Mutant T7 RNA polymerase is capable of catalyzing DNA primer extension reaction

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Abstract The mutant T7 RNA polymerase (T7 RNAP), containing two substitutions (Y639F, S641A) was earlier shown to utilize both rNTP and dNTP in a transcription-like reaction. In this report the ability of the enzyme to catalyze DNA primer extension reaction was demonstrated. The efficiency of the reaction essentially depended on the type of the primer sequence, and was significantly higher if the primer coincided with the T7 promoter non-coding sequence. In this case the primer extension reaction proceeded along with de novo RNA synthesis. The length of the product did not exceed 8 nucleotides, indicating that the primer extension reaction proceeds according to the mechanism of the T7 RNAP-catalyzed abortive transcription.

Key words: Bacteriophage T7 RNA polymerase; Mutant; Primer extension

1. Introduction

Main differences between DNA- and RNA-polymerase-catalyzed reactions are not only the enzyme specificity toward dNTPs and rNTPs but also the structural organization of template and product and the output mode of the latter. DNA-polymerase reaction is primer-dependent with single-stranded DNA and double-stranded DNA as template and product, respectively. The reaction ceases after a single cycle of polymerization and the product yield is limited by concentrations of dNTPs and a template [1]. In contrast, RNA polymerase reaction is promoter-dependent, the template and product are double-stranded DNA and single-stranded RNA, respectively. The template can be used repeatedly and the product yield is limited only by rNTP concentrations [2].

Recently we described the T7 RNA polymerase mutant capable to utilize both rNTP and dNTP as substrates and to synthesize mixed ribo-/deoxy-containing single-stranded polynucleotides in the transcription-like reaction [3,4]. The incorporation of deoxynucleotides into the nascent chain proceeds rather efficiently in positions +7 and further after the transcription start when T7 RNA polymerase overpasses the stage of abortive transcription (7–10 nucleotides). The incorporation of deoxynucleotides in positions 1–6 is hampered. Besides these stages are accompanied by misincorporations in the respective positions when a 'canonical' dNMP is replaced by wrong rNMP (usually G to A) [4].

A number of papers described the attempts to use the wild-type T7 RNA polymerase in the template/primer systems. The de novo synthesis of oligoribonucleotides was moderately efficient only when complementary regions of the template and primer contained T7 promoter sequences. The products obtained usually did not exceed 20–30 nucleotides (nt) and were shown to have the 5′-end sequence complementary to that of the template. No primer extension reaction in the 'DNA-polymerase-like' manner was demonstrated [5,6].

Some scattered and rather conflicting data exist concerning the T7 RNAP ability to carry out the 'promoterless' primer-dependent RNA synthesis. Thus, the RNA primer extension (with DNA or RNA templates) was demonstrated for various model systems [7–9]. In contrast to numerous sources [5–11], only two papers reported the DNA primer extension by T7 RNAP [12,13]. In both cases the experimental conditions considerably differed from those usually applied; extremely long incubation time and large concentrations of substrates and enzymes were used.

As mentioned above, mutant T7 RNAP [3,4] is capable of utilizing dNTPs as substrates. It seemed intriguing to find out whether this property of mutant T7 RNAP affects its interaction with template or, in other words, whether the mutant is capable to synthesize oligonucleotides in the 'DNA-polymerase' manner.

2. Materials and methods

Unlabeled rNTPs and dNTPs and RNasin ribonuclease inhibitor were purchased from Promega or Boehringer. Radioisotopes were obtained from Amersham.

The deoxyribooligonucleotides were synthesized on a DNA Synthesizer ASM-102V (Biosan Ltd., Russia) by the phosphoamidite method [14] and purified on the Hypersil ODS 5-mm column (4.6×250 mm) in 0–50% acetonitrile gradient in 0.1 M ammonium acetate. 5′-^{32}P-end-labeling of oligos was carried out with T4 polynucleotide kinase according to [15].

