Processing of mammalian tRNA transcripts *in vitro*: different pre-tRNAs are processed along alternative pathways that contain a common rate-limiting step

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ABSTRACT

We have analyzed the pathways and kinetics of processing of mouse tRNA gene transcripts *in vitro*. Different transcripts are processed along two alternative pathways. The 3' trailer sequence of the tRNA^{His} primary transcript is excised before the 5' leader sequence. In contrast, for the tRNA^{Gly} primary transcript, the 5' leader sequence is excised before the 3' trailer sequence, as has been found for other monomeric eukaryotic tRNA gene transcripts.

Computerized analysis of the kinetics of processing indicates that tRNA^{Asp}, tRNA^{Gly}, tRNA^{Glu} and tRNA^{His} transcripts are processed in a substrate concentration-dependent manner and also reveals the existence of a common rate-limiting step, the rate constant of which is equivalent for three of the four transcripts tested. The processing of one pre-tRNA transcript can be competitively inhibited by addition of another pre-tRNA transcript to the processing reaction. The common rate-limiting step is associated with the conversion of the primary transcript to an intermediate and is independent of sequence and the particular processing pathway of the transcript.

INTRODUCTION

Primary transcripts of eukaryotic tRNA genes undergo a number of processing reactions prior to the appearance of mature tRNA in the cytoplasm. These reactions include the removal of sequences external to both the 5' and 3' ends of the mature tRNA, nucleotide modifications and addition of CCA to the mature 3' terminus (1-5). In addition, a subset of tRNA gene transcripts requires the removal of intervening sequences subsequent to terminal maturation (4-6). The 5' and 3' termini of tRNA molecules appear to result from specific endonucleolytic cleavages of the primary transcript (7-11). The excision of 5' leader sequences is catalyzed by RNase P, an endonuclease most extensively studied in *E. coli* (reviewed in ref. 12), but also found in a variety of eukaryotes including HeLa cells (11-17). Enzymatic activities responsible for the maturation of 3' termini have been purified to varying degrees from several eukaryotic sources (see refs. 1,7,10,). The most extensively characterized of these activities, the 3' pre-tRNase from *Xenopus laevis* ovaries, displays a strict substrate requirement for tRNA precursor molecules from which the 5' leader sequence has already been removed (10). Processing activities present in *Drosophila* Kc cell and human KB cell extracts also exhibit this substrate specificity (7,18). To our knowledge, all published analyses of monomeric eukaryotic tRNA precursor processing, in which the pathway has been determined unambiguously, indicate that the 5' leader sequence is excised before the 3' trailer sequence. Exceptions to this are seen for some, but not all

(6), multimeric tRNA transcripts (19,20) and for transcripts from a tRNA gene mutated *in vitro* in which sequence complementarity extends from the amino acid acceptor stem into the leader and trailer sequences (21). A major question in regard to tRNA processing is thus whether the pathway in which the 5' leader sequence is excised first is obligatory for wild type monomeric transcripts. In addition, there are relatively little data regarding the relative rates of processing of transcripts of different sequence, although some of the sequence requirements for efficient processing have been analyzed for genes mutated *in vitro* (21-27).

In this paper we address issues relevant to an understanding of the mechanisms of tRNA biosynthesis by analyzing in detail the pathways and kinetics of processing of four different mouse tRNA gene transcripts. We show that some transcripts are processed via a pathway in which the 3' trailer sequence is excised before the 5' leader sequence. In addition, a mathematical analysis of processing kinetics reveals the existence of a common rate-limiting step, the rate of which is independent of sequence or pathway for three of the four transcripts tested.

MATERIALS AND METHODS

Preparation of Transcription Templates

λ clones containing tRNA genes were isolated from a mouse recombinant DNA library. The tRNA genes and flanking regions were subcloned into phage M13mp7 or M13mp9 vectors and sequenced as described (28,29). The M13 subclones used in this paper are as follows: mAsp has a 510 bp Sma I insert containing a tRNA^{Asp} gene, mGly has a 141 bp Nar I insert containing a tRNA^{Gly} gene, mGlu has a 1120 bp Alu I insert containing a tRNA^{Glu} gene, mHis has a 296 bp Sau 3A insert containing a tRNA^{His} gene. Subclones mAsp, mGly and mGlu were derived from the mouse DNA insert of the recombinant λ phage Mt2 (28). Subclone mHis was derived from λ phage Mt1 (29). Supercoiled RF I DNA was isolated from a cleared lysate and further purified by CsCl density gradient centrifugation (30).

