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Drosophila RNase Z processes mitochondrial and nuclear pre-tRNA 3′ ends *in vivo*

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ABSTRACT

Although correct tRNA 3′ ends are crucial for protein biosynthesis, generation of mature tRNA 3′ ends in eukaryotes is poorly understood and has so far only been investigated *in vitro*. We report here for the first time that eukaryotic tRNA 3′ end maturation is catalysed by the endonuclease RNase Z *in vivo*. Silencing of the *JhI-1* gene (RNase Z homolog) *in vivo* with RNAi in *Drosophila* S2 cultured cells causes accumulation of nuclear and mitochondrial pre-tRNAs, suggesting that *JhI-1* encodes both forms of the tRNA 3′ endonuclease RNase Z, and establishing its biological role in endonucleolytic tRNA 3′ end processing. In addition our data show that *in vivo* 5′ processing of nuclear and mitochondrial pre-tRNAs occurs before 3′ processing.

INTRODUCTION

Generation of functional tRNA molecules is an essential step in all organisms. Besides their central role in protein biosynthesis, tRNAs have other important functions in the cell, such as targeting proteins for degradation (1). tRNAs are first transcribed as precursors containing additional 5′ and 3′ sequences which have to be removed to yield functional tRNA molecules. Removal of the 5′ leader sequence by the ubiquitous enzyme RNase P has been studied in detail (reviewed in 2). In contrast, we know little about the generation of the mature tRNA 3′ end (reviewed in 3).

Maturation of pre-tRNA 3′ ends in bacteria differs from organism to organism; in *Escherichia coli*, it is a multistep process involving endo- and exonucleases (4), whereas in *Bacillus subtilis* the mature tRNA 3′ end of CCA-less pre-tRNAs is generated by a single endonucleolytic cut, catalysed by RNase Z (E.C. 3.1.26.11) (5). Archaeal tRNA precursors are also processed at the tRNA 3′ end by an RNase Z activity (6,7). Most eukaryotic systems have been reported to employ endonucleases for cleaving pre-tRNAs directly 3′ to the discriminator, although a few exonucleolytic pathways have also been reported (8–20). Exclusively endonucleolytic processing 3′ to the discriminator has been observed in eukaryotic organelles (mitochondria and chloroplasts) (21–27). In mammals and fruit flies the mitochondrial DNA is transcribed into only two long primary transcripts (one for each strand) which contain the tRNA genes interspersed between the different mRNA and rRNA genes (28). Here tRNA processing is thus also vital for releasing functional mRNAs and rRNAs from the primary transcript, as first suggested for human mitochondria (29).

In plants, nuclear RNase Z is not only required for tRNA maturation but also for snoRNA maturation, at least where a snoRNA is encoded downstream from a tRNA on a dicistronic locus (30), in which case the snoRNA is released from the primary transcript through endonucleolytic cleavage by RNase Z.

The recent identification of the first RNase Z protein and gene sequences now allows the identification of orthologous proteins in all three kingdoms: bacteria, archaea and eukarya (7). These orthologous RNase Z proteins belong to a conserved group of proteins, the ELAC1/ELAC2 group (31). The shorter ELAC1 proteins (ranging from 250 to 350 amino acids) can be found in representatives of all three kingdoms, whereas the longer ELAC2 proteins (700–900 amino acids) are exclusively found in eukaryotic organisms.

In *Drosophila melanogaster*, the sole RNase Z ortholog is an ELAC2 protein encoded by *JhI-1*, a juvenile hormone (JH) regulated gene (32). JH serves a number of important functions in insects, such as regulation of moulting during pre-adult development, and control of reproductive maturation and sexual behaviour in adults (33,34). At the cellular level, JH is implicated in cell proliferation and differentiation (35,36). The molecular mechanism of JH action is not yet known, and there have been few genes described so far whose expression is directly affected by JH (37). The *JhI-1* gene is rapidly and specifically induced in *Drosophila* cultured cells in the presence of JH. Accumulation of *JhI-1* transcripts requires the continuous presence of hormone, suggesting direct regulation.

All data published to date concerning the eukaryotic tRNA 3′ processing enzyme RNase Z have been *in vitro* studies. Here we show for the first time that the biological role of eukaryotic RNase Z is indeed tRNA 3′ processing and that the *Drosophila JhI-1* locus encodes the mitochondrial and nuclear tRNA 3′ processing enzyme RNase Z. Furthermore, we find that tRNA 5′ end processing precedes tRNA 3′ end processing *in vivo*.

