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Genetics

Yeast tRNA precursor mutated at a splice junction is correctly processed *in vivo*

(intervening sequences/RNA processing/yeast tRNA)

DIANE COLBY, PHOEBE STARFIELD LEBOY*, AND CHRISTINE GUTHRIE†

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

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ABSTRACT **Yeast mutants with decreased expression of a $tRNA^{Tyr}$ gene were obtained by selection for functional inactivation of the tyrosine-inserting ochre suppressor *SUP4* and subsequent screening for production of the tRNA gene product *in vivo*. One mutant with reduced suppressor activity was characterized by a decreased quantity of the suppressor-specific tRNA; a precursor to this tRNA, matured at both 5′ and 3′ termini but still containing a 14-nucleotide intervening sequence, was present in an amount >7-fold that in the parent. By RNA sequence analysis of the accumulated precursor, we have identified the mutation as an A→G transition at the 5′ splice junction. Similar analysis of the mature tRNA produced in this mutant demonstrated that the intervening sequence was accurately excised. We conclude that the specific sequence of nucleotides at this splice junction affects the efficiency but not the fidelity of processing.**

Many eukaryotic genes are not colinear with the mature, functional products that they encode (for a review, see ref. 1). In the yeast *Saccharomyces cerevisiae*, a subset of tRNA genes, including those for $tRNA^{Tyr}$, $tRNA^{Phe}$, $tRNA^{Ser}_{UCG}$, $tRNA^{Trp}$, and $tRNA^{Leu}_3$, contain intervening sequences (IVS) (2–8). Precursors with these additional internal nucleotides have been identified (4–6) in a temperature-sensitive mutant of yeast with an unknown defect in RNA metabolism (9). These molecules are mature at both 5′ and 3′ termini, suggesting that IVS excision is a late step in the biosynthetic pathway.

A first step in understanding the biological significance of tRNA splicing is the identification of those features of the precursor that determine the accuracy and efficiency of IVS processing. A comparison (8) of primary and proposed secondary structures of five such precursors reveals large variations in IVS size (13 to ≈60 nucleotides) and sequence. In striking contrast is the conserved location of the IVS relative to the mature sequences: in all cases it is found one nucleotide in the 3′ direction from the anticodon. Furthermore, each of the precursors can be fit to a common secondary-structure model in which the anticodon is base-paired to a complementary sequence within the IVS. With the exception of this "extended anticodon stem," all other characteristic features of the cloverleaf are maintained. This picture is consistent with a mechanism in which the specificity of the splicing reaction is determined by recognition of common structural features within conserved portions of the precursor.

To test these predictions we have used a genetic approach. By analogy to results from prokaryotic systems (10), selection for reduced activity of a nonsense suppressor tRNA should yield a high proportion of mutations within the tRNA structural gene that generate defective substrates for processing. The *SUP4* locus, one of eight genes in *S. cerevisiae* that code for the cytoplasmic species of $tRNA^{Tyr}$ (11, 12), includes a 14-base-pair IVS. The ochre suppressor *SUP4* arises through a G·C to T·A base pair change in the position corresponding to the 5′ nucleotide of the anticodon (2). Mutants selected for decreased suppressor activity were screened for production of the precursor and mature *SUP4* gene product *in vivo*. In one such mutant, the IVS-containing precursor was present in an amount >7-fold that in the parent. By RNA sequence analysis of the accumulated precursor, we have identified the mutation as an A→G transition at the 5′ splice junction. Similar analysis of the mature suppressor tRNA (present in reduced amounts) demonstrated that the intervening sequence was accurately excised. We conclude that this sequence alteration affects the efficiency but not the fidelity of processing.

MATERIAL AND METHODS

Strains. The *SUP4* and ochre-suppressible alleles used in this study were obtained from the Berkeley Stock Center, Berkeley, CA. All strains contain the ochre-suppressible alleles *ade2-1*, *lys1-1*, *his5-2*, and *trp5-48* as well as *can1-100*, an ochre mutation in the structural gene for arginine permease.

Mutant Selection. Cells capable of expressing the *SUP4* $tRNA^{Tyr}$ gene are sensitive to the toxic effects of the arginine analogue canavanine; mutants defective in the function or expression of the suppressor will have a resistant phenotype. Canavanine-resistant colonies were selected as described by Rothstein (13). Only those mutants that showed reduced expression of at least one auxotrophic marker were further considered.