Transcription and Processing Assays

HeLa cell S100 extracts were prepared by the method of Weil et. al. (31) as modified by Talking-ton et. al. (32). Freezing and thawing of the cell lysate prior to high speed centrifugation (32) substantially and reproducibly increased the processing activities in the S100 extract. Optimal transcription was obtained for each clone at 0.8 μ g DNA/ 25 μ l reaction, containing 10 mM HEPES (pH 7.9), 60 mM KCl, 1.5-2.0 mM MgCl₂, 12% glycerol, 500 μ M each ATP, CTP, UTP, 25 μ M GTP and 2.5 mM creatine phosphate. Analytical transcription reactions were performed in a volume of 25 μ l containing 10 μ Ci of [α -³²P]GTP at 30°C. Preparative scale transcriptions were performed in a volume of 75 μ l containing 100 μ Ci of [α -³²P]GTP. Non-radioactive tRNA transcripts were synthesized under preparative conditions with 500 μ M GTP.

RNA transcripts were isolated by phenol extraction in the presence of 8M urea followed by ethanol precipitation (33). The transcripts were resolved on 12% polyacrylamide gels (20 $\times$ 20 $\times$ 0.4 mm) containing 7M urea and TBE buffer (34), visualized by autoradiography of the dried gel, excised and

quantitated by liquid scintillation counting. Equivalent areas above and below the bands were also excised and counted to determine background.

In experiments in which purified tRNA gene transcripts were added exogenously to the S100 extract, processing was performed using analytical scale transcription conditions (unless otherwise noted) except that DNA template and [α - 32 P]GTP were not present. RNA endogenous to the S100 extract, used as controls in Fig. 6, was isolated from mock transcription reactions containing no DNA template after incubation for 40 minutes at 30°C.

RNA Isolation and Sequence Analysis

RNA transcripts run on polyacrylamide gels were isolated by the crush and soak method in 50 mM Tris (pH 8.0), 300 mM NaCl, 1mM EDTA, 1% phenol followed by phenol extraction and ethanol precipitation (7). Nonradioactive RNA transcripts were located by co-migration of radioactive markers. Further purification was performed by chromatography on Whatman CF11 cellulose (H. Robertson, personal communication).

RNA transcripts synthesized *in vitro* with [α - 32 P]-GTP were digested with RNase T_1 and fingerprinted according to the procedure of Volckaert and Fiers (35) but using Homomix C15 according to Barrell (36) in the second dimension. The identity of each oligonucleotide was inferred from its relative position in the fingerprint (37) and the known DNA sequence and, in the case of mHis transcripts, by comparison with a published T_1 fingerprint of tRNA^{His} (38). The presence or absence of large T_1 generated oligonucleotides specific to the 3' trailer sequences in mGlu and mHis transcripts was confirmed by electrophoresis in 20% polyacrylamide gels containing 8 M urea (39) or by one dimensional homochromatography on PEI-cellulose plates (35). Nucleoside 5'-triphosphate 3'-monophosphates were identified in transcripts digested with RNases A, T_1 and T_2 by TLC on PEI-cellulose plates (Polygram CEL 300 PEI, Brinkmann Instruments Co.) developed in 0.75 M potassium phosphate, pH 3.5 (7).

Mathematical Analysis of Processing Kinetics

As shown in Results and by others (e.g. refs. 6,7,18), the sequential maturation of tRNA termini occurs via a pathway in which the primary transcript is processed through an intermediate form to yield a final tRNA-sized molecule. In its simplest form the processing pathway can be considered to consist of two consecutive first-order reactions, represented by $A \xrightarrow{k_1} B \xrightarrow{k_2} C$, in which A is the primary transcript, B, the intermediate and C, the fully processed transcript. The behavior of reactants and products in this pathway can be described by the following rate equations:

$$\frac{d(A)}{dt} = -k_1(A) \quad (1)$$

$$\frac{d(B)}{dt} = k_1(A) - k_2(B) \quad (2)$$

$$\frac{d(C)}{dt} = k_2(B) \quad (3)$$

Figure 1. In vitro transcription products of four mouse tRNA genes.