Wild-type T7 RNAP and its mutant form (Y639F, S641A) [3] were purified as described [4,17]. To analyze the T7 RNAP-catalyzed reaction, two assay systems were used:

1. Assay mixture (10 μl) contained 50 mM Tris-HCl, pH 7.8; 20 mM $MgCl_2$; 10 mM DTT; 0.1 mg/ml BSA; 200 U/ml RNasin; 0.05 μg wild-type or mutant T7 RNAP; 2×10^6 cpm of [α-^{32}P]ATP or [α-^{32}P]GTP, and variable concentrations of template/primer and/or rNTP/dNTP.
2. The same assay mixture containing no [α-^{32}P]NTP and variable concentrations of template/5′-^{32}P-end-labeled primer.

The samples were incubated for 30 min at 37°C. The reactions were stopped by addition of 4 μl of sample buffer (10 ml of formamide containing 1 mg/ml each of bromophenol blue and xylene cyanol, 1 mM EDTA), heated for 5 min at 95°C and subjected to PAGE in 20% polyacrylamide-7 M urea gels in TBE buffer [15]. After electrophoresis the gels were dried and subjected to autoradiography.

For quantitative evaluation of primer extension reaction and for the determination of apparent K_m values for templates/primers or

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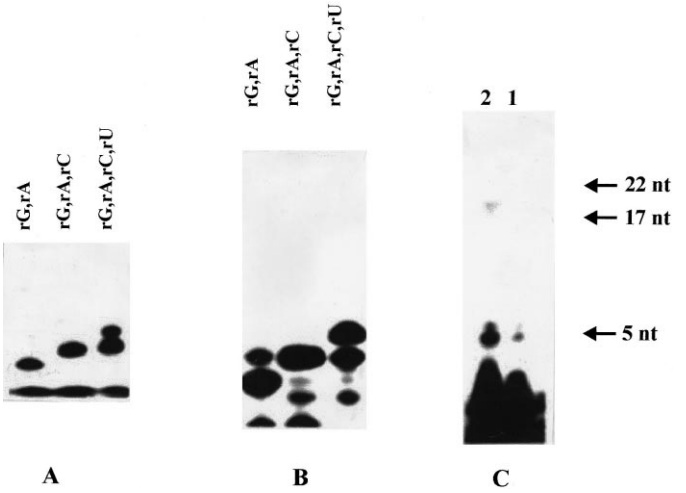

Fig. 1. RNA synthesis on the different templates/primers. A: Wild-type enzyme, template/primer I; B: wild-type enzyme, template/primer IIa. C-mutant enzyme, template/primers I (line 1) and IIa (line 2). Incubation mixture contained various compositions of rNTPs, as indicated. The positions of oligonucleotide markers are indicated by arrows. Labeled substrate [α-^{32}P]ATP.

r/dNTPs, the respective autoradiography patterns were scanned with FAST SCAN 300A densitometer (Molecular Dynamics). The apparent K_m values were calculated using the MICROCAL ORIGIN 3.5 program (MicroCal Software).

Templates/primers used:

I - 3′-ATTATGCTGAGTGATATCCTGA
5′-TAATACGACTCACTATAGGACT

a synthetic DNA duplex with blunt ends containing the T7 RNAP consensus promoter and complementary 5 nt long message

IIa - 3′-ATTATGCTGAGTGATATCCTGA
5′-TAATACGACTCACTATA

a synthetic DNA duplex with 5′ overhang containing the T7 RNAP consensus promoter and 5 nt long message corresponding to the coding strand

IIb - 3′-ATTATGCTGAGTGATATCCTGA
5′-TAATACGACTCACTATAG

a synthetic DNA duplex analogous to IIa except the promoter sequence non-coding strand contained 3′-terminal dGMP corresponding to the 5′-end nucleotide of the message

IIIa - *pTZ18R*---ATTATGCTGAGTGATATCCCTT----*pTZ18R*
5′-TAATACGACTCACTATA

a single-stranded form of plasmid pTZ18R containing the T7 promoter coding strand annealed with complementary synthetic deoxyoligonucleotide primer corresponding to the noncoding strand

IIIb - *pTZ18R*---ATTATGCTGAGTGATATCCCTT----*pTZ18R*
5′-TAATACGACTCACTATAG

the template/primer analogous to IIIa except the primer contained 3′-terminal dGMP corresponding to 5′-end nucleotide of the message

IV - *pSWT*------GGCTACTCTTGTGACCACTTTAGA----- *pSWT*
5′-CCGATGAGAACACTGGTG

a single-stranded form of plasmid pSWT (the derivative of plasmid pSELECT (Promega) [16], annealed with complementary synthetic deoxyoligonucleotide non-containing promoter sequence.

