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Modified polynucleotides. IV. Template activity of 5-alkyluracil-containing poly[d(A-r^{5}U)] copolymers for DNA and RNA polymerases

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ABSTRACT

The change of templating properties for E. coli DNA and RNA polymerases of poly[d(A-r^{5}U)] copolymers on substitution of the uracil for 5-methyl-, 5-ethyl-, 5-n-propyl-, 5-n-butyl- or 5-n-pentyluracil, resp., was studied. All modified polymers proved to be more effective template-primers than poly[d(A-U)] or poly[d(A-T)] for the DNA polymerase. Direct dependence of the template-primer efficiency (v_{max}/K_m) value on the thermal stability of the polymers was observed. For transcription the r = ethyl and n-propyl polymers were also more effective templates than poly[d(A-T)].

INTRODUCTION

For an examination of DNA polymerase substrate specificity and the dependence of properties of DNA on modification of its components we performed model structure-activity relationship studies. This included the study of incorporation of r^{5}dUTPs[1] into polydeoxynucleotides by enzyme[2,3,4], dependence of incorporation rate on the type of DNA polymerase used[5] and the study of physical (thermal stability)[6] and biochemical properties (resistance to different nucleases)[7] of the modified polymers, the poly[d(A-r^{5}U)]s prepared by DNA polymerase, where r was a hydrogen atom, or a methyl, ethyl, n-propyl, n-butyl or n-pentyl group, resp. Results of the experiments showed close correlation with known physical and biochemical properties of some naturally occuring modified (bacteriophage) DNas[8,9]. As part of these studies we present here results of the template properties of poly[d(A-r^{5}U)]s for enzymatic ribo- and deoxyribopolynucleotide synthesis.

EXPERIMENTAL

E. coli DNA polymerase I large fragment enzyme (6900 units/mg), RNA polymerase from E. coli MRE 600 (1200 units/mg), bovine pancreatic DNase, dTTP and dATP were from Boehringer, UTP and ATP were from Reanal (Hungary). [^{3}H]dATP (17 Ci/mmol) was from New England Nuclear and [^{3}H]ATP (27 Ci/mmol)

was from The Radiochemical Centre, Amersham.

Poly[d(A-r^{5}U)] copolymers, including poly[d(A-T)] and poly[d(A-U)] were prepared according to our earlier paper[2] with the large fragment enzyme taken from the same tube. Copolymers excluded by gel filtration on a Sephadex G-200 column were only used for the template experiments. Comparison of the molecular weight of the polymers was carried out by " half" end-group determination (see below) with [^{3}H]dATP. Concentration of the polymers are expressed in nucleotide units, μM(P), using the ε(P) values of the polymers[7].

DNA polymerase reactions were carried out in 60 mM phosphate buffer (pH 7.4), 6 mM $MgCl_2$, 1 mM 2-mercaptoethanol using 0.25 mM of [^{3}H]dATP (10.8 dpm/pmol), 0.25 mM of dTTP, 0-350 μM(P) of the poly[d(A-r^{5}U)]s and 0.065 μg of the enzyme (lacking 5'$\rightarrow$3' exonuclease activity) in a final volume of 82.5 μl. Reactions were started by addition of $MgCl_2$ to the preincubated mixtures at 37^{o}C and after 30 min. 50 μl-s of the mixtures were spotted onto GF/C filters (Whatman), precipitated in 5 % TCA, washed, dried and counted. Reproducibility of the results was $\pm$2 to $\pm$6 %. Slopes of s/v - s plots were calculated by the linear regression method of least squares.

RNA polymerase reactions were carried out in 40 mM TRIS.HCl (pH 7.9), 150 mM KCl, 10 mM $MgCl_2$, 0.4 mM KH_2PO_4, 0.1 mM EDTA, 0.1 mM dithiotreitol, 0.5 mg/ml BSA, 130 μM [^{3}H]ATP (6.8 dpm/pmol), 130 μM UTP, 0-500 μM(P) of poly[d(A-r^{5}U)]s and 4 μg of the enzyme in a final volume of 82.5 μl. Reactions were started by addition of enzyme to the preincubated mixture at 37^{o}C and after 10 min. 50 μl-s of the mixtures were taken and processed as above. Results were reproducable $\pm$2 to $\pm$10 %.

