SELECTIVE CHANGES IN METHIONINE tRNA SPECIES DURING DEVELOPMENT OF THE POSTERIOR SILKGLANDS OF BOMBYX MORI

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SUMMARY

Transfer RNA with methionine acceptor activity isolated from two distinct physiological stages of the developing posterior silkgland of the silkworm, Bombyx mori, was examined. The tRNA from both stages could be fractionated on benzoylated DEAE-cellulose column into two isoaccepting species, $tRNA_1^{Met}$ and $tRNA_2^{Met}$. The molar quantity per gland of $tRNA_1^{Met}$ species, which was also formylatable with the E. coli enzymes, increased twelve-fold as the gland differentiates to produce a large amount of a single protein, silk-fibroin. Since methionine is not a part of silk-fibroin, the preferential increase in $tRNA_1^{Met}$ content would reflect the increased biological activity and the rapid rate of protein synthesis during the terminal differentiation of posterior silkgland.

The process of cell differentiation in the posterior silkgland of the fifth instar larvae of the silkworm Bombyx mori, involves a dramatic change from its pattern of synthesis of a variety of proteins to a state in which a single portion, silk-fibroin, is synthesized (1,2). Of particular interest is the change in tRNA population that undergoes an "adaptation" during the early stages of gland development such that the distribution of tRNA specific for the amino acids glycine, alanine, serine and tyrosine, which make up more than 90% of silk-fibroin, matches the composition of these amino acids in the protein (3-6). Several laboratories including ours (4-9) have examined the quantitative changes in the silkgland tRNA species, specially for $tRNA^{Gly}$, the most abundant of all tRNA, and found

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that the changes in relative amounts of the iso-accepting species reflected the changes in the protein synthetic activity during the terminal differentiation of silkgland. Such an "adaptation" of tRNA population resulting in production of high level of selective tRNA species was suggested to be part of a control mechanism that ensures maximum efficiency of silk-fibroin synthesis (10). In a recent communication (11, and unpublished observations), the intracellular levels of aminoacylated tRNA were examined and it was observed that the levels of four major tRNA, namely $tRNA^{Gly}$, $tRNA^{Ala}$, $tRNA^{Ser}$, $tRNA^{Tyr}$ went up approximately 30-fold within 4-6 days of larval development, whereas the increase in the levels of other tRNA specific for the amino acids not involved in silk-fibroin synthesis was substantially low, with the exception of $tRNA^{Met}$ which recorded a 24-fold increase measured under identical conditions. Since methionine is not part of silk-fibroin it was speculated that the observed increment for $tRNA^{Met}$ might be due to an increase in the $tRNA^{Met}$ species required for polypeptide chain initiation during fibroin synthesis. In the following, we have examined the levels of iso-accepting species of $tRNA^{Met}$ in the posterior silkgland by fractionation on benzoylated DEAE-cellulose columns and found that the massive increase in the amount of $tRNA^{Met}$ in each gland was clearly due to the tRNA species with properties consistent with those expected for initiator tRNA. The potential of this selective tRNA synthesis is discussed.

MATERIALS AND METHODS

The conditions for raising the B. mori larvae, methods for preparing tRNA and synthetases from the silkglands were described previously (8,11, 12). tRNA was isolated from the posterior silkglands of the first or second (V-1 glands) and on the seventh and eighth day (V-8 glands) of the fifth instar by removal of ribosomal RNA by precipitation at 1M NaCl, followed by chromatography on DEAE-cellulose as described by Yang and Novelli (13). tRNA was then stripped of amino acids by incubation in 0.2 M Tris-Cl, pH 8.7 at 37°C for 1 hr. Crude synthetases from E. coli were prepared according to Muench and Berg (14), which also served as a source for formylase. Labelled formyl-donor was a gift from Dr. S. Kerwar of Roche Institute of Molecular Biology.

Methionine acceptance was measured by incorporation of [^{14}C] or [^{3}H] methionine into trichloroacetic acid-insoluble material using Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), and as described prev-

iously (8). The formylation of charged tRNA was done according to Dickerman and Smith (15) and the radioactivity for both formylation and aminoacylation assays was measured in a Nuclear Chicago Scintillation counter with 18% and 77% efficiency for [^{3}H] and [^{14}C] respectively. Fractionation of tRNA on benzoylated DEAE-cellulose column was done essentially as described by Gillam et al. (16).

