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Substrate recognition by RNase P and by the catalytic M1 RNA: identification of possible contact points in pre-tRNAs

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Modified bases were introduced into pre-tRNAs during *in vitro* RNA synthesis or by chemical modification. These RNAs were used as substrates for the catalytic M1 RNA and the RNase P holoenzyme from *Schizosaccharomyces pombe*. The synthetic approach permitted the insertion of 100% m^{7}GTP into pre-tRNAs and this resulted in complete inhibition of the specific 5′ processing reactions. Partially modified RNAs were obtained by chemical modifications of purines and uridines in the pre-tRNAs. This allowed detailed analyses of specific bases excluded in the products. With pre-tRNA^{Ser} and initiator pre-tRNA^{Met}, strong effects were observed in the T arm and weaker effects in the anticodon stem. Only minor base exclusions were detected in the acceptor stem of pre-tRNA^{Ser} and in the D arm of pre-tRNA^{Met}.

Key words: modified bases/ribozyme/tRNA processing

Introduction

Mature tRNAs are obtained only after a series of processing and modification reactions of the primary transcripts (Deutscher, 1984). The 5′ termini are generated by the endoribonuclease RNase P, a ribonucleoprotein (Altman *et al.*, 1988). *In vitro*, the isolated RNA component of eubacterial enzymes can perform precise enzymatic cleavages (Guerrier-Takada *et al.*, 1983; Gardiner *et al.*, 1985). In pre-tRNAs, the sequences of the flanks and the mature tRNA domain vary considerably and the major recognition features must be localized in conserved structural elements of the mature domain (Altman, 1978; Engelke *et al.*, 1985; Willis *et al.*, 1986; Leontis *et al.*, 1988; Nichols *et al.*, 1988; Carrara *et al.*, 1989). Mutation of the conserved trinucleotide sequence UUC in the T arm of pre-tRNA^{Tyr} did not reveal any important Watson–Crick base pairs between this segment and the catalytic M1 RNA (Baer *et al.*, 1988). More information about essential tRNA elements was obtained by the analysis of truncated pre-tRNAs and tRNA-like viral RNAs, where conserved tRNA elements had been removed (McClain *et al.*, 1987; Guerrier-Takada *et al.*, 1988; Green *et al.*, 1988).

A different approach for the analysis of substrate recognition mechanisms is based on the interference of base modifications with the enzymatic reaction. This technique has been used previously for the analysis of RNA processing reactions (Conway and Wickens, 1987; Rymond and Rosbash, 1988; Spacciapoli *et al.*, 1989). With a combination of this method and other approaches, we have shown previously that one guanosine methylation (m^{7}G) at a specific site in pre-tRNA^{Ser} can prevent processing by RNase P holoenzymes and catalytic M1 RNA (Kahle *et al.*, 1990). The modified bases were introduced by *in vitro* RNA synthesis in the presence of m^{7}GTP. We have extended these studies and included a second pre-tRNA, the initiator tRNA^{Met} from *Schizosaccharomyces pombe*. The analysis of chemically modified RNAs allowed a detailed study of specifically affected purines and uridines. This revealed potential areas of contact between the enzymes and the RNA where base modifications had very strong effects, whereas in other segments, only weak effects of base modification were detected.

Results

Effects of 100% modified nucleosides in pre-tRNAs

The term pre-tRNA^{Met} is used for the dimeric tRNA^{Ser}–tRNA^{Met}, because only the 3′ terminal tRNA was analyzed here (Figure 1). It was synthesized in the presence of three unmodified nucleoside triphosphates and m^{7}GTP. In the resulting pre-tRNA, all guanosines were replaced by m^{7}G; these modifications inhibited specific 5′ processing. The pre-tRNA^{Met} was completely degraded in the presence of RNase P or M1 RNA, respectively (not shown).

Detection of potential contact areas in pre-tRNAs

This section briefly describes our experimental approach. Recently, chemically modified RNAs have been used for the identification of nucleotides which are directly involved in mRNA polyadenylation (Conway and Wickens, 1987), mRNA splicing (Rymond and Rosbash, 1988) and maturation of tRNAs by tRNA nucleotidyl transferase (Spacciapoli *et al.*, 1989). In our studies, the 3′ ^{32}P-endlabeled pre-tRNAs were chemically modified prior to cleavage reactions with RNase P or M1 RNA. The modification procedures were initially developed for chemical RNA sequencing. Accordingly, most RNA molecules contain only one modified base and only a minor fraction has more than one. The modified bases are randomly distributed and RNA molecules with all possible modification sites are present. In the subsequent cleavage reaction, all RNA molecules that are still active substrates for the enzyme can be cleaved. Some RNA molecules will contain modified bases at positions that are involved in contacts with the enzyme. If the modifications (partially) disrupt these interactions, the cleavage of these RNA molecules by RNase P activities will be (partially) inhibited. As a result, RNA molecules with inhibitory base modifications will be (partially) excluded from the cleaved products and they will be enriched in the remaining, uncleaved pre-tRNA molecules. With low enzyme levels, only the best substrates with no or only slightly inhibitory base modifications will be cleaved, and the exclusion of inhibitory modified bases will be evident in the small fraction of 5′ processed tRNAs.

