Mutants of T7 RNA polymerase that are able to synthesize both RNA and DNA

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Abstract A mutant T7 RNA polymerase (T7 RNAP) having two amino-acid substitutions (Y639F and S641A) is altered in its specificity towards nucleotide substrates, but is not affected in the specificity of its interaction with promoter and terminator sequences. The mutant enzyme gains the ability to utilize dNTPs and catalyze RNA and DNA synthesis from circular supercoiled plasmid DNA. DNA synthesis can also be initiated from a single stranded template using a DNA primer. Another T7 RNAP mutant having only the single substitution S641A loses RNA polymerase activity but is able to synthesize DNA.

Key words: T7 RNA polymerase; Mutagenesis; dNTP utilization; DNA polymerizing activity

1. Introduction

Recent crystallographic studies have revealed that, in spite of their apparent sequence dissimilarities, such diverse polymerases as *E. coli* DNA polymerase I Klenow fragment (KF) [1], HIV-1 reverse transcriptase (HIV RT) [2] and T7 RNAP [3] share similar structural elements required for the same catalytic functions of template-directed nucleic acid polymerization (reviewed in [4]). Most of the amino-acid residues that are essential for a catalysis are arranged in three well-conserved sequence motifs (A, B, and C as defined by Delarue et al. [5]), and are found in the majority of DNA-dependent DNA and single-subunit RNA polymerases. For T7 RNAP, five of the invariant residues (D537, D812, K631, Y639, and G640) cluster to form a putative catalytic pocket within the larger template-binding cleft [3]. The last three residues mentioned above are found in motif B and it has been suggested that this structural element may be in association with the template strand [3,6,7]. There is also evidence suggesting that this motif B may be implicated in NTP binding in both T7 RNAP [6–12] and KF [4,12–15].

Mutations of Y766 in KF (which is analogous to Y639 in T7 RNAP) have been reported to influence the fidelity of dNTP insertion [12–15] and affect the discrimination between deoxy and dideoxy nucleotide substrates [4]. In considering the structure of T7 RNAP in the region of Y639 (Fig. 1), we noted that in RNA polymerases this motif is characterized by a unique distribution of hydroxyl-containing amino-acid residues. Specifically, a serine residue is present at position 641 in T7 RNAP (and at corresponding positions in related RNAPs), while in DNA polymerases (DNAP) no such regularity is observed [5]. As S641 is the hydroxyl-bearing amino-acid residue closest to Y639 we have asked whether the hydroxyl groups of these two residues may be involved in the interactions of enzyme with NTP and, specifically, in discrimination between dNTP and rNTP as potential substrates. To test this, we have generated mutant enzymes with phenylalanine in place of tyrosine at position 639, alanine in place of serine in position 641 and a double mutant bearing both of these substitutions. The substrate specificity and other features of the latter two proteins were found to be quite surprising.

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2. Materials and methods

Oligonucleotide-directed mutagenesis was carried out according to [16]. The mutations were verified by dideoxy-sequencing. Wild-type T7 RNAP and mutants were purified as in our previous work [17].

The activity of wild-type T7 RNAP and mutants on pGEMT template (pGEMT (3.3 kbp) is the derivative of the plasmid pGEM4 (Promega) obtained by the insertion of a *KpnI–PvuII* fragment (0.53 kbp) of phage T7 DNA containing terminator TΦ [17]) was assayed in 40 mM Tris-HCl buffer (pH 7.8) plus 10 mM $MgCl_2$ (Buffer A) for 45 min (37°C). Samples (20 μl) contained 0.25 mM of each four rNTPs or dNTPs, 5×10^5 cpm [α-^{32}P]ATP or [α-^{32}P]dATP, 20 μg/ml pGEMT, 0.05 μg enzyme. Reactions products were precipitated with 0.1 volume of 7.5 M ammonium acetate plus 2.5 volumes of ethanol and left overnight at −20°C. Precipitates were resuspended in the mixture (16 μl) containing 1.6 μl of 0.1 M Na-phosphate buffer (pH 7.0), 8 μl dimethylsulfoxide, 2.7 μl of 6 M glyoxal and 3.7 μl water, incubated for 1 h (50°C) and subjected to 2% agarose gel electrophoresis according to [18].

