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3' processing of eukaryotic precursor tRNAs

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Abstract

Biogenesis of eukaryotic tRNAs requires transcription by RNA polymerase III and subsequent processing. 5' processing of precursor tRNA occurs by a single mechanism, cleavage by RNase P, and usually occurs before 3' processing although some conditions allow observation of the 3'-first pathway. 3' processing is relatively complex and is the focus of this review. Precursor RNA 3' end formation begins with pol III termination generating a variable length 3' oligo(U) tract that represents an underappreciated and previously unreviewed determinant of processing. Evidence that the pol III-intrinsic 3' exonuclease activity mediated by Rpc11p affects 3' oligo(U) length is reviewed. In addition to multiple 3' nucleases, pre-tRNA processing involves La and LSM, distinct oligo(U)-binding proteins with proposed chaperone activities. 3' processing is performed by the endonuclease RNase Z or the exonuclease Rex1p (possibly others) along alternate pathways conditional on La. We review a *S. pombe* tRNA reporter system that has been used to distinguish two chaperone activities of La protein to its two conserved RNA binding motifs. Pre-tRNAs with structural impairments are degraded by a nuclear surveillance system that mediates polyadenylation by the TRAMP complex followed by 3' digestion by the nuclear exosome which appears to compete with 3' processing. We also try to reconcile limited data on pre-tRNA processing and LSM proteins which largely affects precursors but not mature tRNAs. A pathway is proposed in which 3' oligo(U) length is a primary determinant of La binding with subsequent steps distinguished by 3' endo- vs. exo- nucleases, chaperone activities and nuclear surveillance.

Keywords

RNase P; tRNase Z; La protein; LSM8; Lhp1; Sla1; Rex1; Rrp6; Rpc11; Trf4; TRAMP complex

Introduction

Nuclear tRNA genes are transcribed by RNA polymerase III (pol III) as nascent precursor tRNAs (pre-tRNAs) that undergo multiple processing and modification steps. Collective data indicate significant variability in the temporal order of cleavages and modifications among eukaryotes, for different tRNAs, and under different conditions. Since there is inherent variability in the sequence and structure of different tRNAs it should be expected that some will differ from others in their activity as substrate for a given enzyme, binding protein or process. Complexity in the collective 'pathway' presumably reflects that some events differ in the extent to which they are abundance-driven, kinetics-limited, spatially organized, and to which they can be substituted by alternate mechanisms that lead toward the end product.

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Early steps in tRNA biosynthesis are common to most tRNAs while steps individualized for specific tRNAs more often occur later (1). The universal steps in tRNA biogenesis are transcription, 5' and 3' end processing, the common nucleotide modifications, addition of CCA to the processed 3' end, and nuclear export. Individualized steps include splicing of those pre-tRNAs that contain introns, modifications that occur on specific subsets of tRNAs, and aminoacylation. Some tRNA biogenesis steps are temporally ordered and/or segregated to different subcellular locales. These aspects of the tRNA maturation process including tRNA gene localization, subcellular compartmentalization of tRNA processing activities, as well as tRNA nuclear export and retrograde transport (from cytoplasm to nucleus), have benefited from a recent review (1) as well as a more comprehensive review (2). A recent review of the role of modifications in tRNA stability and function is also available (3).

This review will focus on end processing, the removal of 5' leaders and 3' trailers from nascent pre-tRNAs, which occur in the nucleus relatively early during tRNA maturation. It is well accepted that all pre-tRNAs undergo 5' leader removal by the highly conserved endonuclease, RNase P, and that for most nuclear pre-tRNA species, this appears to occur before 3' end processing (2). Detailed reviews of 5' processing by RNase P are available (4-6). Our focus on 5' processing will be limited to conditions that appear to alter the temporal order of 5' and 3' end processing. At the other end, 3' trailers are removed by the endonuclease RNase Z. However, in contrast to a single mechanism of 5' leader removal, 3' trailers can also be removed by one or more 3' exonucleases and the determinants of the endo vs. exo pathways appear to be the availability and access of certain ancillary factors and other conditions.

The pre-tRNA processing factors La and RNase P have been found associated with tRNA genes in human cells (7,8), suggesting continuity between transcription and end processing. However, in contrast to mRNA biogenesis in which 5' capping and splicing occur in a polar manner during synthesis by pol II, we are unaware of evidence that any 5' processing event begins prior to release of the nascent transcript by pol III. As will be detailed below, pol III produces nascent RNAs with oligo(U) tracts on their 3' ends which is a sequence-specific binding site for the conserved La protein (9) and for the Lsm protein complex, both of which have been shown to be involved in pre-tRNA metabolism in yeast. Considerably more work has been on the effects of La protein on pre-tRNA processing than the Lsm proteins, the latter of which is limited to one solid publication. We will focus only on the early steps in eukaryotic pre-tRNA 3' processing, removal of the 3' trailer.

Does the 5'-before-3' processing pathway overshadow a simultaneous 3'-before-5' pathway?

The 5'-before-3' cleavage order appears to reflect a major pathway for tRNA processing (10-12, reviewed in 13). In addition to the endonuclease RNase Z, the exonuclease Rex1p (and perhaps others) can also process 3' ends (14,15). The 5'-before-3' order is reversed in yeast mutants that lack the La protein (16). However, this is not a simple reversal since in this case the 3' ends are matured by exonuclease(s) rather than RNase Z (16). La protein-RNA crystal structures reveal that La sequesters the RNA 3' end in its binding pocket (17), apparently imposing a steric block to the 3' exonucleases. The 5'-before-3' end processing appears to apply most clearly when RNase Z is used for 3' processing.

