JOURNAL OF BACTERIOLOGY, Apr. 1985, p. 55–60
0021-9193/85/040055-06$02.00/0

Vol. 162, No. 1

5-Methyl-2-Thiouridine in the tRNA of *Candida tropicalis* and Its Localization in Lysine tRNA

SATISH PATWARDHAN† AND JOSEPH D. CHERAYIL*

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Received 19 July 1984/Accepted 12 January 1985

^{35}S incorporation studies showed that *Candida tropicalis* tRNA contained two thionucleosides, one of which was identified as 5-methyl-2-thiouridine. The other thionucleoside was alkali labile, and it appeared to be an ester. Pulse-chase experiments suggested that the two thionucleosides were structurally related. 5-Methyl-2-thiouridine was present in one of the lysine tRNAs. This is the first report of the presence of this nucleoside in a yeast tRNA.

Transfer RNA (tRNA) contains a large number of modified nucleosides, including unique thionucleosides. The latter, with the exception of 2-thiocytidine, are derivatives of uridine and adenosine. A number of 2-thiouridine derivatives with modification at the position 5 carbon atom have been reported and have been shown to be present at the first position of the anticodon of tRNAs such as those of lysine, glutamic acid, and glutamine (5). One exception to this is 5-methyl-2-thiouridine (m^5s^2U), whose presence has been shown in the TψC arm of tRNAs from thermophilic bacteria (16). The presence of only one thionucleoside, 2-thiouridine acetic acid methyl ester or 5-methoxy-carbonylmethyl-2-thiouridine (mcm^5s^2U), has been reported in the tRNA of yeasts (5). We report here the presence of two thionucleosides in the tRNA of the yeast *Candida tropicalis* and present evidence for a possible biosynthetic relationship between the two thionucleosides. One of the thionucleosides was identified as m^5s^2U and shown to be present in one of the lysine tRNAs.

MATERIALS AND METHODS

The *C. tropicalis* strain, used in the present studies was from the Microbiology and Cell Biology Laboratory of this Institute. DEAE-cellulose, DEAE-Sephadex, BD-cellulose, the enzymes, and m^5s^2U were from Sigma Chemical Co., St. Louis. Reverse-phase chromatography (RPC)-5 material was a gift from A. D. Kelmers, Oak Ridge National Laboratory. Cellulose thin-layer plates were from J. T. Baker Chemical Co., Phillipsburg, N.J. Carrier-free $H_2{}^{35}SO_4$, $H_3{}^{32}PO_4$, and [^{14}C]lysine (specific activity, 240 mCi/mmol) were from Bhabha Atomic Research Centre, Bombay, India. All other reagents were of analytical grade.

Preparation of labeled tRNA. The synthetic medium for the growth of *C. tropicalis* contained (per liter) 4 g of NH_4Cl, 1 g of K_2HPO_4, 0.25 g of $MgSO_4$, 20 g of glucose, 2 mg of biotin, 1 mg of thiamine hydrochloride, 1 mg of calcium pantothenate, and 0.1 ml of the trace elements mixture of Vogel (18). For labeling with ^{35}S (2.5 mCi/100 ml of culture), the concentration of sulfate in the growth medium was reduced to 1/10 of the amount used in the normal synthetic medium by replacing $MgSO_4$ with $MgCl_2$. Similarly, the phosphate concentration was reduced to 1/10 of the normal for labeling with ^{32}P (1 mCi/100 ml of culture). tRNA was isolated by extraction of the cells with phenol, followed by chromatography on DEAE-cellulose. The sample was deacylated by incubation in buffer (pH 8.8) for 30 min.

tRNA was hydrolyzed to nucleotides by digestion with RNase T_2 (10 U of enzyme for 15 A_{260} units of tRNA) in 0.1 ml of 0.05 M ammonium acetate buffer (pH 4.5) containing 1 mM EDTA at 37°C for 16 h. Digestion with RNase A (20 μg/10 A_{260} units) was carried out in 0.05 M Tris-hydrochloride (pH 7.5) at 37°C for 10 h. The same incubation conditions were used for digestion with RNase T_1, except that 2 U of the enzyme per A_{260} unit of tRNA were used.

