SYNTHESIS BY RIBONUCLEASE A OF CODONS CONTAINING MODIFIED NUCLEOSIDES IN THE 'WOBBLE' POSITION

H.G. GASSEN

Arbeitsgruppe Biochemie, Max-Planck-Institut für experimentelle Medizin, 34 Göttingen, Germany

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1. Introduction

In the course of studies of codon–anticodon recognition with model compounds, it was necessary to synthesize trinucleoside diphosphates containing certain structural analogues of the natural bases at different positions in the codon. The first attempts to synthesize these components by chemical means were unsuccessful because of the instability of the glucosyl linkage of the modified nucleosides to the alkaline and acidic conditions used in the chemical synthesis [1]. Therefore, we turned to the ribonuclease-catalysed synthesis of oligonucleotides. Compared to other reports [2, 3] we used a 1000-fold higher concentration of pancreatic ribonuclease and found that these high concentrations catalyse the synthesis of oligonucleotides effectively. The somewhat lower yields obtained by this method compared to chemical synthesis are offset by the ease of preparation and the high degree of purity of the products obtained. We report here the millimole-scale synthesis by ribonuclease A of U–N and U–U–N, where N can be uridine (I), 4-deoxyuridine (II), 3-deazauridine (III) and 3-deaza-4-deoxyuridine (IV)*.

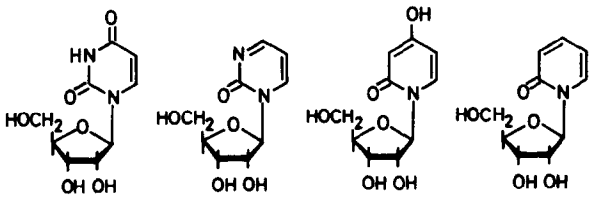

Fig. 1. Formulas I–IV.

2. Materials and methods

2.1. *Chemicals*

Ribonuclease A (15331 ERAA) was purchased from Boehringer, Germany. All other chemicals were of the purest grade available. The modified nucleosides were synthesized according to the method of Niedballa et al. [4].

2.2. *General procedure for the ribonuclease-A-catalyzed synthesis of oligonucleotides*

A solution containing 1 mmole U > p, 3 mmoles nucleoside and 2 ml 0.1 M Na_2HPO_4 pH 7.0** in a 25 ml flask was chilled to 0°. After the addition of 50 mg of RNase A the flask was connected to a rotary evaporator [3]. A vacuum of 0.1 torr was applied, and the flask was kept in an ice-water bath with a trap cooled to −20°. In 4 hr, the solution was slowly concentrated to a highly viscous fluid. The re-

* These compounds are symbolized, in harmony with the Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (European J. Biochem. 15 (1970) 203), as follows:

(II) 1-β-D-Ribofuranosyl-2-pyrimidone (4-deoxyuridine): h^4U

(III) 1-β-Ribofuranosyl-4-hydroxy-2-pyridone (3-deazauridine): c^3U

(IV) 1-β-D-Ribofuranosyl-2-pyridone (3-deaza-4-deoxyuridine): c^3h^4U

** In the case of 3-deazauridine as acceptor the pH of the reaction mixture is lowered to pH 5.0.

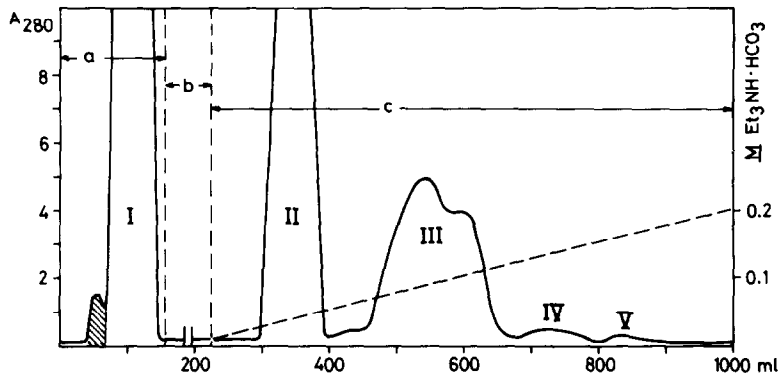

Fig. 2. Separation of the reaction products of 1 mmole U>p, 3 mmole U and 50 mg RNase on a DEAE-cellulose DE 32 ($HCOO^-$) column (15 cm × 2 cm). Flow rate: 1 ml min^{-1}. a) 20 mM $HCOONH_4$ pH 2.5 b) H_2O wash c) gradient 1000 × 1000 ml, 20 mM to 500 mM $(Et)_3NH_2CO_3$ pH 7.5. The shaded area shows the evolution volume of the ribonuclease.