A change in 5' and 3' processing order may be explained by the relative robustness of these processes in the presence or absence of La. It would appear that La binding stabilizes nascent pre-tRNAs and delays their processing, since in its absence the 3' exonuclease(s) pathway is so robust that nascent transcripts with intact leaders and trailers do not accumulate as distinct species (16). A consideration raised in this review is that La-dependent and La-independent pathways of pre-tRNA processing occur simultaneously for

different subsets of pre-tRNAs in normal cells. Some of the individual transcripts from a single tRNA gene may use the La-dependent pathway while others from the same gene use the La-independent pathway. Observing pre-tRNAs of the La-independent pathway is challenging because the intermediates differ by only a few nucleotides at their 3' ends (13,16,18,19). While distinct species of the La-dependent pathway are concentrated as a band on Northern blots, those of the La-independent pathway are dispersed into a smear, attributed to variable 3' exonucleolytic nibbling (16), some of which may be masked by the overlying distinct band. The 5'-before-3' order is most clearly reversed in mutants lacking La (16). However, we suspect that the La-independent pathway is simultaneously active for some pre-tRNAs in La replete cells but these intermediates are more difficult to discern in the presence of an intact La-dependent pathway.

The 5'-before-3' order is reversed for *S. cerevisiae* pre-tRNA^{Trp} (13), and possibly other pre-tRNAs, even when La is present. Since La binds pre-tRNAs in a 3' oligo(U) length-dependent manner, we suggest that the 5'-before-3' order would be reversed for pre-tRNAs that do not bind La due to a limitation of La and/or an insufficient number of 3' terminal U residues (20) because these would be subjected to robust 3' exonucleolytic processing to indistinct intermediates difficult to discern. As will be reviewed in detail below, current data argue that La is indeed limiting for the maturation of some pre-tRNAs and that some pre-tRNAs are synthesized with too few Us for La binding when La is limited (20).

In a later section we will return to the issue of 5'-before-3' processing, considering other kinetic aspects of the interactions between La, RNase P, RNase Z and the pre-tRNA, and then discuss the limited published data on Lsm proteins and pre-tRNA metabolism and offer suggestions for future studies. Before that however, since a significant portion of this review will focus on the relationship between pol III termination, La function and early pre-tRNA processing, we will first introduce some features of the *S. pombe* system that has been used to examine this.

***S. pombe* suppressor tRNA alleles for the study of pol III termination and early processing**

An opal suppressor tRNA gene whose functional readout is suppression of a UGA stop codon in *ade6-704* mRNA and suppression of red pigment can be used to follow tRNA biogenesis (21-23). Various alleles of the suppressor tRNA have been examined (24). An allele was developed such that only accurate pol III termination leads to functional tRNA (25). Some allelic variants of this require only the 3' end protection activity of the La protein (either *S. pombe* La protein, Sla1p, or human La), while other tRNA alleles also require La's secondary chaperone-like activity, for functional suppressor tRNA (24). Thus a tRNA pathway that operates in *S. pombe* independently of La is nonfunctional for these pre-tRNAs which are processed exclusively along the La-dependent pathway(s).

It is important to note that despite the popular impression that La is an abundant protein, the normal amount of endogenous Sla1p is limiting for maturation of suppressor pre-tRNA. This is evident since overexpression of ectopic Sla1p increases mature suppressor tRNA levels with concomitant increase in *ade6-704* suppression activity (20,22). This limitation presumably reflects the ability of the many other endogenous cellular pre-tRNAs and other pol III products to out compete the suppressor pre-tRNA for Sla1p.

Pre-tRNA 3' end formation begins with pol III transcription termination

The earliest event in the processing pathway is the point at which the transcription phase ends and the nascent pre-tRNA is released from the pol III enzyme complex. Although the way pol III creates RNA 3' ends prior to transcript release is very similar in vertebrates, *S. pombe* and *S. cerevisiae*, relevant differences will be reviewed. The relevant issues

discussed in this and the following three sections are species-specific length differences in the oligo(dT) termination signals recognized by pol III, the minimal oligo(U) length required for recognition by La protein, which also appears to be species-specific, and 3' oligo(U) length heterogeneity as a conserved intrinsic property of pol III and a possible mechanism for its generation.

A distinguishing feature of pol III is its termination mechanism, which occurs at a simple oligo(dT) tract at the 3' ends of its transcribed genes (26). It is imperative to note relevant differences among several model organisms regarding nascent RNA 3' end formation that result from pol III termination. While an oligo(dT) tract of four Ts leads to highly efficient termination by vertebrate pol III, four Ts are insufficient for *S. pombe* and *S. cerevisiae* pols III which require progressively longer dT tracts (25). This is reflected in the distribution of the oligo(dT) lengths of tRNA gene terminators by genome-wide analysis. Most tRNA genes end with four or five dTs in *H. sapiens*, five or six dTs in *S. pombe*, and six or more dTs in *S. cerevisiae* (27), nicely matching the oligo(dT) length dependent termination activities of these pols III *in vitro* (25).