Electrophoresis on DEAE-cellulose and Whatman 3 MM paper was carried out at 50 to 60 V/cm in pyridine-acetate buffer (pH 3.5) (3). Two-dimensional thin-layer chromatography was performed according to the method of Saneyoshi et al. (15). The phosphocellulose column for the separation of nucleosides was run as described earlier (14). Fractionation of tRNA by column chromatography on various columns, DEAE-Sephadex (1), BD-cellulose (7), and RPC (12) was carried as described earlier with minor modifications. Gel electrophoresis was conducted on 12% polyacrylamide gels at pH 8.3 (6).

RESULTS

Identification of the thionucleotides. ^{35}S-labeled tRNA isolated from *C. tropicalis* grown in the presence of radioactive sulfate to log phase was completely digestible with RNase T_2, as revealed by the Sephadex G-150 gel filtration patterns obtained before and after treatment with the enzyme, showing that the tRNA preparation was free from mucopolysaccharides. On electrophoresis on Whatman 3 MM paper at pH 3.5, the RNase T_2 digest showed the presence of two major thionucleotides moving close together between guanosine 3′-phosphate (Gp) and uridine 3′-phosphate (Up) and a minor one moving faster than Up (Fig. 1a). Sometimes spots 1 and 2 overlapped, and only one broad spot could be detected. The paper pieces corresponding to the spots were cut out, and the radioactivity in each was determined. The relative proportions of spots 1, 2, and 3 were 52.3, 45.4, and 2.2, respectively. Upon subjecting the RNase T_2 digest to two-dimensional thin-layer chromatography on a cellulose

* Corresponding author.
† Present address: Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110.

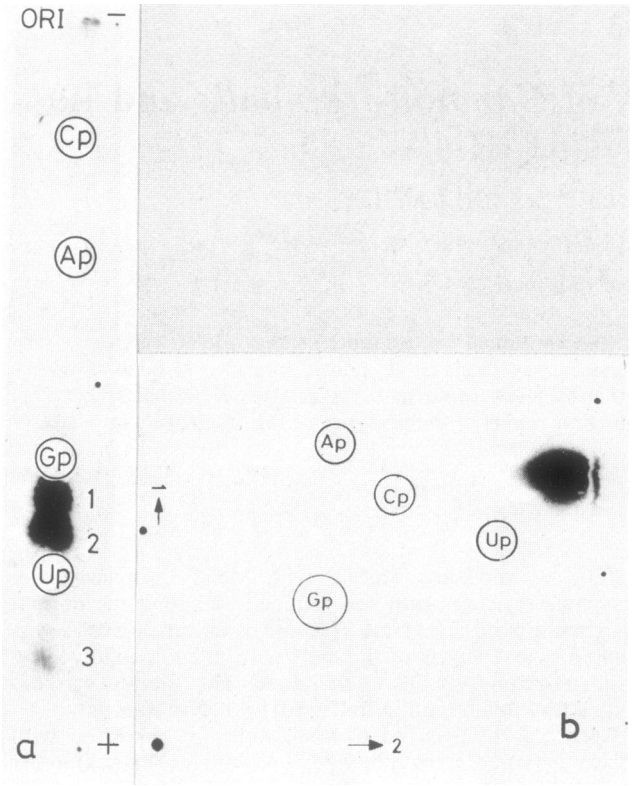

FIG. 1. Autoradiogram showing the separation of thionucleotides from *C. tropicalis* tRNA. (a) ^{35}S-labeled tRNA was digested with RNase T_2 and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. (b) ^{35}S-labeled nucleotides were subjected to two-dimensional thin-layer chromatography on cellulose plates (20 by 20 cm). First dimension, isobutyric acid–0.5 M ammonia (5:3 [vol/vol]); second dimension, isopropanol-concentrated HCl-water (70:15:15 [vol/vol/vol]).

