Oligo(dG)$_{12-18}$ aggregates result in non-homogeneity of oligo(dG)$_{12-18}$·poly(C) type primer·template

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ABSTRACT

Studies on the absorption spectra of equimolar solutions of oligo(dG)$_{12-18}$ and poly(C), poly(C$_m$) or poly(C$_e$) showed that only 13%, 3% and 3% of base pairs, respectively, form complexes. Upon centrifugation of oligo(dG)$_{12-18}$ with a molar excess of poly(C) of poly(C$_m$) in an analytical and a preparative centrifuge, it was found that only a part of oligodeoxynucleotide sediments with the polynucleotide, i.e. more rapidly than oligo(dG)$_{12-18}$, poly(C) or poly(C$_m$). Products of binding of oligo(dG)$_{12-18}$ with poly(C), poly(C$_m$) or poly(C$_e$) direct the synthesis of poly(dG) by AMV reverse transcriptas in accordance with the reported characteristics of these primer·templates, as well as of the enzyme. These observations suggest that the solutions of oligo(dG)$_{12-18}$ with (polyC) or its analogues, commonly used as primer·templates of RNA- and DNA-directed DNA polymerases, contain a polynucleotide, to which oligo(dG)$_{12-18}$ aggregates are bound through a few nucleotides chains shorter than 12-18 residues. These chains of oligo(dG)$_{12-18}$ containing the 3'-OH ends are capable of initiating the reaction with DNA polymerases.

INTRODUCTION

The tendency of oligo(dG)$_3$[a], oligo(dG)$_4$ and oligo(dG)$_5$ towards formation of aggregates,[1] as well as the "narcism" of poly(dG),[2] signifying that the very strong interactions between strands of this polynucleotide prevent normal complex formation with poly(C) and poly(dC) upon mixing, have been known for about 10 years. However, insufficient attention is devoted to these facts when oligo(dG)$_{12-18}$ mixed with poly(C) is applied as primer·template for DNA- or RNA-dependent DNA polymerases. The same applies to oligo(dG)$_{12-18}$·poly(C$_m$), which according to Gerard et al.[3,4] is a specific primer·template permitting the differentiation

of reverse transcriptase from cellular DNA polymerases. In this last case the mode of action of the oligodeoxynucleotide raises doubts, because poly(C_m) which was first synthesized for investigation of the role of the 2'-hydroxyl in the structure of polynucleotides,[5] fails, like poly(C_e) to form a complex even with poly(dI).[6,7]

The present study was aimed at an evaluation of the interaction of oligo(dG)$_{12-18}$ with poly(C), poly(C_m) and poly(C_e) under conditions applied to preparation and replication of primer·template with the aid of AMV DNA polymerase.

MATERIALS AND METHODS

Deoxynucleoside triphosphates were products of Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and ^3H -labelled deoxynucleoside triphosphates were obtained from the Radiochemical Centre (Amersham, England).

Oligo(dG)$_{12-18}$ and oligo(dT)$_{12-18}$ were purchased from P-L Biochemicals (Milwaukee, Wis., U.S.A.), and poly(C), poly(A) and poly(I) - from Miles Laboratories (Elkhart, Ind., U.S.A.). The preparation and physico-chemical properties of poly(Cm) and poly(Ce) have been described elsewhere;[5,8] both exhibited sedimentation coefficients $S_{20,w}$ of about 7-8. Poly(dI), prepared with the aid of terminal transferase,[10] was a gift of Dr. F.J. Bollum.

Primer-template duplexes for the enzymic reaction were prepared by mixing of the two components at a concentration of 200 µg/ml in 5 mM Tris-HCl buffer (pH 7.8) containing 10 mM NaCl, heating to 80°C, and then annealing at 37°C for 1 h and at 4°C for 18 hrs.

