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5'-Derivatives of oligonucleotides as primers of DNA polymerization catalyzed by AMV reverse transcriptase and Klenow fragment of DNA polymerase 1

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The K_m and V_{max} values for d(pT)$_8$ and its derivatives containing various 5'-end groups were estimated in the reaction of polymerization catalyzed with AMV-RT and FK. The change in affinity of modified primers was more pronounced in the case of AMV-RT than in the case of FK. Introducing in d(pT)$_8$ of intercalators such as phenazinium, ethidium and daunomycin residues results in 2.7-, 8.7- and 11-fold increases in the primer affinity to AMV-RT, respectively. However, in the case of hemin and cholesterol derivatives the K_m values were 3 and 5 times higher than those for d(pT)$_8$. Compared to d(pT)$_8$, the affinity of FK to all the above analogs was 2.3–3.6 times higher with the exception of cholesterol derivative to which it was 2.4-fold lower. The effect of the 5'-end residues on the V_{max} values of d(pT)$_8$ was small and ranged from 44% to 120% of that for d(pT)$_8$. Therefore such reactive derivatives of oligonucleotides can be used as effective primers of AMV-RT and FK. Possible reasons for various effects of the 5'-end residues of the primer on its interaction with FK or AMV-RT in the presence of poly(A) are discussed.

Klenow fragment; AMV reverse transcriptase; 5'-Derivatives of d(pT)$_8$; Affinity

1. INTRODUCTION

According to a general model of the template-primer interaction with DNA polymerases suggested in [1–2] the 3'-end of oligonucleotides makes a crucial contribution to the binding of DNA polymerases with primers. That is why the 5'-end is a convenient point for introducing various groups in order to prepare modified primers and use them as tools for the analysis of DNA replication by AMV-RT.

The efficiency of A-T and G-C base pairing in enzyme–template–primer complex is essentially lower ($\Delta G = -0.3$ to -0.5 kcal/mol) than that in solution ($\Delta G = -1.2$ to -2.4 kcal/mol) [1–8]. It is likely that the efficiency of complementary DNA interaction is decreased by the enzyme. Stabilization of DNA duplexes in solution may be achieved by introducing intercalating and hydrophobic groups into DNA chains [9–11]. Coupling the Chs, Dnm, Phe and Et residues to the 5'-end of d(pN)$_{7-8}$ increases melting temperatures of R-d(pT)$_{7-8}$ complexes with complementary chains by 1–3°C [12], 8–10°C [13], 15–20°C [14] and 20–25°C [15], respectively. The influence of introducing intercalating and hydrophobic residues to the 5'-end of d(pT)$_8$ on its catalytic efficiency and primer affinity was analysed.

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Abbreviations: FK, Klenow fragment of *E. coli* DNA polymerase 1; AMV-RT, reverse transcriptase from avian myeloblastosis virus; Et-, Phn-, Hem-, Chs-, Dnm-, derivatives of ethidium, *N*-(2-hydroxyethyl)-phenazinium, hemin, cholesterol and daunomycin in d(pT)$_8$ analogs, respectively

2. MATERIALS AND METHODS

Klenow fragment of DNA polymerase I ($3.7 \cdot 10^4$ U/mg) and AMV reverse transcriptase ($\alpha\beta$-form, 8–10 U/μl) were obtained according to [16,17] and were homogeneous as follows from electrophoresis data. Poly(dA) and BSA were from Sigma, poly(A) from Boehringer Mannheim, dTTP from NIKTI BAV (USSR), $MgCl_2$ from Merck, [^{3}H]-dTTP ($3 \cdot 10^3$ Ci/mol) from Izotop (USSR). Other reagents were of analytical grade.

Synthesis, characterization and methods of purification to homogeneity of d(pT)$_8$ and its derivatives were reported elsewhere [12–15].