Thus, I corresponded to 'normal' condition for T7 RNAP reaction; II–III mimic 'DNA polymerase' conditions and simultaneously contain T7 promoter sequences, and IV represents 'pure' template/primer system lacking T7 promoter.

Isolation of plasmids and single-stranded circular DNA as well as annealing with respective oligonucleotides were performed according to protocols [15,17].

3. Results and discussion

Fig. 1 demonstrates the results of experiments with synthetic promoters (I–II) and ^{32}P-labeled ATP. As can be seen, in the case of wild-type T7 RNAP only short oligonucleotides of 3–5 bases (depending on the set of rNTPs added) were formed and no detectable primer extension was revealed (Fig. 1A,B). These data suggest that in the presence of promoter in the duplexes both with the blunt and sticky ends only de novo synthesis is manifested. This observation is in accordance with other results [5,6].

When mutant T7 RNAP was used with the blunt-end duplex I, identical short products were obtained (Fig. 1C, line 1). With the sticky-end duplexes II, in addition to the predom-

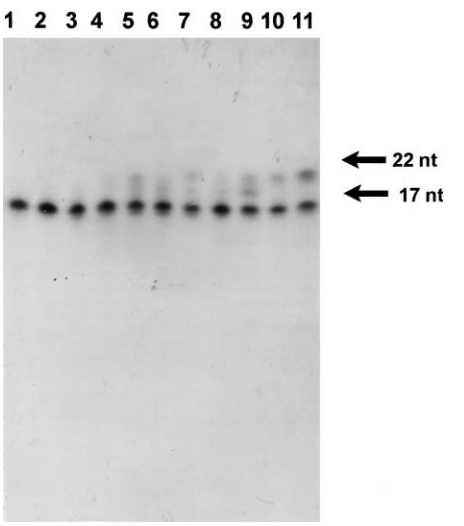

Fig. 2. Mutant T7 RNAP-catalyzed reaction (5′-^{32}P-end-labeled primer assay) with template/primer IIa. Lines: 1, control; 2, wild-type T7 RNAP+4 rNTPs; 3–11, mutant T7 RNAP+following combinations of NTPs: rG,rA (3); rG,rA,rC (4); rA,rC,rU (5); rG,rC,rU (6); rG,rA,rC,dT (7); rG,rA,dT,dC (8); dG,rA,rC,rU (9); rG,dA, rC,rU (10); rG,rA,rC,rU (11). The arrows denote the lengths of oligonucleotide fragments.

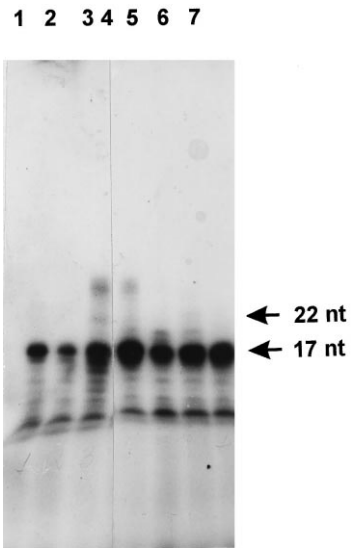

Fig. 3. Mutant T7 RNAP-catalyzed reaction (5′-^{32}P-end-labeled primer assay) with template/primer IIIa. Lines: 1, control; 2, wild-type T7 RNAP+4 rNTPs; 3–7, mutant T7 RNAP+following combinations of NTPs: rG,rA,rC,rU (3); rG,rA,rC,dT (4); rG,dA,dT,dC (5); dG,rA,dT,dC (6); dG,dA,dC,dT (7). The arrows denote the lengths of oligonucleotide fragments.