AMV reverse transcriptase, purified according to Kacian and Spiegelman,[9] was a gift of Dr. J.W. Beard. The enzyme was further dialysed against 50 mM Tris-HCl (pH 7.5) containing 2 mM mercaptoethanol, 0.5% Triton X-100 and 50% glycerol. The final enzyme content was 41 units/ml.

UV absorption spectra of primer-template were measured as a function of temperature in a Unicam SP-500 spectrophotometer fitted with a special temperature-controlled cuvette compartment, through which an aqueous glycol solution fed from a Hoeppler ultrathermostat circulated. Cuvette temperatures were measured with a thermistor in a dummy cuvette.

The sedimentation of oligo- and polynucleotides was analysed using: 1) a Spinco model E analytical ultracentrifuge fitted with ultraviolet optics; and 2) a Beckman L5-75 preparative ultracentrifuge with a Beckman prep. UV scanner in an ANF rotor. The value of E_{280} as a function

of the distance from the rotation axis was recorded every 30 min.

Samples of oligo(dG)$_{12-18}$, polynucleotides and mixtures of oligo(dG)$_{12-18}$ with polynucleotides in 0.01 M Tris-HCl (pH 8) containing 0.1 M NaCl, were analysed spectrophotometrically, heated to 80°C, and annealed at 37°C for 1 h and at 4°C for 18 hrs.

Samples of poly(C), and of a mixture of oligo(dG)$_{12-18}$ with poly(C), treated as mentioned above, were centrifuged in a Beckman L5-75 preparative ultracentrifuge, in a SW 65L rotor, at 20°C for 24 hrs, at 50 000 rpm. After centrifugation, the upper part of the supernatant was analysed spectrophotometrically.

Reverse transcriptase activity was assayed in 50 µl reaction volumes containing the following: 40 mM Tris-HCl (pH 8), 60 mM KCl, 1.6 mM DTT, 0.05% Triton X-100, 0.2 mM $MnCl_2$ or 12 mM $MgCl_2$, 0.04 units of enzyme, 10 µM substrate, 30 µM poly(A), poly(C), poly(C_m) or poly(C_e) as templates and, respectively, 15 µM oligo(dT)$_{12-18}$, 10 µM oligo(dG)$_{12-18}$, 30 µM poly(I) or 30 µM poly(dI) as primer. Specific activity of substrates: dTTP 320 cpm/pmole, dGTP 300 cpm/pmole. The mixture was incubated at 37°C for 20 min. Acid-precipitable radioactivity was collected and washed on Whatman GF/C glass fiber filters as described by Bollum.[11] One unit of enzyme activity is defined as that leading to incorporation of 1 nmole of dTMP into acid-precipitable material, with oligo(dT)$_{12-18}$•poly(A) as primer•template.

RESULTS

Absorption spectra. Examination of the absorption spectrum of the poly(dG)•poly(dC) complex obtained with the aid of DNA polymerase from E. coli,[12,13] and of the poly(dG)•poly(C) complex prepared from the former by displacement of the deoxycytidyl chain by a cytidyl chain[12] indicates that complex formation with oligo(dG)$_{12-18}$ can be followed most conveniently by observation of the absorption changes at 275-280 nm. From the value of the hyperchromicity parallelling the thermal denaturation of poly(dG)•poly(C), which at 276 nm amounts to 61%,[12,13] it can be calculated that the hypochromicity accompanying formation of this complex ought to be about 38%. In the case of complexes with oligo(dG)$_{12-18}$ a closely similar value of hypochromicity can be expected, since, as shown by Tazawa, Tazawa and Ts'o[14] for the poly(C), oligo(I) and poly(I) model - complexes of polynucleotide with complementary oligonucleotide containing more than six nucleotides, exhibit a spectrum nearly identical with that for

complexes of two polynucleotides.

