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Biosynthesis of tRNA in histidine regulatory mutants of Salmonella typhimurium

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ABSTRACT

HisU mutants of *Salmonella typhimurium* are depressed in the histidine operon since they have lower intracellular concentration of histidyl-$tRNA^{His}$. In this paper we present evidences showing that a strain carrying a *hisU* mutation *(hisU1206)* is altered in a nucleolytic enzyme involved in tRNA maturation process. The analysis of several *hisU* mutants indicates that *hisU* region of bacterial genome may account for more than one function involved in tRNA biosynthesis.

INTRODUCTION

Studies on the regulation of biosynthetic operons in bacteria have led to the conclusion that, in some cases (histidine, leucine, isoleucine-valine), tRNA is an essential element in the mechanism of repression (1-5). Lewis and Ames (3) have shown that the level of derepression of histidine operon is inversely proportional to the intracellular concentration of histidyl-$tRNA^{His}$. To be an efficient signal for repression, histidyl-$tRNA^{His}$ must contain two pseudouridines in the anticodon region; *hisT* mutants, isolated as derepressed in the histidine operon, lack an enzyme which converts uridine into pseudouridine in the anticodon region of many tRNA species (6,7). $tRNA^{His}$ lacking pseudouridine is perfectly able to accept histidine and to function in protein synthesis (8), but is unable to repress the histidine operon (5,6).

Exploiting the property that any decrease in the absolute concentration of $tRNA^{His}$, accompanied by a decrease in the intracellular concentration of histidyl-$tRNA^{His}$, leads to derepression of the histidine operon, constitutive mutants - mapping in three different regions of *S. typhimurium* chromosome, called *hisR*, *hisU* and *hisW* - have been selected, which have lower intracellular concentration of $tRNA^{His}$ (1).

Previous work (3,9) has shown that it is possible to distinguish the

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phenotype of *hisR* mutants from that of *hisU* and *hisW*: the former is altered only in the content of $tRNA^{His}$, whereas *hisU* and *hisW* are pleiotropic mutants with lower concentration of several different tRNA species (3). Furthermore, evidences have been provided showing that *hisR* is the structural gene for $tRNA^{His}$ (2,10), whereas the pleiotrophy of *hisU* and *hisW* mutants implies that the mutation must be in genes responsible for functions related to the biosynthesis of many, if not all, tRNAs.

The biosynthesis of a tRNA molecule starts with the transcription of an RNA precursor which is converted to tRNA through a multistep process implying nucleolytic cleavages progressively reducing its length and chemical modifications of several nucleosides along the sequence (11). In the present paper we report an investigation on the steps of this process in which the alteration of *hisU* mutants occurs.

MATERIALS AND METHODS

Chemicals. (8-^{3}H)GDP (5 Ci/mmol) and (5-^{3}H)uridine (28 Ci/mmol) were purchases from the Radiochemical Centre, Amersham. All other reagents used were reagent grade.

Bacterial strains. TA265 is *Salmonella typhimurium* wild-type LT2 strain; histidine regulatory mutants used are: TA797 *(hisU1817)*, TA798 *(hisU1818)*, TA799 *(hisU1820*, TA802 *(hisU1819)*, TA803 *(hisU1823)*, DA11 *(hisU1206)*, JL250 *(hisW3333)*, *hisU6322* and *hisU6397* (the last two are *hisU* mutants according to unpublished results of D.S. Straus). All strains used were obtained from Dr. B.N. Ames.

Pulse-chase experiments. Cells were grown in a medium containing 14.5 g Tris, 1.07 g NH_4Cl, 0.203 g $MgCl_2$, 0.029 g $CaCl_2{\cdot}H_2O$, 0.136 g KH_2PO_4, 0.00054 g $FeCl_3$, 0.00149 g KCl, 0.071 g Na_2SO_4, 0.40 g bactopeptone (Difco) per liter, 0.02% glucose. The pH was adjusted to 7.5 with HCl. At about 0.2 A_{650} units at temperatures specified in the legends to the figures, 30 µCi/ml (5-^{3}H) uridine (28 Ci/mmol) were added. After various fixed intervals the labeling was stopped by addition of one-volume of 0.1 M Tris•HCl-saturated phenol, pH 7.5, containing 50 µg/ml cold carrier *S. typhimurium* tRNA. When chase was required, prior to phenol extraction, rifampicin at a final concentration of 300 µg/ml was added together with cold uridine to a final concentration of 500 µg/ml. After phenol extraction 1/10 volume of 2M sodium acetate was added to the aqueous phase and RNA precipitated overnight at -20°C in 2.5 volumes of ethanol. RNA was applied on 10% or 5% polyacrylamide slab gel

(15 x 30 x 0.15 cm) and fractionated by electrophoresis according to the procedure of Peacock and Dingman (12). Electrophoresis was carried out for 18 h at 150 volts at room temperature on 10% polyacrylamide gel and for 14 h at 100 volts on 5% gel.