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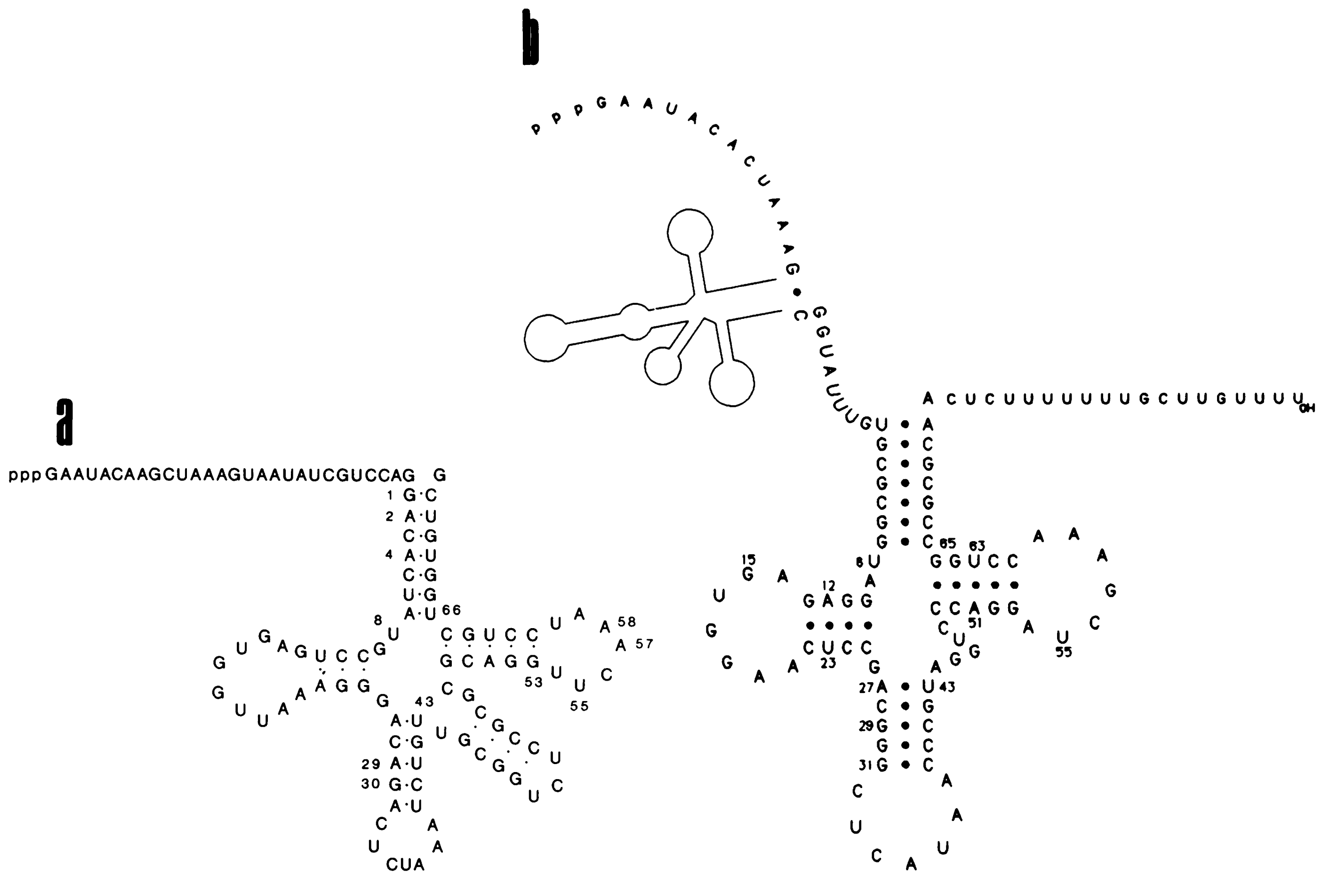

Fig. 1. Cloverleaf models of pre-tRNA transcripts. The numbering is according to the yeast $tRNA^{Phe}$ system (Sprinzl *et al.*, 1989) and numbers were assigned for affected bases only (see Figures 6 and 7). **Panel a**: *S.pombe* pre-$tRNA^{Ser}$ (derived from the gene *supSI*). **panel b**: *S.pombe* dimeric pre-$tRNA^{Ser}$−$tRNA^{Met}_i$ (from the gene *sup3-e*), containing a long 3′ terminal sequence. Only the 3′ terminal $tRNA^{Met}_i$ was analyzed. The intron-containing $tRNA^{Ser}$ is indicated by a line drawing. In the 3′ ^{32}P-endlabeled pre-tRNA, only the 3′ terminal $tRNA^{Met}$ domain could be analyzed; in the text, the term pre-$tRNA^{Met}$ is used for the dimeric precursor.

Only minor accumulation effects would be expected in the major fraction of the still unprocessed pre-tRNAs. With increasing enzyme levels, poorer substrates could also be cleaved. This means that in the processed tRNAs, some of the exclusion of tRNAs containing modified bases can be overcome. Finally, the small amount of remaining pre-tRNAs will contain only molecules with inhibitory base modifications and accumulation effects become more prominent.

Pre-tRNAs and processed tRNAs can be isolated by gel electrophoretic separation. The presence of modified bases can be detected by the sensitivity of these bases to aniline, which causes RNA cleavage. The base exclusion effects can be monitored by comparing quantitative analyses of the nucleoside modifications present in a control pre-tRNA with analyses of the cleaved tRNA products and the remaining, uncleaved pre-tRNAs. The control pre-tRNAs were incubated without enzyme and also gel-purified, to avoid possible artifacts arising from these steps (Spacciapoli *et al.*, 1989). We infer that modified nucleosides, that were (partially) excluded from the cleaved products, interfered with the enzymatic cleavage. This interference of specific base modifications could result from the inhibition of the first reaction step, the binding of the substrate, or by reducing the rate of the subsequent cleavage reaction. The excluded bases could be located at essential contact points between substrate and catalyst, or their modification could disrupt important structural motifs in the pre-tRNA. Based on the following arguments, such structural changes seem unlikely.

In our modification reactions, the purines were N7-alkylated with diethylpyrocarbonate, and in uridines, a treatment with hydroxylamine replaced the oxygen at C4 by an oximido-group. Neither modification interferes with Watson−Crick base pairing (Saenger, 1984) and 5′ processing reactions were still possible with the modified pre-tRNAs. The effect of cytidine modification could not be analyzed because modification of these bases disrupts base-pairing and eliminates all processing reactions. Possible disruptions of tertiary structure interactions will be considered below (see Discussion). The presence and amount of these modified bases can be conveniently monitored by aniline cleavage of 3′ ^{32}P-endlabeled RNAs (Peattie, 1979; Waldmann *et al.*, 1987). In general, for the reaction products analyzed here (Figures 2−5), ~50−80% cleavage was obtained in the preceding processing reactions with M1 RNA and ~20% (pre-$tRNA^{Met}$) to 50% (pre-$tRNA^{Ser}$) with *S.pombe* RNase P. As discussed above, exclusion effects should be more evident for lower amounts of cleaved products with RNase P, whereas with M1 RNA, accumulations in the still unprocessed pre-tRNAs should become more prominent. To evaluate the observed effects, we compared peaks for neighboring bases in densitometer curves of control RNAs and processed RNAs. To differentiate between weak and strong effects, the integrated peak area of an unaffected

