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Tyr-571 is involved in the T7 RNA polymerase binding to its promoter

V.O. Rechinsky, V.L. Tunitskaya, S.M. Dragan, D.A. Kostyuk and S.N. Kochetkov

V.A. Engelhardt Institute of Molecular Biology, The Russian Academy of Sciences, Moscow, Russian Federation

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The in vitro studies of three T7 RNA polymerase point mutants suggest that substitutions of Ala and Thr for Pro-563 and of Ser for Tyr-571 have little effect on the enzyme catalytic competence, but result in its inability to utilize the promoter. Both P563A and P563T mutants retain the promoter-binding ability, whereas the promoter affinity of the Y571S mutant drops drastically.

Bacteriophage T7; RNA polymerase; Mutagenesis; RNA polymerase promoter interaction

1. INTRODUCTION

T7 RNA polymerase (T7 RNAP), as well as the other members of a family of homologous monomeric RNA polymerases, has a stringent specificity for its own promoters. Considerable recent attention has been focused on revealing the bases within the promoter that are essential for its functioning. Two structural domains have emerged from an extensive mutational analysis of the promoter: a binding domain located 4–17 base pairs upstream from the start site and an initiation domain that takes up positions from −4 to +5 (see Fig. 1). Base substitutions in the initiation domain have little if any effect on the enzyme binding but decrease the rate of the transcription initiation, whereas changes in the binding domain affect mainly the polymerase binding [1–5]. Discrimination between T3 and T7 promoters by the enzyme has been shown to depend primarily on three base pairs at positions from −10 to −12 within the binding domain [3]. In contrast to a detailed characterization of the promoter in itself parts of the RNA polymerase that provide ability to bind the promoter are scantily known. In this regard the revealing of amino acid residues that are responsible for discriminating between T3 and T7 promoters represents a considerable step forward. Joho et al. [6] demonstrated that a region of T7 RNAP that spans amino acids 674–752 and is likely confined to residues 749–751 serves this function. No other part of the enzyme that is implicated in the promoter binding has been identified to date, although there is evidence that T7 RNAP has another domain taking part in promoter selection [6]. In relation to this topic, it seems pertinent to note that Patra et al. [7] isolated a T7 RNA polymerase mutant (containing an insertion of Tyr residue after Asp-240) that has lost a promoter utilization capacity as a result of a reduction in its affinity for the initiating NTP. In this paper we present evidence that Tyr-571 is involved in T7 RNAP binding to its promoter.

Correspondence address: S.N. Kochetkov, V.A. Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov st. 32, Moscow B-334, 117984, Russia. Fax: (7) (095) 135-1405.

Abbreviations: T7 RNAP, bacteriophage T7 DNA-dependent RNA polymerase; w.t., wild type; BSA, bovine serum albumin.

2. MATERIALS AND METHODS

W.t. and mutant T7 RNAPs were purified and assayed as described by Tunitskaya et al. [8] from heat-induced *E. coli* cells harboring a plasmid pACT7 or its mutagenized derivatives pACT7 -M6, -M9, -M8 which carry Thr and Ala substitutions for Pro-563, and a Ser substitution for Tyr-571, respectively [9]. Transcription assays were carried out with one of the following templates: T7 promoter-containing plasmid pGEM-2 (Promega), poly(dC), and poly[d(I-C)]. The apparent K_m^{GTP} values were determined as the GTP concentrations required for half- maximal activity of the enzymes in question on poly(dC).

The capacity of the mutants to bind the promoter was examined by the nitrocellulose binding [10] and band-retardation [11] techniques. The ability of mutant T7 RNAP to compete with the w.t. enzyme for the promoter under the reaction conditions was tested in the following way. Conventional transcription assay was carried out for mixtures of the mutant in question with the w.t. enzyme that varied in their ratios but had a constant total protein concentration. As a control BSA was used for T7 RNAP dilution.

