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Role of the extra G-C pair at the end of the acceptor stem of $tRNA^{His}$ in aminoacylation

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ABSTRACT

All sequenced histidine tRNAs have one additional nucleotide at the 5' end compared with other tRNA species. To investigate the role of this unique structure in aminoacylation, we constructed *in vitro* transcripts corresponding to the *E. coli* histidine tRNA sequence and its variants at the G_{-1}-C_{73} base pair, by using T7 RNA polymerase transcription system. A transcript having a wild-type sequence with no modified bases was a good substrate for histidyl-tRNA synthetase (HisRS), and aminoacylation activity was affected by introduction of a triphosphate at the 5' terminus. Base replacements at position 73 caused a marked decrease of Vmax, and deletion and substitution of the G_{-1} had a remarkable effect on the aminoacylation. A mutant having an A_{-1}-U_{73} pair was also not a good substrate for HisRS. Comparison among G_{-1}-deficient mutants showed that A was preferable rather than C as the base at position 73. These data demonstrate that the set of the G_{-1}-C_{73} pair at the end of the acceptor stem of histidine tRNA is crucial for the catalytic process of aminoacylation.

INTRODUCTION

All $tRNA^{His}$ molecules are known to be one nucleotide longer at the 5' end than other tRNA species (1,2). In eucaryotic cells, an extra G is added post-transcriptionally during tRNA maturation (3). In procaryotic cells and in chloroplasts (4-7), the extra G is encoded within the gene, which is subjected to an unusual type of processing by RNase P. In addition to protein synthesis, $tRNA^{His}$ is known to participate in other cellular processes, such as the ubiquitin- and ATP-dependent proteolytic systems (8,9). However, it is not clear why the extra G exists only in $tRNA^{His}$ species, and the purpose for which the extra G is required. The opposing base, the fourth base from the 3' end of this tRNA, is well conserved as A in eucaryotes and C in procaryotes or organelles (1). In *E. coli*, only $tRNA^{His}$ possesses C at this position (1).

Histidyl-tRNA synthetase is an α_2 dimer with a subunit molecular weight of 45,000 (10,11). This enzyme seems to have been derived from a different progenitor from other aminoacyl-tRNA synthetases (ARSs), since it lacks the conserved, $H^{I}/_{L}GH$ sequence.

The problem of recognition of tRNAs by ARSs has been intensively discussed

for over 20 years. Much information concerning tRNA identity has been obtained from genetic studies using suppressor tRNAs (reviewed in refs. 12 and 13). Recently, Sampson and Uhlenbeck demonstrated that an in vitro transcript corresponding to yeast phenylalanine tRNA with no modified nucleotides was a good substrate for phenylalanyl-tRNA synthetase (14), and that the five nucleotides containing all the anticodon bases and the fourth base from the 3' end were sufficient for specific aminoacylation with phenylalanine (15). At present, this method seems to be one of the best approaches for clarifying the molecular basis of tRNA recognition. With this method, Schulman and Pelka showed that the anticodon contained sufficient information for distinguishing methionine and valine tRNAs (16).

In this study, we investigated the importance of sequence and structural requirements of the extra G or the extra G_{-1}-C_{73} base pair of $tRNA^{His}$ for aminoacylation by HisRS. For this purpose, we prepared in vitro transcripts corresponding to the normal sequence and mutants of E. coli $tRNA^{His}$ in order to compare their kinetic parameters for aminoacylation.

MATERIALS AND METHODS

Materials

E. coli strain BL21 harboring a T7 RNA polymerase-overproducing plasmid, pAR1219, was a generous gift from Dr. F. W. Studier. EcoT22I was purchased from Toyobo Co., LTD.. Native $tRNA^{His}$ (1350 pmol/A_{260}) was obtained from Subriden RNA (Rolling Bay, WA). L-[U-^{14}C]histidine and [α-^{32}P]ATP were purchased from Amersham.

Construction of plasmid DNA

Synthetic genes of E. coli $tRNA^{His}$ were joined to a T7 RNA polymerase promoter, inserted into pUC19 and cloned in E. coli strain JM109. Transformants harboring the plasmid DNA were screened by restriction analysis, and the DNA sequence was confirmed by the dideoxy sequencing method (17).

