The Modified Wobble Base Inosine in Yeast tRNA^{Ile} Is a Positive Determinant for Aminoacylation by Isoleucyl-tRNA Synthetase[†]

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ABSTRACT: Earlier work by two independent groups has established the fact that anticodons GAU and LAU of *Escherichia coli* tRNA^{Ile} isoacceptors play a critical role in the tRNA identity. Yeast possesses two isoleucine transfer RNAs, a major one with anticodon IAU and a minor one with anticodon ΨAΨ which are derived from the post-transcriptional modification of AAU and UAU gene sequences, respectively. We present direct evidence which reveals that inosine is a positive determinant for yeast isoleucyl-tRNA synthetase. We also show that yeast tRNA^{Met} with guanosine at the wobble position becomes aminoacylated with isoleucine while methionine acceptance is lost. As inosine and guanosine share the 6-keto and the N-1 hydrogen groups, this suggests that these hydrogen donor and acceptor groups are determinants for isoleucine specificity. The role of the minor tRNA^{Ile} anticodon pseudouridines in tRNA isoleucylation could not be tested directly but was deduced from a 40-fold decrease in the activity of the unmodified transcript. The presence of the NHCO structure in guanosine, inosine, pseudouridine, and lysidine suggests a unifying model of wobble base recognition by the yeast and *E. coli* isoleucyl-tRNA synthetase. In contrast to lysidine which switches the identity of the tRNA from methionine to isoleucine [Muramatsu, T., Nishikawa, K., Nemoto, F., Kuchino, Y., Nishimura, S., Miyazawa, T., & Yokoyama, S. (1988) *Nature 336*, 179−181], pseudouridine-34 does not modify the specificity of the yeast minor tRNA^{Ile} since U-34 is a strong negative determinant for yeast MetRS. Therefore, the major role of Ψ-34 (in combination with Ψ-36 or not) is likely in isoleucine AUA codon specificity and translational fidelity.

About 75 different modified nucleosides have been identified in tRNAs, some of them present in all three phylogenetic domains. Besides these conserved nucleosides which are believed to increase the stability of the tRNA, each tRNA molecule has its own set of modified nucleosides introduced post-transcriptionally by different modifying enzymes. Early genetic work with mutants defective in individual modifying enzymes has established the fact that the primary function of tRNA modification is enhancement of the rate and accuracy of translation (Björk *et al.*, 1987; Björk, 1992), and this general role is achieved by decreasing the rate of dipeptide synthesis and increasing the rate of rejection with noncognate codons (Harrington *et al*., 1993). Modifications located in the anticodon appear, however, to have a predominant role in codon−anticodon recognition (Björk, 1992). Inosine (6-deaminated adenosine) and 2-thiouridine are two modified nucleosides found exclusively in the first position of the anticodon (position 34). These nucleosides have been selected either for their ability to wobble with the third base of the codon or to avoid ambiguous recognition of codons ending with G (Agris, 1991).

Initial experiments with unmodified tRNA transcripts did not implicate modified nucleosides in tRNA aminoacylation (Sampson & Uhlenbeck, 1988). Lysidine (L-cytosine with the carbonyl group replaced by lysine), present at the wobble position of the minor *Escherichia coli* tRNA^{Ile}, was the first example of a base modification playing a major role in tRNA discrimination (Muramatsu *et al.*, 1988). Lysidine acts as a negative determinant for *E. coli* methionyl-tRNA synthetase and is able to change the identity of the tRNA from methionine to isoleucine because, in the absence of base modification, *E. coli* methionyl-tRNA synthetase interacts specifically and strongly with cytosine-34 (Schulman & Pelka, 1983, 1988). Previously, substitution of the anticodon pseudouridine-35 of yeast tRNA^{Tyr} by adenosine was shown to increase by 10-fold the rate of misacylation by yeast phenylalanyl-tRNA synthetase (Bare & Uhlenbeck, 1985). More recently, two other modified bases have been implicated in tRNA recognition. 5-[(Methylamino)methyl]-2-thiouridine (mnm^{5}s^{2}U) at position 34 of *E. coli* tRNA^{Glu} interacts positively with *E. coli* glutamyl-tRNA synthetase (Sylvers *et al.*, 1993), whereas 1-methylguanosine (m^{1}G) at position 37 of yeast tRNA^{Asp} is a negative determinant for yeast arginyl-tRNA synthetase (Putz *et al.*, 1994).