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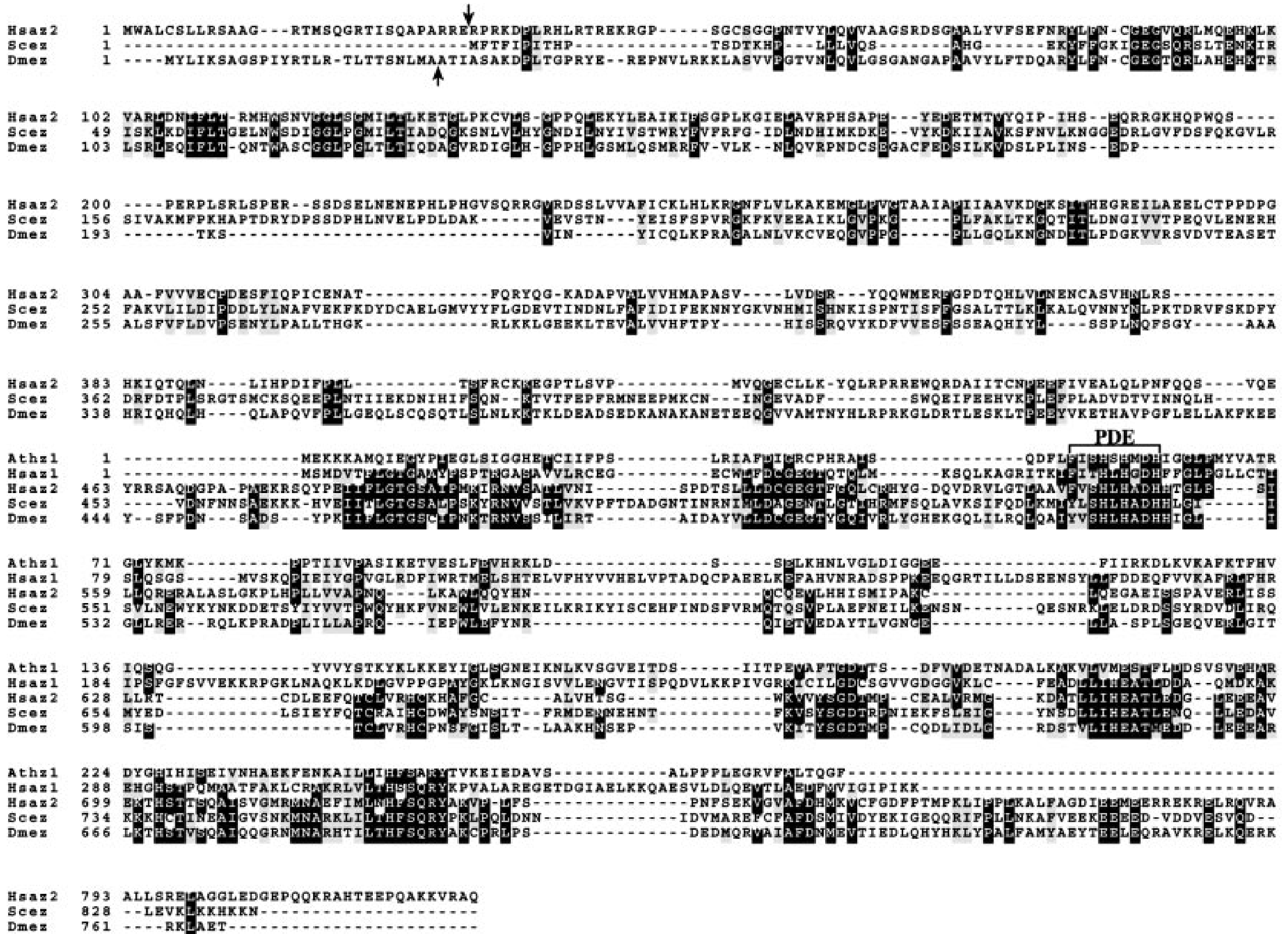

Figure 1. Alignment of ELAC1 and ELAC2 protein sequences. The RNase Z like ELAC1 protein sequences of *A.thaliana* (Athz1, accession no: AAG52354) and *H.sapiens* (Hsaz1, accession no: AAH01939) were aligned with the ELAC2 protein sequences from *S.cerevisiae* (SceZ, accession no: NP_013005), *H.sapiens* (Hsaz2, accession no: AAG24441) and *D.melanogaster* (DmeZ, accession no: AAF99588), to investigate similarities. The PDE domain, which was first described in phosphodiesterases where it is the catalytic domain, is indicated with a line above the amino acid sequences. The predicted cleavage sites for the mitochondrial target sequences for the fruit fly and human ELAC2 proteins are indicated with arrows. No mitochondrial N-terminal target sequence is found in the yeast ELAC2 protein. The *Drosophila* RNase Z contains two nuclear localization signals, ^{405}RPRK^{498} and ^{759}RKRK^{762}. In addition, it contains a second methionine at amino acid 24 close to the predicted cleavage site of the target sequence at amino acid 26.

MATERIALS AND METHODS

Expression of *Drosophila* RNase Z

The *D.melanogaster* homolog of *Arabidopsis thaliana* RNase Z (accession no. AY119279) was amplified from the cDNA (accession no. BI633079) SD27051 (Invitrogen) using the Advantage-HF2 PCR kit (Clontech) (for primer sequences see Supplementary Material Table 1; PCR conditions are available upon request). The PCR fragment generated contains the coding sequence for *Drosophila* RNase Z starting at the second methionine (amino acid 25) to exclude the mitochondrial targeting sequence. The PCR fragment was subcloned into the EcoRI–XhoI sites of *E.coli* expression vector pET32a (Novagen). Accuracy of amplification and subcloning was confirmed by sequencing. Expressed *E.coli* proteins were purified according to the manufacturer's instructions (Novagen). To improve expression, the cDNA was subcloned into the corresponding sites of the baculovirus expression vector pFastbacHTA (Invitrogen) for expression in insect Sf9 cells in serum-free medium. His tag purification of the recombinant enzyme was performed by NP40 lysis of infected cells followed by affinity purification of the soluble expressed protein using Ni-NTA Superflow (Qiagen). Expressed *D.melanogaster* RNase Z (dRNase Z) was stable at –70°C.

