Function of the Extra 5′-Phosphate Carried by Histidine tRNA

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ABSTRACT: Among elongator tRNAs, tRNA specific for histidine has the peculiarity to possess one extra nucleotide at position −1. This nucleotide is believed to be responsible for recognition by histidyl-tRNA synthetase. Here, we show that, in fact, it is the phosphate 5′ to the extra nucleotide which mainly supports the efficiency of the tRNA aminoacylation reaction catalyzed by *Escherichia coli* histidyl-tRNA synthetase. In the case of the reaction of *E. coli* peptidyl-tRNA hydrolase, this atypical phosphate is dispensable. Instead, peptidyl-tRNA hydrolase recognizes the phosphate of the phosphodiester bond between residues −1 and +1 of $tRNA^{His}$. Recognition of the +1 phosphate of $tRNA^{His}$ by peptidyl-tRNA hydrolase resembles, therefore, that of the 5′-terminal phosphate of other elongator tRNAs.

Several studies have indicated that, in the case of eubacteria, the phosphate at the 5′-end of an elongator tRNA could serve as an identity element in the generic recognition of this molecule by translational elongation factors such as elongation factor Tu (EF-Tu)[1] or peptidyl-tRNA hydrolase (PTH) (*1*−*4*). However, to obtain the positive effect of the 5′-phosphate, the nucleotide +1 of the elongator tRNA must be involved in a canonical base pairing (*1*, *2*, *4*, *5*). This constraint accounts for the exclusion of Met-$tRNA^{fMet}$ and fMet-$tRNA^{fMet}$ by EF-Tu and PTH, respectively. Indeed, bacterial initiator tRNAs systematically display mismatched nucleotides at positions +1 and +72 (*6*−*12*).

Among elongator tRNAs, tRNA specific for histidine has the peculiarity to possess one extra nucleotide at position −1 (*13*). Clearly, this atypical character affects the relative location of the 5′-phosphate with respect to the end of the acceptor arm of the tRNA and can be expected to interfere with the recognition of $tRNA^{His}$ by EF-Tu or PTH. Yet, kinetic studies of Tu−GTP−aminoacyl-tRNA complexes have not revealed any discrimination of the elongation factor toward His-$tRNA^{His}$ (*14*). Moreover, upon transfer of a *pth*^{ts} strain at nonpermissive temperature, accumulation of $tRNA^{His}$ as a peptidyl-tRNA was described (*15*). Such an observation suggests that, in vivo, peptidyl-$tRNA^{His}$ behaves as a correct substrate of the hydrolase, being therefore functionally indistinguishable from other elongator tRNAs.

Recently, on the basis of the crystallographic structure of *Escherichia coli* PTH (*3*), the putative binding site of the 5′-phosphate of *N*-acetyllysyl-$tRNA^{Lys}$ could be mapped by changing cationic residues at the surface of PTH into alanyl ones and scoring the catalytic performances of the obtained enzyme variants (*4*). The conclusion was that the phosphate site is composed of an electropositively charged clamp involving residues K105 and R133. A similar arrangement, with two cationic residues interacting with the 5′-phosphate, can also be deduced from examination of the 3-D structure of *Thermus aquaticus* EF-Tu complexed to phenylalanyl-$tRNA^{Phe}$ [PDB accession number 1ttt (*16*)].

The first aim of the present study was to examine the capacity of our PTH mutants to use *N*-acetyl-His-$tRNA^{His}$ as a substrate and eventually to assess whether recognition of this tRNA can be achieved in a way similar to that described with N-blocked lysyl-$tRNA^{Lys}$. The availability of various $tRNA^{His}$ substrates for peptidyl-tRNA hydrolase also gave us an opportunity to reinvestigate the role of the −1 extra nucleotide in the efficiency of the aminoacylation reaction catalyzed by histidyl-tRNA synthetase (HisRS). In agreement with an early communication by Allen and Parsons (*17*), we observed an effect of the only dephosphorylation of the tRNA. In addition, comparison of the kinetic parameters of the modified $tRNA^{His}$ molecules to those of authentic $tRNA^{His}$ showed that, in $tRNA^{His}$, the 5′-phosphate acts as one major positive determinant in the reaction of the synthetase. This finding enables us to propose that, in the recognition by HisRS, the key role usually attributed to the full extra nucleotide at the 5′-terminus of $tRNA^{His}$ (*18*−*20*) can be, in fact, accounted for by the 5′-phosphate end alone. Implications of this conclusion in the capacity of plant viral RNAs to be histidylated are discussed.

