УДК 615.281.8.014.42

M. A. Surzhik, A. L. Timkovsky

AFFINITY SORPTION ANALYSIS OF THE STRUCTURE OF POLY(G)·POLY(C) COMPLEX OBTAINED BY MEANS OF TEMPLATE-DEPENDENT SYNTHESIS

The analysis of the structure of poly(G)·poly (C) complexes obtained by the enzymatic synthesis on poly(C) template was carried out using the method of affinity sorption. This latter was used also for the purification of the synthesized so far complexes from the redundant template as well as for the investigation of the structure of poly(G)× ×poly(C) complexes obtained by the «traditional» mixing of complementary homopolynucleotides. It was shon that the degree of template transcription depends on the quality of RNA polymerase and the incompleted transcription is connected with the inhibition of the elongation of poly(G) chain.

Introduction. The preparation of double-stranded polyribonucleotide complex poly(G)·poly(C) by means of poly(G) synthesis from GTP on the poly(C) template with the use of bacterial DNA-dependent RNA polymerase as well as the evidence of its high structural regularity had been described by us earlier [1]. But it was shown in the course of following investigations that the degree of radioactive GTP incorporation into the macromolecular fraction depended on the used lot of commercial RNA polymerase. This may be the effect of either the action of contaminating RNAses partially hydrolized the template in the course of synthesis or the incomplete template transcription. This work is devoted to the analysis by means of affinity sorption of the structure of poly(G)·poly(C) complexes obtained in the course of template-dependent synthesis. This method was applied also for the purification of resulting complexes from redundant unused template as well as for structural investigation of «traditional» poly(G)·poly(C) complexes obtained by the mixing of complementary polynucleotides in solution [2].

Materials and methods. Commercial preparations of DNA-dependent RNA polymerase from *Escherichia coli* (Scientific Research Design-Technology Institute of Biologically Active Sabstances, Berdsk, Novosibirsk district, Russia) were used.

Sodium guanosine-5′-triphosphate (GTP) («Reanal», Hungary) was purified directly before use from contaminating mono- and diphosphates by the ion-exchange chromatography on Dowex 1×8 resin in Cl^--form. Radioactive [8-^{3}H]-GTP with specific activity of 814 TBq/mole was produced by the All-Union Concern «Isotope».

Guanosine-5′-diphosphate (GDP) and cytidine-5′-diphosphate (CDP) were obtained from Scientific-Productive Formation «Biolar» (Olajne, Latvia). Sodium salts of poly(G) and poly(C) were synthesized from GDP and CDP correspondingly by means of continuous-flow synthesis with the use of bacterial polynucleotide phosphorylase immobilized on macroporous glass [3].

Preparative scale synthesis of poly(G)·poly(C) with the use of RNA polymerase was performed on poly(C) template as described earlier [1].

Our own modification of the described in the literature method of DNA immobilization on cellulose powder [4] was used for the preparation of poly(G)-cellulose (PGC) which was used as affinity adsorbent for poly(C). Microcrystalline cellulose, qualification «LK — for column chromatography» was purified from fine components by repeated decanting of its water suspension and washed with 0,5 N NaOH on paper filter till the absence of yellow colour of filtrate. Then it was washed by small aliquotes of 96 % EtOH and air-dried overnight at room temperature. Poly(G) was dissolved at a concentration of 2 mg/ml in 1 mM EDTA with 10 mM NaCl. Purified dry cellulose was added with continuous stirring to the poly(G) solution — 1 g per 15—20 mg of poly(G).

After thorough mixing on magnetic stirred for 1 hr the mixture was dried in rotational evaporator and suspended in 7—10 ml of 96 % EtOH per 1 g of cellulose. This suspension was UV irradiated with vigorous stirring at 4 °C for 18 hrs at a distance of 15 cm from 3 parallel sterilizing 15 W UV-lamps BUV-15, then dried on paper filter, suspended in 2M NaCl solution containing 10 mM EDTA, stirred for 30—60 min and washed with the same solution at paper filter till the disappearance of the optical density at 260 nm in filtrate. Final steps were washing with water, then with 96 % EtOH and air-drying.

