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Chemical modification of cytosine residues of U_6 snRNA with hydrogen sulfide (Nucleosides and nucleotides. Part 49 [1])

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ABSTRACT

Sulfhydrolysis of cytosine residues to 4-thiouracil residues in mouse U_6 snRNA was carried out to examine the secondary structure of U_6 snRNA. The cytosine residues at positions 6, 42 and 68 were modified significantly, and at positions 11, 19 (or/and 25), 61 and 66 in moderate extent. Based on the result, the plausible secondary structure of U_6 snRNA is discussed.

INTRODUCTION

Recently, the structures of several small nuclear RNAs (snRNA) have been elucidated and a role of these snRNAs in the splicing of RNA precursors has been postulated. SnRNAs contain the sequences which are complementary to the junction sequences of the intron of RNA precursors to form the splicing complex between these terminal regions of the intron sequences of RNA precursor and snRNAs (2-4). In relation to these postulations, it becomes important to elucidate the higher order structure of snRNA.

We have described that sulfhydrolysis is an excellent method for study of higher order structure of RNAs. Sulfhydrolysis of $tRNA^{Val}$ and 5S ribosomal RNA resulted in the modification of the cytosine residues to 4-thiouracil residues located in the exposed sites (5,6). Therefore, this chemical approach for an examination of secondary structure of other RNAs is useful.

In this report, we discuss the secondary structure of U_6 snRNA by means of the chemical modification of the cytosine residues with hydrogen sulfide. The modification of the cytosine residues to the 4-thiouracil residues occurred at C_6, C_{11}, C_{19} or/and 25, C_{42}, C_{61}, C_{66} and C_{68} as will be described.

MATERIALS AND METHODS

Enzymes and reagents used in this study were obtained as described previously (6). Procedures for polyacrylamide gel electrophoresis, two-dimensional electrophoresis for preparing fingerprint and cellulose thin layer chromatography were also as described (7-10).

Preparation of ^{32}P-labeled U_6 snRNA

^{32}P-Labeled snRNA was prepared from mouse kidney cell (C3H2K) cultured in the presence of ^{32}P-phosphate and the U_6 snRNA was isolated by two-dimensional polyacrylamide gel electrophoresis as described previously (11).

Sulfhydrolysis of ^{32}P-snRNA

To a solution of ^{32}P-U_6 snRNA (about 100,000 cpm) containing 50 ug of yeast tRNA as a carrier in 1 ml of 20 mM HEPES-KOH (pH 7.2)-0.15 M NaCl-10 mM $MgCl_2$ was added 4 ml of liquid hydrogen sulfide and 2 ml of pyridine in a stainless steel container at -70°C. The container was sealed and kept at 28°C or 35°C for an appropriate time (-5days). Then the reaction mixture was worked up as described previously (6). The recovery of ^{32}P-snRNA was about 80%.

Determination of the modification sites

The modificaiton sites in H_2S-treated U_6 snRNA were determined by comparison of the respective fingerprints of the digests with RNases A and T_1 of untreated and treated U_6 snRNAs and analysis of oligonucleotides that appeared in the RNase hydrolysates of H_2S-treated U_6 snRNA.

RESULTS

Sulfhydrolysis was carried out at 28°C or 35°C and the sites of the modification were determined by preparation of the fingerprints and subsequent analysis of oligonucleotides. On digestion with RNase A of H_2S-treated U_6 snRNA, four new spots, As-1 to As-4 were obtained (Fig. 1a). RNase T_1 digestion gave six new spots, Ts-1 to Ts-6 (Fig. 1b).

As-1, 2 and 3 were identified as 4-thiouridylate (Sp), ApSp and GpSp, respectively, by direct comparison with authentic samples on two-dimensional electrophoresis as described previously (6). However, after extraction of spots As-1, 2 and 3 from

