Processing of transcripts of a dimeric tRNA gene in yeast uses the nuclease responsible for maturation of the 3′ termini upon 5 S and 37 S precursor rRNAs

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The *rna82* mutation of *Saccharomyces cerevisiae* inactivates an RNA processing activity responsible for maturation of 3′-terminal sequences upon 5 S and 37 S ribosomal RNA precursors. This study describes a difference in the processing of transcripts of an *S. cerevisiae* dimeric tRNA gene (tRNA^{Arg}-tRNA^{Asp}) in RNA polymerase III in vitro transcription extracts prepared from *rna82* and wild-type cells. The mutant extract accumulated additional processing intermediates containing tRNA^{Arg} sequences as compared to the extract from wild-type cells. The structure of these intermediates revealed a defect in removal of the 10 nucleotides left 3′ to the tRNA^{Arg} sequence by the RNase P cleavage immediately 5′ to tRNA^{Asp}. This is the first demonstration of a mutational defect affecting maturation of 3′ sequences upon a eukaryotic tRNA precursor.

tRNA precursor; Nuclease deficiency; Sequence maturation, 3′-; (*S. cerevisiae*)

1. INTRODUCTION

Many of the functions of the different RNA processing enzymes of *Escherichia coli* have been determined through detailed study of mutants lacking one or more of these activities (reviewed by Deutscher [1,2]). Despite the availability of these mutants, it is still unclear which activities process the 3′ end of tRNA precursors (pre-tRNAs). An endonucleolytic cleavage downstream of the 3′ end of the mature tRNA sequence is thought to be followed by an exonucleolytic trimming that stops at the encoded -CCA [1]. However, an *E. coli* strain deficient in at least four exonucleases known to be capable of removing 3′ nucleotides from pre-tRNA in vitro (RNase D, RNase II, RNase BN and RNase T) can still synthesise tRNAs [3]. Recent studies of the processing of pre-tRNAs in bacterial

extracts have revealed a fifth activity, a phosphate-requiring exonuclease (RNase PH), that can participate in pre-tRNA 3′ maturation [4,5].

No comprehensive series of mutants causing defects in the enzymes of stable RNA processing has yet been isolated in a eukaryote. One *Saccharomyces cerevisiae* mutation (*rna82*) has been shown to cause complete loss of an endonucleolytic activity required for correct 3′ sequence maturation upon 5 S and 37 S rRNA precursors [6,7]. It has no effect on cell viability or growth. Also, no altered pre-tRNA maturation could be detected in *rna82* cells in in vivo pulse-labelling studies, indicating the enzyme affected by this mutation to be nonessential for tRNA biosynthesis [8]. However, these studies in vivo could not exclude the possibility that *rna82* affects one of several enzymes involved in 3′ sequence maturation on yeast tRNA gene transcripts. The maturation of 3′ sequences on pre-tRNAs in eukaryotes is unlikely to be exactly identical to the same process in *E. coli* because the 3′-CCA of eukaryotic