Preparation of fruit fly cell culture extracts active in tRNA 3′ processing

Cell culture and extract preparation were performed as described (38); the fraction used for tRNA 3′ processing is the S-Sepharose flow-through.

Generation of pre-tRNA substrates

The nuclear pre-tRNA^{His} template was prepared as described (38). The mitochondrial pre-tRNA^{Ile} template was amplified from *Drosophila* genomic DNA yielding template Fmile. *Drosophila* genomic DNA was isolated from S2 cultured cells

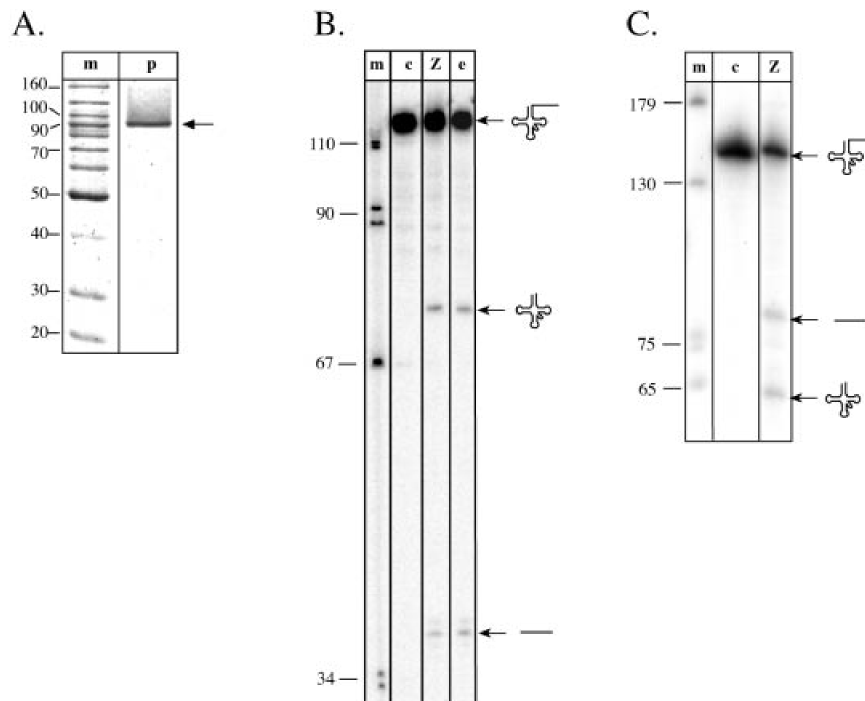

Figure 2. *Drosophila* RNase Z catalyses nuclear and mitochondrial pre-tRNA 3′ processing. (**A**) Expression of *Drosophila* RNase Z; 200 ng of recombinant *Drosophila* RNase Z expressed in insect cells was loaded on a 10% SDS–PAGE (lane p), which was subsequently stained with Sypro Orange (Molecular Probes). According to the molecular weight marker shown at the left in kDa (lane m), dRNase Z (arrow at right) runs at an apparent molecular weight of 92 kDa. (**B**) Processing of a nuclear pre-tRNA by dRNase Z. Nuclear pre-tRNA^{His} was incubated with recombinant RNase Z from *Drosophila* (lane Z) and an RNase Z active fraction from fruit fly extracts (lane e). Lane c: control reaction without addition of proteins (lane c). A DNA size marker is shown at the side in nucleotides. Both protein fractions generated the same reaction products, a mature tRNA (72 nt) and a 3′ trailer (36 nt). (**C**) A mitochondrial pre-tRNA is also processed by dRNase Z. Mitochondrial pre-tRNA^{Ile} was incubated with *Drosophila* RNase Z (lane Z). Lane c: control reaction without proteins, lane m: DNA size marker in nucleotides. The enzyme cleaves the precursor yielding the two products, the tRNA (65 nt) and the 3′ trailer (75 nt).

using DNeasy spin columns (Qiagen) according to the manufacturer's instructions. PCR products were cloned and sequenced to confirm the accuracy of amplification. Pre-tRNA templates were transcribed as described (24,38).

In vitro processing

In vitro processing with fruit fly cell extract was performed as described (38). For processing reactions with recombinant *Drosophila* RNase Z, 10 ng *Drosophila* RNase Z were incubated with substrates in *Drosophila* processing buffer (25 mM Tris–HCl pH 8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 5% glycerol) for 15 min at 28°C.

Cell culture and RNAi treatment

Drosophila S2 cells were cultured in Schneider's medium supplemented with 10% FBS at 25°C. The following primers were used to PCR amplify a fragment encoding JhI-1 (AF215894) protein: 192-5′-GGATCCTTTAACAGGACC-3′-209 and 1338-5′-AAGAGTGCGATCCAGACC-3′-1321. The fragment cloned in both orientations linked to a T7 promoter was used to generate dsRNA with the T7 MEGAscript Kit (Ambion). S2 cells were transfected with 20 µg of dsRNA as described (39).