Activation of polymers up to their maximal activity was carried out in 60 mM phosphate buffer (pH 7.4), 6 mM $MgCl_2$, reacting 20 nmol(P) of the polymers with 0.25 μg of pancreatic DNase at 37^{o}C for 15 min. in a final volume of 66 μl. Enzyme was denatured at 78^{o}C for 5 min.

End-group determination of poly[d(A-r^{5}U)]s was carried out under conditions described above for DNA polymerase assay. Differences were: no dTTP was present and specific activity of [^{3}H]dATP and amount of enzyme were increased (182.7 dpm/pmol and 0.13 μg, resp.) and 200 μM(P) of polymer concentration was used. In 30 min. of incubation 3.0, 3.5, 2.4, 2.4, 1.5 and 3.2 pmol of [^{3}H]dAMP were incorporated into the 10.0 nmole(P) of $\underline{r}$ = h, me, et, pr, bu and pe, resp., polymers. Assuming that every second polymeric chain was terminated by r^{5}dUMP, number of nucleotides per polymeric molecule

were 1400-3300.

RESULTS AND DISCUSSION

Template activity for DNA polymerase

Comparison of [^{3}H]dAMP incorporation rates on poly[d(A-r^{5}U)] template analogues of 0-350 μM(P) concentration is presented on Figure 1. /Concentrations are expressed as molar(P)./

Highest activity was displayed by the r = pr, bu and pe copolymers, it was 2-2.5 times higher at 200 μM(P) than that of the poly[d(A-T)]. At lower concentrations differences were even higher: at 25 μM(P) relative activities were 3.6-6 fold.

Kinetic parameters were also calculated, namely from s/v - s plotting[10] of the data (Fig. 2 and Table 1). Modified polymers had much lower Michaelis constants than poly[d(A-T)], especially when molecular weights, taken from end-group determinations, were taken into account. The

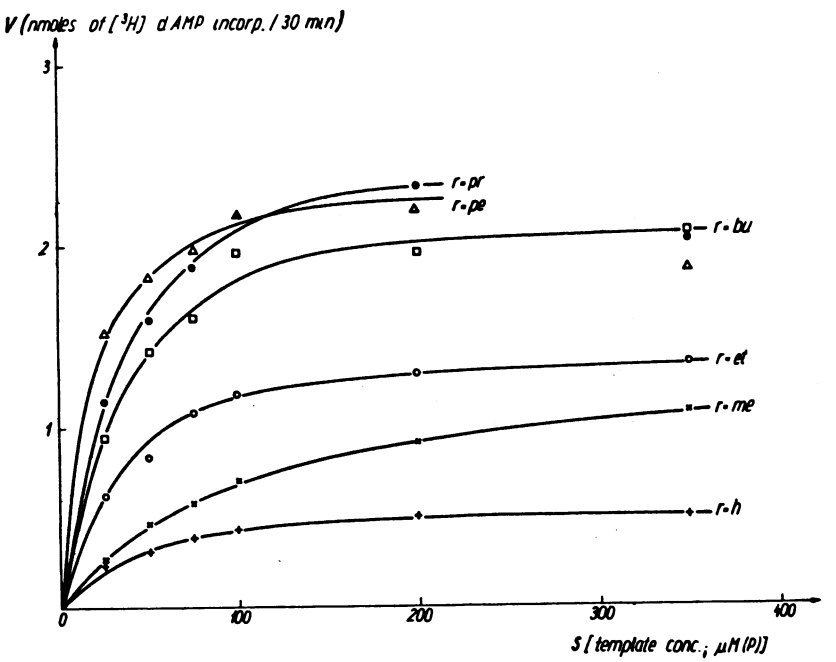

Figure 1. Template activity of poly[d(A-r^{5}U)] copolymers for E. coli DNA polymerase I large fragment enzyme