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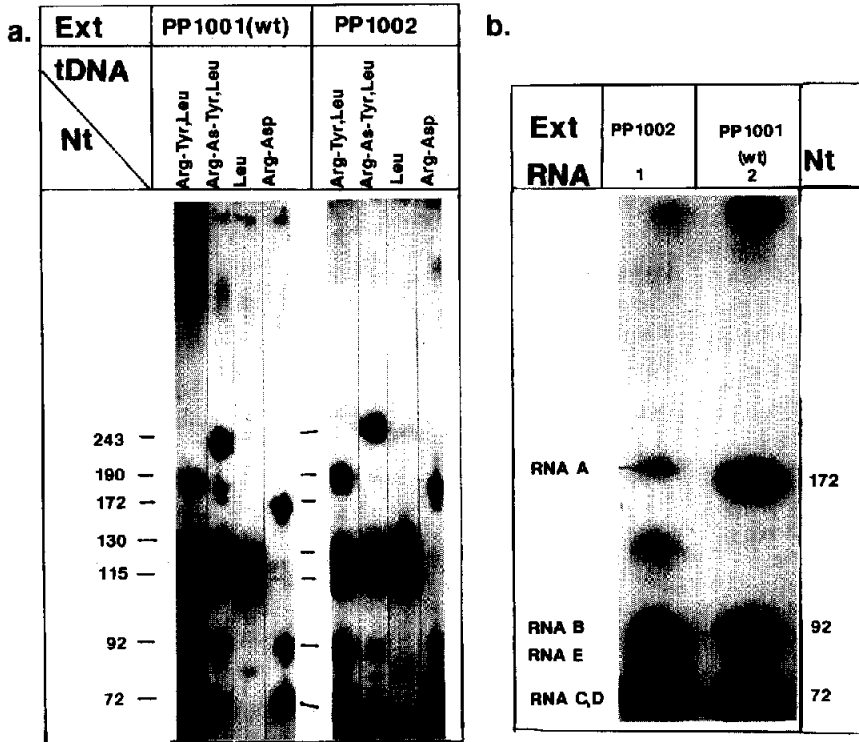
Fig.1. RNAs synthesised in RNA polymerase III transcription extracts (Ext) from yeast strains PP1001 and PP1002. Transcription and RNA processing (see section 2) were under conditions of 40 mM or 80 mM KCl (as indicated below). The transcription reactions in (a) were with 40 mM KCl in the PP1001 and PP1002 extracts, and [α-^{32}P]UTP as isotope. The plasmid DNAs used as templates are described in [10] and were (left to right in (a)): pYCEN3ARS2-107 (tDNA^{Arg}-tDNA$^{SUP6(Tyr)}$ and tDNA^{Leu}); pYCEN3ARS2-506 (tDNA^{Arg}-5' part of tDNA^{Asp}-tDNA$^{SUP6(Tyr)}$ and tDNA^{Leu}); pYCEN3ARS2 (tDNA^{Leu}); and pJB19f (tDNA^{Arg}-tDNA^{Asp}). Band patterns similar to those from pYCEN3ARS2-107 and pYCEN3ARS2-506, but lacking the bands of 115 and 130 nucleotides (Nt), were obtained (data not shown) with plasmids pKS102 and pKS14, both of which [10] lack the tDNA^{Leu} of the former plasmids. (b) Preparative 10% polyacrylamide gel used in purification of processing products from the transcription of pJB19f (tDNA^{Arg}-tDNA^{Asp}). In panel 1, the KCl concentration in the extract was 80 mM; in panel 2, it was 40 mM. Although in this PP1002 extract 40 mM KCl was optimal for in vitro transcription, the higher KCl concentration resulted in more efficient transfer (see section 2) of RNA A to RNA E (panel 1).

tRNAs, unlike the -CCA of bacterial tRNAs, is not generally gene-encoded [1]. We have therefore investigated how pre-tRNA processing in yeast RNA polymerase III transcription extracts is affected by the presence of rna82 in the strain used for extract preparation.
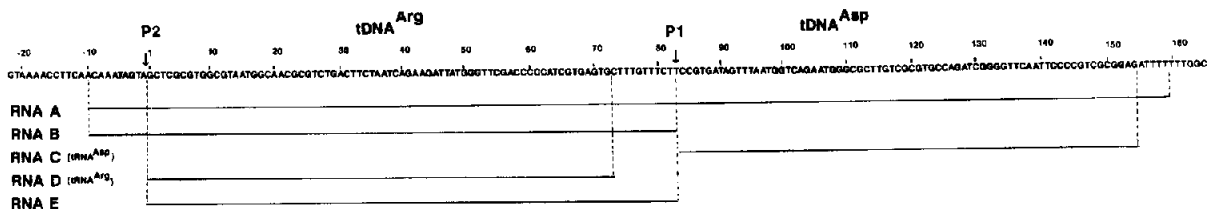

Fig.2. The S. cerevisiae tRNA^{Arg}-tRNA^{Asp} gene [11]. RNA A, the primary transcript, is processed in extracts from wild-type yeast [9] by the RNase P cleavages P1 and P2 indicated with vertical arrows. Analysis of processing in rna82 mutant extracts revealed a defect in removal of the intergenic spacer (nucleotides 73 to 82) from RNAs B and E, implicating an endonuclease already known to function in rRNA 3' sequence maturation in the removal of these spacer nucleotides.