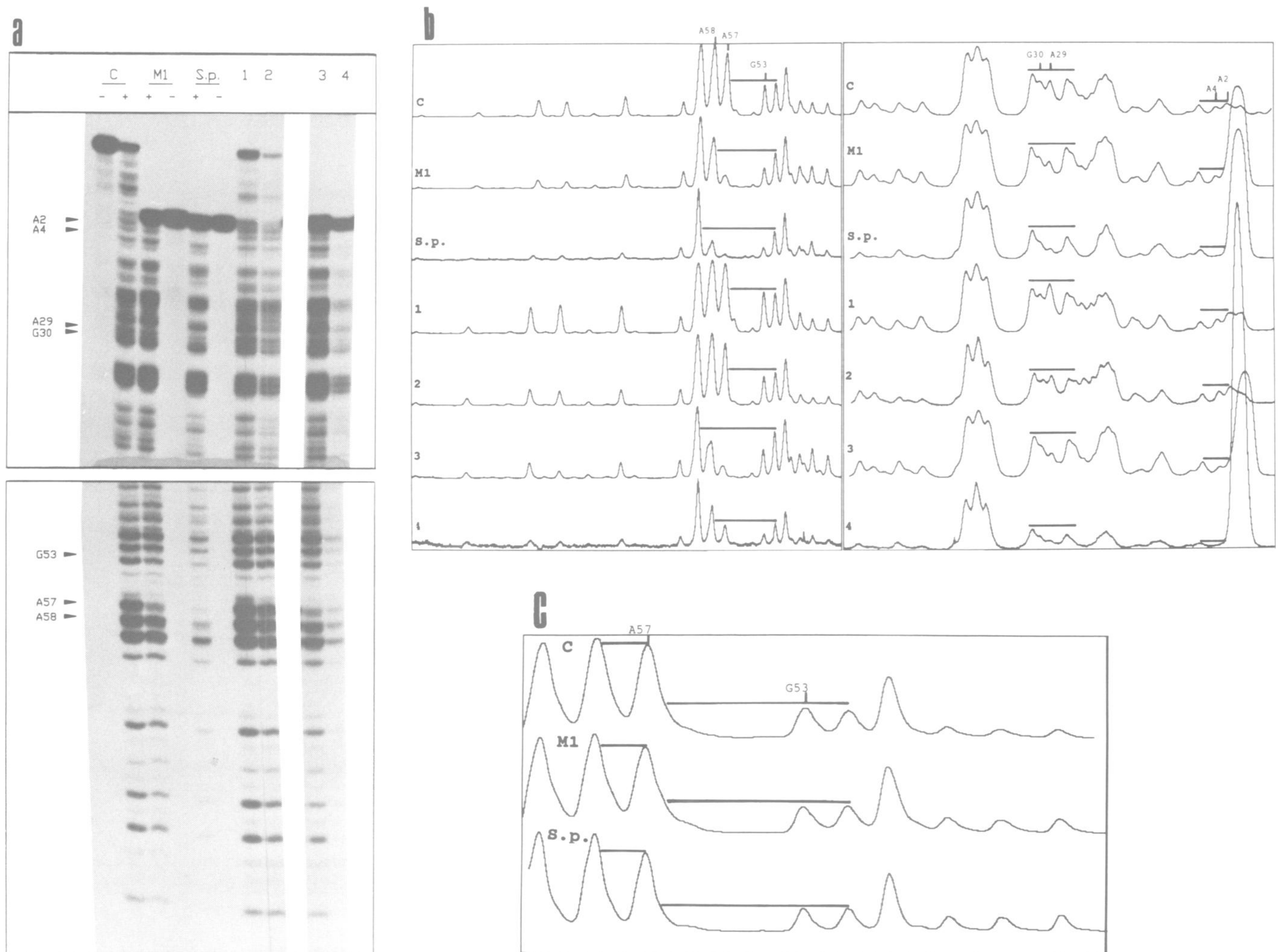

Fig. 2. Base exclusions of alkylated purines in pre-tRNA^{Ser}. **Panel a**: The 3′ ^{32}P-labeled and alkylated RNAs were gel-purified after 5′ processing reactions. The RNAs were applied directly (a − sign above the lanes), to detect degradation products, and after aniline cleavage at the alkylated purines (a + sign above the lanes and lanes 1−4). Lanes C, analysis of the pre-tRNA after a control 'processing' reaction without catalyst; lanes M1, 5′ processed tRNA after incubation of 1 pmol pre-tRNA with 200 ng M1 RNA (1.6 pmol); lanes S.p., 5′ processed tRNA after incubation with 10 μl *S.pombe* RNase P. Only partial processing had occurred and the unconverted pre-tRNAs were analyzed in lane 1 (with M1 RNA) and lane 2 (with *S.pombe* RNase P). In lane 3 (with M1 RNA) and lane 4 (with *S.pombe* RNase P), the 5′ processed tRNAs were obtained with tenfold lower enzyme amounts. The analysis on a denaturing (7M urea), 8% polyacrylamide gel is shown at the top, the lower half was obtained with a 20% gel. Bases are assigned only where exclusions were observed. Here, A2 was not resolved from the strong band of intact, processed tRNA. **Panel b**: Densitometer tracings for comparing with individual lanes. The curves were assigned as the lanes in panel a and only the samples cleaved with aniline are shown. Only relevant bases are assigned and marked by a short vertical line. The horizontal lines should make it easier to compare relative intensities. **Panel c**: Additional curves with tRNAs from processing reactions with the same amounts of catalysts, but 5-fold lower amounts of pre-tRNA (200 fmol).

base was set to 100% in the control RNA and in the processed RNA. Then the relative intensities of the peak for an affected, neighboring base were compared. To serve as a guideline, we introduced an arbitrary definition of a 'strong' effect: the relative peak area for the affected base in the processed RNAs should be <60% of the value for this base in control RNA. All results presented below were obtained after three independent experiments with freshly synthesized pre-tRNAs.

