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Chemical Modification of Cytosine Residues of $tRNA^{Val}$ with Hydrogen Sulfide (Nucleosides and Nucleotides. XL[1])

KAZUNOBU MIURA,*,[a] TATSUYOSHI IWANO[a], SHIGEKI TSUDA,[a] TOHRU UEDA,[a] FUMIO HARADA,[b] and NOBUYUKI KATO[b]

Faculty of Pharmaceutical Sciences, Hokkaido University,[a] Kita-ku, Sapporo, 060, Japan and Virology Division, National Cancer Center Research Institute,[b] Tsukiji, Chuo-ku, Tokyo, 104, Japan

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The conversion of cytosine residue of transfer ribonucleic acid (tRNA) to 4-thiouracil residues was carried out with a hydrogen sulfide–pyridine–water system. In the case of $tRNA^{Val}$ from mouse cells, the cytosine moieties located in unpaired and looped-out regions of the molecule, such as the anticodon and CCA-end regions, were converted. This result shows that the secondary and tertiary structures of tRNA are maintained even in medium containing liquid hydrogen sulfide and pyridine, and hence, this system should be suitable for studies of the higher-order structure of nucleic acids.

Keywords——Sulfhydrolysis; 4-thiouracil residue; modification of $tRNA^{Val}$; mouse cells-$tRNA^{Val}$; hydrogen sulfide; chemical modification

Since 4-thiouridine was identified as a minor component of transfer ribonucleic acid (tRNA) from *Escherichia coli*,[2] many studies have been carried out to clarify the biological role of this nucleoside moiety. For example, intramolecular linking between the 4-thiouracil residue and a cytosine residue of $tRNA^{Val}$ of *Escherichia coli* by photoirradiation gave information about the relation between the tertiary structure and the function of tRNA.[3–5] Specific modifications of the 4-thiouracil residue with various reagents have also been carried out.[6,7]

In relation to chemical and biophysical studies of 4-thiouracil residue in tRNAs, we have reported an excellent method for the conversion of cytosine nucleosides and nucleotides to their 4-thiouracil counterparts by means of sulfhydrolysis.[8] This method has been successfully applied to the preparation of oligonucleotides containing 4-thiouracil residues from oligonucleotides containing cytosine residues.[9] Introduction of 4-thiouracil residues into polynucleotides, such as yeast RNA, yeast tRNA and heat-denatured calf thymus deoxyribonucleic acid (DNA) has also been achieved.[10] Moreover, the sulfur-isotope labeling of nucleic acids containing 4-thiouracil residues has been performed by treatment of the nucleic acids with cyanogen bromide followed by substitution with ^{35}S-Na_2S.[1] This reaction should be useful not only for the introduction of 4-thiouracil residues but also for elucidation of the secondary and tertiary structures of nucleic acids, provided that the original ordered structure is not significantly altered in the reaction medium, which contains large amounts of liquid hydrogen sulfide and pyridine.

We report here the highly selective sulfhydrolysis of $tRNA^{Val}$ from mouse kidney cells. The results indicate that this reaction system is suitable for studies of higher-order structures of various nucleic aicds.

Materials and Methods

Enzymes and Materials for Electrophoresis——RNases T_1, T_2 and U_2 were obtained from Sankyo Co., Ltd. Nuclease P_1 was obtained from Yamasa Shoyu Co., Ltd. RNase A was purchased from Sigma Chemical Co., Ltd. Cellulose acetate (Separax) was obtained from Fuji Film Co., Ltd. Procedures for electrophoresis and fingerprinting were as described in the previous reports.[11–13]

Preparation and Sequence Analysis of ^{32}P-$tRNA^{Val}$——The ^{32}P-labeled RNAs were prepared by phenol

extraction from mouse kidney cells (C3H2K) cultured in the presence of ^{32}P-phosphate for 24 h as described elsewhere[13] and the ^{32}P-tRNA^{Val} was isolated by two-dimensional gel electrophoresis (Fig. 1, an arrow denotes the spot of tRNA^{Val}). The product was further purified by gel electrophoresis on 16% polyacrylamide gel containing 7 M urea and was identified as tRNA^{Val} by the comparison of the fingerprints of RNases T_1 and A with those reported for tRNA^{Val} from mouse myeloma cells.[14] Further analysis of the sequence in detail was carried out by further digestion of oligonucleotides with appropriate RNases or nuclease followed by separation and identification of the hydrolysis products by two-dimensional cellulose thin layer chromatography (TLC).

