IN VIVO MATURATION OF AN UNDERMODIFIED ESCHERICHIA COLI LEUCINE TRANSFER RNA

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SUMMARY: Two chromatographically unique leucine tRNAs which accumulate during leucine starvation of relaxed control E. coli have been shown to be undermodified forms of the major, normal isoacceptor species, $tRNA_I^{Leu}$. Results from ^{32}P label-chase experiments demonstrate that the undermodified species, deficient in specific dihydrouridine and pseudouridine residues, can be converted to chromatographically normal $tRNA_I^{Leu}$ but, only slowly. The slow rate of conversion suggests that the undermodified isoacceptors are not normal intermediates in the biosynthetic process and provides additional support to the view that certain of the post-transcriptional modification reactions occur in a sequential manner.

INTRODUCTION: It is well documented that changes in the isoacceptor patterns of tRNA can occur under a variety of metabolic conditions including: viral infection, cellular differentiation and alterations in culturing conditions (1-7). In most cases, the biochemical basis for the changes observed is not known, but is believed to be related to the extent of post-transcriptional modification of the tRNA rather than modulation of tRNA gene expression. Modification deficiency has been shown to be the biochemical basis for the changes in the spectra of isoacceptor tRNAs from relaxed control (rel^-) E. coli starved of methionine, threonine or cysteine. These amino acids are involved in the synthesis of methylated nucleosides (8), threonylcarbamoyladenosine (9) and sulfur-containing nucleosides (10) respectively; methionine is also a precursor to 3-(3-amino-3-carboxypropyl) uridine ($4abu^3U$-ref. 11).

Changes in tRNA isoacceptor patterns have also been observed for a number of rel^- tRNAs from bacterial cells starved of amino acids not known to be

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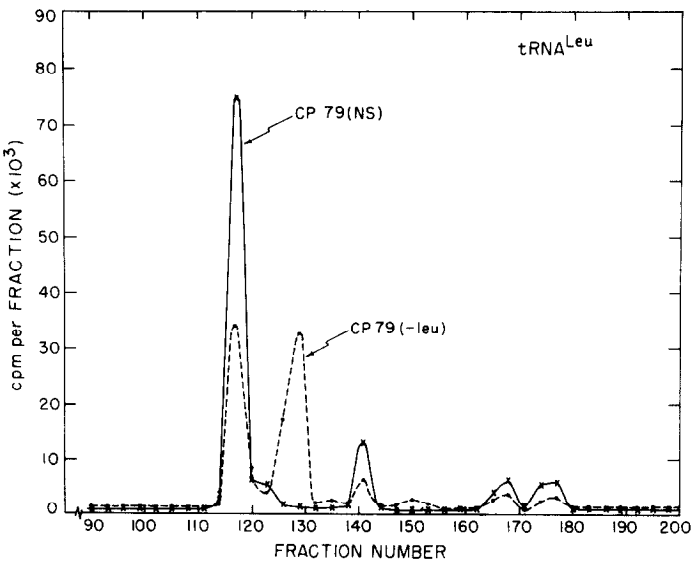

Figure 1. Effect of leucine starvation on the leucine tRNA from relaxed control E. coli. Samples of tRNA from leucine-starved (6 hrs) and nonstarved (mid-log) cultures of E. coli CP79 (arg⁻, his⁻, leu⁻, thr⁻, thi⁻, rel⁻) were aminoacylated with ^{3}H- and ^{14}C-leucine respectively, mixed and analyzed by RPC-5 chromatography. Fractions were precipitated with 5% trichloroacetic acid and the insoluble material collected on Millipore glass fibre filters. The ^{3}H- and ^{14}C-activity profiles were determined by liquid scintillation spectrometry and normalized to the same total activity. (X ——— X) $tRNA^{Leu}$ from nonstarved cells (NS), [^{14}C] leucine; (● - - - ●) $tRNA^{Leu}$ from leucine-starved cells (-leu), [^{3}H] leucine.

involved in tRNA base modification (4,5,7,12) and from cells treated with inhibitors of protein synthesis (2,3,5,12). The effect of leucine starvation on the isoacceptor patterns of $tRNA^{Leu}$ from a rel⁻ strain of E. coli is shown in Fig. 1. Nonstarved cells contain five or six leucine isoacceptors while the tRNA from cells deprived of leucine contains an additional major, chromatographically unique species of $tRNA^{Leu}$ (fractions 123-132). Frequently, a minor, unique $tRNA^{Leu}$ isoacceptor is also observed in the tRNA from starved cells (fractions 148-155). In earlier work, it was determined that the apparently homogeneous major unique species consists of a mixture of subspecies which can be partially resolved by RPC-5 chromatography following partial purification of $tRNA^{Leu}$ (5).

