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Antiviral Research 38 (1998) 131–140

Antiviral Research

Template-dependent biosynthesis of poly(G)·poly(C) and its antiviral activity in vitro and in vivo

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Received 13 November 1997; accepted 9 March 1998

Abstract

Experimental conditions for poly(G) synthesis from GTP on a poly(C) template with the aid of *Escherichia coli* DNA-dependent RNA polymerase were investigated. The reaction product was purified without the use of RNase. On the basis of spectral data, gel permeation chromatography, affinity adsorption and electron microscopic visualization, the poly(G)·poly(C) product was assumed to possess a high degree of structural regularity. Its in vitro and in vivo antiviral activities were compared with those of traditional poly(G)·poly(C) and poly(I)·poly(C). Template-dependent poly(G)·poly(C) was similar in its in vitro activity to poly(I)·poly(C) or even surpassed it, whereas the 'traditional' poly(G)·poly(C) was only slightly active in vitro. However, 'traditional' poly(G)·poly(C) and poly(I)·poly(C) had similar activity in vivo, whereas template-dependent poly(G)·poly(C) was much less active in vivo. The role of intramolecular structural regularity in the in vitro and in vivo antiviral activity of polyribonucleotide duplexes is discussed.

Keywords: Antiviral activity; In vitro; In vivo; Poly(G)·poly(C); Polyribonucleotide duplexes; Structure; Template biosynthesis

1. Introduction

Synthetic complementary polynucleotide duplexes have been thoroughly investigated not only as model nucleic acids, but since 1967 also as interferon inducers and antiviral agents. These trials had been initiated by the discovery of the antiviral and interferon-inducing activities of poly(I)·poly(C) and poly(A)·poly(U) (Field et al., 1967). These properties of poly(G)·poly(C) duplex were also found by Zeitlenok et al. (1968). The results of our subsequent studies with poly(G)·poly(C) (Subbotina et al., 1972, Aksenov

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0166-3542/98/$19.00
PII S0166-3542(98)00018-7

et al., 1973, Timkovskii et al., 1973, Novokhatsky et al., 1975) were confirmed and further elaborated by Torrence and De Clercq (1984). Duplexes of different nucleotide composition were obtained and investigated and were shown to be active as antiviral, antitumor and immunomodulating agents. Usually they were the products of 'traditionally' mixing solutions of complementary polynucleotides synthesized beforehand with the use of polynucleotide phosphorylase (PNP) (for refs. see De Clercq, 1974, 1981, Vilner et al., 1977).

It is obvious that the polydispersity of synthetic polynucleotides and the absence of their strict reciprocal arrangement in the course of interaction are unavoidable in the traditional preparation method. This results inevitably in the distortions (loops, branches, one-stranded areas and so on) in the macromolecular structure of duplexes (Vilner et al., 1977, Timkovskii, 1981, Timkovskii et al., 1994). Nevertheless, these duplexes retain their importance as immunocorrecting (Lacour, 1985) and antiviral, especially anti-HIV (Suhadolnik et al., 1991, Laurent-Crawford et al., 1992, Krust et al., 1993), remedies.

The possibility of poly(G)·poly(C) duplex formation and its biological activity remained undecided for a long time due to the restrictions in the interaction between poly(G) and poly(C). These difficulties were the result of a stable secondary structure of poly(G) (Fresco and Massoulié, 1963, Pochon and Michelson, 1965, Englander et al., 1972, Subbotina et al., 1972, Aksenov et al., 1973, Timkovskii, 1981). In attempts to overcome this problem the poly(G) strand sometimes was synthesized on the poly(C) template with the use of bacterial RNA polymerases (Haselkorn and Fox, 1965, Hirschbein et al., 1967, Colby and Chamberlin, 1969). The biological activity of poly(G)·poly(C) thus obtained was measured in vitro only in one case (Colby and Chamberlin, 1969) and was found to be essentially lower than that of the poly(I)·poly(C) duplex obtained by the traditional mixing method. This could be the result of RNase treatment (Gray et al., 1972) in the course of product purification.