Northern blot hybridizations

For northern blot analysis of mRNAs (see Fig. 3), RNAs were fractionated on a 1% agarose–formaldehyde gel and transferred to a nylon membrane (MSI). Probes, PCR-labelling and hybridization conditions are described elsewhere (40). For northern blot analysis of tRNAs (see Figs 4–6), RNAs were fractionated on a 6% polyacrylamide, 8 M urea gel and transferred to Zetaprobe nylon membrane (Bio-Rad). Blots were hybridized as described (41). Oligonucleotides used to detect mature and precursor tRNA^{His} (accession no. K02462) were 5′-GAACCTGGGTTACCACGGCCA-3′, as the internal probe; 5′-CCCAACTCCGTGACAATGTTTGTTTCAAC-3′, as the 3′ trailer probe; and 5′-ACGGCTACATCGGGTGAT-3′, as the 5′ leader probe. To detect mitochondrial tRNAs (accession no. NC_001709) four probes were used. Oligonucleotides 5′-TGCAGAAAACTGCATGATTTA-3′ and 5′-TGGTGTATGATGCACAAAAGT-3′ detected mature and precursor tRNA^{Ile}, respectively, and oligonucleotides 5′-ATCGCCTAAACTTCAGCCACT-3′ and 5′-TAATTAATT-TGTCCTTATTTG-3′ detected mature and precursor tRNA^{Tyr}, respectively. All probes were 5′-end-labeled with [γ-^{32}P]ATP (>5000 Ci/mmol, Amersham).

RESULTS AND DISCUSSION

Identification of *Drosophila* RNase Z

The isolation of RNase Z from wheat germ (7) allowed us to identify the sole homologous gene in the *D.melanogaster* genome (Fig. 1). *Drosophila* RNase Z is encoded by the *JhI-1* gene, which was previously identified as JH inducible (32). *Drosophila* RNase Z contains two nuclear localization signals

(405RPRK408 and 759RKRK762); additionally an N-terminal target sequence routes the protein to mitochondria according to two sorting servers (iPSORT, MitoProt). A second methionine residue is present at position 24 close to the predicted cleavage site of the N-terminal target sequence (position 26), suggesting that the *JhI-1* gene may encode both a nuclear and a mitochondrial form of RNase Z. With 766 amino acids, the protein is almost three times as long as the originally identified RNase Z enzymes from the plant *A.thaliana* and the archaeon *Methanococcus janaschii*, and is thus a representative of the long form of RNase Z enzymes (ELAC2 proteins). Interestingly, the long RNase Z variants are only found in eukaryotic organisms and the relative distribution of ELAC1/ELAC2 proteins varies among eukaryotes. *Saccharomyces cerevisiae* and *D.melanogaster* contain only a single RNase Z protein (an ELAC2 protein), while *Homo sapiens* has one ELAC1 and one ELAC2 and *A.thaliana* encodes two ELAC1 and two ELAC2 proteins. ELAC1 and ELAC2 proteins belong to a single group since the C-terminal part of the ELAC2 proteins shows high sequence similarity to the ELAC1 proteins (including the highly conserved PDE domain (Fig. 1) (31). While it has been shown that the ELAC1 proteins and the C-terminal parts of the ELAC2 proteins have tRNA 3′ processing activity (7,42), the function of the long N-terminal domain of the ELAC2 proteins is not clear. Long ELAC2 proteins appear to have evolved from duplication of the short ELAC1 proteins (31). Consistent with this theory, representatives of the ELAC1 proteins [*E.coli* RNase Z (43) and *A.thaliana* RNase Z (B.Späth and A.Marchfelder, unpublished data)] are homodimers, and the yeast ELAC2p N-terminal part (which possesses a slightly altered PDE domain) alone can process tRNAs at their 3′ ends (B.Späth *et al*., in preparation).

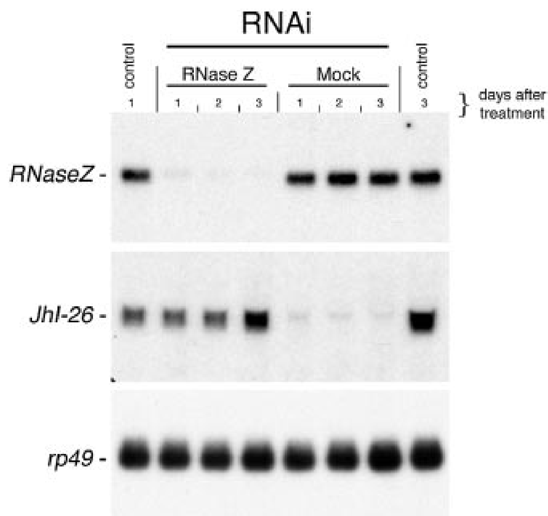

Figure 3. RNAi-mediated silencing of gene expression. S2 cells were transfected with dsRNA specific to *dRNase Z* (RNase Z lanes) or *JhI-26* (Mock lanes). Control cells were non-transfected. After 1, 2 and 3 days of incubation with dsRNA, control and RNAi cells were collected, total RNA was isolated and subjected to northern blot analysis. Hybridization probes are designated on the left. The upper panel shows that dsRNA mediated interference against *dRNase Z* was efficient and specific: *dRNase Z* mRNA abundance was greatly reduced even after 1 day of *dRNase Z* dsRNA treatment, whereas *JhI-26* dsRNA was not inhibitory at all. No RNAi induced cytotoxicity was observed in our experiments.

