A Single Base Change in the Intron of a Serine tRNA Affects the Rate of RNase P Cleavage in Vitro andSuppressor Activity in Vivo in Saccharomyces cerevisiae*

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Differences in the processing of dimeric tRNASer-*tRNAMet precursors derived from the Schizosaccharomyces pombe sup9 wild-type and opal suppressor genes can be attributed to conformational alterations in the tRNASer anticodon/intron domain. A comparison of the patterns obtained upon transcription of the sup9* (wild-type) and sup9-e (opal suppressor) genes in a coupled transcription-processing extract from Saccharomyces cerevisiae reveals that the latter exhibits a greatly reduced efficiency of 5' end maturation and is susceptible to specific endonucleolytic cleavage(s) within the intron. Free energy calculations indicate that these effects coincide with a destabilization of the wild-type anticodon/intron stem and suggest that the predominant sup9-e conformer lacks secondary structure in this region. Evidence in support of this hypothesis was obtained by (i) analyzing the processing of sup9* and sup9-e precursors carrying the intron base substitution, G37:10, which destroys and restores, respectively, the base-pairing potential of the proposed secondary structure and (ii) comparing the strength and temperature sensitivity of sup9-e and sup9-e G37:10 suppression in vivo in S. cerevisiae. The data indicate that the anticodon/intron structure of tRNA precursors can influence the rate of RNase P cleavage in vitro and affect tRNA expression in vivo.

In the genomes of eukaryotes, tRNA genes occur almost exclusively as single transcription units (Sharp et al., 1985). Only five examples are known where tandemly arranged tRNA genes give rise to multimeric tRNA precursors. Two of these are in Saccharomyces cerevisiae and involve a tRNAArg-tRNA*Arg gene pair (Schmidt et al., 1980). The others occur in Schizosaccharomyces pombe at three genetically defined suppressor loci (sup3, sup9, and sup12) and comprise different tRNAser genes, each separated from a tRNA*Ser gene by an identical 7-base pair spacer sequence (Hottinger et al., 1982; Willis et al., 1984; Mao et al., 1980). In all five cases, the synthesis of a dimeric tRNA precursor has been demonstrated using in vitro transcription systems from Xenopus and/or S. cerevisiae (Mao et al., 1980; Schmidt et al., 1980; Willis et al., 1984; Pearson et al., 1985). In addition, Northern analysis of RNA from an S. cerevisiae strain harboring the S. pombe sup9 tRNAser-tRNAMet gene pair has shown that dimeric transcripts are also synthesized in vivo (Willis et al., 1984).

In recent years considerable effort has been applied to the investigation of tRNA precursor processing in eukaryotic systems (for a recent review see Deutscher, 1984). These studies have employed both monomeric and dimeric tRNA precursors as substrates to examine (i) the number and nature of tRNA processing nuclease in different eukaryotic cells, (ii) the tRNA structural requirements of these enzymes, and (iii) whether tRNA precursor processing follows an ordered sequence of events. In the most studied systems (yeast, Xenopus, and Drosophila) the relevant processing enzymes have been identified and in some cases purified (Garber and Altman, 1979; Kline et al., 1981; Nelson et al., 1982; Peet et al., 1983; Greer et al., 1983; Engelke et al., 1985; Pearson et al., 1985; Castano et al., 1985). The removal of 5' leader sequences and the separation of tRNAs in a dimeric transcript appears to be carried out by an endonucleolytic activity similar to Escherichia coli RNase P (Koski et al., 1976; Akaboshi et al., 1980; Kline et al., 1981; Engelke et al., 1985; Pearson et al., 1985). Processing of 3' trailer sequences on the other hand can be accomplished either by a 3'→5' exonuclease as found in yeast (Engelke et al., 1985; Pearson et al., 1985) or by an endonucleolytic 3' pre-tRNA* as in Drosophila (Frendewey et al., 1985) and Xenopus (Castano et al., 1985). Finally, the removal of intervening sequences from those tRNA precursors which contain them requires splicing endonuclease and ligation activities (Peet et al., 1983; Greer et al., 1983). From these and other studies (Deutscher, 1984 and references therein) it is apparent that the processing of all the tRNA precursors in a given eukaryote is probably achieved by a relatively small number of nucleolytic activities. In accordance with this expectation, studies on the processing of mutant tRNA precursors have shown that the endonucleases involved in tRNA maturation (including the splicing endonuclease) recognize overall tRNA conformation and conserved features of tRNA structure rather than specific sequences (Nishikura et al., 1982; Willis et al., 1984; Pearson et al., 1985). In the case of the intron-containing tRNA precursors from S. cerevisiae, this theme has been extended to include conserved structural features outside the mature tRNA domain (Swerdlow and Guthrie, 1984; Lee and Knap, 1985). These precursors can be folded into secondary structures where part or all of the anticodon is base paired with complementary sequences in the intron (Ogden et al., 1984). The formation of such structures is consistent with calculations of free energy minima and has been confirmed for six of the nine precursors (only