In this work we studied the template-dependent biosynthesis of poly(G)·poly(C) and determined its in vitro and in vivo antiviral activities compared with those of traditional poly(G)·poly(C).

2. Materials and methods

Commercial preparations of *Escherichia coli* DNA-dependent RNA polymerase were obtained from the Technological Institute of Biologically Active Substances (Berdsk, Novosibirsk District, Russia). GTP (Reanal, Hungary) was purified from mono- and diphosphates directly before use by means of ion-exchange chromatography on Dowex AG 1x8 resin in Cl^- form. [U-^{14}C]GTP with a specific activity of 18 GBq/mol (Institute of Radioproducts, Prague, Czech Republic) or [8-^{3}H]GTP (814 TBq/mol; ISOTOP, Russia) was used without additional purification. CDP was obtained from the Scientific-Productive Union Biolar (Olaine, Latvia). The sodium salt of poly(C) was synthesized with immobilized bacterial PNP (Glazunov and Chernaenko, 1985). Optimal conditions for RNA polymerase reaction were determined in incubation mixtures with the addition of [^{14}C]GTP at 37°C. We took into account also the earlier results of Niyogi and Stevens (1965). These mixtures contained, in 0.1 ml: Tris–HCl, pH 7.8, 2 μmol; 2-mercaptoethanol, 2 μmol; sodium salt of EDTA, 0.1 μmol; [^{14}C]GTP, 0.16 MBq; $MnCl_2$, 0.2 μmol. Samples of 5 μl in volume were treated as follows: 50 μl of bovine serum albumin (Calbiochem, Switzerland) in aqueous solution at a concentration of 1 mg/ml and 3 ml of 5% aqueous solution of trichloroacetic acid (TCA) chilled to 4°C were added to each probe. The solutions were maintained for 10 min in an ice bath and filtered through the nitrocellulose membrane filter SYNPOR 6 with 0.45 μm pore size (CHEMAPOL, Czech Republic). The filters were then washed three times with 3 ml of 2% TCA water solution and air-dried under gentle heating by an IR lamp. The radioactivity of the sediments on filters was counted in a scintillation radiometer (SL-30; Intertechnique, France). The preparative syntheses were carried out in 20-ml volumes of incubation mixture. After completion of the synthesis, the reaction mixture was deproteinized with water-saturated phenol as described else-

where (Surzhik and Timkovskii, 1993). Then the polymer fraction was precipitated from the water phase with a double volume of 96% ethanol at 4°C. We did not use RNAase in the product purification. The precipitate was dried under vacuum, dissolved in 0.013 M EDTA, pH 8.0, and dialysed against several changes of buffer containing 0.005 M sodium phosphate, pH 7.5, with 0.1 M NaCl until the complete disappearance of residual GTP. Gel chromatography of an aliquot from the incubation mixtures and purified product was carried out on Sephadex G-50 superfine and Sepharose 2B by a common method (Timkovskii et al., 1973). The method of measuring the degree of poly(C) template utilization with the use of poly(G)-cellulose has been described elsewhere (Surzhik and Timkovskii, 1993).

The preparation of samples for electron microscopy was carried out according to a modified Kleischmidt method (Davis et al., 1971). The hyperphase for the protein film formation contained: Tris–HCl, pH 8.0, 50 mM; Na_2EDTA, 5 mM; cytochrome *c*, 100 μg/ml; polynucleotide samples, 10 μg/ml; formamide, 5% (v/v). Double-distilled water was used as the hypophase. After stabilization the films were mounted on grids covered with collodium. The grids were washed out by 90% ethanol, stained with a 0.05 mM solution of uranyl acetate in 90% ethanol, shadowed by evaporation of Pt/Pd alloy at an angle of 8 degrees and inspected under an electron microscope (JEM 6A; JEOL, Japan). Each sample was put on 4–6 grids and 50–60 areas on each grid were inspected. From these we selected for micrographs two to three areas most covered with the molecules.