Each tRNA gene was transcribed under analytical conditions for 60 minutes at 30°C (see Materials and Methods). Lanes: a, mAsp; b, mAsp + 5 µg/ml α-Amanitin; c, mAsp + 300 µg/ml α-Amanitin; d, mGly; e, mGly + 5 µg/ml α-Amanitin; f, mGly + 300 µg/ml α-Amanitin; g, mGlu; h, mGlu + 5 µg/ml α-Amanitin; i, mGlu + 300 µg/ml α-Amanitin; j, mHis; k, mHis + 5 µg/ml α-Amanitin; l, mHis + 300 µg/ml α-Amanitin; m, M13mp7 (vector DNA).

By solving each rate equation, the amount of A, B or C at any time (t) is expressed by:

$$(A) = (A)_0 e^{-k_1 t} \quad (4)$$

$$(B) = \frac{k_1(A)_0}{k_2 - k_1} \begin{bmatrix} e^{-k_1 t} & -e^{-k_2 t} \end{bmatrix} \quad (5)$$

$$(C) = (A)_0 \left[1 + \frac{1}{k_1 - k_2} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right] \quad (6)$$

where $(A)_0$ is the amount of (A) at $t = 0$. If reactant A is being synthesized at a constant rate such that $d(A)/dt = k_T$ and simultaneously undergoing conversion to B , then in equations 4, 5 and 6, $(A)_0$ can be replaced by $(k_T t^*)$ where k_T is the rate of synthesis and t^* is the time of *de novo* synthesis. This

transforms equation 6 into:

$$(C) = k_1 t^* \left[1 + \frac{1}{k_1 - k_2} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right] \quad (7)$$

This equation is used to describe mature tRNA accumulation during transcription (see Results).

Computerized curve fitting and error analyses were performed using the COTFIT program (40,41) on a Vax-11/780 computer (Digital). Function 20 of COTFIT, used to evaluate the data, was generously written by Dr. Robert F. Murphy (Carnegie-Mellon University).

RESULTS

Transcription of Four Mouse tRNA Genes in Vitro

We have previously described the isolation and sequence analysis of mouse genes encoding tRNA^{Asp}, tRNA^{Gly}, tRNA^{Glu} (28) and tRNA^{His} (29). The M13 subclones containing these genes are designated mAsp, mGly, mGlu and mHis, respectively, as described in Materials and Methods. As shown in Fig. 1, each clone yields a distinctive pattern of transcripts in a HeLa cell S100 extract. Transcription is completely inhibited by 300 µg/ml α-Amanitin (Fig. 1, lanes c,f,i,l) but is relatively unaffected by 5 µg/ml α-Amanitin (lanes b,e,h,k), indicating that the genes are transcribed by RNA polymerase III (42). M13 vector DNA alone does not support the synthesis of any discernable transcripts (lane m).

Each gene produces three different sized transcripts (or groups of transcripts), as indicated in Fig. 1. The size of the largest RNA species (RNA 1) from each gene is approximately 96 (mAsp), 93 (mGly), 148 (mGlu) and 110 (mHis) nucleotides, respectively, as judged by electrophoretic mobility in comparison to a DNA sequencing ladder. All RNA 3 species are between 72 and 75 nucleotides in length, as expected for fully processed tRNA transcripts. The intermediate sized transcripts labelled RNA 2 appear only after production of RNA 1 and remain in relatively low abundance during transcription, suggesting that the RNA 2 molecules are processing intermediates (see also the sequence analysis, below).

In experiments in which the transcription of each gene was stopped after 30 minutes by addition of α-Amanitin and processing allowed to continue during further incubation, RNA 1 of each gene disappears and a transient increase in the amount of each RNA 2 occurs. Incubation for 120 minutes results in the loss of RNAs 1 and 2 and the concomitant accumulation of RNA 3. An exception to this involves the long mGlu RNA 1 transcript. This transcript is processed at an intrinsically slower rate than the others, requiring 5 hours to be fully chased into the RNA 3 band. RNA 3 does not chase into a smaller band (data not shown). From these results, we conclude that each gene is transcribed to produce RNA 1 which is processed through an intermediate form (RNA 2) to mature sized tRNA (RNA 3).

Sequence Analysis of in Vitro Transcripts

The RNA transcripts described above were synthesized in preparative scale reactions containing [α-³²P]GTP, isolated and characterized by RNA fingerprint analysis as described in Materials and

Figure 2. Fingerprint analysis of mHis transcripts.

mHis RNA 1 (panel A), RNA 2 (panel B) and RNA 3 (panel C), synthesized using [α - 32 P]GTP, were digested with RNase T_1 and fingerprinted. The T_1 products are aligned with the mHis RNA 1 sequence in panel D. The residues in bold print correspond to the mHis tRNA coding region.