The major unique species of leucine tRNA is also observed in rel⁻ cells

starved of arginine and in cells treated with chloramphenicol or puromycin (5). The unique tRNAs produced under these different culturing conditions are chromatographically similar, suggesting a common biochemical basis for their formation. It was suggested (3-5, 12) that the unique species of tRNA from leucine-starved or chloramphenicol-treated cells were undermodified forms of the normal isoacceptor species, the appearance of which stemmed from the loss of activity of certain of the base modifying enzymes. Direct sequence analyses of the major unique $tRNA^{Leu}$ and $tRNA^{Phe}$ from leucine-starved rel^- cells (13 and Kitchingman and Fournier, manuscripts in preparation) and the unique $tRNA^{Phe}$ from chloramphenicol-treated cells (14) have revealed that the unique species are indeed, modification-deficient forms of the major, normally occurring isoacceptors. Relative to the fully modified tRNAs, the unique $tRNA^{Leu}$ and $tRNA^{Phe}$ species are deficient in specific dihydrouridine and pseudouridine residues and the unique $tRNA^{Phe}$ is additionally deficient in $4abu^3U$ and the thiomethyl moiety of 2-thiomethyl-N^6-(Δ^2-isopentenyl) adenosine (13). While it has been established conclusively that the unique and normal isoacceptors are related, it was not clear from earlier studies whether the hypomodified forms are normal intermediates in the tRNA biosynthetic process.

Suggestive evidence that a true precursor-product relationship exists for the unique and normal species was derived from recovery studies in which the relative amount of the unique species of $tRNA^{Leu}$ and $tRNA^{Phe}$ were observed to decrease upon restoration of leucine to a culture of leucine-starved cells (5) or, in the case of $tRNA^{Phe}$, removal of chloramphenicol from the growth medium (3). The redistribution of isoacceptor species occurred only slowly, however, a fact which could be explained by a slow rate of conversion or, rather, simple dilution of the unique species by newly synthesized, fully modified tRNA. Results from a pulse-chase experiment indicated that the unique rel^- $tRNA^{Phe}$ could be converted to a chromatographically normal form (15).

To determine if the unique $tRNA^{Leu}$ species could be converted to normal $tRNA^{Leu}_I$ and to gain more information about the post-transcriptional modification

of tRNA, we have performed a label-chase experiment in which the unique rel^- tRNAs formed during leucine starvation were labeled with $^{32}PO_4^{3-}$, and the distribution of label in the unique and normal $tRNA^{Leu}$ isoacceptor species determined at various times after restoration of leucine to the starved culture. The results show that the unique species of $tRNA^{Leu}$ can, in fact, be converted *in vivo* to $tRNA_I^{Leu}$ by a two step process but, that the maturation occurs only slowly.

MATERIALS AND METHODS: Materials were obtained from the following sources: benzoylated-DEAE cellulose (BD-cellulose, 50-100 mesh), from Schwarz-Mann; diethylaminoethylcellulose (Whatman DE-52), from Reeve Angel; RPC-5 sorbent was prepared according to Method C of Pearson *et al.* (16); ^{32}P (H_3PO_4) was obtained from New England Nuclear.

Culture and labeling conditions. *E. coli* CP79 (arg^-, his^-, leu^-, thi^-, thr^-, $relA^-$) was the strain used in these studies. Conditions for culturing and amino acid starvation have been described previously (4).