Pol III termination-associated RNA 3' oligo(U) length heterogeneity

For most intents here, RNA 3' oligo(U) length heterogeneity refers to the fact that the pol III transcripts produced from a single gene make up a population of RNAs, usually consisting of U₍₁₋₃₎U-3'OH. The distribution pattern of oligo(U) length can vary in a gene-specific manner, limited by the length of the oligo(dT) tract. High resolution gels or two-dimensional systems revealed 3' length heterogeneity after *in vitro* transcription (28-34). Although some of this could be due to contaminating exonuclease activities in the extract used, 3' heterogeneity was also observed using purified pol III (in some cases noted not to have contaminating nuclease activity) (20,35,36). Nascent RNAs with 3' oligo(U) length heterogeneity of 1-3 nt were synthesized and released by *S. cerevisiae* pol III observed using fast kinetics analysis (37,38). Moreover, mutations in the second largest subunit of yeast pol III cause greater variations in the 3' oligo(U) length distribution relative to wildtype in the RNAs released from pol III (see figure 7 in 38), providing additional support for the idea that RNA 3' oligo(U) heterogeneity is a conserved intrinsic property of pol III itself.

Nascent RNA 3' oligo(U) metabolism by pol III subunit Rpc11p

RNA 3' oligo(U) length heterogeneity is generated during the slowing down or pausing phase of pol III termination. A well-characterized, pol III-intrinsic processive 3' exoribonuclease activity cleaves RNA in the active center of pol III during pausing (38-41). This 3'-5' nt removal activity is distinct (and 'opposite') from the principal activity of pol III, which is 5'-3' nt polymerization, and is not simply a chemical reversal of the forward reaction. This 3' exoribonuclease activity is mediated by a 12.5 kDa subunit of pol III, Rpc11p, that is homologous to TFIIIS a factor that helps pol II overcome pausing and other blocks to elongation (42). Mutants defective for pol III termination show alterations in this activity (38). In a report that examined tRNA maturation monitored by functional suppression, Huang et al. proposed that 3' terminal oligo(U) length distribution results in part from cleavage by Rpc11p (38). This was supported by the observation that *rpc11*-mutants differing in their deficiency of pol III-associated 3'-5' RNA cleavage activity showed progressive lengthening of the 3' oligo(U) tracts in the nascent transcripts (20). This lengthening presumably reflects alterations of Rpc11p-mediated 3'-5' RNA cleavage activity during the pausing phase of transcription termination that precedes transcript release.

Some pol III transcripts contain 3' oligo(U) tracts too short for competitive La binding

La protein is a molecular chaperone that protects newly synthesized nascent pol III transcripts from 3' exonucleases and also assists in pre-tRNA folding (43-45). As noted

above, La binds RNA in an 3' oligo(U) length-dependent manner (9). Human La binds stably to RNAs that contain 3 or more Us at their 3' end (9). This was confirmed for human La by Huang et al. who also showed by comparison that the *S. pombe* La protein, Sla1p, requires 4 or more Us (20).

Using purified pol III Cambell & Setzer found that a majority of *Xenopus* nascent 5S rRNAs contained only two Us at the 3' end, confirming earlier data from Bogenhagen & Brown and Cozzarelli et al, who reported a major pol III transcription stop after 2 Us and a minor stop after 3 Us from a highly efficient 4 T terminator (26,36). Studies on pol III transcription of adenovirus VA1 RNA revealed more transcripts with 1 or 2 Us at the 3' end than with 3 Us (46). It is noteworthy that the vertebrate VA1 and 5S RNA genes used for these studies contain highly efficient oligo(dT) terminators that are only 4 Ts in length.

The pol III primary transcript of a mouse B1 RNA gene terminates at its natural 5 T terminator, AATTTTAA, and is stabilized by La protein. Replacement of this terminator with the *Xenopus* 5S rRNA efficient terminator of 4 Ts, GCTTTTGC, produces a primary transcript not stabilized by La, that undergoes quick 3' processing *in vitro* and *in vivo* (47,48). These data are consistent with the earlier results showing that most transcripts from the 5S gene contain only 2 Us (26,36) and strongly support the idea that some pol III terminators produce transcripts with too few Us for efficient La binding.

Mathews & Francouer confirmed that VA1 RNA transcripts made by pol III *in vitro* that ended in 2 Us were more abundant than those with 3 Us, and further, that only the 3U RNA species was immunoprecipitable with La while the 2U RNA species was left intact in the supernatant (29). This binding preference agrees with results of Stefano who used a mixture of tRNAs with varying numbers of terminal Us and showed that the tRNAs with 3 or more terminal Us are most readily immunoprecipitable with La protein (9).

In *S. pombe*, nascent pol III transcripts with 4 or more terminal Us competed significantly better for Sla1p than did the RNAs with 3 or fewer Us (20). In a side-by-side analysis Sla1p required a longer oligo(U) tract for competitive binding than did human La (20). The same difference between human La and Sla1p was observed using chemically synthesized RNAs that differed in 3' oligo(U) length (20). Thus Sla1p prefers nascent transcripts with 4 or more terminal Us while leaving disproportionately more of the RNAs with the shorter U tracts in the supernatant. The requirement of Sla1p for an oligo(U) tract that is slightly longer than is required by human La protein matches the slightly longer oligo(dT) tract lengths required for termination by the *S. pombe* pol III relative to human pol III noted earlier (25). Examination of *S. cerevisiae* Lhp1p minimal oligo(U) length has not been reported.

