonucleotide fingerprint and nucleotide separation analysis of tRNA^{Gly} species of posterior silk gland. Purified [^{32}P]tRNA^{Gly} species, obtained by two-dimensional polyacrylamide gel electrophoresis, were subjected to oligonucleotide fingerprint and nucleotide separation analysis as described in Materials and Methods. T_1-oligonucleotide fingerprints are on the left and T_2-hydrolysate separation is on the right.

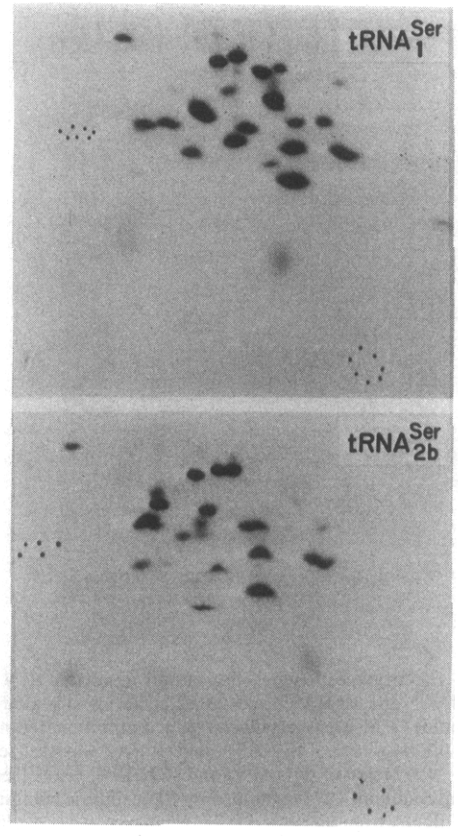

FIGURE 6: Oligonucleotide fingerprint analysis of tRNA^{Ser} species of posterior silk gland. Purified [^{32}P]tRNA^{Ser} species obtained by two-dimensional polyacrylamide gel electrophoresis were subjected to oligonucleotide fingerprint analysis in Figure 4.

were identical with the exception of a single nucleotide; the oligonucleotide Cm^{1}IΨGmCG in tRNA$^{Ala}_{2b}$ was substituted by Cm^{1}IΨGmUG in tRNA$^{Ala}_{2a}$ in the anticodon stem. A diagrammatic representation of the fingerprint patterns of the tRNAs along with the base sequences of each oligonucleotide derived from the sequence analysis of Sprague et al. (1977) is shown in Figure 8. Although the tRNA$^{Ala}_{2a}$ species, tRNA$^{Ala}_{2a1}$ and tRNA$^{Ala}_{2a2}$, appear very similar based on RNase T_1 and T_2 analyses, the presence of minor differences such as Ψ → U substitution in tRNA$^{Ala}_{2a2}$ may be possible, which may explain the mobility difference on gel. One significant feature common to all major tRNA^{Ala} species is the absence of ribothymidine (T). The oligonucleotide fingerprints also did not show the presence of T-Ψ-C-G in the tRNA^{Ala} species examined; instead A-Ψ-C-G was substituted for the tetranucleotide, a feature which is characteristic of eukaryotic initiator tRNAs (see Sprague et al., 1977).

The oligonucleotide fingerprints for tRNA^{Gly} and tRNA^{Ser} species are shown in Figures 5 and 6, respectively. The RNase T_1 patterns of tRNA$^{Gly}_1$ (spot 14) and tRNA$^{Gly}_{1a}$ (spot 13) were identical, whereas the pattern of tRNA$^{Gly}_2$ was different. The major tRNA$^{Gly}_1$ and tRNA$^{Gly}_2$ species have one RNase T_2 stable dinucleotide UmpCp located in the acceptor arm (Garel et al., 1976a), whereas the minor tRNA$^{Gly}_{1a}$ appears to lack the 2′-O-methylation in Um (Table II). Figure 8C shows the T_1-oligonucleotide pattern for tRNA$^{Gly}_1$ along with the sequence for each oligonucleotide based on the sequence analysis of Garel and Keith (1977).

The fingerprint maps of tRNA^{Ser} species (Figure 6) showed more variations relative to those of tRNA^{Gly} and tRNA^{Ala} isoacceptor species. The T_2 analysis (not shown) indicated that i^{6}A was present in tRNA$^{Ser}_2$ as reported earlier (Hentzen et al., 1976). An interesting feature which became evident by the

