Histidine Regulation in Salmonella typhimurium

IX. HISTIDINE TRANSFER RIBONUCLEIC ACID OF THE REGULATORY MUTANTS*

(Received for publication, September 23, 1971)

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SUMMARY

Four classes of mutants constitutive for the histidine operon of Salmonella typhimurium, hisR, hisT, hisU, and hisW, were examined for a possible role in the production of histidine tRNA. The content of tRNAHis in tRNA isolated from each of the mutant types was compared to that of the wild type, and the chromatographic behavior of the tRNAHis obtained from the mutants and the wild type was examined. Chromatography of the tRNAHis of the wild type gave no evidence for multiple species.

Two factors suggest that hisR is a structural gene for histidine tRNA. First, hisR strains were found to have 25 to 45% less tRNAHis than the wild type; and second, introduction of an episome carrying the hisR gene into the wild type resulted in a 2.5-fold increase in the quantity of tRNAHis.

The tRNAHis of a hisT mutant chromatographed differently from that of the wild type. Since the hisT gene has previously been shown to code for a dispensable protein, we conclude that this protein is involved in tRNAHis maturation. The tRNAHis from hisT mutants is present in normal amounts, is charged normally, and appears to behave normally in protein synthesis, although not in repression control.

The tRNA from a cold-sensitive hisW mutant was found to have an altered acceptance for several amino acids. This indicates that hisW also codes for a tRNA maturation enzyme. No increase in tRNAHis levels was obtained when an episome carrying the hisW gene was inserted into the wild type, and the tRNAHis from a hisW mutant behaved as the tRNA from the wild type on chromatography.

No difference was found between the tRNAHis of hisU mutants and that of the wild type.

The histidine operon of Salmonella typhimurium LT-2 is composed of nine contiguous genes which specify the enzymes of histidine biosynthesis. The operon is known to be under repression control (reviewed in Reference 2), but the mechanism of the control is not clear. Although mutations in any of six loci (hisO, hisS, hisR, hisT, hisU, and hisW) are capable of producing constitutivity, the functions of only two of the six genes are known. HisO is an operator gene (or promoter gene, or both): mutations in hisO are cis-dominant (3, 4), and map at the beginning of the operon (5). The second gene, hisS, codes for the histidyl-tRNA synthetase (1, 6), the enzyme which attaches histidine to tRNAHis. The properties of the hisS mutants, and also some analogue studies (7), have shown that histidine itself does not act directly in the repression mechanism. Instead, charged histidine tRNA may provide the controlling signal.

Because of the probable importance of histidine tRNA in the repression mechanism, a series of experiments were undertaken in an attempt to clarify a suggested relationship (2) between histidine tRNA and the remaining regulatory mutants, hisR, hisT, hisU, and hisW. Using column chromatography, a search was made for multiple (isoaccepting) species of histidine tRNA in the wild type. Column chromatography was also used in an attempt to detect alterations in the tRNAHis from mutant strains. Finally, a more thorough study was made of the level of histidine-accepting activity of tRNA isolated from the mutant strains.

MATERIALS AND METHODS

The crystalline sodium salt of ATP, and bovine serum albumin, type V, were obtained from Sigma. Glass fiber filters (type A, 1 inch in diameter) were purchased from Gelman Instrument Co. and filter paper discs (25 mm) from Schleicher and Schuell. [3H]- and [14C]histidine, [14C]glycine, [3H]lysine, and [3H]valine were purchased from New England Nuclear; [3H]and [14C]valine were also obtained from ICN; [14C]arginine was obtained from tracerlab; [3H]arginine from Schwarz BioResearch; and [3H]leucine from Amersham-Searle. Homogeneous histidyl-tRNA synthetase (specific activity 5000 units per mg) was supplied by F. De Lorenzo. Aminoacyl-tRNA synthetases for arginine, glycine, leucine, lysine, and valine were obtained as by-products of the purification of the histidyl-tRNA synthetase (5): the column fractions containing no histidyl-tRNA synthetase activity were pooled, concentrated by vacuum dialysis, and stored at -20° in 50% glycerol. Benzoylated DEAE-cellulose was a gift of W. Holloman. This material was superior in packing, flow rate, and resolution to some commercial preparations tried (Schwarz BioResearch). Chromosorb W, acid washed, dimethyl dichlorosilane treated (100 to 120 mesh), was purchased from...
Varian Aerograph. Triocetylpropylammonium bromide was obtained from Eastman Organic Chemicals.