MATERIALS AND METHODS

Enzymes and Substrates. Alkaline phosphatase and DNA modification enzymes were from Roche Diagnostics (Meylan, France); L-[^{14}C]histidine (12 GBq/mmol) and [γ-^{33}P]-ATP (111 TBq/mmol) were from NEN-DuPont (Paris, France). Oligonucleotides were synthesized by Genset (Paris, France).

Preparation of Full-Length $tRNA^{His}$ and Mutant $tRNA^{His}$-ΔG_{-1}. E. coli full-length $tRNA^{His}$ and mutant $tRNA^{His}\Delta G_{-1}$ genes were constructed and overexpressed in JM101TR cells from pBSTNAV derivatives according to the method described in ref *21*. Crude tRNA extracts from these cells accepted 1000 ± 50 and 400 ± 50 pmol of L-histidine/A_{260}

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[1] Abbreviations: EF-Tu, elongation factor Tu; PTH, peptidyl-tRNA hydrolase; HisRS, histidyl-tRNA synthetase.

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unit, respectively. These samples were used without further purification.

Native *E. coli* $tRNA^{His}$ was partially purified from bulk *E. coli* tRNA (Roche Diagnostics, Meylan, France) through C_4 hydrophobic chromatography, as previously described (*22*). The resulting sample accepted 70 pmol of histidine/A_{260} unit.

Dephosphorylations of $tRNA^{His}$ and $tRNA^{His}\Delta G_{-1}$ were carried out as described previously for $tRNA^{Lys}$ (*3*). The extents of dephosphorylation, followed as in ref *3*, were better than 99.9%. Dephosphorylated tRNAs accepted 900 ± 50 and 400 ± 50 pmol of L-histidine/A_{260} unit, respectively.

Reintroduction of a phosphate into dephosphorylated $tRNA^{His}$ was performed at 37 °C for 30 min in a reaction mixture (20 μL) containing 50 mM Tris-HCl (pH 8.2), 1.35 μM dephosphorylated tRNA, 1 mM ATP, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM spermidine, and 500 units/mL of polynucleotide kinase (Roche). The amount of phosphate incorporation into tRNA was followed with the help of two experiments: In one experiment, [γ-^{33}P]-ATP (3 GBq/mL) was used, and the radiolabeled tRNA obtained was precipitated with 5% trichloracetic acid, filtered, and counted. Recovered precipitable radioactivity indicated that more than 90% of the tRNA had been rephosphorylated under the above assay conditions. In a second experiment, reconstitution of a functional 5′-phosphate was followed through the capacity of the tRNA to recover the kinetic properties of full-length $tRNA^{His}$ in the reaction catalyzed by HisRS. For this purpose, after completion of the polynucleotide kinase action (30 min at 37 °C), the above assay was further incubated for 5 min at 60 °C. Then, aliquots (5−10 μL) were analyzed in the standard tRNA aminoacylation assay described below.

All tRNA samples were stored at −20 °C as ethanol precipitates in 300 mM sodium acetate (pH 5.5). Before use, each precipitated sample was centrifuged, and the pellet was redissolved in 20 mM Tris-HCl (pH 7.5), containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10 mM $MgCl_2$. At this stage, the final concentration of the tRNA was about 100 μM. Before use, the resulting sample was heated for 5 min at 60 °C (*23*).

Preparation of PTH Substrates. Concentrations of $tRNA^{His}$ and of its derivative were derived from values of the histidylability of samples. Twenty-five picomoles of either 5′-phosphate-$tRNA^{His}$, 5′-OH-$tRNA^{His}$, 5′-phosphate-$tRNA^{His}$-ΔG_{-1}, or 5′-OH-$tRNA^{His}\Delta G_{-1}$ was aminoacylated at 28 °C for 45 min in a reaction mixture (2 mL) buffered with 20 mM Tris-HCl (pH 7.5), containing 7 mM $MgCl_2$, 2 mM ATP, 20 μM L-[^{14}C]histidine (1.85 GBq/mmol), 0.1 mM dithiothreitol, and either 0.2, 2, 2, or 4 μM purified HisRS, respectively. After acetylation and purification through Chelex 100 (Bio-Rad) and Trisacryl GF05 (IBF, France) chromatographies (*4*), the recovered tRNA samples were precipitated with ethanol and stored at −20 °C. Before use in the presence of PTH, each sample was dissolved and heated at 60 °C as described above.