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In Vitro Transcription and Processing—The genes encoding sup9 and sup9-e were cloned and sequenced as described by Willis et al. (1984). Whole cell extracts from S. cerevisiae were prepared by the method of Klekamp and Weil (1982) and were used for all transcription and processing experiments. Analytical transcription/processing reactions were carried out under the conditions described by Willis et al. (1984) for 1 h at 25 °C. The products were purified and resolved by electrophoresis on 6% polyacrylamide, 8.3 M urea gels.

Primer-directed Mutagenesis—The methods described by Messing (1983) were used under the conditions of Stewart et al. (1985) with a synthetic 15-mer to introduce the point mutation G57:T10 into the intron of the sup9 and sup9-e genes. M13 mp8 clones of sup9 and sup9-e and DNA-sequencing procedures are described by Willis et al. (1984). The 1.5-kilobase HindIII/EcoRI fragment from M13 mp8 clones containing each sup9 allele was recloned into the centromeric plasmid vector YCp50 for in vitro transcription and transformation (Struhl et al., 1979) into S. cerevisiae strain 3A84 (Schaack and Soll, 1985).

RESULTS

Reduced RNase P Cleavage and Unusual Endonucleolytic Cleavages in the Intron of sup9-e Precursors—Wild-type and opal suppressor alleles of the S. pombe sup9 serine tRNA differ by only a single base pair in the anticodon region (Willis et al., 1984), yet upon transcription in an S. cerevisiae extract pronounced differences appear in the type, number, and abundance of the products (Fig. 1). For sup9-e, five major deviations from the parent sup9 transcription pattern may be distinguished: (i) only two dimeric tRNA precursors (PRE-1 and PRE-2) instead of the usual three, are detectable; (ii) two new products (PRE-3a and PRE-3b) appear; (iii) there is an increase in the amount of PRE-4; (iv) a reduction in the amount of tRNAser+I1V5's species and mature tRNAser; and (v) a change in the number, size, and amount of tRNAser-half-molecules. To understand the nature of these differences we determined the primary structures of the major sup9 and sup9-e products. The results of this work are summarized in Fig. 7. Based on these data and the relative abundance of each species (see Fig. 1), the altered processing of sup9-e precursors may be explained by effects on RNase P cleavage and splicing. Three features of the sup9-e processing pattern indicate that the removal of the 5' leader sequence from these precursors occurs less efficiently than for sup9*: (i) the absence of a third dimeric tRNA precursor (i.e. one matured at the 5'-end of the tRNAser as well as at the 3' end of tRNAser, see Fig. 1); (ii) the accumulation of the 5' leader-containing tRNAser+I1V5 precursor (PRE-4) and the corresponding reduction in the amount of end-matured tRNAser+I1V5 product (Fig. 1); and (iii) the presence of tRNAser 5' half-molecules which contain the 5' leader sequence (half-molecules c and d). In contrast the effect on splicing primarily concerns the appearance of unexpected intron cleavage products. Process-

Fig. 1. Transcription and processing of sup9 and sup9-e in an S. cerevisiae extract. The RNA products obtained were fractionated on a denaturing 6% polyacrylamide gel and visualized by autoradiography. sup9 tRNA precursor products were identified initially by comparison with RNAs of known primary structure (derived from the supl23 tRNA-5tRNA gene (Mao et al., 1980). These assignments were confirmed by RNase T1 fingerprinting, nearest-neighbor analysis, and 5'-end group analysis (Fig. 5 and data not shown).

