DETECTION AND ISOLATION OF $(Gp)_nXp$ SEQUENCES FROM POLYRIBONUCLEOTIDES BY MEANS OF RESTRICTED RIBONUCLEASE T_2 HYDROLYSIS

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1. Introduction

It is known that poly A sequences may be isolated from polyribonucleotides after digestion of polymer with a mixture of pyrimidyl and guanyl ribonucleases. No such method exists for isolation of poly G sequences which may also play an essential role in structure and function of natural polynucleotides. Kethoxalation is known to be a selective procedure for modification of guanine bases [1] in nucleic acids. This change in G structure may produce a restriction in splitting of phosphodiester bond between Gp and 3′ prime neighbour irrespectively of the chemical nature of the latter.

In this work we examined the possibility of producing resistance against ribonuclease T_2 hydrolysis with natural and synthetic polyribonucleotides. On the basis of the data obtained a procedure is suggested for isolation of the $(Gp)_nXp$ sequences from any polyribonucleotides.

2. Experimental

2.1. *Materials*

RNA from tobacco mosaic virus (TMV) was kindly provided by Dr. V.K. Novikov (Moscow State University), 16 S ribosomal RNA from *E.coli* was a kind gift of Dr. V.A. Spiridonova (Moscow State University). Poly (U, G) was synthesized by polynucleotide phosphorylase in the Prof. M. Grunberg-Manago laboratory and kindly sent to us.

Ribonucleases T_1 and T_2 (Sabkyo Ltd.) were obtained through the courtesy of Prof. S. Nishimura and Prof. H. Zachau. Alkaline phosphatase from *E.coli* was presented as a gift to us by Dr. R.I. Tatarskaya (Institute of Molecular Biology). [^{12}C]- and [^{14}C]-kethoxal (β-ethoxy-α-kethobutyraldehyde) was synthesized as described previously [12]. In [^{14}C]kethoxal synthesis a $^{14}C_2H_5OH$ was used with specific activity 1.0 Ci/mol.

FND cellulose (Filtrak) was used for thin-layer chromatography, Aminex UA-8 and DEAE-cellulose, 0.47 meqV/gr was purchased from Reanal, DEAE-Sephadex A-25 was from Pharmacia.

2.2. *Measurements*

Absorption at 254 nm was recorded with Uvicord 4701A densitometer (LKB), UV spectra were recorded with Specord UV-Vis (Carl Zeiss Jena). Radioactivity in fractions was measured after taking aliquots into dioxan scintillating fluid and counting them in a Intertechnique spectrometer SL-30.

2.3. *Reactions of polynucleotides with [14]kethoxal*

Twenty A_{260} units of polynucleotide, 20 μmoles of sodium cacodilate buffer pH 7.0, 5 μmoles of EDTA, 140 μmoles of [14]kethoxal were incubated 4 hr at 37°C in the total volume of 1 ml. The reaction was terminated by cooling and adding 0.1 ml of 2 M KCH_3COO and 2.5 ml of ethanol. The precipitation was repeated three times and the residue after the last precipitation was dried in vacuum dissicator under P_2O_5.

2.4. *Hydrolyses*

2.4.1. Ribonuclease T_2

One A_{260} of polynucleotide was dissolved in 0.05 ml of ribonuclease T_2 solution pH 4.5 containing 1 unit of enzyme activity and kept 2 hr at 37°C. The in-

cubation mixture was enlarged proportionally if the quantity of the hydrolysable material has increased.

2.4.2. Phosphatase

To 0.05 ml of incubation mixture (2.4.1) 5 μl of 0.1 M $Na_2B_4O_7$ pH 8.0 and the amount of phosphatase sufficient for complete removal of phosphate from 1 A_{260} unit of nucleotide were added and the mixture was kept 2 hr at 37°C.

2.4.3. Ribonuclease T_1 + phosphatase

0.5 A_{260} unit of oligonucleotide was added to 5 enzyme activity units of ribonuclease T_1 and phosphatase (2.4.2) in a total volume of 20 μl, pH was adjusted to 8–8.5 with 0.5 M NH_4OH and the mixture was incubated 2 hr with 37°C.