*Bacterial Strains*—*JL250*, a cold-sensitive hisW mutant, was supplied by J. E. Brenchley. At 37°, the mutant has a normal growth rate in nutrient broth, but grows slightly more slowly than the wild type in minimal salts-glucose. When shifted to 20° the organism stops growth immediately, regardless of the growth rate in nutrient broth, but grows slightly more in minimal salts-glucose medium at a concentration of 10^{-4} M glucose. When required, histidine was added to the growth medium (9). HisRl230 (TA1536), and adenine and thiamine added at 2 μM each, were grown in 15-liter New Brunswick fermentors with vigorous aeration in a minimal salt medium (10) containing 0.5% glucose. His01828 was obtained from P. E. Hartman, and His01825 was isolated by G. W. Chang by mutagenesis of LT-2 (same origin as hisT1508) are described in Reference 3. The mutant hisW1595 (same origin as hisT1508) is described in Reference 5. Episomes F'14 and F'32 and the episome-carrying strains hisRl230 metE88/F'14 and ibsC401 metE88/F'14 are described in Reference 5. Strains LT-2, ara-9, his01242, hisU1817, hisU1820, hisW1821, hisW1825, hisRl228, hisRl818 (TA796), hisH107, hisRl203 (TA778), and hisT1504 were obtained from M. Brenner and B. N. Ames.

*Preparation of tRNA*—The tRNA was prepared by a modification of the method of Silbert et al. (11) and stripped according to the method of Sari and Zamecnik (12). The following procedure is given for 100 g of cells, wet weight, but has been scaled up and down with comparable results. All steps are carried out at 4°, unless otherwise noted. The frozen bacterial paste is cut into chunks and suspended in 200 ml of 10 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂. Then 200 ml of phenol (Malinckrodt) saturated with the same buffer are added and the mixture shaken vigorously for at least 1 hour. The resulting suspension is centrifuged at 14,000 × g for 30 min, the aqueous (top) phase sucked off and passed directly onto a column of DEAE-cellulose (Selectacel 70, Schleicher and Schuell Co.) with a bed volume of 100 ml (2.3 × 24 cm).

The column had been packed under about 50 p.s.i. in a buffer containing 1 mM Tris-HCl, pH 7.5, and 10 mM MgCl₂ containing 1 mM NaCl, and then originally equilibrated with TM buffer containing 0.02 M NaCl. The aqueous phase from the phenol extraction is passed onto the column at a flow rate of 2 to 4 ml per min. The column is washed with the equilibrating buffer at 5 ml per min until the A₂₆₀ of the effluent decreases to about 0.1, and then is washed at the same flow rate with TM buffer containing 0.1 M NaCl until the A₂₆₀ of the effluent decreases to about 0.05. The tRNA is then eluted at a flow rate of 1 ml per min with TM buffer containing 1 M NaCl, and fractions of about 20 ml are collected. The specific activity of tRNAH is 15% higher than 1.0, over 95% of the total A₂₆₀ is obtained, and material with constant specific activity is insured. The pooled fractions are precipitated with 2 volumes of ethanol, left in the cold overnight, and then most of the supernatant removed by suction and the precipitate collected by centrifugation at 14,000 × g for 30 min. The precipitate is dried under vacuum at room temperature, and then dissolved in 10 ml of 1.8 M Tris-acetate, pH 8.0. The solution is incubated at 37° for 90 min, filtered through a glass fiber filter, and the tRNA precipitated by the addition of 25 ml of 95% ethanol kept at -20°. After 60 min at -20°, the precipitate is collected by centrifugation at 27,000 × g for 10 min. The precipitate is washed twice by resuspension in cold 75% ethanol. The final precipitate is dried under vacuum at room temperature. Yields are from 150 to 250 mg per 100 g of cells, wet weight, with there being about a 15% loss in A₂₆₀ units between the pooled column fractions and the final yield. The tRNA has 15 to 18 A₂₆₀ units per mg, with approximately 15 pmoles of tRNAH per A₂₆₀ unit.

The purity of all tRNA's is increased about 30% if fresh, rather than frozen, cells are used, presumably because freezing and thawing the cells produces some cell breakage, leading to greater contamination of the tRNA with ribosomal RNA. Growing the cells on nutrient broth (Difco), rather than in minimal medium, produced no significant change in the tRNA contents (done for wild type only). The inclusion of 2 mM thiosulfate in the buffers has no detectable effect on the kinetic or chromatographic properties of a number of tRNA species tested; however, Singer and Smith have found that the 4-thiouridine present in *Salmonella* tRNAH is about 20% oxidized in the absence of the thiosulfate.

*Preparation of Charged tRNA*—Charged tRNA was prepared as described elsewhere.

*Operation of Benzoylated DEAE-cellulose Column*—A column bed, 1.5 × 34 cm, was packed by gravity flow of a slurry of the adsorbent in a buffer of 10 mM MgCl₂ and 10 mM sodium acetate at pH 4.5, which was 1 M in NaCl. Columns were initially equilibrated with the MgCl₂-sodium acetate buffer 0.5 M in NaCl, and the tRNA applied in a volume of several milliliters. A salt gradient running from 0.5 to 1 M NaCl in the MgCl₂-sodium acetate buffer was used to develop the columns. The gradient was followed by a wash of 2 M NaCl in 15% ethanol. When used, nonlinear gradients were generated with a Dialagrad model 190 (ISCO, Lincoln, Neb.). All column operations were conducted at room temperature. Recovery of A₂₆₀ units was usually about 90%, and recovery of histidine-accepting activity was about 85%.