Fluorography of ^{3}H-labeled RNA. In order to detect, by X-ray film, the tritium-labeled RNA species fractionated on polyacrylamide gel, we applied the procedure for scintillation autography (fluorography) described by Bonner and Laskey (13), with slight modifications. The gel was soaked in 20 times its volume of dimethylsulphoxide (Me_2SO) for 20 min; this treatment was then repeated twice using fresh dimethylsulphoxide. The Me_2SO-impregnated gel was then transferred in 4 volumes of 18% (w/v) PPO solution in Me_2SO and left for three hours. After this period the gel was washed in a large excess (more than 20 times) of water for 1 h and finally vacuum dried on Whatman 3MM paper. X-ray Regulix BB54 Kodak film was used for fluorography at $-80^{o}C$. When needed it was possible to recover RNA from single bands on the gel after fluorography by swelling the gel slice in water, removing PPO by washing with Me_2SO and then stirring the homogenized slice in 0.5 M NaCl, 0.01 M EDTA, 0.1 M Tris.HCl pH 8.5. Further details of this procedure will be published elsewhere (L. Bossi and M.S. Ciampi, manuscript in preparation).

In vitro *processing of precursor tRNA.* For the preparation of the crude *S.typhimurium* cell-free extract cells were grown in a medium containing 8% Nutrient Broth (Difco) and 1.5% NaCl till 0.7 A_{650} units and harvested by centrifugation. The pellet was resuspended in 4 volumes of buffer containing 0.05 M Tris.HCl pH 7.5, 0.06 M NH_4Cl, 0.1 M $MgCl_2$ and 0.006 M 2-mercaptoethanol. After sonication (15 s for 4 times), the cells were centrifuged for 1 h at 30,000 x g. The resulting supernatant (S 30), containing about 15 mg/ml proteins, was used for *in vitro* processing of tRNA precursors. This reaction was carried out in 400 µl volume of a mixture containing 5 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 M NH_4Cl, 10 mM Tris.HCl pH 8, 20 µg of cold *S.typhimurium* tRNA and 400 µg of S 30 proteins. The reaction was stopped by phenol extraction of RNA which was then applied on the gel.

RESULTS AND DISCUSSION

When (^{3}H)uridine is added to growing cells, it is rapidly incorporated in all nascent RNA chains which will be labeled to an extent depending on their steady-state concentration; all those molecules, e.g., tRNA, that

are not immediate transcriptional products, will appear labeled only after a short lag period, during which their maturation occurs. Mutants altered in the maturation of tRNA show qualitative and quantitative alterations in the pattern of newly made RNA chains fractionated by polyacrylamide gel electrophoresis (14,16). In order to investigate whether the histidine regulatory mutants show this type of alterations, we performed a series of pulse experiments comparing the pattern of pulse-labeled RNA synthesized in *hisU* with the wild-type pattern.

