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Characterization of a *Salmonella typhimurium hisU* Mutant Defective in tRNA Precursor Processing

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The DA11 mutant of *Salmonella typhimurium*, originally isolated as derepressed for the histidine operon, carries a temperature-dependent alteration in a nucleolytic enzyme specifically involved in the maturation of tRNA. As a consequence of this alteration, no detectable synthesis of any mature tRNA species occurs in DA11 upon shift at 43°C, whereas many tRNA precursors, whose sizes range between 80 and 750 nucleotides, do accumulate. Kinetic studies on the synthesis and processing of these maturation intermediates show that these molecules represent different stages in the maturation pathway, most of them being the products of previous nucleolytic events. These RNA molecules are in vivo substrates of methylation and thiolation enzymes and can be cleaved in vitro to 4S RNA by wild-type but not by DA11 cell-free extract. Evidence is presented that DA11 is very probably a ribonuclease P mutant.

The immediate transcriptional products of tRNA genes in prokaryotes are polynucleotide chains longer than tRNA (2, 28, 29), sometimes carrying multiple copies of identical or different tRNA species (1, 9, 13, 25). The conversion of these precursor molecules into functional tRNA's occurs via a multistep "maturation," including size reduction of the polynucleotide chains by means of endo- and exonucleolytic cleavages and chemical modifications of several nucleosides along the tRNA sequence. There is now only preliminary knowledge of the nature of these steps, their order, and the relationships between processing and modification, the difficulty residing in the short half-life of the maturation intermediates. Most of the knowledge of tRNA maturation derives from studies on systems in which the steady-state concentration of tRNA precursors is exceptionally higher than normal. For instance, phage T4 during infection of *Escherichia coli* cells induces the biosynthesis of phage-coded tRNAs whose dimeric precursors have a relatively long half-life, so their structure as well as some of their properties could be determined (11, 12, 17, 18, 26). A considerable amount of information has also been obtained by studying some $tRNA^{Tyr}su3^+$ precursors which show a slow maturation as a consequence of mutations in their nucleotide sequence (2, 29) or mutations in maturation-specific nucleases (22, 24). The isolation of these two types of mutants is based on the same principle: selection of conditional lethal mutants which, at a nonpermissive temperature, will not synthesize mature tRNA molecules able to function in protein synthesis.

Mutants in tRNA biosynthesis have also been obtained by selecting for alterations of tRNA-mediated regulation of operon transcription. The regulation of transcription of some biosynthetic operons in bacteria depends on the intracellular concentration of tRNA, this function of tRNA being independent of its role as amino acid adapter in protein synthesis (15); some mutants selected as derepressed for the histidine operon prove to be altered in the biosynthesis of tRNA (8, 27).

We have previously reported (6) that one of these mutants, a *Salmonella typhimurium* strain carrying a *hisU* mutation (*hisU1206*), is temperature sensitive in the conversion of tRNA precursors to mature tRNA's. We provided evidence that the strains might be altered in a nuclease involved in the tRNA maturation process. In this paper we report further on *hisU1206*.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]uridine (25,000 mCi/mmol), L-[methyl-^{3}H]methionine (250 mCi/mmol), and L-[^{35}S]cysteine hydrochloride (54 mCi/mmol) were purchased from Radiochemical Centre, Amersham, England. T1 ribonuclease, pancreatic ribonuclease (RNase), alkaline phosphatase, and snake venom phosphodiesterase were purchased from Sigma Chemical Co. Thin-layer chromatography foils were pur-

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chased from Merck (product no. 5563/0001). All other reagents were reagent grade.

Bacterial strains. TA265 is a *S. typhimurium* LT2 wild-type strain. Strain DA11 is a histidine regulatory mutant belonging to the class of *hisU* (*hisU1206*) (3). *E. coli* A49 mutant strain and phage ϕ80pSu_3 were a gift of J. D. Smith. Phage $T4^+$ and *E. coli* B^E strain were kindly provided by A. Cascino.

Preparation of RNA. The [5-^{3}H]uridine pulse labeling, rifampin chase, and phenol extraction of RNA were all carried out as previously described (6). In vivo labeling of methylated and thiolated nucleosides was performed as follows: cells were grown at 30°C in a minimal tris(hydroxymethyl)aminomethane (Tris)-glucose medium (10) supplemented with 20 μg of all the amino acids per ml with the exception of methionine, cysteine, and aspartic acid. At an absorbancy at 650 nm of 0.2 units, cell cultures were transferred to 43°C, and after 35 min, 2.5 mCi of [*methyl*-^{3}H]methionine or 0.5 mCi of [^{35}S]cysteine were added separately to 100-ml culture portions. After 20 min of labeling, the cells were quickly cooled and killed by addition of a frozen solution of 10 mM Tris-hydrochloride (pH 7.3)–5 mM $MgCl_2$–10 mM sodium azide–250 μg of chloramphenicol per ml. Cells were centrifuged, and the pellet was resuspended in 2 ml of a mixture of 0.1 M Tris-saturated phenol and water (1:1, vol/vol). Phenol extraction was repeated four times to remove any trace of protein; 0.1 volume of 2 M sodium acetate was added, and RNA was precipitated with 2.5 volumes of ethanol at −20°C.