The effect of mixing of poly(C), poly(C_m) and poly(C_e) with an equimolar amount of oligo$(dG)_{12-18}$, in the presence of 0.1 M NaCl, is recorded in Fig. 1. The slight drop in the absorption of the solution, as compared with the arithmetical sum of the absorption of components at 270-280 nm, was reproducible in successive experiments; for the solution containing poly(C) it is about 5%, and for solutions with poly(C_m) and poly(C_e) - less than 1% (i.e. at the limit of accuracy of the method). Heating of the solutions to 80°C, slow cooling, and storing for 18 hrs at 4°C (and additionally, in the case of solutions containing poly(C_m) and

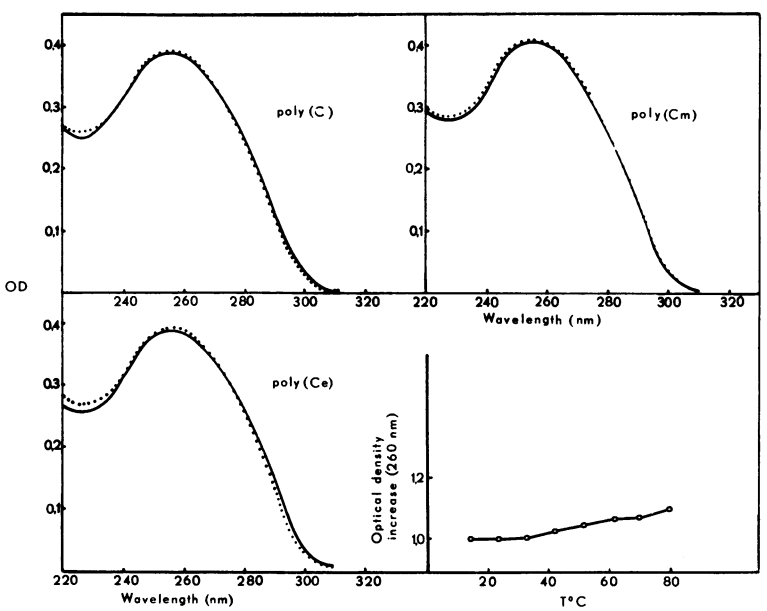

Fig. 1. The effect of mixing of poly(C), poly(C_m), poly(C_m) or poly(C_e) with oligo$(dG)_{12-18}$ on UV spectrum. Arithmetical sum of the absorptions of polynucleotide and oligodeoxynucleotide (——); spectrum of a mixture of polynucleotide and oligodeoxynucleotide in 0.01 M Tris-HCl buffer (pH 7.9) containing 0.1 M NaCl (·····). Concentration of polynucleotides and oligodeoxynucleotide was 26 μM. Insert: Absorbance-temperature profile for 26 μM oligo$(dG)_{12-18}$ in 50 mM phosphate buffer (pH 7.8).

poly(C_e), alkalization to pH 12 and neutralization) caused no further changes in the absorption spectrum. An analogous result was obtained when the concentration of poly(C) and oligo(dG)$_{12-18}$ was increased from 26 μM to 310 μM. If, by analogy with poly(dG)•poly(dC), it is assumed that formation of oligo(dG)$_{12-18}$•poly(C), oligo(dG)$_{12-18}$•poly(C_m) and oligo(dG)$_{12-18}$•poly(C_e) should be associated with hypochromicities of about 38%, then the spectral changes observed in this study point to formation of complexes to the extent of only 13%, 3% and 3%, respectively.

Centrifugation. The interaction of oligo(dG)$_{12-18}$ with cytidylyl polynucleotides was examined by analytical and preparative centrifugation. The pattern of the sedimentation boundaries obtained in an analytical centrifuge for oligo(dG)$_{12-18}$, poly(C) and a mixture of oligo(dG) with poly(C) in molar ratio 1:1.2 is presented in Fig. 2. It is evident that in the cuvette containing a mixture of both compounds their interaction product appeared; it was more heterogeneous than the homogeneous poly-