The assay conditions for DNA polymerase activity of mutant T7 RNAPs were the same except ssDNA/primer (200 μg/ml) was used instead of pGEMT. ssDNA/primer was prepared by annealing a synthetic 18mer oligodeoxynucleotide (5′-GGCACTGGCTGGTCAATG-3′) to the single stranded form of plasmid pSWT (8.5 kbp), which is the derivative of plasmid pSELECT-1 (Promega) that contains *NruI–SalI* fragment of plasmid pACT7 [9].

The assay for DNA polymerase activity in SDS-polyacrylamide gel electrophoresis was carried out according to [19].

Immunoblotting with monoclonal antibodies against T7 RNAP [20] was carried out as in [21].

3. Results and discussion

Fig. 2 presents the results of examinations of the products synthesized by mutant T7 RNAPs on a plasmid pGEMT containing both T7 promoter and terminator with either rNTPs or dNTPs as substrates. As seen in Fig. 2A, with rNTPs both

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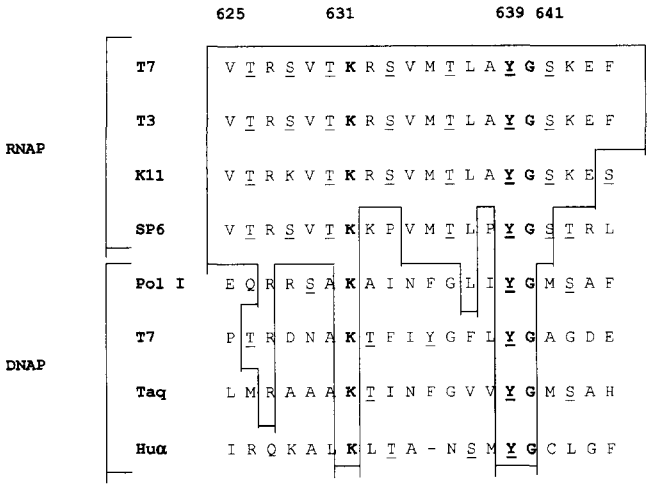

Fig. 1. Motif B sequences in T7, T3, K11, SP6 RNA polymerases and *E. coli* I (Pol I), T7, *T. aquaticus* (Taq), human α (Hu α) DNA polymerases. The residues identical to those of T7 RNAP are boxed; residues invariant in the majority of single-subunit RNA and DNA polymerases [5] are in boldface. The hydroxyl-bearing residues are underlined. Numbering is as in T7 RNAP.

Y639F and Y639F,S641A can synthesize RNA products of the same length as that of the wild-type T7 RNAP, indicating that the ability of the enzyme to recognize the specific promoter and terminator sequences is not impaired by either of the two mutations. The S641A mutant appears to be devoid of RNA polymerizing activity.

With dNTPs as substrates, completely different results were obtained. Whereas wild-type and Y639F T7 RNAPs exhibit no detectable DNA polymerizing activity, both S641A and Y639F,S641A can synthesize a DNA product as long as the specific T7 RNAP transcript, and in so doing the double mutant is more efficient than the single mutant. (For mutant enzymes the minor bands are also seen with both rNTPs and dNTPs.) Hence, the single substitution S641A results in a switch in enzyme specificity from rNTPs to dNTPs, while the double mutation leads to an enzyme that is able to utilize both types of NTPs.