Meiotic Recombination Analysis. Mutations lying within the *SUP4* structural gene were identified by measuring the frequency at which ochre suppression was regenerated by meiotic recombination between the *SUP4* mutation, at the first (5′) nucleotide in the anticodon, and the anti-suppressor mutation. Individual diploid colonies produced by matings of mutants with sup^+ strains were replica-plated to sporulation plates (14). After 3 days the sporulated cells were replica-plated to media lacking histidine, tryptophan, or lysine. Control (sup^+/sup^+) diploids gave rise to colonies at a frequency of 5 per 10^7 tetrads.

Preparation of ^{32}P-Labeled $tRNA^{Tyr}$ and $PretRNA^{Tyr}$. Individual RNA species were prepared as described (4), except that cells were grown at 30°C and labeled for 1 hr.

RESULTS

The canavanine-resistant mutant a122 was phenotypically ade^- but exhibited residual suppression of the other ochre alleles *lys1-1*, *trp5-48*, and *his5-2*. This argues that the anti-suppressor

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Abbreviation: IVS, intervening sequence.

* Present address: Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104.
† To whom reprint requests should be addressed.

mutation is not a reversion from *SUP4* to *sup*$^+$. Recombination between a122 and *SUP4* could not be reproducibly detected over the background of spontaneously arising ochre suppressors. However, when crossed with other *SUP4* mutants whose genetic distances relative to the anticodon have been determined, a122 gave rise to *SUP4* recombinants at frequencies that allowed us to construct an internally consistent linear map (unpublished data). These findings argue that the a122 phenotype resulted from a single mutation very closely linked to the *SUP4* anticodon.

To determine whether a122 is defective in a step of tRNA expression prior to function of the mature fully processed product, we analyzed RNA from mutant and parent cells labeled for 1 hr with ^{32}P. Two-dimensional gel electrophoresis (cf. ref. 4) yielded precursor to tRNA^{Tyr} (pretRNA^{Tyr}) of ≥90% purity. Quantitating the amount of precursor derived from the *SUP4* locus was complicated by the fact that the corresponding products of the other seven tRNA^{Tyr} loci comigrated with the suppressor species. This problem could be circumvented by exploiting the G→U mutation in the *SUP4* anticodon. With the elimination of this RNase T1-sensitive site, the anticodon of the suppressor was then contained within a single RNase T1 oligonucleotide (P1) 19 residues in length, which could be readily separated

sup$^+$ (5′)A-C-U-G-U-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp (3′)
P2

SUP4 A-C-U-*U*-*U*-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp
P1

from the 15-nucleotide product (P2) of the *sup*$^+$ RNA by homochromatography on DEAE-cellulose. The results obtained with precursor from the *SUP4* parent are shown in Fig. 1*a*. Quantitation of these oligonucleotides (Table 1) indicated that the *SUP4*-specific product comprises ≈10% of the total pretRNA^{Tyr} population, in good agreement with the theoretical prediction of 12.5%.

RNase T1 digestion of pretRNA^{Tyr} from a122 yielded a unique pattern. The *SUP4*-specific product P1 was missing, and two novel oligonucleotides (P4 and P5) appeared in high yield (Fig. 1*b*). This suggests that a122 arose by the creation of an RNase T1 site within the sequence of P1. By sequence analysis of these products (Table 1), the alteration in a122 could be identified as an A→G transition one nucleotide in the 3′ direction from the anticodon. The *sup*$^+$, *SUP4*, and a122 sequences are thus related to each other as follows:

sup$^+$ (5′)A-C-U-G-U-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp (3′
P2

SUP4 A-C-U-*U*-*U*-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp
P1

a122 A-C-U-*U*-*U*-A-G-U-U-U-A-U-C-A-C-U-A-C-Gp
P4 P5

The a122 product P5 was present in 0.63 molar yield relative to the analogous oligonucleotides from the seven wild-type tRNA^{Tyr} loci (Table 1). Because the *SUP4* parent contained P1 in 0.09 molar yield, we estimated that the level of the mutant precursor was increased 7-fold relative to that of *SUP4* pretRNA^{Tyr}.