A hisU *mutant* (hisU1206) *is altered in a nuclease responsible for the conversion of tRNA precursors into tRNA-size molecules. HisU* mutants have been mapped by conjugation between *ilvC* and *pyrE* genes at around 120 min on the *S. typhimurium* chromosome (17). Most of our biochemical approaches were carried out on strain DA11 (*hisU1206*). Although this mutant was not selected as temperature sensitive (17), we found that its alteration was much more evident if cells were labeled at 43°C rather than at 37°C. Therefore we carried out all pulse-chase experiments on DA11 after shifting the cells to 43°C. Fig. 1 shows the pattern of RNA synthesized during 2 min and 10 min pulses of (5-^{3}H)uridine and during 10 min pulse followed by 10 min chase, respectively in strain TA265 (*S. typhimurium* wild-type) and in strain DA11. The RNA pattern shown by the wild-type (*lanes 1,2* and *3*) is practically identical to the one obtained in similar experimental conditions with *E. coli* cells (18). After 2 min a conspicuous amount of tRNA and 5S rRNA have already been synthesized; in the 6S region an RNA species is present which we have tentatively identified as the precursor of 6S RNA (18), whereas 6S RNA is detectable only after 10 min labeling. Many other RNA species larger than 5S appear in 2 min pulse, but their amount remains the same also after 10 min pulse, thus suggesting that they are unstable RNAs which have reached their steady-state concentration after 2 min. During a chase of 10 min there is a strong decrease of 6S RNA precursor to the advantage of 6S mature form. The RNA bands in the upper part of the gel which were evident after the pulses disappear completely, whereas 5S and 4S RNAs remain unchanged. DA11 samples are in *lanes 4,5* and *6*. After 2 min pulse only negligible quantity of 4S RNA is detectable in the mutant, whereas on the contrary the amount of 5S RNA and 6S RNA precursor is almost the same as in wild-type. In addition, several RNA bands migrating slower than 5S are uniquely present in DA11. After 10 min pulse these RNAs appear in very intense bands and new ones become evident. In the 4S

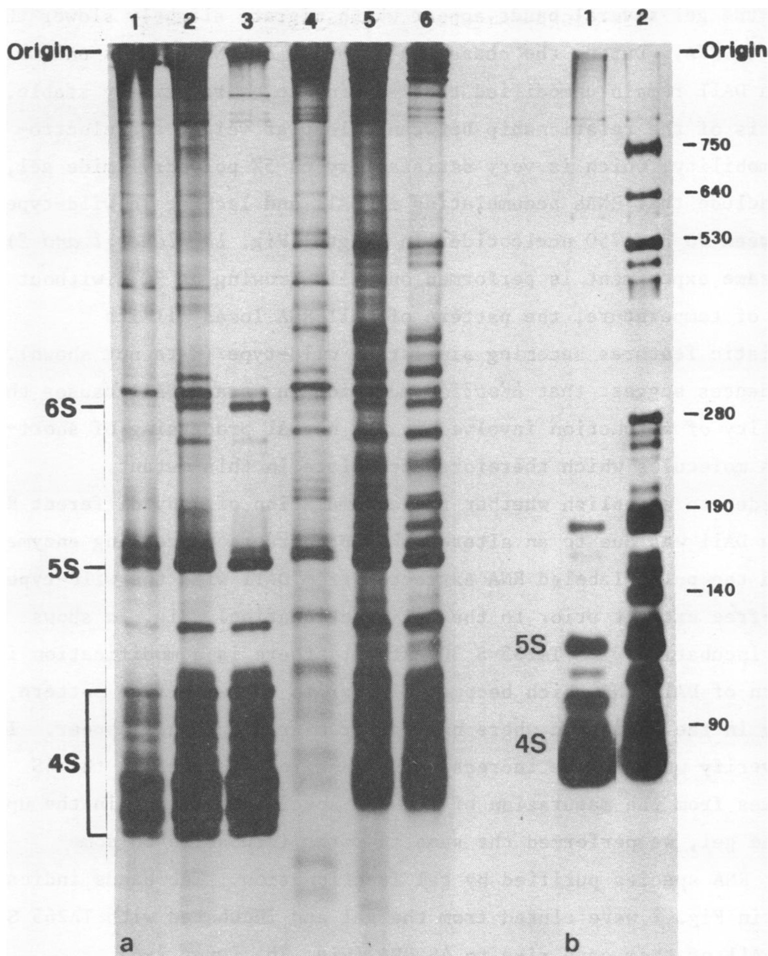

Fig. 1. *Fluorogram of polyacrylamide gel electrophoretic fractionation of ^{3}H-labeled RNA from strain TA265 (wild-type) and strain DA11 (*hisU1206). Cells were grown at 30°C until 0.15 A_{650} units and then transferred at 43°C. At 35 min after temperature shift (5-^{3}H)uridine was added and newly synthesized RNA was phenol extracted from 2 ml portions of bacteria after 2 min and 10 min of labeling. After 10 min labeling rifampicin and cold uridine were added to another 2 ml aliquot of cells as described in Materials and Methods, and a chase was carried out for 10 min before phenol extraction. RNA was fractionated by electrophoresis on 10% *(panel a)* and 5% *(panel b)* polyacrylamide slab gels. After the electrophoretic run gels were treated for fluorographic detection of ^{3}H-labeled RNA bands. *a,* 10% gel: *lanes 1,2,3,* TA265 RNA labeled respectively for 2 min pulse, 10 min pulse, 10 min pulse+10 min chase; *lanes 4,5,6,* DA11 RNA labeled for 2 min pulse, 10 min pulse, 10 min pulse+10 min chase. *b,* 5% gel: *lane 1,* TA265 RNA after 10 min pulse, *lane 2,* DA11 RNA after 10 min pulse.