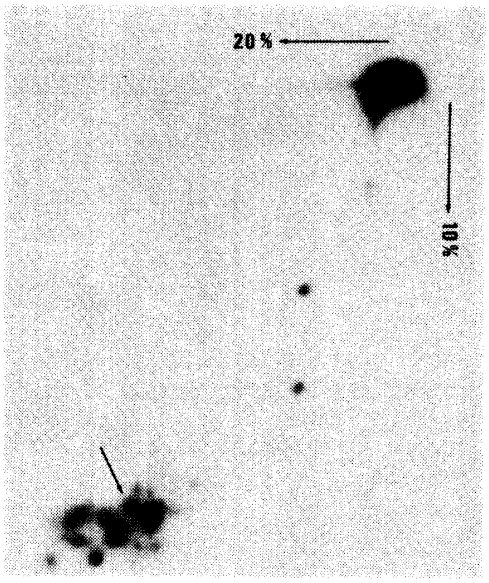

Fig. 1. Two-dimensional Polyacrylamide Gel Electrophoretic Pattern of Cytoplasmic RNAs of Mouse Kidney Cells

The arrow indicates tRNA^{Val}.

Sulfhydrolysis of tRNA^{Val}——^{32}P-tRNA^{Val} (about 500000 cpm, containing 50 μg of yeast tRNA as a carrier) was dissolved in 1 ml of 20 mM HEPES-KOH buffer (pH 7.2)–0.15 M NaCl–10 mM $MgCl_2$ in a stainless steel container. The container was cooled to −70°C and a mixture of liquefied H_2S (4 ml) and pyridine (2 ml) was added at −70°C. The container was sealed and kept at 28 or 35°C for an appropriate time, then opened. The H_2S was evaporated off, and 4 M NaCl was added to the residual mixture to make 0.8 M NaCl solution, then 2.5 vol of ethanol was added and the suspension was kept overnight at −20°C. The resulting precipitate was collected by centrifugation and dissolved in water, and the precipitation-centrifugation cycle was repeated. The recovery of tRNA was 80—90%.

Determination of the Modification Sites——The modification sites in H_2S-treated tRNA^{Val} were determined by comparison of the fingerprints of RNases T_1 and A digests of untreated and treated tRNA^{Val} and by analyses of newly appeared oligonucleotides formed by hydrolysis of H_2S-treated tRNA^{Val} with RNases.

Results

The Nucleotide Sequence of tRNA^{Val} from Mouse Kidney Cells

The RNases A and T_1 fingerprints of tRNA^{Val} from mouse kidney cells are shown in Figs. 2a and 3a. The fingerprints were almost identical with those reported by Piper for tRNA^{Val} from mouse myeloma cells.[14] Further analysis of oligonucleotides on the fingerprints showed that the isolated tRNA was a mixture of two species differing at the wobble position of the anticodon, CAC and IAC, in an approximate ratio of 6 : 4. Although it was reported[14] that the tRNA^{Val} from myeloma cells contained only IAC as the anticodon, the RNase T_1 fragment containing CAC anticodon (t-20, C–C–U–C–A–C–A–C–Gp) was also detected at the appropriate position on the fingerprint of Piper.[14] The other partial methylation and partial exchange of the bases at positions 4 (Um), 32 (Cm), 38 (m^5C), and 43 (G) have been observed previously.[14] It should be noted that the methylation at positions 32 and 38 was observed only in the species having IAC anticodon. The total sequence of tRNA^{Val} from kidney cells was deduced from these results and is shown in Fig. 4.

Since the two species migrate very closely and could not be separated completely even by repeated gel electrophoresis, the mixture was used for the next step.