If the a122 gene were transcribed with the same (i.e., not higher) efficiency as was *SUP4*, this elevated level would suggest that the mutant precursor may be processed with reduced efficiency. Yet because a122 expressed residual suppressor activity, some amount of functional suppressor tRNA must be formed. Moreover, because the a122 mutation involves the phosphodiester bond that is cleaved in the normal splicing reaction, the substituted nucleotide will appear in the mature tRNA if the mutant precursor is recognized with the same fidelity as is the wild-type substrate. The sequences within the anticodon loops of the processed products then would be related as follows:

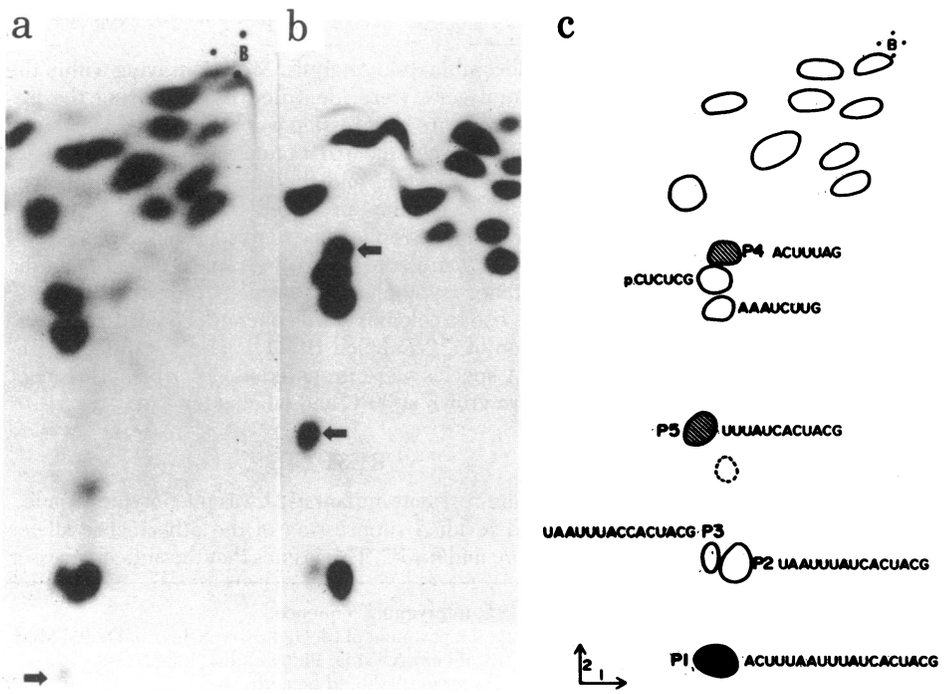


FIG. 1. RNase T1 digestion products of pretRNA^{Tyr}. Precursor from *SUP4* (*a*) and mutant a122 (*b*) were digested with RNase T1 as described by Barrell (15). Oligonucleotide fractionation was by electrophoresis at pH 3.5 on cellulose acetate in the first dimension and by homochromatography on DEAE-cellulose plates at 60°C in the second dimension. Oligonucleotides unique to *SUP4* or a122 are indicated by arrows. (*c*) A composite representation of the autoradiographs shown in *a* and *b* in which the dark circle is the oligonucleotide (P1) specific to *SUP4* pretRNA^{Tyr}, hatched circles are a122-specific, and open circles are common to both species. The dashed circle indicates a product that occurs in variable yield in both *SUP4* and a122 pre-tRNA^{Tyr}; it is distinct in both composition and mobility from P5. Molar yields of the numbered products are given in Table 1. P1 migrated 3 cm from the origin in the second dimension. B, The reference dye xylene cyanol.

Table 1. Composition and molar yields of ribonuclease T1 digestion products of $tRNA^{Tyr}$ and $pretRNA^{Tyr}$