Polyacrylamide gel electrophoresis and fluorography. Ten percent polyacrylamide slab gels (15 by 30 by 0.15 cm) were prepared according to the procedure of Peacock and Dingman (19). Electrophoresis was carried out at room temperature in a continuous buffer system containing (per liter) 10.8 g of Tris base, 0.93 g of disodium-ethylenediaminetetraacetic acid, and 5.5 g of boric acid, pH 8.3, for 18 h at 150 V.

To detect ^{3}H-labeled RNA components in polyacrylamide gel, we applied the procedure of Bonner and Laskey (5) with slight modifications as previously described (6).

Elution of RNA from gel. The region of the dried gel corresponding to each RNA band was excised by using an exposed X-ray film as template. On the resulting gel slice, a procedure was carried out to remove 2,5-diphenyloxazole trapped in gel lattice (this treatment was required since we found that elution of RNA from gel was hampered by the presence of 2,5-diphenyloxazole. The dried gel slice was swollen in water for 15 min and then soaked in dimethyl sulfoxide 20 times its volume. This last treatment was repeated twice more, and then the slice was washed in water (10 min), homogenized, and stirred overnight in 0.5 M NaCl–0.01 M ethylenediaminetetraacetic acid–0.1 M Tris-hydrochloride, pH 8.5.

In vitro processing of tRNA precursors. Preparation of S 30 cell extract was carried out as previously described (6). ^{3}H-labeled RNA species eluted from gels were incubated in a reaction mixture containing 5 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, 0.1 mM ethylenediaminetetraacetic acid, 0.1 M NH_4Cl, 10 mM Tris-hydrochloride, pH 8, 20 μg of unlabeled *S. typhimurium* tRNA, and 400 μl of S 30 extract which was prewarmed at 43°C for 35 min before incubation. The reaction was carried out at 43°C and stopped by addition of 1 volume of 0.1 M Tris-hydrochloride-saturated phenol, pH 7.5, containing 50 μg of cold carrier *S. typhimurum* tRNA per ml.

Analysis of methylated nucleosides. [*Methyl*-^{3}H]RNA was hydrolyzed by the combined action of pancreatic RNase, alkaline phosphatase, snake venom phosphodiesterase, and T1 ribonuclease, and the resulting nucleosides were analyzed by two-dimensional thin-layer chromatography on cellulose-coated aluminum foils according to the procedure of Rogg et al. (21). The first dimension was in *n*-butanol-isobutyric acid-concentrated ammonium hydroxide-water (75:37.5:2.5:25, by volume), and the second dimension was in saturated ammonium sulfate–0.1 M sodium acetate, pH 6–isopropanol (79:19:2, by volume). Chromatograms were treated with a 7% (wt/vol) 2,5-diphenyloxazole solution in diethyl ether for fluorography as described by Randerath (20).

RESULTS

The alteration of tRNA maturation in DA11 mutant involves most, if not all, tRNA species. Inactivation of a nucleolytic enzyme involved in tRNA maturation, occurring in DA11 mutant upon shift at 43°C, leads to the accumulation of tRNA precursor molecules (6). A question raised in the preliminary characterization of *hisU*-linked lesion in strain DA11 is whether the DA11-altered enzyme plays a central role in the maturation of all tRNA species or rather is specialized in the processing of a restricted number of tRNA precursors.

The analysis on 10% polyacrylamide gel of pulse-labeled RNA extracted from DA11 upon shift at 43°C revealed the presence of many RNA components which widely differ in size (Fig. 1). A careful comparison between mutant and wild-type RNA patterns and their densitometric scanning leads to the conclusion that none of the RNA species present in the wild type is also present in DA11 with the exception of 5S rRNA (band 21), 6S RNA (band 14), and band 13, which probably is a 6S RNA precursor (13). Also, when 4S regions of wild type and DA11 are compared, it can be seen that no single bands comigrate, and since mature wild-type tRNA molecules migrate in this region, one can hypothesize that either no mature tRNA is made in DA11 or it is made in amounts below the detection threshold. To prove that DA11 small RNA species migrating in the 4S region of the gel are not mature tRNA's, we extracted these RNAs from gels and used them as substrates for in vitro incubation with wild-type cell-free extract. As we reported in a previous work (6), this treatment of the high-molecular-weight RNAs