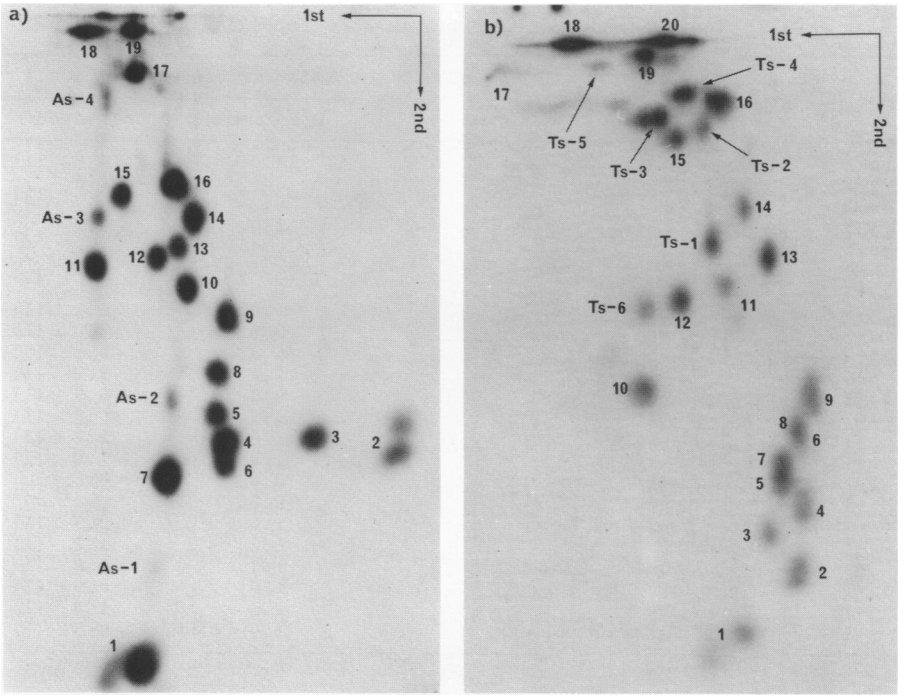

Fig. 1. Fingerprints of U_6 snRNA treated with H_2S at 35°C for 116 hours.
a) RNase A digest, b) RNase T_1 digest. The nucleotides sequences of oligonucleotides are summarized in Table I and II.

DEAE-cellulose paper, they were detected as Up, ApUp and GpUp, the 4-thio group being hydrolyzed during extraction after second electrophoretic run in 7% formic acid, as described in previous paper (6). This was the case with all oligonucleotides containing Sp.

As-4 was digested with RNase T_2 to give Gp and CmpUp in a ratio of 2:1. Therefore, As-4 was determined as GpGpCmpSp.

The sequences of Ts-1 to Ts-6 were determined by further digestion with RNases. Ts-1 was digested by RNase T_2 to give Up, Ap and AmpGp in an equimolar ratio, which showed that Ts-1 was SpApAmpGp. Ts-2 was digested with RNase T_2 to give Gp, CmpUp and CmpCmpUp. Therefore, the sequence of Ts-2 was determined as CmpSpCmpCmpUpGp. RNase A digestion of Ts-3 gave Cp, Up and Gp in

Table I. Oligonucleotides produced by RNase A digestion of U_6 snRNAs. Sulfhydrolysis was carried out at 35°C for 116 hours.

Spot No.	Sequence	Molar ratio (theoretical)	
		H_2S-treated	Untreated
a-1	Up + Ψp	14.5	14.8 (11)
a-2	Cp	3.6	4.8 (5)
As-1	Sp	0.3	-
a-3	ApCp	1.4	3.3 (3)
As-2	ApSp	0.4	-
a-4	GpCp	3.3	5.0 (5)
As-3	GpSp	0.5	-
a-5	AmpGmpCp	1.0	1.1 (1)
a-6	CmpCmpUp	0.9	1.0 (1)
a-7	ApUp	6.3	5.5 (5)
a-8	ApGpCp	1.1	1.2 (1)
a-9	GpApCmpApCp	0.8	0.9 (1)
a-10	ApApApUp	1.1	0.9 (1)
a-11	GpUp	2.6	2.2 (2)
a-12	GpGpCp	1.0	1.0 (1)
a-13	GpGpCmpCp	0.6	0.8 (1)
As-4	GpGpCmpSp	0.2	-
a-14	ApApApApΨp	1.0	0.8 (1)
a-15	GpApΨp	1.2	1.2 (1)
a-16	GpGpApApCp + GpApApGpCp	1.9	1.6 (1+1)
a-17	ApAmpGpm^{2}GpApUp	1.0	0.9 (1)
a-18	XpppGpUp	1.2	1.2 (1)
a-19	m^{6}ApGpApGpAmpApGpApUp	1.0	0.8 (1)

