responsible for these observed differences in seasonal cycles. Nonetheless, we believe that the enhancement of trace gas concentrations in the Southern Hemisphere that is due to emissions from biomass burning in Africa and, to a lesser extent, South America, is the mechanism that determines much of the seasonality of these trace species.

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Identity Elements for Specific Aminoacylation of Yeast tRNA^{Asp} by Cognate Aspartyl-tRNA Synthetase

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The nucleotides crucial for the specific aminoacylation of yeast tRNA^{Asp} by its cognate synthetase have been identified. Steady-state aminoacylation kinetics of unmodified tRNA transcripts indicate that G34, U35, C36, and G73 are important determinants of tRNA^{Asp} identity. Mutations at these positions result in a large decrease (19- to 530-fold) of the kinetic specificity constant (ratio of the catalytic rate constant k_{cat} and the Michaelis constant K_m) for aspartylation relative to wild-type tRNA^{Asp}. Mutation to G10-C25 within the D-stem reduced k_{cat}/K_m eightfold. This fifth mutation probably indirectly affects the presentation of the highly conserved G10 nucleotide to the synthetase. A yeast tRNA^{Phe} was converted into an efficient substrate for aspartyl-tRNA synthetase through introduction of the five identity elements. The identity nucleotides are located in regions of tight interaction between tRNA and synthetase as shown in the crystal structure of the complex and suggest sites of base-specific contacts.

THE CORRECT AMINOACYLATION OF tRNAs by their cognate synthetase is crucial for accurate transmission of genetic information and is determined by certain structural features of the tRNA, which in certain systems include nucleotides in the anticodon loop, acceptor stem, and D-loop (*1*). Regions of contact between yeast tRNA^{Asp} and yeast aspartyl-tRNA synthetase (AspRS) have been previously characterized with chemical and enzymatic footprinting methods (*2, 3*). A high-resolution x-ray structure of this complex (*4*) has confirmed that the anticodon loop and stem as well as portions of the acceptor stem are sites of interaction with AspRS. We describe steady-state aminoacylation kinetics for a series of mutant transcripts of yeast tRNA^{Asp} in order to delineate the determinant nucleotides important for aminoacylation by yeast AspRS.

The aminoacylation kinetics of mutant tRNAs were compared to that of the unmodified transcript of yeast tRNA^{Asp} in which U1-A72 base pair was changed to G1-C72 (Fig 1A); both transcripts have equivalent kinetic parameters for aspartylation as the fully modified molecule (*5*). Since the transcript of *Escherichia coli* tRNA^{Asp} is an equivalent substrate for aspartylation by yeast AspRS as the yeast transcript (Table 1), a number of nucleotide positions could be eliminated as potential identity elements (Fig. 1B). Moreover, single-stranded nucleotides protected in footprinting experiments (*2, 3*), G-U base pairs (*6*), and nucleotides identified by computer sequence analysis (*7*) as specific for tRNA^{Asp} were tested explicitly. For simplicity, the effects of mutations (Fig. 1C) on the steady-state aminoacylation kinetics (Table 1) are described below in terms of the four structural domains of the tRNA molecule.

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Change of position 73 at the 3′ end of the acceptor helix to any other nucleotide resulted in a significant decrease in the specificity constant (k_{cat}/K_m) for aspartylation; thus this position, the discriminator base (*8*), is a determinant for aspartylation. Increases in the Michaelis constant (K_m)(about a five- to sevenfold increase) are similar, but changes in the catalytic constant (k_{cat}) vary significantly. Mutant U73 is the most active, with a decrease of k_{cat}/K_m of only 36-fold, whereas mutant A73 decreases 160- and mutant C73 decreases 200-fold. Position 73 is a determinant in other systems; in particular, G73 is important for the aspartylation of *E. coli* $tRNA^{Asp}$ by *E. coli* AspRS (*9*). The crystal structure of the yeast $tRNA^{Asp}$-AspRS complex suggests tight interactions between the protein and G73, from the major-groove side of this stacked residue. Possible interaction with AspRS at O-6 of G73 on the major-groove side of the stacked CCA extremity may explain the relatively high activity of the U73 mutant; indeed, the uridine presents an identical chemical group (O-4) that may maintain an interaction with the protein on the major-groove side.