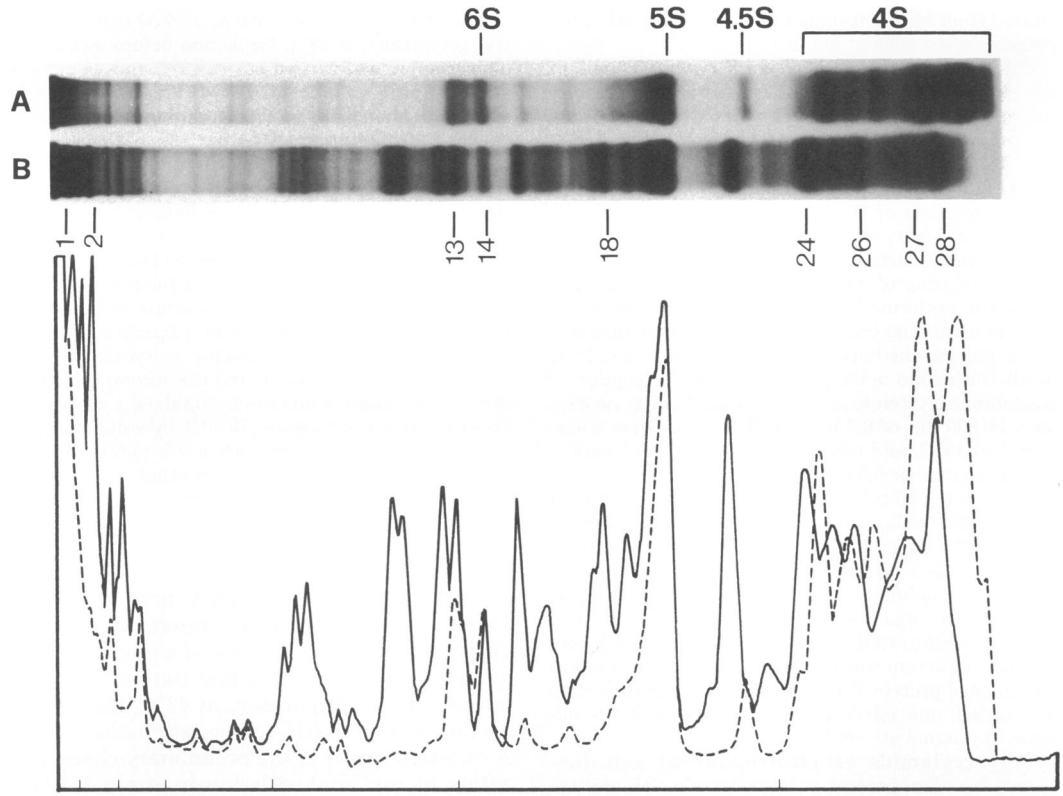

FIG. 1. *Ten percent gel separation of newly made RNA from strains TA265 (wild type) and DA11 (hisU1206). Cells were grown at 30°C to an absorbancy at 650 nm of 0.15 units and then transferred to 43°C. Thirty-five minutes after the temperature shift, 2-ml cultures were pulse labeled for 10 min with 60 μCi of [5-^{3}H]uridine; then a 2,000-fold excess of cold uridine (500 μg/ml) and rifampin (300 μg/ml) were added, and after 2 min, RNA was phenol extracted and fractionated on a 10% polyacrylamide gel. The gel was processed for fluorographic detection of ^{3}H-labeled RNA as described in the text. Fluorograms were scanned in a Gilford spectrophotometer (model 2400). Lane A and the densitometric tracing in dotted line represent RNA from TA265; lane B and the densitometric tracing in continuous line represent RNA from DA11.*

accumulating in DA11 always results in the production of tRNA-size molecules. Figure 2 shows that small RNAs like component 24 (lane 1) and component 28 (lane 4) are converted to even smaller RNA species by incubation with TA265 30,000 × *g* supernatant (lanes 3 and 6, respectively), whereas incubation with DA11 extract leaves them unmodified (lanes 2 and 5). The same result was obtained for other 4S RNA bands from DA11 (unpublished data). This finding indicates that all newly made 4S RNA components in DA11 are tRNA precursors only slightly longer than their mature forms. Moreover, since no stable tRNA bands were detectable, the conclusion can be drawn that *hisU*-linked lesion in DA11 impairs the maturation of probably all tRNA species.

It is interesting to note that mature 4.5S RNA was also missing in DA11 (Fig. 1, lane B), whereas an RNA species accumulated (band 18 in Fig. 1) which could be cleaved in vitro to 4.5S RNA (our unpublished results). This is in agreement with the notion that 4.5S RNA, which has no known relationships with tRNA, shares with it a common maturation pathway (7).

tRNA precursors in DA11 show different kinetics in pulses and chases. During [5-^{3}H]uridine labeling of the DA11 mutant, the label is incorporated into the growing chains of tRNA precursors at a rate presumably dependent only upon the frequency of transcription initiation of the tRNA genes. Therefore, an RNA species which is either an immediate transcriptional product or an early maturation product will quickly appear on gel fluorograms after a short labeling; on the other hand, RNA molecules deriving from a late step of the maturation pathway will be detectable only after longer labeling periods or after a chase with unlabeled precursor. In Fig. 3, we have plotted the kinetics of labeling of various RNA bands during pulses of radioactivity of various length (2, 4, 6, and 8