The degree of poly(G) fixation was checked in the following manner: 100 mg of PGC were suspended in 4 ml of 0,4 N NaOH and incubated at 37 °C for 48 hrs, then 4 ml of 2,5 % $HClO_4$ were added for 10 min in the ice bath. After centrifugation the UV absorbance spectrum in supernatant was registered and the amount of alkaline-splitted GMP was calculated on the basis of molar spectral data. The amount of immobilized poly(G) was equal usually to 1,0—1,1 μ moles per 100 mg of PGC. The interaction of poly(C) or complexes poly(G)·poly(C) with PGC was performed at 20 °C with stirring

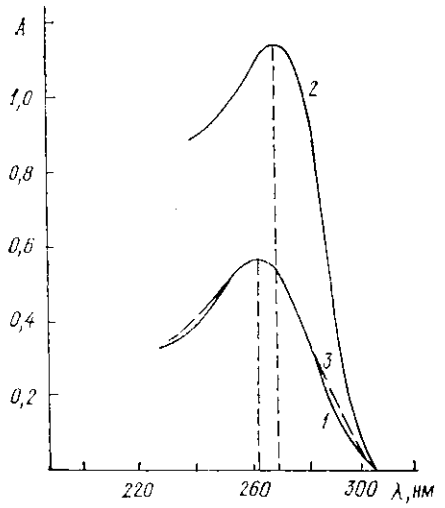

Fig. 1. UV absorption spectra. *1* — complex poly(G)·poly(C); *2* — complex poly(G)·poly(C) with added free poly(C) before interaction with poly(G)-cellulose; *3* — complex poly(G)·poly(C) with added free poly(C) after interaction with poly(G)-cellulose

for 4 hrs in 5 mM sodium phosphate, pH 7,5, containing 100 mM NaCl.

The interaction of poly(G)·poly(C) complexes after template-dependent synthesis was performed as follows. To 0,42 ml of the reaction mixture containing 0,15 mM poly(C), 1,5 mM ^{3}H-GTP, and RNA polymerase [1], 25 μ l of 4 M NaCl and H_2O till 1 ml were added. Then 50 mg of PGC containing 0,5—0,55 μ moles of poly(G) were added and incubation was proceeded for 4 hrs at 20 °C. Radioactivity of acid-insoluble fraction in the supernatant was measured [1] before and after incubation with PGC.

Column gel chromatography of samples on Sepharose 2B was performed in usual manner [5].

The method of determining free poly(C) concentration in solution using the differential pulse polarography at the mercury dropping electrode was described earlier [6]. Automated polarographic recorder model 174 A (PARC, USA) was used.

The radioactivity of samples was measured in the liquid scintillation counter SL-30 («Intertechnique», France).

Results and discussion. First of all the control experiments were carried out in order to check the binding of poly(C) with PGC in the presence of complex poly(G)·poly(C). Fig. 1 shows UV absorbancy spectra of the complex and its mixture with added extra poly(C) both before and after the interaction with PGC. We see that the presence of complex did not prevent full binding of free poly(C) with PGC.

Then we checked that PGC could bind not only the excess of free poly(C) but also the imperfect complex molecules bearing sites of poly(C) uncoupled with poly(G). In table 1 the composition of poly(G) and poly(C) mixtures prepared for complex formation is shown (column 1), in parallel with the degree of changes in the optical density after the contact of resulting complexes with PGC (column 2). In addition to the equimolar ratio poly(G)/poly(C) we used also the excess of poly(C), up

to 70 % in molar concentration. All absorbance spectra were almost identical in their form to the spectrum of complex at the equimolar ratio of poly(G) to poly(C), with the maximum of OD at 260–261 nm, so we do not give them here. The decrease in total absorbancy after PGC treatment was found to be proportional to the molar excess of poly(C). As far as the mechanical addition of poly(C) to the finished complex resulted in drastic change of the form of summary absorbance spectrum (cf.