Lysidine also changes the decoding specificity of the minor *E. coli* tRNA^{Ile}. By hindering C-34 pairing with G of the third codon position, the minor *E. coli* tRNA^{Ile} reads isoleucine codon AUA and not methionine codon AUG (Muramatsu *et al.*, 1988), while isoleucine codons AUU and AUC are decoded by tRNA^{Ile} with the GAU anticodon. The

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two wobble bases, G-34 and L-34, have since been implicated in the positive selection by *E. coli* IleRS[1] (Pallanck & Schulmann, 1991; Muramatsu *et al.*, 1992). As guanosine and lysidine have different chemical structures, it is unclear how the first anticodon base is recognized by *E. coli* IleRS.

To better understand the molecular basis of wobble base recognition by IleRS, we analyzed the role of these bases in yeast $tRNA^{Ile}$ aminoacylation. Indeed, there are several examples of conservation of tRNA identity determinants in evolution [i.e. $tRNA^{Phe}$ (Sampson *et al.*, 1989), $tRNA^{Ala}$ (Hou & Schimmel, 1989), and $tRNA^{Met}$ (Senger *et al.*, 1992)] giving credence to our assumption that the $tRNA^{Ile}$ anticodon, and particularly the wobble base, is recognized by yeast IleRS. The study of yeast $tRNA^{Ile}$ recognition is particularly interesting in that the two yeast isoleucine acceptors have wobble bases that differ from their *E. coli* counterparts. The major $tRNA^{Ile}$ from yeast has an IAU anticodon (Pixa *et al*., 1984), whereas the minor species has ΨAΨ (Szweykowska-Kulinska *et al*., 1994). Here, we show that inosine-34, introduced post-transcriptionally by enzymatic means in a $tRNA^{Ile}$ transcript, is a positive determinant for yeast IleRS interaction. The role of the two anticodon pseudouridines in tRNA aminoacylation could not be demonstrated directly, but an unmodified version of the minor $tRNA^{Ile}$ shows a substantial decrease in the catalytic efficiency of the tRNA. On the basis of these results and on the successful cross-charging of *E. coli* $tRNA^{Ile}$ (GAU) by the yeast IleRS, we suggest recognition determinants for IleRS that are common to guanosine, lysidine, inosine, and pseudouridine.

MATERIALS AND METHODS

Construction of tRNA Genes. $tRNA^{Met}$ variants were obtained as described in Senger *et al*., (1992). Construction of the wild-type $tRNA^{Ile}$ gene was obtained from overlapping oligonucleotides and cloned into phagemid pUC119 to yield plasmid pTIM1. Single-stranded pTIM1 DNA containing deoxyuridine was isolated from *E. coli* CJ 236-transformed cells according to Kunkel *et al*. (1991) and was used as the template for the annealing of mutagenic oligonucleotides containing single- or double-point mutations. Second strand synthesis was performed by addition of dNTPs, T4 DNA polymerase, and T4 DNA ligase (Boehringer Mannheim). The resulting double-stranded DNA was used to transform *E. coli* MV 1190 cells. The clones were identified by DNA sequencing using the dideoxy method. DNA oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer.

In Vitro Transcription. tRNA transcripts from plasmid-encoded tRNA genes were enzymatically synthesized using T7 RNA polymerase. Runoff transcription was obtained by creating a *Bst*NI restriction site at the 3′-terminal end of the tRNA variants. T7 RNA polymerase was isolated from *E. coli* strain BL 21 containing the plasmid pAR1219 and purified according to Grodberg and Dunn (1987).

Forty micrograms of linearized plasmid DNA was transcribed under the following conditions: 60 mM Tris-HCl (pH 8), 30 mM $MgCl_2$, 7 mM dithiotreitol, 1.4 mM spermidine, 14 mM Triton X-100, 20 mM GMP, 4 mM ribonucleotide, and 4 mM T7 RNA polymerase. After 3 h of incubation at 37 °C, the reaction was stopped by addition of 100 μL of 0.5 M EDTA and phenol extraction. The RNA was precipitated by ethanol, resuspended in 250 μL of 50% formamide, and purified by polyacrylamide gel electrophoresis under denaturing conditions. The bands were visualized by UV shadowing, excised, and electroeluted.

Aminoacylation Assays. Transcripts were submitted to a renaturation step under the following conditions: heating in a 90 °C water bath for 1 min, cooling at room temperature for 5 min, and incubation at 30 °C for 5 min after addition of the reaction mixture containing magnesium, ATP, and isoleucine. Aminoacylation was carried out at 25 °C in a reaction mixture of 30 μL containing 150 mM Tris-HCl (pH 7.8), 10 mM dithiothreitol, 100 mM KCl, 2 mM ATP, 15 mM $MgCl_2$, 10 μM [^{3}H]isoleucine (30 Ci/mmol; 3000 cpm/pmol), and IleRS ranging from 5 to 350 nM.