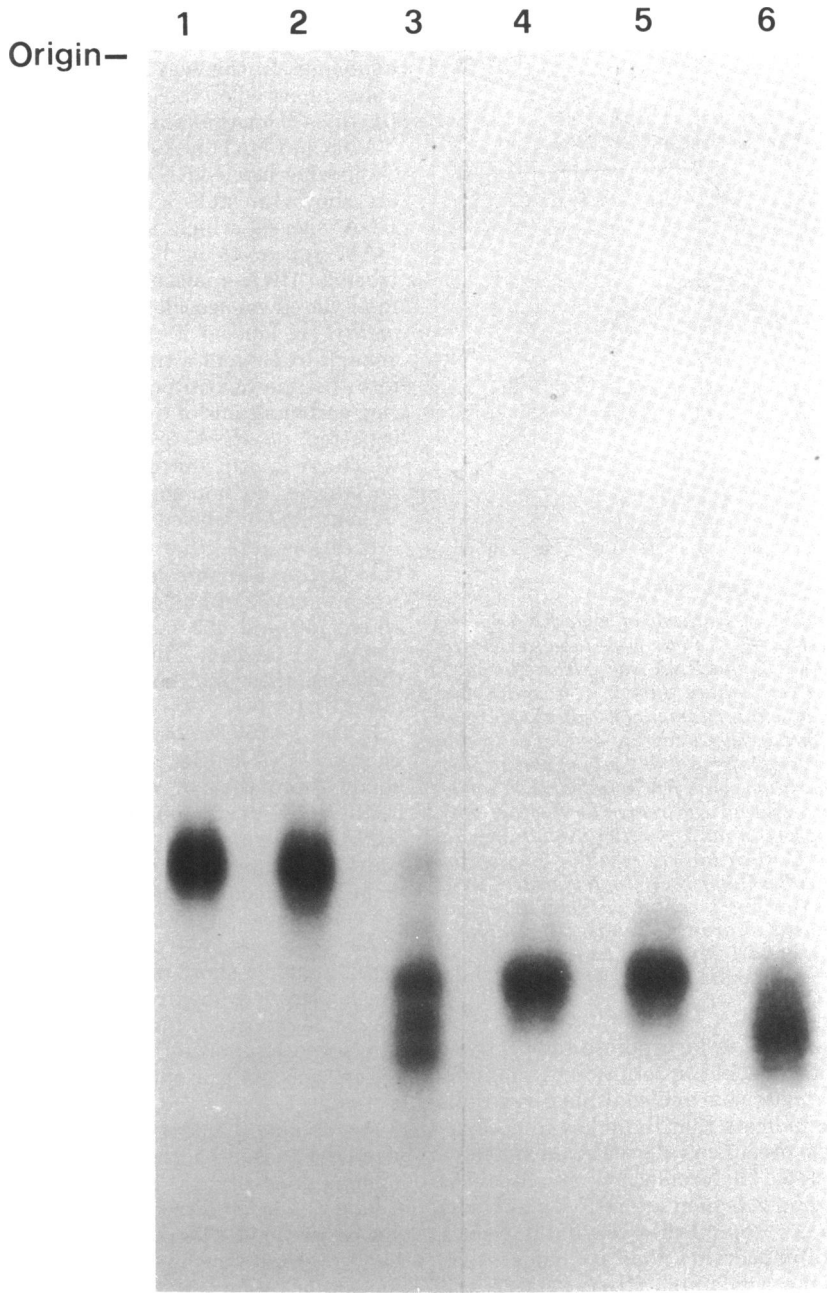

FIG. 2. *In vitro processing of band 24 RNA and band 28 RNA. Gel components 24 and 28 of Fig. 1 were eluted from a gel and incubated in vitro at 43°C with the 30,000 × g supernatant of TA265 or a Da11 extract as described in the text. After 30 min of incubation, RNA was phenol extracted, dried, fractionated on a 10% polyacrylamide gel, and detected by fluorography. Lane 1, untreated band 24 RNA; lane 2, band 24 RNA incubated with DA11 cell extract; lane 3, band 24 RNA incubated with TA265 cell extract; lane 4, untreated band 28 RNA; lane 5, band 28 RNA incubated with DA11 cell; lane 6, band 28 RNA incubated with TA265 cell extract. The radioactivity applied was the same for all the samples (3,000 counts per min).*

min) and rifampin chases of 2 and 9 min (starting after 8 min of labeling). We observe that some RNA species show a lag in the kinetics of [5-^{3}H]uridine incorporation, especially in the case of the small tRNA precursors (bands 26, 27, and 28 of Fig. 1); moreover, RNA components