3. RESULTS AND DISCUSSION

Random mutagenesis approach coupled to a simple phenotypic selection enabled us to isolate several virtually inactive in vivo T7 RNAP mutants [9]. All the mutations revealed map to one of the two short regions, both being highly conservative in known sequences of monomeric RNA polymerases. Recent data suggest that one of these regions (residues 625–652) or motif B as defined by Delarue et al. [12] forms part of the elongating NTP-binding site [10,12–15]. The second region (see Fig. 2) was found to be located adjacent to the other motif A (residues 532–555) which has been shown to

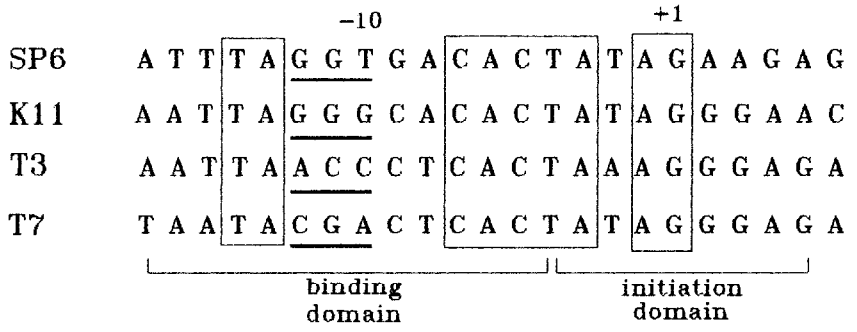

Fig. 1. Comparison of sequences of phage promoters. The consensus sequences of promoters for the T7, T3, SP6, and K11 RNA polymerases [19–22] are presented. Identical bases are boxed. Bases that were shown to determine the promoter specificity [6] are underlined.

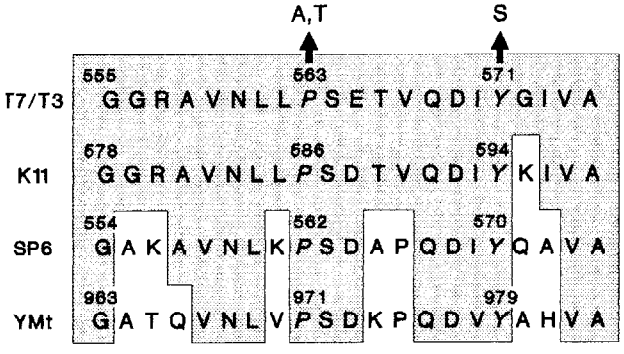

Fig. 2. Homology between the amino acid sequence of T7 RNAP in the region of mutations and sequences of other monomeric RNA polymerases. Numbers represent the positions of amino acid residues. Identical residues are boxed.

contain catalytically significant amino-acid residues [14,15]. In order to elucidate the functional role of the Pro-563 and Tyr-571 residues in T7 RNAP we purified P563A, P563T, and Y571S mutants to homogeneity and examined their properties in vitro.

Table I lists the relative activities of mutant enzymes on diverse templates. The plasmid pGEM-2 was used to assay a promoter-dependent activity, whereas synthetic polynucleotides known to be readily transcribed by the w.t. T7 RNAP [16] were utilized to identify mutants that retain catalytic competence but have lost their capacity for promoter-dependent transcription. Special attention must be given to the fact that the P563A and, to a much lesser extent, the P563T mutants exhibit a sizable activity on the promoter-containing template as soon as they are purified, since nothing of the kind was detected when assaying T7 RNAP in crude extracts immediately after cell lysis [9]. However, this activity decays rapidly (in a matter of hours or days at most) to near-zero. Electophoretic analysis (data not shown) demonstrated that the drop in activity did not result from proteolytic degradation and appears to be a consequence of structurally disruptive effects of Ala and Thr substitutions for Pro at position 563 in T7 RNAP which are suggested by either of the two methods of the secondary structure analysis that were applied [17,18] (see Fig. 3).