In vitro transcription

T7 RNA polymerase was purified from E. coli strain BL21 (18, 19). Transcripts of the tRNA genes were prepared in a reaction mixture containing 40 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, 2 mM spermidine, 10 mM $MgCl_2$, bovine serum albumin (50 μg/ml), 2.0 mM each NTP, 20 mM 5' GMP, EcoT22I-digested DNA (0.2 mg/ml), 2 units of inorganic pyrophosphatase (Sigma) and pure T7 RNA polymerase (70 μg/ml). Transcripts initiate with 5' pppG were prepared in the absence of 5' GMP and inorganic pyrophosphatase (14). Transcripts initiate with an A were prepared in the reaction mixture containing 20 mM 5' AMP instead of 5' GMP. The transcripts were purified by 15% polyacrylamide gel

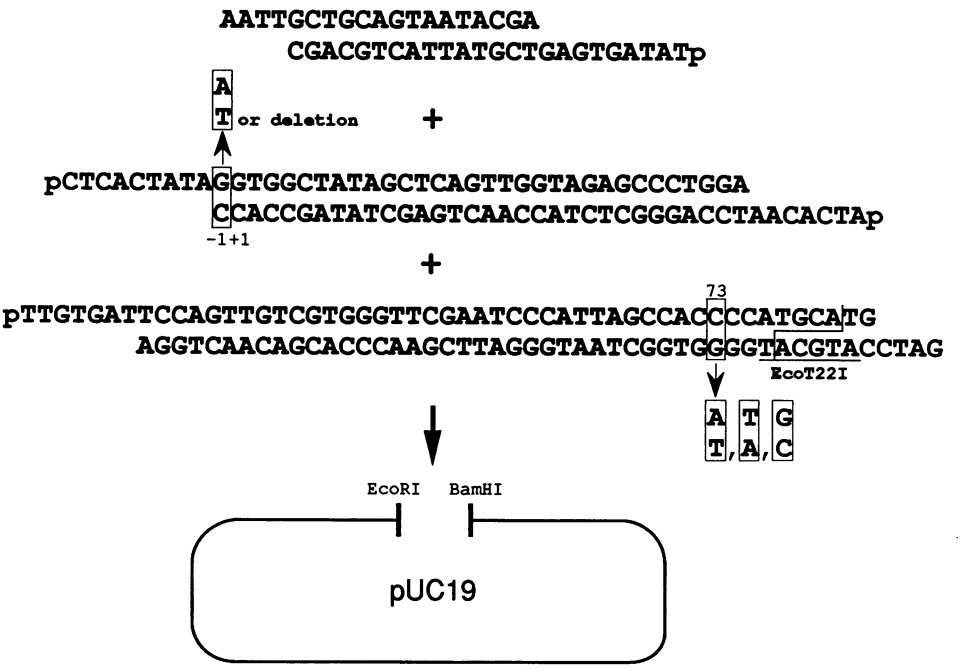

Fig. 1 Schematic diagram of the plasmid construction. Six synthetic DNA oligomers were ligated and inserted into EcoRI/BamHI sites of pUC19. Arrows indicate the sustitutions or deletion at positions -1 and 73. Transcription start point is at position -1 (or +1 for the G_{-1} deficient mutants).

electrophoresis. Lengths and purities of transcripts were verified by denatured 15% polyacrylamide gel electrophoresis.

5' end analysis of transcripts

Transcripts were dephosphorylated by bacterial alkaline phosphatase (Toyobo), followed by 5' end labeling with [γ-^{32}P]ATP (3000 Ci/mmol) and polynucleotide kinase from E. coli A19 (Takara). After purification by polyacrylamide gel electrophoresis, the transcripts were digested with nuclease P1. ^{32}P-labeled nucleotides were subjected to two-dimensional thin-layer chromatography (20), and detected by autoradiography.