Source of tRNA	Oligonucleotide	Sequence	Molar yield	R_B	Products of digestion with RNase A	Molar yield*
SUP4	P1	A-C-U-U-U-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp	0.09	0.1	A-A-Up,A-Up,A-Cp,Cp,Gp,Up	ND
	P2	U-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp	1.0†	0.22	A-A-Up,A-Up,A-Cp,Cp,Gp,Up	ND
	P3	U-A-A-U-U-U-A-C-C-A-C-U-A-C-Gp		0.22	A-A-Up,A-Cp,Cp,Gp,Up	ND
a122	P2	U-A-A-U-U-U-A-U-C-A-C-U-A-C-Gp	1.0†	0.22	A-A-Up,A-Up,A-Cp,Cp,Gp,Up	ND
	P3	U-A-A-U-U-U-A-C-C-A-C-U-A-C-Gp		0.22	A-A-Up,A-Cp,Cp,Gp,Up	ND
	P4	A-C-U-U-U-A-Gp	1.03	0.70	A-Gp,A-Cp,Up	ND
	P5	U-U-U-A-U-C-A-C-U-A-C-Gp	0.63	0.42	A-Up,A-Cp,Gp,Up,Cp	ND
SUP4	M1	A-C-U-U-Ψ-A-i^6A-A-Ψ-C-U-U-Gp	0.13		A-i^6A-A-Ψp,	0.17
					A-Cp,	0.14
					Gp,Cp,Up	ND
	M2	Ψ-A-i^6A-A-Ψ-C-U-U-Gp	1.0		A-i^6A-A-Ψp,	1.0
					Gp,Cp,Up	ND
	M4	D-D-D-A-A-Gp	1.05		A-A-Gp,Dp	ND
a122	M2	Ψ-A-i^6A-A-Ψ-C-U-U-Gp	1.0		A-i^6A-A-Ψp,	1.0
					Cp,Up,Ψp	ND
	M3	A-C-U-U-Ψ-A-m^1Gp	[0.18]‡		A-m^1Gp,§	0.10
					A-Cp,	0.08
					Up,Ψp	ND
	M4	D-D-D-A-A-Gp + A-Ψ-C-U-U-Gp	1.11		A-A-Gp,	1.04
					A-Ψp,	0.09
					Cp,Gp,Up,Dp	ND

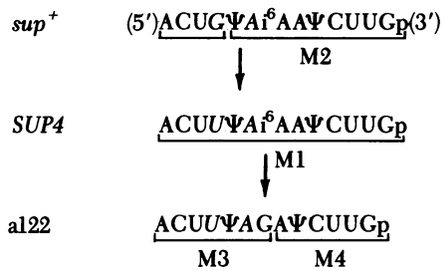
All RNase T1 and RNase A products of $tRNA^{Tyr}$ and $pretRNA^{Tyr}$ from *SUP4*- and a122-containing strains have been quantitated and characterized by modified base analysis and by analysis of redigestion products with RNases A and T1, respectively. We report in this Table only those RNase T1 oligonucleotides that differ among these RNAs. The oligonucleotides listed are the digestion products shown in Figs. 1 and 2. Molar yields of T1 oligonucleotides were calculated by normalizing the radioactivity in each product to the sum of that from the sup^+ products P2 and P3 (in the case of $pretRNA^{Tyr}$) or to the sup^+ fragment M2 (in the case of $tRNA^{Tyr}$). R_B is the mobility relative to that of the indicator dye xylene cyanol. Products of redigestion with RNase A were characterized by their electrophoretic mobility and composition as determined by complete digestion with RNase T2. Modified base analysis by two-dimensional ascending chromatography was performed as described by Saneyoshi *et al.* (16).

* ND, not determined.

† The eight genes coding for $tRNA^{Tyr}$ are identical except for the presence of a sequence polymorphism within the IVS (2, 5).

‡ The molar yield value of M3 is probably an overestimate due to contamination by nearby spots.

§ The RNase A digestion product A-m^1Gp differs in its mobility on DEAE-paper at pH 3.5 (R_B = 0.70) from that of standard A-Gp (R_B = 0.59).



The *SUP4*-specific RNase T1 oligonucleotide M1 could be resolved from the sup^+ counterpart M2 (Fig. 2*a*). This oligonucleotide was not found in the a122 fingerprint (Fig. 2*b*). There was instead a spot (M3) with mobility and composition consistent with the predicted T1 cleavage. The rest of the anticodon was contained in a T1 oligonucleotide (M4) which comigrated with D-D-D-A-A-Gp (D = dihydrouridine). These results (Table 1) are consistent with the conclusion that mature $tRNA^{Tyr}$ arises in a122 by cleavage of the mutant precursor at the same position as wild-type $pretRNA^{Tyr}$—i.e., next to the mutated nucleotide in the 3′ direction.