region of the gel several bands appear which migrate slightly slower than wild-type 4S RNA. During the chase the majority of RNA species uniquely present in DA11 remain unmodified thus showing to be relatively stable. On the basis of the relationship between molecular weight and electrophoretic mobility, which is very satisfactory on 5% polyacrylamide gel, we can conclude that RNAs accumulating in DA11 and lacking in wild-type range between 90 and 750 nucleotides in length (Fig. 1*b, lanes 1 and 2*). When the same experiment is performed on cells growing at 30°C without any shift of temperature, the pattern of DA11 RNA loses all its characteristic features becoming similar to wild-type (data not shown). These evidences suggest that *hisU1206* mutation in strain DA11 causes the heat-lability of a function involved in the normal processing of short-living RNA molecules which therefore accumulate in this mutant.

In order to establish whether the accumulation of many different RNA species in DA11 was due to an altered tRNA precursors processing enzyme, we treated the pulse-labeled RNA extracted from DA11 with the wild-type S 30 cell-free extract prior to the gel fractionation. Fig. 2*a* shows that upon incubation with TA265 S 30 extract, there is a modification in the pattern of DA11 RNA which becomes similar to the wild-type pattern, especially in the 4S region where new faster migrating bands appear. In order to verify whether the increase of tRNA-size molecules in the 4S region comes from the maturation of the RNA species migrating in the upper part of the gel, we performed the same *in vitro* incubation on some individual RNA species purified by gel fractionation. The bands indicated by arrows in Fig. 2 were eluted from the gel and incubated with TA265 S 30 extract. All of them gave rise to 4S RNA (Fig. 2*b, lanes 1-6*).

These results show that higher molecular weight RNA species accumulated in DA11 mutant are transformed into tRNA-size molecules by an enzyme lacking in DA11 and normally present in wild-type cytoplasm.

So far a well characterized nuclease involved in tRNA maturation process is RNase P (19), which cleaves tRNA precursors generating mature 5' terminus (14,19). Mutants in RNase P have been isolated which accumulate tRNA precursors (15,16).

In order to ascertain whether DA11 is also mutated in RNaseP we did a comparative experiment of in vitro maturation using P32 labeled RNA and S-30 cell extract from DA11 and A49 (obtained from J.D. Smith), the latter being a well characterized RNase P^- mutant strain (15). The results are shown in fig. 3. In panel a is a 10 polyacrylamide fractionation of P^{32}