Sulfhydrolysis of tRNA^{Val}

First, the sulfhydrolysis of ^{32}P-tRNA^{Val} was performed at 28°C for 72 h. The presence of 4-thiouracil residues in H_2S-treated tRNA was confirmed by nuclease P_1 digestion followed by two-dimensional cellulose thin layer chromatography (Fig). 5, pS=4-thiouridylate). A fingerprint of the RNase A digest of H_2S-treated tRNA^{Val} is shown in Fig. 2b. Three new spots, As-1 to -3, were observed. As-1 was identified as 4-thiouridylate by direct comparison of the mobility with that of authentic 4-thiouridylate on the fingerprint. On the fingerprint

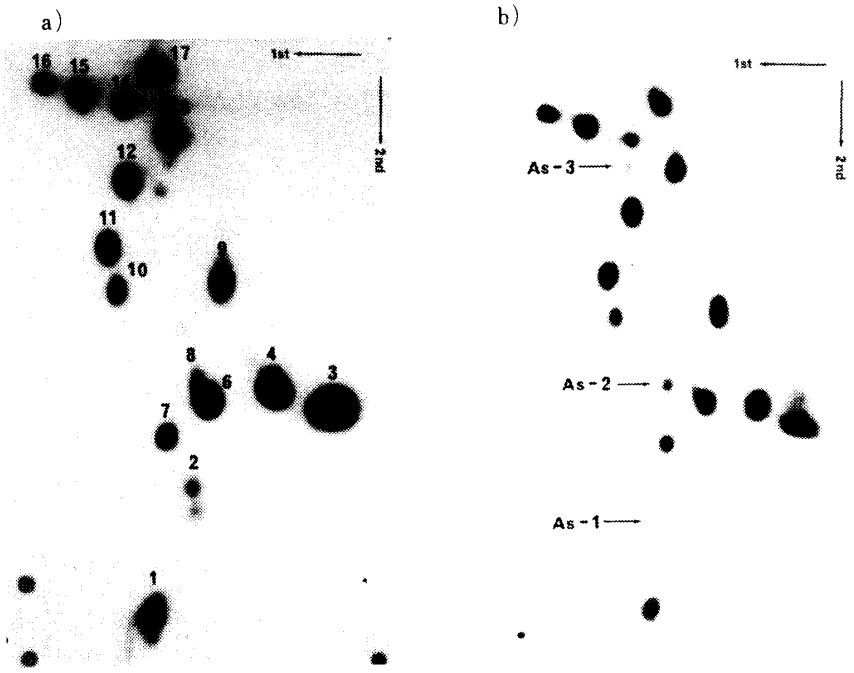

Fig. 2. RNase A Fingerprints of Untreated (a) and H_2S-treated (35°C) (b) $tRNA^{Val}$

The sequences of oligonucleotides are summarized in Table II.

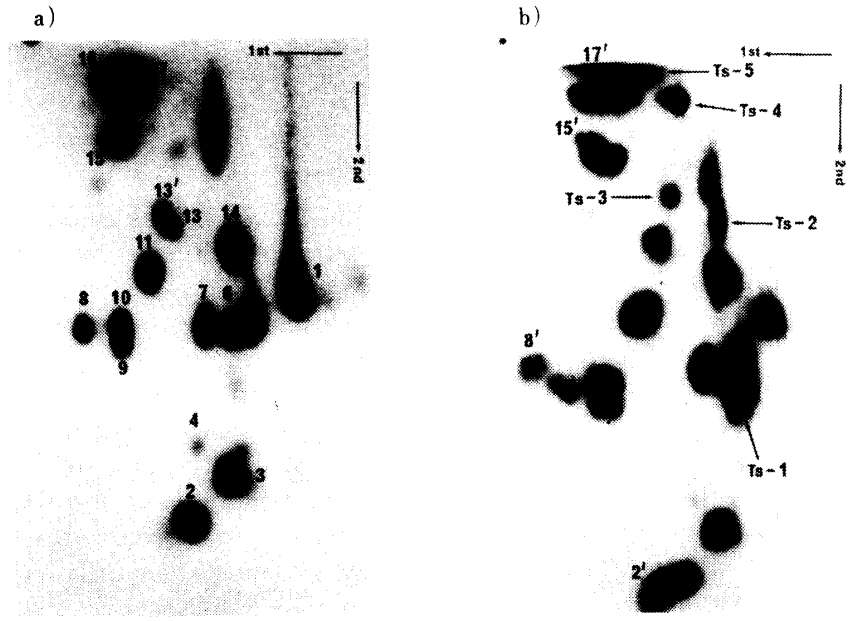

Fig. 3. RNase T_1 Fingerprints of Untreated (a) and H_2S-treated (35°C) (b) $tRNA^{Val}$

The sequences of oligonucleotides are summarized in Table III.

of the RNase T_1 digest, four new spots of oligonucleotides (Ts-1, -3, -4, and -5) were observed.