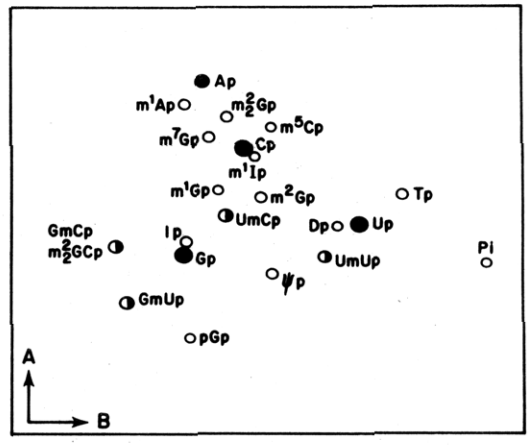

FIGURE 7: Diagrammatic representation of nucleotides and RNase T_2-stable dinucleotides separation by two-dimensional thin-layer chromatography. The positions of nucleotides and dinucleotides obtained after RNase T_2 hydrolysis of tRNA depicted in the diagram are based on separations obtained with the major and a few minor nucleotides in our laboratory and on separations reported by Nishimura (1972) and Hashimoto et al. (1975). Only those nucleotides and dinucleotides present in tRNA^{Gly} and tRNA^{Ala} species are shown here. The position of U* is not presently known. First dimension, bottom to top; second dimension, left to right.

structural studies is the exclusive presence of a few specific modified bases in the major tRNA species of the posterior silk gland, which would be useful as markers for a rapid identification of these species especially during the studies involving in vitro processing of the corresponding precursor tRNA molecules. For example, m^{1}G was present in all tRNA^{Gly} species whereas m^{2}G, m$^2_2$G, m^{7}G, Gm, I, and m^{1}I were con-

TABLE II: Nucleotide Composition of $tRNA^{Ala}$ and $tRNA^{Gly}$ Species from the Posterior Silk Gland of *Bombyx mori* L.[a]

Nucleotide	$tRNA^{Ala}_{2a2}$	$tRNA^{Ala}_{2a1}$	$tRNA^{Ala}_{2b}$	$tRNA^{Gly}_{1a}$	$tRNA^{Gly}_{1}$	$tRNA^{Gly}_{2}$
A	12 ± 1	12 ± 1	12 ± 1	9 ± 1	9 ± 1	13 ± 1
G	23 ± 1	22 ± 1	22 ± 1	25 ± 1	25 ± 1	23 ± 1
U	9 ± 1	9 ± 1	8 ± 1	11 ± 1	10 ± 1	11 ± 1
C	21 ± 1	21 ± 1	22 ± 1	20 ± 1	20 ± 1	20 ± 1
m^1A	1	1	1	1	1	1
m^1G				1	1	1
m^2G	1	1	1			
m^2_2G	1	1	1			
m^7G	1	1	1			
Gm	1	1	1			
I[b]	1	1	1			
m^1I[b]	1	1	1			
Ψ	2	2	2	1	1	2
D	1	1	1	1	1	1
U*[b]						1
Um	1	1	1		1	1
T				1	1	1
m^5C	1	1	1	4	4	2
Total (±1)	76	76	76	76	76	77

[a] Nucleotide composition of [^{32}P]tRNA species purified by two-dimensional gel electrophoresis was determined by separation of the RNase T_2 digests on thin-layer chromatography as described by Nishimura (1972). These values are in agreement with the values obtained previously using nonlabeled tRNA species (Garel et al., 1976b). [b] These nucleotides, which are not identifiable by the method above, were analyzed as nonlabeled nucleosides, according to the mapping of Rogg et al. (1976).

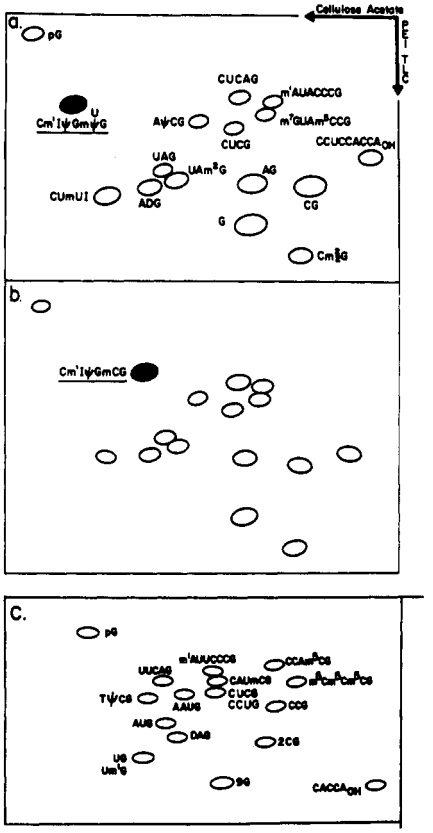

FIGURE 8: Diagrammatic representation of T_1 oligonucleotides fingerprints of $tRNA^{Ala}_{2a}$, $tRNA^{Ala}_{2b}$, and $tRNA^{Gly}_{1}$. The T_1 oligonucleotide fingerprint patterns for $tRNA^{Ala}_{2a}$ (a), $tRNA^{Ala}_{2b}$ (b), and $tRNA^{Gly}_{1}$ (c) were reproduced from Figures 4, 5, and 6, respectively. The base sequence for $tRNA^{Ala}$ species was derived from the sequence data of Sprague et al. (1977) and for $tRNA^{Gly}_{1}$ was based on that of Garel and Keith (1977).

stituents of major $tRNA^{Ala}$ species and four residues of m^5C were present in $tRNA^{Gly}_{1}$. In addition, m^3C and ac^4C were found in all $tRNA^{Ser}$ species (Garel et al., 1976b; Hentzen et al., 1976) and mt^6A was present in $tRNA^{Ser}_{1}$, i^6A in $tRNA^{Ser}_{2}$, and t^6A in $tRNA^{Tyr}$ (see also Brambilla et al., 1976).