Huang et al. mapped the oligo(U) length of *in vivo* synthesized nascent suppressor pre-tRNAs in wild type *S. pombe* and in *rpc11* mutants (20). In wild type *S. pombe* only ~25% of the nascent suppressor pre-tRNAs had 4 or more 3' Us, while this was increased to 46% and 57% in the two *rpc11* mutants examined (see above). Moreover, for the La-dependent suppressor tRNA allele used the increases in 3' oligo(U) length were accompanied by corresponding increases in the efficiency of conversion of pre-tRNA to mature tRNA, mature suppressor tRNA levels, and suppression activity (20). A straightforward model that emerged is that a relatively small fraction (~25%) of suppressor pre-tRNAs is bound by La in wild type cells while a larger fraction is bound in the *rpc11* mutants, with congruent effects on tRNA maturation and functional suppression (20). The unbound fraction presumably has too few 3' U residues for La binding which apparently leads them to be degraded (20). Thus, by affecting 3' oligo(U) length, the presence or absence of the RNA 3' cleavage activity of Rpc11p can exert a most early effect on nascent RNA 3' end formation and subsequent pre-tRNA metabolism (Fig. 1). This model predicts that tRNA genes with

longer oligo(dT) tracts may produce nascent transcripts that compete better for La protein than other tRNA genes with relatively short oligo(dT) tracts. Another hypothesis would be that genes with the shortest oligo(dT) tracts may have adapted to be less dependent on La function than genes with longer oligo(dT) tracts.

La, RNase Z and RNase P interactions during pre-tRNA processing

La protein and RNase P have been found localized with the pol III machinery and at tRNA genes (7,8,49,50), consistent with their function in the early phases of processing (28,51-55). Early binding by La fits nicely with the facts that La exhibits RNA chaperone-like activity possibly to assist pre-tRNA folding *in vivo* and that RNase P requires some tRNA-like structure in its substrates (23,56-59). Mutations that affect pre-tRNA folding or structure can decrease the substrate activity of pre-tRNA for RNase P (4-6), even when the mutation is in the intron of the pre-tRNA (60).

That La protein is linked to the earliest phase of pre-tRNA biogenesis is consistent with the phenotypes of two classes of *S. pombe* mutants. In nonphosphorylatable La mutants in which La exhibits increased affinity for the nascent suppressor pre-tRNA, the latter accumulates to high levels relative to the phosphorylatable La control, but with inefficient 5' processing by RNase P and failure to produce mature suppressor tRNA^{Ser}UCA (22). This is consistent with earlier data that showed that nonphosphorylated human La binds to the 5' end of pre-tRNA (as well as the UUU-3'OH end) and interferes with processing by RNase P, an inhibitory activity that is attenuated by phosphorylation on serine-366 (61). However this would appear not to reflect an activity that is used during tRNA biogenesis *in vivo* since most nuclear La is in the phosphorylated form and it is this form that is found associated with pre-tRNAs in HeLa cells (62). Nonetheless, these mutants provide evidence that La gains control of the nascent pre-tRNA even before what is normally considered the first processing step, 5' cleavage by RNase P. Also in support of this conclusion, a second class of mutants, in which subcellular trafficking of La is aberrant, La-associated pre-tRNAs are prematurely exported from the nucleus before RNase P or Z cleaves the pre-tRNA (63,64). Thus although La and RNase P are localized at tRNA genes, it would appear in these cases that La gains control over the nascent pre-tRNAs.

Why RNase P cleaves before RNase Z when La is present is not entirely understood. Purified recombinant La protein can impede 3' cleavage by RNase Z in a purified *in vitro* system, dependent on UUU-3'OH trailer binding (65). Initially it might seem that inhibition of RNase Z activity by La would be somewhat at odds with the conclusion that yeast La protein is required for pre-tRNA 3' endonucleolytic cleavage (16). However, we believe that it fits nicely with the *in vivo* data if we consider that La binding stabilizes nascent pre-tRNAs, as evidenced by their accumulation as distinct species (16). A model to accommodate these data is that La binding to pre-tRNA would be inhibitory both to 3' exo- and 3' endo-nucleolytic processing, but more so to the 3' exonuclease(s) than to RNase Z. This hierarchy seems quite plausible knowing that La sequesters the 3'-OH end of oligo(U) RNA in a largely hydrophobic binding pocket (17). In either case, inhibition of 3' end processing might allow RNase P to act first. Moreover, it is conceivable that by sequestering the 3' trailer La could actually enhance reactivity of the 5' leader with RNase P (for example see Fig. 6 in 23). Other aspects of the relative affinities of RNases P and Z for a given pre-tRNA substrate may contribute to processing order in some cases.

Isoforms of RNase Z

Both short and long forms of RNase Z are present in eukaryotes, designated tRNase ZS and tRNase ZL respectively, whereas bacteria and archaea have only the short form (reviewed in 66). tRNase ZL is ~850 amino acids in length and contains mitochondrial or chloroplast

targeting sequences while the ZS form is ~350 amino acids in length (67). The C-terminal part of tRNase ZL has sequence homology with tRNase ZS. Understanding the roles of tRNases Z is also important because independent roles for tRNase ZL in gene silencing, including involvement of siRNA-related enzymes, have emerged (68-71) and because human tRNase ZL is encoded by ELAC2, a prostate cancer susceptibility gene (72,73).

Humans and *S. cerevisiae* have one copy each of tRNase ZS and tRNase ZL genes, with different isoforms of tRNase ZL localized to nucleus and mitochondria. Curiously, *S. pombe* has no apparent RNase ZS but contains two tRNase ZL genes, *sptrz1⁺* and *sptrz2⁺* (66). These localize to nuclei and mitochondria respectively, suggesting that the RNase Z functions of nuclei and mitochondria may be more readily dissected in *S. pombe* than in other organisms (66). Both *sptrz1⁺* and *sptrz2⁺* are required for cell growth (66) and consistent with its nuclear localization, *sptrz1⁺* was shown to promote 3' processing of a nuclear encoded suppressor-tRNA^{Ser}UCA resulting in a functional increase in the mature tRNA and suppression of red pigment (66).