plate, only one radioactive spot on the upper right side of Up was observed (Fig. 1b). A number of thionucleotides, like 5-methyl-2-thiouridine 3′-phosphate (m^5s^2Up), mcm^5s^2Up, and 4-thiouridine 3′-phosphate, and known to move to the region of Up (15). The identities of spots 1 and 2 (Fig. 1a) were established by further analysis. Spot no. 1 was eluted from the paper and converted to the nucleoside, and a sample was subjected to column chromatography on a phosphocellulose column along with an authentic sample of ms^5s^2U. The radioactivity and the optical density peaks eluted together from the column, showing that spot no. 1 was m^5s^2Up. (data not presented). The identity of spot no. 1 as m^5s^2Up was further confirmed by chromatography of the nucleoside in four solvent systems along with the authentic sample. The radioactivity moved with the authentic sample in all the solvent systems (Table 1).

TABLE 1. Identification of spot no. 1[a]

Solvent system[b]	R_f value	
	Spot no. 1 nucleoside	Authentic m^5s^2u
A	0.69	0.69
B	0.55	0.55
C	0.68	0.68
D	0.77	0.77

[a] Radioactivity in spot no. 1 (Fig. 1a) was eluted from the paper, and the spot was digested with alkaline phosphatase.

[b] Samples were subjected to ascending paper chromatography on Whatmans 3 MM paper in the following solvent systems: butanol-acetic acid-water (5:3:2 [vol/vol/vol]) (A), sopropanol-concentrated ammonia-water (55:10:35 [vol/vol/vol]) (B), *n*-propanol-concentrated ammonia-water (55:10:35 [vol/vol/vol]) (c), and 95% ethanol–water (4:1 [vol/vol]) (D).

A sample of the eluant from spot no. 2 (Fig. 1a) was heated with 0.2 M KOH at 100°C for 1 h; the sample was neutralized, and it was subjected to electrophoresis at pH 3.5. The autoradiogram showed that the radioactivity moved faster than Up (Fig. 2). These results suggested that spot no. 2 was an ester, possibly mcm^5s^2Up. Another sample of the eluant from spot no. 2 was digested with alkaline phosphatase and subjected to paper chromatography in the solvent system *n*-butanol-acetic acid-water (5:3:2 [vol/vol/vol]). It moved with a R_f value of 0.55, in near agreement with the R_f value of 0.6 reported for mcm^5s^2Uin this solvent system (2). From these results and the location of spot no. 2 at the position expected for mcm^5s^2Up (Fig. 1b) in the two-dimensional thin-layer chromatographic system (9, 15), it appeared to be mcm^5s^2Up.

Biosynthetic relationship among the thionucleotides. Since both the thionucleotides were modified at the fifth position of uridine, it was of interest to know whether they had a common pathway for their biosynthesis. *C. tropicalis* tRNA was pulse-labeled with [^{35}S]sulfate for 10 min, phenol was added to half of the cells, and tRNA was isolated. Excess nonradioactive sulfate was added to the other half of the cells, and the cells were grown for a further period of 30 min. The tRNA samples isolated from the two cultures were analyzed for thionucleotides by paper electrophoresis. Because of the low level of incorporation, the paper was cut and counted. In the 10-min pulse-labeled sample the propor-

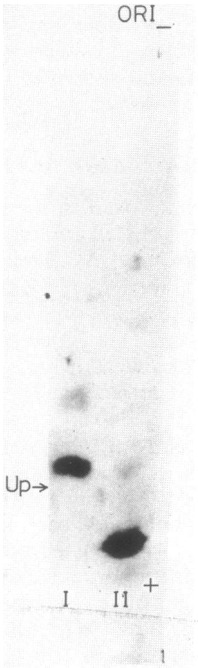

FIG. 2. Alkali digestion of spot no. 2. Radioactivity in spot no. 2 (Fig. 1a) was eluted, concentrated, and heated with 0.2 M NaOH in a boiling water bath for 1 h. The sample was neutralized and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. Lane I, Untreated sample; lane II, alkali-treated sample.