Enzymatic Introduction of Inosine into the Major $tRNA^{Ile}$ Transcript. The transcript (40 μg) was synthesized and purified as described above. It was then incubated at 30 °C for 2 h in a mixture (1 mL) containing 100 mM Tris-HCl (pH 8), 10 mM $MgSO_4$, 0.1 mM EDTA, 100 mM ammonium acetate, 2 mM DTT, and 8 mg of enriched tRNA adenosine deaminase preparation. The modified transcript was then completely digested with ribonuclease P1, and the resulting mononucleosides were analyzed by thin layer chromatography.

Verification of Inosine Biosynthesis. For a qualitative verification of the inosine incorporation, we used a postlabeling technique. Inosine (0.5 g) containing transcript was digested with 0.5 unit of RNase T2 in 50 mM ammonium acetate at pH 5.3 for 5 h at 37 °C. The enzyme is then inactivated at 95 °C for 2 min, and the mixture is lyophilized. The mononucleotides obtained are then phosphorylated with 2.5 units of T4 polynucleotide kinase (New England Biolabs) and 3 μCi [α-^{32}P]ATP. The excess of ATP is removed by addition of apyrase (Sigma) using 0.01 unit of enzyme and 0.08 mg/mL AMP and incubation at 37 °C for 30 min. The reaction mixture is lyophilized again. In order to obtain species that are identifiable by thin layer chromatography, the 3′−5′-diphosphonucleotides were treated with 1 unit of RNase P1 at 37 °C for 4 h in the same buffer as that for RNase T1. The 5′ mononucleotides are then separated by thin layer chromatography.

Thin Layer Chromatography Analysis. Identification of the labeled nucleotides was performed by one-dimensional chromatographic analysis on cellulose thin layer plates as described by Auxilien *et al*. (1996) using 0.1 M sodium phosphate (pH 6.8)/solid ammonium sulfate/*n*-propanol (68:17.6:14.4, v/w/v) as the solvent. The efficiency of inosine formation was measured by cutting out the labeled spots from the thin layer chromatography plates (TLC) and counting the radioactivity by liquid scintillation techniques. Taking into account the amount of label in each of the nucleotides spots (AMP and IMP) and knowing the relative number of labeled nucleotides per RNA substrate, we easily calculated the molar ratio of inosine per mole of tRNA.

RESULTS AND DISCUSSION

The T7 RNA Transcript of Yeast Major $tRNA^{Ile}$ Is a Poor Substrate for Yeast IleRS. Inosine-34 of the major $tRNA^{Ile}$ species (Pixa *et al*., 1984) results from the post-transcriptional modification of the gene-derived adenine (Felici & Cesarini,

[1] Abbreviations: I, inosine; Ψ, pseudouridine; L, lysidine; MetRS, methionyl-tRNA synthetase; t6A, *N*-[(9−13-D-ribofuranosylpurin-6-yl)carbamoyl]threonine; IleRS, isoleucyl-tRNA synthetase.

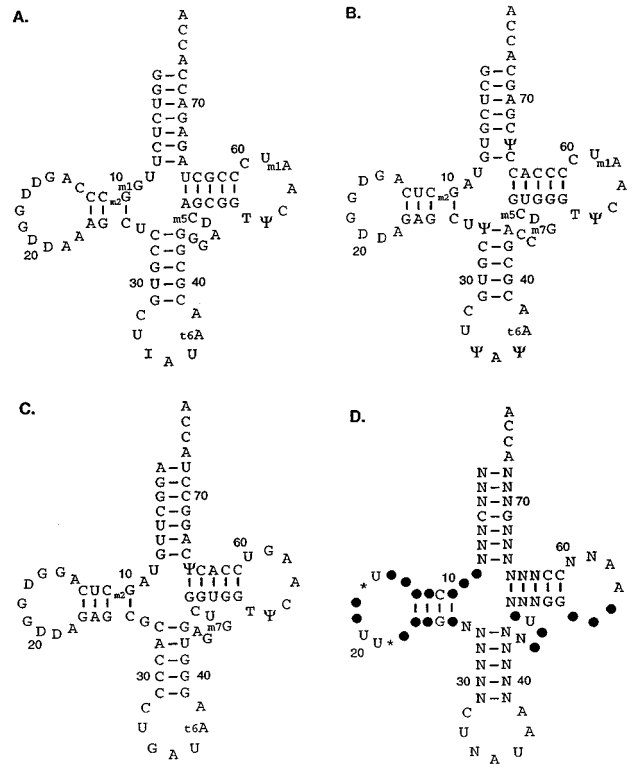

FIGURE 1: Homologous and heterologous $tRNA^{Ile}$ isoacceptors aminoacylated by yeast IleRS. Native forms of $tRNA^{Ile}$ are represented: (A) major yeast $tRNA^{Ile}$, (B) minor yeast $tRNA^{Ile}$, (C) major *E. coli* $tRNA^{Ile}$ and (D) consensus sequence of common nucleotides derived from $tRNA^{Ile}$ sequences aminoacylated by the yeast enzyme. In panel D, nucleotides which are involved in the tRNA tertiary structure are indicated by black dots.