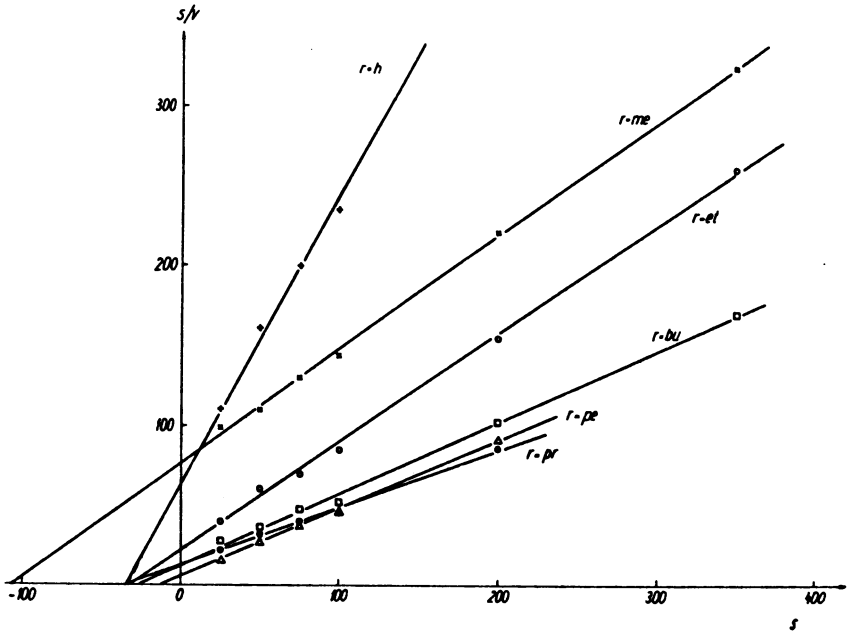

Figure 2. s/v - s plotting of the data presented in Fig. 1

Table 1. Kinetic parameters of poly[d(A-r^{5}U)] copolymers as template primers in DNA polymerase reactions

r in poly[d(A-r^{5}U)]	n	K_m a. molar [μM(P)]	K_m b. molecular [nM(P)]	v_{max} (nmol of [^{3}H]dAMP incorp. in 30 min.)	v_{max}/K_m(b)x10^2
h	0	34.8	21	0.56	2.67
me	1	107.0	75	1.40	1.87
et	2	31.6	15	1.48	9.87
pr	3	34.3	16.5	2.76	16.7
bu	4	28.0	8.4	2.26	26.9
pe	5	13.3	8.5	2.37	27.9

v_{max} values of r = pr, bu and pe polymers were close to each other and were 1.5-2 times higher than that of the r = me polymer. The v_{max}/K_m value, substrate efficiency of poly[d(A-r^{5}U)] seems to depend on the size of the alkyl substituent, i.e. on the number of carbon atoms (n) in the r substituent of the polymer. Poly[d(A-bu^{5}U)] and poly[d(A-pe^{5}U)] proved to be the most effective template-primers, the v_{max}/K_m values were 14-15 times as high as that of the poly[d(A-T)].

Earlier we observed an other function of the n, it was the thermal stability (T_m) of these polymers[6]. In this way v_{max}/K_m and T_m values can be interrelated: $v_{max}/K_m = 1/(k_1/T_m + k_2)$ for the n = 0-5 copolymers (Fig. 3.) This correlation can be obtained with T_m series measured at any