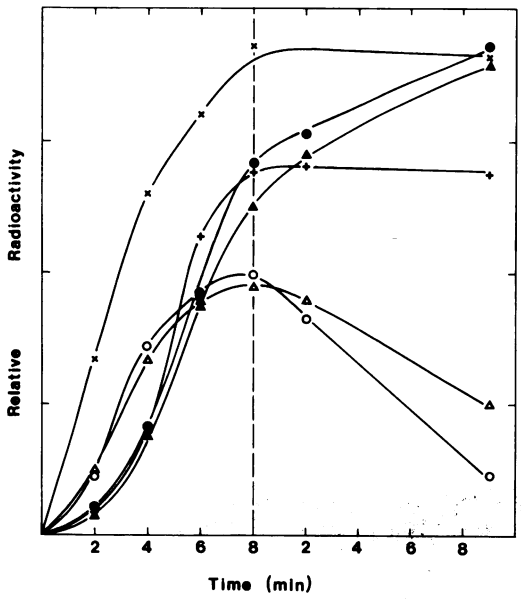

FIG. 3. *Kinetics of synthesis of some RNA species in strain DA11 at 43°C. Cells were labeled with [5-^{3}H]uridine at 43°C, and RNA was extracted from 2-ml portions of cell culture after 2, 4, 6, and 8 min. After 8 min of labeling, rifampin (300 μg/ml) and cold uridine (500 μg/ml) were added to 4 ml of cells, and RNA was extracted after 2 and 9 min of chase. Then all RNA preparations were fractionated on 10% polyacrylamide gel. The gel was treated for fluorography, and after exposure of an X-ray film (Kodak Regulix BB54) at −80°C, each lane of resulting fluorogram was scanned with a Gilford spectrophotometer. Areas of some peaks (see Fig. 1, continuous line) were measured and plotted in arbitrary units.* (×), *5S rRNA (band 21);* (○), *band 1 RNA;* (△), *band 2 RNA;* (+), *band 26 RNA;* (▲), *band 27 RNA;* (●), *band 28 RNA.*

27 and 28 continue to incorporate radioactivity even after the arrest of transcription by rifampin. Both lag of appearance and continued synthesis during chase indicate that these low-molecular-weight tRNA precursors derive from previous maturation steps. High-molecular-weight RNAs like bands 1 and 2 (which are tRNA precursors according to our unpublished results) present a shorter lag of appearance than components 26, 27, and 28. During rifampin chase, bands 1 and 2 decrease, showing that either they are immediate transcriptional products or that the pool of their macromolecular precursors is very small.

Methylation and thiolation occur in vivo on tRNA precursors. Radioactive methyl groups originating from [*methyl*-^{3}H]methionine are incorporated into RNA by transmethylation reactions involving *S*-adenosylmethionine as methyl group donor (15). We pulse labeled DA11 and TA265 with [*methyl*-^{3}H]methionine and then fractionated the RNA on 10% polyacrylamide gel. In this way, methylated RNA molecules appeared on the gel fluorogram. In Fig. 4a, [*methyl*-^{3}H]methionine-labeled RNAs from TA265 and DA11 are shown (lanes 1 and 2); [5-^{3}H]uridine-labeled RNAs from TA265 and DA11 are shown in lanes 3 and 4. The majority of RNA species which are uniquely present in DA11 appear to be both 5-^{3}H and *methyl*-^{3}H labeled. RNA molecules not containing any methylated nucleoside are expected not to be *methyl*-^{3}H labeled if the pulse period is short enough to prevent a turnover of methyl groups into the one-carbon pool which is precursor to ring carbon atoms of the purine ring. In fact, as expected, 6S RNA, 5S rRNA, and 4.5S RNA, which are known not to contain any methylated nucleoside, do not appear labeled in [*methyl*-^{3}H]methionine-labeled samples.

It can be noted that even the higher-molecular-weight precursors detectable in DA11 (bands 1 to 5 in lane 2 of Fig. 4a), which range between about 750 and 450 nucleotides in length, are *methyl*-^{3}H labeled. This suggests that, despite their size, these molecules already carry tRNA-like structures.

In the TA265 [*methyl*-^{3}H]RNA pattern (lane 1), some RNA species (a, b, and c), whose size is about twice that of mature tRNA's, appear methylated. Very probably these are wild-type tRNA precursors having "physiological" steady-state concentrations in *S. typhimurium* high enough to become detectable in our experimental conditions. Besides being methylated and thiolated (see below), they are unstable (disappearing after a short rifampin chase), and they can be cleaved in vitro into 4S fragments by TA265 cell extracts (unpublished results). It is of interest that none of them is present in DA11. We will speculate on this point in the Discussion section.