Purification of Enzymes. HisRS was purified from *E. coli* strain JM109 {*recA1 endA1 thi gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36*]} transformed by overproducing plasmid pHRS7 (*24*). Bacteria were grown overnight in 1 L of 2 × TY medium containing 50 μg of ampicillin/mL and 0.3 mM IPTG. After centrifugation for 35 min at 5000*g*, cells were resuspended at a final concentration of 100 A_{650} units/mL in 50 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoehanol, 0.1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride and submitted to ultrasonic disintegration (5 min, 0 °C). Cell debris was removed by centrifugation for 30 min at 8000*g*. Nucleic acids were precipitated by addition of steptomycin sulfate (30 g/L) to the supernatant, which was then centrifuged for 30 min at 8000*g*. The resulting supernatant was brought to 70% ammonium sulfate saturation and centrifuged (20 min, 8000*g*). After dialysis for 2 h against 2 L of buffer A (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoehanol, 50 mM KCl), the protein sample (8 mL) was loaded on a Superose 6 molecular sieve column (1.6 × 50 cm, from Amersham-Pharmacia Biotech) equilibrated in buffer A. Chromatography was developed at a flow rate of 0.2 mL/min. Fractions containing HisRS activity were pooled and loaded on a Q-Hiload anion-exchanger column (1.6 × 10 cm, from Amersham-Pharmacia Biotech) equilibrated in buffer A. The column was eluted using a linear KCl gradient in buffer A (2.5 mL/min, 100 mM KCl/h). Recovered enzyme (54 mg) was dialyzed against 20 mM Tris-HCl, pH 7.5, 0.1 mM dithioerythritol, and 60% glycerol (v/v) and stored at −20 °C. According to SDS−PAGE analysis, purified HisRS was at least 95% homogeneous. Enzyme concentrations were calculated using a molar extinction coefficient at 280 nm of 1 unit·g^{-1}·L and a molecular ratio of 94 000.

Each PTH variant was purified from the *E. coli* strain K37ΔpthTr transformed with the appropriate recombinant pUC18 vector (*4*). According to SDS−PAGE analysis, all enzyme mutants were at least 95% homogeneous.

Enzymatic Assays. HisRS activity was measured at 28 °C in a 100 μL assay containing 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 2 mM ATP, 0.1 mM dithiothreitol, 3 μM L-[^{14}C]histidine (12 GBq/mmol), 0.03−4 μM tRNA under study, and catalytic amounts of HisRS (60 pM to 1.2 μM). The reaction was quenched by the addition of 1 mL of cold TCA (5% w/w) and 20 μL carrier RNA from yeast (4 mg/mL). The radioactivity retained after filtration on GF/C filters (Whatman) was measured by scintillation counting, as described (*25*).

PTH activity was followed at 28 °C in a 100 μL assay containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.25−1 μM of the tRNA substrate under study, and catalytic amounts of PTH ranging from 2 nM to 2.5 μM. The reaction was quenched by the addition of 100 μL of cold TCA (10% w/w) and 20 μL of carrier RNA from yeast (4 mg/mL). The sample was then centrifuged, and the soluble radioactivity was measured in the supernatant by scintillation counting, as described (*5*).

K_m and k_{cat} values were derived from iterative nonlinear fits of the theoretical Michaelis equation to the experimental values, using the Levenberg−Marquardt algorithm (*26*).

RESULTS

Aminoacylation of $tRNA^{His}$ by Histidyl-tRNA Synthetase Strongly Depends on the Presence of a 5′-Phosphate on the Top of Extra Base G_{-1}. Properties of full-length $tRNA^{His}$ overexpressed in JM101TR were compared with those of native $tRNA^{His}$ partially purified from bulk tRNA. In the aminoacylation reaction, the k_{cat} and K_m values obtained were

Table 1: Catalytic Parameters of $tRNA^{His}$ Variants in the Histidylation Reaction Catalyzed by *E. coli* HisRS[a]