1987). Inosine-34 is found in six other yeast tRNAs specific for alanine, proline, serine, threonine, valine, and arginine and is enzymatically produced by hydrolytic deamination of adenosine-34 (Auxilien *et al.*, 1996). Besides inosine, the major yeast $tRNA^{Ile}$ contains five dihydrouridines at positions 16, 17, 20, 20A, and 47 and m^1G, m^2G, t^6A, m^5C, T, Ψ and m^1A at positions 9, 10, 37, 48, 54, 55, and 58, respectively (Figure 1). A completely unmodified version of this tRNA generated by T7 RNA transcription yielded tRNA molecules poorly recognized by yeast IleRS. The plateau level of aminoacylation is only 3%, and the catalytic efficiency k_{cat}/K_m is reduced 400-fold with respect to that of native tRNA (Table 1, experiment 2). Usually the difference in k_{cat}/K_m values between a completely unmodified tRNA and the native form is within 1 order of magnitude (Sampson & Uhlenbeck, 1988). This suggests that the decrease of the catalytic efficiency results from the loss of critical contacts between IleRS and, at least, one of the modified bases.

Enzymatic Incorporation of Inosine-34 into the Yeast $tRNA^{Ile}$ Transcript and Aminoacylation Activity of the Inosine-Containing Transcript. Proving that a modified base plays a role in the tRNA identity is not easy. The role of Ψ-35 in yeast $tRNA^{Tyr}$ (Bare & Uhlenbeck, 1985) or L-34 in $tRNA^{Ile}$ (Muramatsu *et al.*, 1988) could be clarified using a six- or seven-step procedure developed in Uhlenbeck's laboratory (Bruce & Uhlenbeck, 1982). In both cases, the sensitivity of the anticodon loop to nuclease digestion was exploited to create a gapped tRNA that was used as the donor in a RNA ligase reaction. Recent progress in recombinant RNA technology has facilitated the introduction of specific modified bases in a tRNA. For example, the contribution of 5-[(methylamino)methyl]-2-thiouridine-34 (mnm^5s^2U-34) in *E. coli* $tRNA^{Glu}$ aminoacylation was tested by mixing 5′- or 3′-halves of native tRNA (obtained by oligonucleotide-addressed RNase H cleavage) with the complementary *in vitro* T7 transcripts (Sylvers *et al.*, 1993), and the rejection of the yeast $tRNA^{Asp}$ transcript by the heterologous yeast arginyl-tRNA synthetase (Putz *et al.*, 1994) was shown by mixing a T7 5′-half with a complementary 3′-half (containing a single N-1 methyl guanosine at position 37) obtained by chemical RNA synthesis. Chemical RNA synthesis of more than 40−50 nucleotides is still not a routine way to produce biologically active RNA molecules, especially when the activity requires a modified base.

To introduce inosine at position 34 in yeast $tRNA^{Ile}$, we enzymatically modified a T7 RNA transcript, taking advantage of the recently characterized yeast tRNA adenosine-34 deaminase (Auxilien *et al.*, 1996). This enzyme requires no cofactor except magnesium to convert A-34, present in a tRNA gene, into inosine. The absence of added *S*-adenosylmethionine prevents guanosine methylation of the yeast $tRNA^{Ile}$ (the major species contains four methyl-G, see Figure 1) and explains why only two tRNA modifications are enzymatically produced with a S100 extract, I-34 and Ψ-55 (Szweykowska-Kulinska *et al.*, 1994; Auxilien *et al.*, 1996). Ψ-55 is not produced when the S100 extract is subjected to five additional chromatographic steps (Auxilien *et al.*, 1996). We used this purified enzyme preparation to introduce post-transcriptionally I-34 in the major yeast $tRNA^{Ile}$ T7 transcript. Conditions for a stoichiometric formation of inosine were worked out at a small scale with uniformly [α-^{32}P]ATP-labeled T7 transcript as described in Auxilien *et al.* (1996). This transcript, after incubation with the modifying enzyme, was hydrolyzed with ribonuclease P1 to generate labeled 5′-AMP or its modified counterpart IMP. We used one-dimensional thin layer chromatography on cellulose plates to separate the two labeled nucleosides (Figure 2), and quantification of the radioactive IMP spot established a stoichiometry of 1 mol of inosine per mole of tRNA. The kinetics of inosine formation indicate that this value is reached within 1 h of incubation and remains constant for at least an 1 additional h. Identical conditions were applied on a large scale to unlabeled yeast $tRNA^{Ile}$ transcript, and the presence of inosine was qualitatively demonstrated (results not shown) by resolving the [α-^{32}P]ATP postlabeled $tRNA^{Ile}$ T2 digest in a two-dimensional chromatographic system that separates modified nucleotides from their unmodified counterparts (Auxilien *et al.*, 1996).