Table 1
The K_m(NTP) values (μM) for the RNA- and DNA-polymerizing reactions catalyzed by the wild-type (WT) and mutant T7 RNAPs. Wild type or mutant enzymes (0.01 μg) were incubated for 10 min at 37°C in Buffer A (see section 2) containing variable amounts (1–200 μM) of one of rNTPs or dNTPs, 0.25 mM of other three rNTPs or dNTPs, 5×10^5 cpm [α-^{32}P]ATP or [α-^{32}P]dATP, 20 μg/ml pGEMT. After incubation samples were processed as described in [9]. Kinetic parameters were calculated using MicroCal Origin 3.0 program (MicroCal Software)

Substrate	WT	Y639F	S641A	Y639F,S641A
ATP	40 ± 5	90 ± 10	–	50 ± 8
GTP	160 ± 30	380 ± 40	–	180 ± 40
CTP	75 ± 16	145 ± 20	–	80 ± 20
UTP	40 ± 8	100 ± 20	–	40 ± 10
dATP	–	–	7.5 ± 3	8 ± 3
dGTP	–	–	20 ± 7	20 ± 8
dCTP	–	–	12 ± 4	10 ± 3
dTTP	–	–	8 ± 2	8 ± 3

Table 1 shows the apparent K_m values calculated for T7 RNAP and mutants on pGEMT template. For Y639F a slight increase of K_m is observed (in agreement with data of Osumi-Davis et al. [10]). The K_m(rNTP) for Y639F,S641A are close to