The in vitro antiviral activity of duplex preparations was determined in primary chick embryo fibroblast cultures with vesicular stomatitis virus (VSV) as a challenge (Vilner et al., 1985). Duplexes were adsorbed on cells for 1.5 h in the presence of DEAE–dextran, 50 μg/ml (Pharmacia, Sweden), and then the cultures were incubated for an additional 20–22 h at 37°C and infected with VSV. After virus adsorption on the cells the monolayers were covered with 2% agar. After incubation at 37°C for 72 h, the number of sterile plaques was counted and the decrease of VSV titer following treatment was compared with the control. Poly(I)·poly(C) (Calbiochem, Switzerland) was used for comparison. The in vivo antiviral activity was determined as described elsewhere (Vilner et al., 1985).

3. Results

The kinetics of ^{14}C label incorporation from GTP into the acid-insoluble fraction catalysed by RNA polymerase on the poly(C) template (not shown here) indicated that the reaction proceeded effectively at 0.15 mM of poly(C) (with respect to mononucleotide) and the tenfold molar surplus of GTP. Increasing the poly(C) concentration up to the equivalent of GTP decreased the reaction efficiency sharply. After the completion of the polymerization reaction, the calculated molar quantity of labeled GTP incorporated into the acid-insoluble fraction was shown to be nearly equal to that of poly(C) template. This meant that under our assay conditions RNA polymerase transcribed the template almost completely. Additional evidence of the completeness of template utilization was obtained from the experiments with poly(G)-cellulose. The radioactivities of the macromolecular (acid-insoluble) fraction after the completion of the reaction with [8-^{3}H]GTP in 0.4 ml of incubation mixture before and after incubation with poly(G)-cellulose were compared and appeared to be 1705 ± 12 CPM and 1715 ± 16 CPM, respectively. This indicated that the macromolecular fraction did not contain a significant amount of free poly(C) that could interact with immobilized poly(G).

Gel chromatography on Sepharose 2B of the samples from the reaction mixture of preparative synthesis showed a successive increase of the macromolecular peak reflecting accumulation of the reaction product (Fig. 1, curve 2). The gel chromatographic pattern of the zero-time mixture containing poly(C), GTP and enzyme is also shown (curve 1). It is necessary to emphasize that the reaction proceeded effectively at a tenfold molar surplus of GTP over the template. Therefore, up to 90% of GTP remained unused and was found as a low-molecular-weight peak. Curve 3 in

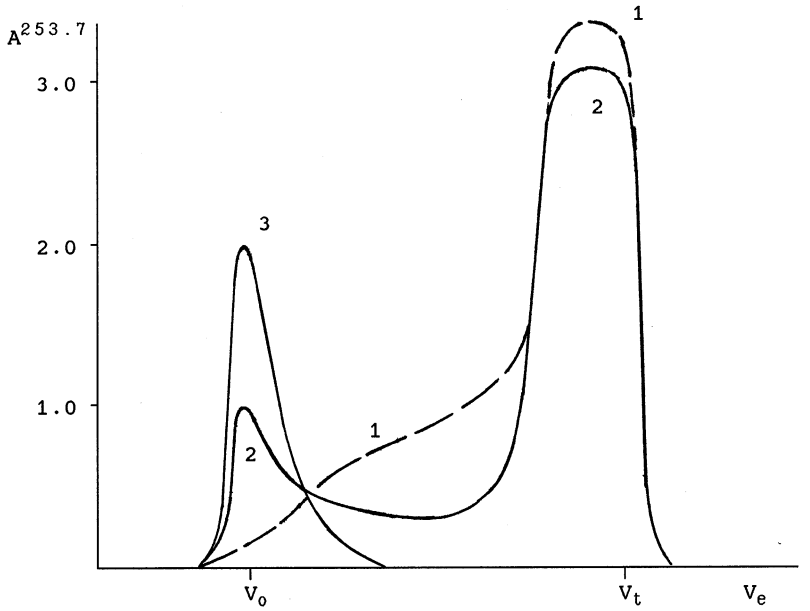


Fig. 1. Sepharose 2B gel-chromatographic patterns of the RNA polymerase incubation mixtures and of the purified reaction product. Curve 1, incubation mixture before the beginning of incubation; curve 2, incubation mixture after 120 min of incubation; curve 3, the reaction product purified by means of deproteinization and dialysis (see Section 2).