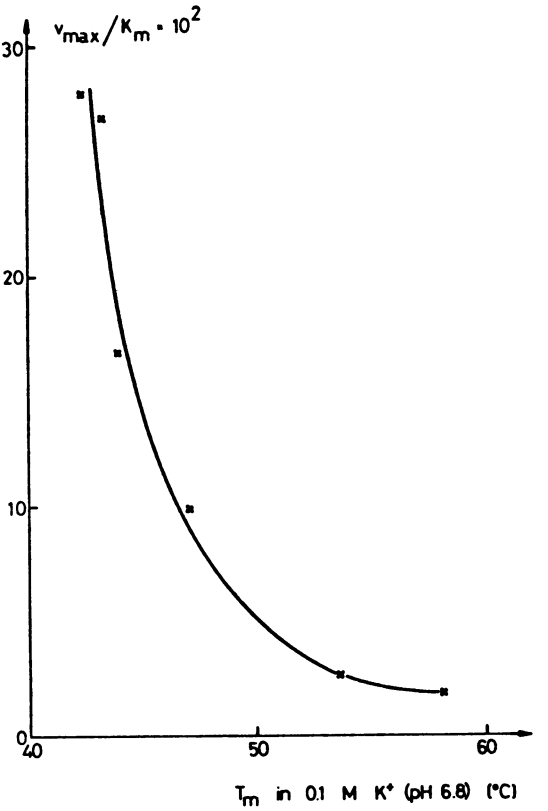

Figure 3. Dependence of template efficiency values (v_{max}/K_m) of poly[d(A-r^{5}U)]s on the thermal stability (T_m) of the polymers

salt concentration in the range of 0.01 - 0.2 M K^+, or with ΔT_m values, where ΔT_m = 0 in the case of poly[d(A-T)]^6. According to the data the lower the thermal stability the greater the template-primer efficiency value in this series of copolymers. The physical background may be that the lower thermal stability of the longer alkyl chain-substituted analogues is more preferable for the funtioning enzyme-template-primer complex and also unwinding of the hairpin duplex in 5'→3' direction needs less energy.

Activation of the copolymers by pancreatic DNase increased the template activities at 100 μM(P) concentrations up or over to the v_{max} values of the unactivated polymers (Table 2). The differences in relative rates almost disappeared, they were ±20 % of the activity of poly[d(A-T)]. This may be the result partly of the creation of new free 3'-OH ends and partly of the increased flexibility (which also derives from nicking and degradation) of the molecules: template activity of the activated r = h, me and et polymers were much higher than the v_{max} value of the corresponding unactivated polymers. In this way the r = pr, bu and pe polymers have, already in their unactivated forms the structural or conformational parameters which are optimal for dAMP and dTMP incorporation by the E. coli DNA polymerase I large fragment enzyme.

Template activity for RNA polymerase

Template activity of poly[d(A-r^{5}U)]s for E. coli RNA polymerase showed a different pattern of substitution dependence from that observed for DNA

Table 2. Template activity of 100 μM(P) pancreatic DNase-activated poly[d(A-r^{5}U)] copolymers for E. coli DNA polymerase and RNA polymerase enzymes

r in poly[d(A-r^{5}U)]	DNA polymerase nmoles of [^{3}H]dAMP incorp. in 30 min.	RNA polymerase nmoles of [^{3}H]AMP incorp. in 10 min.
h	2.66	0.17
me	2.74	0.14
et	3.29	0.26
pr	2.93	0.16
bu	3.03	0.12
pe	2.36	0

polymerase (Fig. 4). Poly[d(A-et^{5}U)] and poly[d(A-pr^{5}U)] also proved to be more effective templates than poly[d(A-T)], but poly[d(A-bu^{5}U)], with the exception of its low concentration value was less effective, whereas poly[d(A-pe^{5}U)] was almost without activity. Poly[d(A-U)] had only 10 % relative activity compared to poly[d(A-T)]. The template of transcription is no substrate of the RNA polymerase in the classical sense therefore no kinetic parameter was calculated. Nevertheless, plotting of relative transcription rate againts the n of poly[d(A-r^{5}U)] at different template concentrations shows well the structure-dependence of template activity (Fig. 5). Activation of the polymers, except for poly[d(A-U)], decreased [^{3}H]AMP incorporation rates (Table 2).