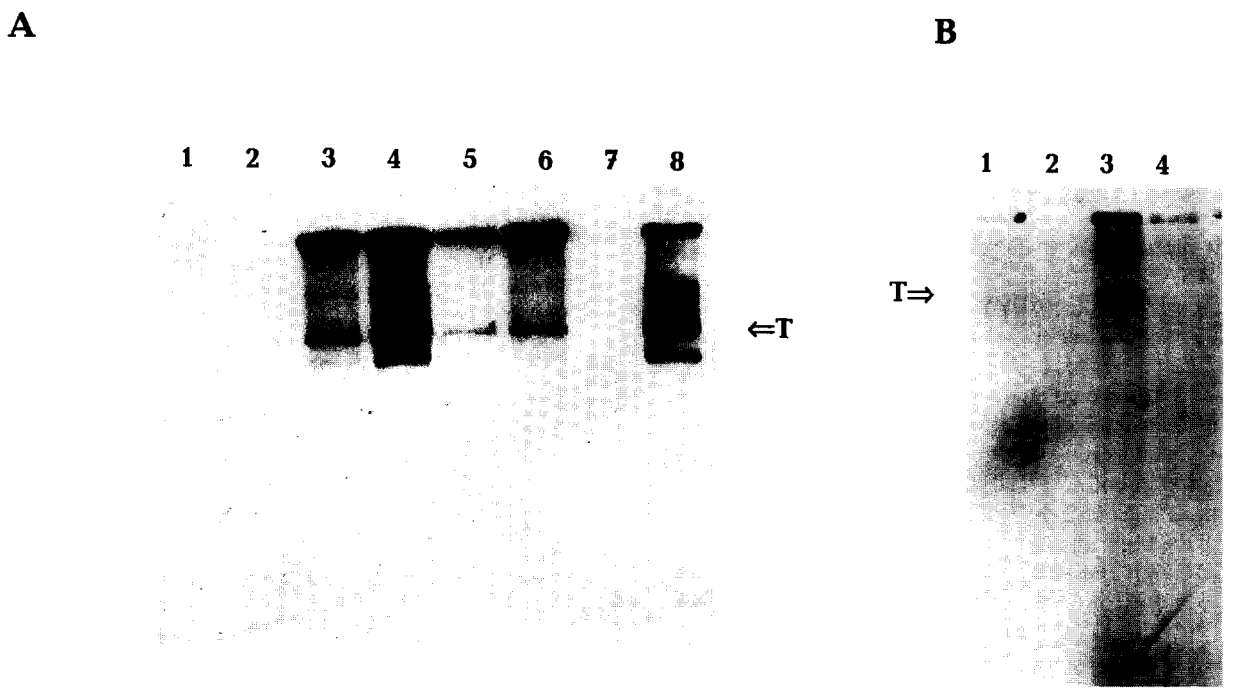

Fig. 2. Polynucleotide synthesis on pGEMT template. (A) Agarose gel electrophoresis of glyoxal-denatured products of mutant T7 RNAPs reactions on pGEMT template (see section 2). Lines 1–4 correspond to reaction with dNTPs; lines 5–8 with rNTPs. 1,5 – wild-type T7 RNAP; 2,6 – Y639F; 3,7 – S641A; 4,8 – Y639F,S641A. Transcription products terminated at TØ are indicated (T). (B) Synthesis of mixed polynucleotides by Y639,S641A. The experimental conditions were the same as described above except assay mixture contained three GTP, CTP, UTP and [α-^{32}P]dATP as substrates. Other combinations of rNTPs and dNTPs were also tested and provided similar results (data not shown). Lines: 1, wild-type T7 RNAP; 2, Y639F; 3, Y639F,S641A; 4, S641A.

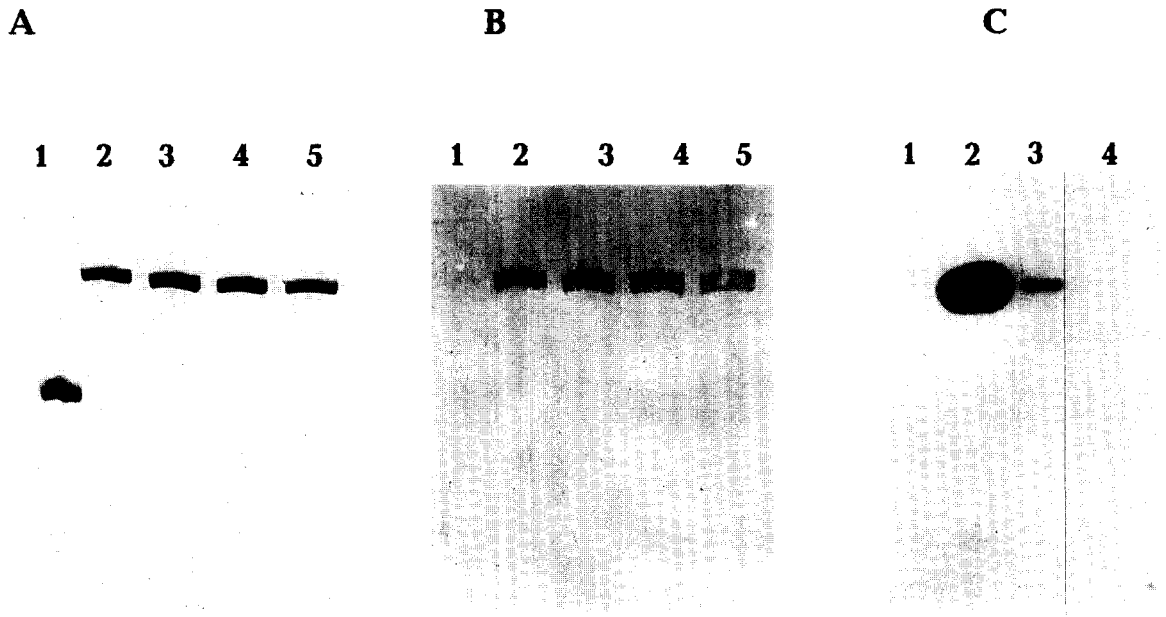

Fig. 3. SDS-polyacrylamide gel electrophoresis of T7 RNAP mutants (A) Coomassie-stained 6% PAGE of T7RNAP mutants. (B) Immunoblotting of T7 RNAP mutants. Lines 1, BSA (control); 2, wild-type T7 RNAP; 3, Y639F; 4, S641A; 5, Y639F,S641A. (C) 'Staining' of DNA polymerase activity in polyacrylamide gel according to [14]. Lines 1, wild-type T7 RNAP; 2, Y639F,S641A; 3, S641A; 4, Y639F.

that of wild-type T7 RNAP, while K_m(dNTP) for both mutants are close to those of *E. coli* DNA polymerase I [22]. The data in Fig. 2B exemplifies another striking characteristic of Y639F,S641A, i.e. with three rNTPs and the missing dNTP in a substrate mixture, the double mutant synthesizes a mixed polynucleotide.