RESULTS

Two distinct physiological stages of silkgland development were chosen for comparison of $tRNA^{Met}$ levels; the first and second days of the fifth larval instar (V-1) which represent the stage of active growth of the gland

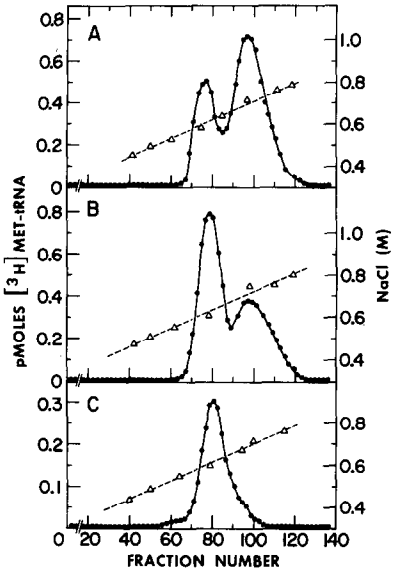

Figure 1. Fractionation of [^{3}H]methionyl-tRNA on BD-cellulose column. tRNA was acylated with [^{3}H]methionine, Sp. act. 11.0 Ci/mM, with homologous enzyme preparation (A & B) and with E. coli enzyme (C) as described in "Materials and Methods". 37,500 TCA-insoluble cpm for V-1 tRNA (A), 28,900 cpm for V-8 tRNA (B) and 21,600 cpm for V-8 tRNA charged with E. coli enzyme (C) were applied separately on BD-cellulose columns, 0.5 x 40 cm, and the chromatography was done at 4°C using a linear gradient produced by 150 ml each of 0.35 and 1.0 M NaCl in 10 mM NaOAc, pH 4.5 buffer containing 10 mM $MgCl_2$ and 1 mM β-mercaptoethanol. Fractions of 1.5 ml each were collected at a flow rate of 13 ml/hr. Radioactivity was measured by counting 1.0 ml aliquots in Bray's solution as before (8). The first peak eluting at low salt concentration is referred to as met_1 and the second peak as met_2 in text. ●, [^{3}H]; Δ (M) NaCl.

resulting in a remarkable increase in cellular components but little silk-fibroin is synthesized; the seventh and eighth days of development (V-8), a stage where an exclusive synthesis of silk-fibroin occurs reaching to a saturation level just prior to larval maturation (1). When tRNA from these stages were fractionated on BD-cellulose columns after acylation with [^{3}H]-methionine two distinct peaks, Met_1 and Met_2 were obtained in each case, but the relative amounts of these peaks changed significantly (Fig. 1A&B). During V-1 stage the ratio of peak Met_1/peak Met_2 was 0.52, but within 5-6 days of development the ratio was increased to 1.63 (Table 1). Since the tRNA species from both stages were eluted at the same salt concentration, there appeared to be no qualitative alterations in the tRNAs, although structural differences not resolvable by the chromatographic conditions cannot be excluded. The quantitative changes were apparent when the data from Fig. 1 was replotted to represent the total amount (in picomoles) of

TABLE I

RELATIVE AMOUNTS OF $tRNA^{Met}$ ISO-ACCEPTING SPECIES IN THE POSTERIOR SILKGLAND

Vth Instar	$tRNA^{Met}$ isoaccepting species %		$\frac{tRNA_1^{Met}}{tRNA_2^{Met}}$
	Met_1	Met_2	
1st day (V-1)	34	66	0.52
8th day (V-8)	62	38	1.63

$tRNA^{Met}$ from V-1 and V-8 glands was fractionated on BD-cellulose columns as described in the Legend to Figure 1. The relative amounts of each peak were calculated by adding the cpm for the peak fractions. It was assumed that deacylation during the chromatography was the same for both $tRNA^{Met}$ species.

each species in each gland for the two tRNA populations (Fig. 2). $tRNA_1^{Met}$ recorded about twelve-fold increase whereas the $tRNA_2^{Met}$ content was up by four-fold.

We have previously reported (11) a twenty-four-fold increase in the in vivo level of unfractionated methionyl-tRNA, based on aminoacylation after periodate oxidation, which was almost as high an increase as that observed for $tRNA^{Gly}$ and $tRNA^{Ala}$. Since methionine is not part of silk-fibroin, it was assumed that the specific increase in methionyl-tRNA content might reflect an active participation of this tRNA in the initiation mechanism of the specialized protein synthesis that occurs at a remarkably rapid rate (17). Ishizuka et al. (18) have recently shown that the N-terminal amino acid residue of nascent peptides in the posterior silkgland is methionine. In order to examine whether the $tRNA_1^{Met}$ species of the silkgland had properties similar to initiator tRNA in eukaryotes, the tRNA of V-8 glands was fractionated on BD-cellulose column as before and assayed for methionine acceptance using the silkgland and E. coli synthetases separately. Kawakami et al. (19) have reported earlier that one of the two