The activity of AMV-RT and FK was determined at 37°C according to [18]. The reactio mixture (50–100 μl) for AMV-RT contained 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 1 mg/ml BSA, 60 μM [^{3}H]dTTP, 0.2 A_{260}/ml poly(A) and one of the primers. The reaction mixture for FK contained: 50 mM Tris-HCl buffer (pH 7.5), 0.2 mg/ml BSA, 3 mM KCl, 2.5 mM $MgCl_2$, 5 mM NaF, 0.6 A_{260}/ml poly(dA), 30 μM [^{3}H]dTTP and one of the primers. The primer concentrations were varied over a wide range. The reaction was started by adding FK or AMV-RT. Further treatment of the reaction mixtures was done as in [2–6] using a method of acid insoluble

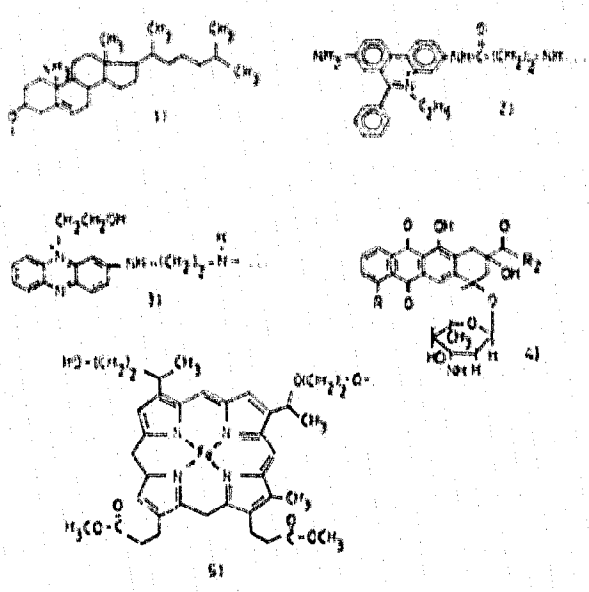

Fig. 1. Structure formulas of 5'-end radicals in d(pT)$_8$: 1, cholesterol (Chs); 2, ethidium (Et); 3, *N*-(2-oxyethyl)phenazinium (Phn); 4, daunomycin (Dnm); 5, hemin (Hem).

precipitates. The K_m and V_{max} values were determined according to Eisenthal and Cornish-Bowden [5,19]. Errors in K_m and V_{max} were within 20–40%.

3. RESULTS AND DISCUSSION

Structural formulas of R-residues attached to the 5'-end of d(pT)$_8$ are given in Fig. 1. The size of the residues is comparable with that of the nucleotide units of the primer. The introduction of such residues in the primer can lead to an increase in the primer's affinity due to formation of additional contacts of R with template or enzyme. At the same time, the affinity of the primer derivatives can decrease because of steric reasons. As follows from [2,20] the difference between the K_m and K_d values for the primers in the case of DNA polymerases is negligible, so that K_m may be used as a measure of their affinity. The effect of 5'-terminal R-residues of d(pT)$_8$ on the interaction of the primer with the enzyme and template was estimated using the K_m and V_{max} values for d(pT)$_8$ and its derivatives. Fig. 2 presents the dependencies of initial rates of the polymerization reaction on concentrations of d(pT)$_8$, Chs-d(pT)$_8$ and Phn-d(pT)$_8$ primers. The K_m and V_{max} values of all the investigated derivatives of d(pT)$_8$ are given in the Table.

Phn-, Dnm-, Et- and Hem-residues stabilize complexes of d(pT)$_8$ with poly(dA) and FK about 2.4–3.6-fold; a more pronounced stabilization (by a factor of 3–9) of the above complexes takes place in the case of AMV-RT. However the introduction in d(pT)$_8$ of cholesterol residues decreases the primer's affinity to both FK and AMV-RT by a factor of 2–3. According to [1,2] only the 3'-terminal nucleotide of primers interacts with DNA polymerases. All other nucleotide units of the primer interact only with the template. Extending of the oligonucleotide chain by 1 unit brings about 1.82- and 3-fold increases in the d(pT)$_n$ primer affinity to FK and AMV-RT, respectively. Therefore the stabilization of primer–template–enzyme complexes by 5'-end R-residues is similar to that caused by lengthening of the primer by 1–2 nucleotide units. The V_{max} values for both enzymes in the reaction of R-d(pT)$_8$