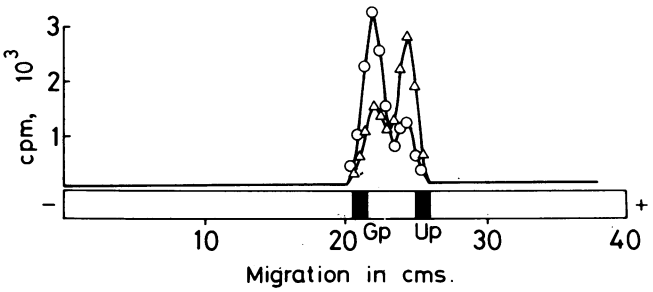

FIG. 3. Pulse-chase experiment. *C. tropicalis* cells were grown in ^{35}S-containing medium for 10 min, phenol was added to half of the cells, and tRNA was isolated (○). The other half of the cells were grown for a further period of 30 min with excess cold sulfate, and tRNA was isolated (△). Nucleotides prepared from these tRNA samples were subjected to electrophoresis on Whatman 3 MM paper. The paper was cut into 0.5-cm lengths and counted.

tion of radioactivity in the m^{5}s^{2}Up spot was two to three times more than that in the presumed mcm^{5}s^{2}Up spot, whereas in the chased sample the proportion of the label was higher in mcm^{5}s^{2}Up than that in m^{5}s^{2}Up (Fig. 3). From these results it was concluded that m^{5}s^{2}Up may be a precursor of mcm^{5}s^{2}Up.

Location of the thionucleotides in the tRNA. Since m^{5}s^{2}U or 2-thioribothymidine has been reported to be present at the TψC region of the tRNA of thermophilic bacteria (5) and no tRNA has been reported to contain m^{5}s^{2}U at the anticodon in any of the published sequences, it was of interest to know the position of this nucleoside in *C. tropicalis* tRNA. All tRNAs have a pyrimidine at the 5′ side of the anticodon (5). Hence m^{5}s^{2}U, if present at the first position of the anticodon of a tRNA, should be released as a mononucleotide on RNase A treatment. On the other hand, if it is present at the TψC region, it might be released as GT*, AT*, or an oligonucleotide on RNase A treatment (see reference 5). When a RNase A digest of ^{35}S-labeled tRNA was subjected to electrophoresis on a DEAE-cellulose paper at pH 3.5, a prominent radioactive spot moving slower than Gp was observed (Fig. 4a). The position of the spot indicated that it was at least a dinucleotide. The radioactivity from the spot was eluted, and samples were digested separately with RNases T_2 and T_1 and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. RNase T_2 produced a spot corresponding to the mononucleotide position of the thionucleotides (Fig. 4b, lane II). RNase T_1, however, did not change its mobility (Fig. 4b, lane III). Since RNase T_1 specifically cuts after Gp, the latter result suggested that Gp did not precede the thionucleotide. It was observed that spots 1 and 2 (Fig. 1) could not always be separated. Hence the RNase T_2 digestion products (Fig. 4b, lane II) were eluted, treated with nuclease P1 to convert them to the nucleosides, and chromatographed in the solvent system isopropanol-concentrated ammonia-water (7:1:2 [vol/vol/vol], in which m^{5}s^{2}U and mcm^{5}s^{2}U separate well. The autoradiogram showed the presence of both the species in

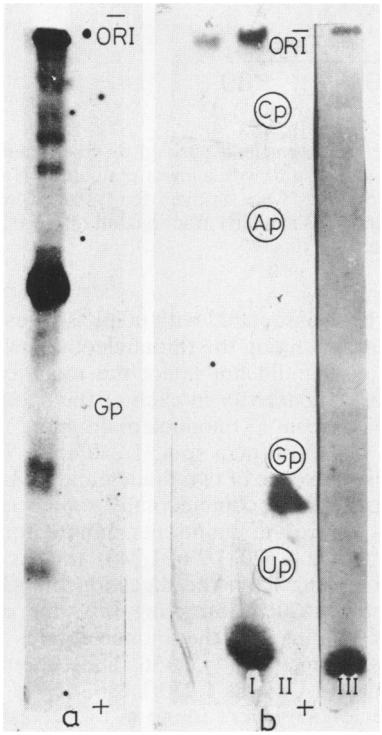

FIG. 4. Analysis of RNase A digestion products of ^{35}S-labeled tRNA. (a) ^{35}S-labeled tRNA was digested with RNase A and subjected to electrophoresis on DEAE-cellulose paper at pH 3.5. (b) The major spot in (a) moving slower than Gp was eluted, digested with RNase T_2, and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. Lane I, Undigested samples; lane II, sample digested with RNase T_2; lane III, sample digested with RNase T_1.