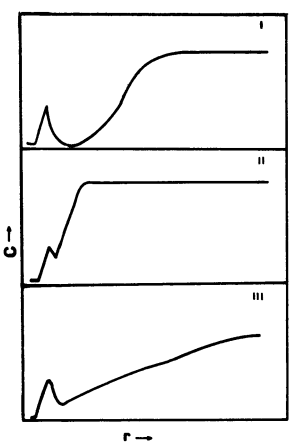

Fig. 2. Interaction of oligo(dG)$_{12-18}$ with poly(C) in an analytical ultracentrifuge. Sedimentation boundaries of poly(C) (I), oligo(dG)$_{12-18}$ (II) and mixture of oligo(dG)$_{12-18}$ with poly(C) (molar ratio 1:1.2) (III). Diagrams are densitometer traces of absorption after 40 min sedimentation at 60 000 rpm, at 20°C. c is proportional to the concentration, and r is the distance from the rotation axis.

nucleotide, and sedimented more rapidly than oligo(dG)$_{12-18}$ or poly(C) (sedimentation constant $S_{20,w} = 6.4$).

On the other hand, measurements of absorption in this cuvette in the vicinity of the meniscus indicated that, in contrast to the cuvette with poly(C) where, after 40 min centrifugation, the concentration of the preparation dropped to zero, a portion of the material underwent no sedimentation. Since in this region of the cuvette oligo(dG)$_{12-18}$ alone also failed to undergo complete sedimentation, it can be assumed that the non-sedimented material in the cuvette containing both components represented the fraction of oligo(dG)$_{12-18}$ which did not react with the polynucleotide, despite the excess of poly(C).

Similar results were obtained when a mixture of oligo(dG)$_{12-18}$ with poly(C) (molar ratio 1:3) or with poly(C_m) (molar ratio 1:1.2) was centrifuged in a preparative ultracentrifuge with a UV scanner. Records obtained in both experiments are presented in Figs. 3A and 3B, respectively, for centrifugation times at which free polynucleotides, and the more so the products of their interaction with oligo(dG)$_{12-18}$, were removed by sedimentation from the region near the meniscus. Sedimentation boundaries in this region - in the cuvettes containing a mixture of oligo- and polynucleotides - reflected, as previously, the fraction of oligo(dG)$_{12-18}$ which did not react with poly(C) or poly(C_m). In both cases, as in the control cuvettes containing the oligodeoxynucleotide alone, the heterogeneity of the oligo(dG)$_{12-18}$ preparation, a part of which began to undergo slow sedimentation, became evident.

Since the content of oligo(dG)$_{12-18}$ in the control cuvettes, and in those containing a mixture of oligodeoxynucleotide and polynucleotides, were identical in each experiment, the amount of oligo(dG)$_{12-18}$ interacting with poly(C) may, from the record in Fig. 3A, be estimated at approximately 50%. Because of the low accuracy of the absorption measurement in the ultracentrifuge, the amount of oligo(dG)$_{12-18}$ interacting with poly(C_m) could not be determined (Fig. 3B). However, this interaction was demonstrated by the appearance of the product sedimenting more rapidly than the polynucleotide (Fig. 3B).

Centrifugation in a preparative ultracentrifuge under conditions permitting complete removal of polynucleotide provided further evidence that only a part of the oligo(dG)$_{12-18}$ preparation reacted with poly(C). The supernatant of a control sample containing poly(C) did not absorb at 230-300 nm, whereas the supernatant of a sample containing oligo(dG)$_{12-18}$

and poly(C) (molar ratio 1:1.2) exhibited an absorption representing about 10% of the initial absorption of oligo(dG)$_{12-18}$ itself. This level of the absorption probably corresponded to only that part of oligo(dG)$_{12-18}$ which was not bound with poly(C), since (with the long centrifugation time applied in this experiment) a part of the preparation could have undergone sedimentation.