Recombinant fruit fly RNase Z catalyses 3′ end maturation of nuclear pre-tRNAs

To analyse whether the *JhI-1* gene indeed codes for a tRNA 3′ processing endonuclease, we expressed the cDNA in insect Sf9 cells. The resulting recombinant protein has an apparent

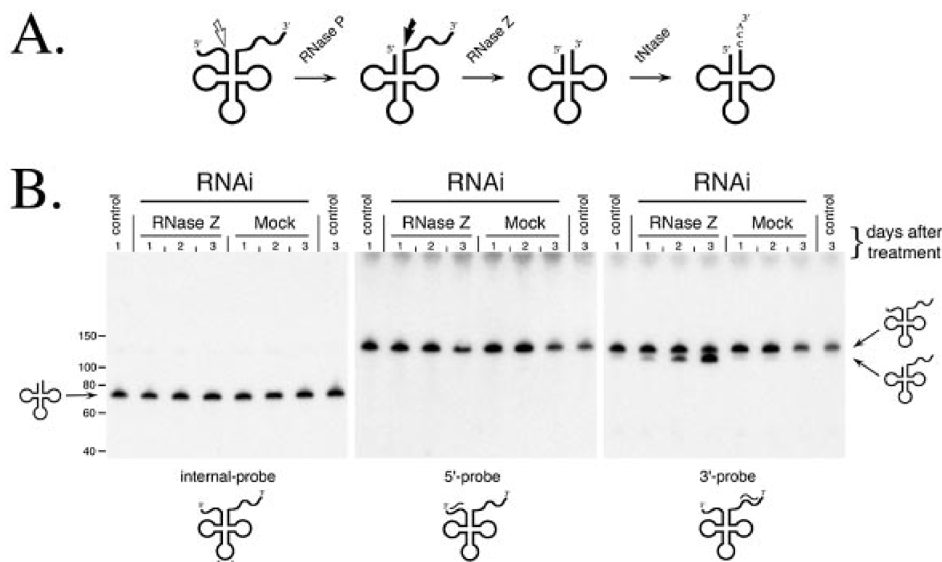

Figure 4. Loss of dRNase Z results in tRNA processing arrest *in vivo*. (**A**) A schematic illustration of the tRNA end processing pathway in eukaryotes. A tRNA primary transcript contains 5′ leader and 3′ trailer sequences, which are removed by RNase P and RNase Z, respectively, via endonucleolytic cuts. CCA is then added to the 3′ end by tRNA nucleotidyl transferase (tNtase) yielding a functional tRNA molecule. (**B**) RNA samples identical to those probed in Figure 3, from control and RNAi cells, were separated in polyacrylamide–urea gels and tested in northern blot hybridization with three oligonucleotide probes specific to $tRNA^{His}$ (indicated below each panel). The internal probe, which was designed against the anticodon domain, was used to detect mature $tRNA^{His}$; 5′ and 3′ probes were complementary to the 5′ end leader and 3′ end trailer, respectively. These probes detected the $tRNA^{His}$ primary transcript and an intermediate processing product. RNA size markers (Ambion) are designated at left; sizes are given in nucleotides. Mature $tRNA^{His}$ as well as its precursor and the intermediate processing product are indicated schematically to the left and right of the panel.