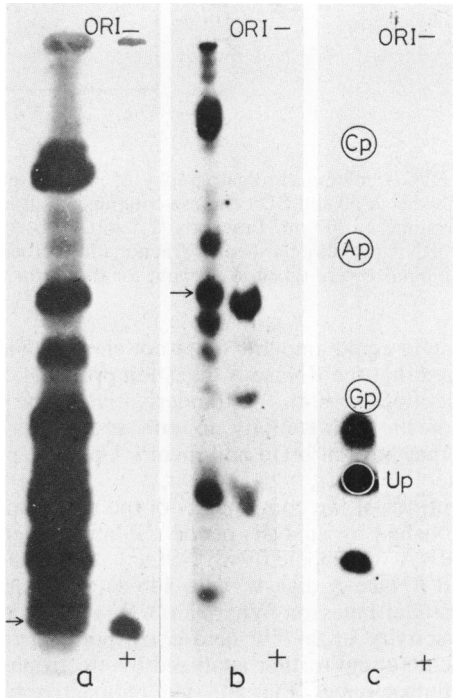

FIG. 5. Analysis of ^{32}P fragments of tRNA containing thionucleotides. (a) ^{32}P- and ^{35}S-labeled tRNA samples were digested separately with RNase A and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. The ^{32}P fragment corresponding to the ^{35}S band (arrow) was eluted. (b) The eluted ^{32}P band was reelectrophoresed on DEAE-cellulose paper, and the spot corresponding to the major ^{35}S spot (arrow) was eluted. (c) The purified spot was digested with RNase T_2 and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5.