The label-chase experiment was performed as described previously (15). Briefly, twenty millicuries of [^{32}P] H_3PO_4 were added to a 300 ml culture of *E. coli* CP79 in low phosophate (LP) medium 20 to 30 minutes after the onset of leucine starvation. After 6 hours of starvation, the cells were harvested by centrifugation, washed in LP medium, resuspended in 600 ml fresh, fully supplemented medium minus ^{32}P and incubated under normal culturing conditions. At 0, 3.75 and 5.25 hours after resuspension in the fresh medium, one-third of the culture was removed for analysis of the $tRNA^{Leu}$.

Purification of ^{32}P-labeled $tRNA^{Leu}$. Bulk ^{32}P-tRNA was prepared by phenol extraction and chromatography on DEAE-cellulose (4). $tRNA_I^{Leu}$ and the major unique $tRNA^{Leu}$ were purified by electrophoresis through a slab of 10% polyacrylamide gel and chromatography on benzoylated DEAE-cellulose as described by Dube *et al.* (17). Approximately 350 µg of unlabeled bulk tRNA from leucine-starved cells were added in the purification process. The

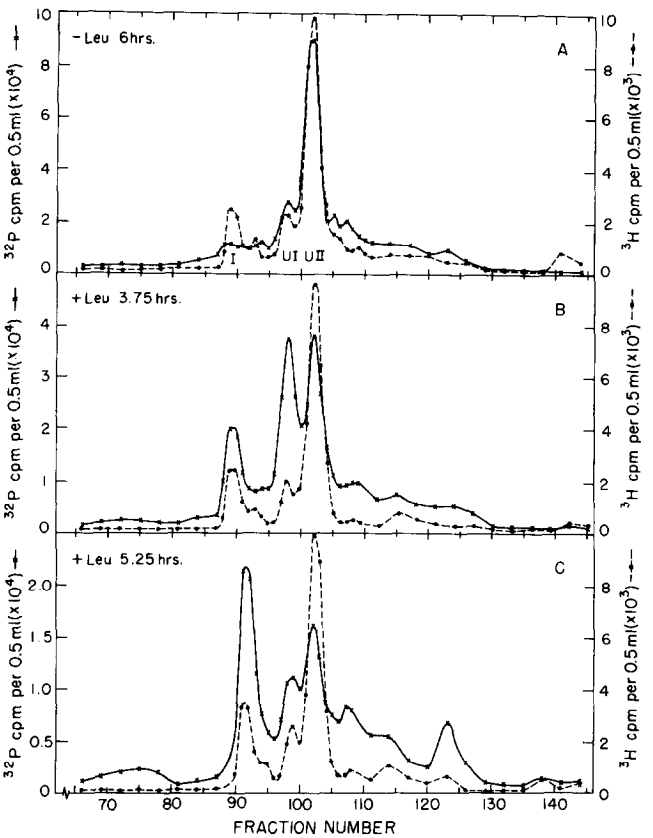

Figure 2. Effect of restoring growth on the unique $tRNA^{Leu}$ produced in rel$^-$ E. coli during leucine starvation. Labeling of the cells with [^{32}P] PO_4^{3-} and purification of $tRNA_U^{Leu}$ and $tRNA_I^{Leu}$ were performed as described in Methods. The $tRNA^{Leu}$ was aminoacylated with [^{3}H] leucine and fractionated by RPC-5 chromatography as described in Fig. 1. Radioactivity in each fraction was determined by counting portions of each fraction in 5 ml Liquifluor (New England Nuclear)/toluene/Triton X-100 (84:916:500) as described previously (5). The resulting RPC-5 elution profiles are shown. (● - - - ●) [^{3}H] leucyl-tRNA; (X ——— X) [^{32}P] tRNA. $tRNA_I^{Leu}$ is identified as species 'I'; the unique tRNA sub-species are designated UI and UII. The material eluting in the region defined by fractions 120-130 corresponds to $tRNA_{III}^{Leu}$ which is co-purified to some extent with $tRNA_I^{Leu}$ and the unique tRNAs in the procedure used here. Analysis of the fingerprint of $tRNA_{III}^{Leu}$ showed that this species is not related to $tRNA_I^{Leu}$ although there are a number of oligonucleotides common to both isoacceptors. Perhaps the label in $tRNA_{III}^{Leu}$ is derived from the minor unique $tRNA^{Leu}$ which elutes between $tRNA_{III}^{Leu}$ and $tRNA_{IV}^{Leu}$ (fractions 147-154) in the profile of total $tRNA^{Leu}$ shown in Fig. 1.