A preliminary analysis on the nature of methylated nucleosides accounting for the *methyl*-^{3}H labeling of several DA11 RNA precursors (Fig. 4, lane 2) and of TA265 bands a and b (lane 1) was carried out. The results are summarized in Table 1. From the distribution of methylated nucleosides among the various tRNA precursors, it can be noted that only ribothymidine or both ribothymidine and N^7-methyl guanosine are synthesized at the level of longer precursor molecules, whereas the majority of other kinds of methylation reactions take place on the smaller precursors and therefore at a late step of the maturation pathway.

Figure 4b shows the 10% gel fractionation pattern of RNA extracted from TA265 (lane 1)

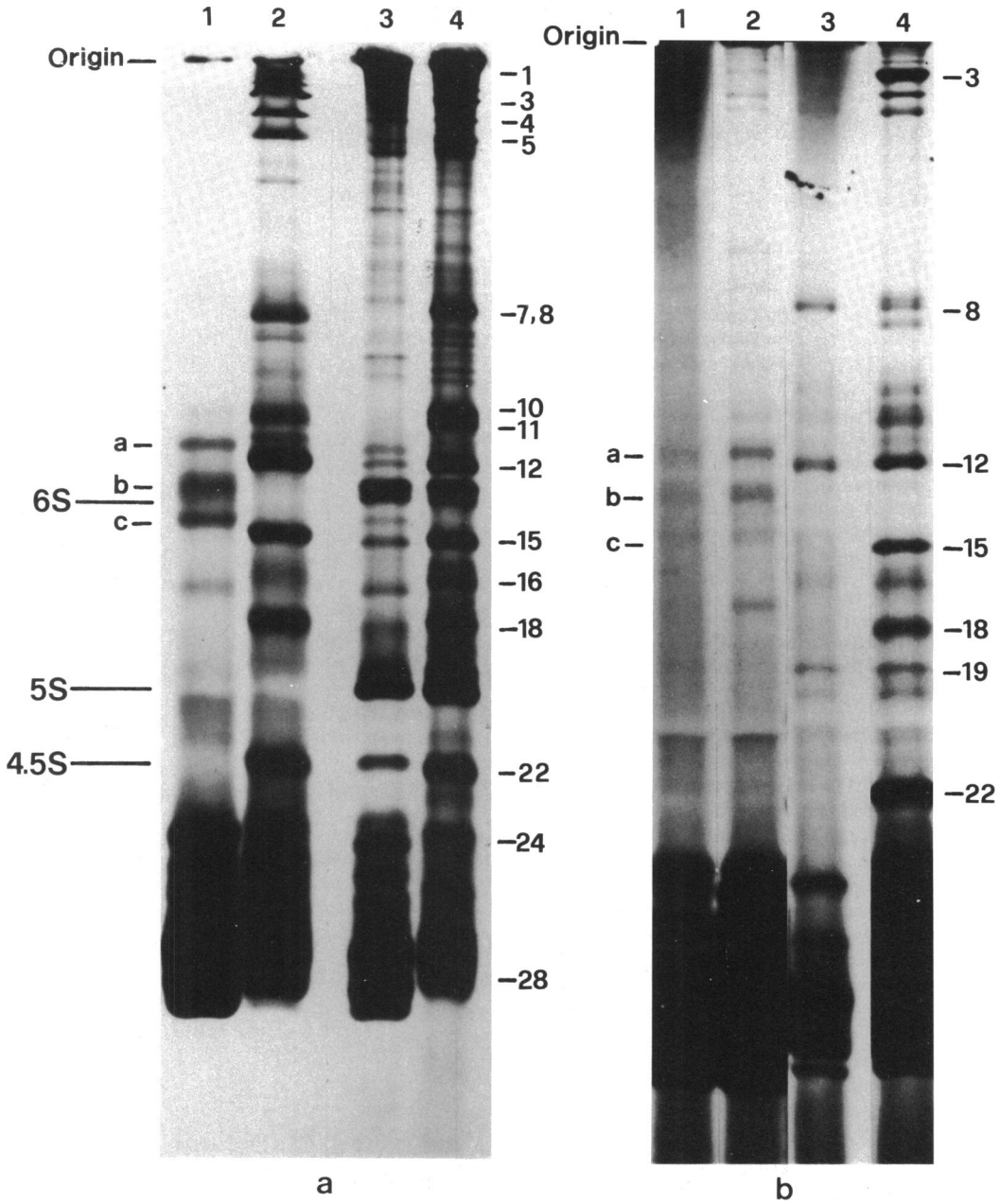

FIG. 4. *Ten percent gel separation of TA265 and DA11 RNAs selectively labeled in methylated and thiolated nucleosides. Cells were labeled with [methyl-^{3}H]methionine or [^{35}S]cysteine for 20 min at 43°C, and RNA was extracted as described in the text. Then RNA was fractionated on 10% polyacrylamide gel and detected by fluorography. Panel a, [methyl-^{3}H]methionine-labeled RNA from TA265 (lane 1) and DA11 (lane 2) and [5-^{3}H]uridine-labeled RNA (as control) from TA265 (lane 3) and DA11 (lane 4). Panel b, [^{35}S]cysteine-labeled RNA from TA265 (lane 1) and DA11 (lane 3) and [methyl-^{3}H]methionine-labeled RNA (as control) from TA265 (lane 2) and DA11 (lane 4).*

and DA11 (lane 3) after a pulse experiment performed using [^{35}S]cysteine as label. From the presence of ^{35}S labeling in some precursor RNA bands (e.g. bands 8, 12, and 19), one can argue that thiolation enzymes, like methylases, can act on tRNA precursors before nucleolytic process-