Discussion

The discovery of phage specific tRNA in *E. coli* and the information on structural characterization including complete sequences has contributed to the rapid knowledge of many steps in maturation of tRNA in *E. coli* (Altman, 1975; Smith, 1976). Similar characterization of tRNA and tRNA precursors in eukaryotes is not yet available since it is difficult to obtain radiochemically pure species in sufficient amounts for structural analysis. The unique adaptation of tRNA population in the posterior silk gland of *B. mori* resulting in an accumulation of specific tRNA species makes the silk gland a promising system for isolation, quantitation, and characterization of the preponderant tRNA in the gland and their counterpart precursor molecules.

The two-dimensional acrylamide gel electrophoresis described above provide a remarkably high resolution for tRNA separation. The number of tRNA spots, 53 detectable in Figure 2, represents approximately the actual number of tRNA species in *B. mori*, assuming that eukaryotic tRNA contains 55 ± 5 species (Gallo and Pestka, 1970). Electrophoresis under identical conditions of yeast and *E. coli* tRNA produced 40 and 30 spots, respectively (unpublished observations). The resolution for these tRNA separations was similar to that reported by Feldmann (Fradin et al., 1975; Varricchio and Last, 1976). The posterior silk gland tRNA gel maps also provide a quantitative measurement for the distribution of the individual tRNA species (see Table I). The three major tRNA species account for 70% of total tRNA tRNA ($tRNA^{Ala}$, 19%; $tRNA^{Gly}$, 41%; $tRNA^{Ser}$, 10%). This ratio is similar to that derived by acylation assays and that expected if the adapted tRNA population is to reflect the amino acid composition of silk fibroin, which constitutes 75% of total protein during the fibroin secretion larval stage. Assuming a uniform labeling of tRNA in vivo our measurements on tRNA labeled over a period of 24–48 h would reflect the newly synthesized tRNA species, and the measurements derived from acylation assays

would show the total accumulated tRNAs that are acylatable.

The quantitative estimates derived from the two-dimensional gels provide additional information about the composition of the isoaccepting tRNA species in the posterior silk gland and its correlation with the codon frequency in fibroin mRNA (Garel, 1976). Based on codon responses reported for the posterior silk gland $tRNA^{Ala}$, $tRNA^{Gly}$, and $tRNA^{Ser}$ (Garel et al., 1974; Chen and Siddiqui, 1974) and on sequence analysis of $tRNA^{Gly}_1$ (Garel and King, 1977) and of $tRNA^{Ser}_2$ (Hentzen et al., 1976) combined with the identification of the tRNA peaks from BD-cellulose columns and countercurrent distribution with the spots on the two-dimensional gels, it was possible to assign codons for the main tRNA spots and compare their quantitative ratios with the corresponding codon frequency in fibroin mRNA (Suzuki and Brown, 1972; see Table I). The anticodon IGC present in all major $tRNA^{Ala}_2$ species (spots 1, 2, and 5) is capable of recognizing GCU, the main alanine codon of fibroin mRNA. The ratio of $tRNA^{Gly}_1$, which decodes GGU, GGC, to $tRNA^{Gly}_2$, which responds to GGA, was 1.28, close to the ratio of 1.4 proposed by Suzuki and Brown (1972). Also $tRNA^{Ser}_{2b}$ (spot 43) containing an IGA anticodon can decode the main serine codon UCA.

The mechanism by which such an efficient adaptation of tRNA population occurs in the silk gland is not known. It was reported that there was no differential degradation of mature tRNA species since the half-lives of tRNA were similar (Fournier et al., 1976). It was also observed that tRNA genes in the silk gland are not amplified during the growth and secretion phases (Gage, 1974). One could, therefore, speculate that enrichment in certain tRNA species might be due to a rapid and differential transcription of the respective tRNA genes or a selective mechanism operating posttranscriptionally during maturation and processing of precursor tRNA might be responsible for the dramatic changes in mature tRNA distribution. It would be of interest, therefore, to ascertain whether the ratio of adapted tRNA population is reflected in distribution of the recently described precursor tRNA population of the posterior silk gland (Chen and Siddiqui, 1975; Tsutsumi et al., 1974, 1976). Our present experiments on gel mapping of precursor tRNA are directed to this end.

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