Mutations that conserve base pairing (Fig. 1C and Table 1) within the acceptor stem have little effect on aspartylation, showing that these positions are not determinants for aminoacylation by yeast AspRS. Despite the distinct nature of U1-A72 in yeast $tRNA^{Asp}$ (*7*), change to G1-C72 had no effect on aspartylation, in contrast to the importance of this pair in *E. coli* $tRNA^{Gln}$ for recognition (*10*) and aminoacylation (*11*) by GlnRS. However, in the Gln system, the U1-A72 pair is broken upon complex formation, whereas in the yeast $tRNA^{Asp}$-AspRS complex protein contacts are observed in the major groove to this base pair, but base pairing and stacking of the acceptor helix and 3′-CCA are maintained. Mutation of U1-A72 to G1-C72 conserves the same chemical groups in the inner region of the major groove (*12*); the similar interaction of AspRS with these groups in the wild type (U1-A72) and mutant (G1-C72) may explain the lack of an effect on aminoacylation upon mutation. Therefore, direct contact of synthetase with base pairs or single-stranded nucleotides does not necessarily guarantee their role in sequence discrimination.

Changes at any of the three anticodon positions resulted in a significant decrease in aspartylation activity (k_{cat}/K_m decreased 19- to 530-fold); thus these three positions (G34, U35, and C36) are determinants for aspartylation. Changes at position 35 resulted in the largest range of activities. Mutant G35 had only a 19-fold decrease in k_{cat}/K_m, whereas C35 resulted in a 91-fold decrease, and A35 a 530-fold decrease. Chemical groups conserved upon mutation (such as the O-4 of U35 and O-6 of G35) may be sites of direct interaction with AspRS.

The crystal structure of the complex shows that tight interactions occur with the anticodon nucleotides. The increase of K_m for all of the mutations at the three anticodon nucleotides suggests loss of specific interactions in the enzyme-substrate complex. Interestingly, change of nucleotide 37 resulted in little change in aspartylation activity, despite footprinting (*13*) and crystallographic evidence for tight interactions with this nucleotide. The footprinting results, as well as the present resolution of the crystal structure, cannot distinguish between base-specific contacts and those with the ribose-phosphate backbone. The crystal structure shows a major conformational change of the anticodon loop upon binding to the synthetase. The conformational difference between uncomplexed and complexed tRNA, also observed in the crystal structure of $tRNA^{Gln}$ with GlnRS (*10*), emphasizes the role of both tRNA structure and conformational flexibility in recognition by AspRS. Unstacking of the anticodon nucleotides may provide an improved presentation of chemical groups to the synthetase. Whether nucleotide sequence affects this large induced conformational change for productive interaction of yeast $tRNA^{Asp}$ with AspRS remains an important unanswered question.

As in the acceptor stem, all of the changes in the anticodon stem had little effect on aspartylation. U40 was changed to C40 to give a G30-C40 pair with only a twofold decrease in k_{cat}/K_m. Pairwise changes in the G27-C43, G28-C42 base pairs, identified by computer sequence analysis [(7); see figure 4 in (*4*)] as relatively distinct to $tRNA^{Asp}$ among yeast tRNAs, to either C27-G43, C28-G42, or U27-A43, U28-A42 also had virtually no effect on aspartylation. Preliminary results suggest that change of C31-G39 to A31-U39 has little effect on activity (*14*). In the *E. coli* $tRNA^{Asp}$ transcript, the C29-G41 pair is changed to U29-A41. Previous footprinting experiments showed protec-