*Operation of Reversed Phase Column No. 3*—The adsorbent was prepared and the columns packed and operated as described by Weiss et al. (13). When tRNA was chromatographed uncharged, columns were operated at 37° using a buffer of 10 mM MgCl₂ and 10 mM Tris-HCl, pH 7.0. When charged tRNA was chromatographed, columns were operated at either room temperature or 37°, with a buffer of 10 mM MgCl₂ and 10 mM sodium acetate, pH 4.5. Columns were developed with a NaCl gradient generated with the Dialagrad model 190. Recovery of histidine-accepting activity from columns run with the tRNA uncharged was about 80%. Recovery of charged histidine tRNA from columns was roughly that expected from the half-lives for decacylation of histidyl-tRNAH in the column buffer: 17.3 hours at room temperature, and 5.5 hours at 37°. Recoveries from specific runs are given in the figure legends.


Issue of February 25, 1972
Analysis of Double Labeled Columns—The tRNA was precipitated by the addition of 0.1 volume of 100% trichloroacetic acid (1 g per ml), and the precipitate collected on a glass fiber filter. Filters were washed several times with ethanol, dried about 10 min at 110°C, and counted in 7.5 ml of a mixture of toluene, 2,5-diphenyloxazole (PPO), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (Spectrafluor, Amersham-Searle) in a Nuclear Chicago Mark I liquid scintillation counter.

Concentration and Desalting of Column Fractions—Fractions of columns run with unlabeled tRNA were simultaneously concentrated and desalted by precipitating the tRNA with 2.5 volumes of ethanol. The alcohol solutions were left overnight at -20°C, and the precipitates then collected on Millipore filters type HA, 0.45 pm (Millipore Filter Corp., New Bedford, Mass.). The filters were air dried, and the tRNA eluted into 1 ml of buffer containing 0.167 M sodium cacodylate at pH 7.5 and 13.3 mM MgCl2. These buffer components were chosen so that they would satisfy the standard assay requirements for sodium cacodylate (0.1 M) and MgCl2 (8 mM) when the tRNA sample made up 40% of the final reaction volume. The elution was carried out in glass scintillation vials by shaking for 30 min at 37°C. Recovery of 100 units, which was monitored in each case, was generally between 85 and 95%.

Assays for tRNA—Two methods were used to process the reaction mixture. In the glass fiber filter assay, the reaction was stopped by the addition of 3 ml of cold 10% trichloroacetic acid, and the resulting suspension was chilled in ice. The precipitate was collected on a glass fiber filter and washed four times with 5 ml of 10% trichloroacetic acid, four times with 5 ml of 95% ethanol, and twice with 95% ether. The precipitate was counted in the toluene-PPO-POPOP fluid described above in a Nuclear-Chicago Mark I liquid scintillation counter at an efficiency of 30% for 1H and 90% for 14C.

The second assay method utilized filter paper discs (14). Aliquots of reaction mixture were pipetted onto filter paper discs which were held on a straight pin. The wet filter was immediately immersed in cold 10% trichloroacetic acid, and the resulting suspension was chilled in ice. The precipitate was washed three times for 10 min in cold 10% trichloroacetic acid, and then 5 min each in ethanol and ether. Stirring was accomplished by a magnetic stirring bar separated by a wire mesh from the filter paper discs. The air-dried discs were counted as above. This assay was not used for precise quantitative work, as it was found washing in trichloroacetic acid slowly removed counts from the filters (about 10% in 10 min).

Assay of Column Fractions for Histidine Acceptance—A partially purified histidyl-tRNA synthetase, supplied by J. R. Roth, was used in the assays of the benzoylated DEAE-cellulose columns. The enzyme had been purified about 8-fold by protamine sulfate precipitation of nucleic acids and Sephadex G-150 chromatography. Assays of the reversed phase columns No. 3 were conducted with pure histidyl-tRNA synthetase, supplied by De Lorenzo and Ames (8).

In assays for histidine acceptance the column fractions were either assayed directly or after they had been concentrated and desalted as described above. When the column fractions were used directly, the aliquot of fraction assayed constituted up to 50% of the final reaction volume. This gave NaCl concentrations of up to 0.4 M in the reaction mixture. However, the aminoclaylation reaction with histidine is reasonably insensitive to salt, with no reduction in the final level of incorporation being found at 0.25 M NaCl, and less than a 25% reduction at 0.5 M. In assays of either the concentrated or uncentrated column fractions, the reaction mixture contained 8 mM MgCl2, 4 mM ATP, 50 μM [1H] or [14C]histidine, 0.1 M sodium cacodylate at pH 7.5, and sufficient histidyl-tRNA synthetase to bring the reaction to completion in the time allotted. The reactions were run at 37°C, usually for 20 min. The reaction volume was 0.25 ml for assays of the benzoylated DEAE-cellulose columns, and 0.1 ml for assays of the reversed phase columns No. 3. The reaction mixtures were processed by either the filter paper or glass fiber filter methods.