Portions of this paper (including part of "Materials and Methods, " part of "Results," Figs. 2-6, Tables 1 and 2, and Footnote 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopics are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3469, cite the authors, and include a check or money order for $6.00 per set of photocopics. Full size photocopics are also included in the microfilm edition of the Journal that is available from Waverly Press.
**Fig. 7. Summary of the primary structures of the major sup9-e products.** The figure shows a secondary structure representation of the primary sup9-e transcript (PRE-1). Nucleotides are numbered from the 5'-end of the transcript. Arrows mark the boundaries between flanking sequences or the intervening sequence and nucleotides of the mature tRNAs. The anticodon in the tRNAsec half of the precursor is boxed. Nucleotides of the initial transcript which were deduced from the structural data to be present in the various sup9-e products are indicated below the dimeric precursor. Note that the products PRE-2, PRE-3b, PRE-4, and tRNAa which have been 5' processed may contain part or all of the sequence —CCAAAG.

- Pre-1: 1-188
- Pre-2: 1-181
- Pre-3a: 55-188
- Pre-3b: 55-181
- tRNAa: 1½-c-1-43
- tRNAd: 1½-d-1-42

**Fig. 8. Potential anticodon/intron conformers of sup9+ and sup9-e.** The anticodon stem and loop and intervening sequence of sup9+ and sup9-e precursors are shown schematically in different secondary structure conformations. Solid arrows mark the 5' and 3' boundaries of the intron. The open arrow marks the 5' terminus of PRE-3a and b and tRNAsec half-molecules a and b. The anticodons are boxed and the position of the G37:10 base substitution mutation is shown for both precursors.

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5 I. Willis, unpublished results.
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Fig. 9. Coupled transcription and processing of sup9+ and sup9-e containing the intron base substitution mutation G37:10. Transcription and processing conditions are the same as those used in Fig. 1. The autoradiogram has been overexposed to show the amounts of PRE-3a and b and tRNA^ser half-molecules a–d produced.

We have identified the primary dimeric transcript of the sup9-e tRNA^ser-tRNA^met gene and the major nucleolytic products which result from the processing of this precursor in an S. cerevisiae cell-free extract. From these data and a comparison of the processing patterns of the dimeric sup9+ and sup9-e transcripts, it is clear that sup9-e tRNA^ser precursors (either with or without tRNA^met attached) are less efficiently matured at their 5′-end than their wild-type counterparts and are susceptible to specific endonucleolytic cleavage(s) within the intron by unknown activities in the extract. In order to explain these effects we have proposed that the predominant sup9+ and sup9-e conformers differ in regard to their structure in the anticodon/intron region. Several independent lines of evidence are consistent with this conclusion. Calculations of free energy minima for the proposed structures (Fig. 8) as well as comparative data for intron-containing tRNA precursors from S. cerevisiae (Ogden et al., 1984) argue that sup9+ precursors adopt primarily a 4-base pair anticodon/intron stem structure. A comparison between this structure (Fig. 8) and that deduced for S. cerevisiae pre-tRNA^Ser from structure-probing studies (Swedlow and Guthrie, 1984) is particularly interesting. Pre-tRNA^Ser has been shown to form a 3-base pair extended anticodon stem and hairpin comprising one G-C pair, two A-T pairs, and a 9-nucleotide loop. However, the low thermodynamic stability of this structure together with data obtained using different structure-specific probes suggest that a large unpaired anticodon/intron loop is also present as one of the conformers of pre-tRNA^Ser (Swedlow and Guthrie, 1984). Clearly, the 4-base pair stem and loop of sup9+ precursors (which includes a terminal G-C base pair) is more stable than the corresponding pre-tRNA^Ser structure. However, the base change which generates the sup9-e suppressor eliminates the terminal G-C base pair in this stem (Fig. 8) leaving a potential structure (comprising three A-T pairs) which has a lower stability than that for pre-tRNA^Ser (Ninio, 1979).