2.5. *Chromatography*

2.5.1. Separation of ribonuclease T_2, phosphatase hydrolysates were made on the Chromex UA-8 microcolumns as described [3].

2.5.2. Separation of oligonucleotides

Mixture of U_p, ApGp, ApG^*_p, GpGpCp, and $G^*_pG^*_pCp$ (0.7 A_{260} unit of each) was applied on the DEAE–Sephadex microcolumn (volume 130 μl) equilibrated with 0.02 Tris–borate buffer, pH 7.5 in 7 M urea. 0.04 M → 0.4 M NaCl gradient was used; total volume of the effluent 6.2 ml, flow rate o.2 ml/hr. Analytical chromatography of the ribonuclease T_2 hydrolysates of the kethoxalated polynucleotides was performed by the same way. For preparative purposes 20–30 A_{260} units of hydrolysate after ribonuclease T_2 action were applied on the DEAE–Sephadex column (volume 1.2 ml) equilibrated and eluted as described for analytical procedure. Total volume of the gradient 90 ml, flow rate 1.6 ml/hr, fraction volume 1.6 ml.

2.5.3. Desalting of oligonucleotides

Fractions containing separated oligonucleotides (2.5.1) were diluted 5 times with water and passed through a DEAE-cellulose (CO_3^{2-}) column (volume 0.1–0.5 ml). The column was washed with 0.01 M NH_4HCO_3 until complete disappearance of Cl^- ions and nucleotide material was eluted with 0.6 M NH_4HCO_3. The eluate was evaporated several times until complete removal of bicarbonate was achieved.

2.5.3. Thin-layer chromatography

Solvent system A: isobutyric acid: 0.5 M NH_1OH (10:6 v/v), pH 3.7. Solvent B: *n*-butanol, saturated with water.

2.6. *Removal of modification*

For complete removal of kethoxal oligonucleotide solutions were kept in 0.1 M NH_4OH during 24 hr at 20°C followed by evaporation to dryness.

3. Results

Exhaustive hydrolysis of TMV RNA by ribonuclease T_2 followed by phosphatase treatment causes a complete splitting of the polymer chain and the single products obtained are nucleosides (fig. 1A). The chromatographic profile changes profoundly if RNA has been subjected to kethoxalation before hydrolysis (fig. 1B). Guanosine disappears completely and that means complete modification of RNA leaving no unmodified G. The area of A,U and C diminishes significantly and two new peaks appear, a big one proceeding the U and corresponding to ~40% of the total material applied on the column, and small one of modified G^*. This pattern (fig. 1B) is consistent with the appearence of a non-hydrolysable fraction after kethoxalation of RNA.

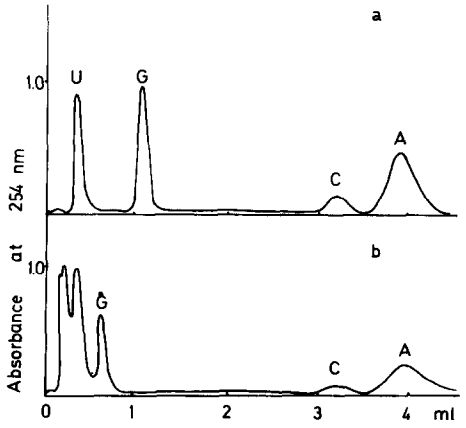

Fig. 1. Chromex UA-8 chromatography of ribonuclease T_2 and phosphatase hydrolyzates of the non-modified (a) and modified (b) TMV RNA.