Assays of Column Fractions for Glycine, Lysine, and Valine Acceptance—Due to their greater sensitivity in salt concentration assays for glycine, lysine, and valine acceptance were done with the desalted, concentrated fractions only. In a total of 0.1 ml the reaction mixtures contained 8 mM MgCl2, 4 mM ATP, 0.1 M sodium cacodylate at pH 7.5, 50 μM [1H]- or [14C]-amino acid, and sufficient aminoacyl-tRNA synthetase to bring the reactions to completion in the time allotted. The reactions were conducted at 37°C, usually for 20 min. Aliquots of 75 μl were taken for the filter paper assay.

Assay of Bulk tRNA for Histidine, Arginine, Leucine, lysine, and Valine Acceptance—In a final volume of 0.1 ml the reaction mixtures contained 0.1 M sodium cacodylate at pH 7.5, 8 mM MgCl2, 4 mM ATP, 4 to 5 A590 units of tRNA; and sufficient enzyme to drive the reaction to completion (as judged by no further incorporation of label with time and proportionality of counts incorporated to tRNA added). The reaction contained the appropriate amino acid at a concentration of 50 μM, except for histidine, which was at 15 μM. The specific activities of the amino acids were adjusted to yield a minimum of 10,000 cpm incorporated per assay. The reactions were performed at 37°C for 20 min, then quenched by the addition of several milliliters of cold 10% trichloroacetic acid. The tRNA precipitate was collected on glass fiber filters and counted as described above.

Determination of tRNA14H Content: Method of Standardization—In the course of preparing a number of batches of wild type tRNA it had been observed that the purity of the preparations varied significantly. Hence, acceptance per A590 unit was not a good basis for comparing the relative amount of tRNA14H in the several regulatory strains. Instead, the acceptance of four other amino acids served as internal standards. These four amino acids were arginine, leucine, lysine, and valine. The acceptances of the four standard amino acids were used to correct for differences in the purity of the tRNA preparations. This was done by choosing a factor to multiply the acceptance for each of the four amino acids so that the adjusted values would be in the closest possible agreement with the wild type standard values. This same factor was then used to multiply the acceptance for histidine, thereby providing a value adjusted for the relative purity of the preparation. The basis for calculating the correction factor was that the adjusted acceptance of each of the four standard amino acids should be as close as possible to their acceptance in the wild type standard. Hence, if the measured values are represented as MArg, MLeu, MHis, and MV, and the standard values as SArg to SV, then the factor f was chosen such that fM = S, or by simple rearrangement, so that fM/S = 1. Since four standards were used, the factor f was selected so that the average fM/S equaled one.
of several different preparations of tRNA from wild type Salmonella. As can be seen, very good reproducibility is obtained for the same preparation assayed at different times. However, the different preparations show some variation from each other. This variation might arise during either the growth of the bacteria or the isolation of the tRNA, as different batches of cells were used for each preparation. These variations determine the sensitivity with which the tRNA content of the various mutants can be distinguished, any difference less than ±15% is probably not significant. Because of this variation we have used as a standard value an average of the values obtained from all the wild type tRNA preparations.

**RESULTS**

**Histidine tRNA Content of Histidine Regulatory Mutants**

Reproducibility of Assays and of tRNA Preparations: Selection of Wild Type Standard—Table I shows data obtained from assays of different preparations of tRNA from wild type Salmonella. As can be seen, very good reproducibility is obtained for the same preparation assayed at different times. However, the different preparations show some variation from each other. This variation might arise during either the growth of the bacteria or the isolation of the tRNA, as different batches of cells were used for each preparation. These variations determine the sensitivity with which the tRNA content of the various mutants can be distinguished, any difference less than ±15% is probably not significant. Because of this variation we have used as a standard value an average of the values obtained from all the wild type tRNA preparations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Preparation</th>
<th>Relative purity</th>
<th>Relative content of tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>His</td>
<td>Arg</td>
</tr>
<tr>
<td>LT-2</td>
<td>Silbert</td>
<td>1.10</td>
<td>0.90</td>
</tr>
<tr>
<td>LT-2</td>
<td>Silbert</td>
<td>0.95</td>
<td>1.12</td>
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<tr>
<td>LT-2</td>
<td>Standard</td>
<td>1.02</td>
<td>1.05</td>
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<tr>
<td>LT-2</td>
<td>Thiosulfate</td>
<td>1.02</td>
<td>0.85</td>
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<tr>
<td>LT-2</td>
<td>Thiosulfate</td>
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</table>

**Table II**

**tRNA Content of ara-9, hisO, hisS, hisT, hisU, and hisW strains**—The relative histidine acceptances of tRNA isolated from the histidine regulatory mutants, *hisO*, *hisS*, *hisT*, *hisU*, and *hisW*, are shown in Table II. The tRNA isolated from *hisO* and *hisS* strains may be considered as derepressed controls, as these two genes are known not to be involved in tRNA production. The tRNA*His* content of the *hisO* and *hisS* tRNA falls in the normal range of variation.