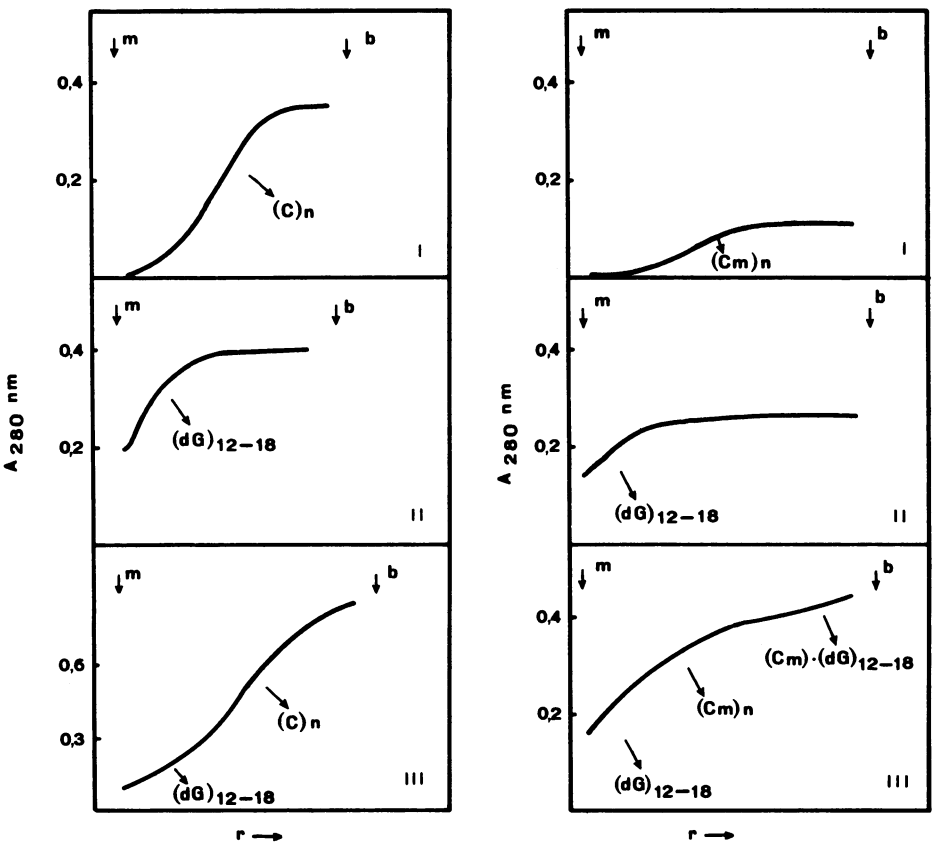

Fig. 3. Study of the interaction of oligo(dG)$_{12-18}$ with poly(C) and poly(C$_m$) in a preparative ultracentrifuge with prep. UV scanner: Left, sedimentation boundaries of poly(C) (I), oligo(dG)$_{12-18}$ (II), and mixture of oligo(dG)$_{12-18}$ with poly(C) (molar ratio 1:3) (III), after 2 hrs of centrifugation at 45 000 rpm, at 9°C; Right, sedimentation boundaries of poly(C$_m$) (I), oligo(dG)$_{12-18}$ (II) and mixture of oligo(dG)$_{12-18}$ with poly(C$_m$) (molar ratio 1:1.2) (III), after 1 hr of centrifugation at 50 000 rpm, at 18°C. Symbol r - distance from the rotation axis.

Enzymatic reactions. The preparation of oligo(dG)$_{12-18}$, annealed with poly(C), poly(C$_m$) or poly(C$_e$) under conditions analogous to the conditions of the spectrophotometric measurements and centrifugation, were tested as primer•templates for AMV reverse transcriptase. Data from Table 1 indicate that, with the first two templates, the dGMP incorporation proceeded similarly and represented about 60 and 40%, respectively, of the dTMP incorporation found with oligo(dT)$_{12-18}$•poly(A) as primer•template. This is consistent with findings of many other authors, regarding the efficient replication of the poly(C) type templates with the aid of DNA polymerase of oncornaviruses.[15,16,17] A slight, but reproducible, reaction was also obtained on carrying out replication with the use of oligo(dG)$_{12-18}$•poly(C$_e$).[7]

In a subsequent experiment attempts were made to replace the aggregated oligo(dG)$_{12-18}$ primer by poly(dI) and poly(I). The use of oligo(dI) seemed inadvisable; namely, since in 0.1 M sodium phosphate buffer the T_m of poly(dI)•poly(C) was 35°C,[13] the stability of the presumed complex with poly(C) would be too low.