2. MATERIALS AND METHODS

RNA polymerase III transcription extracts employed for this study were from the *S. cerevisiae* strains PP1001 (*αade2,leu2,pep4-3*) and PP1002 (*αade2,leu2,pep4-3,rna82*). Extract preparation and in vitro transcription were as described previously [9,10]. Transcription and processing employed either 40 mM or 80 mM KCl, and 0.1–0.2 μg template DNA per 20 μl reaction. The optimal KCl concentration for in vitro transcription in PP1001 and PP1002 extracts was 40 mM, and the difference in the way that these two extracts processed dimeric tRNA gene transcripts was clearly apparent at this KCl concentration (fig.1a). However, at 80 mM KCl, there was more efficient transfer of the primary transcript of the dimeric tRNA^{Arg}-tRNA^{Asp} gene (RNA A, fig.2) to processing products, and this KCl concentration was therefore employed to facilitate isolation of RNA E (figs 1b and 2) from PP1002 extracts for fingerprint analysis. RNAs were eluted from the gels and analysed as described in [6,8].

3. RESULTS AND DISCUSSION

Monomeric and dimeric *S. cerevisiae* tRNA genes were used as transcription templates in the PP1001 and PP1002 nuclear extracts. Primary transcripts made in the two extracts were identical for all the transcription templates tested. The products of processing of these transcripts were analysed by gels and RNA fingerprinting. Monomeric tRNA gene transcripts did not show any major differences in processing in the PP1001 and PP1002 extracts. However, when dimeric tRNA gene transcripts were synthesised, we observed a reproducible difference in the processing catalysed by wild-type and mutant extracts. With the tRNA^{Arg}-tRNA^{Asp} [11] or tRNA^{Arg}-tRNA^{Tyr} [10] genes as transcription templates, the PP1002 extract accumulated additional processing intermediates (fig.1a). The structural analysis of these extra intermediates from the tRNA^{Arg}-tRNA^{Asp} transcript is described below.

The processing of tRNA^{Arg}-tRNA^{Asp} gene transcripts in yeast RNA polymerase III in vitro transcription extracts has been described previously [9,12]. Transcription initiates at position −10, with the major site of termination being at 160. The resultant 172-nucleotide RNA (RNA A, figs 1 and 2) is then converted by processing to the 72-nucleotide tRNA^{Arg} and tRNA^{Asp} (RNAs D and C, respectively) RNAs, which do not always acquire a complete 3′-CCA in these extracts [9]. The major intermediate of this processing is a 92-nucleotide RNA (band B). Its structure (fig.2)

reveals the removal of the 5′ leader and 3′ trailer sequences of tRNA^{Arg} to be the slowest steps of processing in the in vitro extracts. Engelke et al. [9] have proposed that this may be due to the apparent propensity for limited base pairing of these sequences within RNA B (fig.2). Consistent with such base pairing conferring a transient stability to RNA B is the fact that wild-type extracts do not accumulate appreciable amounts of other intermediates containing tRNA^{Arg} sequences, suggesting almost simultaneous 5′ and 3′ sequence maturation upon RNA B in the production of tRNA^{Arg} [9].