The *hisW* mutant displaying the severest phenotypic effects is JL250, a cold-sensitive *hisW* strain isolated in the laboratory of J. L. Ingraham (9). Analysis of the tRNA content of this strain is presented in Table II.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Preparation</th>
<th>Relative purity</th>
<th>Relative content of tRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>His</td>
<td>Arg</td>
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<tr>
<td>01242</td>
<td>Standard</td>
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<td>0.76</td>
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<td>S1595</td>
<td>Standard</td>
<td>0.82</td>
<td>1.280</td>
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<td>JL250</td>
<td>Thiosulfate</td>
<td>0.69</td>
<td>0.960</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>W1885(a)</td>
<td>Standard</td>
<td>0.75</td>
<td>1.020</td>
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<td>W1885(a)</td>
<td>Thiosulfate</td>
<td>0.85</td>
<td>1.171</td>
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<td>U1802(a)</td>
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<td>U1802(a)</td>
<td>Thiosulfate</td>
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<td>1.128</td>
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</tr>
</tbody>
</table>

**Table II**

**tRNA content: ara-9, hisO, hisS, hisT, hisU, and hisW**

Independent preparations of tRNA are denoted by repetition of the strain designation. Values have been normalized to the wild type standards as described under "Materials and Methods." The various preparations are as follows: "standard," prepared as described under "Materials and Methods"; "thiosulfate," prepared as "standard" except 2 mM thiosulfate was present in all buffers; "Silbert," prepared by method of Silbert et al. (11).
The remaining hisW mutants, and also the of the other strains tested (except for tRNAHis in an episome-Roth, unpublished experiments referred to in Reference 15). The same preparations used in that study were also analyzed in the present one (Table I. Line 1: Table II. Lines 8 and 10) with the mutant and wild type preparations. The apparent reduction in internal standards no significant difference was found between the preparations. The apparent reduction in internal standards no significant difference was found between the elution position of uncharged tRNAHis, or for its charging.

The hisT gene codes for an enzyme which forms pseudouridine in the anticodon loop of histidine tRNA (see below). The data in Table I show that this modification is not required for in vivo stability of tRNAHis, or for its charging.

**Table III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Preparation</th>
<th>Relative acceptance of His</th>
<th>A60</th>
<th>Leu</th>
<th>Lys</th>
<th>Val</th>
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</thead>
<tbody>
<tr>
<td>R1900</td>
<td>Silbert</td>
<td>0.77 ± 0.75</td>
<td>0.00</td>
<td>0.98</td>
<td>0.03</td>
<td>1.03</td>
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<tr>
<td>R1203</td>
<td>Silbert</td>
<td>0.70 ± 0.73</td>
<td>0.00</td>
<td>0.98</td>
<td>0.03</td>
<td>1.03</td>
</tr>
<tr>
<td>R1203</td>
<td>Thiosulfate</td>
<td>1.00 ± 0.56</td>
<td>0.00</td>
<td>0.97</td>
<td>0.06</td>
<td>1.05</td>
</tr>
<tr>
<td>R1205</td>
<td>Standard</td>
<td>0.63 ± 0.58</td>
<td>0.00</td>
<td>0.92</td>
<td>0.18</td>
<td>0.91</td>
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<tr>
<td>R1813</td>
<td>Silbert</td>
<td>0.90 ± 0.58</td>
<td>1.00</td>
<td>0.80</td>
<td>0.99</td>
<td>1.20</td>
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<tr>
<td>R1203/14 (hisR+)</td>
<td>Silbert</td>
<td>0.90 ± 0.53</td>
<td>0.94</td>
<td>0.86</td>
<td>1.18</td>
<td>1.02</td>
</tr>
<tr>
<td>HisR/F2/14 (hisR+)</td>
<td>Silbert</td>
<td>0.89 ± 0.54</td>
<td>0.90</td>
<td>1.12</td>
<td>0.70</td>
<td>0.92</td>
</tr>
<tr>
<td>HisR/F2/14 (hisR+)</td>
<td>Thiosulfate</td>
<td>0.85 ± 0.49</td>
<td>1.00</td>
<td>1.17</td>
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<td>0.81</td>
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<tr>
<td>HisR/F2/14 (hisR+)</td>
<td>Thiosulfate</td>
<td>0.74 ± 0.43</td>
<td>1.01</td>
<td>1.24</td>
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<td>0.90</td>
</tr>
<tr>
<td>HisR/F2/32 (hisW+)</td>
<td>Thiosulfate</td>
<td>0.80 ± 0.47</td>
<td>1.09</td>
<td>1.15</td>
<td>0.90</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* Complete genotype given under "Materials and Methods."