the ratio of 1:2:1, and nuclease P_1 digestion gave pU, pG and inorganic phosphate (Pi) in the ratio of 2:1:1. Therefore, the 5'-end nucleotide was determined as Cp and the possible sequence of Ts-3 was either CpUpSpGp or CpSpUpGp. Since only CpUpCpGp was a possible original oligonucleotide to give Ts-3, the Ts-3 could be CpUpSpGp. The RNase T_2 digestion of Ts-4 gave Ψp and m^{6}Ap in addition to Ap, Up and Gp which showed that Ts-4 was ApΨpApSpm^{6}A-pGp and therefore, original oligonucleotide was ApΨpApCpm^{6}ApGp. This was further confirmed by RNase A digestion of Ts-4 which gave ApΨp, ApUp and m^{6}ApGp in an equimolar ratio. Ts-5 was digested to Cp, Up and Gp in a ratio of 1:3:1. Since at least one molar of Up was derived from Cp via Sp, an original oligonucleotide was deduced as CpUpUpCpGp. Nuclease P_1 digestion of Ts-5 gave pU, pG and Pi in a ratio of 3:1:1. Therefore, Ts-5 was identified as CpUpUpSpGp. Ts-6 was determined as SpGp by RNase A digestion giving Up and Gp in an equimolar ratio.

Thus, the oligonucleotides containing Sp derived by sulfhydrolysis were identified (Table I and II). From these results,

Table II. Oligonucleotides produced by RNase T_1 digestion of U_6 snRNAs. Sulfhydrolysis was carried out at 35°C for 116 hours.

Spot No.	Sequence	Molar ratio (theoretical)		
		H_2S-treated	Untreated	
t-1	Gp + m^2Gp	4.2	5.1	(4)
t-2	CpGp	1.7	3.2	(2)
Ts-6	SpGp	0.6	-	
t-3	ApGp	1.2	1.1	(1)
t-4	CpApGp	1.0	1.0	(1)
t-5	AmpApGp	1.0	0.9	(1)
t-6	CpApAmpGp	0.2	0.9	(1)
Ts-1	SpApAmpGp	0.6	-	
t-7	ApApGp	1.0	1.0	(1)
t-8	ApApCpGp	0.7	0.6	(1)
t-9	ApCmpApCpGp	0.6	0.8	(1)
t-10	UpGp	2.0	2.0	(2)
t-11	CpUpCpGp	0.1	1.1	(1)
Ts-3	CpUpSpGp	0.7	-	
t-12	ApUpGp	1.1	1.2	(1)
t-13	CmpCpCmpCmpUpGp	0.6	1.1	(1)
Ts-2	CmpSpCmpCmpUpGp	0.3	-	
t-14	ApØpApCpm^6ApGp	0.4	0.9	(1)
Ts-4	ApØpApSpm^6ApGp	0.8	-	
t-15	CpUpUpCpGp	0.7	1.1	(1)
Ts-5	CpUpUpSpGp	0.2	-	
t-16	CpApApApUpØpCpGp	0.7	1.0	(1)
t-17	XpppGp	1.0	1.4	(1)
t-18	UpUpCpCpApUpApUpUpUpUpU	1.5	1.4	(1)
t-19	ApUpUpAmpGmpCpApUpGp	0.9	0.9	(1)
t-20	CpApCpApUpApU(Ø)pApCpUpApApApApØpUpGp CpApSpApUpApU(Ø)pApCpUpApApApApØpUpGp CpApCpApUpApU(Ø)pApSpUpApApApApØpUpGp CpApSpApUpApU(Ø)pApSpUpApApApApØuUpGp	1.0	0.6	(1)

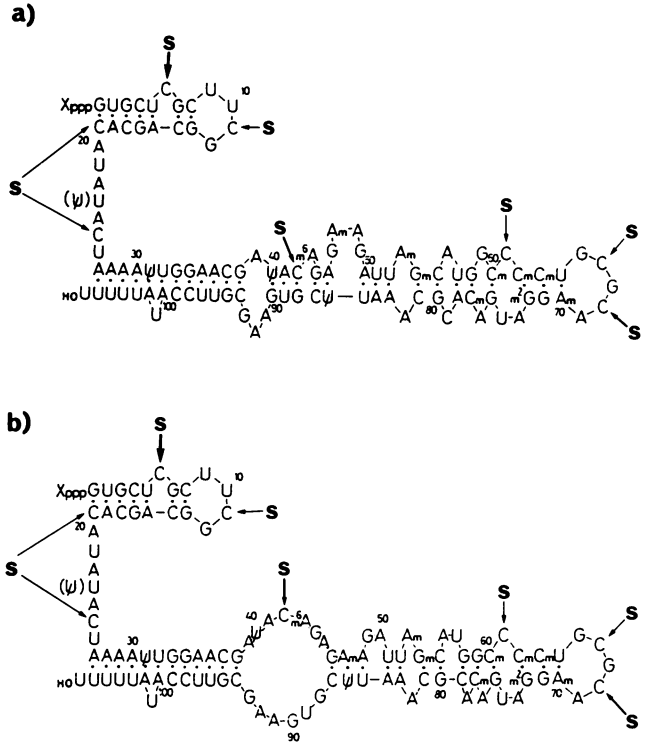

Fig. 2. The secondary structures of U_6 snRNA drawn by Harada et al. (a) and by Epstein et al.(b) (11,12). Modification sites are shown by a capital S with an arrow. A thick arrow indicates a cytosine residue to be more susceptible.

it was determined that C_6, C_{11}, C_{42}, C_{61}, C_{66} and C_{68} were modified to 4-thiouracil residues.