$tRNA^{His}$ variant	accepting capacity of tRNA samples (pmol of His/A_{260} unit)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	relative k_{cat}/K_m[b]
native $tRNA^{His}$	70	1.4	0.14	10	
overexpressed $tRNA^{His}$ (5′-phosphate)	1000	1.3	0.10	13	100
overexpressed $tRNA^{His}$ dephosphorylated (5′-OH)	900	>0.14	>5	0.028	0.2
overexpressed $tRNA^{His}$ after dephosphorylation and repair (5′-phosphate)	900			11	85
overexpressed $tRNA^{His}\Delta G_{-1}$[c] (5′-phosphate)	400			0.010	0.08
overexpressed $tRNA^{His}\Delta G_{-1}$[c] dephosphorylated (5′-OH)	400			0.0027	0.02

[a] Catalytic parameters were measured as described in Materials and Methods. Standard errors on the values shown are less than 10%. [b] Relative k_{cat}/K_m values were given an arbitrary value of 100 to the measurement obtained with overexpressed 5′-phosphorylated $tRNA^{His}$. [c] In these $tRNA^{His}$ mutants, the G_{-1} nucleotide is lacking.

very similar (Table 1). This therefore indicates that $tRNA^{His}$ produced with this in vivo system is fully competent in the presence of *E. coli* HisRS. The value of the K_m for $tRNA^{His}$ (0.14 μM), as found here, appears to be better than that (3.5 μM) described in ref *27*. However, the ionic conditions in the two assays are very similar. This discrepancy may be explained by our observation that, after ethanol precipitation, $tRNA^{His}$ had to be renatured by heat in the presence of magnesium to obtain aminoacylation kinetics with a Michaelian behavior. In the absence of this treatment, $tRNA^{His}$ samples looked heterogeneous, with one fraction being fairly aminoacylatable and another one slowly accepting histidine. This situation is reminiscent of several studies where poorly chargeable aggregates of tRNA could be evidenced (see, for instance, refs *28* and *29*). Dissociation of such aggregates required heat treatment under appropriate conditions (*23*).

A remarkable feature of $tRNA^{His}$ is the presence of an atypical additional base G_{-1} at its 5′-end. From the available literature, this nucleotide is believed to correspond to one major identity element in the productive interaction with HisRS (*18*, *27*, *30*, *31*). To assess the role of G_{-1} in the interaction of PTH with $tRNA^{His}$, we also produced the mutant $tRNA^{His}\Delta G_{-1}$. The aminoacylation capacity of this mutant, as shown in Table 1, confirmed the crucial role of the G_{-1} position. In agreement with previous studies (*27*), the k_{cat}/K_m value was decreased 1200-fold upon simple removal of the full nucleotide residue.

Since we also wanted to examine the substrates of PTH after 5′-dephosphorylation, the two tRNAs above, with or without the G_{-1} residue, were submitted to alkaline phosphatase digestion as described in ref *3* and then assayed for aminoacylation in the presence of HisRS. Upon the loss of its 5′-phosphate, full-length $tRNA^{His}$ became an extremely weak substrate of the synthetase. The resulting drop in the k_{cat}/K_m value was 500-fold, a factor comparable to that ensuing the removal of the full G_{-1} nucleotide. Values in Table 1 show that a marked change in K_m accompanies the observed drop in k_{cat}/K_m.

To exclude any artifactual inactivation of the tRNA during the dephosphorylation procedure, a phosphate was reintroduced at the 5′-end of the dephosphorylated polynucleotide with the help of T4 polynucleotide kinase, and the aminoacylation capacity of the resulting tRNA was measured. By using [γ-^{33}P]ATP, we could verify that the extent of the repair of the tRNA exceeded 90%. As shown in Table 1, upon the action of polynucleotide kinase, the k_{cat}/K_m value associated with dephosphorylated $tRNA^{His}$ increased from 0.028 to 11 $\mu M^{-1}\cdot s^{-1}$. This measurement establishes that, within ±10%, the repaired tRNA was indistinguishable from the starting material (13 $\mu M^{-1}\cdot s^{-1}$).

From the results above, one can propose that the 5′-phosphate associated to the additional base of $tRNA^{His}$ plays a role almost equivalent to that of the full nucleotide G_{-1} in the productive complex formation of HisRS with its cognate tRNA. The idea of a specific role of the 5′-phosphate at the top of nucleotide G_{-1} could be reinforced by comparing the aminoacylation of $tRNA^{His}\Delta G_{-1}$ with that of its dephosphorylated derivative. As summarized in Table 1, the k_{cat}/K_m value associated to the mutant tRNA deprived of phosphate was decreased 3−4-fold only as compared to the starting mutant tRNA. Clearly, the contribution to the aminoacylation reaction of the phosphate of the phosphodiester bond between residues G_{-1} and G_{+1} (P_{+1}) is negligible as compared to the very strong positive effect of the 5′-phosphate (P_{-1}) on the top of G_{-1}.