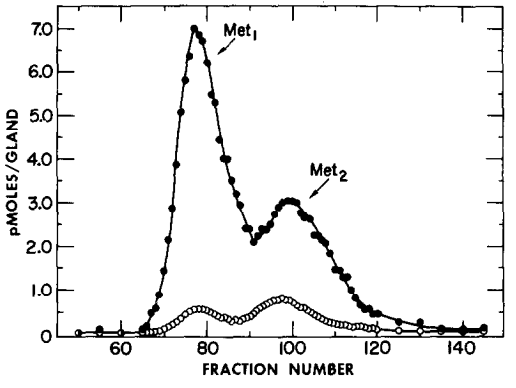

Figure 2. Composite BD-cellulose elution profiles of [^{3}H]methionyl-tRNAs. The data from Fig. 1 A & B are plotted here to represent pmoles of tRNA per gland based on measurements of total tRNA content in each gland (1.1 A_{260} units in V-1 gland and 8.0 A_{260} units in V-8 gland). 0, V-1; ●, V-8.

iso-accepting species of $tRNA^{Met}$ from V-5 stage glands of B. mori, fractionated on a DEAE-sephadex column, was recognized by E. coli synthetase for amino acylation. The same $tRNA^{Met}$ species was also formylated by E. coli transformylase. Others have previously shown that methionyl-tRNA synthetases from E. coli do recognize specifically the initiator species of several eukaryotic $tRNA^{Met}$ which are also formylated whereas the non-formylatable $tRNA^{Met}$ species are not (20 , and refs. cited therein). Our results shown in Fig. 1C (see also Fig. 4) are in agreement with these findings and show that the $tRNA_1^{Met}$ species hetero-acylated by E. coli enzymes was eluted as a symmetric peak at the position corresponding to $tRNA_1^{Met}$ fractionated under identical chromatographic conditions (Fig. 1A & B). In order to compare the aminoacylation and formylation properties of the two iso-accepting $tRNA^{Met}$ species, 107 A_{260} units of V-8 tRNA was fractionated on a BD-cellulose column and the fractions representing $tRNA_1^{Met}$ and $tRNA_2^{Met}$ were pooled, as shown in Fig. 3, to recover the tRNA. Each one of these $tRNA^{Met}$ species was then examined for methionine acceptor activity with the silkgland and E. coli synthetases and for formylation

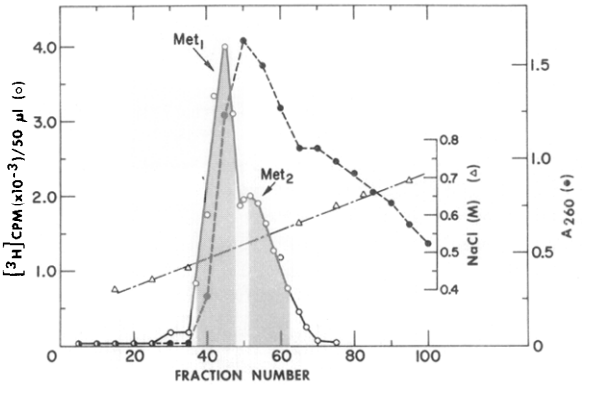

Figure 3. Fractionation of uncharged $tRNA^{met}$ on BD-cellulose. 107 A_{260} units of tRNA from V-8 glands were applied on a BD-cellulose column (0.5 x 40 cm) as described in "Materials and Methods" and in the legend to Figure 1. 50 µl aliquots from alternate fractions were tested for [^{3}H] methionine acceptance using the standard Hepes assay and fractions representing the $tRNA^{met}$ species (shaded area) were pooled to recover tRNA by ethanol precipitation.

using the E. coli crude synthetase preparations which contained transformylase activity. The kinetics of aminoacylation of $tRNA_1^{Met}$ and $tRNA_2^{Met}$ and those of formylation of $tRNA_1^{Met}$ are shown in Fig. 4. The saturating level of E. coli enzyme present in the reaction mixture apparently yielded methionyl- and formyl-methionyl-tRNA very rapidly and the product was stable up to 50 min of incubation. Clearly, only a fraction (12%) of $tRNA_2^{Met}$ was acylatable by the E. coli synthetase and a mere 3% of the total was formylated. $tRNA_1^{Met}$ on the