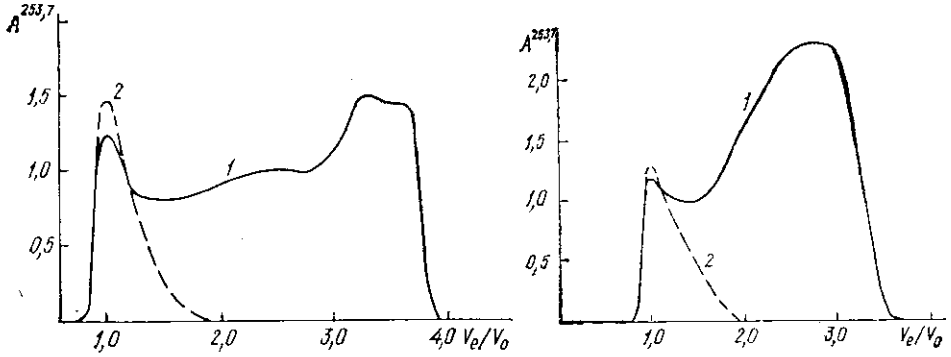

Fig. 2. Gel-chromatographic pattern on Sepharose 2B of the complex poly(G)·poly(C) obtained with the 70 % molar excess of poly(C) (tabl. 2, sample *4*): *1* — before interaction with poly(G)-cellulose; *2* — after interaction with poly(G)-cellulose

Fig. 3. Gel-chromatographic pattern on Sepharose 2B of the product of incomplete template-dependent synthesis: *1* — before interaction with poly(G)-cellulose; *2* — after interaction with poly(G)-cellulose

Fig. 1), we conclude that in the samples *2*, *3*, and *4* almost all redundant poly(C) is involved into the interaction with poly(G). This interaction is incomplete, but absolutely free molecules of poly(C) are apparently absent.

In Fig. 2 gel chromatography patterns of the sample N 4 with 70 % molar excess of poly(C) before and after its interaction with PGC is shown. Most real explanation of their features consists in the extraction from this sample in the course of affinity sorption of imperfect complex molecules with the sections of uncoupled poly(C).

After this the analysis of the products of template-dependent synthesis of poly(G)·poly(C) was performed. The presence of ^{3}H-GTP in incubation mixtures allowed us to determine the degree of the synthesis on the basis or radioactive label incorporation into the acid-insoluble fraction. In fig. 3 the results of gel chromatography on Sepharose 2B of the sample with the degree of completeness of the synthesis near 50 % from maximal level before and after the treatment with PGC is shown. The sample was polydisperse before the treatment with PGC and contained high amount of relatively low-molecular-weight components, but af-

Table 1

Optical density and differential pulse polarography data before and after the interaction with PGC for model complexes obtained at different molar ratios poly(G)/poly(C)

Molar ratio poly (G)/poly (C)	OD_{260} decrease after interaction with PGC, %	DPP current at a potential of -1,4 V, μA	
		before PGC	after PGC
N 1. 1/1,0	10	0,04	<0,01
N 2. 1/1,1	23	0,07	<0,01
N 3. 1/1,3	45	0,25	<0,01
N 4. 1/1,7	60	0,57	<0,01

Table 2

The results of radioactivity measurements in the acid-insoluble fraction of the product of template-dependent synthesis before and after the interaction with PGC

The radioactivity in acid-insoluble fraction, cpm			
Incomplete (50 %) synthesis		Complete synthesis	
Before PGC	After PGC	Before PGC	After PGC
2184±18	1034±12	1705±16	1715±16

ier the affinity sorption its gel chromatographic pattern consisted of macromolecular fraction only. It is unclear newertheless what are the components of the reaction mixture removed in this way — are they incompletely transcribed template molecules of poly(C) (i. e. «partial» complexes) or they consist of poly(C) molecules that had not contacted with RNA polymerase and remained free.