To estimate what proportion of the a122 precursor was accurately processed, we first compared the yields of mature *SUP4* and a122 $tRNA^{Tyr}$. In the parent strain, *SUP4*-specific $tRNA^{Tyr}$, was present in 14–17% of the amount of $tRNA^{Tyr}$ products from the other seven loci. The amount of a122-specific $tRNA^{Tyr}$, estimated by analyzing the RNase A redigestion products of M3 and M4 (Table 1), was 8–10%. This suggests that the amount of tRNA accurately matured from the mutant precursor was only 30–50% less than that from *SUP4* $pretRNA^{Tyr}$.

Conceivably this reduction can be accounted for solely by a decreased efficiency of processing. Alternatively, some precursor might have been degraded, or inaccurately cleaved at multiple sites, or both. The ratio of mature $tRNA^{Tyr}$ to sup^+ or *SUP4* precursor is 40:1. In a122, however, this ratio was reduced to 7:1, suggesting that the mutant precursor was in fact processed more slowly. Under the conditions of our experiments, mature $tRNA^{Tyr}$ and its IVS-containing precursor were the only two $tRNA^{Tyr}$ gene products detected in significant yield. In the parent strain the two *SUP4*-specific products accounted for 14 ± 2% of the products from all $tRNA^{Tyr}$ genes. Because the products of the a122 allele constituted 11 ± 1% of the total, they cannot be substantially less stable than those of the *SUP4* gene. In summary, then, we estimated that at least 63% (10% vs. 16%) and perhaps as much as 100% of the a122 precursor was not degraded and was accurately processed.

The A→G mutation in a122 $tRNA^{Tyr}$ should have resulted in formation of the dinucleotide A-Gp after RNase A digestion of the RNase T1 oligonucleotide M3 (Table 1). The observed dinucleotide exhibited an altered electrophoretic mobility; RNase T2 digestion revealed that the guanosine residue had been modified to 1-methylguanosine (Table 1). As a result of the a122 mutation, therefore, the modified adenosine (i^6A) adjacent to the anticodon was replaced by a methylated guanosine.

DISCUSSION

We have employed a strategy based on the rationale that mutations which reduce the expression of a suppressor tRNA gene will be recovered in a genetic selection for mutants with decreased suppressor function. In the mutant a122, precursor to