The hisT gene codes for an enzyme which forms pseudouridine in the anticodon loop of histidine tRNA (see below). The data in Table I show that this modification is not required for in vivo stability of tRNAHis, or for its charging.

**Content of hisR Mutants and of Some Episome-carrying Strains**—A previous study by Silbert et al. (11) showed that hisR mutants have a reduced quantity of histidine tRNA. That result is confirmed here (Table III). In addition, the histidine tRNA content has been determined of strains carrying an extra dose of the hisR+ gene on an episome. If hisR is a structural gene for histidine tRNA, then such strains should have an increased level of tRNAHis. Introduction of the episome into either a hisR- or a hisR+ strain results in a large increase in the level of histidine tRNA (Table III). On the other hand, insertion of an episome covering the hisW region into a hisW strain has no effect on the tRNAHis levels (compare Table III with Table I). These data suggest that hisR is a structural gene for histidine tRNA, while hisW is not.

**Chromatographic Properties of tRNA from Wild Type and Regulatory Mutants**

**Chromatography on Benzoylated DEAE-cellulose—**Chromatography of tRNA from wild type Salmonella on a benzoylated DEAE-cellulose column gave the elution pattern shown in Fig. 1. As shown in the figure, there is no evidence for multiple species of histidine tRNA.

**Chromatography on Reversed Phase Column No. 8—**The reversed phase column No. 3 of Weiss et al. (13) was used to analyze Salmonella tRNA, as the column had been shown to resolve two species of histidine tRNA from Escherichia coli B, an organism very similar to Salmonella. However, as shown in Fig. 2, wild type Salmonella tRNA yielded only one major peak of tRNAHis. A very small second peak contained a few percent of the total histidine-accepting activity. The tRNA of this second peak was not studied further because it was present in such a small amount, and because it was present in tRNA isolated from each of the regulatory strains as well as from the wild type. Experiments in which the tRNAHis was previously labeled with radioactive histidine before being applied to the column suggest that the small peak is a column artifact, and not a different species of tRNAHis (see below).

The large number of sharp A260 peaks indicated that the column was resolving well; however, as a control, tRNA from E. coli B was fractionated to show the presence of two tRNAHis peaks. Surprisingly, commercially prepared tRNA from E. coli B yielded only a single peak, whereas E. coli B tRNA supplied by A. D. Keimers gave two peaks, exactly as published (13). The reason for this discrepancy is not known.

In addition to tRNA from wild type Salmonella, tRNA from hisR, hisU, hisW, and hisT mutants was analyzed on the reversed phase column. Since the aminocyl linkage of histidine to tRNA is one of the most labile, it was considered possible that if chromatography was performed with the tRNA previously labeled an altered species of tRNAHis might be missed if it were preferentially deacylated. Accordingly, an initial set of chromatographic analyses were conducted in which the elution position of uncharged tRNAHis was determined by charging column fractions with radioactive histidine. Since the A260 pattern varied from column to column, the peak positions of three nearby tRNA's, glycine, lysine, and valine, were used as internal standards. No significant difference was found between the elution
Fig. 1. Fractionation of tRNA from Salmonella typhimurium LT-2 on a benzoylated DEAE-cellulose column. A sample of 182,426 units was applied to the column, which was then developed with a linear gradient of NaCl in a buffer of 10 mM MgCl₂ and 10 mM sodium acetate, pH 4.5. The initial volumes of the reservoir and mixing chambers were each 500 ml. The flow rate was 0.5 ml per min, and fractions of 12 ml were collected.

Fig. 2. Fractionation of tRNA from Salmonella typhimurium LT-2 on a reversed phase column No. 3. A sample of 1200 A₄₅₀ units was applied to the column, which was then developed with a gradient of NaCl in a buffer of 10 mM MgCl₂ and 10 mM Tris-HCl, pH 7.6. The flow rate was 1.5 ml per min, and fractions of 15 ml were collected. Fractions were assayed for histidine acceptance as described under "Materials and Methods."

Comparison of Histidine tRNA from Wild Type and Mutant Strains by Double Label Chromatography—Except for the ubiquitous minor peak, the tRNA_His of each of the mutant strains migrated as a single peak in about the same position as the tRNA_His from the wild type. To more critically compare the elution positions of the tRNA_His from the mutants to that from the wild type, double label columns were run. In these experiments tRNA isolated from a mutant strain was charged with [αH]histidine, that from the wild type with [14C]histidine (or vice versa), and the two preparations cochromatographed on a reversed phase column No. 3. The tRNA_His from the hisU and hisW strains tested (hisU180 and the cold-sensitive hisW, JL25) eluted exactly with the wild type. The tRNA_His from the hisT1604, however, eluted after the wild type tRNA_His (Fig. 3). This difference in elution positions remained when independent preparations of wild type and hisT1604 tRNA were analyzed, and also when the labels used to charge each tRNA were interchanged. Histidine tRNA prepared from a hisR1813 mutant eluted slightly ahead of the wild type tRNA (Fig. 4). However, the sequence of the tRNA_His of hisR1813 has subsequently
Fig. 4. Comparison of the elution position of histidyl-tRNAHis from the wild type (LT-2) and hisR1813 on a reversed phase column No. 3. The applied sample contained 1.7 mg of tRNA from LT-2 charged with [14C]histidine, 0.2 mg of tRNA from hisR1813 charged with [3H]histidine, and 5 mg of uncharged LT-2 tRNA as carrier. The column was developed at 37°C with a gradient of NaCl in 10 mM MgCl₂ and 10 mM sodium acetate, pH 4.5. The flow rate was 1.5 ml per min, and 10-ml fractions were collected. The tRNA was precipitated from the fractions and counted as described under “Materials and Methods.” Peak tubes of [14C] (LT-2) and [3H] (hisR1813) had 2055 and 3054 cpm, respectively. Recoveries were 8.7% for 14C (LT-2) and 8.7% for 3H (hisR1813).