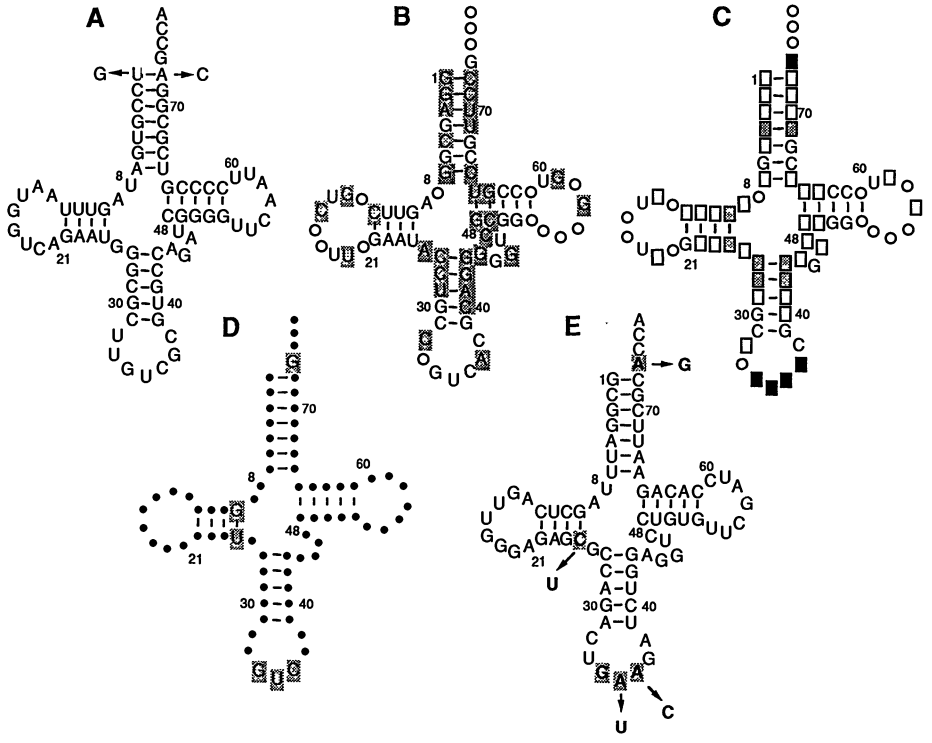

Fig. 1. (**A**) Sequence of yeast $tRNA^{Asp}$ (*25*) transcript showing change of the first base pair; nucleotides are numbered according to (*15*). (**B**) Sequence of *E. coli* $tRNA^{Asp}$ transcript (*26*). Nucleotides that differ from yeast $tRNA^{Asp}$ are shaded. Nucleotides conserved in all tRNAs are indicated by circles. (**C**) Summary of nucleotide positions in yeast $tRNA^{Asp}$ tested for their effect on aspartylation activity. Positions at which other nucleotides were substituted (either three, two, or one) are indicated by solid, shaded, and open squares, respectively. Nucleotides conserved in all tRNAs are indicated by circles. Only those nucleotides not tested by our mutational analysis are shown explicitly. (**D**) Positions of major determinants for aspartylation by yeast AspRS deduced by mutational analysis. (**E**) Sequence of yeast $tRNA^{Phe}$ transcript (*27*) in which the Asp determinants, indicated by arrows, have been integrated at the shaded positions. The $tRNA^{Asp}$ genes were constructed from ten synthetic DNA oligomers (*5, 28*) or were obtained by site-directed mutagenesis by using the procedure of Eckstein (*29*). After transcription with T7 RNA polymerase (*5, 28*) transcripts were purified to single-nucleotide resolution with denaturing polyacrylamide gels.