Fig. 1 shows the elution profile of a macromolecular component in the incubation mixture after the completion of preparative synthesis, deproteinization, and dialysis (see Section 2). This pattern confirmed the complete purification of the product from all other components of the incubation mixture. This macromolecular fraction was used for further investigation. Its UV absorbance spectrum (Fig. 2) was identical to the characteristic spectrum of poly(G)·poly(C) duplex (Pochon and Michelson, 1965, Englander et al., 1972, Subbotina et al., 1972).

The reaction product that appeared under the electron microscope was quite similar to double-stranded DNA or RNA (Fig. 3a). The branching and aggregation of this fraction were minimal in comparison with the samples of poly(G)·poly(C) duplex obtained by mixing poly(G) with poly(C) (Timkovskii et al., 1973, 1994) (Fig. 3b). It should be mentioned here that we used the same poly(C) lots in parallel both as templates for the polymerase-mediated synthesis and for the preparation of the 'traditional' duplexes.

A comparison of the antiviral activities in chick embryo fibroblasts of the products obtained with the use of RNA polymerase, traditional poly(G) and poly(C) mixing and a commercial preparation of poly(I)·poly(C) was carried out. At a dose of

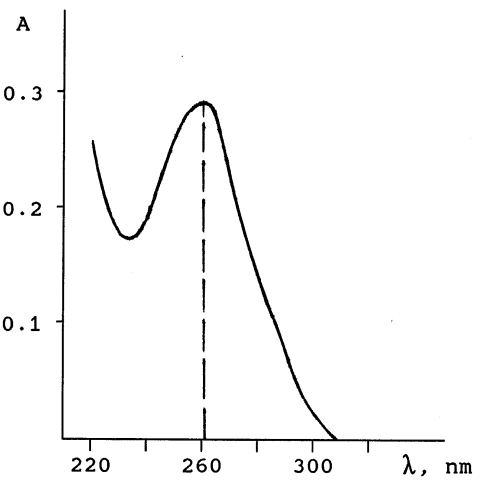


Fig. 2. UV absorbance spectrum of the product purified from the incubation mixture with RNA polymerase.

