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Substrate structural requirements of *Schizosaccharomyces pombe* RNase P

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RNase P from *Schizosaccharomyces pombe* has been purified over 2000-fold. The apparent K_m for two *S. pombe* tRNA precursors derived from the *supS1* and *sup3-e* $tRNA^{Ser}$ genes is 20 nM; the apparent V_{max} is 2.5 nM/min (*supS1*) and 1.1 nM/min (*sup3-e*). Processing studies with precursors of other mutants show that the structures of the acceptor stem and anticodon/intron loop of tRNA are crucial for *S. pombe* RNase P action.

RNase P; tRNA; Substrate recognition; (*Schizosaccharomyces pombe*)

1. INTRODUCTION

The ribonucleoprotein, RNase P, is a key enzyme involved in tRNA biosynthesis in prokaryotes and eukaryotes [1]. This enzyme catalyzes the endonucleolytic cleavage of nearly all tRNA precursors to produce 5′-end-matured tRNA. We have examined the ability of many mutant *S. pombe* tRNA precursors to be processed in vivo and in vitro using heterologous systems derived from *S. cerevisiae*. Mismatches at or near the top of the acceptor stem can prevent removal of the 5′-leader sequence [2]. Mutations in the anticodon stem and the anticodon/intron loop [3–5] can also influence the rate of cleavage in vitro. In addition, sequences outside the mature tRNA may affect the cleavage rate. For instance, *S. cerevisiae* mitochondrial RNase P is sensitive to the 5′-leader sequence of a mitochondrial tRNA precursor [6].

In this report we have used a highly purified preparation of *S. pombe* RNase P for in vitro studies of the kinetics and substrate specificity of this enzyme. Cleavage rates for different wild-type and mutant *S. pombe* tRNA precursors were compared to advance our understanding of the substrate structural features that are important for RNase P catalysis.

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2. MATERIALS AND METHODS

2.1. *Enzyme purification*

S. pombe RNase P was purified from cell extracts by chromatography over two successive DEAE-cellulose columns and a final phosphocellulose column. These steps follow a protocol described earlier [7], but include several modifications to column dimensions and elution gradients which improve significantly the overall purification of the enzyme. The details of this purification will be published elsewhere.

2.2. *Assay for RNase P activity*

The substrates for RNase P assays were in vitro labeled transcripts of the *S. pombe* $tRNA^{Ser}$ genes *supS1*, *sup3-e* and *sup9-e*. Enzyme assays were carried out at 37°C in a 20 μl reaction containing 30 mM Tris-HCl (pH 8.0), 100 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol. The concentration of RNA substrate and concentration of protein in the assay mixture were 1.35 fmol/μl and 2 ng/μl, respec-

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tively. In kinetic studies the protein concentration was 0.5 ng/μl. Reaction products were isolated and resolved on denaturing 8% polyacrylamide gels [7]. Activity was quantified by Cerenkov counting of excised gel slices.

2.3. *Construction of tRNA genes*

Plasmids containing tRNA genes for in vitro transcription were constructed in pSP64 (Promega Biotechnology). The *supS1* plasmid and several of the *sup3-e* mutant constructs have been described previously [2,7]. The *sup3-e* mutants, U_{18}, Δ_{35}, and A_{67} were constructed similarly. The *sup3-e* constructs were cut with *Ban*II prior to transcription. The *sup9-e* plasmid was constructed as follows. An *Aha*II site was created at the 3′-end of the tRNA gene by primer-directed mutagenesis, converting the GT at positions 74 and 75 to TC. The procedure for primer-directed mutagenesis was described earlier [2]. The mutant gene was inserted into pSP64 by cutting with *Sac*I followed by *Alu*I and ligating to *Hin*dIII-cut, filled-in, and then *Sac*I-cut pSP64. Cleavage with *Aha*II produces transcripts truncated after nucleotide 73 of the mature tRNA. The sequences immediately 5′ of the *sup9-e* and *supS1* genes were switched by substituting the 5′-precursor flank of one gene with that of the other via primer-directed mutagenesis [2]. The pSP64 and distal *S. pombe* flanking sequences remain unchanged. For *supS1*, GTCCAG was replaced with AAGAA. The opposite substitution was made for *sup9-e*. Primers for mutagenesis were GTAATATCAAGAAGACACTAT for the *supS1* flank switch and TAGCTTTCGTCCAGGTCACTAT for the *sup9-e* flank switch. The strategy for cloning these genes in to pSP64 was identical to that for the *supS1* and *sup9-e* genes, respectively. In vitro transcription with bacteriophage SP6 RNA polymerase was carried out as described previously [2].