The $tRNA^{Ile}$ transcript with I-34 modification shows significant aminoacylation activity and has a catalytic efficiency only separated by a factor of 26 (Table 1, experiment 3) from that of a wild-type tRNA, indicating that inosine makes a positive contribution to the $tRNA^{Ile}$ identity but does not by itself restore completely the charging activity. This would mean that another or several additional bases present in the wild-type tRNA can be considered as sources of possible activity differences. The additional modified base(s) may act directly or indirectly (see below) on synthetase tRNA recognition.

The GAU Anticodon Is an Identity Element for Yeast IleRS. Inosine is chemically close to guanosine since both purine rings share the 6-keto and the N-1 hydrogen groups. Replacement of the adenosine-34 by guanosine leads to a

Table 1: Kinetic Parameters for Aminoacylation of tRNAs[a]

expt	tRNA	K_m (μM)	k_{cat} ($\times 10^{-3}$) (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	loss (*x*-fold)
1	*Saccharomyces cerevisiae* $tRNA^{Ile}$ (IAU) transcripts	0.9	370	410000	1
2	$tRNA^{Ile}$ (AAU)	1	1	1000	410
3	$tRNA^{Ile}$ (IAU)	0.8	12.5	16000	26
4	$tRNA^{Ile}$ (GAU)	3	14	4600	90
5	$tRNA^{Ile}$ (UAU)	4	9	2250	180
6	$tRNA^{Ile}$ (CAU)	–	–	<130	>3200
7	$tRNA^{Ile}$ (GAA)	–	–	<460	>1000
8	$tRNA^{Ile}$ (CUA)	–	–	<50	>8000
9	*S. cerevisiae* $tRNA^{Ile}$ ($\Psi A\Psi$)	–	–	250000	1
10	$tRNA^{Ile}$ UAU (transcript)	5	32	6400	39
11	$tRNA^{Met}$ (GAU) (transcript)	2.5	3.5	1400	300
12	$tRNA^{Met}$ (GAU) C_4G_{69}	1.8	3.8	2100	200
13	*E. coli* $tRNA^{Ile}$ (GAU)	2.1	70	33000	12
	Aminoacylation with Yeast MetRS				
13	wild-type $tRNA^{Met}$ (transcript)	10	300	30000	1
14	$tRNA^{Ile}$ (CAU)	11	180	16000	1.9
15	$tRNA^{Met}$ (GAU)	–	–	–	>1700

Aminoacylation with yeast MetRS was performed as described in Senger *et al.*, (1992). The anticodon sequence of each tRNA is indicated in parentheses. T7 transcripts were systematically treated with ATP (CTP) tRNA nucleotidyl transferase for 5 min at 30 °C as described in Aphasizhev *et al.* (1996). Aminoacylation was carried out using 0.2−40 μM transcript and 5−300 nM pure IleRS. Native forms of $tRNA^{Ile}$ were tested using 6 nM IleRS and 10 μM [^{3}H]isoleucine (30 Ci/mmol; 3000 cpm/pmol). The amount of $tRNA^{Ile}$ (YAY) was limiting so that k_{cat}/K_m was not measured from the individual kinetic parameters but estimated from the slope of initial rates *versus* tRNA concentration (1−4 μM). The major and minor yeast isoleucine tRNAs had a charging capacity of 1260 and 1550 pmol/A_{260}, respectively. The IleRS used in the aminoacylation tests had a specific activity of 1100 units/mg (1 unit corresponds to 1 nmol of amino acid incorporated into tRNA per minute at 37 °C).

transcript whose k_{cat}/K_m (4.6×10^3 M^{-1} s^{-1}) only differs by a factor of 3 from that of the $tRNA^{Ile}$(IAU) transcript (Table 1, experiment 4) and whose plateau level of aminoacylation reaches 50%. This result suggests that the functional groups common to inosine and guanosine are in contact with yeast IleRS. We also found that *in vitro* replacement of the CAU anticodon of yeast $tRNA^{Met}$ by GAU confers to this tRNA an isoleucine identity while methionine acceptance is lost (Table 1, experiments 11 and 15; Despons *et al.*, 1992). A factor of 3 still separates the catalytic efficiency of this transcript from that of a $tRNA^{Ile}$ (GAU) transcript (4.6×10^3 *versus* 1.4×10^3 M^{-1} s^{-1}), reflecting the absence of positive and/or the presence of negative determinants elsewhere in the molecule. From the examination of the composite structure of $tRNA^{Ile}$ sequences common to yeast and *E. coli* and which are aminoacylable by the yeast enzyme (Figure 1), we noticed the presence of a conserved C4G69 pair in the acceptor stem of isoleucine tRNAs. However, introduction of this pair in the $tRNA^{Met}$ framework did not modify significantly the catalytic efficiency of the transcript (Table 1, experiment 12), suggesting that the C4G69 pair has little influence in yeast $tRNA^{Ile}$ aminoacylation as is the case for *E. coli* $tRNA^{Ile}$ (Nureki *et al.*, 1994).