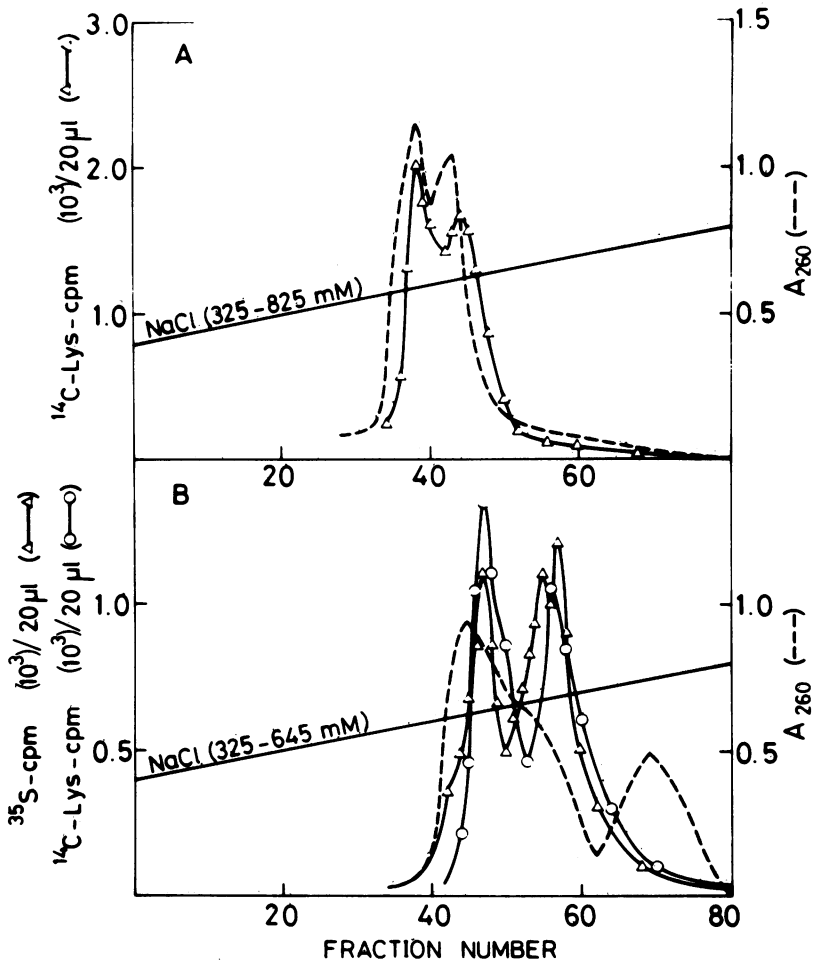

FIG. 6. RPC-5 column chromatography of partially purified lysine tRNA. (A) Lysine tRNA partially purified by fractionation through DEAE-Sephadex A-50 and BD-cellulose columns was fractionated on a RPC-5 column (0.8 by 24 cm) with a linear gradient of NaCl (pH 4.5) in a total volume of 200 ml, Fractions (2.5 ml) were collected at the rate of 30 ml/h. Samples were assayed for lysine acceptance. (B) ^{35}S-labeled tRNA partially purified by fractionation through a DEAE-Sephadex A-50 column was similarly fractionated on a RPC-5 column. Appropriate blanks were taken to account for the sulfur present when assaying for lysine.

approximately equal amounts (data not shown). The results thus showed that the RNase A digestion products contained the dinucleotides of both the thioderivatives. The presence of most of the radioactivity in one spot (Fig. 4a) also indicated that both m^5s^2Up and mcm^5s^2Up were present in the dinucleotide spot.

The identities of the components of the two dinucleotides were established by analysis of the ^{32}P-labeled derivatives. Transfer RNA was labeled with $^{35}SO_4^{2-}$ and $^{32}PO_3^{3-}$ separately, and RNase A digests were subjected to electrophoresis in parallel lanes on Whatman 3 MM paper (Fig. 5a). The radioactivity in the ^{32}P lane corresponding to the ^{35}S spot was eluted and further analyzed by electrophoresis on DEAE-cellulose paper (Fig. 5b). The radioactive band from the ^{32}P lane corresponding to the ^{35}S-dinucleotide was eluted, digested with RNase T_2, and subjected to electrophoresis on Whatman 3 MM paper at pH 3.5. Three spots, one between Gp and Up corresponding to the mixture of m^5s^2Up and mcm^5s^2Up, the second corresponding to Up, and the third corresponding to pGp, were observed (Fig. 5c). It was surprising that this RNase A product did not contain Gp or adenosine 3'-phosphate (Ap). The results at once suggested that the thionucleotides were associated with Up. They could not have been associated with pGp, as digestion of the RNase fragment containing the thionucleotides with RNase T_1 carried out earlier did not affect the mobility (Fig. 4). Measurement of radioactivity in each of the spots (Fig. 5c) gave the following results: thionucleotide spot, 1,270 cpm; Up spot, 1,756 cpm; and pGp spot, 1,240 cpm. The results fitted well for the presence of two dinucleotides, U*pUp and pGpUp, the latter having coincidentally similar electrophoresis properties. Since the counts per minute equivalent of one phosphate in pGp is 620 (1/2 of 1,240), the excess counts in the Up spot arising from the digestion of pGpUp with RNase T_2 must be 620. Subtracting this, the counts per minute in Up combining with the thionucleotides was calculated as follows: 1,756 − 620 = 1,136. Thus, the molar ratio of thionucleotides to Up was 1,270:1,136, nearly 1:1. Since both the thionucleotides were found in the RNase A-resistant fragment (see above), the results established that both of the thionucleotides were associated with Up only. The mobilities of the dinucleotides on DEAE-cellulose and Whatman 3 MM paper were in agreement for a dinucleotide of the type U*pUp or UpU*p. Observations similar to this have been made by other research workers, who encountered a RNase-resistant dinucleotide, mcm^5s^2Up followed by uri-

dine-3′-phosphate, in the course of sequence analysis of tRNAs containing mcm^{5}s^{2}Up at the first position of the anticodon (10, 11, 13, 17). The present results thus suggest the presence of mcm^{5}s^{2}UpUp in the RNase A digest of *C. tropicalis* tRNA. Further, they show that the dinucleotide, m^{5}s^{2}UpUp, is also resistant to RNase A.