been found to be identical with that of the wild type.¹ The reason for the different elution position found here is not known.

Some difficulty was encountered in the performance of the reversed phase column No. 3 in the double label experiments. Freshly packed columns yielded two peaks of radioactivity, one near the position at which unlabeled tRNAHis elutes, and a much larger peak at a higher salt concentration. On succeeding runs, however, the late peak steadily diminished, until after about five runs the histidyl-tRNAHis eluted entirely in the early position. In the experiment illustrated in Fig. 5 (the fourth run of that particular column), labeled tRNA was run with a 20-fold excess of unlabeled tRNA. The tRNA in even-numbered tubes was precipitated with trichloroacetic acid to determine the position of the previously labeled tRNAHis, and the tRNA in odd-numbered tubes was aminoacylated with [3H]histidine to determine the position of the unlabeled tRNAHis. As shown previously (Fig. 2), unlabeled tRNAHis yielded an early major peak and a late minor peak. The proportion of histidine tRNA eluting in the second peak was greatly increased, however, when the tRNA was aminoacylated before chromatography. Since the magnitude of this late peak depends on whether or not the tRNA is charged, and since it decreases (with a corresponding increase in the early peak) as the column ages, we consider it to be an artifact of the column, rather than representing multiple isoaccepting species for histidine tRNA. The experiment also shows that the position of the early peak is sensitive to aminoacylation of the tRNA.


Fig. 5. Comparison of the elution position of charged and uncharged tRNAHis from hisT1504 on a reversed phase column No. 3. The applied sample contained 2 mg of tRNA from hisT1504 charged with [3H]histidine, and 18 mg of the same tRNA carried through the identical charging procedure except that the histidine was omitted. The column was developed at 37°C with a gradient of NaCl in 10 mM MgCl₂ and 10 mM sodium acetate, pH 4.5. The flow rate was 1.5 ml per min, and 10-ml fractions were collected. The tRNA in even fractions was precipitated and collected on glass fiber filters to determine the elution position of charged tRNAHis. The tRNA of odd fractions was assayed by the standard procedure (“Materials and Methods”) to determine the elution position of uncharged tRNAHis. The recovery of counts applied to the column was 12%, and the recovery of histidine acceptance was 94%. The peak tube of precharged tRNAHis had 3069 cpm, and the peak tube of postcharged tRNAHis (50-μl samples assayed) had 4710 cpm.

DISCUSSION

As a result of the experiments presented here, the function of three of the four histidine regulatory genes examined can be more clearly delineated: hisR appears to be a structural gene for histidine tRNA, and hisT and hisW appear to be genes involved with tRNA maturation. No positive evidence has been obtained regarding the nature of hisU.

It had previously been shown by Silbert et al. that hisR mutants have 40 to 50% less histidine tRNA than the wild type (11). Those data have been essentially confirmed here, with reductions of from 25 to 45% being found in the histidine tRNA content of hisR mutants. The reduced histidine tRNA content of hisR mutants shows that the hisR gene is required for formation of active histidine tRNA. The finding does not prove that hisR is a structural gene for histidine tRNA, however, since the histidine tRNA levels could be reduced if hisR were a gene regulating the expression of a structural gene located elsewhere on the chromosome, or if hisR encoded an tRNA-modifying enzyme whose activity was necessary to produce the full complement of active tRNAHis. These alternate possibilities are made less likely by the finding of an increased tRNAHis level in strains carrying an
additional wild type hisR gene on an episome. This increase in the tRNA H18 level suggests that even in the wild type Salmonella expression of the hisR1 gene must be rate limiting in the production of histidine tRNA. Such a rate-limiting role is not expected for a modifying enzyme or an unlinked regulatory unit.

Below, reasons will be given for believing that the hisR1 locus is the only one coding for histidine tRNA. Accepting this to be true, it remains to explain the reduction in tRNA H18 content of hisR1 mutants. We consider two possibilities likely. One possibility is that the hisR1 locus is comprised of two histidine tRNA genes in tandem, similar to the case for tyrosine tRNA (16). In this case the hisR1 mutations would result in one of the tRNA genes being inactive. The other possibility is that there is a single hisR1 gene, and that mutations in the gene render the tRNA unstable. Mutations in a tyrosine suppressor tRNA gene of E. coli have been found to have such an effect (17).