Methods. The fingerprints of mHis transcripts are shown in Fig. 2. The fingerprint of mHis RNA 1 (Fig. 2A), was compared with the pattern of T_1 generated oligonucleotides predicted from the DNA sequence of mHis. All of the oligonucleotides of RNA 1 can be identified and positioned unambiguously in and around the tRNA^{His} coding region. The primary structure of mHis RNA 1 is shown in Fig. 2D. Examination of the fingerprint of RNA 1 reveals the presence of two T_1 oligonucleotides, 8 and 17, which extend past the 5' and 3' ends of the tRNA coding region (Fig. 2A). Oligonucleotide 8 has the sequence UCCG and is diagnostic for the presence of the 5' leader sequence. The G residue of oligonucleotide 8 is the first residue of the tRNA^{His} coding region (see Fig. 2D). Oligonucleotide 17 has the sequence CAUUAUCCUCUG and is diagnostic for the presence of the 3' trailer sequence.

The CA residues at the 5' end of oligonucleotide 17 are the last two residues of the tRNA^{His} coding region. That initiation of transcription occurs with pppA at position -12 (relative to the start of the tRNA^{His} coding region) was determined as follows. The relative molar yield of oligonucleotide 4 from RNA 1 indicates the presence of several UG fragments in addition to those in the coding region. These can only come from the 5' leader sequence at positions -6, -8, and -10 (in the DNA sequence), placing the initiation point 5' to position -10. Upon digestion of [α -³²P]GTP labelled RNA 1 to mononucleotides, analysis of the mixture by PEI-TLC reveals radioactively labelled pppAp (data not shown), indicating that the transcript starts with pppApGp. This sequence only occurs in the 5' flanking region of the gene at position -12, fixing initiation at that point. Transcription termination appears to occur in the stretch of 6 T residues beginning at position +88, a RNA polymerase III terminator (43). The oligonucleotide UCACUUUUUG, which would be generated by *T*₁ digestion of a transcript continuing past this point, is absent in the fingerprints.

The fingerprint of the intermediate transcript, mHis RNA 2, is shown in Fig. 2B. Oligonucleotide 8 (UCCG) is present, indicating that RNA 2 still retains the 5' leader sequence. This is corroborated by the continued relative excess of oligonucleotide 4 (UG). In contrast, oligonucleotide 17 (CAUUAUCCUCUG) is absent, indicating the loss of the 3' trailer sequence.

The fingerprint of mHis RNA 3 (Fig. 2C) lacks both oligonucleotides 8 and 17 and appears to be identical to a published fingerprint of mammalian tRNA^{His} (38). We conclude from these results that excision of the 3' trailer from the primary transcript (RNA 1) generates an intermediate (RNA 2) which, upon excision of the 5' leader, yields mature sized tRNA. Thus, during the maturation of tRNA^{His}, processing of the 3' end preceeds that of the 5' end.

The fingerprints of mGly transcripts are shown in Fig. 3. The *T*₁ generated oligonucleotides identified in the fingerprint of mGly RNA 1 (Fig. 3A), match those predicted from the DNA sequence in and around the tRNA^{Gly} coding region. The primary structure of mGly RNA 1 is shown in Fig. 3D. Oligonucleotide 5 (CAG) spans both the 5' and 3' termini of the coding sequence. The relative molar yield of this fragment decreases incrementally from RNA 1 to RNA 2 to RNA 3 (compare the difference in intensity between oligonucleotides 5 and 6 in Fig. 3, panels A, B and C), indicating the structure of RNA 1 extends past both these points. This, and the absence of the oligonucleotide UCCCAG, which is found between positions -8 and -13 in the 5' flanking region of the gene, indicates transcription initiates between positions -2 and -13 from the 5' end of the coding region. TLC analysis of [α -³²P]GTP labelled RNA 1 digested to mononucleotides shows two initiating nucleotides, pppAp and pppGp (data not shown). The use of two initiation sites explains the somewhat heterogeneous size of mGly RNA 1 apparent in Fig. 1. The only possible site of initiation with pppAp between positions -2 and -13 is position -9. Initiation with pppGp could occur at positions -3 or -6 if we impose the constraint that the initiating purine is preceded by a pyrimidine, as is the case for other tRNA transcripts (see ref. 44). The 3' end of RNA 1 terminates in a stretch of 4 T residues which starts 12 bp downstream from the tRNA coding sequence at position +84. This is indicated by the presence of oligonucleotide 12 (CCCACG), which is characteristic of the 3' trailer, and by the absence