Table 1. Replication of poly(C) and its analogous with the aid of AMV reverse transcriptase primed by oligo(dG)$_{12-18}$, poly(I) and poly(dI).

Primer	Template	Incorporation in the presence of indicated cation (pmole)			
oligo(dT)$_{12-18}$	poly(A)	40	Mn	—	—
oligo(dG)$_{12-18}$	poly(C)	23	Mn	—	—
oligo(dG)$_{12-18}$	poly(C$_m$)	16	Mn	—	—
oligo(dG)$_{12-18}$	poly(C$_e$)	1	Mn	—	—
poly(I)	poly(C)	—	—	6	Mg^x
poly(I)	poly(C$_m$)	5	Mn^x	0	Mg
poly(I)	poly(C$_e$)	—	—	0	Mg
poly(dI)	poly(C)	—	—	300	Mg^x
poly(dI)	poly(C$_m$)	0.8	Mn^x	1.8	Mg
poly(dI)	poly(C$_e$)	—	—	0.01	Mg^{xx}
oligo(dG)$_{12-18}$	———	0.1	Mn	—	—
poly(I)	———	0	Mn	0	Mg^x
poly(dI)	———	0.7	Mn^x	0.8	Mg
———	poly(C)	—	—	6	Mg^x
———	poly(C$_m$)	1.6	Mn^x	0	Mg
———	poly(C$_e$)	0.1	Mn	—	—

^{x}plus 10 μM dCTP

^{xx}plus 0.5 μM ^{3}H-dGTP (9300 cpm/pmole) and 0.1 M KCl, 20°C

The incorporation of dGMP in the presence of poly(dI)·poly(C) was characterized by a high yield (Table 1), consistent with the findings of Spiegelman;[18] it proceeded better than with the oligo(dG)$_{12-18}$ primer, and more satisfactorily than dTMP incorporation with oligo(dT)$_{12-18}$·poly(A). However, the superiority of poly(dI) as a primer relative to oligo(dG)$_{12-18}$ was not confirmed in the case of the poly(C$_m$) and poly(C$_e$) replication; this might have been expected, since spectrophotometric measurements indicated that these polymers form no complexes with poly(dI).[6,7]

Poly(I), which readily forms complexes with poly(C), poly(Cm) and poly(Ce),[6,8] proved to be an unsatisfactory primer; namely, in the case of these complexes, dGMP incorporation did not exceed that obtained with each single polynucleotide (Table 1). The low efficiency of poly(I)·poly(C) as primer·template for reverse transcriptase was previously reported by Hurwitz and Leis,[19] contrary to Spiegelman et al.[18].

DISCUSSION

The present results indicate that though only 13% of oligo(dG)$_{12-18}$ forms a complex with poly(C), up to 50% of the oligodeoxynucleotide migrates together with polynucleotide during centrifugation. More detailed considerations of the presumable behaviour of oligo(dG)$_{12-18}$ in solution permit us to elucidate this apparent inconsistency, and to form some estimate of the behaviour of a mixture of poly(C) and oligo(dG)$_{12-18}$, as revealed by physico-chemical and enzymic studies.