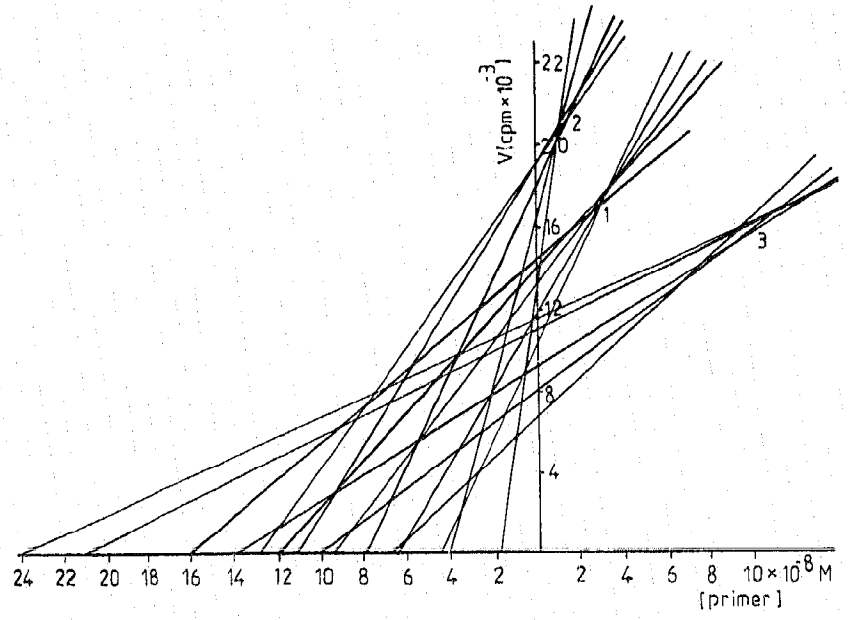

Fig. 2. Initial rate of polymerization catalyzed by AMV-RT versus concentrations of d(pT)$_8$ (1), Chs-d(pT)$_8$ (2) and Phn-d(pT)$_8$ (3) in the Cornish-Bowden and Eisenthal coordinates.

Table I

K_m and V_{max} values for d(pT)$_8$ and its analogs, determined in polymerization reaction catalyzed by FK and AMV-RT

Primer	FK		AMV-RT	
	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)
d(pT)$_8$	0.40	100	0.033	100
Phn-d(pT)$_8$	0.14	102	0.012	114
Dnm-d(pT)$_8$	0.11	110	0.003	107
Et-d(pT)$_8$	0.17	105	0.0038	120
Chx-d(pT)$_8$	0.91	34	0.1	84
Hem-d(pT)$_8$	0.16	70	0.15	115

elongation are equal to 44–120%, compared with V_{max} for d(pT)$_8$. Such little effects of various residues of R-d(pT)$_8$ on the V_{max} values suggest that there is no strong interaction of their R-radicals with the enzymes. Therefore such kind of derivatives can be used as efficient substrates of these enzymes.

It is interesting to compare the effects of various R-residues in solution and in complexes with the enzymes. ΔG for A·T paring on polymerases (-0.35 kcal/mol) and in solution (-1.2 to -1.3 kcal/mol) also show that the interaction between complementary bases of DNA within a complex with the enzyme is by 2–3 orders of magnitude less efficient than in solution. All above data on the template interaction with primers and their analogues in solution and on enzymes correlate rather well.

The results obtained demonstrate for the first time that the interaction of template and primer in a complex with the DNA polymerases and reverse transcriptases may be stabilized by attachment of intercalating residues to the 5′-phosphates of oligonucleotides. Such kind of oligonucleotide derivatives can be efficient tools for the further analysis of the template-primer interaction with these enzymes.

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