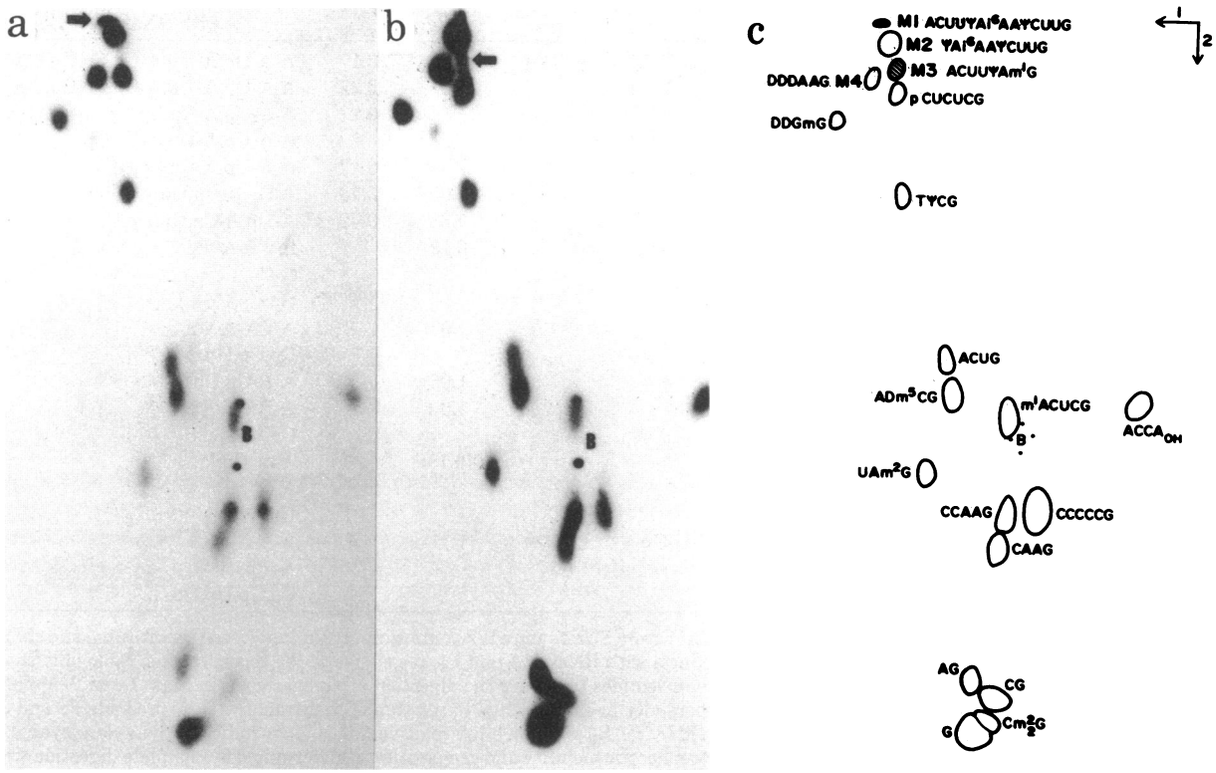


FIG. 2. RNase T1 products of tRNA^{Tyr}. Autoradiographs of RNase T1 oligonucleotides from mature tRNA^{Tyr} of *SUP4* (*a*) and mutant a122 (*b*) are schematically represented in the composite line drawing (*c*). Oligonucleotide fractionation was by electrophoresis at pH 3.5 on cellulose acetate in the first dimension, and on DEAE-paper in 7% (vol/vol) formic acid in the second dimension. All other details are as in the legend to Fig. 1.

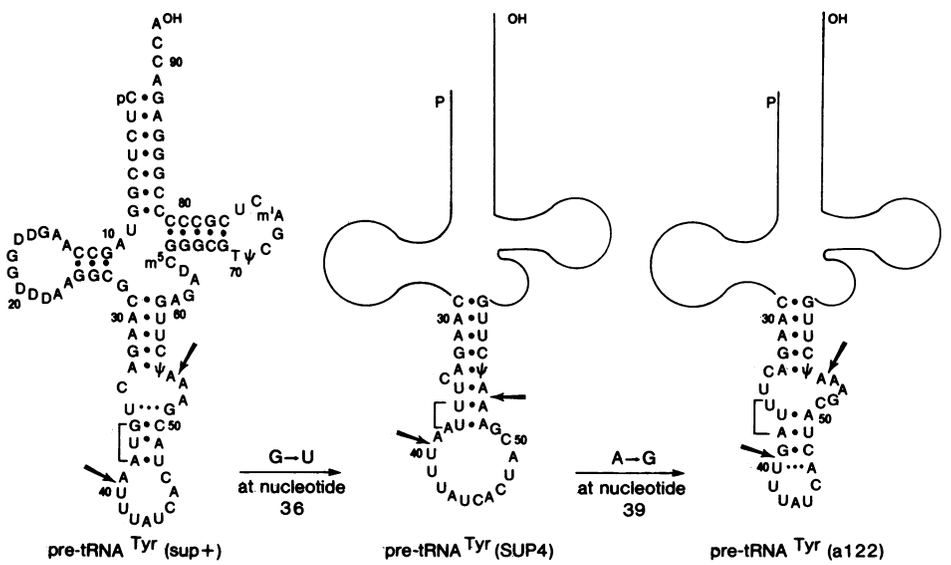


FIG. 3. Nucleotide sequences of pretRNA^{Tyr} from *sup*$^+$, *SUP4*, and a122. The primary sequence of the wild-type precursor was determined by Knapp *et al.* (5). The DNA sequence of the *SUP4* locus has been shown (2) to be identical to that of the *sup*$^+$ allele except for the G→U mutation at position 36. Our data (Table 1) confirm this nucleotide alteration in the RNA sequence and indicate that the sequence of a122 pretRNA^{Tyr} differs from that of *SUP4* only at position 39. For simplicity, the solid lines have been used to represent sequences apparently identical to those in the wild-type precursor. The anticodon is indicated by brackets. Large arrows identify the sites of excision and ligation (see text). The secondary structures shown are those predicted at free energy minima according to rules derived by Tinoco *et al.* (19) and Borer *et al.* (20). They were generated by using a computer program written by J. E. McMahon of Lawrence Berkeley Laboratory. These rules predict the existence of two predominant structures differing only by the presence or absence of the base pair indicated by small dots. The best estimates for the difference in G° between these two forms is 0–1 kcal. These values suggest that the base-paired structure would comprise 30–50% of the population.