3' end analysis of transcripts

[^{32}P]pCp ligated transcripts were purified by polyacrylamide gel electrophoresis, followed by digestion with RNase T2. ^{32}P-labeled nucleotides were subjected to two-dimensional thin-layer chromatography (20), and detected by autoradiography.

Aminoacylation assay

Aminoacylation reactions were carried out at 37 °C in 40μl of reaction mixture

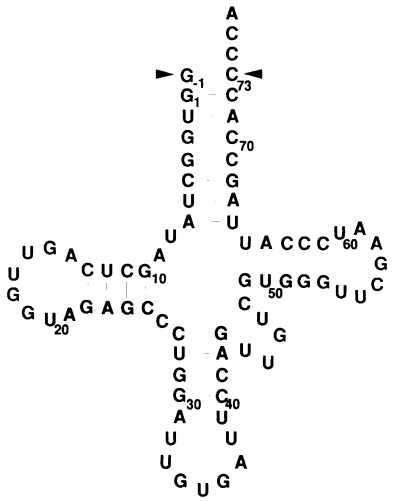

Fig. 2 The $tRNA^{His}$ transcript folded into a cloverleaf structure. The native E. coli $tRNA^{His}$ has s^4U_8, D_{16}, D_{17}, D_{20}, Q_{34}, m^2A_{37}, Ψ_{38}, Ψ_{39}, m^7G_{46}, T_{54} and Ψ_{55}. Arrows indicate the extra G-C pair, on which the present study was focused.

containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 4 mM 2-mercaptoethanol, 2.5 mM ATP, 20 μM L-[U-^{14}C] histidine (312 mCi/mmol) and 8 units of partially purified HisRS from E. coli A19.

RESULTS

The transcripts of E. coli $tRNA^{His}$ derivatives were prepared by the method of Sampson and Uhlenbeck (14) with slight modification. In order to obtain a transcript having CCA at the 3' end, EcoT22I was used instead of BstNI (Fig. 1), because the gene of E. coli $tRNA^{His}$ has an internal BstNI site. Digestion of the plasmid carrying the $tRNA^{His}$ gene by EcoT22I results in a 3' protruding linear DNA template. End analyses and electrophoresis pattern indicate that run-off transcription with this template gave a considerable amount of unmodified $tRNA^{His}$ of the expected sequence (Fig. 2). Generally, the transcription by T7 RNA polymerase is initiated with a G next to the promoter sequence. It has been reported that one of the T7 class II promoters also initiates with an A (21). We then tried to prepare mutant transcripts in which G_{-1} was substituted with A_{-1}. First, the base next to the T7 RNA promoter was changed to A from G on the plasmid (Fig. 1). Instead of 20 mM GMP, 20 mM AMP was added to the reaction mixture. The mutant transcripts of an identical length were obtained with a slightly lower efficiency. From 5' end analysis of the transcript having A_{-1}-C_{73}, more than 99% of the transcript had a 5' pA at

Table I. Kinetic parameters of *E. coli* $tRNA^{His}$ and the transcripts of $tRNA^{His}$ derivatives.

	Apparent Km[a] (μM)	Vmax (pmol/min·mg)	Vmax/Km (relative)
native $tRNA^{His}$	3.5	1.3×10^{4}	1.5
transcript			
$tRNA^{His}$ (G_{-1}-C_{73})	4.0	1.0×10^{4}	1.0
$tRNA^{His}$ ($pppG_{-1}$-C_{73})	3.5	6.1×10^{2}	7.0×10^{-2}
$tRNA^{His}$ (G_{-1}-A_{73})	3.7	7.8×10^{2}	8.4×10^{-2}
$tRNA^{His}$ (G_{-1}-U_{73})	4.8	1.5×10^{2}	1.3×10^{-2}
$tRNA^{His}$ (G_{-1}-G_{73})	nd[b]	nd	$<1.0\times10^{-4}$
$tRNA^{His}$ (C_{73})[c]	nd	nd	4.1×10^{-3}
$tRNA^{His}$ (A_{73})[c]	10	6.1×10^{2}	2.4×10^{-2}
$tRNA^{His}$ (U_{73})[c]	nd	nd	7.3×10^{-4}
$tRNA^{His}$ (G_{73})[c]	nd	nd	$<1.0\times10^{-4}$
$tRNA^{His}$ (A_{-1}-C_{73})	10	4.1×10^{2}	1.6×10^{-2}
$tRNA^{His}$ (A_{-1}-A_{73})	nd	nd	2.9×10^{-3}
$tRNA^{His}$ (A_{-1}-U_{73})	nd	nd	8.9×10^{-4}
$tRNA^{His}$ (A_{-1}-G_{73})	nd	nd	$<1.0\times10^{-4}$