3. RESULTS AND DISCUSSION

Previous studies in our laboratory have shown that alterations of tRNA secondary and tertiary structure affect *S. cerevisiae* RNase P cleavage of *S. pombe* tRNA precursors [2,5,8]. To extend these investigations and to compare these results with those obtained in an homologous system, we examined the action of a highly purified *S. pombe* RNase P preparation on selected *S. pombe* tRNA precursors that were altered structurally by mutations (fig.1B). We also examined the efficiency of RNase P cleavage of different serine suppressor tRNAs (*supS1*, *sup3-e* and *sup9-e*, see fig.1) to quantify differential processing, and of *supS1* and *sup9-e* pre-tRNAs in which the 5′-leader sequences had been exchanged.

All experiments used an *S. pombe* RNase P fraction that had been purified over 2000-fold relative to the crude extract. Processing of the acceptor stem mutants (A_1, C_2, U_{72} and G_{71}) showed that all are cleaved at a reduced rate. An example of such an experiment is given in fig.2 and the data are

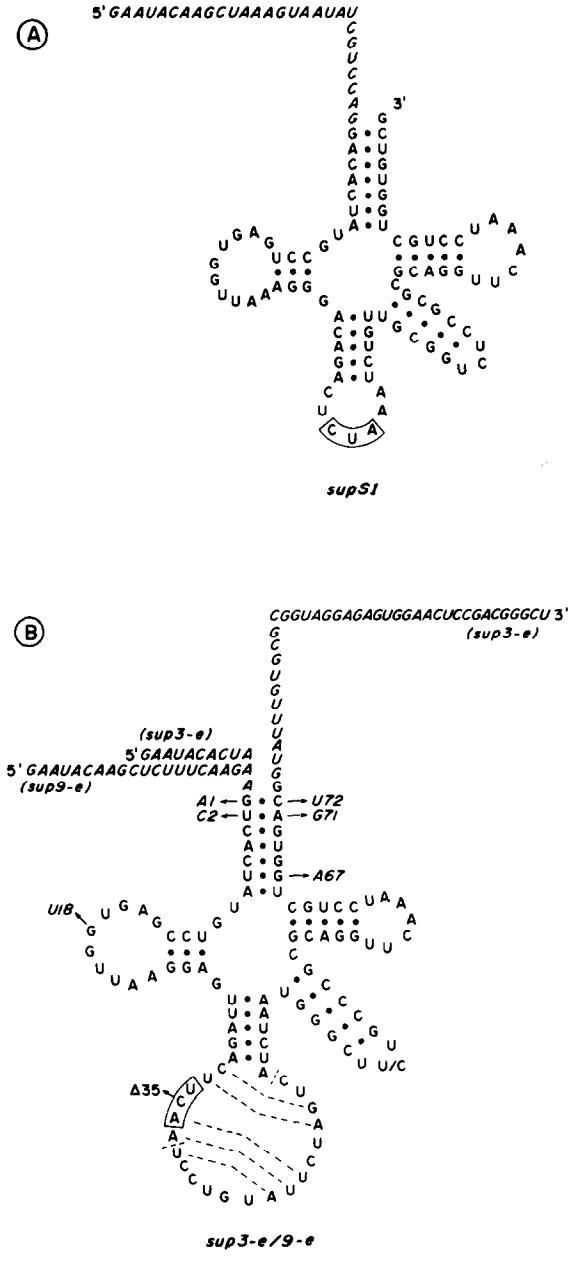

Fig.1. Secondary structure representation of the transcribed tRNA precursors. 5′-leader and 3′-trailer sequences are shown in italics; the anticodons are boxed, and the intervening sequences are shown between dashed lines. (A) The sequence of the *supS1* transcript. (B) The sequence of the *sup3-e/9-e* transcripts. The two genes differ by a U to C substitution at the tip of the extra arm and the 5′-flank. The positions of the *sup3-e* mutations are indicated by arrows. Both *supS1* and *sup9-e* transcripts end just short of the CCA found in mature tRNA.

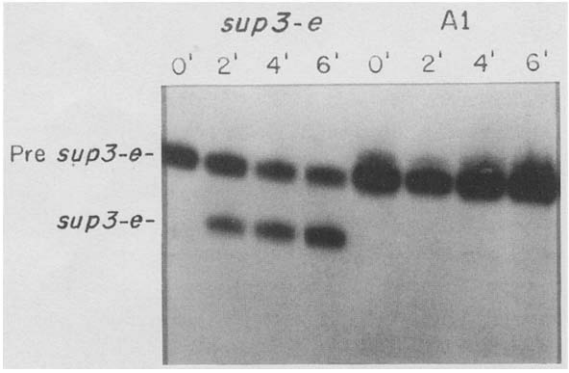

Fig.2. Autoradiogram of RNase P cleavage of the *sup3-e* precursors with altered acceptor stem sequence. Labeled transcripts were synthesized in vitro and were subjected to cleavage with RNase P (see section 2). Reactions were carried out at 37°C in 70 μl volumes containing 1.35 mM substrate and 0.14 μg protein. 20 μl aliquots were removed at 2 min intervals. Reactions were quenched and reaction rates determined as described in section 2.