$tRNA^{Leu}$ from BD-cellulose was aminoacylated with [^{3}H] leucine and fractionated by RPC-5 chromatography as described below.

RPC-5 chromatography. tRNA samples to be analyzed by RPC-5 chromatography

were aminoacylated, phenol extracted and prepared for column analysis as described previously (4,5). The sample was applied to a 0.9 x 69 cm column equilibrated in buffer that was 10 mM sodium acetate, pH 4.5, 1 mM disodium EDTA, 10 mM $MgCl_2$ and 0.4 NaCl and eluted with a linear NaCl gradient with limits of 0.5M and 1.2M. Total volume of the gradient was 600 ml; 2 ml fractions were collected. The radioactivity profiles were determined by scintillation spectrometry.

RESULTS AND DISCUSSION: The results of the label chase experiment are shown in Fig. 2. The upper panel shows the RPC-5 elution profile of [^{3}H]-leucyl [^{32}P] $tRNA^{Leu}$ prepared from rel^- cells starved of leucine for six hours. The near-coincidence of the ^{3}H- and ^{32}P-profiles indicates that the $tRNA^{Leu}$ is of relatively high purity. The ^{3}H-profile, due to the presence of added carrier tRNA from leucine-starved cells, reflects the occurrence of at least three isoacceptor species. The earliest eluting species (fractions 87-92) is $tRNA_I^{Leu}$. The major peak of activity (fractions 100-105) corresponds to the major unique $tRNA^{Leu}$ shown in Fig. 1. A second, minor unique $tRNA^{Leu}$ eluted in a position intermediate (fractions 96-99) to that of $tRNA_I^{Leu}$ and the major unique species. The earlier and later eluting unique species are identified as $tRNA_{UI}^{Leu}$ and $tRNA_{UII}^{Leu}$ respectively.

Both $tRNA_{UI}^{Leu}$ and $tRNA_{UII}^{Leu}$ were labeled during the starvation period, accounting for 18% and 73% of the total ^{32}P activity respectively. Little, if any, label was seen in the region of $tRNA_I^{Leu}$ (<10%) indicating that at best, only a small amount of $tRNA_I^{Leu}$ was formed under the starvation condition. Incubation of the starved cells in fresh medium resulted in major changes in the pattern of labeled species. After 3.75 hours, a considerable proportion of the ^{32}P-label (22%) coeluted with $tRNA_I^{Leu}$, indicating that conversion of unique species to a chromatographically normal form had occurred. The relative amount of label in $tRNA_{UI}^{Leu}$ also increased, doubling from 18% to 36%. The increased labeling of $tRNA_I^{Leu}$ and $tRNA_{UI}^{Leu}$ occurred at the expense of $tRNA_{UII}^{Leu}$

which decreased from 72% to 41% of the total. The labeling pattern continued to change between 3.75 and 5.25 hours of recovery with $tRNA_I^{Leu}$ becoming the most highly labeled isoacceptor. Over 42% of the ^{32}P-label was associated with $tRNA_I^{Leu}$, while $tRNA_{UI}^{Leu}$ and $tRNA_{UII}^{Leu}$ accounted for 19% and 39% of the ^{32}P-activity respectively.

The major unique species of $tRNA^{Leu}$ can thus be converted to a form chromatographically indistinguishable from $tRNA_I^{Leu}$. However, as structural analyses of the $tRNA_I^{Leu}$ produced in this experiment were not performed, it cannot be concluded that the ^{32}P-labeled $tRNA_I^{Leu}$ formed was completely modified. The conversion of the unique species to $tRNA_I^{Leu}$ appears to have occurred in at least two steps. The labeling patterns suggest that $tRNA_{UII}^{Leu}$ was converted to UI, then to $tRNA_I^{Leu}$. The results also reveal that a significant proportion of the unique species persisted even after 5.25 hours of recovery. During this time the number of viable cells (estimated at better than 90% of the population after six hours of starvation--ref. 5) increased by 150%.