action was stopped by the addition of 10 ml 1 M HCOOH and the mixture was adsorbed on a DEAE-cellulose column and eluted with a linear gradient of triethylammonium bicarbonate [$(Et)_3NH.HCO_3$]. For details, see fig. 2. The peaks were pooled and desalted by repeated evaporation from 50% methanol. The fractions were further purified by paper electrophoresis in 0.1 M $(Et)_3NH.HCO_3$ pH 7.5 on Schleicher and Schüll no. 2216 (2000 V, 60 min). Table 1 shows the results obtained with uridine as a nucleoside.

2.3. *Analysis of the oligonucleotides*

Oligonucleotides were hydrolyzed with 0.3 M NaOH or appropriate enzymes and the products obtained were analyzed by the method of Uziel, Koh and Cohn [5]. Details are published elsewhere [6]. As an example, the results of the analysis of U–U–c^3U are listed in table 2.

3. Results

The synthesis of oligonucleotides with modified

Table 1

Oligonucleotides obtained by RNase-catalyzed synthesis. The fifth column lists the μmoles obtained after separation by paper electrophoresis.

Peak	A_{260}-units	Component	A_{260}-units	μmoles	% donor
I	27,000	U	27,000	2,700	
II	7,700	U>p	2,400	240	24
		U–U	3,600	180	18
		U–p	2,750	275	27
III	3,400	U–U–U	740	25	5.0
		U–U>p	620	30	6.0
		U-U-U>p	54	2	0.6
IV	86	U–U–U–U	24		
V	100	U–U–Up + higher			

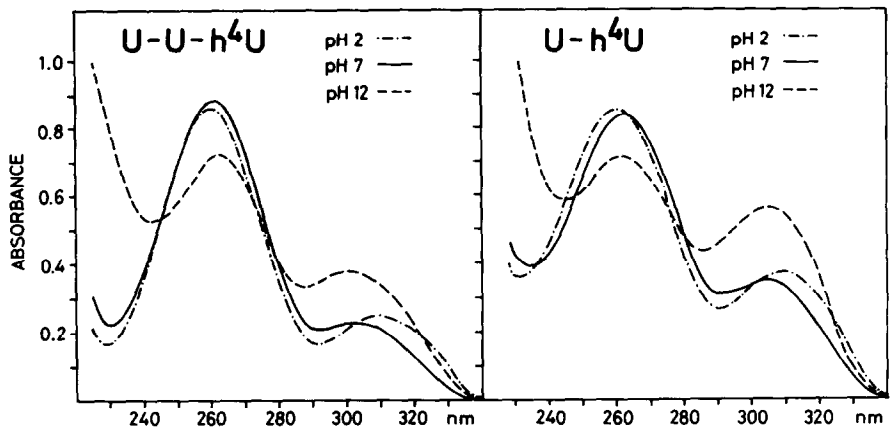

Fig. 3. The UV-spectra of U–h^{4}U and U–U–h^{4}U at different pH-values. The spectra were recorded with a Cary model 14 spectrophotometer.

Table 2
Analysis of 20.0 nmoles U–U–c^{3}U. SVD = snake venom phosphodiesterase (EC 4.1.4.1). BAC = alkaline phosphatase (EC 3.1.3.1).

Method of hydrolysis	Nucleosides obtained (nmoles)	
	U	c^{3}U
(1) NaOH	–	19.0
(2) RNase A	1–2	19.8
(3) SVD	16.5	–
(4) RNase + BAC	41.0	19.5
(5) SVD + BAC	38.5	19.0

nucleosides as acceptors gave results similar compared to those obtained with uridine (table 1). The yields obtained for the di-, tri- and tetranucleotides with analogues of uridine in the 3′-position are listed in table 3. Starting with 1 mmole of donor and using a threefold excess of acceptor, about 100 μmoles of dinucleoside phosphate and 20 μmoles of triplet may be obtained. The oligonucleotides are pure after two separation steps. They are completely hydrolyzed pancreatic RNase; thus translocation of the phosphodiester bond is less than 3%. Table 4 shows the spectral properties of the oligonucleotides; UV-spectra of U–h^{4}U and U–U–h^{4}U are shown in fig. 3.