summarized in table 1. Clearly, disruption of the two base pairs proximal to the cleavage site reduces cleavage by *S. pombe* RNase P significantly. Of the two double mutants tested (A_1U_{72}, C_2G_{71}), only A_1U_{72} was processed at a rate equal to the parent *sup3-e* precursor (table 1). In agreement with the results obtained for the *S. cerevisiae* enzyme [2], restoration of the penultimate base pair in the acceptor stem (C_2G_{71}) did not restore wild-type processing efficiency. This indicates that simple maintenance of base pairing interactions in the acceptor stem is not sufficient for maximal rates of RNase P action.

A base deletion from the anticodon loop, (Δ_{35}), dramatically reduces the rate of 5′-end maturation (table 1). While disruption of potential Watson-Crick base pairing in the anticodon/intron loop is known to inhibit the rate of RNase P cleavage in *S. cerevisiae* [3,4], this explanation is not likely to apply here since position 35 does not take part in base pairing (at least according to the proposed scheme [3], see fig.1). Nevertheless, the deletion probably causes a significant alteration in precursor tRNA structure: the sequence created by the deletion (CUUAAUC) is complementary to part of the D-loop. This competing pairing interaction may underlie the reduced susceptibility of this precursor to RNase P cleavage.

The mutation in the D-loop (U_{18}) does not affect RNase P efficiency. This is an interesting result because of the sequence invariance at this position (and at the adjacent position, nucleotide 19) and the involvement of these bases in universally conserved tertiary interactions with bases in the T-loop. Two mutations in the D-loop of *sub3-e* are known (A_{19}, G_{22}), which reduce processing by *S. cerevisiae* RNase P [8]. Since the U_{18} mutation destroys the potential tertiary pair with U_{55} but does not reduce processing, we conclude that loss of this particular interaction does not alter the tRNA structure sufficiently to affect RNase P binding or catalysis.

The A_{67} mutation, like U_{18}, does not affect processing by RNase P (table 2). This mutation in the acceptor stem has been shown to compensate for inactivation mutations in other parts of the tRNA [5]. Our results on the processing of this mutant precursor with *S. pombe* RNase P are in agreement with the in vitro studies using the *S. cerevisiae* en-

Table 1

RNase P processing of precursors derived from *sup3-e* mutants

sup3-e allele	% RNase P processing[a]
sup3-e	32
A_1	0
U_{72}	12
A_1U_{72}	30
C_2	0
G_{71}	7
C_2G_{71}	8
Δ_{35}	5
A_{67}	34
U_{18}	35

[a] Represents % conversion of precursor tRNA into mature product after reaction for 2 min

Table 2

RNase P processing of precursors derived from suppressor $tRNA^{Ser}$ species

Serine suppressor tRNAs	% RNase P processing[a]
supS1	39
sup3-e	26
sup9-e	0
sup9-e with *supS1* flank	0
supS1 with *sup9-e* flank	40

[a] Represents % conversion of precursor tRNA into mature product after reaction for 2 min

zyme [5]. This mutation seems to affect RNase P catalysis only when it is combined with other mutations. Overall, a comparison of the in vitro specificity of the *S. pombe* and *S. cerevisiae* RNase P enzymes reveals no differences.

Of the three serine suppressor tRNAs tested (*supS1*, *sup3-e* and *sup9-e*, fig.1), the *supS1* transcript proved to be the best substrate for *S. pombe* RNase P (fig.3). The *supS1* precursor tRNA is processed at 1.5 times the rate of *sup3-e*, while *sup9-e* is processed to a negligible extent under our assay conditions (table 2). To test whether the nucleotide sequence in the 5′-flank influences processing in these cases, we switched the 5′-flanks on the synthetic *supS1* and *sup9-e* precursors. The new substrates have the same pSP64 leader sequence and adjacent *S. pombe* sequences as in the parent plasmids but have exchanged the 5′-leader sequences of the authentic precursors (see section 2). As table 2 shows, there is no detectable difference in the rate of processing, indicating that a small number (5–6) of nucleotide changes in the 5′-flank, proximal to the site of cleavage, does not interfere with 5′-end-maturation of *supS1* or improve *sup9-e* processing. The unique substrate properties of the suppressor $tRNA^{Ser}$ species are undoubtedly due to differences in the structures of their precursors. The experiments described here and elsewhere [3] suggest that the intervening sequence in *sup3-e* and