Figure 3. Fingerprint analysis of mGly transcripts. mGly RNA 1 (panel A), RNA 2 (panel B) and RNA 3 (panel C), synthesized using [α - 32 P]GTP, were digested with RNase T_1 and fingerprinted. The T_1 products are aligned with the mGly RNA 1 sequence in panel D, (parentheses surround those nucleotides present only in the RNA 1 transcripts initiating with pppA). The residues in bold print correspond to the mGly tRNA coding region.

of the oligonucleotide UUUUG, which would be encoded by the terminator sequence.

The fingerprint of the intermediate transcript, mGly RNA 2, is shown in Fig. 3B. The decrease in relative intensity of oligonucleotide 5 (CAG) indicates that at least one of the flanking regions has been removed. The presence of oligonucleotide 12 (CCCAAG), which is well resolved from oligonucleotide 13 (CCAACG) in this fingerprint (4B), demonstrates that RNA 2 still retains the 3' trailer sequence. In contrast, TLC analysis reveals the absence of both initiating nucleotides, indicating the loss of the 5' leader sequence (data not shown). Therefore, RNA 2 is generated by excision of the 5' leader sequence from the primary transcript. This means that the heterogeneous size of mGly RNA 2 (see Fig. 1, lane d) is not due to the two different initiation events. TLC analysis shows that neither the larger nor smaller transcripts of RNA 2 contain the initiating mononucleotides. Also, T_1 generated oligonucleotides from either RNA 2 transcript produce the same fingerprint (data not shown). We therefore assume that the size heterogeneity of mGly RNA 2 is due to a variable number of 3' terminal uridylate residues and/or base modifications or alternative tertiary structures which affect the transcript's electrophoretic mobility.

Figure 4. Kinetics of synthesis and processing of mGly transcripts.

The reaction contained 0.8 μ g mGly DNA in a total volume of 50 μ l. Aliquots (5 μ l) were taken at the times indicated, run on 7 M urea gels and the precursor and tRNA bands quantitated as described in Materials and Methods.

A. Synthesis and accumulation of mGly transcripts. Total transcripts, X; precursor transcripts, ■; tRNA sized transcripts, □.

B. Semi-logarithmic plot of the accumulation of mature tRNA^{Gly} from the data in panel A. The curve through the points was generated by COTFIT as the best fit solution to Eq. 7 (see Results). Transcription rate = 40.0 cpm/min.

The fingerprint of mGly RNA 3 (Fig. 3C) shows that the 3' trailer sequence has been removed (as indicated by the absence of oligonucleotide 12) as well as the 5' leader sequence (TLC analysis not shown). From these results we conclude that excision of the 5' leader from the primary transcript

(RNA 1) generates an intermediate (RNA 2) from which the 3' trailer is excised to yield mature sized tRNA (RNA 3). Thus, during the maturation of tRNA^{Gly}, processing of the 5' end preceeds that of the 3' end.

Analysis of Processing Kinetics

In its simplest form the synthesis and terminal maturation of a tRNA transcript can be represented by:

where A is the primary transcript, B, an intermediate and C, the mature tRNA molecule. In this expression, k_T is the transcription rate constant and k_1 and k_2 are processing rate constants. As described in Materials and Methods, rate equations describing the kinetics of this pathway can be derived and their solution yields equations that allow the accumulation of A, B or C to be expressed as a function of time. Thus, the amount of mature tRNA species (C) at any time (t) during transcription can be expressed by:

$$C = k_T t^* \left[1 + \frac{1}{k_1 - k_2} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right] \quad (7)$$

where t^* is the time of *de novo* synthesis or transcription.

An analysis of the *in vitro* kinetics of tRNA^{Gly} processing is shown in Fig. 4. The accumulation of mGly transcripts is plotted in Fig. 4A. Total transcripts accumulate in a linear fashion for approximately 150 minutes, after which *de novo* synthesis stops. The rate of accumulation of mature sized tRNA begins slowly but then increases with time, suggesting an exponential rate of appearance. Note that after *de novo* synthesis stops (150-180 min.), the rate of accumulation decreases, suggestive of a substrate concentration-dependent reaction.