TABLE 1. *Methylated nucleosides in tRNA precursor bands*[a]

Band	Nucleosides
3	T
4	T
5	T
9	T
11	T
12	T, m^7G
15	T, m^7G
16	T, m^7G
18	T, m^7G, m^1G,[b] mA[b]
22	T, m^7G, m^1G,[b] mA[b]
24–28[c]	T, m^7G, m^1G, mA, Cm, Um[b]
a	T, m^7G
b	T, m^7G

[a] Analysis of *methyl*-^{3}H-labeled nucleosides in RNA species eluted from gel in Fig. 4a (lanes 1 and 2) was carried out according to the procedure of Rogg et al. (21). T, Ribothymidine; m^7G, N^7-methyl guanosine; m^1G, 1-methyl guanosine; Cm, 2′-*O*-methyl cytidine; Um, 2′-*O*-methyl uridine. mA is a methylated adenosine (2-methyl adenosine and/or N^6-methyl adenosine). 2-Methyl adenosine and/or N^6-methyl adenosine). 2-Methylthio N^6-isopentenyl adenosine was not detectable by our experimental procedure.

[b] These nucleosides occurred in very low amount.

[c] RNA from these bands was pooled before nucleoside analysis.

ing. On the other hand, comparison between ^{35}S-labeled RNA (lane 3) and [*methyl*-^{3}H]RNA (lane 4) in DA11 shows that some RNA bands are methylated but not thiolated (e.g., bands 3, 15, 18, and 22). The absence of any thiolated nucleoside in some precursors might be explained by assuming that these RNAs are at a stage of maturation preceding the thiolation step or that these are precursors of tRNA species not containing thiolated nucleosides within their nucleotide sequence (all mature tRNA's are methylated, but few of them lack thiolated nucleoside, e.g., $tRNA^{Leu}$ [4] and $tRNA^{Gly}$ [30]).

$tRNA^{Tyr}$ precursor is not cleaved by extracts from DA11 mutants. The first tRNA precursor characterized was the monomeric precursor to $tRNA^{Tyr}$ (2, 28). It is a 128-nucleotide-long molecule which is undetectable in wild-type cells but accumulates in some $tRNA^{Tyr}$ mutants