To test directly the hypothesis that an unpaired anticodon/intron loop was responsible for the altered processing of sup9-e precursors we introduced the base substitution G37:10 to restore the potential for the 4-base pair anticodon/intron stem. The dramatic effect of the G37:10 mutation on sup9-e precursor processing in vitro and also on sup9-e suppressor activity in vivo confirmed these expectations. Interestingly, the G37:10 mutation did not result in complete reversion to a sup9+ type processing pattern. Furthermore, the effect of the G37:10 mutation on the processing of sup9+ precursors was not as significant as in the wild-type or opal suppressor structures that they adopt.
tempt to mimic. Thus, the equilibrium between alternative structures must be different for each of the four precursors. In a comparison between sup9+ and sup9-e G37:10 (Figs. 8 and 9) different base-stacking energies resulting from the terminal G-C or C-G base pair may account for the differences observed in in vitro processing. To explain the differences between sup9-e and sup9+ G37:10, we suggest that the latter may involve a nontraditional purine-purine interaction (G35 with G37:10) or simply, purine base stacking.

These data have obvious implications for RNase P cleavage. We have previously shown that S. cerevisiae RNase P recognizes conserved features of tRNA structure including the anticodon stem region (Pearson et al., 1985). We now add that the conformation in the anticodon/intron domain of tRNA precursors is also important in determining, in vitro and possibly in vivo, the efficiency of RNase P catalysis. Note that in the in vivo data reflects the overall efficiency of suppressor biosynthesis which, in a comparison of sup9-e and sup9+ G37:10, may comprise the individual contributions of RNase P cleavage and splicing. However, recent in vitro analysis of the splicing of sup3-e (which is homologous to sup9-e) and sup9+ (which is structurally analogous to sup9-e G37:10) precursors shows that these substrates are indistinguishable at this level. Thus, in this case, differential RNase P cleavage may be the major factor contributing to the different suppressor activity observed in vivo. A possible explanation for the reduced efficiency of 5'-end maturation for substrates lacking a stable anticodon/intron stem structure may involve a steric hindrance of RNase P. Thus, we predict that if a stable anticodon/intron interaction cannot be formed, then the rate of RNase P cleavage will decrease above a critical loop size. The unpaired 22-nucleotide loop of sup9-e has clearly already exceeded this limit. Also noteworthy is that the relationship between anticodon/intron structure and RNase P cleavage provides a functional constraint in addition to splicing for the conservation of anticodon/intron complementarity among tRNA precursors in S. cerevisiae.

The identification of the sup3-e-processing products described here also serves to identify the corresponding products of the homologous sup3-e tRNA\textsuperscript{tetra} - tRNA\textsuperscript{met} precursor (Pearson et al., 1985). Surprisingly, a comparison of the processing of these dimeric precursors reveals different rates of 5'-end maturation. sup3-e precursors appear to be more efficiently processed by RNase P than their sup9-e counterparts. This is illustrated by the appearance of three dimeric sup3-e precursors (similar to sup9+), and by the relative amounts of 5'-flanked and 5'-matured tRNA\textsuperscript{tetra} - tRNA\textsuperscript{met} precursors (compare Fig. 1 to Pearson et al., 1985, Figs. 2 and 3). These different rates of processing must occur in response to the nature of the 5' leader sequence or to the base at the tip of the extra arm since these are the only differences between sup3-e and sup9-e tRNA\textsuperscript{met} precursors. Since the analysis of a number of point mutations in the extra arm of both sup3-e and sup9-e has shown that the conformation of this region is unimportant for RNase P cleavage, we favor the former explanation. The availability of intergenic convertants in which the 5' flanking sequences of sup3 or sup9 have been transferred to the sup12 tRNA\textsuperscript{tetra} - tRNA\textsuperscript{met} gene will allow us to examine these possibilities (Amstutz et al., 1985; Heyer et al., 1986).

The data described here together with a time course experiments have been used to elaborate a preferred order of nucleolytic processing steps for the sup3-e tRNA\textsuperscript{tetra} - tRNA\textsuperscript{met} precursor (Pearson et al., 1985). Similarly, a subcellular extract from S. cerevisiae has been used to examine the processing of the tRNA\textsuperscript{tetra} - tRNA\textsuperscript{met} precursor (Engelke et al., 1985).