The kinetic behavior seen in Fig. 4A is consistent with the processing pathway depicted above, which consists of a zero-order synthetic reaction followed by two consecutive first-order processing reactions. To determine whether the data of Fig. 4A are described accurately by Eq. 7, above, we have analyzed the data using the COTFIT program (40,41). COTFIT uses a non-linear least squares, iterative method to determine values for parameters of a function which enable the function to best fit experimental data. In this case, the function is Eq. 7 (COTFIT function 20). The rate of transcription and the time of *de novo* synthesis (parameters k_T and t^* , respectively) are set to experimentally determined values. The processing rate constants, k_1 and k_2 , are calculated by the computer program.

A description of the accumulation of mature tRNA^{Gly} by Eq. 7 is shown in Fig. 4B. Using k_T and t^* derived from the data in 4A, COTFIT generated a curve that fits the measured accumulation of mature sized tRNA. The overall RMS error of the fit is 6% (chi square value/Ymean=0.1063, 4 parameters, 9 degrees of freedom). Equation 7 provides a good fit to the data and, therefore, accurately describes the kinetics of tRNA processing during *in vitro* transcription.

The processing kinetics of precursor transcripts from all four tRNA genes were analyzed by

Table I. Transcription and Processing Rate Constants

	k_T^a	k_1^b	k_2^b
mAsp	366	0.00811	0.959
mGly	166	0.00613	0.909
mGlu	83	0.00250	1.490
mHis	66	0.00788	0.313

^aExperimentally determined transcription rate (cpm/min).^bProcessing rate constants (min^{-1}) calculated by the COTFIT program.

COTFIT. In parallel reactions, transcripts of mAsp, mGly, mGlu and mHis were synthesized and processed. Using the transcription rates measured in each reaction, curves were generated from Eq. 7 which best fit the data obtained from each reaction and values for the processing rate constants of each transcript were generated (the 5 minute lag in synthesis normally seen in the beginning of transcription has been taken into account). The computer-generated values for the processing rate constants (k_1 and k_2) are given in Table I. The smaller of the two rate constants (k_1) describes the rate-limiting step of the pathway and is the critical parameter with regard to determining the shape and fit of the curve.

The rate-limiting values of k_1 for mAsp, mGly and mHis transcripts are quite similar (0.00737 ± 0.00088). This suggests that the rates of processing of mAsp, mGly and mHis transcripts are equivalent. Thus, any relative differences in the accumulation of mature sized tRNA from these three genes in the *in vitro* system are due solely to differences in the concentrations of unprocessed transcripts (as determined by transcription rates).

The rate-limiting value of k_1 for mGlu transcripts (0.0025) is significantly lower than those of the other three tRNAs; mGlu transcripts exhibit an intrinsically slower rate of processing regardless of the concentration of unprocessed transcripts. This had also been noticed in α -Amanitin pulse-chase experiments (as discussed above).

It is important to note that for all four gene transcripts the rate-limiting processing step involves k_1 (and not k_2). Although the k_1 and k_2 values for each tRNA transcript are interchangeable in Eqs. 6 and 7, they cannot be exchanged for each other in Eqs. 4 and 5 (see Materials and Methods). The rate-limiting value in each set of processing rate constants must be used as k_1 in Eqs. 4 and 5 to derive values for (A) and (B) which are non-negative and increase with time (calculations not shown). This point is corroborated by the fact that the slow rate of mGlu transcript processing is due to the persistence of the primary transcript, mGlu RNA 1, not the intermediate transcript.

The experimental data presented in Fig. 5 confirms the fact that processing is a substrate concentration-dependent reaction. Precursor tRNAs were synthesized *in vitro*, purified, added back to the S100 extract and the rate of processing determined as a function of the initial concentration of precursor. The results show that the relationship between the processing rate and precursor concentration is linear for mAsp, mGly and mHis transcripts, indicating a first-order rate-limiting step. Furthermore,

Figure 5. Effect of tRNA precursor concentration on rate of processing.