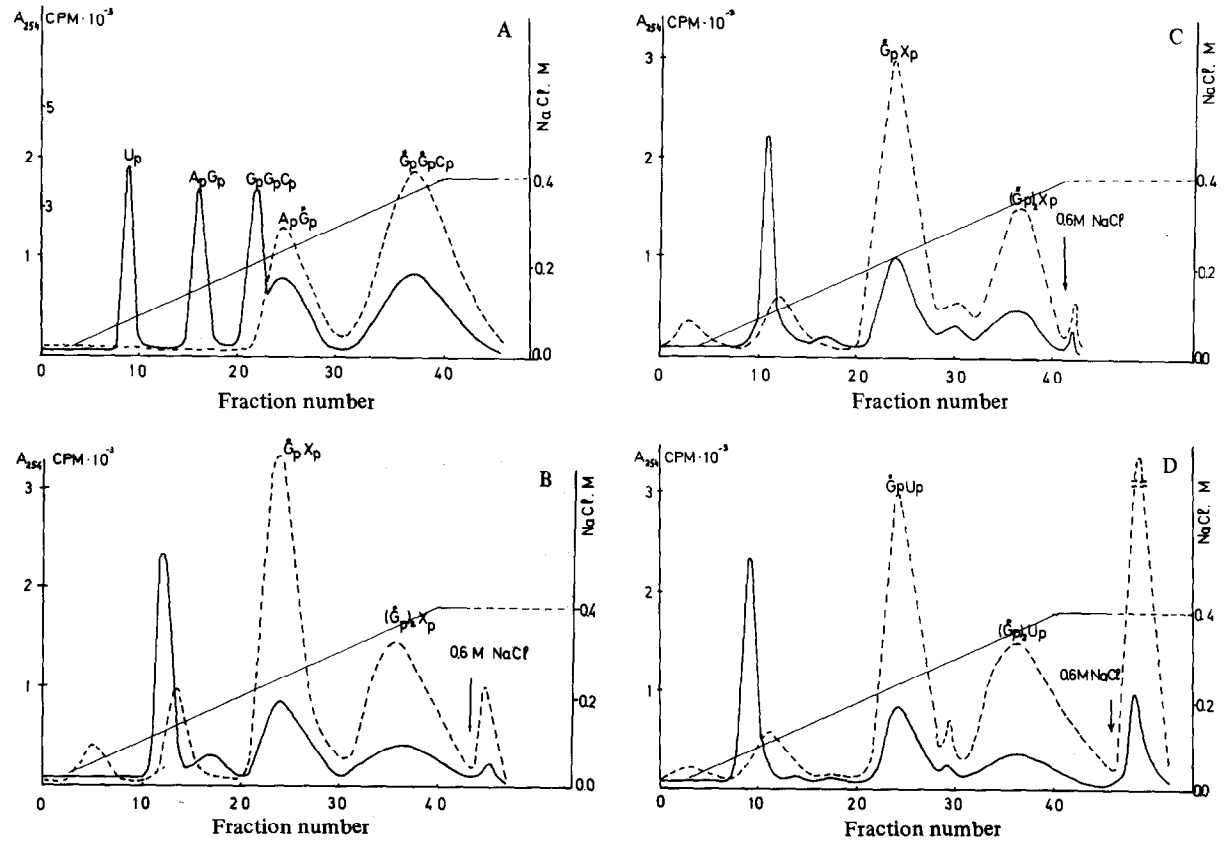

Fig. 2. DEAE-Sephadex chromatography in 7 M urea of the oligonucleotides. A, model mixture of mono- di- and tri-nucleotides B,C and D, ribonuclease T_2 hydrolysate of the kethoxalated TMV RNA, *E.coli* 16S ribosomal RNA and poly (U,G), respectively. Absorbance ——; radioactivity – – –.

For oligonecleotide analysis of the products resistant to ribonuclease T_2 hydrolysis, DEAE-Sephadex column chromatography in the presence of urea was used which is known to separate oligonucleotides under these conditions according to charge [4]. Fractions of the model mixture is shown in fig. 2A. The shift of the modified oligonucleotides relatively to the non-modified ones is accounted for two factors: 1) in the presence of BO_3^- ions a complex is formed between *cys*-glycol groups of the adduct and the borate ion followed by appearance of new negative charges of the oligonucleotide the number of which is equal to the total number of G^* in the given oligonucleotide [5]; 2) increase in the hydrophobicity of the G^* as compared with the nonmodified G increases the affinity of the modified oligonucleotides to the DEAE-Sephadex.