When the temperature of sulfhydrolysis was raised to 35°C an additional spot (Ts-2) was observed on the RNase T_1 fingerprint (Fig. 3b).

Sequence Analysis of Oligonucleotides containing 4-Thiouracil Residues

Oligonucleotides produced by RNase A Digestion——Spot As-1 was identified as 4-thiouri-

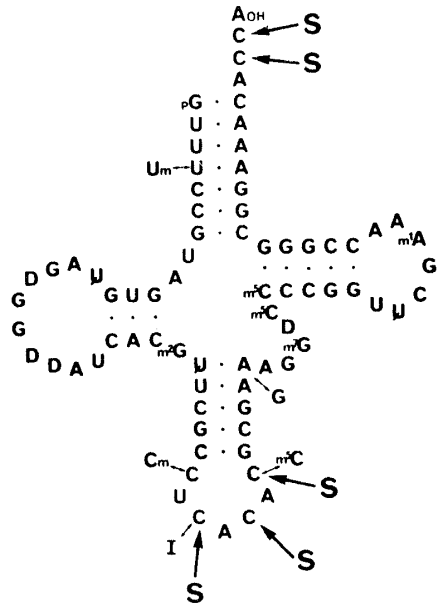

Fig. 4. Cloverleaf Structure of $tRNA^{Val}$ and the Sites of Sulfhydrolysis

A capital 'S' with an arrow indicates each site of sulfhydrolysis.

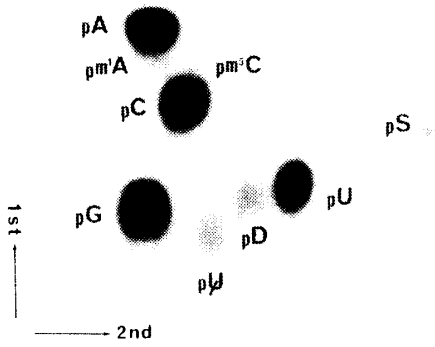

Fig. 5. Two-dimensional Cellulose Thin Layer Chromatographic Pattern of Nuclease P_1 Digest of H_2S-treated $tRNA^{Val}$

dylate (Sp) as described above. However, after extraction of the nucleotide from DEAE-cellulose paper after the second eletcrophoresis, most of As-1 behaved as uridylate rather than Sp on a two-dimensional cellulose thin layer chromatogram. This is due to acid hydrolysis of the 4-thiono function to the 4-keto function in Sp to give Up during the work-up after the second electrophoresis in 7% formic acid on DEAE-cellulose paper. Similarly, all other oligonucleotides containing 4-thiouridylate were analyzed as the uridylate counterpart on the final cellulose thin layer chromatogram.

As-2 was identified as A–Sp from the migration position of As-2 on the fingerprint, which was identical with that of an authentic A–Sp. The extract from the fingerprint migrated as A–Up on the two-dimensional cellulose thin layer chromatogram. Further digestion of this oligonucleotide with RNase T_2 gave Ap and Up in a ratio of 1 : 1 (data not shown).

As-3 was digested with RNase T_2 to give Ap, Up and Ip in an equimolar ratio (data not shown). Since Up is derived from Sp, the original nucleotide should be I–A–Sp or A–I–Sp. As I–A–Cp was the only possible digestion product of $tRNA^{Val}$ with RNase A, the sequence of As-3 was assigned as I–A–Sp.

Oligonucleotides produced by RNase T_1 Digestion——The sequencing of the five newly appeared spots (Ts-1 to -5) was carried out by further enzymatic digestions and characterization of the digests. The results are shown in Table I.