Unexpected finding in *S. pombe* La and RNase Z double mutant

La promotes pre-tRNA 3' endonucleolytic processing (16) by blocking 3' exonucleases (14,15). Because the 3' exonuclease Rex1p and/or other exonucleases can functionally process pre-tRNAs in the absence of La (14,15), it may have been predicted that nuclear RNase Z, i.e., encoded by *sptrz1⁺*, would be nonessential in La-deleted yeast. Intriguingly, this is not the case (66). This suggests the possibility that some pre-tRNAs must be processed by RNase Z in the absence of La and if so, La is not required for 3' endonucleolytic cleavage of some pre-tRNAs (16). However, another interpretation is considered below.

S. cerevisiae tRNase ZL (*scTRZ1*) and human ELAC2 were shown to function in *S. pombe* for 3' processing of nuclear encoded pre-tRNAs (66). It is therefore intriguing that *scTRZ1* and ELAC2 complement the temperature sensitive growth defect of *sptrz1-1*, but not the *sptrz1-null* mutant. These data suggest that *sptrz1⁺*, *scTRZ1* and ELAC2 exhibit a conserved activity for the 3' processing of those pre-tRNAs that were examined (66) but that *sptrz1⁺* also exerts an additional essential function that is species-specific. It is formally possible that an essential *S. pombe* pre-tRNA requires *sptrz1⁺* but can not be processed by *scTRZ1* or ELAC2. It also seems plausible that the presumed additional essential function of *sptrz1⁺* is independent of tRNA production. Since other ribonuclease activities for RNase Z have been demonstrated (67,69,74), *sptrz1⁺* may be required for the processing of another essential RNA, perhaps guided by a protein or RNA partner that can not interact with *scTRZ1* or ELAC2. In any case, the distinctive genetics of the *S. pombe* tRNA 3' processing factors may be well suited to uncover the essential function of tRNase ZL and lead to a better understanding of the relationship between La and RNase Z.

Pre-tRNA 3' exonucleases

As reviewed previously, 3' end maturation of pre-tRNAs takes place in the nucleus and can occur via multiple mechanisms (75). Although it appears that endonucleolytic cleavage by RNase Z reflects the major pathway, the 3' exonucleases Rex1p and Rrp6p perform 3' end processing under some conditions and it is suspected that additional exonucleases may also contribute (14,15). Rex1p appears to be the major 3' exonuclease that contributes to 3' end maturation in yeast cells containing La (14). Substitution mutations to Rex1p residues that are predicted to be important for catalysis indeed inactivate its exoribonuclease activity *in vitro* and *in vivo* (15). Some data suggested that Rex1p may specifically process tRNA^{Met} precursors with long 3' but not short 3' trailers (15).

Although deletion of RRP6 from *S. cerevisiae* appeared to restore the pattern of intron-containing pre-tRNA^{Ser} intermediates that is otherwise aberrant in a *Lhp1-Δ Rex1-Δ* double mutant, it is not clear if RRP6 contributes positively to production of mature tRNA (14). Deletion of *S. pombe rrp6*⁺ had no negative effect on suppressor tRNA maturation; for suppressor tRNA^{Ser} alleles with little or no structural impairment, deletion of *rrp6*⁺ did not reduce suppressor activity in the presence or absence of *sla1*⁺ suggesting that Rrp6p does not make significant contributions to tRNA maturation (24). It would appear for most pre-tRNAs not cleaved by RNase Z, that Rex1p processes their 3' ends and that although Rrp6p may contribute under some conditions it probably serves more to degrade pre-tRNAs as a component of nuclear surveillance (14).

Pre-tRNA nuclear surveillance competes for 3' end metabolism

Nuclear surveillance was first proposed as a means by which cells perceive defects in tRNA processing or nuclear export and react by decreasing translation initiation mediated by tRNA^{iMet} (76). It was later found that some mutants that reduce tRNA^{iMet} levels do so by nuclear degradation of hypomodified pre-tRNA^{iMet} caused by lack of modification of A58 of pre-tRNA^{iMet} to m¹A58 (77). It was discovered that the degradation of hypomodified pre-tRNA^{iMet} requires Rrp44p, a 3'-5' exoribonuclease subunit of the exosome complex as well as Trf4p, a poly(A) polymerase (78). The hypomodified pre-tRNA^{iMet} is first polyadenylated on its 3' end by Trf4, creating a single-stranded landing pad for exonuclease loading, and is then degraded in 3'-5' direction by the Rrp6p-containing nuclear exosome (78,79). Trf4p is a member of the TRAMP (Trf4/Air2/Mtr4p Polyadenylation) complex comprised of poly (A) polymerase (Trf4 or Trf5), RNA helicase (Mtr4) and a Zinc knuckle protein (Air1 or Air2) which interacts with the nuclear 3' exoribonuclease, Rrp6p of the nuclear exosome (80,81). This intranuclear pathway is also operational for other aberrant RNAs that bear a structural perturbation or result from an error in processing (reviewed in 82). However, the details of how this pathway preferentially targets aberrant RNAs are not clear.

Nuclear surveillance would appear to be in competition with pre-tRNA 3' end processing. It was reported that while hypomodified pre-tRNA^{iMet} is subjected to degradation by nuclear surveillance, the yeast La homologous protein, Lhp1p could offset this (77,83). In *gcd10/14*-mutants defective for m¹A58 modification of pre-tRNA^{iMet}, over expression of Lhp1p leads to increased levels of mature tRNA^{iMet} and suppresses their phenotypes (77). Deletion of LHP1 in the *gcd10/14*- mutants exacerbates pre-tRNA^{iMet} 3' exonuclease-mediated degradation and the associated phenotypes (77,83).