The slow rate of conversion could be interpreted to mean that the unique species of $tRNA^{Leu}$ are not proper substrates for the modifying enzymes which convert $tRNA_{UII}^{Leu}$ to $tRNA_I^{Leu}$ and that these species may not be normal intermediates in the biosynthesis of $tRNA_I^{Leu}$. Perhaps some of the nine base modification reactions involved in the maturation of $tRNA_I^{Leu}$ occur in an ordered manner, while others can occur at any time during the modification process. If one or more of the ordered modifications occurs out of sequence, the resulting tRNA may not be a good substrate for further modification. Modification of certain bases may be accompanied by subtle changes in tRNA conformation which affect the formation of other modified nucleotides. Another, related explanation would be that certain of the base modification reactions may usually occur at the level of a larger, precursor tRNA and, once cleaved, the mature-sized tRNA is a poor substrate. Yet another possibility is that the hypomodified, unique species mature slowly because they are not readily available for modification owing to compartmentalization of the modifying enzymes.

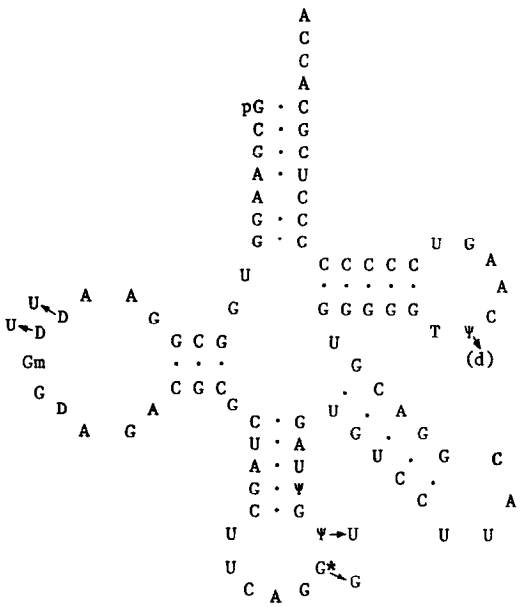

Figure 3. Structure of the major, unique leucine tRNA from rel$^-$ E. coli. The structures of $tRNA_I^{Leu}$ and the major unique $tRNA^{Leu}$ from leucine-starved cultures of E. coli CP79 were determined according to the method of Brownlee and Sanger (18) and Griffin (19) and will be described in detail elsewhere. The structure shown is of $tRNA_I^{Leu}$ (17, 20). The arrows indicate the positions at which the unique rel$^-$ $tRNA^{Leu}$ differs from fully modified $tRNA_I^{Leu}$. (d) indicates a deficiency; the Ψ in the Ψ loop is a mixture of U and Ψ in both the rel$^-$ $tRNA_I^{Leu}$ and unique $tRNA^{Leu}$. G* is an unidentified derivative of G.

Results from sequencing studies to be presented in detail elsewhere (manuscript in preparation) have revealed that the major unique species of $tRNA^{Leu}$ differs from $tRNA_I^{Leu}$ at positions 16, 17, 38 and 39 from the 5' terminus (see Fig. 3). Unmodified uridine (U) occurs in place of dihydrouridines (D) 16 and 17 and pseudouridine (Ψ) 39. The unique $tRNA^{Leu}$ has an unmodified guanosine residue adjacent to the anticodon instead of the modified G which normally occurs at position 38 in fully modified $tRNA_I^{Leu}$. G* 38 and Ψ39 were also partially deficient in our preparations of $tRNA_I^{Leu}$, so the shift in elution position for the unique species is probably due to the absence of the two dihydrouridine residues in the D-loop. Results of oligonucleotide analyses of subfractions of the unique tRNA indicate that the in vivo conversion probably occurs by the formation of D16 first (UII → UI), then D17 (UI → I).

These results provide direct in vivo evidence for a precursor-product relationship for the chromatographically unique species of rel$^-$ tRNA^{Leu} and a normal isoacceptor. As indicated earlier, a similar relationship has been indicated for the major unique and normal species of tRNA^{Phe} from leucine starved rel$^-$ cells (15).

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