Ts-1 was found to be derived from the 3′-terminal nucleotide, since RNase T_2 digestion did not produce guanylate and, in addition, nuclease P_1 digestion released neither guanylate nor inorganic phosphate. From the RNase A digests two sequences, A–A–A–C–A–S–C–A_{OH} and A–A–A–C–A–C–S–A_{OH}, were deduced which are formed by the sulfhydrolysis of either cytosine moiety of the CCA end. Ts-2 was also found to be derived from the 3′-terminal nucleotide and determined as A–A–A–C–A–S–S–A_{OH}, since the RNase A digestion gave Up, A–Up and A–A–A–Cp. As described before Ts-2 appeared only when the sulfhydrolysis was carried out at higher temperature (35°C). Although the nucleotide composition analyzed by RNase T_2 digestion of Ts-2 is inconsistent with this structure, there may be problems in the digestion procedure, such as incomplete digestion or incomplete conversion of Sp to Up due to disulfide

formation from Sp. Similar inconsistencies have been encountered in other cases.

Oligonucleotide Ts-3 was assigned as A–S–A–C(m^5C)–Gp or A–C–A–S–Gp derived from A–C–A–C(m^5C)–Gp (t-6) from the results of RNases A and T_2 digestions. Although C-38 was partially replaced with m^5C, its corresponding 4-thio derivative was not detected in the RNase T_2 digest. It is reasonable to expect that the displacement of the 4-amino function of m^5Cp should be retarded by the presence of the 5-methyl group as compared to that of Cp.

Ts-4 was identified as a mixture of 3 species of monothiated t-20. Limited digestion of Ts-4 with RNase U_2 gave C–C–U–C–A>p and C–C–U–U–A>p, C–A>p and U–A>p, and C–Gp and U–Gp which means that cytosine at three positions was modified. The nucleotide composition of Ts-4 is consistent with this assumption. The sequence of Ts-5 was determined to be as shown in Table I, and one cytosine residue of Ts-4 is further modified. RNase U_2 digestion gave the same oligonucleotide fragments as in the case of Ts-4. Although the nucleotide compositions as determined by RNase T_2 digestion were not satisfactory, the direction of migration of the spots of t-20→Ts-4→Ts-5 on the fingerprint is reasonable if the exchange of C to S has occurred.

It is assumed that the first consecutive sequence of cytidylate in t-20 is unmodified, since the sequence of C–C–(Cm)–U–Ip (t-13, the RNase T_1 digest of other species having IAC anticodon) was found to be unmodified.

All nucleotide fragments produced by RNases A and T_1 digestions of $tRNA^{Val}$ and H_2S-treated $tRNA^{Val}$ are summarized in Table II and III.

The extent of conversion of cytosines at the anticodon region (positions 34, 36 and 38) was estimated as 30—35% and that of cytosines at the 3′-terminal was 50% at 35°C. The

Table I. Nucleotide Compositions determined by RNase T_2 Digestion, and Products of Further Digestion of RNase T_1 Fragments from H_2S-treated $tRNA^{Val}$

Spot No.	Products of further digestion with					Deduced sequence
	RNase T_2	Composition[a]	Nuclease P_1	RNase A	RNase U_2	
Ts-1	Ap	3.9(4)	pA	Cp		
	Cp	2.3(2)	pC	Up		A-A-A-C-A-S-C-A_{OH}
	Up	0.8(1)	pU	A-Cp		
				A-Up		A-A-A-C-A-C-S-A_{OH}
				A-A-A-Cp		
Ts-2	Ap	3.5(4)	pA	Up		
	Cp	2.1(1)	pC	A-Up		A-A-A-C-A-S-S-A_{OH}
	Up	1.3(2)	pU	A-A-A-Cp		
Ts-3	Ap	2.0(2)		Gp		A-S-A-C(m^5C)-Gp
	Cp+m^5Cp	1.3(1)		A-Cp(A-m^5Cp)		
	Up	0.8(1)		A-Up		A-C-A-S-Gp
	Gp	0.9(1)				
Ts-4	Ap	2.1(2)	pA	Cp	C-A>p	
	Cp	4.4(4)	pC	Up	U-A>p	C-C-U-S-A-C-A-C-Gp
	Up	1.4(2)	pU	Gp	C-Gp	
	Gp	1.0(1)	pG	A-Cp	U-Gp	C-C-U-C-A-S-A-C-Gp
			Pi	A-Up	C-C-U-C-A>p	C-C-U-C-A-C-A-S-Gp
					C-C-U-U-A>p	
Ts-5	Ap	1.9(2)	pA	Cp	C-A>p	
	Cp	3.7(3)	pC	Up	U-A>p	C-C-U-S-A-S-A-C-Gp
	Up	2.4(3)	pU	Gp	C-Gp	
	Gp	1.0(1)	pG	A-Cp	U-Gp	C-C-U-S-A-C-A-S-Gp
			Pi	A-Up	C-C-U-C-A>p	C-C-U-C-A-S-A-S-Gp
					C-C-U-U-A>p	