The Trf4p poly(A) polymerase component of the TRAMP complex can polyadenylate substrates with single strand 3' overhangs of no less than 3 nucleotides (84). The endonuclease RNase Z leaves only one unpaired nucleotide at the 3' end and would not generate good substrates for Trf4p (84). This fits with the idea that 3' trailers subjected to partial processing by 3' exonuclease(s) as well as those pre-tRNAs with too few 3' terminal Us for La binding would be preferential substrates of Trf4p. Knowing that La binds and protects pre-tRNA 3' trailers from 3' exonuclease, the data suggest that La competes with the TRAMP/nuclear exosome surveillance system for the 3' end of the hypomodified pre-tRNA^{iMet}.

Opposite effects of La and nuclear surveillance components were demonstrated for suppressor pre-tRNA^{Ser}UCA alleles of *S. pombe* (24). For the subset of alleles that were affected, deletion of *rrp6*⁺ increased suppressor activity whereas deletion of *sla1*⁺ decreased activity (24). The degree to which *rrp6*⁺ and *sla1*⁺ exerted their effects was greater for some suppressor tRNA^{Ser} alleles than for others, in general accordance with the degree of structural impairment, i.e., containing one, two or three base substitutions that weaken

basepairing potential by changing a G:C pair to a G:U. The most severely affected of the pre-tRNAs^{Ser}UCA were shown to be polyadenylated and deletion of *rrp6*⁺ increased the mature suppressor tRNA^{Ser}UCA levels twelve-fold (24). The activity of the U40 allele, which contains a single G:C to G:U change in the anticodon stem, was unaffected by *rrp6*⁺ or *sla1*⁺ deletion (24). Activity of another allele, C37:10, was diminished by *sla1*⁺ deletion but fully restored by *rrp6*⁺ deletion in the double mutant. Since Rrp6p acts to degrade pre-tRNAs after polyadenylation by TRAMP, it would seem that La and TRAMP compete for the 3' ends of at least some pre-tRNAs although it is also possible that Rrp6p and La protein compete more directly as well.

Wolin and colleagues analyzed a more complete set of factors that may be involved in pre-tRNA 3' end metabolism and extended the list of individual pre-tRNAs that succumb to nuclear surveillance (14). They also showed that competition with La for pre-tRNA 3' ends extends to the 3' exonuclease Rex1p. Moreover, Rex1p-dependent 3' trimming appears to target some pre-tRNAs for decay by TRAMP (14). Anderson and colleagues found that loss of Rex1p results in polyadenylation of pre-tRNA^{iMet}, suggesting that defects in 3' end processing can activate the nuclear surveillance pathway. Thus, direct competition between La and Rex1p may determine the subsequent fate of a pre-tRNA. The conclusion that Rex1p contributes to pre-tRNA 3' end maturation even in cells containing La protein (14) is consistent with the idea that both 5'-before-3' and 3'-before-5' processing occur simultaneously.

It would seem that while nuclear surveillance targets defective pre-tRNAs for decay, La protein can protect those same pre-tRNAs and promote their processing to functional mature tRNAs. A question that arises is why should there be these opposing activities? It is intriguing that the defects in hypomodified pre-tRNA^{iMet} and impaired suppressor tRNA^{Ser}UCA appear to subject them to degradation in the nucleus more so than preventing them from functioning at the ribosome in the cytoplasm. Appreciation that RNA degradation may compete with productive RNA processing has arisen in part because of the prevalence and efficiency of RNA degradation. While popular belief once suggested that cells should not waste resources on RNA degradation, a prevailing view that cells make RNA that is targeted for degradation questions this (85-87).

Lsm proteins affect pre-tRNA metabolism

Although a report indicates convincingly that some of the Lsm proteins are required for the normal pathway of early pre-tRNA metabolism in *S. cerevisiae* and for pre-tRNA association with La-homologous protein Lhp1p, it has not been clear how this requirement fits into the pre-tRNA processing pathway (19). Deletion of Lsm2p to Lsm5p and Lsm8p led to alterations in pre-tRNA intermediates while mature tRNA levels were unaffected, somewhat reminiscent of the effects of *sla1*⁺ or LHP1 deletion in *S. pombe* and *S. cerevisiae* respectively. Lsm6-Δ and Lsm7-Δ also exhibited a similar pre-tRNA phenotype but in this case only for one of the transcripts examined, pre-tRNA^{Ala} (19). Thus, the previously characterized seven subunit Lsm2p-8p complex is presumably involved in pre-tRNA metabolism. The Lsm2p-Lsm8p forms a heptameric ring structure that plays a major role in the metabolism, stabilization, nuclear retention and function of the U6 snRNP (88-91).

The Lsm2p-Lsm8p complex also functions in the degradation of pre-mRNAs in the nucleus (92). By contrast, the Lsm1p-7p complex functions in cytoplasmic mRNA degradation (reviewed in 93). Genetic experiments indicate that the only essential function of LSM8 is related to its role in U6 snRNA metabolism or function (94).

Surprisingly, Lsm1-Δ cells also showed accumulation of pre-tRNA^{Ala} (19). Although Lsm1p is cytoplasmic, its dysregulation (overexpression) has been shown to perturb U6

snRNA accumulation presumably by disrupting the normal stoichiometric equilibrium of the other Lsm components of the Lsm2p-8p complex (95).