Ralph, Connors and Khorana[1] found that in 0.25 M phosphate buffer (pH 6.8) oligo(dG)$_3$ forms aggregates which fail to melt completely even at 95°C; Lefler and Bollum[2] observed that poly(dG) occurs in solution in the form of two-stranded or higher stranded structures resistant to thermal denaturation and alkali. In consequence, it can be assumed that oligo(dG)$_{12-18}$ also does not occur in a single-stranded form. Because of the length of the deoxyoligonucleotide chain, the lack of an effect of temperature on the structure of oligo(dG)$_{12-18}$, as shown in Fig. 1 IV, is well-founded. Rapid interactions between the oligo(dG)$_{12-18}$ molecules can interfere with complete formation of the secondary structure and may cause the maintenance of short one-strand deoxyoligonucleotide chains. It can be assumed that these chains represent the 13% of oligo(dG)$_{12-18}$ forming a complex with poly(C), and that they are responsible for the hypochromicity presented in Fig. 1.

Moreover, it can be expected that, in connection with the very strong interaction between deoxyguanylic and cytidylic polynucleotides

(T_m in 1 mM phosphate buffer containing 0.1 mM EDTA is 71°C[14]), chains much shorter than 12-18 G,C pairs are sufficient to bind oligo(dG)$_{12-18}$ with poly(C) during centrifugation in 0.1 M NaCl at 20°C. If it is borne in mind that an aggregate with a chain able to form complexes with poly(C) can comprise more than one oligo(dG)$_{12-18}$ chain, it becomes clear why the amount of oligo(dG)$_{12-18}$ sedimenting with the polynucleotide greatly exceeds that of deoxyoligonucleotide participating in the complex.

Analogous considerations are applicable to mixtures of oligo(dG)$_{12-18}$ with poly(C_m) or poly(C_e), though it is noteworthy that in these cases the extent of interactions between the components is even lower, as compared with poly(C). The available data do not clarify how this fact is related to the presence, in the polynucleotide, of the 2'-O-methyl- and 2'-O-ethyl-substituents, instead of the 2'-OH group. The previous failures to prepare complexes of poly(C_m) and poly(C_e) with poly(dI)[6,7] should be borne in mind.

The present suggestions about the type of the interactions between oligo(dG)$_{12-18}$ and poly(C) or its analogues are extremely relevant to the application of these compounds as primer•template for DNA polymerases. Consistently with the generally accepted characteristics of these enzymes, it is obvious that only the fractions of the double helices of oligo(dG)$_{12-18}$•poly(C), oligo(dG)$_{12-18}$•poly(C_m) and oligo(dG)$_{12-18}$•poly(C_e) having free 3'-OH ends of oligo(dG)$_{12-18}$, can react as primer•template. In fact, it can be anticipated that if the length of some of these double helices is smaller than the primer length required by DNA polymerase, then not even all of them will initiate the polymerization. Under these circumstances, quantitative considerations regarding the concentrations of templates, primer or 3'-OH ends of primer (e.g. the determination of the Michaelis constant) are meaningless.

The experimental finding that solutions of oligo(dG)$_{12-18}$ with poly(C), poly(C_m) or poly(C_e) stimulate the dGMP incorporation in the presence of DNA polymerase (in this study the RNA-dependent DNA polymerase of AMV virus) cannot conceal the fact that for the most part the oligo- and polynucleotide present in the reaction mixture does not occur in the form of a helical complex. The presence of oligo(dG)$_{12-18}$ aggregates can exert an effect on the process of binding of the primer•template with the active centre of enzyme, or on the course of polymerization. Moreover, the free polynucleotide may inhibit DNA polymerase.[7] Finally, it seems that a comparison of the results obtained for oligo(dG)$_{12-18}$ mixed with

poly(C), poly(C_m) or poly(C_e) with the results concerning the primer•template whose double helix structure is proven, e.g. poly(dT)•poly(A)[20] or poly(dI)•poly(C),[13] can lead to erroneous conclusions.

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[a]Abbreviations employed conform to those recommended by the Commission on Biochemical Nomenclature, IUPAC. In addition: poly(C_m), poly 2'-O-methylcytidylate; poly(C_e), poly 2'-O-ethylcytidylate; AMV, avian myeloblastosis virus; DTT, dithiothreitol.

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