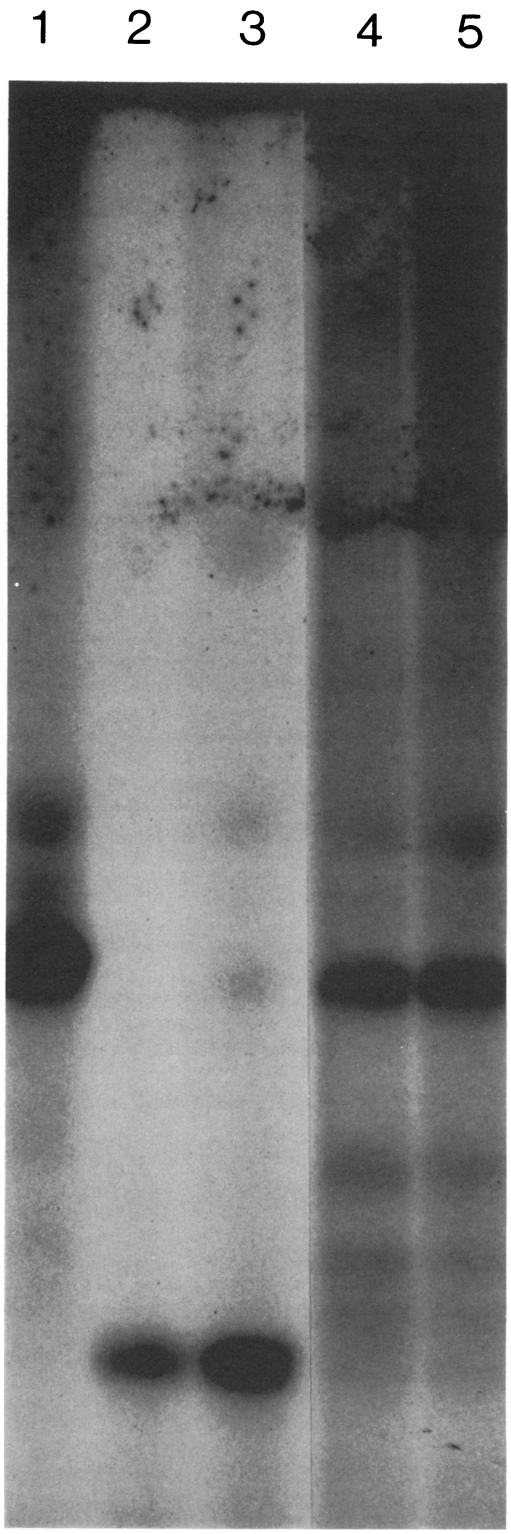

FIG. 5. *In vitro cleavage of $tRNA^{Tyr}$ precursor. ^{32}P-labeled $tRNA^{Tyr}$ precursor was incubated for 20 min at 43° in the presence of extracts from strains TA265, DA11, and A49. The reaction was stopped by addition of 0.1 M Tris-saturated phenol (pH 7.5), and cleavage products were analyzed on 10% polyacrylamide gel. Lane 1, $tRNA^{Tyr}$ precursor; lane 2, mature $tRNA^{Tyr}$; lane 3, after incubation with TA265 extract; lane 4, after incubation with DA11 extract; lane 5, after incubation with A49 extract.*

or in RNase P⁻ mutants (2, 24). In vitro, this molecule is a substrate of RNase P which cleaves off the extra 41 nucleotides at the 5′ end, thus generating a mature 5′ terminus. We have prepared purified ^{32}P-labeled tRNA^{Tyr} precursor by infecting *E. coli* A49 with ϕ80psu3$^+$ phage, followed by RNA fractionation on a polyacrylamide gel and elution of the desired band according to the procedure described (2, 6). Figure 5 shows the results of the in vitro maturation of tRNA^{Tyr} precursor (run uncleaved in lane 1), using extracts from strains TA265 (lane 3), DA11 (lane 4), and A49 (lane 5). Purified, mature tRNA^{Tyr} (a gift of J. D. Smith) is shown as control in lane 2. These results indicate that *S. typhimurium* wild-type cell extract contains an enzymatic activity equivalent to RNase P. DA11 mutant strain lacks this activity and behaves, in the in vitro maturation, like the well-characterised RNase P⁻ mutant *E. coli* A49.

DISCUSSION

We observed that in DA11, the alteration of a nucleolytic enzyme caused the complete stop of mature tRNA synthesis at 43°C, with production of large amounts of several tRNA precursors, whose sizes range from 80 to 100 nucleotides (small tRNA precursors) to several hundred nucleotides (long tRNA precursors). The small precursors differed from mature tRNA's only by a few extra nucleotides, and their kinetics of appearance in DA11 indicated that they were late-maturation products. During rifampin chase, the labeling of some small precursors, e.g., band 27 RNA and band 28 RNA, becomes 50% higher than in the corresponding pulse (Fig. 3). This fact indicates that the pool of macromolecular radioactivity flowing into bands 27 and 28 during the chase is rather large, thus showing that the small precursors are generated from longer RNA chains which also accumulate in DA11. This phenomenon does not exist in wild-type cells where no significant increase of individual RNA species occurs during the chase, since the steady-state concentrations of all the maturation intermediates are normally very low (data not shown).

The large tRNA precursors (e.g., bands 1 and 2) accumulate in DA11 during the pulse, but decrease during the chase, and it is likely that at least some of them are cleaved into small precursors during the chase period (Fig. 3). Therefore we observe that, in DA11, small tRNA precursors accumulate whose maturation is completely blocked at 43°C and which derive from longer RNA species, also accumulating, whose maturation is not completely blocked but only slowed down.

tRNA precursors can be detected also in wild-type cells, presumably because their steady-state concentration is "physiologically" high enough to be detectable also in absence of any alteration in the maturation pathway. These wild-type precursors are clearly visible in the *methyl*-labeling experiment (Fig. 4a, lane 1), but they can be identified also in the parallel gel slot containing [5-^{3}H]uridine-labeled RNA (lane 3). We think that these precursors are detectable since they are the substrate of a maturation step which is physiologically rate limiting in the wild type. In DA11, none of these precursors was present; this suggests that their processing was not altered or slowed in the mutant (otherwise, they would accumulate), but rather their synthesis was altered or slowed, as if they were maturation products of the precursors which accumulate in DA11.

One point concerning the nature of the enzyme altered in DA11 still remains to be clarified. The pattern of precursors accumulating in this mutant shows some differences from that shown by RNase P⁻ mutants, but this might be due to minor differences between *E. coli* and *S. typhimurium*. A known substrate for RNase P is the 128-nucleotide-long tRNA^{Tyr} precursor (2, 28). With respect to the in vitro maturation of this molecule, DA11 behaves like *E. coli* strain A49, a well-characterized RNase P mutant.

On the basis of these results, we conclude that DA11 is very likely mutated in the RNase P gene. A definite answer to this question, however, can only be provided by sequence analysis of tRNA precursors accumulating in DA11 mutant.

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