Characteristics of the relationship between the Lsm2-8p complex and Lhp1p in pre-tRNA metabolism are clearly distinguishable from Lsm2-8p participation with Lhp1p in the metabolism of another pol III transcript, U6 snRNA (88,94). While La/Lhp1p is transiently associated with a small fraction of U6 snRNA that represents the nascent pol III transcript, the Lsm2-8p complex is associated with mature U6 snRNA as part of the stable U6 snRNP (90,91,96). La protein requires a 3' hydroxyl group on its RNA ligands which is present only on newly synthesized yeast U6 snRNA but not mature U6 snRNA which contains a 3' phosphate and is bound by Lsm2-8p (94). This order of binding to U6 snRNA, Lhp1p before Lsm2-8p (94) is consistent with the idea that Lhp1p functions to stabilize U6 snRNA for assembly into the U6 snRNP while the Lsm2-8p complex participates in the function of the U6 snRNP (91,96).

Metabolic pulse-chase experiments indicate that pre-tRNA processing was delayed in cells that previously underwent transcriptional shut off of Lsm3p (19). Steady state levels of pre-tRNAs but not mature tRNAs were altered in *lsm*-deletion mutants (19). In this case, a significant increase in pre-tRNA levels occurred in *lsm*-mutants, consistent with delay or obstruction of their processing or degradation. Moreover, while coprecipitation of the pre-tRNAs with Lsm3p suggest a direct role for the Lsm proteins in pre-tRNA metabolism, the relatively low efficiency of coprecipitation suggested a transient association (19).

Aberrant pre-tRNAs with extended 3' ends also accumulated in the *lsm*-mutants. These aberrant 3'-extended pre-tRNAs appear to represent transcripts produced by read-through of the normal pol III transcription termination site of the tRNA gene, that would be rapidly degraded in wild-type cells but accumulate in the *lsm*-mutants (19). However, that the elevated levels of the other pre-tRNAs in the *lsm*-mutants might represent misfolded or defective pre-tRNAs that would otherwise be targeted for degradation was considered unlikely because it implied a substantial amount of pre-tRNA decay in wild-type cells and because similar accumulation was not observed in exosome-deficient strains (19). On the other hand, the authors did note two-hybrid interactions between Lsm8p and the exosome subunit Mtr3p and between Lsm8p and the 5' exonuclease Xrn1p, as well as physical association of Lsm8p with another exosome subunit Rrp42p (see citations in 19), consistent with the association of Lsm2p-Lsm8p with the RNA degradation machinery. Subsequent work by this group revealed that the Lsm2p-8p complex indeed targets nuclear RNA, albeit capped pre-mRNA, for degradation (92). These observations suggest that Lsm2-8p involvement in pre-tRNA metabolism may be related to degradation more than productive processing.

As noted, the transient association between the Lsm complex and the pre-tRNAs with the concomitant reduction of Lhp1p binding to its substrate pre-tRNAs demonstrated by Kufel et al., is consistent with the Lsm complex functioning as a pre-tRNA chaperone (92), possibly involved in quality control. The limited available evidence is consistent with a role for the Lsm2-8p complex in either pre-tRNA degradation or processing. The experimental data do not address whether the Lsm proteins involved in pre-tRNA metabolism contributes positively or negatively to the production of mature tRNA. However, this may be addressed using the *S. pombe* suppressor-tRNA system described above. In this case, increases or decreases in suppressor tRNA levels and with concomitant alteration of suppression activity may be monitored and compared in strains carrying various *lsm*-alleles, *slal*-alleles and suppressor tRNA alleles.

Molecular chaperone and RNA chaperone activities

As referred to in the previous section, the Lsm2-8p complex is believed to possess chaperone activity during U6 snRNP assembly and/or function and may also provide this for pre-tRNAs (92). Multiple sources of evidence of chaperone activity for the La proteins from several species has been accumulating. First, it should be noted that two types of chaperone activity have been distinguished. That La meets the definition of a ‘molecular chaperone’ as a protein that binds and stabilizes a substrate and exhibits controlled binding and release was first documented for U6 snRNA and suggested for nascent pol III transcripts in general (see 88). A second definition, that for ‘RNA chaperone activity’ refers to participation of a given protein in the RNA folding process specifically, often by preventing the RNA from becoming trapped in a misfolded conformer (97). Evidence that La fulfills this activity, that it is required for the efficient folding of a pre-tRNA *in vivo* is also available (56). In addition, recombinant human La protein has been shown to exhibit RNA chaperone activity *in vitro* using a self-splicing intron RNA misfolding trap assay (23,57). This RNA chaperone activity has been mapped to one of the two conserved RNA binding motifs of human La protein, the canonical RNA binding surface of the RNA recognition motif-1 (RRM1) (23). An intact RRM1 binding surface of La protein (both human La and Sla1p) has also been shown to be required for the maturation of a structurally impaired pre-tRNA^{Ser}UCA (24).

Two activities differentially map to the two conserved RNA binding motifs of La proteins, the La motif (LAM) and RNA recognition motif-1 (RRM1) (23,24). The principal activity of La, binding to the 3' oligo(U) tract, which is mediated mostly by its La motif (LAM) and serves to protect pre-tRNAs from exonucleases and as reviewed in an earlier section imposes a 5'-before-3' end processing pathway. As noted above, this 3' oligo(U) binding activity can offset the TRAMP & 3' exonuclease Rps6p nuclear surveillance system which leads to degradation of aberrant pre-tRNAs.

For the maturation of other, more severely impaired pre-tRNAs, there is a requirement for La's principal activity to be accompanied by its second activity (activity-2), mediated by the La RRM β -sheet surface, that functions to offset pre-tRNA decay that would otherwise result from an as yet uncharacterized surveillance system distinct from Rps6p (24). It remains unclear if this second activity, as examined for human La and Sla1p in *S. pombe*, offsets or sterically blocks a nuclease activity that degrades the structurally impaired pre-tRNA or if it helps the pre-tRNA avoid or overcome misfolding (24). In support of the second possibility, La RRM, the same RNA binding motif required for maturation of the structurally impaired pre-tRNA *in vivo* is also required for RNA chaperone activity *in vitro* (23,24). It is tempting to speculate that this activity is also responsible for the requirement of Lhp1p in pre-tRNA^{Arg}CCG folding (97).