When we investigated the processing of the same dimeric tRNA gene transcript in an in vitro transcription extract from the *rna82* mutant, we observed an additional 82-nucleotide band (RNA E, fig.1) as well as a series of weaker bands between this RNA E and the RNA C+D position. RNA E was not detected in wild-type extracts (fig.1a and b; also data not shown), in agreement with the earlier studies. Also, minor RNA bands between the RNA B and RNA C+D positions in the wild-type extract (reported to be due to 3′ exonuclease action on RNA B [9]) were much weaker in RNA samples from PP1001 extracts relative to PP1002 RNAs. The dimeric tRNA^{Arg}-tRNA^{Tyr} gene contains the same 10-base-pair spacer as the tRNA^{Arg}-tRNA^{Asp} gene [10]. When it was used as template in PP1001 and PP1002 extracts, it also gave extra RNA bands in the latter extract (fig.1a), their lengths being comparable to the extra RNAs obtained using the tRNA^{Arg}-tRNA^{Asp} gene as template. The structures of these products from tRNA^{Arg}-tRNA^{Tyr} have not been investigated in detail. RNA E synthesised using the tRNA^{Arg}-tRNA^{Asp} gene pair as the in vitro transcription template was analysed further by RNA fingerprinting. When [α-^{32}P]UTP was the isotope in RNA synthesis, its fingerprint (fig.3a) showed all of the T1 RNase digestion fragments given by mature UTP-labelled tRNA^{Arg}, but none of the tRNA^{Asp} fragments (table 1). There were two additional spots not given by mature tRNA^{Arg} (nos 4 and 7, fig.3a). Nearest neighbour analysis of these, by identification and quantitation of labelled RNase T2 digestion products (table 1), gave results completely consistent with the fragments (CUUUGp (spot 7) and UUUCU (spot 4)) that should result from the sequence between the tRNA coding
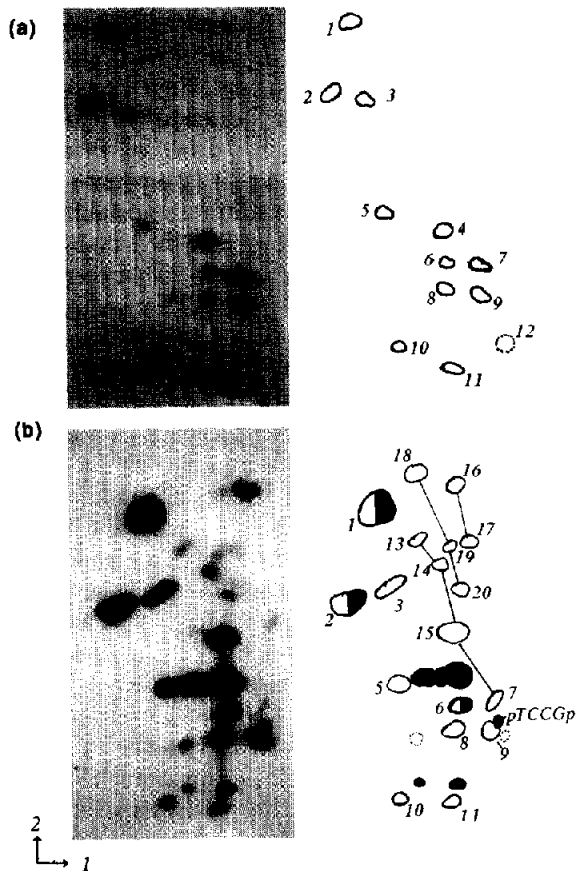
(a)

(b)

Fig.3. Fingerprints of (a) RNA E; and (b) RNAs 72–80 nucleotides long synthesised in PP1002 extract using [α-^{32}P]UTP as isotope and plasmid pJB19f as template. After extraction of these RNAs from the gel in fig.1b, they were digested with RNase T1 and their digestion products were separated in the first (1) and second (2) dimensions of RNA fingerprinting as in [8]. Both autoradiographs of the fingerprints and plans of numbered oligonucleotides listed in table 1 are shown. Numbered open circles in (b) are oligonucleotides from RNAs B and E; filled circles are oligonucleotides originating from the sequence of mature tRNA^{Asp}, and half-filled circles are fragments given by both RNA E and tRNA^{Asp}. The 5'-terminal fragment of tRNA^{Asp} (pTCCGp) is indicated. Lines in (b) connect products arising from processive 3'-5' exonuclease action.

regions (73 to 82, fig.2). Compared to the corresponding fingerprint of RNA B [9], the only product absent was pppACAAAUAGp (fig.3a and table 1), indicating that the 5' leader sequence of the tRNA^{Arg} precursor had been removed in RNA E. This was confirmed by fingerprints (not shown) of band E RNA labelled with [α-^{32}P]GTP in *rna82*

extracts which showed: (i) complete absence of labelled UAGp; and (ii) the presence of pGp (not labelled using UTP label) and CUUUGp (which is labelled by UTP), both in yields approximately equimolar to other unique tRNA^{Arg} RNase T1 digestion fragments.