Each parameter was estimated from a Lineweaver-Burk plot. Several mutant tRNA transcripts demonstrated very low levels of histidine acceptance and their initial velocities increased linearly with increasing transcripts up to 15 μM, and therfore Km and Vmax were not able to be determined with accuracy under these conditions. In this case, Vmax/Km was estimated from the slope of the linear plot of initial velocity versus tRNA transcript concentration. Parentheses indicate the bases at positions -1 and 73. [a]HisRS used here was not fully purified, and therefore the apparent Km is presented. [b]Not determined. [c]G_{-1} deficient mutants.

the 5' end (data not shown), indicating that the initiation was just occurring with an A at position -1. This method would thus be widely applicable to many RNAs having an A at the 5' end.

Usually, the wild-type transcript purified by polyacrylamide gel electrophoresis incorporated 1,000-1,100 pmol of histidine per A_{260} unit. However, the transcript having a 5' pppG at the 5' end and the mutant transcripts incorporated only less than 400 pmol of histidine per A_{260} unit.

Judging from the end analyses and the lengths on the polyacrylamide gel, it is reasonable to consider that this reduction of incorporation was mainly caused by a decrease in the initial velocity and enzyme inactivation under the assay conditions used, rather than impurity of the transcripts.

Table I shows the kinetic parameters of the transcripts for the aminoacylation by HisRS. The transcript $tRNA^{His}$ has only a slightly higher Km and only a slightly lower Vmax than those of the native $tRNA^{His}$. Although E. coli $tRNA^{His}$ has 11 modified nucleotides (see legend of Fig. 1), removal of these modifications did not significantly affect the aminoacylation. The Km of the transcript having guanosine triphosphate at the 5' end was much the same, whereas the Vmax was significantly lower than that of the normal sequence. Mutation at position 73 had little effect on the Km but a great effect on the Vmax. The base preference at position 73 was C>A>U>G. Removal of G_{-1} and substitution of G_{-1} with A_{-1} resulted in a great decrease in the Vmax/Km. In the G_{-1}-deficient mutants, A_{73} was preferable to C_{73}. The Vmax/Km of the mutant transcript having an A_{-1}-U_{73} pair was lower by a factor of 10^3 than that of the native $tRNA^{His}$. Little activity was detected in every mutant for which position 73 was occupied by a G.

DISCUSSION

E. coli $tRNA^{His}$ contains four distinctive modified bases, Q_{34}, m^2A_{37}, ψ_{38} and ψ_{39} in the anticodon stem and loop (1). The $tRNA^{His}$ from a his T mutant lacking pseudouridines in the anticodon arm was normally aminoacylated (22). The first letter of the anticodon of $tRNA^{Asp}$, $tRNA^{Asn}$, $tRNA^{His}$ and $tRNA^{Tyr}$ from procaryotes and mammals is occupied by queuosine (Q) or modified Q (1). In mammals, undermodified G-containing $tRNA^{Asp}$ has been found to have slightly lower aminoacylation activity than the native, mannosyl Q-containing $tRNA^{Asp}$ (23). Since the kinetic parameters of the transcript of $tRNA^{His}$ made no great difference from those of the native $tRNA^{His}$, these modifications may not be directly involved in the recognition by HisRS in E. coli system.