Localization of m^{5}s^{2}U in lysine tRNA. As 2-thiouridine derivatives are known to occur in tRNAs for lysine, glutamic acid, and glutamine, it was of interest to know which of the thionucleotide was present in these tRNAs. Fractionation of tRNA was done starting with 100 mg of total tRNA by successive chromatography on DEAE-Sephadex, BD-cellulose, and RPC-5 columns and electrophoresis on a polyacrylamide gel. Lysine tRNA was eluted as a single peak just after the main A_{260} peak both from DEAE-Sephadex and BD-cellulose (data not presented). However, it partially resolved into two peaks on the RPC-5 column (Fig. 6A). ^{35}S-labeled tRNA was similarly fractionated, except that fractionation on BD-cellulose was not done. Upon fractionation on the RPC-5 column, lysine tRNA, as expected, resolved into two peaks (Fig. 6B), as did the nonradioactive tRNA. The radioactivity coincided with the first peak of lysine tRNA, tRNA$_1^{Lys}$. Although ^{35}S label could be detected in the region of the second lysine acceptor peak, the sulfur peak did not coincide with the tRNA$_2^{Lys}$ peak. Hence, only tRNA$_1^{Lys}$ was further analyzed. The latter (nonradioactive as well as ^{35}S-labeled tRNA) was recovered separately by using small DEAE-cellulose columns and subjected to electrophoresis on a polyacrylamide gel (Fig. 7a). The fastest moving band (left lane) accepted lysine when assayed with [^{14}C]lysine. The tRNA in the fastest moving band in the right lane was eluted, digested with RNase T$_2$, and subjected to paper electrophoresis along with authentic radioactive samples of m^{5}s^{2}Up and mcm^{5}s^{2}Up in separate lanes. The paper strips were cut, and the radioactivity in each was determined. Radioactivity in tRNA$_1^{Lys}$ was found to migrate with m^{5}s^{2}Up (Fig. 7b). This established that the purified tRNA$_1^{Lys}$ contained only m^{5}s^{2}Up.

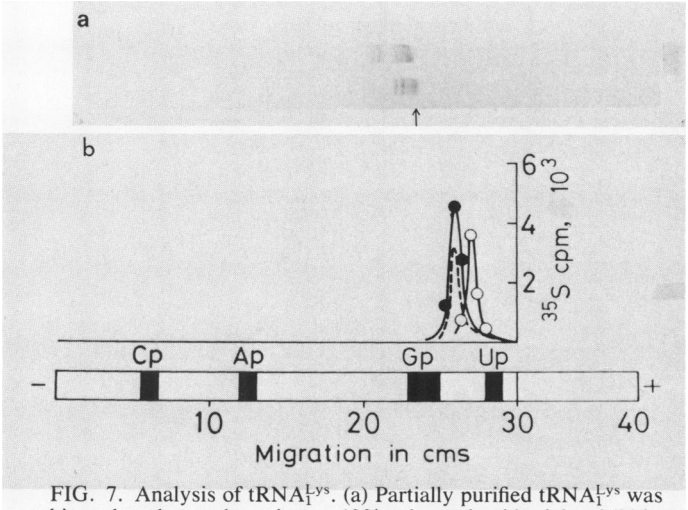

FIG. 7. Analysis of tRNA$_1^{Lys}$. (a) Partially purified tRNA$_1^{Lys}$ was subjected to electrophoresis on a 12% polyacrylamide slab gel (20 by 15 by 0.15 cm) at pH 8.3. The gel was stained with toluidine blue. Left lane, nonradioactive tRNA$_1^{Lys}$; right lane, ^{35}S-labeled tRNA$_1^{Lys}$. (b) The band with lysine acceptance as determined by aminoacylation assay was cut out from the right lane, and the tRNA was extracted, digested with RNase T$_2$, and subjected to electrophoresis on Whatman 3 MM paper along with authentic ^{35}S markers of m^{5}s^{2}Up and mcm^{5}s^{2}Up in separate lanes. The paper in each lane was cut into 0.5-cm pieces, and the radioactivity in each was determined. Symbols: ●, standard ^{35}S-labeled m^{5}s^{2}Up; ○, standard ^{35}S-labeled mcm^{5}s^{2}Up; – – – –, ^{35}S-labeled tRNA$_1^{Lys}$ digested with RNase T$_2$.