More evidence supporting the fact that the GAU anticodon is a major site for IleRS−$tRNA^{Ile}$ interaction comes from the successful *E. coli* $tRNA^{Ile}$ cross-charging by yeast IleRS. Using purified recombinant yeast IleRS, Racher *et al.* (1991) could unambiguously demonstrate that the yeast enzyme specifically aminoacylates *E. coli* $tRNA^{Ile}$ in a total tRNA fraction. We confirmed this result, using a purified *E. coli* $tRNA^{Ile}$ (GAU) species which has a catalytic efficiency of aminoacylation that differs only by a factor of 12 from that of native yeast $tRNA^{Ile}$ (IAU) (Table 1, experiment 13). Comparison of the yeast and *E. coli* $tRNA^{Ile}$ sequences that are substrates for the yeast IleRS indicates that the anticodon loop is the sole element conserved in the three $tRNA^{Ile}$ structures, suggesting a major role for the anticodon and adjacent bases in $tRNA^{Ile}$ recognition. Nureki *et al.* (1994) proposed that another modified base of *E. coli* $tRNA^{Ile}$, t6A-37, is required for efficient aminoacylation by *E. coli* IleRS. However, *in vivo* identity swap experiments in which the anticodon of $tRNA^{fMet}$ was replaced by that of isoleucine tRNA (Pallanck & Schulman, 1991) did not implicate t6A, since position 37 of *E. coli* $tRNA^{fMet}$ is not modified. Since *in vivo* identity data do not always correlate with *in vitro* k_{cat}/K_m values (Komatsoulis & Abelson, 1993), we cannot, however, exclude the possibility that t6A37 modification contributes to the anticodon conformation required for optimal IleRS recognition. It is well established that the biological activity of DNA analogues mimicking the anticodon hairpin of a tRNA (inhibition of aminoacylation activity and ribosome binding) requires base modifications (Guenther *et al.*, 1994; Dao *et al.*, 1994), indicating that conformational determinants are not restricted to the standard A-RNA structure.

The other two anticodon bases, A-35 and U-36, of yeast $tRNA^{Ile}$ also contribute to IleRS recognition. Single or double substitutions of these positions to yield a GAA, CAU, or CUA anticodon have a severe deleterious effect on the efficiency of aminoacylation (Table 1, experiments 7 and 8), thus reinforcing the importance of the anticodon triplet in the isoleucine identity. The analogy with the mode of $tRNA^{Met}$ recognition/discrimination previously seen in the *E. coli* system is now confirmed in the yeast $tRNA^{Met}$/$tRNA^{Ile}$ system. Preservation of the location of identity nucleotides between methionine and isoleucine tRNAs parallels that of the corresponding anticodon-binding domains of MetRS and IleRS. Because MetRS and IleRS are two of the most closely related class I tRNA synthetases (Starzyk *et al.*, 1987; Burbaum *et al.*, 1990; Eriani *et al.*, 1990), the MetRS anticodon-binding domain (Perona *et al.*, 1991, and references therein) was used as a model for the analogous motif in the isoleucine enzyme (Shepard *et al.*, 1992). The significance of this motif for anticodon recognition was

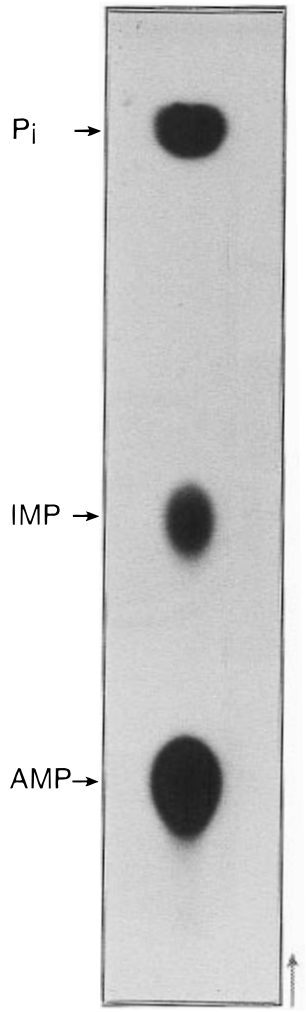

FIGURE 2: Presence of inosine in the enzymatically modified $tRNA^{Ile}$ transcript. The [α^{32}P]ATP-labeled major $tRNA^{Ile}$ transcript was subjected to enzymatic modification with the inosine synthase preparation and completely digested with RNase P1, and the digest was analyzed by thin layer chromatography (see Material and Methods). P_i is free orthophosphate which originates from the degradation of mononucleosides during the incubation at 37 °C with a large excess of commercial nuclease P1.