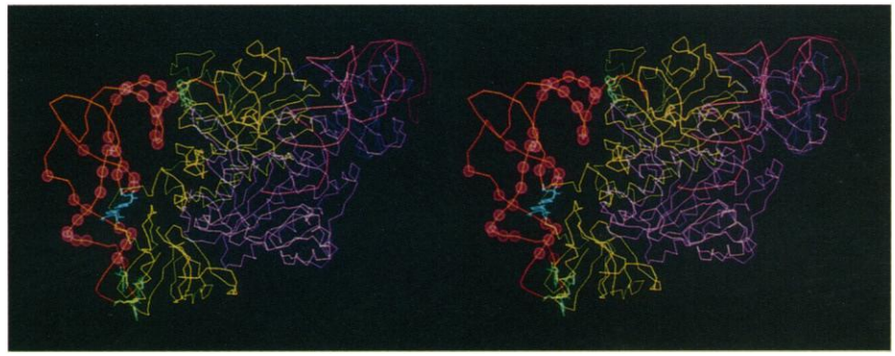

Fig. 2. Stereoview of the complex between the yeast $tRNA^{Asp}$ and AspRS in which determinant nucleotides are highlighted. The α-carbon chains of the two monomeric subunits are yellow and purple. The phosphodiester backbones of the two bound tRNAs appear in red. Determinant nucleotides that had a strong effect on aspartylation are in green. The G10-U25 base pair, which also had an effect, is shown in blue. Phosphates corresponding to nucleotides that were changed without effect on aspartylation are indicated by red spheres. For simplicity these groups are given on only one tRNA.

tions by AspRS at phosphates of G26 to C31 in the anticodon stem (*2*); the N-7 position of G30, located in the major groove of the stem, was also protected by AspRS (*13*). The crystal structure shows interactions in the major groove of the anticodon stem at the base of the anticodon loop, consistent with the footprinting results, although, because of steric or chemical reactivity effects, a greater number of positions are protected in footprinting experiments than are probably in direct contact with the synthetase. In view of the functional data, synthetase contacts with the anticodon stem are probably not base specific.

Although G10 is conserved in almost all elongator tRNAs, a G10-U25 base pair in the D-stem is rare and exists in only 3 of 37 yeast elongator tRNAs (*15*). Mutation of nucleotide U25 to C25 resulted in a reproducible decrease in k_{cat}/K_m (eightfold decrease). This mutation changes G10-U25 to the more common G10-C25 base pair, while in principal conserving the tertiary interaction with G45. The K_m increases by fivefold, whereas k_{cat} decreases less than twofold. Mutation of G10 to A10 allows formation of an A10-U25 base pair but may disrupt the G45 tertiary interaction. The k_{cat}/K_m for this mutant decreased 33-fold mainly because K_m is increased by 28-fold. We conclude that the G10-U25 pair is also an important determinant for aspartylation.

Direct recognition of the G10-U25 pair should occur from the minor-groove side of the D-stem, since the major groove is blocked by the tertiary interaction with G45. The crystal structure suggests possible contact with this region of the D-stem; moreover, footprinting by ethylnitrosourea in the presence of AspRS showed protections of phosphates around G10 but not around U25 (*2*). Recognition may involve the NH_2 group on the guanine, which in a G-U pair is exposed in the minor groove but is hydrogen bonded in a G-C pair (*16, 17*). The large effect on aspartylation of the G10 to A10 mutation is perhaps due in part to the absence of an amino group in the minor groove, although the structure of this mutant is altered (*18*). Furthermore, substitution of a G-C pair may subtly change the local geometry or overall folding of the tRNA (*17*). Changes of other D-stem base pairs did not significantly affect the kinetics of aspartylation. Other changes of the D-loop, variable loop, and T-stem had little effect on aspartylation specificity. The crystal structure of the complex shows that these regions of the tRNA are not in interaction with synthetase, which is consistent with the mutational results.