Distinguishable chaperone activities for pre-tRNAs

Thus, it appears that La can facilitate tRNA processing by two mechanisms: simple 3' end binding with consequent 3' end protection, and by promoting folding and/or the structural integrity of a tRNA. Discerning these as separate activities has been a multifaceted process. By screening for and characterizing mutations that confer yeast synthetic lethality in the absence of La, substitutions were found that disrupt basepairing in the anticodon stem of the single copy gene essential for tRNA^{Ser}CGA (16). It was later reported that substitutions in a suppressor-tRNA in *S. pombe* confer dependence on La for functional maturation (22). Yeast La indeed promotes proper folding of a mutation-containing pre-tRNA^{Arg}CCG with a demonstrable propensity for misfolding (56). By genetic and biochemical dissection it was formally shown that La confers two distinct activities in tRNA maturation, mediated by separate RNA binding surfaces on the two highly conserved motifs of the La protein (24).

The degree to which a pre-tRNA requires or engages La for its principal and secondary activities can vary (24). Some structurally impaired suppressor pre-tRNA alleles trigger decay more than others and are differentially sensitive to Rrp6p and rescue by La. For *S. pombe* pre-tRNA^{Ser}UCA, a G:CTIG:U basepair change in the variable arm causes it to be much more highly dependent on La for maturation than basepair disruptions elsewhere in the pre-tRNA, even in the absence of *rrp6*⁺ (24). It is important to note for the purpose of this review that while these two activities of La protein are distinguishable, both require intact oligo(U) 3' end binding activity. Thus, while activity 2 is distinguishable from activity 1 in pre-tRNA metabolism, it is dependent on activity 1 (23). This is in agreement with a two binding site model for La interaction with pre-tRNA in which the La motif binds 3' oligo(U) and allows the adjacent RRM RNA binding site to mediate activity 2 (23).

We suggest that the 'molecular chaperone' and 'RNA chaperone' activities of La are represented by distinct activities that differentially map to the two conserved RNA binding motifs of La proteins, the La motif (LAM) and RNA recognition motif-1 (RRM1) respectively (23,24). The molecular chaperone activity as initially characterized for U6 snRNA and involving Lsm2-8p appears to be related to UUU-3' end protection and stability during U6 snRNP assembly (94). This activity appears to apply to pre-tRNAs as La can protect their 3' ends from 3' exonucleases and other competing activities such as nuclear surveillance as reviewed above, and keep them on a productive maturation pathway. The *in vitro* RNA chaperone activity and the *in vivo* activity required for the maturation of structurally-impaired pre-tRNA requires an intact RRM1 β -sheet surface. By contrast an intact RRM1 β -sheet surface is not required for the maturation of other, less impaired pre-tRNAs that succumb to nuclear surveillance that do require an intact LAM but not RRM1 β -sheet surface (23,24).

It is noteworthy that processing of the apparently nonimpaired pre-tRNA^{Lys}CUU was also affected in La activity-2 mutants (24). This is consistent with the observation that the RNA binding surface of RRM1 involved in activity-2 participates in, but is not required for, the metabolism of normal pre-tRNA. In this case, La uses both of its conserved RNA binding surfaces, the LAM and RRM, to distinguish pre-tRNAs from 3' processed tRNAs (23). Both surfaces engage different parts of a contiguous pre-tRNA for high affinity stable binding whereas after 3' cleavage, each site exhibits a faster off rate and more readily releases its RNA (23).

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Abbreviations

pre-tRNA	precursor tRNA
pol III	RNA polymerase III
nt	nucleotide

Figure 1.

Alternative pathways for pre-tRNA processing begin during transcription termination by RNA polymerase III. This figure incorporates protein nomenclature from multiple organisms (see text). **A)** Pol III pauses at the tRNA gene terminator. According to structure modeling the integral pol III subunit, Rpl11p, contains a N-terminal domain that resides on the surface of pol III and an acidic hairpin that enters through a pore to the catalytic center. The acidic tip of the Rpl11p hairpin (designated “-”) mediates RNA 3' cleavage of nascent pre-tRNA transcripts during pausing by pol III. **B)** Nascent pre-tRNA transcripts with different length 3' oligo(U) termini are released from pol III and sorted into those that bind La and those that do not, depicted on the right and left sides of the vertical dashed arrow respectively. **C)** Lsm proteins (presumably Lsm2p-8p) can affect the degree to which nascent pre-tRNAs are associated with La and may function at this point (see text). Pre-tRNA transcripts with sufficient 3' oligo(U) length maintain stable association with La protein. **D)** Pre-tRNAs move along alternative pathways, numbered 1-4, depicted for normal pre-tRNAs (pathways 1 & 2) and structurally-impaired pre-tRNAs (pathways 3 & 4), distinguished by 3' endonucleolytic (pathways 1 & 3), or 3' exonucleolytic (pathways 2 & 4), as well as nuclear surveillance involving TRAMP and Rpl6p (pathway 4). La protein gains early dominance over the pre-tRNAs with sufficient 3' oligo(U) length (see text). The exact point in this scheme at which the RNA chaperone-like activity of La (La activity 2) appears to function is unclear from current data but occurs sometime before RNase Z separates the trailer from the tRNA body.