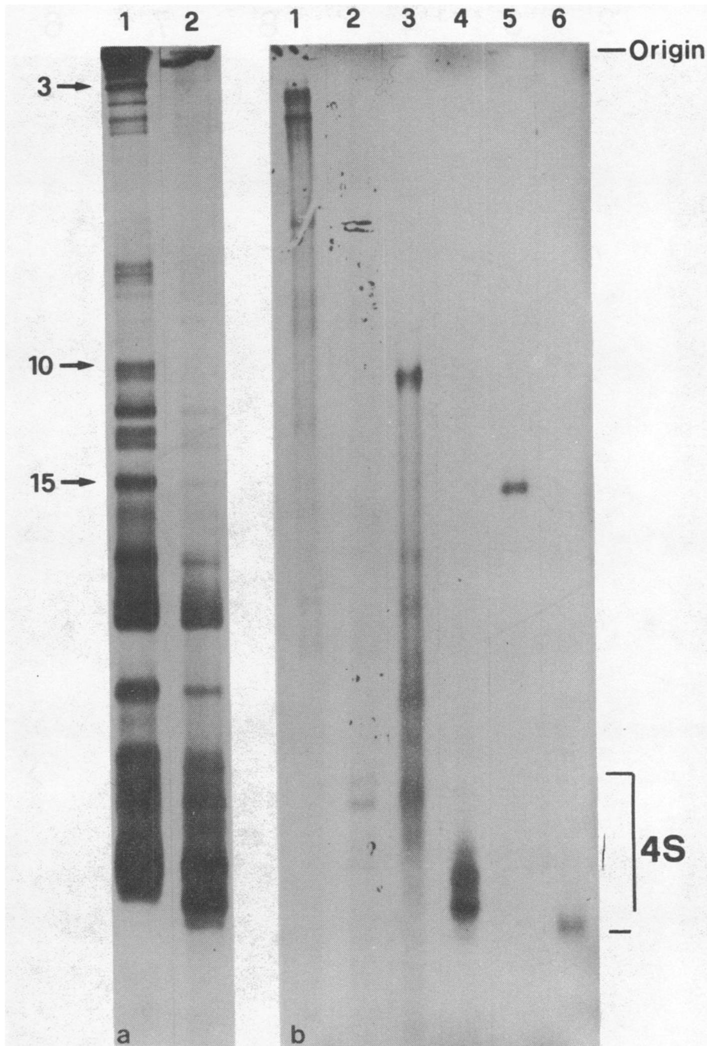

Fig. 2. *Fluorogram of 10% polyacrylamide gel electrophoresis of products generated by* in vitro *treatment of RNA from DA11 with TA265 cell-free extract.* DA11 cells were labeled with (5-^{3}H)uridine for 10 min at 43°C and RNA phenol extracted and precipitated with ethanol; the pellet was dried, resuspended in the buffer described in Materials and Methods, and divided in two aliquots: one was incubated for 20 min at 37°C with TA265 30,000 x g supernatant before phenol extraction and gel electrophoresis *(a, lane 2)*, the other was incubated for the same time with buffer as control *(a, lane 1)*. Analogous *in vitro* experiment was carried out on the three RNA species indicated by arrows in *panel a*, after their extraction from the gel. Conditions were the same except that incubation was carried out for 35 min. *Panel b: lanes 1 and 2*, respectively RNA band 3 before and after incubation; *lanes 3* and *4*, band 10 before and after incubation; *lanes 5* and *6*, band 15 before and after incubation.