Fractionation of the ribonuclease T_2 hydrolysate of the kethoxalated TMV RNA is shown in fig. 2B. Peak 1 contains no UV absorbing material and represents traces of the free kethoxal, peak 2 occupies on the profile the position that corresponds to the $G\overset{*}{p}$. Since the radioactivity of this peak is less than 10% of the total in all cases (fig. 2,B,C,D) one may conclude that contribution of the non-specific splitting of $(G\overset{*}{p})_nXp$ sequences to the general pattern is insignificant. Peak 3 (~60% of the total radioactivity) corresponds on the elution profile to $G\overset{*}{p}Xp$ and peak 5 to $(G\overset{*}{p})_2Xp$. Peak 6 eluted with 0.6 M NaCl is a oligonucleotide mixture with general formula $(G\overset{*}{p})_nXp$ where $n \geqslant 3$. Peak 4 which is very small both in UV and in radioactivity was not identified.

A similar but not identical picture was obtained with 16 S ribisomal RNA (fig. 2C). Differences may be seen in relative ratios of the peaks. For example, the proportion of the relatively long oligoguanylic

Table 1
Composition of the oligonucleotides isolated from the kethoxalated polyribonucleotides after ribonuclease T_2 hydrolysis.

Polymer	TLC chromatography in solvent A after desalting and removal of kethoxal	Analysis of individual spots obtained in solvent A
		Hydrolysis with ribonuclease T_1 + phosphatase followed by TLC chromatography is solvent B. Nucleotide material from individual spots was eluted and UV spectra recorded
TMV RNA	GpXp – 3 spots	GpUp(G/U = 1/1); GpAp(G/A = 1:1); GpCp(G/C = 0.9:1)
	$(Gp)_2Xp$ – 2 spots	$(Gp)_2Ap$(G/A = 2.1/1); $(Gp)_2Op$(G/U = 2/1)
16 S RNA	$(Gp)_2Xp$ – 3 spots	$(Gp)_2Ap$(G/A = 2:1); $(Gp)_2Up$(G/O = 2:1); $(Gp)_2Cp$(G/C = 2:1)
Poly (U,G)	GpXp – 1 spot	GpUp(G/U = 1:1)
	$(Gp)_2Xp$ – 1 spot	$(Gp)_2Up$(G/U = 1.8/1)

sequences is much higher in 16 S RNA than in viral RNA (cf. peaks 6 of fig. 2B and C).

Poly (U,G) after kethoxalation and hydrolysis has qualitatively similar distribution of the peaks although the proportion of the longer sequences is much higher as expected.

In summary one may conclude that the pattern of oligonucleotide distribution obtained is in agreement with that one may expect if to assume a resistance of $(G\overset{*}{p})_nXp$ sequences to ribonuclease T_2 hydrolysis. To prove further this conclusion composition of the peaks isolated by column chromatography was analyzed with specific ribonuclease T_1 hydrolysis, followed by TLC and UV spectroscopy (table 1).

Practically no GpGpCp was revealed in the $(Gp)_2$ Xp fraction isolated from TMV RNA. This is not the case for 16 S ribosomal RNA from *E.coli*. Therefore differences in primary structure of these two polymeric RNA's are evident even at the level of trinucleotide patterns.

4. Discussion

The observations described open new possibilities in investigation of primary structures of polyribonucleotides.

A comparative study of various RNAs may be performed based on relative proportions of $(Gp)_nXp$ isopliths and composition of each $(Gp)_nXp$ fraction. Set of codons for glycine (GpGpAp, GpGpCp, and GpGpUp) may be obtained without difficulties (cf. fig. 2C, peak 5). Polyguanylic sequences may be revealed and isolated with its 3′ neighbours if necessary.

Studies of primary sequences of ribonucleic acids are based on complete hydrolyses with pyrimidyl and guanyl ribonucleases and partial hydrolysis is used for overlapping fragments. However, this latter procedure is very time-consuming and needs careful choice of appropriate experimental conditions. Since 3′ neighbours of kethoxalated G bases are not splitted with ribonuclease T_2 correct arrangements of the fragments obtained both with pyrimidyl and guanyl ribonucleases are greatly facilitated. We suppose that in many cases incomplete hydrolysis with ribonucleases may be substituted with complete hydrolysis of kethoxalated substrate with ribonuclease T_2. Determination of the number of G in oligo G stretches is also simple with the proposed method.

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