The activity of each of the P563A, P563T, and Y571S T7 RNAP mutants on both single-stranded poly(dC) and double-stranded poly[d(I-C)] templates, as distinct from the promoter-containing pGEM-2 DNA, was found to be fairly high and storage time invariant (see Table I). K_m^{GTP} values for the mutants and for the w.t. enzyme proved to be close (ca. 1 mM) (within the experimental error) when poly(dC) was used as a template. For NTPs other than GTP it was not possible to determine K_m values because of the vanishingly low activity of mutants on a heteropolymer pGEM-2 DNA template. These results show that the revealed amino acid changes at positions 563 and 571 in T7 RNAP have little effect on the catalytic competence of the enzyme, but results in its inability to initiate RNA synthesis at the promoter, provided that a common catalytic machinery is used during transcription of all templates. Capacity

Table I

Activity (%) of the w.t. and mutant T7 RNAPs on various templates

Enzyme	pGEM-2			Poly(dC)			Poly[d(I-C)]		
T7 RNAP w.t.	100*	100**	98***	100*	100**	100***	100*	100**	97***
P563A	30	10	<1	50	50	40	80	78	70
P563S	5	2	<1	100	98	90	100	100	97
Y571S	<1	<1	<1	70	65	60	>100	>100	>100

Measurements were taken immediately after the purification (*), in 3 days (**) and one week (***) of storage.

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          550        560        570        580
wt        LRDEVGGRAVNLLPSETVQDIYGIVAKKVNE
1         XXXXXXX------++---------XXXXXXX
2         XXX++++++--++++++------XXXXXXXX

          550        560        570        580
P563A     LRDEVGGRAVNLLASETVQDIYGIVAKKVNE
1         XXXXXXXXX---XXXXXXXX----XXXXXXX
2         XXX++++XXXXXXXX+++-------XXXXXX

          550        560        570        580
P563T     LRDEVGGRAVNLLTSETVQDIYGIVAKKVNE
1         XXXXXXXX----------------XXXXXXXX
2         XXX+++++XX---++++++------XXXXXX

          550        560        570        580
Y571S     LRDEVGGRAVNLLPSETVQDISGIVAKKVNE
1         XXXXXXX------++----X-XXXXXXXXXX
2         XXX++++++--++++++++++-----XXXXXX
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Fig. 3. Semi-graphical representation of the secondary structure of the w.t. and mutant T7 RNAPs in the region of mutations predicted by Garnier's [17] (1) and GGBSM [18] (2) methods. Symbols: (×) helix; (−) extended chain; (+) coil.

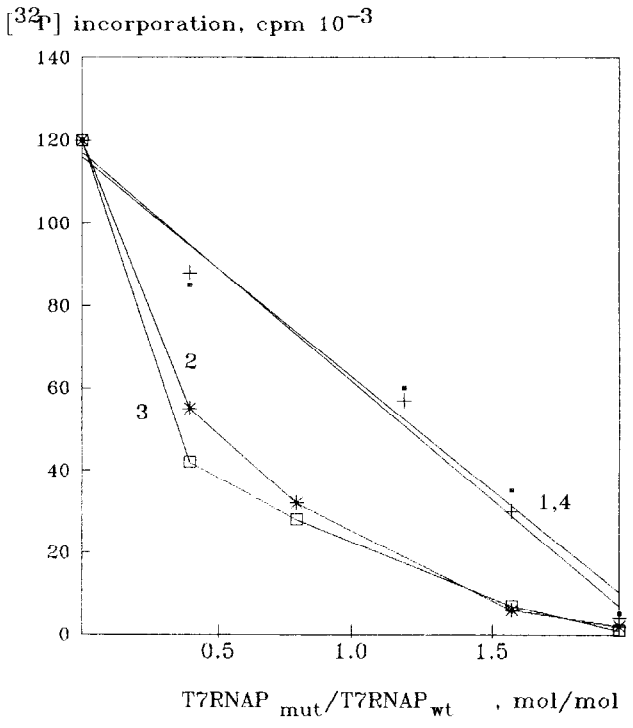

Fig. 4. Competition of mutants with w.t. T7 RNAP for the promoter. (1) Y571S; (2) P563A; (3) P563T; (4) BSA (μg/μg) (control).