Potential contact points in pre-tRNA^{Ser}

The results with alkylated purines are shown in Figure 2, and those with modified uridines in Figure 3. Autoradiograms of sequencing gels are shown in panels a; the corresponding densitometer tracings in panels b allow a more quantitative analysis. This can be illustrated by one example: in Figure 2, no obvious effects were found for the purines A51, G52 and A59. The bands for these bases were used as references in the different lanes and G52 was set to 100%. In the control lane, the peak ratios for A51:*G52*:G53:A57:A58:A59 were 180:*100*:88:268:345:317. After processing with M1 RNA, the corresponding ratios were 160:*100*:54:55:200:280 for lane M1 and 154:*100*:57:41:154:246 for lane 3. After cleavage with *S.pombe* RNase P, the ratios were 166:*100*:52:30:107:279 for lane S.p. and 177:*100*:65:114:208:300 for lane 4. These data substantiate the visual impression that strong exclusion effects were found for A58 and primarily A57. The corresponding peak areas in the processed samples were reduced to ≤60% of the control values. G53 was less severely affected, with a maximal reduction to ~60%. Other results are summarized below.

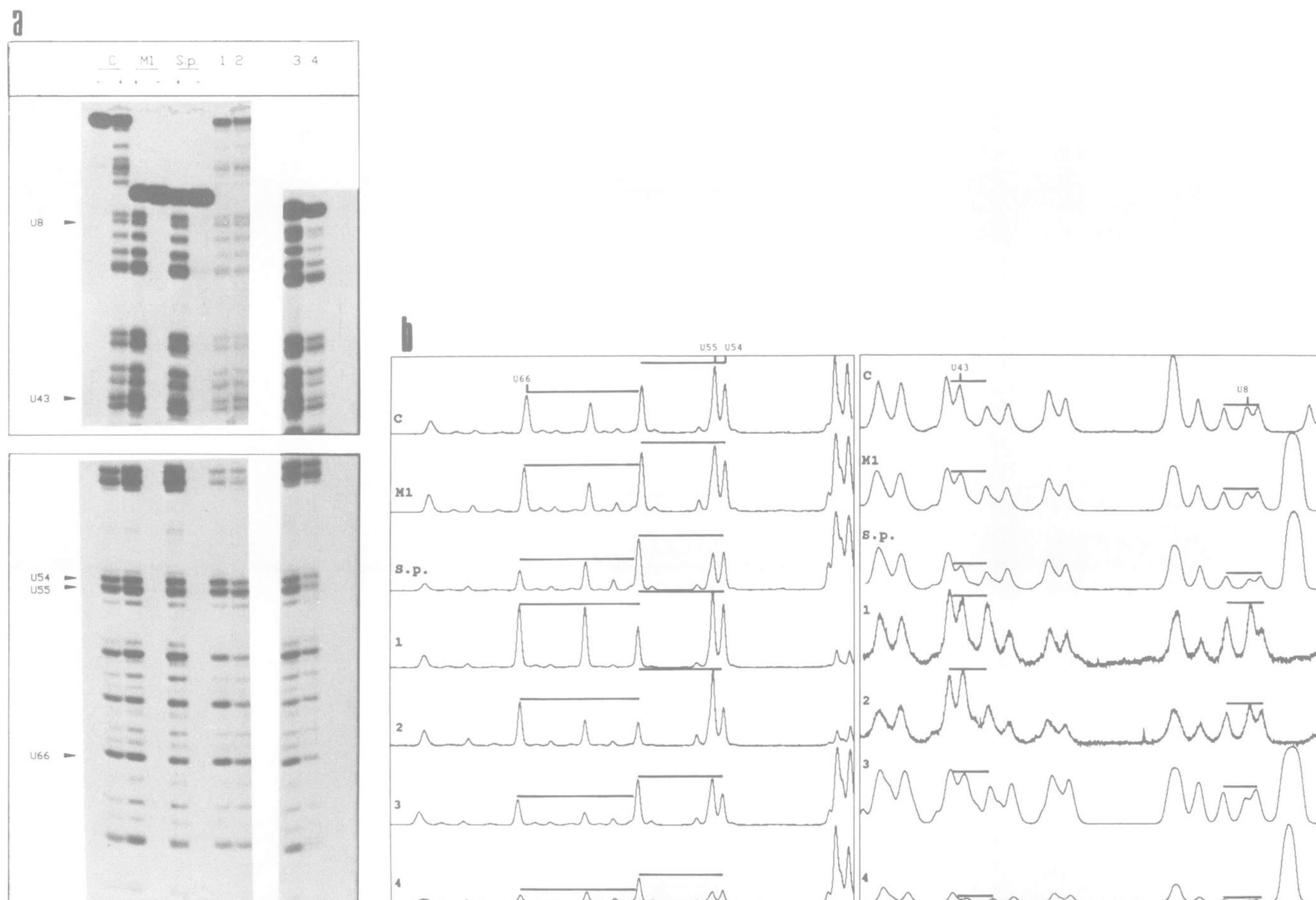

Fig. 3. Exclusions of modified uridines in pre-tRNA^{Ser}. The results are presented as described for Figure 2.

In the acceptor stem, significantly weaker bands for the bases A2, A4 and U66 were found in all processed tRNA samples (Figures 2 and 3; lanes M1, S.p., 3 and 4). The same modified bases accumulated in the corresponding, still unprocessed pre-tRNAs and stronger bands were present (lanes 1 and 2). On the autoradiogram shown here, the band for A2 was not resolved from the strong band for the intact processed tRNA and the exclusion effect was found in an independent experiment (not shown). With M1 RNA, similar behavior was observed for the two uridines U66 and U63 (Figure 3; lanes M1, 1 and 3), but with *S.pombe* RNase P, essentially no effects were visible for U63 (lanes S.p., 2 and 4). This indicates a small difference between both catalysts and U63 was not included in the compiled results (Figure 6).

With U8, which is at the hinge between the two helical segments in the L-shaped tRNA structure, only very weak exclusion effects were observed (Figure 3; lanes M1 and S.p.). However, very high accumulations of U8 were found in the still unprocessed pre-tRNAs (lanes 1 and 2). Therefore, we did not disregard the effects and U8 was marked in Figure 6.

In the anticodon stem, the adenosine A29 was excluded from the cleavage products of both catalysts (Figure 2), whereas an effect with U43 was observed only with *S.pombe* RNase P (Figure 3). The result with the holoenzyme is marked in Figure 6.