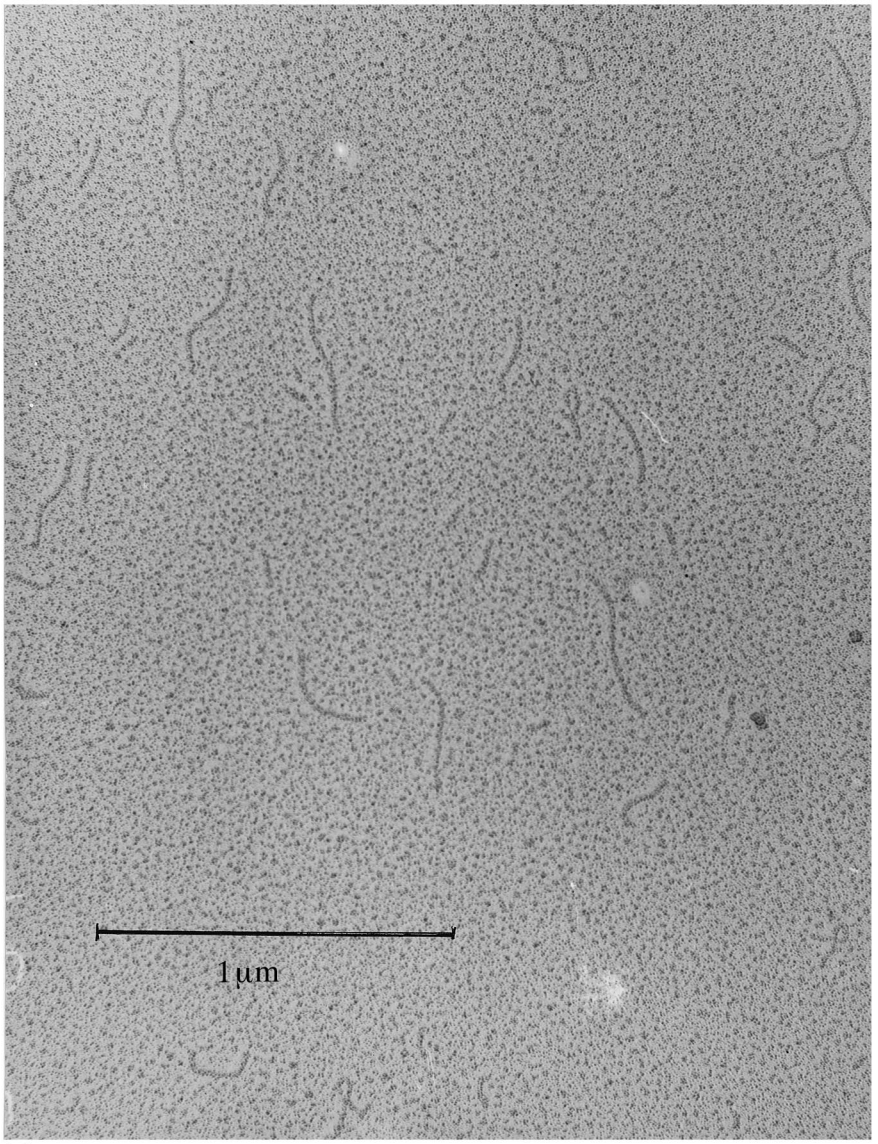
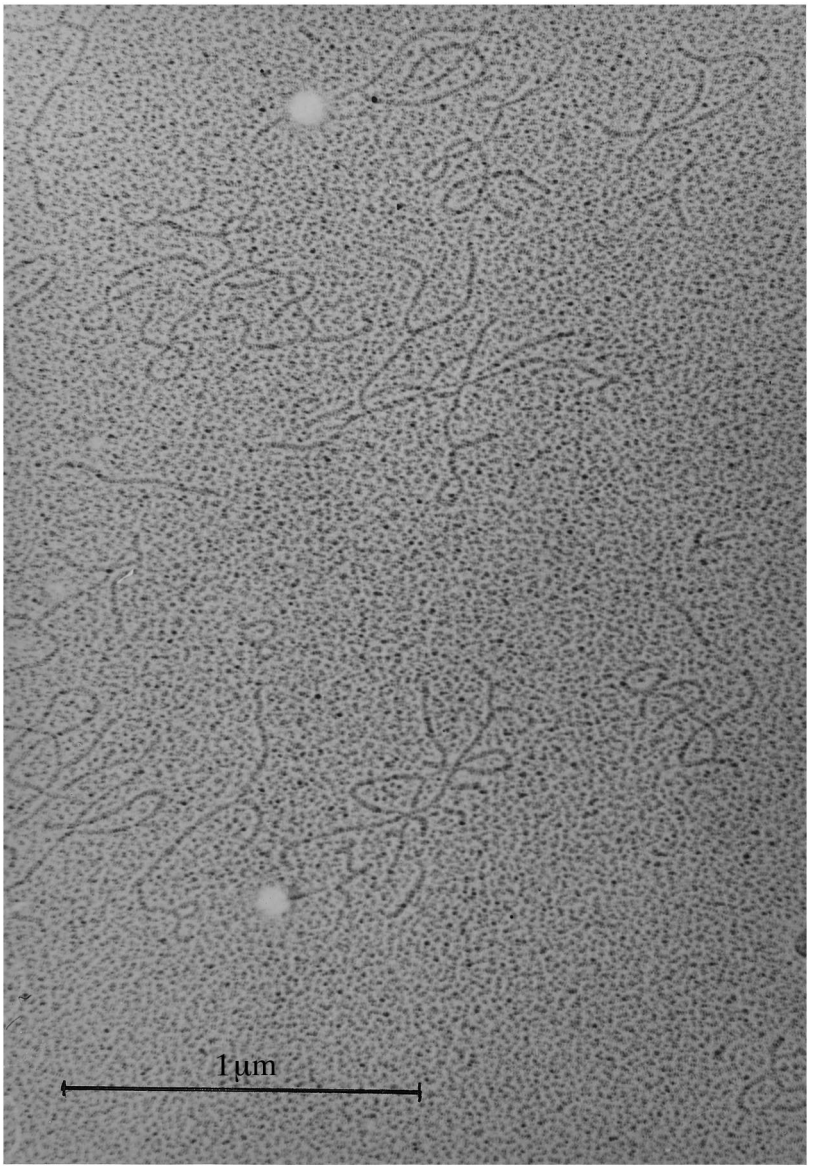