We are sure that the results of following experiments answer this question. We used now the specific curcumstance that radioactive ^{3}H-label is present in the macromolecular component in the poly(G) strand only. It is obvious therefore that combining the measurements of OD and radioactivity distributions permits the discrimination of completely free poly(C) molecules from free poly(C) mojeties in the imperfect poly(G) × ×poly(C) complexes. The results are presented in table. 2. We see that incubation with PGC of reaction mixture with about 50 % level of poly(G) synthesis resulted in two-fold decrease of ^{3}H amount in the acid-insoluble fraction. At the same time the amount of radioactive label in the analogous fraction of the product of complete template-dependent synthesis did not change after the incubation with PGC.

We conclude that after the incomplete template-dependent synthesis the reaction mixture contains considerable amount of imperfect complex molecules resulting from the incomplete template transcription and having rather lengthy free poly(C) sequences. These imperfect complex molecules just bind with the affinity sorbent. Our results show also that the method of affinity sorption permits the purification of poly(G)·poly(C) preparations, both from template dependent synthesis and traditional, from contaminating imperfect molecules.

It was shown by us earlier [6] that differential pulse polarography (DPP) at a mercury dropping electrode is a sensitive quantitative method for measuring the amount of free poly(C) sequences in the molecules of polyribonucleotide complexes. This method was applied now in order to obtain additional evidence of the purification of our samples from the redundant free poly(C). The results of polarographic current measurements for model complex preparations before and after their interaction with PGC are shown in table 1 (columns 3 and 4). They confirm that free poly(C) completely disappeared after treatment with PGC. The analogous measurements for the products of incomplete template-dependent synthesis gave identical results which by this reason aren't shown here.

We are sure therefore that the affinity sorbent is capable to extract from the samples described here both unused poly(C) template and the imperfect complex molecules. Their possible remainder is obviously behind the frames of the sensitivity of the methods used here. In order to estimate additionally the completeness of this extraction we could use the results of our previous work where the hydrolysis of poly(G)·poly(C) complexes by human blood nucleases was investigated. We used complexes obtained in template-dependent synthesis as well as by traditional mixing and standardized them in accordance with the criteria elaborated by us earlier [5]. It was shown that complexes which had in gel chromatographic pattern on sepharose 2B only one peak eluted directly after void volume had not been by no means destroyed by serum nucleases. We concluded that they did not contain sufficient amounts of one-stranded contaminants and regions which are much more sensitive to RNAses than double-stranded RNA [8]. As far as we see in sepharose 2B after PGC treatment only one peak without retardation in the gel, this could serve as additional evidence of full extraction of one-stranded regions and molecules.

The main conclusion of this work is that the affinity sorption on poly(G)-cellulose allows us to analyze the structure of complexes poly(G) × ×poly(C) and to isolate the most regular part from their preparations. As for the incompleteness of the template-dependent synthesis in case of some lots of RNA polymerase, it is clear now that it is connected rather with the inhibition of poly(G) chain elongation than with the decreasing

the number of initiation centers which allows to some poly(C) molecules to remain unused. We think that this may be the effect of σ subunit deficiency in definite lots of enzyme [9] but additional investigation is needed in order to confirm this assumption.

The authors are grateful to Dr. E. A. Glazunov for his helpful assistance in the synthesis of polynucleotides and to Prof. E. N. Kazbekov for critical reading of the manuscript.