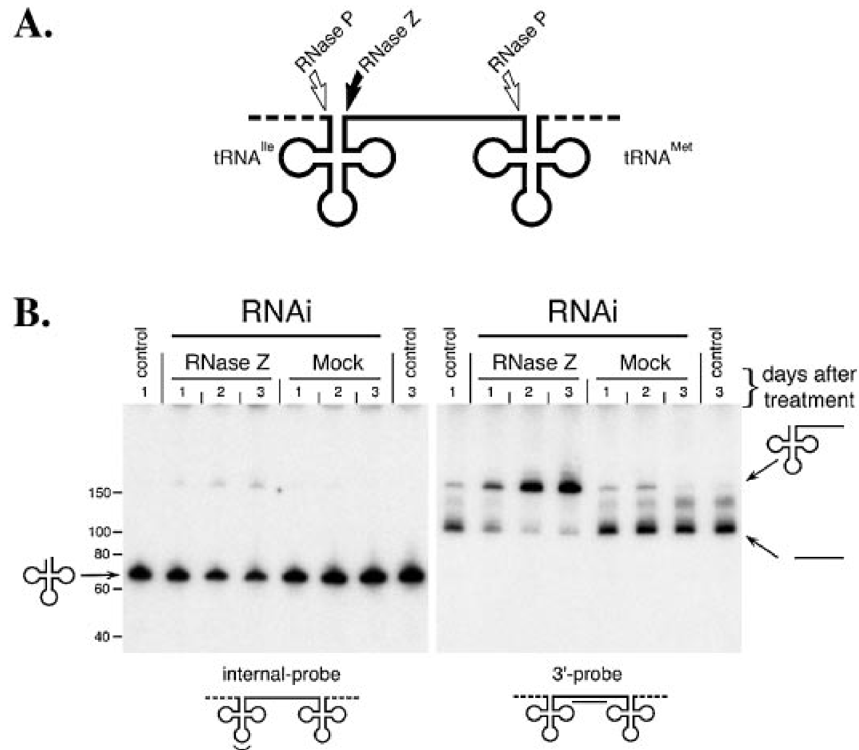

Figure 5. *Drosophila* RNase Z is required for mitochondrial tRNA^{Ile} processing. (**A**) A schematic illustration of the tRNA^{Ile}–tRNA^{Met} precursor organization within the mitochondrial primary transcript. RNase P and RNase Z cleavage sites are indicated by open and filled arrows, respectively. (**B**) RNA samples identical to those probed in Figure 3, from control and RNAi cells, were tested in northern blot hybridization with two oligonucleotide probes specific to tRNA^{Ile}. The internal probe detected mature tRNA^{Ile}. The 3′ probe against the 105-nt spacer between tRNA^{Ile} and tRNA^{Met} detected processing products of the primary transcript. RNA size markers (in nucleotides) are designated on the left. Mature tRNA^{Ile} and processing products are indicated schematically at the sides.

molecular weight of 92 kDa, somewhat larger than the calculated molecular weight of 83 kDa (Fig. 2A). Incubation with *Drosophila* nuclear pre-tRNA^{His} shows that the recombinant enzyme indeed cleaves the pre-tRNA to yield two products, the tRNA and the 3′ trailer (Fig. 2B). Both products are the same size as the cleavage products generated with the fruit fly extract (Fig. 2B), which cleaves pre-tRNAs directly 3′ to the discriminator (20,38,44). Thus recombinant *Drosophila* RNase Z is capable of converting a nuclear pre-tRNA into a 3′ trailer and a 3′ mature tRNA ready for CCA addition. Based on the sequence homology and tRNA 3′ endonucleolytic activity, we conclude that *JhI-1* encodes *Drosophila* RNase Z. We therefore re-designate the *JhI-1* gene as *dRNase Z*, *Drosophila* tRNA 3′-processing endonuclease.

Mitochondrial pre-tRNAs are also cleaved by *Drosophila* RNase Z *in vitro*

The mitochondrial genome of *D.melanogaster* encodes 22 tRNAs which punctuate the mRNA and rRNA genes (45), suggesting that the tRNAs must be precisely excised from the long polycistronic bi-directional primary transcripts. Sorting servers predict that the lone *Drosophila* RNase Z is mitochondrially targeted, thus *Drosophila* RNase Z is likely to also be the mitochondrial tRNA 3′ processing enzyme. To investigate whether mitochondrial tRNAs can be cleaved by *Drosophila* RNase Z, we incubated a mitochondrial pre-tRNA^{Ile} with recombinant dRNase Z (Fig. 2C). The mitochondrial pre-tRNA is efficiently converted into two products, the tRNA and the 3′ trailer.

Targeted disruption of *dRNase Z* expression in S2 cultured cells

Since the recombinant dRNase Z displayed faithful tRNA 3′-end endonucleolytic activity *in vitro*, it was reasonable to investigate whether this enzyme is responsible for tRNA processing *in vivo*. We used RNAi, which in many instances has proven to be an efficient tool for disruption of targeted protein production. RNAi was carried out by transfecting *Drosophila* Schneider S2 (S2) cells with dsRNA corresponding to *dRNase Z* or *JhI-26*. *JhI-26* is an unrelated *Drosophila* gene which, like *dRNase Z,* is expressed in S2 cells at a basal level and can be induced with JH (32). In the current experiment we used *JhI-26* as a mock-transfection control and *rp49* as a loading control. After transfection, cultures were allowed to grow for 1, 2 and 3 days prior to analysis. The efficiency of RNAi-mediated silencing was assessed by northern blot hybridization (Fig. 3). At all time points examined, the cells exposed to specific dsRNA fragments showed selective knockdown of the corresponding gene expression. The level of *dRNase Z* mRNA was significantly reduced in RNase Z RNAi cells after 1 day of treatment with dsRNA and the effect was even stronger when cells were exposed to dsRNA for up to 2–3 days, illustrating the efficiency of RNAi silencing. The level of *JhI-26* mRNA was