This analysis showed RNA E (fig.1) to be the RNA (sequences 1 to 82 in fig.2) that should result from the RNase P cleavage P2 (fig.2) of RNA B. Its presence showed the removal of nucleotides 73 to 82 from pre-tRNA^{Arg} to be much slower in mutant (PP1002) compared to wild-type (PP1001) extracts, indicating the nuclease inactivated by *rna82* as the major activity normally cleaving RNA B at position 72. In wild-type extracts, removal of the 5' leader (-10 to -1) and 3' trailer (83 to 92) of RNA B (fig.2) probably occurs almost simultaneously since the -10 to 72 RNA or the 1 to 82 RNA (RNA E) are virtually undetectable ([9], and this study).

Fingerprints of RNAs slightly shorter than RNA E made by PP1002 extracts provided evidence for a slow exonucleolytic shortening of this RNA. Fig.3b shows a fingerprint of [α-^{32}P]UTP-labelled RNA of 72–80 nucleotides from a PP1002 extract transcribing the tRNA^{Arg}-tRNA^{Asp} gene pair. This RNA sample contained both tRNA^{Arg} and tRNA^{Asp} forms. As in fig.3a, pppACAAAUAGp is virtually absent, consistent with the RNase P cleavage of RNA B having gone to completion. Most significant upon this fingerprint was the large number of T1 RNase fragments (listed in table 1) that lacked a terminal phosphate group, indicating the presence of several 3' termini upon the RNAs fingerprinted. Moreover, CUUUGp (fragment 7) was found, in low yield, to be replaced by a series of fragments (CUUUG, CUUU, CUU, UU and UUU) that must originate from length heterogeneity introduced by exonucleolytic action at the 3' end of the RNA E sequence. All these fragments are in low yield except for CUUUG. The higher abundance (fig.3b) of this pentanucleotide is a strong indication that exonucleolytic digestion of nucleotides 73 to 82 upon RNA E in PP1002 extracts pauses at the solitary purine within this sequence. The exonuclease catalysing this shortening might be identical to the activity that shortens the 3'-extended 5 S rRNA forms of *rna82* cells in vivo since such cells accumulate a major 5 S rRNA form that terminates at the solitary purine in the

Table 1

(a) T1 RNase digestion products on the fingerprint of $[\alpha\text{-}^{32}P]$UTP labelled RNA E and (b) phosphatase-resistant T1 RNase digestion products on the fingerprint in fig.3b

(a) Spot (fig.3a)	Structure(s)	Labelling[a]		Labelled RNase T2 products[b]
		Theor.	Actual	
1	Gp	1	1.0	Gp
2	CGp	3	3.1	Gp
3	AGp	1	1.2	Gp
4[c]	UUUCU	3	3.4	Cp(1); Up(2)
5	CUCGp	1	0.8	Cp
6	UCUGp + UUCGp	2	2.4	Cp(1); Up(2)
7	CUUUGp	4	4.2	Cp(1); Gp(1); Up(2)
8	UAAUGp	1	1.3	Ap
9	AUUAUGp	3	2.7	Ap(2); Up(1)
10	ACCCCCAUCGp	2	1.7	Ap(1); Gp(1)
11	ACUUCUAAUCAGp	4	3.6	Cp(2); Ap(1); Up(1)
12	pppACAAAUAGp	0	<0.05	n.d.