SUP4 tRNA is inefficiently processed. Because residual suppressor activity is retained, the genetic phenotype of the mutant could derive from a reduced rate of formation of fully functional tRNA. However, we have observed only a modest (30–50%) reduction in the apparent concentration of the al22 product. Thus, it is also possible that suppressor activity is reduced because of the substitution of m^1G at the position normally occupied by i^6A. A mutant unable to synthesize the i^6A modification has in fact been identified among *SUP4* anti-suppressors (17). It will be of interest to compare the suppression efficiencies of tRNAs containing m^1G, i^6A, or an unmodified A residue adjacent to the anticodon.

The fact that all five species of yeast tRNA known to contain a guanine residue next to the anticodon in the 3′ direction are also modified to m^1G (18) argues that the presence of this modification in al22 is a secondary consequence of the A→G mutation. Because we do not observe m^1G in the IVS-containing precursor, we conclude that 1-guanine methyltransferase recognizes a G residue 3′ to the anticodon, but only when this region is in the conformation of the mature tRNA.

Splicing of pretRNA^{Tyr} from *sup*$^+$ entails the cleavage of phosphodiester bonds between nucleotides at positions 39 and 40 and positions 53 and 54 (Fig. 3). Knapp *et al.* (5) identified these sites in the wild-type precursor by isolation of the half-molecule intermediates; we have inferred that the locations are the same in the *SUP4* product. From sequence analysis of RNase T1 oligonucleotides in tRNA^{Tyr} and pretRNA^{Tyr} that are uniquely present in the al22 strain, we have shown that the correct sites are still recognized in the mutant precursor. We conclude from these results that a unique sequence of nucleotides at the splice junction is not absolutely required for accurate excision of the IVS. The accumulation of al22 precursor in an amount 7 times that of the *SUP4* product, however, argues that the efficiency of this process is significantly affected by the A→G mutation.

In an attempt to understand how a precursor containing this alteration might be recognized as an inefficient substrate, we have compared probable secondary structures of pretRNA^{Tyr} with those of four other spliced tRNA precursors (8). In all cases the two sites of endonucleolytic cleavage occur a fixed distance from the top of the anticodon stem. Whereas in all five wild-type precursors the anticodon can be base-paired in its entirety to a complementary sequence within the IVS, an examination of the most stable structure for *SUP4* pretRNA^{Tyr} (Fig. 3) suggests that this feature is not essential for efficient processing. Similarly, it would appear that the 3′ splice junction can be recognized efficiently, whether it occurs within a base-paired region, as it does in the case of the *SUP4* precursor, or in a loop. Thus, the only feature of secondary structure that distinguishes the mutant precursor is the location of the 5′ splice junction within a region that can exist in a fully base-paired form (see legend to Fig. 3).

It remains to be seen whether this postulated alteration in secondary structure is, in fact, responsible for the impaired processing of the al22 precursor. We have analyzed a number of additional *SUP4* mutants that also exhibit significant reductions in the efficiency with which the IVS is removed from pretRNA^{Tyr}. Based on fine structure genetic mapping, it appears that the majority of these mutations lie within the structural gene but outside (5′ and 3′ to) the IVS. Although sequence analysis of these mutants is still in progress, these results are already reminiscent of the picture that has emerged from prokaryotic processing studies (1, 21): the primary determinants of recognition appear to be comprised of domains of the precursor destined to become the product of the processing reaction. Thus, biologically significant rates of splicing may depend on recognition of secondary and tertiary structural features of the precursor common to those of mature tRNA. These features also may specify the precise sites at which cleavage occurs, because we have shown that the primary sequence at one such site does not alter the fidelity of the reaction. Although further speculation is premature at this time, it is interesting to compare these results with experiments of others (22, 23) that have emphasized the conservation of sequences across splice junctions in mRNA precursors and the importance of maintaining the integrity of these boundaries. Conceivably this points to a fundamental difference between tRNA and mRNA splicing.

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