Sequence comparison shows that the fourth base from the 3' end of tRNA (discriminator base) is well conserved within every charged amino acid among many organisms (1). This base often serve as an identity element in vivo, as shown for $tRNA^{Tyr}$ (24) and $tRNA^{Ser}$ (12,25), and also serve as a recognition element by cognate ARS in vitro, as shown for $tRNA^{fMet}$ (26), $tRNA^{Phe}$ (15), $tRNA^{Asp}$ (27) and this study. In the latter cases, this base appears to be correlated with Vmax rather than Km, as for $tRNA^{fMet}$ and this study. In E. coli, only $tRNA^{His}$ possesses C at this position. From our present data, base preference is apparent. It has been reported that $tRNA^{His}$ of bacteriophage T5

possesses a U_{-1}-A_{73} pair (1, 28). A is relatively preferable as the base at position 73. In particular, in G_{-1} deficient mutants, A_{73} is preferable rather than C_{73}. If G_{-1} and C_{73} are independently recognized without base-pairing by HisRS, C_{73} should be better than A_{73} in G_{-1} deficient mutants, but it was not the case. This implies that base-pairing is also required for aminoacylation. However, a mutant having an A_{-1}-U_{73} pair is not a good substrate for HisRS. Thus, it is concluded that not only the base-pairing at positions -1 and 73 or merely the nature of the discriminator base, but also the set of the G_{-1}-C_{73} pair is critical for recognition by HisRS. The G_{-1}-C_{73} pair in $tRNA^{His}$ is possibly one of the major identity determinants.

The mechanism of recognition of the vicinity of the 3' CCA end by cognate ARS is still poorly understood. Indeed, it may be difficult to clarify the real feature of this region, because it may stabilize the transition state of the complex. Labouze and Bedouelle have derived a recognition model (29) of the vicinity of the 3'end of $tRNA^{Tyr}$ by tyrosyl-tRNA synthetase (TyrRS) of Bacillus stearothermophilus based on kinetic data obtained from mutants of TyrRS and the crystal structure of B. stearothermophilus TyrRS (30). The model suggests that precise recognition of A_{73} could be an important factor for discrimination by TyrRS. Cocrystals of $tRNA^{Asp}$ with aspartyl-tRNA synthetase (31) and $tRNA^{Gln}$ with glutaminyl-tRNA synthetase (GlnRS) (32) have been obtained, and a structure of the latter complex has been determined at 2.8 Å resolution (33). The structure shows that an intramolecular hydrogen bond formation between 2-amino group of G_{73} and the phosphate of A_{72} of $tRNA^{Gln}$ causes a hairpin turn of 3' CCA tail towards the active pocket of GlnRS. In the case of $tRNA^{His}$, the extra G_{-1} would presumably serve to control the spatial position of the C_{73} for introducing 3' CCA tail into a precise productive binding position in the transition state.

Removal of 5'-terminal phosphate or chemical modification of 5'-terminal region of $tRNA^{fMet}$ (34), and introduction of a 5' triphosphate into a transcript of $tRNA^{Phe}$ (14) had no great effect on aminoacylation. Therefore, it is generally supposed that the 5' terminal phosphate of tRNA does not closely contact with the cognate ARS. In this study, introduction of 5' triphosphate into the transcript had a great effect on aminoacylation, indicating that the 5'-terminal region of $tRNA^{His}$ must be covered with HisRS. From the lack of the $H^{I}/_{L}GH$ sequence conserved among the other ARSs (11), HisRS has presumably a rather unique active pocket.

The fact that the aminoacylation level of the mutant $tRNA^{His}$ having G_{-1}-A_{73} is higher than those of other mutants seems to be interesting on the basis of the evolutionary aspects of the recognition of $tRNA^{His}$ by HisRS. Eucaryotic

$tRNA^{His}$ has a G_{-1}-A_{73}, whereas $tRNA^{His}$ in organelles has a G_{-1}-C_{73}. In addition, it is noteworthy that HisRS in both yeast cytoplasm and mitochondria is encoded by the same gene (35).

This report is the first description of the role of the extra G_{-1}-C_{73} pair of $tRNA^{His}$. However, it still remains an open question as to why only $tRNA^{His}$ species require the extra G_{-1} in all organisms including organelles. To clarify this aspect, further investigations are required.

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