In the loop of the T arm, very strong effects were found for U54, U55 (Figure 3) and A57 and less pronounced effects for A58 (Figure 2). G53 in the T stem was also included, but at a reduced level (Figure 2). The expected accumulations in pre-tRNAs were obvious as very prominent bands for U54 and U55 (in Figure 3; lanes 1 and 2), but not for A57 and A58 (Figure 2; lanes 1 and 2). It is possible, that in the already very strong bands in the control (lane C), a further increase in intensity is not detectable on the autoradiogram. As discussed above, it is possible that the exclusions could be overcome by increased catalyst:substrate ratios. This was analyzed with M1 RNA. A higher conversion rate was obtained with this catalyst ($\sim 80\%$ substrate cleavage), and the exclusion effects were less pronounced than with *S.pombe* RNase P ($\sim 50\%$ cleavage). After a further increase of M1 RNA (5-fold higher ratios were used in Figure 2c), these effects essentially disappeared.

In the D arm and the long variable arm, no significant effects were found.

Potential binding sites in initiator pre-tRNA^{Met}

As in the results with pre-tRNA^{Ser}, very weak exclusion effects were found for U8 (Figure 4; lanes M1, S.p.), whereas accumulation was evident in the remaining unprocessed pre-tRNAs (lanes 1 and 2). In contrast to pre-tRNA^{Ser}, significant effects were found in the D arm for A12, G13, A14, G15 (Figure 5) and U23 (Figure 4).

In the acceptor stem, no effects were detected. This may be due to technical difficulties, related to sequence differences. In pre-tRNA^{Met}, U66 was replaced by a

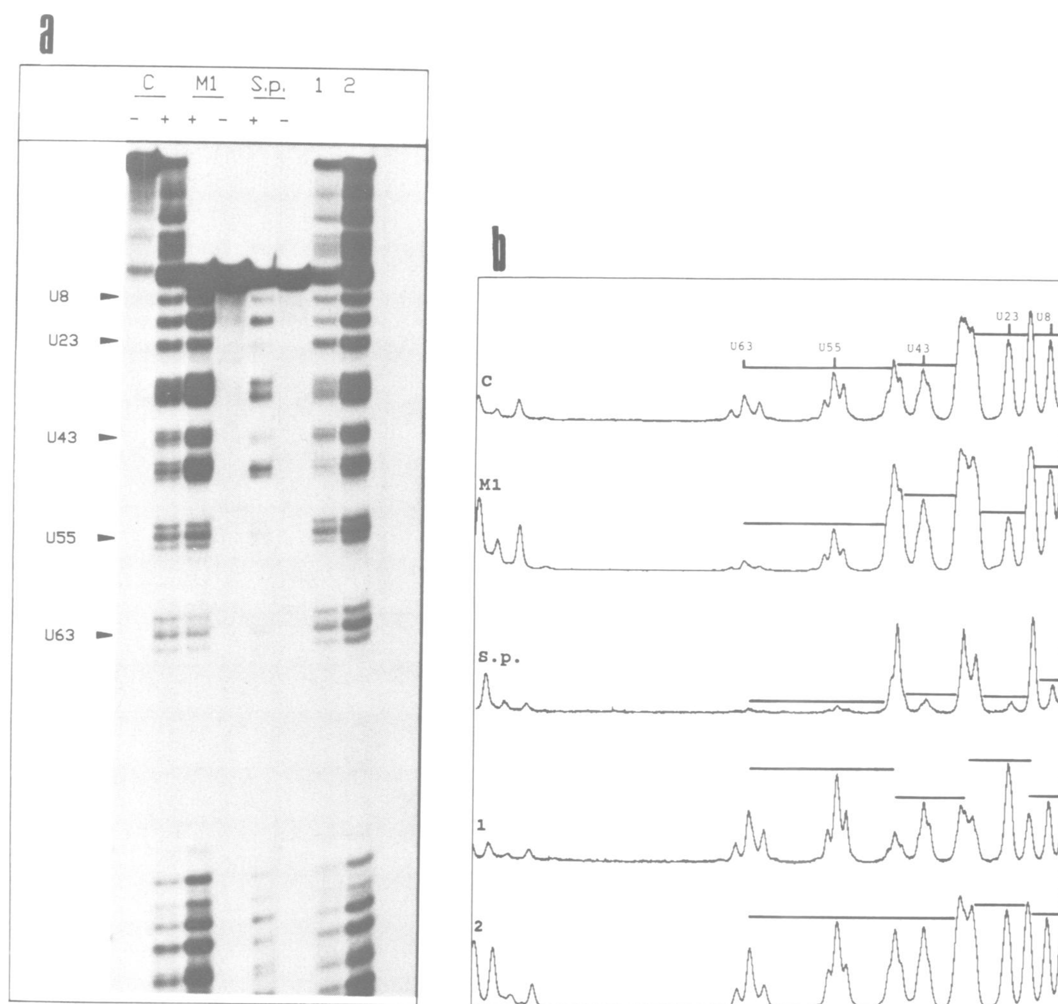

Fig. 4. Uridine exclusions in pre-tRNA^{Met}. The results are shown as described for Figure 2, but lanes 3 and 4 were omitted due to the lower yields of 5′ processed tRNAs. Triple bands occur for the shorter RNA fragments, due to 3′ end heterogeneities caused by imprecise RNA polymerase termination.

cytidine, which could not be analyzed. The adenosines A2 and A4 were substituted by guanosines and weaker bands for G versus A made reductions more difficult to detect.

As in the results with pre-tRNA^{Ser}, bases in the anticodon arm were affected. Here, in addition to U43, pronounced effects were observed for both bases in the pair U43:A27. In contrast to A29 in pre-tRNA^{Ser}, essentially no effect was observed for G29 (Figures 4 and 5).

In both pre-tRNAs, the major exclusions were found in the T arm. In pre-tRNA^{Met}, bases 57 and 58 in the T loop were not affected; an effect was observed with G57 (Figure 5b), but it was not reproducible in independent experiments (e.g., Figure 5c). The total number of affected bases was higher (eight) for the T arm in pre-tRNA^{Met}, including the bases 51–55 (sequence AGGAU) and 63–65 (UGG), than in pre-tRNA^{Ser}, where contacts were found with five bases, positions 53–55 (GUU) and 57–58 (AA). In pre-tRNA^{Met}, the exclusions could not be overcome by increased relative amounts of M1 RNA (Figure 5c). Therefore, we suggest that the inhibition of the specific 5′ processing reactions by modified guanosines (m^7G) was caused by the eliminated contacts with these bases in the T arm of pre-tRNA^{Met}.