Primary transcripts were synthesized with [α - 32 P]GTP from mAsp, mGly and mHis templates under preparative conditions and then gel-purified (see Materials and Methods). Specific amounts of transcripts were added to processing reactions and incubated for 60 minutes at 30°C. The tRNA processing rate is calculated as the increase of radioactivity in the mature tRNA band per 60 minutes and is plotted against the amount of precursor originally added to the reaction. tRNA^{Asp}, $\circ$; tRNA^{Gly}, $\square$; tRNA^{His}, $\diamond$.

data for all three transcripts fall on the same line, indicating that the processing kinetics of the three tRNAs are indeed very similar.

Competitive Inhibition of Processing

The finding that the rate-limiting steps of processing of mAsp, mGly and mHis transcripts have very similar rate constants suggests that the processing pathways of these tRNAs share at least one common component or reaction. If so, unlabelled precursors of one tRNA could competitively inhibit the processing of a different tRNA precursor. The experiment in Fig. 6 demonstrates this. Isolated precursor transcripts of tRNA^{Gly} (synthesized with [α - 32 P]GTP) were incubated for 40 minutes in standard processing reactions in the presence or absence of various forms of gel-purified unlabelled RNA. Incubation in the absence of any additional RNA results in the conversion of a significant amount of precursor tRNA^{Gly} (lane a) to the mature sized form (lane b). In contrast, the addition of a 5-fold excess of unlabelled precursor tRNA^{Gly} to the reaction inhibits the formation of mature sized tRNA^{Gly} (lane c). This result can also be obtained by addition of a 5-fold excess of unlabelled precursor tRNA^{His} to the reaction (lane d). Addition of an equivalent amount of RNA endogenous to the S100 extract that co-purifies with the tRNA precursors does not affect the appearance of mature sized tRNA^{Gly} (lane e). An equivalent amount of RNA in the S100 extract that co-purifies with mature tRNA is also not inhibitory (lane f). This indicates that the inhibition seen in lanes c and d is not caused by the endogenous RNA in the S100 extract. Identical results are obtained when pre-tRNA^{His}

Figure 6. Competitive inhibition of tRNA^{Gly} processing.

Non-radioactive RNAs were synthesized for 40 minutes in preparative scale reactions containing mGly DNA or mHis DNA. Precursor (PT) and mature sized (MT) tRNAs were gel-purified. RNAs from mock transcription reactions were also gel-purified (see Materials and Methods). [³²P]Precursor tRNA was synthesized in preparative scale reactions containing mGly DNA and [α -³²P]GTP and isolated by phenol extraction and ethanol precipitation. All reactions contained one-fifth the amount of [³²P]Pre-tRNA^{Gly} isolated from one preparative scale reaction. The reactions in lanes c-f contained the entire amount of precursor (PT) or mature sized (MT) tRNA in one preparative scale reaction (the amount added was adjusted to reflect a material loss of 40% during purification as judged by the recovery of ³²P-tRNA purified in parallel isolation procedures). Each reaction was 12.5 μ l and was incubated for 0 minutes (lane a) or 40 minutes (lanes b-f) at 30°C.

Each lane contains [³²P]pre-tRNA^{Gly} and: a, No incubation; b, No additional RNA; c, mGly PT; d, mHis PT; e, mock PT (S100); f, mock MT (S100).

is used as the ³²P-labelled tRNA precursor rather than pre-tRNA^{Gly} (data not shown). These results show that the precursors of different tRNAs compete for some component(s) necessary for their maturation in the HeLa cell S100 extract.

DISCUSSION

Processing Pathways

The data reported here for tRNA^{His} provide the first unambiguous evidence to our knowledge that the 3' trailer sequences of some monomeric eukaryotic tRNA gene transcripts are excised before the 5' leader sequences. Preliminary fingerprinting data indicate that this pathway is also followed by tRNA^{Glu} transcripts (data not shown). Thus this pathway is not unique to tRNA^{His}. Other studies (7,11,18) which have examined the processing of monomeric human, *Drosophila* and *Bombyx mori* tRNAs in homologous and heterologous extracts revealed only the pathway in which the 5' leader sequence is removed first (as is also shown here for mouse tRNA^{Gly} transcripts). The only reported deviations from this order of processing involve multimeric tRNA transcripts (19,20) or mutant transcripts with abnormal secondary structures (21). From inspection of the pre-tRNA^{His} and pre-tRNA^{Glu} sequences, we cannot identify any particular motifs that could influence the order of processing in any obvious way. Studies utilizing point mutants of the tRNA genes may identify sequences that influence the choice of processing pathways, although such studies to date have only identified mutants which abolish processing or diminish its rate (18,23,25-27).