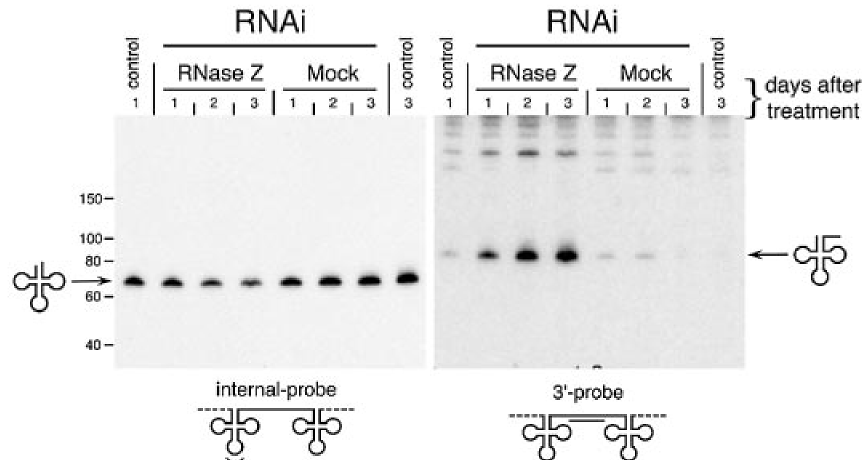

Figure 6. *Drosophila* RNase Z is required for mitochondrial $tRNA^{Tyr}$ 3′ end processing. RNA samples identical to those probed in Figure 3, from control and RNAi cells, were tested in northern blot hybridization with two oligonucleotide probes specific to mitochondrial $tRNA^{Tyr}$. The internal probe detected mature $tRNA^{Tyr}$. The 3′ probe against the 19-nt spacer between $tRNA^{Tyr}$ and $tRNA^{Cys}$ detected processing products of the primary transcript, the most prominent band being the 3′ unprocessed pre-tRNA. RNA size markers (in nucleotides) are designated on the left. Mature $tRNA^{Tyr}$ and processing products are indicated schematically at the sides.

similarly reduced in JhI-26/Mock RNAi cells. Figure 3 also shows the specificity of RNAi, since *JhI-26* dsRNA did not reduce *dRNase Z* mRNA levels, and dsRNA corresponding to *dRNase Z* had no effect on *JhI-26* mRNA levels. RNAi can thus efficiently and specifically silence *dRNase Z* expression.

In vivo tRNA 3′ processing is catalysed by dRNase Z

Next we tested RNAi cells for any defects in the tRNA processing pathway. We examined processing of nuclear encoded $tRNA^{His}$ in control and mock-transfected cells versus cells with inhibited *dRNase Z* expression using a probe specific either for mature $tRNA^{His}$, the 5′ leader or the 3′ trailer (Fig. 4B). The 121 nt long primary transcript of $tRNA^{His}$ consists of a 5′ leader (13 nt), the $tRNA^{His}$ (72 nt) and a 3′ trailer (36 nt) (20,44). As expected, in control and mock-transfected cells, the 5′- and 3′-specific probes both revealed the 121 nucleotide unprocessed $tRNA^{His}$ primary transcript. The dsRNA transfection did not change the pattern of hybridization with the 5′-specific probe: all samples displayed only the 121 nt band even after long exposures. In contrast, the 3′-specific probe detected an additional smaller RNA in *dRNase Z* RNAi cells, a 108 nt processing intermediate with a mature tRNA 5′ end and a 36 nt extension at the 3′ end. Importantly, the tRNA processing arrest was specific for *dRNase Z* RNAi cells: no processing intermediates accumulated in mock-transfected cells. We thus conclude that dRNase Z functions *in vivo* to process tRNA molecules at the 3′ end.

Although the intermediate with a 3′ extension accumulated in *dRNase Z* RNAi cells, the internal probe specific for mature tRNA revealed a 72 nt signal of equal intensity in all samples (Fig. 4B), indicating no alteration in the steady-state level of mature $tRNA^{His}$. Three explanations can be offered for this observation: (i) RNAi treatment was not fully effective, (ii) mature tRNA molecules are stable through several cell generations and (iii) as in yeast (46), there may be a back-up system for the endonucleolytic pathway.

dRNase Z gene also encodes the mitochondrial tRNA 3′ processing enzyme

The dRNase Z gene may be suggested to encode the mitochondrial tRNA 3′ processing enzyme for the following reasons. First, there is only a single RNase Z gene in the *Drosophila* genome; second *Drosophila* RNase Z contains a mitochondrial target sequence, and has a second methionine (amino acid 24) which is suspected to be the first methionine of the nuclear form (Fig. 1) close to the predicted target sequence cleavage site (amino acid 25), and finally, recombinant dRNase Z is capable of processing *Drosophila* mitochondrial pre-tRNAs *in vitro*. Thus we investigated whether it has the same activity *in vivo*. Figure 5A shows the mitochondrial $tRNA^{Ile}$–$tRNA^{Met}$ cluster, which is transcribed as part of a long precursor RNA in which the two tRNAs are separated by a 105 nt spacer. Northern blot hybridization with the 3′ probe specific for the intergenic spacer revealed a 170 nt band that accumulated to a high level in *dRNase Z* RNAi cells, but not in mock RNAi cells (Fig. 5B). This band corresponds to a $tRNA^{Ile}$ processing intermediate with a mature 5′ end and a 105 nt extension at the 3′ end. Thus RNase P efficiently produces the mature 5′ end of $tRNA^{Ile}$ and neighbouring $tRNA^{Met}$, while 3′ end processing of $tRNA^{Ile}$ is inhibited by the dRNase Z knock-down. In conformity with this conclusion, in control and mock-transfected cells the 3′ probe detected a stable 105 nt $tRNA^{Ile}$–$tRNA^{Met}$ intergenic spacer, whose steady-state level decreased with the increased level of the processing intermediate in *dRNase Z* RNAi cells. As expected, in all samples, the internal $tRNA^{Ile}$ probe detected one major band, a 65 nt mature $tRNA^{Ile}$. Two important alterations were observed in the pattern of internal probe hybridization with the *dRNase Z* RNAi samples. First, the intensity of the mature $tRNA^{Ile}$ band decreased progressively during 3 days of treatment with *dRNase Z* dsRNA, suggesting that RNAi against dRNase Z has a stronger effect on the processing of tRNAs encoded in mitochondria than in the