It may be concluded from the results that better templates than the polymers of natural nucleotides can be prepared for both DNA and RNA polymerases. Enzymatic DNA and RNA synthesis has, however, different dependences on the structural modification of the polydeoxynucleotide templates. In the DNA polymerase reaction thermal stability of the polymers

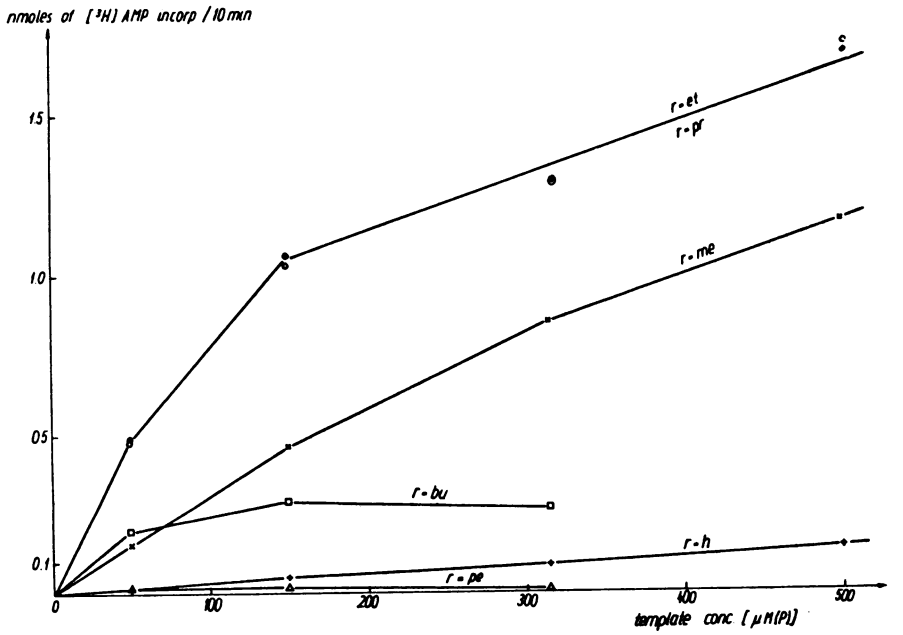

Figure 4. Template activity of poly[d(A-r^{5}U)] copolymers for E. coli RNA polymerase enzyme

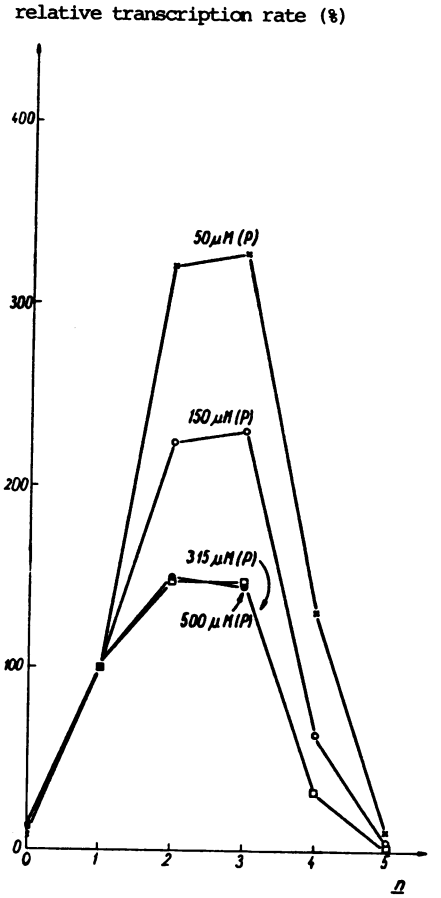

Figure 5. Dependence of relative template activity of poly[d(A-r^{5}U)]s for the RNA polymerase enzyme on the number of carbon atoms (n) in the r substituent; Data are taken from Fig. 4 and activity values of poly[d(A-T)] at different concentrations were taken to be 100 %

may be an important factor in template-primer efficiency.

ACKNOWLEDGEMENT

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REFERENCES

1. Abbrevations: r^5dUTP stands for 5-alkyl-dUTP; two-letter symbols were used for 5-alkyl substitutions of uracil in poly[d(A-r^5U)], where r = alkyl; me, et, pr, bu and pe stand for methyl, ethyl, n-propyl, n-butyl and n-pentyl group, resp., h stands for hydrogen atom; n is the number of carbon atoms in the r substitutent.
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