Discussion

Two pre-tRNAs were analyzed and showed similar patterns of interference by modified nucleosides. The locations of the affected nucleosides in the three-dimensional tRNA structures are shown for pre-tRNA^{Ser} (Figure 6) and pre-tRNA^{Met} (Figure 7).

The strongest interferences were found in the T arm of both pre-tRNAs and in the acceptor stem of pre-tRNA^{Ser}. The importance of these domains is supported by data from point mutations (Altman, 1978; Baer *et al.*, 1988; Nichols *et al.*, 1988; Carrara *et al.*, 1989) and by the analysis of minimal pre-tRNA models containing only these two domains (McClain *et al.*, 1987).

Weaker effects were found in the D arm of pre-tRNA^{Met}, but not with pre-tRNA^{Ser}. The different behaviors of class I and class II tRNAs may be due to the presence of a long variable arm in pre-tRNA^{Ser} (see Figures 1A and 6) which could prevent access to the D arm. Effects of mutations in this domain have also been detected in pre-tRNA^{Gln}, pre-tRNA^{Tyr} (Altman, 1978) and pre-tRNA^{Leu} (Leontis *et al.*, 1988).

In our analysis, we found only minor differences between

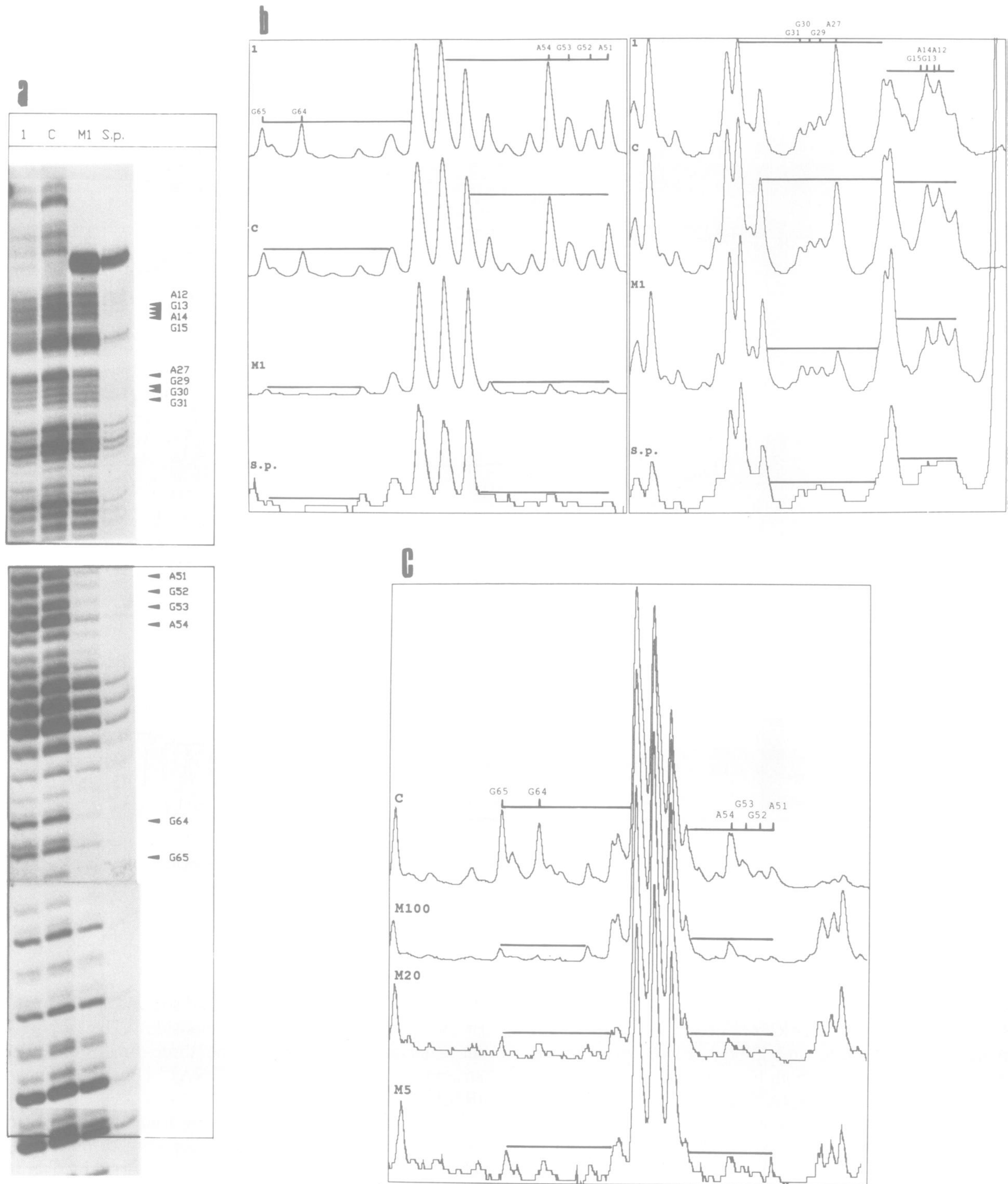

Fig. 5. Excluded purines in pre-tRNA^{Met}. **Panel a**: Lanes C, M1, S.p. and 1 are the same as in Figure 2. Here, the controls without aniline cleavages were omitted. A long (85 cm) 20% polyacrylamide gel was used. **Panel b**: Densitometer tracings of the lanes of panel a. **Panel c**: Additional curves from processing reactions with 10-fold lower amounts of pre-tRNA (100 fmol) and 100, 20 and 5 ng M1 RNA (assigned M100, M20 and M5).

the eukaryotic *S.pombe* RNase P holoenzyme and the catalytic M1 RNA from *E.coli*. In general, the exclusion effects in processed tRNAs were more pronounced with RNase P and stronger accumulations in pre-tRNAs were observed with M1 RNA. As discussed before, this was due to different enzyme ratios and higher percentages of cleaved products with M1 RNA (~65%) versus RNase P (~35%).

It should be considered that neither pre-tRNA contains the 3′ terminal CCA sequence of mature tRNAs. This sequence is important for substrate recognition by prokaryotic RNase P (Guerrier-Takada *et al.*, 1984 and 1989). Direct interactions with this sequence or its indirect effects on other contacts could not be analyzed here.