4. Discussion

With ribonuclease as a catalyst we are able to synthesize triplets containing analogues of uridine in the 'wobble' position. The dinucleoside phosphate is used as a primer for the polynucleotide phosphorylase-catalysed synthesis of the triplets U–N–U containing the analogue in the middle position [7]. In the mmole-scale synthesis the use of RNase concentrations comparable to those reported in the literature [2, 3] gave very poor yields of the dinucleoside phosphate and no

Table 3
Yields obtained in the synthesis of oligonucleotides with modified nucleosides in the 3′-position. Amounts of starting materials and the reaction conditions were the same as described in 2.3.

Nucleoside used as acceptor (N)	U–N		U_2–N		$(U)_3$–N	
	A_{260} units	μmoles	A_{260} units	μmoles	A_{260} units	μmoles
4-Deoxyuridine	1180	103	375	18	135	4
3-Deazauridine	2230	135	124	14.8	–	–
3-Deaza-4-deoxyuridine	1480	110	487	20	230	15

Table 4
Spectral characteristics of the newly synthesized oligonucleotides, at pH values 2, 7 and 12.

Substance	$A_{250}:A_{260}$	$A_{280}:A_{260}$	$A_{290}:A_{260}$	λmax_1	λmax_2
pH					
U–c^3	0.77	0.64	0.34	263	–
U–h^4U	0.80	0.47	0.32	261	310
U–c^3h^4U	0.72	0.66	0.46	264	300
U–U–c^3U	0.74	0.56	0.25	262	–
U–U–h^4U	0.79	0.41	0.20	260	310
U–U–c^3h^4U	0.78	0.51	0.30	262	300
pH 7					
U–c^3U	0.86	0.50	0.21	260	–
U–h^4U	0.77	0.54	0.38	263	305
U–c^3h^4U	0.75	0.63	0.46	264	300
U–U–c^3U	0.81	0.46	0.17	260	–
U–U–h^4U	0.78	0.46	0.25	261	303
U–U–c^3h^4U	0.81	0.50	0.30	261	300
pH 12					
U–c^3U	0.93	0.53	0.33	259	–
U–h^4U	0.82	0.71	0.73	263	305
U–c^3h^4U	0.79	0.74	0.81	265	300
U–U–c^3U	0.89	0.45	0.20	260	–
U–U–h^4U	0.82	0.58	0.48	262	303
U–U–c^3h^4U	0.84	0.68	0.65	263	300

trinucleoside diphosphate. However, upon examination of the reaction at increasing RNase concentrations, we found that 50 mg (40 U/mg) RNase per mmole of donor yielded the optimal amount of both dinucleoside phosphate and trinucleoside diphosphate. Higher concentrations neither improved the yields nor increased the hydrolysis of the cyclic uridylic acid to 3′-uridylic acid. The enzyme was eluted as the first peak from the DEAE-column (fig. 1) and could be recovered separately. It was, however, more convenient to lyophilize the mixture of the enzyme and the nucleoside and to use this mixture for a further preparation. The crucial step in using the reverse of the normal reaction of RNase A is to stop the hydrolytic action of the enzyme and thus prevent degradation of the products formed. Initially we used diethylpyrocarbonate to inactive the RNase [8], but abandoned its use later on when we discovered that it reacts at higher concentrations with a number of nucleosides, especially adenosine. Therefore, we stopped enzyme action by lowering the pH of the reaction mixture to pH 2.5. Although this low pH favors the migration of the phosphodiester bond, the oligonucleotides contained less than 3% of 2′–5′ isomers.

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References

[1] T. Ukita, R. Funakoshi and Y. Hirose, Chem. Pharm. Bull. 12 (1964) 828.
[2] M.R. Bernfield, J. Biol. Chem. 241 (1966) 2014.

[3] S.C. Mohr and R.E. Thach, J. Biol. Chem. 244 (1970) 6566.
[4] U. Niedballla and H. Vorbrüggen, Angew. Chemie 82 (1970) 449.
[5] M. Uziel, Ch. Koh and W.E. Cohn, Anal. Biochem. 25 (1968) 77.
[6] H.G. Gassen and W. Leifer, Z. Anal. Chemie 252 (1970) 337.
[7] H. Schetters, H.G. Gassen and H. Matthaei, manuscript in preparation.
[8] I. Fedorcsák, A.T. Natarajan and L. Ehrenberg, European J. Biochem. 10 (1969) 450.