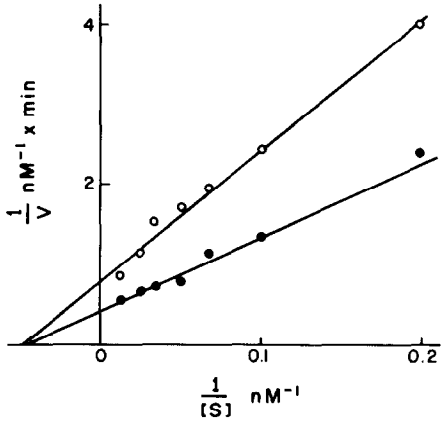

Fig.4. Double reciprocal plots of RNase P activity for *supS1* and *sup3-e* precursor tRNAs. The initial rate of the reaction was measured at different substrate concentrations under standard assay conditions (see section 2). Closed circles, *supS1*; open circles, *sup3-e*. The K_m = 20 nM for *supS1* and *sup3-e*, the V_{max} = 2.5 nM/min for *supS1* and the V_{max} = 1.1 nM/min for *sup3-e*.

sup9-e is largely responsible for the relatively low cleavage efficiency of these substrates. The difference in the rate of cleavage of *sup3-e* and *sup9-e* remains to be explained. Given that these precursors are identical except for flanking sequences and one base in the loop of the extra arm [3], it is possible that the different 5′-leader sequences may affect catalysis as has been found for mitochondrial

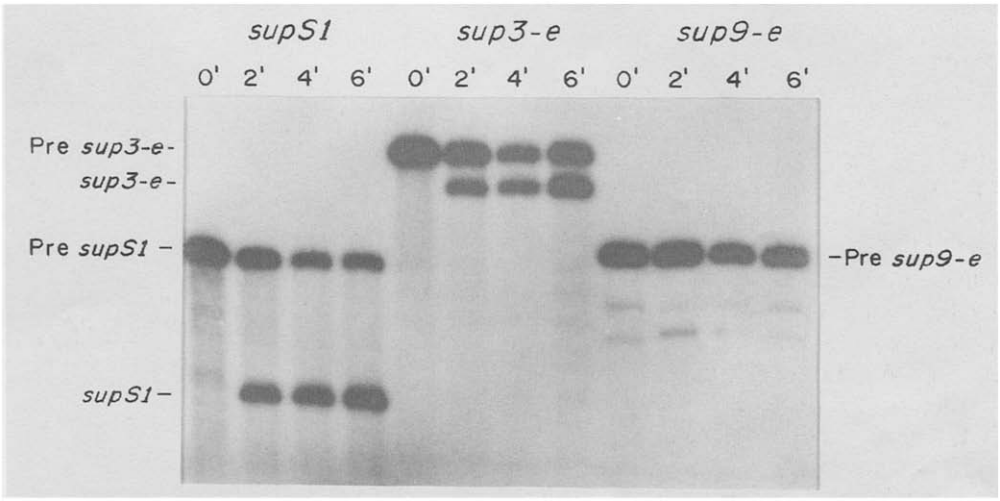

Fig.3. Autoradiogram of the cleavage of *supS1*, *sup3-e* and *sup9-e* tRNA precursors by RNase P. Assay conditions are as in fig.2. Lanes are indicated with the appropriate tRNA precursor used.

tRNA precursors [6]. However, it should be noted that the precursor molecules examined here also differed by the presence or absence of 3′-flanking sequences.

Finally, we calculated the apparent K_m and V_{max} of *S. pombe* RNase P for the *supS1* and *sup3-e* precursors (fig.4). The apparent K_m for both substrates is 20 nM and the apparent V_{max} is 2.5 nM/min for *supS1* and 1.1 nM/min for *sup3-e*. These kinetic constants are similar to those determined for RNase P enzymes isolated from other sources. The K_m (20 nM) compares with 500 nM for the *E. coli* holoenzyme and M1 RNA using a $tRNA^{Tyr}$ precursor [9], 200 nM for the *B. subtilis* P RNA and holoenzyme [10], and 60 nM and 90 nM determined for two substrates of the mitochondrial *S. cerevisiae* enzyme [6]. The V_{max} for the *S. pombe* enzyme is in the same general range as that of the yeast mitochondrial enzyme and the *E. coli* holoenzyme. The fact that *S. pombe* RNase P has the same K_m but a different V_{max} for *supS1* and *sup3-e* precursors indicates that the difference in processing is not attributable to the binding step in the reaction. This observation supports the finding that substrate binding and cleavage are separable functions, as was recently reported for *S. cerevisiae* RNase P based on gel retardation studies [2].

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