Fig. 3. Electron micrographs of: (a) purified product of the RNA polymerase (template-dependent) reaction; (b) the poly(G)·poly(C) complex obtained by the 'traditional' method, i.e. mixing poly(G) with poly(C). Bars correspond to 1 μm length.

0.1 μg/ml (results not shown), the products of template-dependent synthesis (samples 8535, 8639 and 8645) had the same activity as poly (I)·poly(C). They all lowered VSV titers by at least 6–6.5 $\log_{10}$PFU/ml. At the same time, 'traditional' poly(G)·poly(C) preparations (samples 8536, 8638 and 8644) had substantially lower activity and inhibited VSV titers only by 1.2–3.5 $\log_{10}$PFU/ml. It must be noted that all traditional poly(G)·poly(C) preparations had the expected physicochemical characteristics. The antiviral effects of the 'traditional' poly(G)·poly(C) and template-derived poly(G)·poly(C) preparations are presented in Table 1. The data show that, in



(b)

Fig. 3. (*Continued*)

contrast with the results of other authors (Colby and Chamberlin, 1969), the poly(G)·poly(C) product of the template-dependent biosynthesis was not only similar to poly(I)·poly(C) in its antiviral activity but could even surpass it.

We performed a simultaneous comparison of the in vivo and in vitro antiviral activities of 'traditional' poly(G)·poly(C) no. 8644, template poly(G)·poly(C) no. 8645 and poly(I)·poly(C). The results are presented in Table 2. Traditional poly(G)·poly(C) and poly(I)·poly(C) were similar in their activity in vivo, whereas template poly(G)·poly(C) was much less active. Concomitantly, template-dependent poly(G)·poly(C) was

Table 1
Dose dependence of the antiviral activity of poly(G)·poly(C) and poly(I)·poly(C) in chick embryo fibroblast cultures

Preparation	Dose (μg/ml)	Experiment No. 1		Experiment No. 2		Experiment 3	
		VSV titer (log_{10}PFU/ml)	Inhibition of titer (log_{10}PFU/ml)	VSV titer (log_{10}PFU/ml)	Inhibition of titer (log_{10}PFU/ml)	VSV titer (log_{10}PFU/ml)	Inhibition of titer (log_{10}PFU/ml)
Template-dependent poly(G)·poly(C), sample no. 8645	0.2	1.08	3.22	<0.70	>3.60	<0.70	>3.25
	0.04	3.17	1.13	2.21	2.09	2.87	1.08
	0.008	4.17	0.13	4.08	0.22	3.70	0.25
Template-dependent poly(G)·poly(C), sample no. 8750	0.2	<0.70	>3.60	<0.70	>3.60	<0.70	>3.25
	0.04	<0.70	>3.60	<0.70	>3.60	<0.70	>3.25
	0.008	3.42	0.88	2.46	1.84	2.88	1.07
Poly(I)·poly(C)	0.2	<0.70	>3.60	<0.70	>3.60	<0.70	>3.25
	0.04	2.93	1.37	1.48	2.82	2.26	1.69
	0.008	4.00	0.30	3.78	0.52	3.22	0.73
Medium 199	–	4.30	–	4.30	–	3.95	–

Table 2
Comparative in vivo and in vitro antiviral efficacy of 'traditional' and template-dependent poly(G)·poly(C) and poly(I)·poly(C)