nucleus. Second, the internal probe detected the same intermediate as the 3′ probe. The corresponding band is more clearly visible after long exposure, but could not be detected with the internal probe against mature mitochondrial $tRNA^{Met}$ (data not shown). Again, this is consistent with our suggestion that the RNase Z knockdown abrogates cleavage at the 3′ end, leading to the accumulation of tRNA processing intermediates with 3′ extensions.

To test whether the mitochondrial arrest of tRNA 3′ end processing is a general outcome of dRNase Z knockdown, we also examined processing of a second mitochondrial tRNA cluster, $tRNA^{Tyr}$–$tRNA^{Cys}$ (Fig. 6). These two genes are arranged in tandem with a 19 nt spacer and are transcribed as part of a mitochondrial polycistronic transcript analogous to the $tRNA^{Ile}$–$tRNA^{Met}$ cluster (Fig. 5A). With the 3′ probe against the intergenic spacer, we detected accumulation of an 85 nt processing intermediate in *dRNase Z* RNAi cells, but not in wild-type or mock RNAi cells (Fig. 6), while the internal probe only detected 66 nt mature $tRNA^{Tyr}$. The 85 nt intermediate corresponds to a pre-$tRNA^{Tyr}$ with a mature 5′ end and a 19 nt extension at the 3′ end. The progressive decrease in intensity of the mature $tRNA^{Tyr}$ band over 3 days of treatment with dRNase Z dsRNA was not observed in mock-transfected cells. Thus, we conclude that *Drosophila* RNase Z is required for processing of tRNA molecules encoded in both nucleus and mitochondria *in vivo*. Several tRNA modifying enzymes are known which are encoded by a single gene but routed to different cell compartments (47), and data presented here suggest that this is also the case for *Drosophila* RNase Z.

Preferred reaction order in which cleavage by RNase P precedes RNase Z processing

The preferred order of pre-tRNA 5′ and 3′ end processing has been examined with various substrates, organelles and organisms, principally *in vitro*, and results often favour first 5′, then 3′ processing (48). The present study favours this order *in vivo*. An 85 nt nuclear pre-$tRNA^{His}$ with a 5′ end leader and no 3′ end trailer would be expected if 3′ end processing preceded the RNase P reaction. Failure to detect the predicted 85 nt band in control and mock lanes with the 5′ probe (even with long exposures; Fig. 4B and data not shown) suggests a preferred pre-tRNA processing reaction order *in vivo* (first RNase P, then RNase Z), in agreement with earlier *in vitro* results with fruit fly culture cell extracts (20). Furthermore, mitochondrial RNase P apparently cleaves efficiently when 3′ end processing is impaired in RNAi cells (Figs 5 and 6).

tRNA processing and protein biosynthetic capacity

The *Drosophila* gene encoding RNase Z was first detected by differential display of transcripts whose abundance is substantially elevated in cultured cells in the presence of JH (32). In flies, high levels of *dRNase Z* mRNA were found in adult females and in embryos although it is present at all developmental stages.

In adult females, we detected a large amount of *dRNase Z* transcripts produced in ovarian nurse cells and later transferred into the nascent oocyte (E.B.Dubrovsky, unpublished data). By the end of oogenesis, the egg accumulates a stockpile of *dRNase Z* mRNA. We suggest that maternally supplied dRNase Z contributes to the translational capacity of the developing embryo. Recent profiling of gene expression at the genomic level showed that more than 88% of genes whose expression is modulated during the *Drosophila* life cycle have a first peak of transcript abundance early in development, before the end of embryogenesis (49). At least 40% of these transcripts are maternally deposited; they encode proteins that are required for early embryonic events such as the set-up of pattern formation axes and the nuclear division cycles. To accommodate exceptionally high demand for protein synthesis during embryogenesis, the mature egg accumulates components of the translational machinery including the tRNA processing enzyme dRNase Z. Based on this hypothesis, it would be interesting to test the *dRNase Z* gene for a maternal effect.

Do ELAC2 proteins have multiple functions?

The *ELAC2* gene in *S.cerevisiae* has been reported to be vital (31). Since in yeast cells there is a back-up system for the RNase Z-catalysed tRNA 3′ processing pathway, the nuclear tRNA 3′ processing function may not be the essential function of the *ELAC2* gene. Yeast cells can become ρ^0, thus the mitochondrial tRNA 3′ processing activity is also not essential. Consequently there could be another function of the yeast *ELAC2* gene, loss of which leads to cell death.

The human *ELAC2* gene is reportedly connected to the occurrence of prostate cancer (31). Recent reports showed that overexpression of the human ELAC2 protein delays the cell cycle and that human ELAC2 interacts with the γ-tubulin complex (50). ELAC2 protein thus probably has an additional role in the cell besides tRNA 3′ processing, perhaps involving cell cycle regulation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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