Recently, Guerrier-Takada *et al.* (1989) have determined contact points between M1 RNA and pre-tRNAs with UV cross-linking experiments. Crosslinking occurs only between

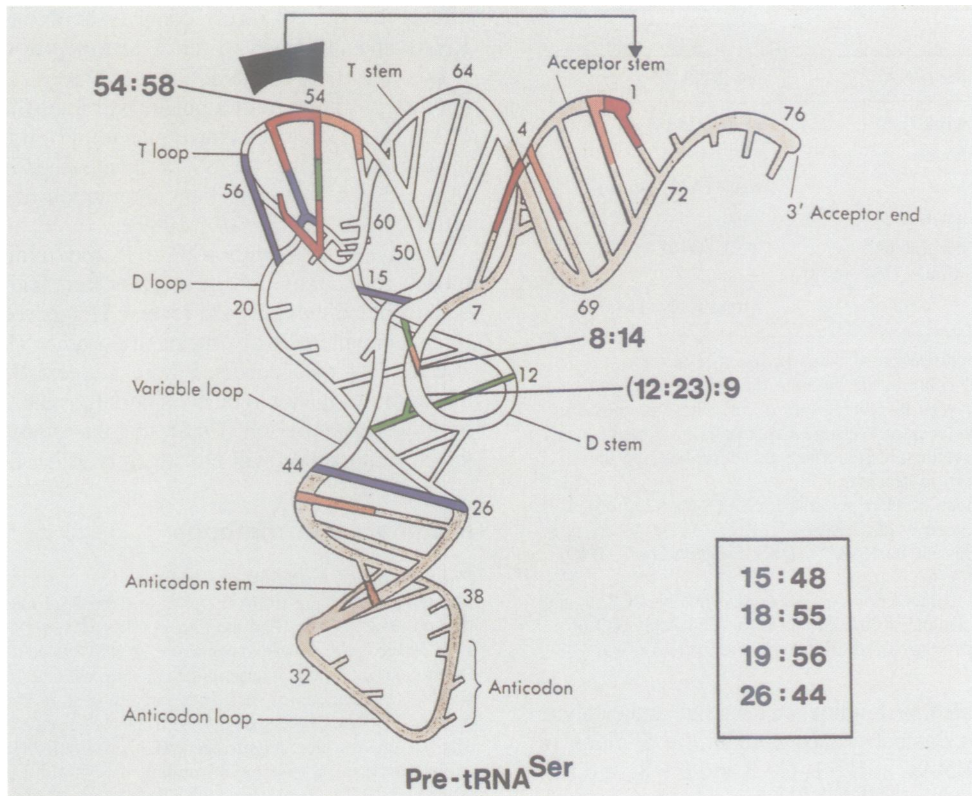

Fig. 6. Affected bases in a schematic three-dimensional model of tRNA^{Ser}. It was drawn according to tRNA^{Phe} (Watson *et al.*, 1987). All affected bases with the RNase P holoenzyme are marked. **In green**, the tertiary interactions are shown and assigned in the model, where N-7 of purines are involved in tertiary hydrogen bonding. **In blue**, the others are shown, and listed in the box. The common interactions of bases (10:25):45 and (13:22):46 are absent in tRNA^{Ser} (Dock-Bregeon *et al.*, 1989) and not shown; no attempt was made to reshape the variable arm. **In red**, bases with strong exclusion effects are marked (see results for a definition); **in pink**, minor effects are shown. U43 was affected only with RNase P, not with M1 RNA. If bases in tertiary pairs (shown as green or blue bars) were affected, part of the bar is shown in red (or pink). The common strong binding at bases 54 and 55 is marked by a black block and an arrow indicates the 'measured' distance to the cleavage site.

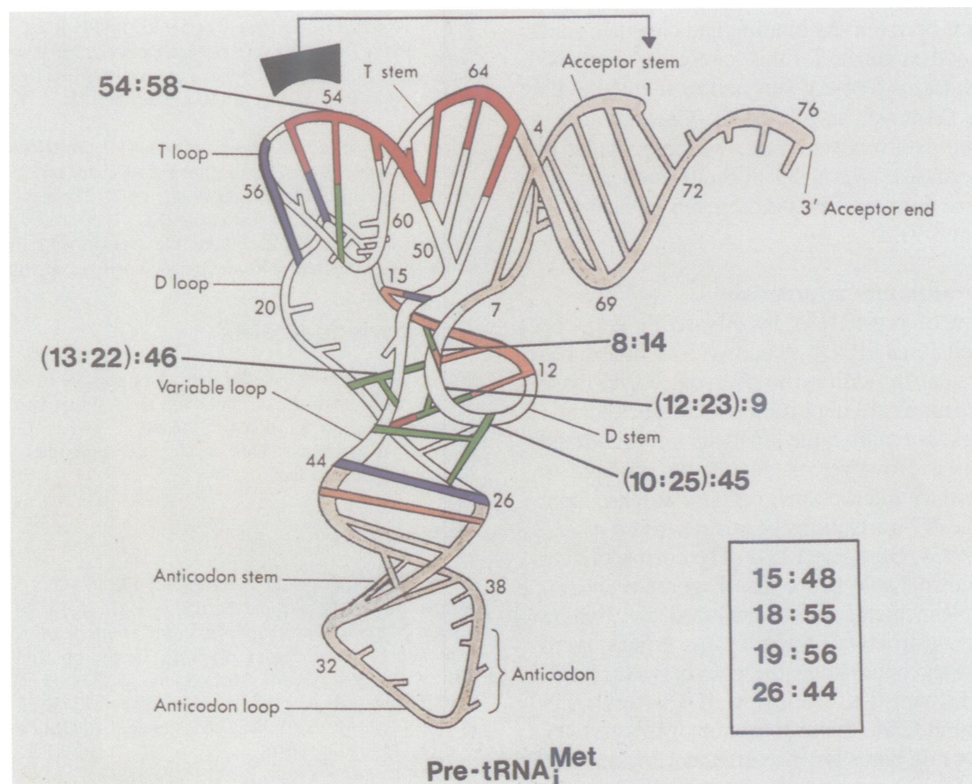

Fig. 7. Base exclusions in a schematic three-dimensional model of pre-tRNA^{Met}. The results are shown as described for Figure 6.

Table I.