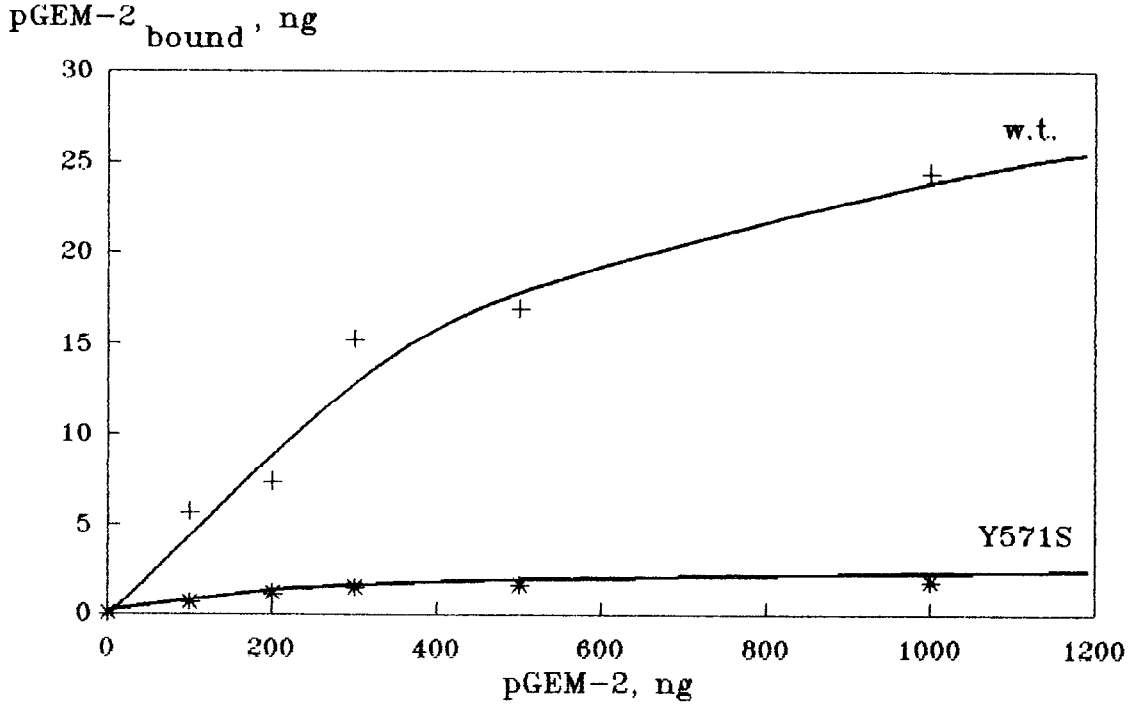

Fig. 5. Binding of the w.t. T7 RNAP and the Y571S mutant to the ^{32}P-end-labeled pGEM-2 linearized with *Eco*RI.

of mutants to compete with the w.t. T7 RNAP for the promoter under the reaction conditions was tested in an effort to substantiate this deduction. As Fig. 4 suggests, both P563A and P563T mutants retained the promoter-binding activity, whereas the promoter affinity of the Y571S mutant decreased drastically. Results of both nitrocellulose filter binding (see Fig. 5) and band-retardation experiments (data not shown) provide evidence in support of this assertion.

At least two conceivable explanations of the observed facts may be offered. One of them implies that Pro-563 and/or Tyr-571 make base-specific contacts with the promoter which are disturbed by mutations. If this is the case, then Tyr-571, substitution for which disturbs the promoter binding, and, evidently, the neighboring Gln-Asp-Ile residues are bound to interact with bases that are invariant in promoter sequences of diverse origin (see Fig. 1), since these residues in themselves are invariant in sequences of distinct RNA polymerases and, hence, cannot determine their promoter specificity. Investigations aimed at finding out whether the Gln-Asp-Ile-Tyr domain represents a structural motif that is important in sequence-specific contacts are now in progress, and involve site-directed mutagenesis of these residues.

Alternatively, it is possible that residues at positions 563 and/or 571 are not in immediate contact with the DNA, but rather, serve adequately to position some other residue(s) essential for T7 RNAP functioning. The latter notion seems highly plausible for the explanation of the part played by Pro-563 when taken into account that its substitutions by both Ala and Thr result in a drastic structural perturbation (see Fig. 3). However, in our view, it would be a mistake to draw ultimate conclusions about structure–function relationships in the absence of crystallographic data.

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