In the RNase T_1 digestion products of U_6 snRNA, there are two long oligonucleotides such as UpUpCpCpApUpApUpUpUpUpU (t-18) and CpApCpApUpApU(Ψ)pApCpUpApApApApΨpUpGp (t-20). Their migrating mobility at the second run on two-dimensional electrophoresis were very slow (Fig. 1b). Accordingly, if cytosine residues in oligonucleotides t-18 and t-20 were modified to 4-thiouracil residues, separation of these long oligonucleotides containing Sp from t-18 and t-20 would not be possible. In order to examine whether spots 18 and 20 contained respective oligonucleotides containing Sp, H_2S-treated U_6 snRNA was digested with RNase T_1 and the digest was subjected to the 20% polyacrylamide gel

Table III. Rate of conversion of cytosine residues

Reaction condition		Rate of conversion (%)						
°C	Hours	C_6	C_{11}	C_{19} or/and 25	C_{42}	C_{61}	C_{66}	C_{68}
28	72	69	9	-	35	15	19	41
35	116	84	21	57	68	34	50	81

electrophoresis. The bands corresponding to t-18 and t-20 were extracted and analyzed by RNases digestion and subsequent electrophoresis or thin layer chromatography. Oligonucleotides t-18 was digested with RNase A to give Cp, Up and ApUp but not Sp. Therefore, the cytosine residue involved in t-18 was not modified. RNase A digestion of t-20 gave ApSp in addition to Cp, Up, Gp, ApCp, ApUp and ApApApApØp. Therefore, the cytosine residues at the positions 19 or/and 25 were partially modified to 4-thiouracil residues.

The modification sites on U_6 snRNA by sulfhydrolysis determined as above are indicated with capital "S" with an arrow in Fig. 2 and the modification rates at each sites are summarized in Table III.

DISCUSSION

Sulfhydrolysis of U_6 snRNA was carried out at different temperatures and the modification sites were determined by preparation of RNases A and T_1 fingerprints and subsequent analysis of newly appeared oligonucleotides containing Sp. RNase T_1 digestion of H_2S-treated U_6 snRNA produced seven oligonucleotides containing Sp. Analysis of such oligonucleotides showed that C_6, C_{11}, C_{19} or/and 25, C_{42}, C_{61}, C_{66} and C_{68} were modified. Analysis of H_2S-treated U_6 snRNA by RNase A digestion also supported above result. Result of the rate of modification showed that the cytosine residues at the positions 6, 42 and 68 were found to be more exposed among seven (or/and eight) susceptible cytosine residues, and the order of reactivity was unchanged by the temperature raise from 28°C to 35°C (Table III). This showed

that the higher order structural change was not significant by elevating temperature from 28°C to 35°C.

Based on the present result two secondary structure models of U_6 snRNA proposed by Harada et al. (11) and by Epstein et al. (12), were examined. Susceptibility of the cytosine residues except for C_{42} can be explained by both secondary structures in which the cytosine residues are located in the single-strand region, loop region and bulged structure (Fig. 2). Between two proposed secondary structures there is a slight difference around the sequence of G_{38} to G_{46} and C_{87} to C_{94} (Fig. 2) and the cytosine residue at the position 42 is involved in the stem region in the case of one model (11) and in the loop region in another model (12). The high susceptibility of C_{42} for sulfhydrolysis can be explained readily by the latter model. Thus, the model proposed by Epstein et al. seems to be more preferable with the results obtained by chemical modification of the cytosine residues with hydrogen sulfide.

On the other hand, the specific sequence (G_{55}-A_{76}, Fig. 2) complementary to the splicing junction of RNA precursor (11, 12) is involved in the relatively stable hairpin structure enriched with G:C pairs.

In addition, a significant difference of the extent of modification was found between C_{66} and C_{68} (Table III) although these are involved in the same loop. This would suggest that, in addition to the complex tertiary structure formation, these secondary structures might change in the course of interaction with RNA precursors.

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