A total of 44 of the 60 nonconserved

Table 1. Kinetic parameters for aspartylation of yeast $tRNA^{Asp}$ variant transcripts with yeast AspRS. All of the variants were constructed from the G1-C72 mutant, which shows equivalent aspartylation parameters to those of fully modified $tRNA^{Asp}$ and U1-A72 transcripts (*5*). Yeast AspRS was purified as described elsewhere (*24*). Aminoacylation tests were performed in 0.1 M Hepes, pH 7.5, 30 mM KCl, 10 mM $MgCl_2$, 5 mM adenosine triphosphate, and 52 μM L-[^{3}H]aspartic acid as described (*5*). Apparent K_m and k_{cat} values for each $tRNA^{Asp}$ variant, obtained at subsaturating concentrations of amino acid (*24*), were derived from a Lineweaver-Burk plot. As a control, each group of mutants was tested in parallel with the $tRNA^{Asp}$ (G1-C72) transcript. The k_{cat}/K_m values for replicate experiments varied at most 15%.

Mutant type	$tRNA^{Asp}$ variant	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (relative)	Loss of specificity (*x*-fold)
$tRNA^{Asp}$	(U1-A72) → (G1-C72)	50	0.52	1	1
E. coli $tRNA^{Asp}$		30	0.39	1.3	0.8
Acceptor stem	G73 → U73	200	0.058	0.028	36
	G73 → C73	320	0.017	0.0051	200
	G73 → A73	290	0.019	0.0063	160
	U5 → C5	80	0.36	0.43	2
	G4-C69 → C4-G69	90	0.36	0.38	3
	G4-C69 → A4-U69	60	0.30	0.48	2
Anticodon loop	G34 → C34	200	0.0052	0.0025	400
	G34 → A34	250	0.036	0.014	71
	G34 → U34	330	0.035	0.010	100
	U35 → C35	210	0.024	0.011	91
	U35 → A35	300	0.006	0.0019	530
	U35 → G35	220	0.122	0.053	19
	C36 → A36	210	0.015	0.0069	150
	C36 → U36	230	0.033	0.014	71
	C36 → G36	250	0.025	0.0096	100
	G37 → U37	70	0.25	0.34	3
	G37 → C37	30	0.16	0.51	2
	G37 → A37	40	0.18	0.43	2
Anticodon stem	U40 → C40	50	0.27	0.52	2
	(G27-C43) + (G28-C42) → (C27-G43) + (C28-G42)	80	0.34	0.41	2
	(G27-C43) + (G28-C42) → (U27-A43) + (U28-A42)	70	0.36	0.49	2
D-stem and loop	G10 → A10	1400	0.44	0.030	33
	U25 → C25	240	0.31	0.12	8
	U13 → C13	50	0.32	0.62	2
	U11-A24 → C11-G24	100	0.38	0.37	3
	C20.1 → U20.1	40	0.38	0.91	1
	[(U12-A23)-A9] → [(C12-G23)-G9]	150	0.54	0.35	3
$tRNA^{Phe \rightarrow Asp*}$		450	0.40	0.086	12

*$tRNA^{Phe}$ with replacement by U25, U35, C36, and G73.

nucleotides in yeast $tRNA^{Asp}$ has been changed to at least one other nucleotide (Fig. 1C). Our results indicate that anticodon nucleotides G34, U35, and C36, discriminator base G73, and the G10-U25 base pair represent the major nucleotides important for specific aminoacylation by yeast AspRS (Fig. 1D). Mutations at these positions resulted in a significant decrease (eightfold or greater) in aspartylation specificity (k_{cat}/K_m); mutations at other positions within the tRNA, including those identified by sequence analysis (except U25), had only small effects on aspartylation (a decrease of threefold or less in k_{cat}/K_m). Untested positions may have minor effects on aspartylation (see below).