(b) Spot	Proposed structure	Labelled RNase T2 products[b]	Probable derivation by 3'-5' exonuclease action on
13[c]	CUU	Cp(1); Up(1)	RNA E
14[c]	CUUU	Cp(1); Up(2)	RNA E
15[c]	CUUUG	Cp(1); Up(2)	RNA E
16[c]	UU	Up	RNA E
17[c]	UUU	Up	RNA E
18[c]	AU	Ap	RNA C
19[c]	AUU	Ap(1); Up(1)	RNA C
20[c]	AUUU	Ap(1); Up(2)	RNA C

[a] Relative to Gp = 1.0; determined by scintillation counting
[b] Numbers in parentheses indicate quantitations of relative labellings from visual inspection of autoradiographs
[c] Phosphomonoesterase-resistant, indicating derivation from 3' terminus of an RNA

pre-5 S rRNA 3' trailer sequence [6]. The RNA sample fingerprinted in fig.3b also contained tRNA^{Asp} forms. The yield of the 5'-terminal tRNA^{Asp} fragment pTCCGp (marked in fig.3b) was equimolar with other unique tRNA^{Asp} products, further evidence for complete RNase P processing of this RNA. The presence of AU, AUU and AUUU in low yield on the fingerprint (fig.3b and table 1) is consistent with a small proportion of the tRNA^{Asp} in the sample having 2–4 extra 3' nucleotides relative to RNA C.

These results, obtained with a crude nuclear extract from the *rna82* mutant, reveal that transcripts of yeast tRNA gene pairs are processed by a mechanism slightly more complicated than in-

dicated by previous studies [9–13]. The data support the earlier conclusion that a single endonucleolytic cleavage (P1, fig.2) generates pre-tRNA^{Arg} species bearing all 10 nucleotides of the intergenic spacer. However, while it has been proposed that this spacer might be removed solely by 3'-5' exonuclease action [9], PP1002 extracts accumulate appreciable levels of RNA E. Thus, the endonuclease inactivated by the *rna82* mutation normally participates in the removal of the spacer nucleotides. Exonuclease action alone may suffice to remove the 3' trailing sequence of several pre-tRNAs, possibly inducing the pre-tRNA^{Asp} generated by RNase P cleavage P1 in fig.2. Other workers have presented biochemical evidence that

maturation of the 3' terminus of certain eukaryotic pre-tRNAs can proceed through endonucleolytic cleavage [14–16]. This study with extracts from the yeast rna82 mutant is the first time the genetic approach has been used to demonstrate such an endonucleolytic step. We have not found evidence for rna82 affecting the processing of monomeric pre-tRNAs and a 3'-endonucleolytic cleavage of pre-tRNAs may therefore only occur either when 5'-leader and 3'-trailing sequences base-pair (as in RNA B) or as an exceptional processing step of dimeric precursors.

Higher eukaryotes (Xenopus and mammalian cells) do not endonucleolytically process the 3' end of 5 S rRNA and this RNA terminus is subject to the exonucleolytic loss of only two or three 3' nucleotides if indeed it is processed at all [17]. Processing at the 3' end of the large rRNA precursor probably also shows differences between lower and higher eukaryotes, though these have yet to be precisely defined. Thus, only lower eukaryotes possess an endonuclease, inactivated by rna82 in yeast, for 3' maturation of 5 S rRNA. The results described here show this endonuclease to be acting in the processing of dimeric tRNA gene transcripts, yet it is clear that removal of the spacer sequence left 3' to tRNA^{Arg} by RNase P cleavage P1 (fig.2) can still take place in rna82 mutant extracts through alternative 3'-5' exonuclease action (fig.3b and table 1). Similarly, Xenopus extracts can still catalyse the processing of yeast dimeric tRNA gene transcripts to mature tRNAs even though they do not possess an endonuclease capable of acting on 5 S rRNA precursors from yeast [17]. Thus, although higher eukaryotes, unlike bacteria, seem not to use multimeric tRNA gene transcripts in tRNA biosynthesis, they still apparently possess enzymes that process the yeast dimeric tRNA precursors.

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