As in the Case of Other Elongator tRNAs, $tRNA^{His}$ Recognition by Peptidyl-tRNA Hydrolase Involves the Phosphate 5′ to Base +1. To study the roles of P_{-1} and P_{+1} in the recognition of $tRNA^{His}$ by *E. coli* PTH, the above tRNA variants, with or without a phosphate, were ^{14}C-histidylated, N-acetylated, and exposed to hydrolysis by PTH.

The k_{cat}/K_m associated with the wild-type substrate was equal to 0.1 $\mu M^{-1}\cdot s^{-1}$, a value comparable to that measured in ref *3* with diacetyllysyl-$tRNA^{Lys}$ (0.6 $\mu M^{-1}\cdot s^{-1}$). Moreover, the catalytic efficiency was insensitive to prior 5′-dephosphorylation of full-length $tRNA^{His}$ (Table 2). The shortening of the tRNA by one full residue to give $tRNA^{His}\Delta G_{-1}$ also had no consequence on the k_{cat}/K_m value, which was measured as 0.11 $\mu M^{-1}\cdot s^{-1}$. At this stage, we could conclude that the entire G_{-1} residue in $tRNA^{His}$ is dispensable in the reaction of PTH.

On the other hand, upon 5′-dephosphorylation, the efficiency of the substrate $tRNA^{His}\Delta G_{-1}$ decreased 7-fold (Table 2). Such a result strongly suggests that PTH can recognize that phosphate, P_{+1}, the location of which

Table 2: k_{cat}/K_m Values Associated with Various 5′-Phosphorylated or 5′-OH Dephosphorylated Substrates in the Hydrolysis Reaction Catalyzed by Wild-Type or Mutant *E. coli* PTHs[a]

	acetylhistidyl-tRNA^{His}		acetylhistidyl-tRNA$^{His}\Delta G_{-1}$[b]		diacetyllysyl-tRNA^{Lys}	
PTH	phosphorylated	dephosphorylated	phosphorylated	dephosphorylated	phosphorylated	dephosphorylated
WT	100	130	100	14	100	5.8
K105A	3.1	2.7	2.0	1.1	1	0.35
R133A	1.4	1.7	1.2	0.24	0.25	0.053
R133A-K105A	0.55	0.51	0.34	0.27	0.061	0.036

[a] k_{cat}/K_m values relative to those obtained with native enzyme (WT) and each of the three 5′-phosphorylated species under study are shown. Data regarding diacetyllysyl-tRNA^{Lys} are taken from ref *4*. An arbitrary value of 100 was given to each of the three reference results. The true k_{cat}/K_m values of these reference results are 0.10, 0.11, and 0.6 $\mu M^{-1}\cdot s^{-1}$ for phosphorylated acetylhistidyl-tRNA^{His}, acetylhistidyl-tRNA$^{His}\Delta G_{-1}$, and diacetyllysyl-tRNA^{Lys}, respectively. Limits of confidence of the relative k_{cat}/K_m values in the table are within ±10%. [b] In this tRNA^{His} mutant, the G_{-1} nucleotide is lacking.

corresponds to that of the standard 5′-terminal phosphate of all elongator tRNAs. With diacetyllysyl-tRNA^{Lys} as substrate, removal of the 5′-phosphate decreased by 17-fold the efficiency of the hydrolysis (*4*).

From the above data, it may be proposed that P_{+1}, not P_{-1}, reaches the cationic clamp formed by both K105 and R133 at the surface of the hydrolase. To assess this idea, the substrate efficiencies of tRNA^{His} and tRNA$^{His}\Delta G_{-1}$, with or without their 5′-phosphate, were compared in the presence of the single-point PTH mutants K105A and R133A. The double mutant K105A-R133A was also included in the analysis. No significant difference was observed between full-length tRNA^{His} and its counterpart lacking the terminal phosphate, no matter what PTH mutant was used (Table 2). The conclusion is that P_{-1} does not need either cationic residue to bind the hydrolase or, more likely, that this atypical phosphate does not make any contact with the hydrolase at all.

