Isolation and analysis of $(Gp)_nXp$ sequences of rat liver 5S RNA by means of restricted ribonuclease T_2 hydrolysis

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ABSTRACT

Essential difficulties arise when base number in oligoguanylic blocks and location of these blocks along the polynucleotide chain need to be determined in the course of determination of the nucleotide sequences in ribonucleic acids. To overcome this difficulty it is suggested to take advantage of a recently discovered resistance of phosphodiester bond between kethoxalated G and its 3'-neighbour against T_2 RNase hydrolysis^{1,2}. The approach is illustrated by analysis of 5S RNA from rat liver. Sequences of general formula $(Gp)_nXp$ were isolated from T_2 RNase hydrolysate of 5S RNA rapidly and quantitatively. The information obtained greatly facilitates the whole procedure of sequencing. It is expected that the method proposed would be effective for analysis of 5S and 4S RNA and for highmolecular weight fragments of ribosomal and viral RNAs.

INTRODUCTION

As well known great progress has been achieved during last years in deciphering the primary structures of ribonucleic acids. However inspite of this rapid development certain objective difficulties still remain. Among them probably one of the most serious is connected with the oligoguanylic stretches: their location in the polynucleotide and the number of monomers. It is remarkable that mistakes appear frequently in sequencing oligonucleotides containing guanylic blocks (for example, cf.³ and⁴).

This situation is reasonably to expect since after T_1 RNase hydrolysis oligoG converts to monomers and structural information is lost. On the other hand after pyrimidyl RNase hydrolysis the resistant purine blocks containing oligoG are still inconvenient for sequencing.

Earlier two of us (T.A. and L.K.) have shown^{1,2} that after reaction of guanine bases in oligo- and polynucleotides with kethoxal the phosphodiester bond between kethoxalated Gp and the

3'-neighbouring nucleotide becomes resistant to T_2 RNase hydrolysis. On the basis of this observation it was proposed¹ to use the phenomenon described for structural investigation of nucleic acids.

The aim of the present work was to illustrate the fruitfulness of this approach taken as an example the structural study of rat liver 5 S RNA with unknown primary structure. In particular we have isolated, separated and analyzed all oligonucleotides of general formula $(Gp)_nXp$ (where $X = U, C$ or A) containing in rat liver 5 S RNA.

MATERIALS AND METHODS.

Isolation of 5 S RNA from rat liver was already described.⁵ Ribonucleases T_1 and T_2 were from Sankyo (Japan). Phosphomonoesterase from *E. coli* came from Worthington (USA). Reaction of 5 S RNA with kethoxal was done as described earlier². 20 A_{260} units of 5 S RNA were dissolved in 1 ml of 0.02 M sodium cacodylate buffer (pH 7.0) containing 5 mM EDTA and 0.14 M kethoxal. After 4 hr incubation at 37°C 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 desiccator under P_{2O_5} .

20 A_{260} units of kethoxalated RNA was dissolved in 0.2 ml of 0.01 M acetate buffer (pH 4.5) containing 20 units of T_2 RNase activity and kept 2 hr at 37°C. After evaporation of the solvent the residue was dissolved in 1 ml of 2 mM $CuSO_4$ to inhibit the T_2 RNase⁶. The solution was passed slowly through a CM-cellulose column (volume 0.4 ml) for complete removal of the RNase. The hydrolysate obtained was subject for demodification reaction: pH of the mixture was adjusted to 10.2 with concentrated ammonia and kept during 24 hr at 20°C.

After removal of kethoxal the hydrolysate was diluted 5 times with 0.01 M tris-Cl (pH 7.5) + 7 M urea and applied on a DEAE-Sephadex A-25 column (0.4 x 120 cm). Chromatography was done in the same buffer with NaCl gradient from 0 to 0.25 M (200 + 200 ml), flow rate 14 ml/hr. Appearance of fractions was monitored at 254 nm with Uvicord II densitometer (LKB). Each fraction was passed through small DEAE-Sephadex column washed

with H_2O and $0.01 \text{ M NH}_4\text{HCO}_3$ and then eluted with $1 \text{ M NH}_4\text{HCO}_3$. The eluates were evaporated several times until complete removal of bicarbonate was achieved.

The residue was dissolved in 0.02 ml tris-Cl ($\text{pH } 8.0$), 0.01 M MgCl_2 , T_1 RNase was added (1 unit of enzyme activity per 1 A_{260}) and phosphomonoesterase sufficient for complete dephosphorylation. After 6 hr at 37°C the hydrolysate was directly applied on the Chromex UA-8 column.

Nucleoside separation on the Chromex UA-8 (Reanal) column based on the ion-exclusion chromatography was made as described for Aminex ⁷. Chromex column ($0.3 \times 35 \text{ cm}$) was covered with thin layer of BioGel P-2. $10\text{--}50 \mu\text{l}$ aliquotes of nucleosides were applied, flow rate 4 ml/hr . The resin was equilibrated and elution performed in the same solution of $0.1 \text{ M NH}_4\text{CH}_3\text{COO}$ ($\text{pH } 9.4$). After elution each fraction was acidified to $\text{pH } 1$ with conc. HCl and the amount of nucleoside material was determined spectrophotometrically.