a) The numbers in parenthesis show the molar ratios expected for the deduced sequence.

Table II. Oligonucleotides produced by RNase A Digestion of H_2S-treated (35°C) and Untreated $tRNA^{Val}$

Spot No.	Sequence	Molar ratio			
		H_2S-treated		Untreated	
		Experim.	Theor.	Experim.	Theor.
a-1	Up+Dp+Ψp	4.0	4—6	5.2	4—6
a-1′	U>p+D>p+Ψ>p	1.1		0.7	
a-2	Cm-Up+Um-Cp	0.4	0—2	0.3	0—2
a-2′	Cm-U>p+Um-C>p	0.2		0.2	
a-3	Cp+m⁵Cp	9.5	10—13	12.7	10—13
As-1	Sp	1.2		—	
a-4	A-Cp+A-m⁵Cp	2.6	3—4	4.1	3—4
As-2	A-Sp	0.8		—	
a-6	G-Cp	1.8	2	2.0	2
a-7	A-Up	1.3	1	0.9	1
a-8	I-A-Cp	0.3	0—1	0.4	0—1
As-3	I-A-Sp	0.15		—	
a-9	G-m¹A-A-A-Cp	1.1	1	0.9	1
a-10	m²G-Ψp	1.1	1	1.1	1
a-11	G-Up+G-Ψp	2.4	1+1	2.2	1+1
a-12	A-G-Up+A-G-Dp	2.6	1+1	2.0	1+1
a-13	G-G-A-A-A-Cp	0.8	1	0.9	1
a-14	G-G-G-Cp	0.4	1	0.5	1
a-15	G-G-Up+G-G-Dp	2.3	1+1	2.1	1+1
a-16	pG-Up	1.1	1	1.0	1
a-17	G-A-A-A-G-m⁷G-Dp	1.1	1	0.9	1

Table III. Oligonucleotides produced by RNase T_1 Digestion of H_2S-treated (35°C) and Untreated $tRNA^{Val}$

Spot No.	Sequence	Molar ratio			
		H_2S-treated		Untreated	
		Experim.	Theor.	Experim.	Theor.
t-1	A-A-A-C-A-C-C-A_{OH}	0.4		1.2	
Ts-1	A-A-A-C-A-S-C-A_{OH}+A-A-A-C-A-C-S-A_{OH}	0.6	1	—	1
Ts-2	A-A-A-C-A-S-S-A_{OH}	0.2		—	
t-2	Gp	2.8	5	5.0	5
t-2′	G>p	2.3		0.3	
t-3	C-Gp	2.0	2	2.4	2
t-4	A-Gp	0.1	0—1	0.1	0—1
t-5	m¹A-A-A-C-C-Gp	0.8	1	0.8	1
t-6	A-C-A-C(m⁵C)-Gp	—	0—1	0.4	0—1
Ts-3	A-S-A-C(m⁵C)-Gp+A-C-A-S-Gp	0.2		—	
t-7	A-A-A-Gp	1.0	0—1	0.6	0—1
t-8	pGp	0.6	1	0.9	1
t-8′	pG>p	0.5		—	
t-9, 10	D-Gp+U-Gp	1.8	1+1	2.1	1+1
t-11	U-A-Gp+Ψ-A-Gp	2.1	1+1	1.8	1+1
t-13, 13′	C-C(Cm)-U-Ip+C-C(Cm)-U-I>p	0.3	0—1	0.4	0—1
t-14	m⁷G-D-m⁵C-m⁵C-C-C-Gp	0.9	1	0.9	1
t-15	U-Ψ-C-Gp+Ψ-U-C-Gp	1.8	1+1	2.1	1+1
t-15′	U-Ψ-C-G>p+Ψ-U-C-G>p	0.1		—	
t-16	U-U-U(Um)-C-C-Gp	1.0	1	1.0	1
t-17, 17′	D-D-A-U-C-A-C-m²Gp+D-D-A-U-C-A-C-m²G>p	0.8	1	1.0	1
t-20	C-C-U-C-A-C-A-C-Gp	0.2		0.6	
Ts-4	C-C-U-S-A-C-A-C-Gp / C-C-U-S-A-S-A-C-Gp+C-C-U-C-A-C-A-S-Gp	0.2	0—1	—	0—1
Ts-5	C-C-U-S-A-S-A-C-Gp / C-C-U-S-A-C-A-S-Gp+C-C-U-C-A-S-A-S-Gp	0.2		—	