In contrast, hydrolysis by the PTH mutants depended on the status of the 5′-end of tRNA$^{His}\Delta G_{-1}$ and on the considered PTH derivative. With native PTH, a 7-fold drop in k_{cat}/K_m accompanied the removal of P_{+1}. The drops were reduced to 2- and 5-fold with the K105A and R133A species, respectively. With the double R133A-K105A mutations, the activity of the hydrolase became nearly insensitive to the presence or the absence of the P_{+1} phosphate in tRNA$^{His}\Delta G_{-1}$. Such a behavior reproduces that already described in ref *4* using N-blocked lysyl-tRNA^{Lys} derivatives. Therefore, we may conclude that the sites of the 5′-phosphate of tRNA^{Lys} or of P_{+1} in tRNA^{His} are identical. Upon destruction of this site, the catalytic advantage specifically conferred by either phosphate vanishes.

DISCUSSION

The above data suggest that, although it is engaged in the phosphodiester bond between G_{-1} and G_{+1}, the P_{+1} phosphate of a full-length tRNA^{His} can establish ionic bonds with the PTH surface, in a specific manner. Such a behavior gives tRNA^{His} the character of the other elongator tRNAs, which all offer to PTH a free 5′-phosphate group at the top of a full 1:72 base pair. It is likely that, upon complex formation with EF-Tu, the same P_{+1} phosphate of tRNA^{His} is recognized.

In the case of *E. coli* HisRS recognition, the 5′-phosphate associated with the additional base of tRNA^{His} plays the role of an identity element in the productive tRNA−enzyme complex formation. This conclusion agrees with the in vivo observation by Yan and Francklyn (*19*) that, provided it keeps the discriminator C_{73} residue, an amber suppressor derivative of tRNA^{His} directs the only incorporation of histidine, whatever the base at position −1 (G, U, or C). Similarly, small helical substrates became aminoacylatable with histidine upon the single introduction of one base pair, $G_{-1}-C_{73}$ (*18, 20*). The above results may support the importance of both C_{73} and P_{-1}. Thus, the keeping of G_{-1} in the amber suppressor derivative of tRNA^{His}, while C_{73} was changed in U, A, or G, did not maintain the histidylability of the tRNA (*19*). Nevertheless, in all of these in vivo studies, alteration of the $G_{-1}-C_{73}$ base pair may first affect the processing by RNase P of precursor tRNA^{His} and generate, at least partly, molecules terminating at the +1 nucleotide. Consequently, the above in vivo experiments could not unambiguously establish the importance of base −1 in the reaction of HisRS. It was also shown that C_{73} alone is not enough to confer a tRNA the capacity to be histidylated. Indeed, amber derivatives of tRNA^{Gln}, tRNA^{Ala}, and tRNA^{Tyr} with a C_{73} could not direct the incorporation of histidine in proteins in vivo and continued to be aminoacylated by their cognate synthetases (*32*, *33*). In the case of these experiments, nonaminoacylation by HisRS may also be contributed to by the absence of a histidine anticodon sequence in the three studied tRNAs. However, conflicting results have been obtained with regard to the importance of the anticodon region of tRNA^{His} from *E. coli*, as reviewed in ref *34*.

In vitro transcripts of full-length tRNA^{His} were also synthesized with various combinations of the −1:+73 pair (*27*). Characterization of these transcripts revealed that a triphosphate at the 5′-end inhibited the reaction of aminoacylation. The reduction of k_{cat}/K_m was 14-fold. In ref *18*, a comparable effect of the presence of 5′-triphosphate on the histidylation of minihelices was noted. These observations support the proposal that optimal tRNA^{His} identity needs an intact P_{-1} group. In the same set of experiments (*27*), a tRNA derivative lacking G_{-1} was studied. In reasonable agreement with our present data, the catalytic efficiency dropped 250-fold upon this modification. Substitution of the base G_{-1} by A in the transcript also had an effect on the aminoacylation. The value of k_{cat}/K_m was reduced 70-fold. One must admit that this weakening of the tRNA activity reflects some specificity toward the G_{-1} base. Transcripts with changes at the 73 position were also studied (*27*). Introduction of an A, a U, or a G opposite to G_{-1} decreased the catalytic efficiency in the aminoacylation reaction by 12, 80, and >10 000 times, respectively. Such results preclude the derivation of any rule

regarding the consequence of base pairing between positions −1 and 73 in the reaction of HisRS.