REFERENCES

1. *Суржик М. Ф., Вильнер Л. М., Качурин А. Л., Тимковский А. Л.* Матричный синтез комплекса поли(Г)·поли(Ц) с помощью ДНК-зависимой РНК-полимеразы *Escherichia coli*. Исследование физико-химических свойств и биологической активности продукта реакции.— Л., 1989.— С. 1—18.— (Препринт / АН СССР. Ин-т ядер. физики; № 1555).
2. *Вильнер Л. М., Тимковский А. Л., Тихомирова-Сидорова Н. С.* Биологическая активность и структурные особенности полинуклеотидных интерфероногенов // Итоги науки и техники.— М. : ВИНИТИ, 1977.— С. 114—159.— (Сер. Вирусология; Т. 7).
3. *Глазунов Е. А., Чернаенко В. М.* Новый способ иммобилизации ферментов на силикатных носителях // Направленный транспорт и иммобилизация биологически активных препаратов для клинической практики.— Киев : Наук. думка, 1984.— С. 11—12.
4. *Гилмор С.* Транскрипция хроматина // Транскрипция и трансляция. Методы.— М. : Мир, 1987.— С. 160—185.
5. *Тимковский А. Л., Аксенов О. А., Бреслер С. Е. и др.* Молекулярно-весовые характеристики комплекса поли(Г)·поли(Ц) и их связь с антивирусной и интерферономенной активностью // Вопр. вирусологии.— 1973.— **18**, № 3.— С. 350—355.
6. *Brabec V., Timkovsky A. L.* Electrochemistry of double-stranded complexes of synthetic polynucleotides having interferonogenic and antiviral activity // Gen. Physiol. Biophys.— 1983.— 2, N 4.— P. 487—497.
7. *Суржик М. А., Дятлова Н. П., Глазунов Е. А. и др.* Устойчивость природных и синтетических полирибонуклеотидных индукторов интерферона к рибонуклеазам человеческой крови // Антибиотики и химиотерапия.— 1992.— **37**.— № 1.— С. 38—45.
8. *Colby C., Stollar B. D., Simon M. I.* Interferon induction: DNA-RNA hybrid or double-stranded RNA? // Nature New Biol.— 1971.— **229**.— P. 172—174.
9. *Niyogi S., Stevens A.* Studied of the ribonucleic acid polymerase from Escherichia coli // J. Biol. Chem.— 1965.— **240**.— P. 2587—2596.

Petersburg Institute of Nuclear Physics,
Academy of Sciences of Russia

28.09.92

УДК 615.357.631:577.157

Ю. И. Губский, Е. Л. Левицкий, Р. Г. Примак, А. Г. Горюшко, Л. К. Ленчевская, В. А. Матюшкин, И. Е. Вистунова, Л. Г. Саченко

ВЛИЯНИЕ ВИТАМИНА Е НА СТРУКТУРНО-ФУНКЦИОНАЛЬНУЮ ОРГАНИЗАЦИЮ ХРОМАТИНА ПЕЧЕНИ В УСЛОВИЯХ ПОВРЕЖДЕНИЯ ТЕТРАХЛОРМЕТАНОМ

Показано частичное корригирующее действие витамина Е на структурно-функциональную организацию ядерного хроматина печени крыс, нарушенную в условиях интоксикации тетрахлорметаном. Оно опосредуется, прежде всего, антиоксидантным действием α-токоферола, что выражается в нормализации течения реакций спонтанного и индуцированного переокисления липидов в активной и низкоактивной фракциях хроматина. Введение витамина Е приводит также к частичной нормализации активности эндогенных РНК-полимераз, ДНК-полимеразы β и к стимуляции активности репликативной ДНК-полимеразы α в активной фракции хроматина. Описан процесс взаимодействия α-токоферола с фракциями хроматина in vitro. Интоксикация тетрахлорметаном мало влияет на способность репрессированного хроматина к комплексообразованию с витамином Е, но усиливает связывание его с активной фракцией хроматина. Выявлены различия в природе химического взаимодействия активной и низкоактивной фракций хроматина с α-токоферолом in vitro, свидетельствующие о различной локализации витамина Е в хроматине. Взаимодействие витамина Е с хроматином частично восстанавливает его структуру, нарушенную в результате интоксикации, что наряду с антиоксидантным эффектом является одним из возможных механизмов его генопротекторного действия.