The question arises as to whether the DNA polymerizing activity observed may be due to some impurity in the protein preparations. However, all mutant proteins were purified to homogeneity following the procedure originally developed for the wild-type T7 RNAP [17] and no contaminating bands expected for *E. coli* DNA polymerases are observed (Fig. 3A). In order to account for the level of DNA polymerase activity that we have observed, a contaminating DNA polymerase would have to have a specific activity several orders of magnitude greater than that of any DNA polymerase presently known. Further, an assay for DNA polymerase activity in SDS-polyacrylamide gel electrophoresis (Fig. 3C) suggests that it is associated with a protein with an electrophoretic mobility and antigenic properties identical to those of T7 RNAP (Fig. 3B). Lastly, the ability of Y639F,S641A to synthesize a mixed polynucleotide (Fig. 2B) confirms the unique properties of the mutant enzyme.

To examine further the DNA polymerizing activity of the mutants, both S641A and Y639F,S641A mutants were tested in conventional DNA polymerase assay with nicked DNA (data not shown) or a single stranded DNA template and a synthetic primer. In this case wild-type T7 RNAP and Y639F are inactive while the double mutant and HIV-1 RT (used as a control) synthesize a product of defined length (Fig. 4A,B).

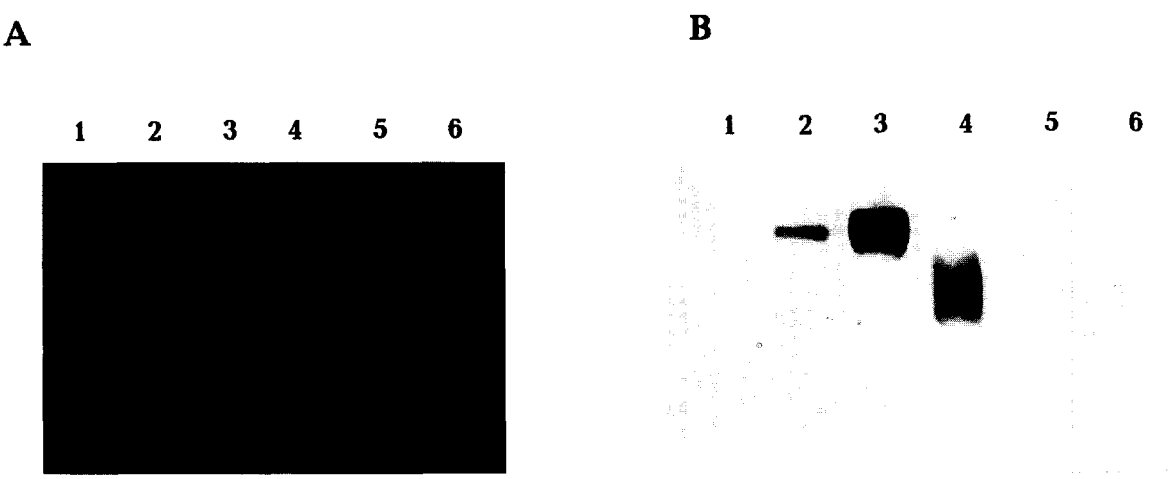

Fig. 4. DNA polymerase activity of T7 RNAP mutants. After electrophoresis the gels were subjected to EtBr staining (A) or autoradiography (B). Lines: 1, no enzyme (control); 2, HIV-1 RT (control); 3, Y639F,S641A; 4, S641A; 5, Y639F; 6, wild-type T7 RNAP.

The product of S641A, seen as a smear, is shorter and heterogeneous in length and may be due an intrinsic nuclease activity present in S641A (data not shown).

In conclusion, we have described a mutant of T7 RNAP that is able to carry out DNA-dependent synthesis of both DNA and RNA, and to initiate a DNA synthesis from a circular supercoiled plasmid in the absence of a primer. We speculate that residues analogous to Ser641 may play a similar role in other single-subunit RNA polymerases. In this connection, one can suppose that double substitution Y6410F,S642A in T3 RNAP should create a mutant enzyme with properties similar to those described above.

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