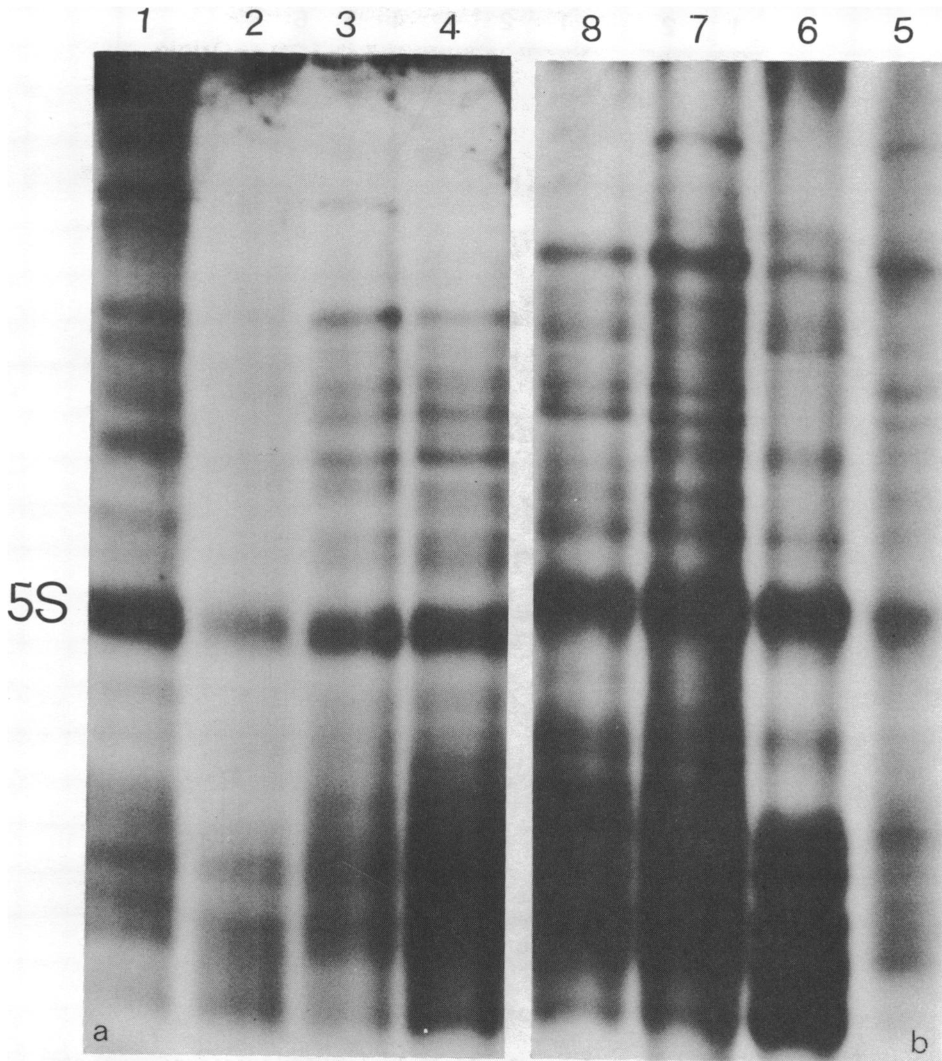

Fig. 3. *Autoradiography of polyacrylamide gel electrophoresis fractionation of P32 labeled RNA from strain DA11 and A49 with and without prior incubation with cell extracts.* DA11 and A49 cells were labeled with P32 for 12 min at 43°C, then phenol extracted and precipitated with ethanol. In *panel a* is P32 RNA from DA11, incubated, prior to electrophoresis, at 42°C for 60 min in the presence of - *lane 1:* buffer as control; *lane 2:* wild-type S. typhimurium S-30; *lane 3:* DA11 S-30; *lane 4:* A49 S-30. In *panel b* is P32 RNA from A49 incubated, prior to electrophoresis, in the presence of - *lane 5:* buffer as control; *lane 6:* wild-type S. typhimurium S-30; *lane 7:* DA11 S-30; *lane 8:* A49 S-30.

pulse labeled DA11 RNA incubated with buffer as control (lane 1) and after incubation with wild-type S. typhimurium S-30 extract (lane 2), or with DA11 S-30 (lane 3) and A49 S-30 (lane 4). In fig. 3, panel b the reciprocal experiment is shown. In lane 5 is P^{32} pulse labeled A49 RNA incubated with buffer as control, in lane 6, 7 and 8, after incubation with wild-type S. typhimurium, with DA11 and A49 S-30 cell extracts respectively. Most of the tRNA precursors, especially those migrating slower than 5S RNA, disappear after incubation with wild-type S-30 cell extract but not with either mutants S-30. This reciprocal experiment indicates that the accumulating precursors must be blocked in their maturation pathway at an analogous step in the two mutants, because both mutants cell extracts are reciprocally unable to maturate their own and the other mutant tRNA precursors. Since A49 has been shown to be an RNase P mutant (15,16) we think that also DA11 is altered in this enzyme. However in order to definitely settle this point it will be necessary to do sequence analysis of the tRNA precursors accumulating in DA11 strain.

HisU *mutants can be distinguished in two classes on the basis of RNA fractionation pattern on polyacrylamide gel.* In order to establish whether other *hisU* mutants are carrying the same alteration shown by strain DA11, we performed pulse-chase experiments on seven distinct mutants mapping in *hisU* region: *hisU6322*, *hisU6397*, *hisU1817*, *hisU1818*, *hisU1819*, *hisU1820* and *hisU1823*. We found that *hisU6322* is very much similar to DA11 since both show identical pattern of tRNA precursors and same temperature dependence in their accumulation (our unpublished results). On the other hand, the newly synthesized RNA patterns obtained from other *hisU* mutants are quite different from those shown by DA11 and *hisU6322*. Fig. 4 shows the fractionation on 10% polyacrylamide gel of RNA labeled during 3 min pulse and 10 min pulse+10 min chase in *hisU1817* and *hisU1820* (*hisU6397*, *hisU1818*, *hisU1819* and *hisU1823* are similar and not shown). It has to be noted that - although the influence of temperature on the RNA patterns of these mutants was very weak - the best conditions to appreciate their differences from wild-type were still obtained by shifting the cells at 43°C before the pulse.