It was originally thought that hisU and hisW were also structural genes for histidine tRNA. As a result, a variety of chromatographic systems have been employed in an attempt to detect multiple species of histidine tRNA. In addition to the studies reported here with the reversed phase column No. 3 and benzoylated DEAE-cellulose, less extensive analyses have been performed on reversed phase columns Nos. 1 and 2, and on columns of methylated albumin kieselguhr, DEAE-cellulose-Sephadex, hydroxyapatite, and Sephadex G-100 (11). In no case has evidence been obtained for more than one species of histidine tRNA. Singer and Smith1 of this laboratory have recently determined a unique sequence for the tRNA H18, and have set up an upper limit for a possible second species as 15% of the total tRNA H18. It is possible that a minor species of histidine tRNA does exist, but that the fractionation methods used to date have failed to resolve it; however, other experiments reported here and elsewhere indicate that hisU and hisW, at least, are not tRNA structural genes.

An association between hisW and histidine tRNA was first suggested by a cross-dominance study of Martin et al. (15). They showed that an additional copy of a hisR1+ gene (on an episome) permitted repression in a hisW strain. In this paper we show that a cold-sensitive hisW1 strain JL250, has aberrant levels of several species of tRNA relative to the wild type. While this confirms a relationship between hisW and tRNA, the pleiotropic effect argues against hisW being a structural gene for tRNA H18. This is also indicated by the finding that a strain carrying an extra dose of the wild type hisW gene on an episome retained the normal level of tRNA H18. The pleiotropic effect could result from the hisW gene being involved in transcription of tRNA genes. More likely is the suggestion that hisW codes for a tRNA maturation enzyme. The cold sensitivity of an unmodified tRNA could be responsible for the immediate cessation of growth when the mutant is shifted to 20°C.

The remaining hisW mutants, and all the hisU mutants, were found to have normal levels of histidine tRNA. This result differs from those of a recent study of Lewis and Ames6. They found the level of histidine acceptance in hisU and hisW mutants to be reduced 20 to 30% relative to the acceptance of valine and arginine. In addition, they found the acceptance of valine and arginine to be reproducibly lowered about 90% relative to the A290 of the hisU and hisW extracts. The conflicting results appear to be due to the different way in which the cells were processed before the tRNA was extracted. The primary objective of the experiments of Lewis and Ames was to examine the level of charging in vivo; consequently, cells were killed very rapidly by the addition of trichloroacetic acid to exponentially growing cultures. The preparations used in this paper were made from cells which were frozen slowly by being placed in a freezer at -20°C. Lewis and Ames have found that if they freeze cells in a similar manner before preparing tRNA by their usual method, then the relative acceptance of histidine by hisU and hisW preparations approaches that of the wild type. A possible explanation for the change upon freezing would be that the hisU and hisW lesions are leaky, and that the mutant phenotype is repaired during the period of slow cooling. If such is the case, then the results reported by Lewis and Ames more correctly represent the tRNA concentrations in vivo, and the hisU gene, as well as the hisW gene, must be involved with tRNA production.

Evidence that hisT codes for a tRNA-modifying enzyme is now very strong. Since hisT1 is known to specify a protein (18), the altered chromatographic mobility for hisT1 tRNA found here is likely the result of a change in a modified base. This suggestion has prompted a comparison of the sequence of histidine tRNA from the wild type and from hisT1504. Unpublished results indicate that 2 pseudouridine residues found in the anticodon loop of the wild type remain as uridine residues in the mutant; the pseudouridine in the ribothymidine-pseudouridine-cytosine loop, however, is intact.

Both this study and that of Lewis and Ames7 find a normal quantity of histidine tRNA to be present in hisT1 mutants. In addition, the tRNA H18 of hisT1 mutants gives the wild type K1 and Vmax in the aminoacylation reaction,8 and is charged in vivo to the same extent as is the wild type.9 Apparently the hisT modification is required for charged histidine tRNA to interact properly in the repression mechanism.

The hisT modification may also be required for regulation of other metabolic pathways. HisT1 mutants are resistant to the leucine analogues trifluoroleucine and β-hydroxyoleucine, and to the tyrosine analogues α-aminotyrosine, 9 suggesting that some element of negative control of the two biosynthetic pathways has been lost. Significantly, a pseudouridine is found in the anticodon loop of a tRNA TH (19) and a tRNA TH (20) of E. coli, an organism which is a very close relative of S. typhimurium.

Acknowledgment—We are happy to acknowledge the cheerful assistance of Mrs. W. L. Chang.

REFERENCES
Histidine Regulation in *Salmonella typhimurium*: IX. HISTIDINE TRANSFER RIBONUCLEIC ACID OF THE REGULATORY MUTANTS

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