Preparation	In vivo activity[a]			In vitro activity[c]			
	Total number of mice	Number of mice surviving[b]	Survival (%)	Experiment no. 1		Experiment no. 2	
				VSV titer (log_{10}PFU/ml)	Inhibition of titer (log_{10}PFU/ml)	VSV titer (log_{10}PFU/ml)	Inhibition of titer (log_{10}PFU/ml)
'Traditional' poly(G)·poly(C), sample no. 8644	20	17	85	5.4	1.2	5.7	1.3
Template-dependent poly(G)·poly(C) sample no. 8645	10	4	40	<0.5	>6.1	<0.5	>6.5
Poly(I)·poly(C)	10	9	90	<0.5	>6.1	<0.5	>6.5
Control[d]	20	0	0	6.6	–	7.0	–

[a] Complexes were injected intraperitoneally at a dose of 25 mg per mouse to BALB/c white mice (12–14 g weight) 24 h before intraperitoneal infection with 100 LD_{50} of tick-borne encephalitis virus (Absettarov strain) (Vilner et al., 1985).
[b] Survival was registered 15 days after infection.
[c] In vitro activity was determined in primary chick embryo fibroblast cultures with VSV as a challenge; preparations were introduced in medium 199 at 0.1 mg/ml with 50 mg/ml of DEAE–dextran.
[d] Control was phosphate-buffered saline for in vivo or medium 199 for in vitro experiments.

similar in its in vitro activity to poly(I)·poly(C), but 'traditional' poly(G)·poly(C) was much less active.

4. Discussion

Our results show the macromolecular product of the template-dependent biosynthesis with RNA polymerase to be a perfect poly(G)·poly(C) duplex. The high degree of structural regularity of the 'template' duplex was confirmed with the aid of affinity adsorption on poly(G)-cellulose as described above and differential pulse polarography on the mercury dropping electrode (Brabec and Timkovsky, 1983, Timkovskii et al., 1994). As for the completeness of the template poly(C) transcription by the enzyme, we have also shown that it depends on the quality of the RNA polymerase, especially on the presence of the σ-subunit (Surzhik and Timkovskii, 1993).

The divergence of the biological activities of 'traditional' poly(G)·poly(C) and poly(I)·poly(C) in different cell cultures, observed earlier by us Brodskaya and Vilner (1976) and confirmed by Torrence and De Clercq (1984), could be attributed to the complicated structure of the poly(G)·poly(C) duplex (Vilner et al., 1977, Timkovskii, 1981, Timkovskii et al., 1994). On the basis of experimental results presented in this work, including electron microscopic investigation, we can now assert that the affinity for cells and antiviral activity in cell cultures of polyribonucleotide duplexes depends on the degree of their intramolecular regularity. We considered recently tertiary structure, the next level of structural organization of polynucleotide duplexes which contains the combination of usual double-stranded regions with branches, loops, etc., hindering the interactions with cellular receptors (Timkovskii and Frenkel, 1997). We also showed that 'traditional' poly(G)·poly(C) duplex could form the most complicated tertiary structure among other duplexes (Timkovskii et al., 1994, Timkovskii and Frenkel, 1997).

We can assume also that there is some difference in the hierarchy of the structural parameters determining the biological activity of the polyribonucleotide duplexes in vitro and in vivo. Complicated macromolecular architecture could be useful for the primary uptake of duplexes in vivo (e.g. by lysosomes or macrophages). Then blood RNases may convert the branched formations into more regular fragments that are active on the target cells. We showed earlier that the tertiary structure is inherent in all traditional polynucleotide duplexes and we could influence its formation by different methods (Brabec and Timkovsky, 1983, Timkovskii et al., 1994, 1995). Our results support the possibility of designing and selecting the desired degree of duplex regularity for cellular and virological studies (Torrence and De Clercq, 1984, Timkovskii et al., 1995). They permit also the use of 'template' poly(G)·poly(C) as a standard reference preparation in the investigation of modified poly(G)·poly(C) and other polynucleotide duplexes.

Acknowledgements

We are grateful to Dr E.A. Glazunov (Laboratory of Molecular Genetics, Petersburg Institute of Nuclear Physics) for his invaluable help in PNP purification and poly(C) synthesis.

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