Two main features of the RNA pattern in Fig. 4 strongly lead to consider *hisU1817* and *hisU1820* (and therefore the other similar ones), as belonging to a class of *hisU* mutants different from DA11 and *hisU6322*: *i)* *hisU1817* and *hisU1820* lack of any detectable tRNA precursor band and *ii)* in *hisU1817* and *hisU1820* mature tRNA molecules are already detectable

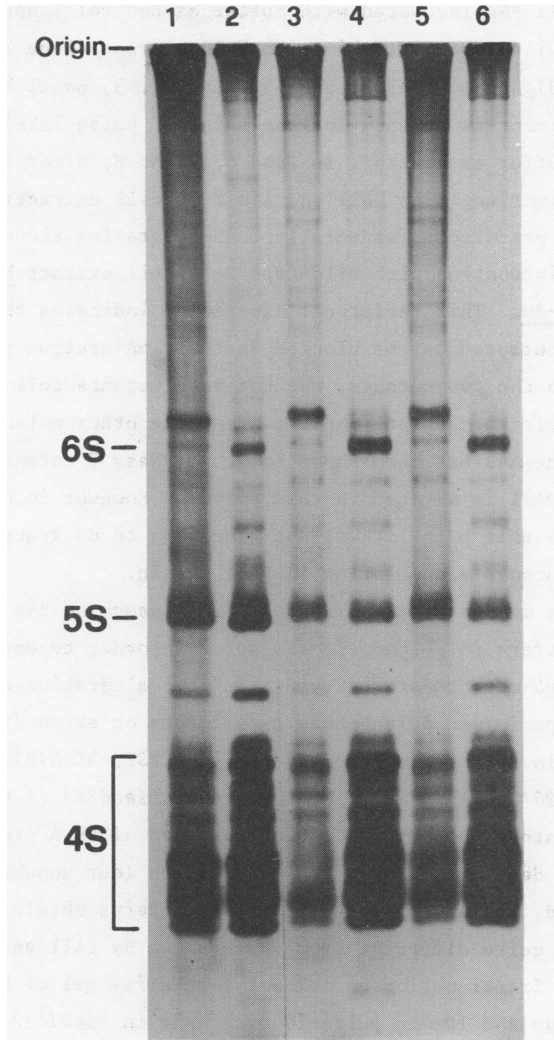

Fig. 4. *Fluorogram of 10% polyacrylamide gel electrophoresis of ^{3}H labeled RNA from TA265 (wild-type), hisU1820 and hisU1817.* Cells were labeled as described in the legend to the Fig. 1. Pulses were for 3 min and chases were for 10 min after a 10 min pulse. *Lane 1:* TA265 3 min pulse; *lane 2:* TA265 10 min pulse+10 min chase; *lane 3: hisU1820* 3 min pulse; *lane 4: hisU1820* 10 min pulse+10 min chase; *lane 5: hisU1817* 3 min pulse; *lane 6: hisU1817* 10 min pulse+10 min chase.

after 3 min pulse whereas in DA11 and *hisU6322* the appearance of mature tRNA is very much delayed. Comparison with wild-type shows that in *hisU1817* and *hisU1820* there is a marked reduction of uridine incorporation in RNA with an apparently different distribution of radioactivity in 4S

region. These quantitative differences between this class of *hisU* mutants and wild-type are difficult to evaluate: the reduced incorporation of label into RNA might indicate an alteration of transcription involving tRNA genes; on the other hand, the lack of accumulation of tRNA precursors does not exclude an alteration of tRNA maturation, if one assumes that maturation intermediates are rapidly degraded by unspecific nucleases. At present we have no further information about this group of *hisU* mutants.

On the basis of our results the distinction between DA11 and *hisU6322* in one class and *hisU1817*, *hisU1818*, *hisU1819*, *hisU1820*, *hisU1823* and *hisU6397* in a second class of *hisU* mutants appears clearly and it is interesting that some indirect genetic evidences (D.S. Straus, unpublished results) give support to this distinction. Using DA11 *(hisU1206)* as recipient it was possible to obtain a high number of wild-type recombinants by transduction at 43°C with phage P22 grown on *hisU6397* or on *hisU1817*; on the contrary very few recombinants were obtained with phage grown on *hisU6322*: this is the result one would expect if DA11 and *hisU6322* were mutated in the same gene but a different one from the gene mutated in *hisU1817* and *hisU6397*. This is in agreement with our biochemical results which allow to classify DA11 and *hisU6322* in one class and *hisU1817* and *hisU6397* in the other.

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Abbreviations. Me_2SO, dimethylsulphoxide; PPO, 2,5-diphenyloxazole; BSA, bovin serum albumin; S-30, 30,000 x g supernatant.

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