In order to verify the set of identity elements for aspartylation of yeast $tRNA^{Asp}$, these nucleotides were transplanted into a transcript of yeast $tRNA^{Phe}$. Aspartylation of the wild-type yeast $tRNA^{Phe}$ transcript was not observed (<1% charge). Four nucleotides were changed as indicated (Fig. 1E): C25 to U25, A35 to U35, A36 to C36, and A73 to G73. The $tRNA^{Phe}$ transcript with these four changes is an excellent substrate for yeast AspRS; this transcript retains a minimal capacity to be phenylalanylated by yeast PheRS (~5% charging plateau) The k_{cat}/K_m for aspartylation is reduced only 12-fold from that of the wild-type $tRNA^{Asp}$; this drop in specificity results from an increase in K_m, since k_{cat} for aspartylation is unchanged from the wild-type value. There are several possible explanations for this increase in K_m. A minor determinant was perhaps not defined by our mutational analysis. However, the increase in K_m upon transplantation may be explained by a greater energetic cost for the $tRNA^{Phe}$ sequence to achieve the conformation required for specific interaction with AspRS. Finally, certain chemical groups in the $tRNA^{Phe}$ sequence, acting as antideterminants (*1, 5*), may interact unfavorably with AspRS. Nevertheless, these results confirm the identity set of major determinants for aspartylation.

The identity nucleotides are all within regions in close interaction with AspRS as defined by the crystal structure of the complex (Fig. 2) (*4*). Our kinetic results probed the nature of these interactions. For certain determinant positions (G73 and U35), the different kinetic effects of nucleotide substitutions suggest chemical groups important for specific aspartylation by AspRS. The contacts between AspRS and yeast $tRNA^{Asp}$ involve nucleotides in addition to the five major identity elements; these are probably sites of non–sequence-specific interactions (for example, with the phosphodiester backbone) since mutations of these nucleotides do not affect aspartylation activity. However, these may include interactions with bases that appear non–sequence-specific, since mutation conserves similar presentation of identical chemical groups. Therefore, the concept of determinant nucleotides should be further refined to the chemical groups responsible for specific aminoacylation.

Care must be taken to distinguish between effects due to removal of specific chemical groups (loss of a direct interaction) and indirect effects due to change in presentation of these chemical groups. Change of U25 to C25 may change the presentation of an important chemical group (the G10 amino group) to the synthetase. Thus, a conserved nucleotide (G10) may be used to discriminate among different tRNAs.

Discrimination among tRNA substrates (reflected by relative values of k_{cat}/K_m) involves both substrate binding and transition-state stabilization. In the yeast Phe system, a single mutation of determinant nucleotides significantly affects only K_m (five- to tenfold increase) (*19*), whereas large effects on k_{cat} have been observed in other systems (*20, 21*). Mutation of determinant nucleotides in the anticodon and discriminator positions in yeast $tRNA^{Asp}$ affect both K_m and k_{cat}, although the kinetic effect predominates. The effect on k_{cat}, particularly for the anticodon determinants far from the enzyme catalytic center, suggests subtle induced conformational changes in the complex during the catalytic process. In contrast, mutations of the G10-U25 pair affect only K_m, suggesting a loss of contact in the enzyme-tRNA complex (see above). that does not influence the catalytic step. For tRNA-synthetase systems studied to date, single mutations of determinant nucleotides have resulted in decreases of k_{cat}/K_m from 10- to as much as 10^4- to 10^5-fold; the effects in yeast $tRNA^{Asp}$ are intermediate. The magnitude of this kinetic discrimination (*22*) contributed by determinant nucleotides may depend on the catalytic mechanism of the particular synthetase (*23*) and on the number of nucleotides needed to specify amino acid identity.

The results presented here and in (*4*) illustrate a confluence of structural and functional studies on specific aminoacylation by yeast AspRS. Further mutational studies of both protein and tRNA are needed to refine the origins of specificity in this system. A detailed molecular view of the RNA-protein interactions responsible for recognition of $tRNA^{Asp}$ by AspRS awaits the ongoing refinement of the crystal structure. Such results should allow discussion of specificity in terms of functional groups on both the tRNA and synthetase.

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