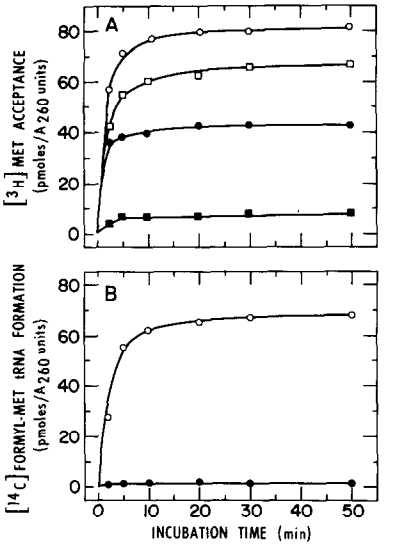

Figure 4. Kinetics of aminoacylation and formylation of $tRNA^{met}$. Partially purified $tRNA_1^{met}$ and $tRNA_2^{met}$ (see Fig. 3), 4.3 and 5.0 A_{260} units respectively, were incubated with [^{14}C] methionine, sp. act. 54 mCi/mM, and enzymes from the silkgland in 1.4 ml of standard Hepes reaction mixture. Aliquots of 200 µl each were taken at times indicated and assayed for radioactivity after TCA precipitation as before (8). For heterologous aminoacylation with E. coli enzymes, incubation was done in 50 mM Tris-Cl, pH 7.4, 12 mM $MgCl_2$, 10 mM KCl, 3 mM β-mercaptoethanol, 10 mM ATP and 0.02 mM [^{14}C] methionine of the above mentioned sp. act. For formylation, the tRNA was incubated for 5 min as for heterologous aminoacylation in the presence of 0.2 mM [^{12}C] methionine and then [^{14}C[formyl-donor, sp. act. 44 cpm/pmole was added to a final concentration of 0.6 mM. Aliquots were withdrawn at times indicated and assayed for radioactivity as above. A, met acceptance; O and ●, homologous charging with silkgland enzymes of $tRNA_1^{met}$ and $tRNA_2^{met}$ respectively; ▭ and ▬ heterologous charging with E. coli enzymes of $tRNA_1^{met}$ and $tRNA_2^{met}$ respectively; B, formylation; O, $tRNA_1^{met}$, ●, $tRNA_2^{met}$.

other hand was acylatable by E. coli enzymes to an extent that was 81% of the activity obtained with the silkgland enzymes. The conditions for both aminoacylation and formylation were optimized to effect a maximum product formation.

DISCUSSION

During the fifth larval instar, the posterior silkgland of B. mori undergoes a remarkably efficient translational specialization that results in the production of 10^{15} silk-fibroin molecules (17). It is believed that each cell of the posterior silkgland contains approximately 10^{10} molecules of fibroin-mRNA which in turn synthesizes silk-fibroin at a rapid rate of about 0.2 fibroin molecule/mRNA/sec. (17). To accomplish this, the cellular components necessary for fibroin synthesis are seemingly organized during the first half of the instar. The tRNA, for example, are synthesized in large amounts such that the relative proportions of the tRNA species match quantitatively the frequency of their codons present in fibroin-mRNA. The resulting high levels of the four major tRNAs, namely $tRNA^{Gly}$, $tRNA^{Ala}$, $tRNA^{Ser}$ and $tRNA^{Tyr}$, capable of decoding fibroin-mRNA was suggested to facilitate a rapid and efficient translation of the mRNA (10). Such a regulation of protein synthesis afforded by a "modulatory role" of tRNA was previously proposed by Stent (21) and others (see ref. 22).

The specific role of methionine-tRNA in initiation of protein synthesis has been well established in prokaryotes and is implicated in the initiation process of eukaryotic cells. There is no direct evidence that $tRNA_1^{Met}$ is involved in chain initiation in the silkgland, but the exclusive aminoacylation and formylation reactions catalyzed by the E. coli enzymes suggests that $tRNA_1^{Met}$ has properties similar to initiator tRNA of other eukaryotes. The significance of a twelve-fold increase in $tRNA_1^{Met}$ content in the silkgland resulting in the production of approximately 5-6 x 10^{10} molecules is not known at present, but clearly it is associated with the increased biological activity in the gland and with the rapid synthesis of fibroin molecules. The initiation step is considered to be a primary target for control of protein synthesis (23). There are numerous examples that suggest the involvement of tRNA

in regulatory function of both normal and neoplastic cell differentiation (see refs. 22, 24 for review). Whether the increased level of $tRNA^{Met}$ prior to the onset of fibroin production in the silkgland plays a role in control of differentiation is conjectural.

Daillie, Garel and co-workers (25, 26) have previously distinguished two groups of posterior silkgland tRNA by examination of the quantitative changes in tRNA by fractionation by counter-current distribution and *in vitro* aminoacylation assays. The tRNA which have a role in fibroin production were found in maximum concentrations at 7-8 days of the fifth larval instar, whereas those which may not be involved in fibroin synthesis reached a maximum at 4th or 5th day. Since there is no evidence for the amplification of tRNA genes in the silkgland (27, 28), the selective changes in tRNA population must be due to a differential gene action or must be the result of a selective mechanism operating post-transcriptionally during maturation and processing of tRNA precursors. The tRNA precursors of 4-5S size from the posterior silkgland have been described recently (12). These pre-tRNA molecules which might be the precursors of late stage during tRNA biogenesis are presently being analyzed for structural and functional characterization. It is not known yet whether the quantitative changes in mature tRNA species presumably needed for the functional decoding of fibroin-mRNA are reflected in the population of precursor tRNA species.

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