RESULTS AND DISCUSSION

Long non-modified oligoribonucleotides are resolved on chromatographic columns slightly better than kethoxalated ones ² probably due to non-specific interaction with the column material of the latter. Therefore it was decided to convert the kethoxalated oligonucleotides derived from 5 S RNA after kethoxalation and T_2 RNase hydrolysis into a non-modified form. Separation of the oligonucleotide mixture is shown in Fig. 1. The first peak which appears before application of the gradient corresponds to CuSO_4 used to inhibit the RNase hydrolysis. It was identified visually due to its blue colour. The next peaks are mono, di, tri and tetranucleotides, respectively. When the rest of material was eluted from the column with 1 M NaCl only traces ($3\text{--}4\%$) of the nucleotide material appear. This fraction was analyzed further and all four nucleosides have been detected, however, in amounts much less than 1 mol per mol of 5 S RNA. Since the relative amount of this fraction was very small it was not taken into account in further analysis. One may assume that the 1 M NaCl fraction is attributed to an incomplete hydrolysis of the kethoxalated RNA.

Fig. 1. Chromatography of $(Gp)_nXp$ oligonucleotides from rat liver 5 S RNA according to chain length
DEAE-Sephadex A-25 column (0.4 x 120 cm),
0.01 M tris·Cl (pH 7.6), 7 M urea
 $(Gp)_nXp$

Fig. 2. Separation of nucleosides on the Chromex UA-8 column
(0.4 x 30 cm) at pH 9.4
The sample was applied in 20-50 μ l of 0.01 M tris·Cl
(pH 8.0), 0.05 M $MgCl_2$. Flow rate 3.5 ml/hr

Table 1

Distribution of $(Gp)_nXp$ sequences in T_2 RNase
hydrolysate of rat liver kethoxalated 5 S RNA

$(Gp)_nXp$	n=0	n=1	n=2	n=3
A_{260} units	10	4.3	2.3	2.7
Calculated number of nucleotides per peak*	61	27.4	14.3	16.8
Expected number of oligomers per peak	61	13-14	5	4
The number of corresponding oligomers in HeLa 5 S RNA**	58	14	5	5

* Assuming chain length for rat liver 5 S RNA to be 120 nucleotides. **Calculated from known sequence for HeLa 5S RNA

Table 2

Expected quantities of individual $(Gp)_nXp$ oligonucleotides
in rat liver 5 S RNA as compared with
5 S RNA from HeLa cells

Oligonucleotides	Rat liver 5S RNA*	HeLa 5 S RNA**
GpCp	6	6
GpUp	3	3
GpAp	3	4
GpGpCp	1.4	2.5
GpGpUp	2.6	1.5
GpGpAp	1	1
GpGpGpCp	2	1
GpGpGpUp	1	2
GpGpGpAp	1	2

* Calculated from A:U:C ratios for each group of isoplit

**Calculated from known primary structure

Since the isoplit pattern was clear enough we analyzed each fraction without preliminary rechromatography. Material of each fraction was hydrolysed with T_1 RNase and phosphatase and nucleosides obtained were subject to ion-exclusion chromatography. As may be seen in Fig. 2 5-6% of G was revealed after analysis of mononucleotide fraction. We think that the presence of a small amount of free G may be mainly accounted for a non-absolute resistance of the phosphodiester bond near modified G against hydrolysis as was already shown ². However at the moment we can not exclude other explanations, e.g. incomplete inhibition and/or removal of T_2 RNase before demodification or partial loss of the adduct in the course of T_2 RNase hydrolysis. At any rate as was shown during further treatment of the data the existence of this fraction has practically no influence on the results summarized in Tables 1 and 2.

Table 1 shows a very good agreement between the amount of nucleotide material analysed (expressed in A_{260} units), the total number of the oligonucleotides and mononucleotides detected and the chain length of the rat liver 5 S RNA molecule.

Comparison of the known primary sequence of the 5 S RNA from HeLa cells ⁸ with the data presented in Tables 1 and 2 clearly shows that the most oligonucleotides in both RNA species are identical. This observation might be expected since human and rat cells should be rather similar in 5 S RNA structure. However distinct differences may be also seen, especially in tetranucleotide fraction.

One cycle of the analysis described here occupies 4-5 days, considerably shorter then the usual way of similar analysis. Furthermore, the quantitativness of the results is much better indeed. It is obvious from the data presented in this and previous papers ^{1,2} that the procedure suggested will greatly facilitate the sequencing of polyribonucleotides.

It is clear that the restriction method proposed as it is described here and elsewhere ^{1,2} are fully applicable for structural investigations of at least 4 S and 5 S RNAs and fragments of high-molecular weight (ribosomal and viral) RNAs. Due to its simplicity and rapidity the method is highly compatible with radioactive polynucleotides to be analysed.

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