shown by a single amino acid swap in a designed peptide which switches recognition between $tRNA^{Met}$ and $tRNA^{Ile}$ (Auld & Schimmel, 1996). The close relation between $tRNA^{Met}$ and $tRNA^{Ile}$ recognition sites seen at the RNA and protein levels further supports the role of the modified wobble base in $tRNA^{Ile}$ recognition.

Role of Anticodon Pseudouridine Modifications in Aminoacylation and Decoding of the Minor Yeast $tRNA^{Ile}$. In *Saccharomyces cerevisiae*, a second tRNA gene for isoleucine has been described with UAU as the anticodon sequence (Ogden *et al*., 1984), making its post-transcriptional modification necessary to avoid misreading of the methionine AUG codon. This tRNA probably evolved because the major isoleucine tRNA is unable to translate AUA *in vivo*. The inability of inosine at the wobble position to pair with A at the third position of the codon has already been demonstrated in genetic experiments using *Schizosaccharomyces pombe*; cells deprived of a $tRNA^{Ser}$ isoacceptor (anticodon UCA) ceased to grow (Munz *et al*., 1981). The native form of the minor yeast $tRNA^{Ile}$ isoacceptor has an unusual ΨAΨ anticodon sequence (Figure 1), and we recently showed that Ψ-34 and Ψ-36 formation are largely dependent upon a structural feature of the pre-tRNA intron (Szweykowska-Kulinska *et al*., 1994). Because of the chemical and structural resemblance between Ψ and U, the absence of Ψ-34 modification is not expected to strongly impair the activity of the tRNA, and in fact, the catalytic efficiency of a T7 transcript is only 40-fold lower than that of the native form (Table 1, experiment 10). The decrease is 180-fold when the UAU anticodon is present in the major $tRNA^{Ile}$ species (Table 1, experiment 5), and this discrepancy may be attributed to context effects since the two isoleucine tRNAs are distinguished by their D-loop−variable loop arrangement.

The absence of Ψ-34 modification in yeast $tRNA^{Ile}$ should not change the identity of the tRNA since the unmodified U-34 is a strong antideterminant for the yeast MetRS (Senger *et al*., 1992). In *E. coli*, lysidine is also associated with a change in the decoding specificity of $tRNA^{Ile}$ from AUG (methionine) to AUA (isoleucine). In this way, translational errors are avoided. In yeast, the absence of Ψ-34 modification in the minor $tRNA^{Ile}$ is more dramatic. *In vivo*, where saturating concentrations of enzyme prevail, an unmodified yeast $tRNA^{Ile}$ (UAU) will be aminoacylated at a significant level by IleRS (values for plateaus of aminoacylation vary from 25 to 65%; results not shown). Therefore, the main role of Ψ modification in yeast $tRNA^{Ile}$ (UAU) is likely the prevention of misreading of AUG codons.

The fact that pseudouridine can modulate codon−anticodon interaction has been observed in suppression assays involving $tRNA^{Tyr}$ from yeast or plants. The activity of yeast *SUP*6 suppressor [$tRNA^{Tyr}$ (UΨA)] is reduced by the change of Ψ-35 to U-35 (Johnson & Abelson, 1983). In plants, the $tRNA^{Tyr}$ suppressor with the GΨA anticodon is used to read through the natural UAG (or mutant UAA) stop codon at the end of the 126K cistron of tobacco mosaic virus RNA, whereas a T7 transcript of the $tRNA^{Tyr}$ suppressor with the anticodon GUA is not (Zerfass & Beier, 1992). This suggests that a strong A−Ψ interaction stabilizes codon−anticodon interactions despite the presence of an unconventional G-G or G-A pair at the wobble position.

The properties of pseudouridine are attributed to the presence of an extra NH moiety able to make more hydrogen bonds uridine. The current model by which stable RNA structures are achieved by pseudouridines has come from the comparison of the crystal structure of unmodified *versus* modified *E. coli* $tRNA^{Gln}$ which has two Ψs in the anticodon loop: Ψ-38 and Ψ-39. The N-1-H atoms of these pseudouridines are hydrogen bonded to a water molecule coordinated to the phosphate backbone (Arnez & Steitz, 1994), and this model also accounts for an identical role of Ψ in single-stranded oligonucleotides, leading to a slow exchange of the imino proton seen by NMR (Davis, 1995). The involvement of the Ψ-34 N-1-H proton of yeast $tRNA^{Ile}$ in coordination of a water molecule may explain the lack of Ψ-34−G codon−anticodon pairing when pseudouridine adopts the RNA *anti* conformation, the conformation found most often in RNA structures.