Castano et al. have recently isolated an endonuclease from *Xenopus* ovaries that excises the 3' trailer sequences from human tRNA^{Met}_I and *Bombyx mori* tRNA^{Ala} transcripts (10). The substrate for this enzyme is a tRNA transcript from which the 5' leader sequence has already been removed; the enzyme does not excise the 3' trailer sequence from primary transcripts of either gene. Activities with similar characteristics have been reported in extracts of human KB cells (18) and *Drosophila* Kc cells (7). These findings suggest that the order of 5' and 3' terminal maturation could be determined by the substrate specificity of the 3' processing nuclease. However, since the tRNA primary transcripts used in the studies by Castano et al. only undergo processing of the 5' end prior to the 3' end (8,18), it will be of interest to determine whether this nuclease can also participate in the processing of tRNA^{His} and tRNA^{Glu} primary transcripts in which the 3' trailer sequence is removed first. If it is not active on these transcripts, then another type of 3' processing nuclease must be present in HeLa cell extracts.

Kinetics of Processing

We show here that the kinetics of synthesis and processing of tRNA gene transcripts can be described quite accurately by an equation that describes a pathway consisting of a zero-order synthetic reaction followed by two consecutive first-order processing reactions. Using this simplified approach, our data suggest that the processing pathway contains a rate-limiting step which has a similar rate constant for three of the four transcripts. In addition, the equation predicts, and the data of Fig. 5 confirm, that for tRNA^{Asp}, tRNA^{Gly} and tRNA^{His}, the rate of processing depends on the concentration of tRNA precursor and is relatively independent of the sequence of the transcripts.

That there can be some effect of sequence on the processing rate is indicated by the fact that tRNA^{Glu} transcripts are processed more slowly than those of the other genes. Inhibitory residues could be present in the tRNA^{Glu} sequence itself. One possible candidate is the U residue at position 61 in the T stem of tRNA^{Glu} (28). A C→T transition at the analogous position in human tRNA^{Met}_I reduces

processing by approximately 50% (27). Alternatively, the mGlu primary transcript may be processed slowly because it contains an unusually long 3' trailer sequence. The transcription termination site of mGlu is 62 bp downstream from the 3' end of the coding region. A survey of 69 eucaryotic tRNA genes shows that termination sites are, on average, 9 bp from the 3' end of the coding region. We have found that long transcripts derived from several tRNA genes from which the normal termination sites have been deleted are processed more slowly *in vitro* than are their normal counterparts (R. Rooney and J. Harding, unpublished). Point mutagenesis at position 61 and deletion mutagenesis of the 3' trailer sequence should resolve this issue.

The similarity of the constant for the rate-limiting processing step for tRNA^{Asp}, tRNA^{Gly} and tRNA^{His} transcripts implies that the processing pathways contain at least one common event or component. The ability of precursor tRNA^{His} transcripts to inhibit the processing of tRNA^{Gly} primary transcripts (Fig. 9) supports this contention. However, the experiments do not prove that pre-tRNA^{His} is actually inhibiting the rate-limiting step revealed by the mathematical analysis of the processing kinetics.

Our analysis suggests that the rate-limiting step is associated with some aspect of conversion of the primary transcript to an intermediate rather than conversion of the intermediate to the mature tRNA. This assertion follows from the mathematical analysis of the processing kinetics (see Materials and Methods and Results) and is exemplified by the refractory nature of mGlu primary transcripts to precursor processing (see Results). A major issue is the identity of this step. In addition, the existence of the rate-limiting step must be reconciled with the fact that different transcripts are processed along different pathways (in regard to the order of 5' and 3' cleavages), as discussed above. One attractive hypothesis which would explain our data is that the rate-limiting step occurs prior to the first endonucleolytic cleavage of the primary transcript. Possibly, the transcript must associate with an additional factor (e.g. the La protein which binds to the 3' oligouridylylate residues of the primary transcript, refs. 45,46) or undergo a particular base modification before a processing nuclease can recognize or interact with it. Alternatively, the 5' and 3' processing enzymes may be present in a complex (although the enzymes clearly can be separated from each other and remain active *in vitro*, refs. 14,18). Once the transcript has bound to the complex (the rate-limiting step) 5' and 3' cuts can perhaps occur rapidly with the particular pathway dictated by the sequence of the transcript.

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