Acquisition of G_{-1} by $tRNA^{His}$ during evolution is likely to reflect adaptation of this tRNA to the specific recognition of HisRS. It may also participate in the rejection of noncognate synthetases. In relation with this idea, Francklyn et al. observed that G_{-1}:C_{73}, the major determinant for histidine identity, was a blocking determinant for alanyl-tRNA synthetase that overrides the G_3:U_{70} base pair (*30*).

Several non-histidine tRNA sequences, as deduced from their gene sequences, such as $tRNA^{Cys}$ of *Mycoplasma genitalium* or *Haemophilus influenzae*, $tRNA^{Asp}$ of *M. genitalium*, $tRNA^{Ile}$ of *Synechocystis* sp., $tRNA^{Arg}$ of *Methanococcus jannaschii*, $tRNA^{Thr}$ of *Stigmatella aurantiaca*, or a few mitochondrial and chloroplastic tRNAs (*35*) show a C as the discriminator base. One can imagine that the synthetases corresponding to these tRNAs also positively recognize the C_{73} in $tRNA^{His}$. However, in the above cells, available $tRNA^{His}$ sequences indicate the systematic presence of a G_{-1}:C_{73} base pair, in which G_{-1} can be suspected to help rejection of any noncognate synthetase.

In the cytoplasm of eukaryotes, there is a $tRNA^{Pro}$ molecule containing a C_{73} residue. Nevertheless, while still presenting the atypic G_{-1}, known cytoplasmic $tRNA^{His}$ sequences never show a C at position 73 (*35*). Instead, eukaryotic $tRNA^{His}$ species have an A_{73}. In the case of the yeast system, efficiency of the synthetase was decreased by more than 2 orders of magnitude upon removal of the −1 residue (*31*, *36*). In contrast, the substitution of G_{-1} by A or C caused a less than 10-fold decrease in histidylability (*36*). Finally, a 20-fold loss of histidylability followed the changing of the A_{73} position into a G (*31*).

Several plant virus RNAs have been characterized as substrates of valyl-, tyrosyl-, or histidyl-tRNA synthetases (reviewed in refs *37* and *38*). To account for this property, structural RNA models mimicking a tRNA have been designed. In the tRNA-like structures of histidine-accepting viral RNAs, the equivalent of residue −1 belongs to a pseudoknot forming a quasi-continuous double helix in the amino acid acceptor branch, and the equivalent of the 5′-phosphate of $tRNA^{His}$ is engaged in a phosphodiester bond. The equivalent of the discriminator base is A or C, depending on the virus considered. Such a degeneracy at this position is in agreement with the relatively low weight of A_{73} in $tRNA^{His}$ (*31*).

Recent studies suggest that many classes of plant viral RNAs with a tRNA-like structure can be acylated by yeast HisRS, although they lack the GUG "pseudo-anticodon" sequence (*39*, *40*). By the way, this behavior may indicate that the recognition by a eukaryotic HisRS of either the anticodon of $tRNA^{His}$ or the pseudo-anticodon of plant viral RNAs tolerates noncognate triplets. Indeed, upon substitution of the anticodon in the *Saccharomyces cerevisiae* system, the decreases in aminoacylation efficiency do not exceed 70-fold (*36*). In another study, the anticodon loop of yeast tRNA was transplanted inside yeast $tRNA^{Asp}$ (*31*). Since histidylation of the obtained mutant was only 2 times more efficient than that of wild-type $tRNA^{Asp}$, the authors concluded that the anticodon region in histidine-specific tRNA is not a strong identity element. The reason for this would be that the specific features of the amino acid acceptor stem play a predominant role in the histidine identity. In the context of the present work, we propose that the property of viral RNAs to accept histidine relates to the capacity of the pseudoknot in each RNA to offer one phosphodiester group capable of mimicking the extra phosphate of $tRNA^{His}$ and that, like prokaryotic HisRS, eukaryotic HisRS recognizes this extra phosphate as a positive determinant. In agreement with this prediction, Nameki et al. observed that the activity of the yeast enzyme responded to the introduction of a triphosphate group at the 5′-end of the cognate tRNA (*36*). On the other hand, as already underlined above (*36*) when G_{-1} is changed to A or C, the yeast enzyme would be rather neutral regarding the base at the −1 position. The latter idea is supported also by the variability of the equivalent of the −1 base in plant RNAs (A or U) and by the observation that, upon mutation of this base, resected pseudoknots derived from brome mosaic virus RNA remain efficient substrates of yeast HisRS (*41*).

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