DISCUSSION

Presence of m^{5}s^{2}Up in *C. tropicalis* tRNA is interesting, as this thionucleotide has not so far been found in any other yeast tRNA. Although this nucleotide has been shown to be present in rat liver total tRNA (8), it has not been reported in any of the rat liver tRNAs sequenced so far (4, 5). The formation of m^{5}s^{2}Up as the major thionucleotide during pulse-labeling with the ^{35}S isotope and its partial conversion to mcm^{5}s^{2}Up after chasing with cold sulfate indicates that m^{5}s^{2}Up is the precursor of mcm^{5}s^{2}Up but not in tRNA$_1^{Lys}$. Since the mature tRNA from cells grown to log phase contains both the thionucleotides more or less in equal proportions (Fig. 1a), it must be assumed that only a part of m^{5}s^{2}Up is finally converted to mcm^{5}s^{2}Up (Fig. 3). This implies that m^{5}s^{2}Up on certain tRNAs only is further modified to mcm^{5}s^{2}Up. This view is substantiated by the detection of only m^{5}s^{2}Up in the purified lysine tRNA$_1$. The modifying enzyme may recognize certain sequences on a tRNA before it converts m^{5}s^{2}Up to mcm^{5}s^{2}Up. The relative proportion of m^{5}s^{2}Up after chase is less than the expected ca. 50% (see Fig. 3). It may be that during pulse-labeling the rates of thiolation of the tRNA species may be different. The amount of 50% may be reached only after a comparatively longer period.

Resistance of the thionucleotides to digestion with RNase A provided a basis for suggestion that these thio derivatives are localized at the anticodon of *C. tropicalis* tRNA. The nucleotide adjacent to the 5′ end of the anticodon of all tRNAs sequenced so far is a pyrimidine, and the tRNAs for lysine (codons AAA and AAG), glutamic acid (codons GGA and GAG), and glutamine (codons CAA and CAG), which are known to contain 2-thiouridine derivatives, will have the anticodon sequences PyU*UU, PyU*UC, and PyU*UG, respectively (5). Therefore the release of U*pUp or Up*Up upon RNase A digestion of *C. tropicalis* tRNA (Fig. 4) suggests that the thionucleotides are at the anticodon.

RNase A digestion of ^{35}S-labeled tRNA gives only one major product, with mobility similar to that of a dinucleotide on DEAE-cellulose paper (Fig. 4a). On further analysis this product revealed the presence of both m^{5}s^{2}Up and mcm^{5}s^{2}Up, indicating the spot was a mixture of two dinucleotides. Koyabashi et al. (10) have found that the dinucleotide mcm^{5}s^{2}UpUp is very resistant to RNase A digestion. The present study confirms this and further indicates that m^{5}s^{2}UpUp also is resistant to RNase A. However, in a recent report it has been shown that RNase A can cut after mcm^{5}s^{2}Up in *sup6* tRNA from *Saccharomyces cerevisiae* (9). However, in this case the sequence is mcm^{5}s^{2}Upψp and not mcm^{5}s^{2}UpUp. Whether the presence of ψp in the sequence helps RNase A to cleave the diester bond is not known. It is possible that the resistance of these dinucleotides to RNase A is not absolute.

Lysine tRNAs from various sources have been of special interest because of the existence of multiple isoacceptors and their high susceptibility to thionucleotide-specific reagents. The present studies have shown that *C. tropicalis* tRNA contains at least two lysine isoacceptor tRNAs, one of which contains m^{5}s^{2}Up. So far only rat liver tRNA$_2^{Lys}$ has been reported to contain m^{5}s^{2}Up (8). All other eukaryotic lysine tRNAs sequenced so far contain mcm^{5}s^{2}Up (5). Thus

this is the first report of the presence of m^5s^2Up in a yeast tRNA.

ACKNOWLEDGMENT

This research project is supported in part by the Indian Council of Medical Research, New Delhi.

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