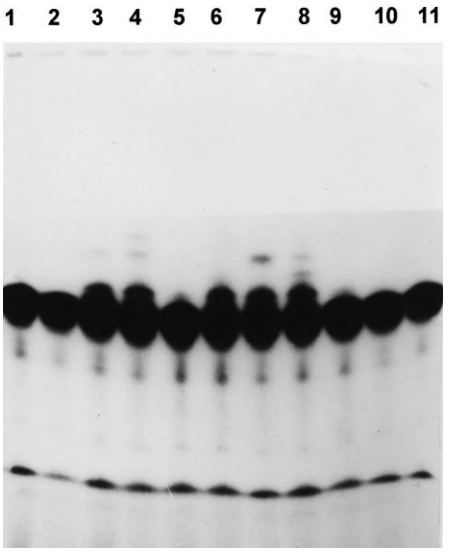

Fig. 4. Mutant T7 RNAP-catalyzed reaction (5′-^{32}P-end-labeled primer assay) with template/primer IV. Lines: 1, control; 2, wild-type T7 RNAP+4 rNTPs; 3–10, mutant T7 RNAP+following combinations of NTPs: rG,rA,rC (3); rG,rA,rC,rU (4); rG,rA,rC,dT (5); rG,rA,dC,rU (6); dG,rA,rC,rU (7); rG,dA,rC,rU (8); rG,dA,dC,dT (9); dG,dA,dC,dT (10).

ination of the de novo synthesis products, oligonucleotides of about 20 nt were also detected (Fig. 1C, line 2). The most feasible explanation of this fact is a primer extension in a 'DNA polymerase-like' manner. To confirm this assumption, the experiments with 5′-^{32}P-end-labeled primers were performed. Fig. 2 clearly demonstrates the primer extension and hereby the formation of the hybrid product, deoxyoligonucleotide containing 4–5 ribo-links at the 3′-end. The presence of the first 'readable' nucleotide (as in IIb) did not affect the pattern (data not shown). The change of rNTPs to dNTPs (lines 7–10) resulted in a decrease in the yields of both transcribed and extended oligonucleotides, thus confirming our earlier observations [4].

With the use of the sticky-end duplexes of type II the length of extended fragment was controlled by the length of coding chain and should not exceed 4–5 nucleotides. To determine the maximal possible length of such a fragment the single-stranded circular pTZ18R DNA annealed with the same deoxyoligonucleotide primers was used (IIIa,b). However, Fig. 3 demonstrates that in this experiment the length of the transcript again does not exceed 7–8 nucleotides.

For duplexes I–III apparent K_m values for templates/primers were determined (Table 1). In the case of de novo synthesis K_m values for template/primer for the mutant rather exceeded that for the wild-type enzyme. For mutant T7 RNAP-catalyzed primer extension reaction the K_m values raised substantially in comparison to that for de novo synthesis. However, in spite of reliable distinctions between these constants, their values lie in the same range; so apparently there are no reasons to consider these differences as important.

The determination of K_m values for each NTP was hampered by the substantially increased misincorporation caused by the Y639F, S641A mutations in the T7 RNAP [4]. Therefore to compare the affinities of NTPs in the de novo and primer extension reactions, a single 'joint K_m', resulting from the varying total concentrations of four NTPs taken in equal quantities was calculated. This joint K_m in general characterizing the enzyme affinity towards nucleotide substrate is again higher for the mutant than for the wild-type enzyme, and it is higher in the primer extension reaction than in the de novo synthesis (Table 1).

The ability of T7 RNAP to carry out the 'promoterless' primer extension reaction was tested with the template/primer IV (see Section 2). Fig. 4 shows that the wild-type T7 RNAP did not utilize this template/primer, whereas the use of the

Table 1
K_m values (μM) for de novo synthesis and primer extension reactions catalyzed by the wild-type and mutant T7 RNAPs

Substrate	Type of the reaction/enzyme			
	De novo synthesis		Primer extension	
	Wild-type	Mutant	Wild-type	Mutant
Template/primer I	0.3 ± 0.1	1.4 ± 0.4	–	–
Template/primer II	0.6 ± 0.2	1.5 ± 0.4	–	4.8 ± 1.1[a] 7.3 ± 0.4[b]
Template/primer III	–	–	–	7.9 ± 1.9[b]
4 rNTPs[c]	1050+250[b]	3200+800[b]		8200+750[b]

[a][α-^{32}P]ATP assay (assay system 1, see Section 2).
[b]5′-^{32}P-end-labeled primer assay (assay system 2).
[c]'Joint K_m' (see text).