In both studies the removal of the spacer sequence between the two tRNAs was shown to be carried out by a 3'→5' exonuclease activity. In the latter case that activity was partially purified. Evidence is now available which suggests that such a 3'→5' exonuclease is responsible for spacer sequence removal in vivo. This is based on the results of Northern analyses of RNAs from S. cerevisiae strains transformed with different alleles of sup3-e or sup9-e. High resolution Northern blots reveal the same ladder-like pattern spacer sequence removal as is seen in vitro. Furthermore, Northern analysis of 29 suppressor-inactive mutant alleles (Willis et al., 1984; Pearson et al., 1985; Heyer et al., 1986) has not produced a single case in which spacer sequence removal is impaired (Willis et al., 1984). We can find no evidence, however, that a 3'→5' exonuclease is responsible for the removal of sequences flanking the 3'-end of tRNA\textsuperscript{met}. In a time course of sup3-e precursor processing no exonucleolytic pattern of bands can be seen between the dimeric tRNA precursors (Pearson et al., 1985). Also, fingerprint analysis of PRE-1 and PRE-2 (Fig. 4) shows that these species do not have heterogeneous 3' termini and might be expected if an exonuclease were involved in their processing. The existence of an endo-nucleolytic 3' pre-tRNase in yeast has been alluded to previously (Frendewey et al., 1985). We have recently found that such an activity is present in the extracts used in this study. We, therefore, suggest that both an endonuclease and an exonuclease are involved in the 3'-end maturation of dimeric tRNA precursors in yeast.

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C. Greer and I. Willis, unpublished results.

V. Chisholm, unpublished results.
Intron Structure of tRNA Precursors Affects RNPase P Cleavage

MATERIALS AND METHODS

Reagents and Plasmid DNA: Ribonuclease (RNase) T1 and T2, tRNAs, and tRNA precursors were purchased from Boehringer-Mannheim. Enzymes and plasmid DNA were obtained from Gibco-BRL and New England Biolabs and from Promega. Restriction enzymes were purchased from New England Biolabs and Pharmacia-LKB.

Preparation of RNA Preparation of Isolated and Cleaved tRNA Precursors: Total RNA was isolated from E. coli cells by the guanidinium thiocyanate/phenol/chloroform method (Chirgwin et al., 1979). Isolated RNA was analyzed on a 5 M formaldehyde agarose gel (Mandel and mall, 1978) and stained with ethidium bromide. The RNA was then precipitated and dissolved in water.

RESULTS

In vitro Processing of tRNA Precursors: tRNA precursors were isolated and purified by the procedure described in Materials and Methods. PCR-1 and 2 were isolated from soluble tRNA precursors by the procedure described in Materials and Methods. RNA from the soluble tRNA precursors was isolated by the guanidinium thiocyanate/phenol/chloroform method (Chirgwin et al., 1979). Isolated RNA was analyzed on a 5 M formaldehyde agarose gel (Mandel and mall, 1978) and stained with ethidium bromide. The RNA was then precipitated and dissolved in water.

Primary Structure Analysis of tRNA Precursors: The RNA species identified in Fig. 1 were labeled in preparation for sequencing (Materials and Methods). RNA-1 and 2 were isolated from the soluble tRNA precursors and sequenced by the dideoxy method (Sanger et al., 1977). RNA-3 was isolated from the soluble tRNA precursors and sequenced by the dideoxy method (Sanger et al., 1977). RNA-4 was isolated from the soluble tRNA precursors and sequenced by the dideoxy method (Sanger et al., 1977).

Fig. 2: Isolated tRNA precursors were isolated and purified by the procedure described in Materials and Methods. RNA-1 and 2 were isolated from soluble tRNA precursors by the procedure described in Materials and Methods. RNA-3 was isolated from the soluble tRNA precursors and sequenced by the dideoxy method (Sanger et al., 1977). RNA-4 was isolated from the soluble tRNA precursors and sequenced by the dideoxy method (Sanger et al., 1977).