Bases	pre-tRNA^{Ser}	pre-tRNA^{Met}
U8:A14	weak (U8)	weak (U8+A14)
G9:(U12:A23)	none*	–
A9:(A12:U23)	–	weak (A12), strong (U23)
(G10:C25):G45	not found**	none
(G13:C22):G46	not found**	weak (G13)$^{+}$
U54:A58	strong (U54+A58)	–
A54:A58	–	strong (only A54)$^{++}$

Observed effects of modification of bases involved in tertiary interactions. In the left column, the bases in the tRNAs are listed, where hydrogen bonds with N-7 of purines occur. These purines are underlined and brackets enclose Watson–Crick pairs in triple interactions. The observed exclusion effects are described and the affected bases are shown in brackets.

*) only a single hydrogen bond is possible for G9(N-7):A23(N-6), if it exists at all (Dock-Bregeon *et al.*, 1989).

**) this interaction is absent in tRNA^{Ser} (Dock-Bregeon *et al.*, 1989) and not shown in Figure 6.

$^{+}$) Unclear geometry: if interaction occurs, does it involve N-7 or not?

$^{++}$) if pair forms, in analogy to the structure for U54:A58, N-7 of both adenosines should be involved. However, the exclusion was observed only for A54.

appropriately oriented pyrimidines in substrate and catalytic RNAs. The major contact points were in the 5′ flank of unprocessed pre-tRNAs, at U−1, C−3 and U−8. A direct comparison with our approach is difficult, because contact points in the cleaved-off 5′ flank were excluded from our analysis.

Apart from general substrate recognition by RNase P, the question of how RNase P determines the specific cleavage site is even more intriguing. Models have been suggested where the active site of the enzyme is in contact with the acceptor stem and tight enzyme binding is established with a 'conserved structural element' in pre-tRNAs. In this way, the physical distance between the binding and cleavage sites is 'measured'. A good candidate for this 'conserved element' is the T arm, which forms a contiguous helical segment with the acceptor stem (Bothwell *et al.*, 1976; Carrara *et al.*, 1989). Based on the data reported here, we suggest that in all pre-tRNAs, the bases 54 and 55 in the T loop are the contact points where tight enzyme binding and 'measuring' occur (Figures 6 and 7).

Important tertiary structure interactions

For the recognition of pre-tRNAs by RNase P, only the general three-dimensional tRNA structure is a conserved motif in all substrates. In addition to Watson–Crick base pairs, tertiary interactions are important structural elements. Our approach does not allow the analysis of individual Watson–Crick pairs. However, it should be possible to detect important tertiary interactions, because at least some are destroyed by the N7-alkylations of purines (Kim *et al.*, 1974; Klug *et al.*, 1974; Saenger, 1984). The corresponding results are compiled in Table I (see also Figures 6 and 7). In almost all examples which could be analyzed, i.e., where N7-alkylations are destructive, weak or no effects were observed. The only exception was the pair between bases 54 and 58 in the T loop. The exclusion of U54 and A58 in pre-tRNA^{Ser} may indicate the importance of this tertiary pair, but the neighboring bases are also affected. In addition, the exclusion of A54 in pre-tRNA^{Met} does not include the invariant A58. Therefore, it is more likely that the observed effects are due to direct contacts between the enzymatic RNAs and the bases in the T arm of pre-tRNAs.

For the triple interactions of bases (13:22):46, the geometry is known for a purine pair at positions 22 and 46. Here, both pre-tRNAs have purine:pyrimidine pairs and it is not clear whether the N7 of the purine is involved or not. In pre-tRNA^{Ser}, this tertiary interaction does not occur at all (Dock-Bregeon *et al.*, 1989).

From the data with RNase P and minimal pre-tRNA models (containing only an acceptor stem and T arm) it could be concluded that only the reverse Hoogsteen pair T54:A58 or its equivalent is of crucial importance (McClain *et al.*, 1987). The data reported here suggest that this is also dispensable. It should be considered, as already pointed out by Reyes and Abelson (1988), that the removal of any single tertiary interaction will not severely affect tRNA structure.

Materials and methods

Preparation of modified pre-tRNAs

The template for pre-tRNA^{Ser}, pSI, was the *Taq*I cleaved plasmid pSSI (Krupp *et al.*, 1986). For the dimeric tRNA^{Ser}–tRNA^{Met}, we used the *Dra*I-cleaved pSP64 plasmid containing an *Alu*I–*Bam*HI fragment of *sup3-e* (Nichols *et al.*, 1988). Transcription reactions were performed as described, with the four unmodified nucleoside triphosphates, or with three unmodified nucleoside triphosphates and m^{7}GTP (obtained from Sigma), respectively. The RNAs were then gel-purified and 3′ ^{32}P-labeled (Kahle *et al.*, 1990).

For the chemical base modifications, $\sim 10^5 - 10^6$ c.p.m. of the 3′ ^{32}P-labeled pre-tRNAs were treated with diethylpyrocarbonate or hydroxylamine (Waldmann *et al.*, 1987). It was essential to replace the carrier tRNA with 5 μg plasmid DNA, to prevent inhibition of the subsequent 5′ processing reactions.

Processing of pre-tRNAs

The catalysts, *S.pombe* RNase P and M1 RNA (the catalytic subunit of *E.coli* RNase P), were prepared as described previously (Kahle *et al.*, 1990). The pre-tRNAs were chemically modified and ethanol-precipitated several times (Waldmann *et al.*, 1987) and then used directly for 5′ processing. The reactions were performed in 100 μl, using ~200–1000 fmol pre-tRNA and 1–10 μl *S.pombe* RNase P or 5–200 ng M1 RNA, respectively (Kahle *et al.*, 1990). The reaction products were precipitated with 7 μl 3 M sodium acetate (pH 5) and 250 μl ethanol and gel-purified (here, the elution was performed in the presence of 10 μg carrier tRNA) (Kahle *et al.*, 1990).

Analysis of modified and 5′-processed pre-tRNAs

The RNAs were cleaved with aniline and separated on 20% or 8% denaturing polyacrylamide gels (Waldmann *et al.*, 1987). The autoradiograms obtained were analyzed with an Ultroscan XL, Enhanced Laser Densitometer (Pharmacia-LKB). For each lane, the average value was determined for seven slightly shifted (800 μm steps), non-overlapping line tracings.

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