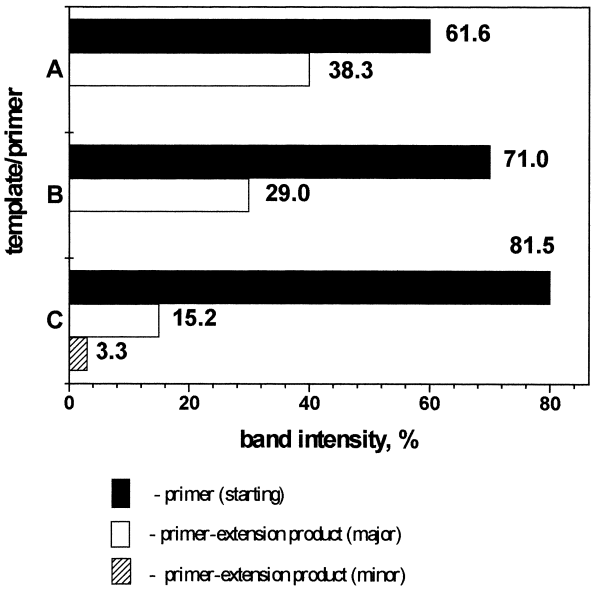

Fig. 5. Relative efficiencies of primer extension reaction. Bars correspond to the intensity of the respective bands from autoradiographs. A: template/primer II (Fig. 2, line 11); B: template/primer III (Fig. 3, line 3); C: template/primer IV (Fig. 4, line 4).

mutant enzyme resulted in a moderate extension of the primer.

Fig. 5 summarizes the results of the mutant T7 RNAP-catalyzed primer extension reactions with different templates/primers. Type II duplexes were the most efficient templates/primers (40% of total radioactivity) leaving behind that of type III (30%). In the 'promoterless' system (IV) the products with one/two 'additional' links constituted about 15% of the radioactivity while the total yield of longer products did not exceed 3%.

It should be noted that promoter-driven primer extension reaction shows the same regularities of dNTP utilization as in the case of de novo synthesis, i.e. incorporation of dGTP is negligible and that of dATP is slender, whereas the pyrimidine nucleotides are readily incorporated [4]. On the contrary, in the 'promoterless' primer extension reaction the incorporation of dTMP was very low while that of dGMP and dAMP was rather fair. This fact is in accordance with our observations that the efficiency of transcription with dNTPs by T7 RNAP mutant depends strongly on the 5′-end sequence of the message [18,19].

This study demonstrates that Y639F, S641A mutations result in a substantial increase in the ability of the enzyme to carry out the primer extension reaction. This process is more efficient when the sequence of the primer coincides with that of the non-coding strand of T7 promoter (templates II, III). The mutant T7 RNAP which is capable to incorporate dNMP links into nascent polynucleotide chain, apparently recognizes the 3′-end of the primer as the attribute of the synthesized product and so continues the reaction.

The data obtained demonstrate that the primer extension product does not exceed 8 ribonucleotide links, independently of the template length. This observation can be explained by the proposed mechanism on T7 RNAP initiation [20] postulating that when the length of the nascent chain is less than 8–10 nucleotides the enzyme/template/product complex is unstable and breaks down readily. The further extension of the product results in additional binding of the latter to the N-terminal domain of the enzyme accompanied by the conformational change and the transition of the complex into a more stable form resulting in the progressive elongation of the product. In the case of the 'DNA polymerase-like' primer-extension reaction described here, when the product is bound to the template and the randomly coiled 5′-end of the product is absent, the fixing of the nascent chain on the enzyme is hardly possible. So, the reaction does not achieve the stable elongation mode and, as the tendency to abort prevails, the length of the product is restricted to 8 nucleotides.

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