Fig. 3: The fingerprint of RNA-1 is shown in Fig. 3. The fingerprint of RNA-2 is shown in Fig. 3. The fingerprint of RNA-3 is shown in Fig. 3. The fingerprint of RNA-4 is shown in Fig. 3. The fingerprint of RNA-5 is shown in Fig. 3. The fingerprint of RNA-6 is shown in Fig. 3. The fingerprint of RNA-7 is shown in Fig. 3.

TABLE I: Identification of RNA-1 and RNA-2

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<thead>
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Fig. 4: The fingerprint of RNA-1 is shown in Fig. 4. The fingerprint of RNA-2 is shown in Fig. 4. The fingerprint of RNA-3 is shown in Fig. 4. The fingerprint of RNA-4 is shown in Fig. 4. The fingerprint of RNA-5 is shown in Fig. 4. The fingerprint of RNA-6 is shown in Fig. 4. The fingerprint of RNA-7 is shown in Fig. 4. The fingerprint of RNA-8 is shown in Fig. 4.

Fig. 5: The fingerprint of RNA-1 is shown in Fig. 5. The fingerprint of RNA-2 is shown in Fig. 5. The fingerprint of RNA-3 is shown in Fig. 5. The fingerprint of RNA-4 is shown in Fig. 5. The fingerprint of RNA-5 is shown in Fig. 5. The fingerprint of RNA-6 is shown in Fig. 5. The fingerprint of RNA-7 is shown in Fig. 5. The fingerprint of RNA-8 is shown in Fig. 5.

Fig. 6: The fingerprint of RNA-1 is shown in Fig. 6. The fingerprint of RNA-2 is shown in Fig. 6. The fingerprint of RNA-3 is shown in Fig. 6. The fingerprint of RNA-4 is shown in Fig. 6. The fingerprint of RNA-5 is shown in Fig. 6. The fingerprint of RNA-6 is shown in Fig. 6. The fingerprint of RNA-7 is shown in Fig. 6. The fingerprint of RNA-8 is shown in Fig. 6.
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Fig. 4: Fingerprint analysis of in vitro transcripts. RNase TI fingerprints are shown for PRE-1, PRE-2, PRE-3b, and PRE-4. Fingerprints are identified in the upper right corner of each panel. The nucleotide used to label each RNA species is shown in brackets. First and second dimensions of the fingerprint are indicated in the lower left corner of the figure. Numbered spots correspond to oligonucleotides present in [α-32P]GTP-labeled PRE-1 (Fig. 3 and Table 1). New oligonucleotides are identified alphabetically (see Table 2).

TABLE 2
Identification of RNase TI Oligonucleotides not Represented in PRE-1

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>[α-32P]GTP</th>
<th>Products of RNase A/</th>
<th>Deduced Sequence*</th>
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<tr>
<td>B1</td>
<td>G or U</td>
<td>A.U.A.GF</td>
<td>pppC</td>
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<td>G</td>
<td>U.G</td>
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<td>A.U</td>
<td>A.UACC</td>
</tr>
</tbody>
</table>

* - no neighbor.
* a - criteria are the same as in Table 1.
* c - data from Fig. 4.
* d - contaminating A and G from unresolved spot 15 (Fig. 6 and Table 1).

Fig. 5: [α-32P]GTP were digested to completion with RNase A/RNase T2 and fractionated by PEI-TLC in 1.75 M ammonium formate. Lane 1, PRE-1; lane 2, PRE-3b; lane 3, PRE-3b; lane 4, PRE-3b; lane 5, TRNA-A; lane 6, TRNA-A and lanes 6 and 7 contain spot B, which was eluted from RNase TI fingerprints of PRE-1-3 and TRNA-A, respectively. (A) PRE-1 (lanes 1 and 3) and PRE-3b (lanes 2 and 4) labeled with [α-32P]GTP were digested with RNase A/RNase T2 and fractionated by PEI-TLC in 1.75 M potassium phosphate (lanes 1 and 2) and 1.75 M ammonium formate (lanes 3 and 4).
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Fig. 6: Fingerprint analysis of sup* transcripts and sup- tRNA^Ser half molecules. The conventions used for the orientation and identification of each fingerprint and the oligonucleotides therein are the same as those used in Figs. 3 and 4.