sites of sulfhydrolysis of $tRNA^{Val}$ are shown in Fig. 4, where S→denotes the conversion to 4-thiouridine.

Discussion

We have developed an excellent method for the introduction of 4-thiouracil residues into RNA and DNA by means of conversion of cytosine residues with liquid hydrogen sulfide.[10] In this paper the effect of this system on the higher-order structure of RNA was studied. $tRNA^{Val}$ was selected as the substrate for sulfhydrolysis, as cytosine residues are present in the anticodon region and the tertiary structure of the tRNA is well elucidated.

The $tRNA^{Val}$ was purified from mouse kidney cells and was found to be a mixture of species containing CAC and IAC as the anticodon. A similar result regarding the anticodon of mammalian $tRNA^{Val}$ was found in the species from human placenta.[15]

The sulfhydrolysis was carried out at 28°C and 35°C to see whether any change in the mode of modification occurred. In a previous study[10] the sulfhydrolysis of unfractionated tRNA was carried out at 40°C for 12 h. The conversion of about 3 cytosine residues, on average, was observed.

Analysis of the site of modification was performed by preparing fingerprints with RNases and by further sequence determination of the separated oligonucleotides. The sequences of new spots of RNase A digest of H_2S-treated $tRNA^{Val}$ (As-1, -2 and -3) were determined as Sp, A–Sp and I–A–Sp, respectively (Fig. 2, Table II). Oligonucleotides from the RNase T_1 digest (Ts-1 to -5) were identified as modified 3′-terminal oligonucleotides (Ts-1 and -2) and three oligonucleotides were derived from the anticodon regions of the species of $tRNA^{Val}$ containing CAC and IAC as the anticodon (Ts-3, -4 and -5, Fig. 3, Table III).

From the results of sulfhydrolysis of $tRNA^{Val}$, the following conclusions can be reached.

1) The cytosine moieties at the anticodon loop and the CCA end were converted to appreciable extents. No other cytosine moiety, including one in the common GTΨC region, was modified.

2) This means that the secondary and tertiary structures of tRNA are maintained in the reaction medium composed of liquid H_2S, pyridine and H_2O in a ratio of 4: 2: 1 by volume. The sulfhydrolysis of $tRNA^{Pro}$ from mouse cells,[16] which has no cytosine moiety in the anticodon loop, has also been performed and in this case no change of cytosines other than those of the CCA end was observed (data not shown).

As reagents for the modification of cytosine residues of tRNAs, aqueous methoxyamine[17] and aqueous sodium bisulfite[18] are known. The characteristic feature of the sulfhydrolysis may be that the 4-thiouracil residue thus introduced can be further converted to other pyrimidine moieties and can undergo various other transformations, such as photodynamic coupling.[3–5]

In conclusion, the present system for the sulfhydrolysis of the cytosine moiety can be useful for studies of the higher-order structures of various RNAs and possibly DNAs. Modification studies of ribosomal 5S and 5.8S RNA from mouse cells are currently being undertaken and the results will be reported separately.[19]

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