A Unifying Model of $tRNA^{Ile}$ Wobble Base Recognition by Yeast and E. coli Isoleucyl-tRNA Synthetase. In a recent paper, Muramatsu *et al*. (1992) proposed that the 2-exocyclic NH group of the lysine moiety of lysidine and one hydrogen of the 2-exocyclic NH_2 group of guanosine are the subsites for *E. coli* IleRS interaction. According to this proposal, the yeast and the *E. coli* IleRS would recognize different

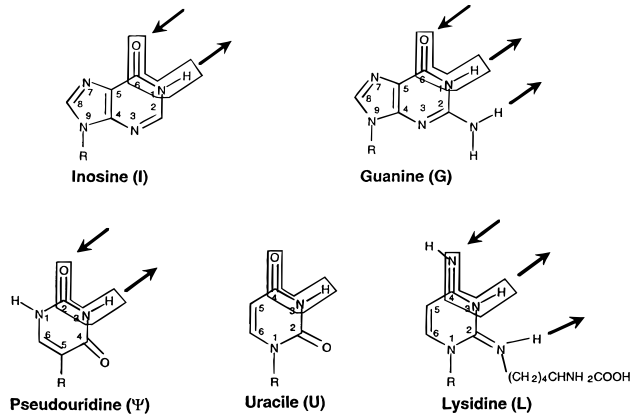

FIGURE 3: Common features shared by the wobble bases in *E. coli* and yeast $tRNA^{Ile}$. The functional groups suggested as the identity determinants for *E. coli* and yeast IleRS are enclosed by thin lines. The arrows indicate the hydrogen donor and acceptor groups involved in codon−anticodon base pairing. The tautomeric structure of lysidine is from Muramatsu *et al.* (1992).

structural features in the guanosine base since the 2-exocyclic NH_2 group of guanosine is absent in inosine. This is in contrast with the successful cross-charging of *E. coli* $tRNA^{Ile}$ by yeast IleRS (Racher *et al.*, 1991; this study). A more unifying model of $tRNA^{Ile}$ wobble base recognition by the two synthetases, consistent with our results, would be one where the *E. coli* IleRS contacts the planar N-4 and N-3 hydrogen groups of the tautomeric form of lysidine that resemble the amide structure of guanosine, inosine, or pseudouridine (Figure 3). Indeed, from a chemical point of view, the N and O atoms are similar with respect to their electronegativity and van der Waals volume.

This model provides a rationale for the species specific wobble base recognition of $tRNA^{Ile}$ by IleRS: guanosine-lysidine in *E. coli* and inosine-pseudouridine in yeast. A similar mode of $tRNA^{Ile}$ recognition may be applied to organisms other than *E. coli* and *S. cerevisiae* on the basis of the presence of the guanosine-lysidine pair for the two $tRNA^{Ile}$ of *Mycoplasma capricolum*, a Gram-positive bacteria (Andachi *et al.*, 1989) and of inosine for the major $tRNA^{Ile}$ from the yeast *Torulopsis utilis* (Takemura *et al.*, 1969), the plant *Lutinus luteus* (Barciszewska *et al.*, 1988), or mouse (Sprinzl *et al.*, 1996). Because wobble base recognition in $tRNA^{Ile}$ is also related to the codon specificity of the tRNA, it is remarkable that two routes coexist in nature to achieve fidelity of AUA codon reading. The relationship between $tRNA^{Ile}$ identity and AUA codon specificity is historically linked to the evolution of the genetic code. We have shown that pseudouridine formation in the yeast minor $tRNA^{Ile}$ is an intron-dependent process and that a structural feature of this intron makes a major, if not exclusive, contribution to Ψ formation (Szweykowska-Kulinska *et al.*, 1994). The general view of intron evolution is that most introns were present in the common ancestor of eukaryotes, eubacteria and archaebacteria, and that the general direction of their evolution has been toward loss (Darnell & Doolittle, 1986; Gilbert *et al.*, 1986; Kuhsel *et al.*, 1990). According to this scheme, the C-modifying (lysylating) enzyme appeared after the loss of the tRNA intron and a concomitant U-to-C substitution in the tRNA gene to reassign AUA as the isoleucine codon since in some chloroplasts and mitochondrial genomes AUA and AUG codons are read as methionine (Jukes & Osawa, 1990).

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