Mol Gen Genet (1995) 247:110–113

ORIGINAL PAPER

Vladimir O. Rechinsky · Boris K. Chernov
Sofia M. Dragan · Dmitry A. Kostyuk
Vera L. Tunitskaya · Sergey N. Kochetkov

Targeted mutagenesis identifies Asp-569 as a catalytically critical residue in T7 RNA polymerase

Received: 25 May 1994 / Accepted: 25 October 1994

Abstract In order to look more closely at a well-conserved region in T7 RNA polymerase (T7 RNAP) containing, as shown earlier, the functionally essential residues Pro-563 and Tyr-571, we used targeted mutagenesis to change those residues within this region that are invariant in all single-subunit RNA polymerases, and characterized the mutant enzymes in vitro. The most interesting finding of this study was the crucial importance of the acidic group of Asp-569. In addition, we have shown that the phenolic ring is the most significant functional group of Tyr-571, with the hydroxy group also contributing to promoter binding.

Key words T7 RNA polymerase · Oligonucleotide-directed mutagenesis · Transcription · Promoter binding

Introduction

The bacteriophage T7 RNA polymerase (T7 RNAP) is a popular model system for structure-function research, being structurally simple and amenable to genetic manipulation. Recent mutational and affinity labeling analyses have identified several regions and individual amino acid residues essential for polymerase function, and sequence alignments of a variety of RNA and DNA polymerases have revealed the conserved motifs containing most crucial residues [see McAllister and Raskin 1993, for review].

In particular, P563 and Y571 reside in a conserved block of amino acids in the single-subunit RNAP family (see Fig. 1), and substitution of P563 by either threonine or alanine and of Y571 by serine yield enzymes that are inactive *in vivo* (Rechinsky et al. 1993a). Two other null mutants, *ins559* and *ins566*, also map to this region (Gross et al. 1992). Both 563 and *ins559* mutants retain promoter-binding ability, whereas *ins566* and Y571S have lost it (Gross et al. 1992; Rechinsky et al. 1993b). However, Y571S is still able to transcribe non-promoter templates such as poly(dC) and poly[d(I-C)] (Rechinsky et al. 1993b).

In light of these findings, we used site-directed mutagenesis to probe the function of this conserved region in T7 RNAP, the sequence of which is shown in Fig. 1 specifically to elucidate whether the strictly conserved block of amino acids QDIY is essential for the enzyme-promoter interaction.

Materials and methods

Oligonucleotide-directed mutagenesis

The plasmid pACT7, coding for T7 RNAP, was subjected to the procedure of Kunkel et al. (1987) as described previously (Maksimova et al. 1991). Oligonucleotides were made on an Applied Biosystems Model 380B DNA synthesizer and purified by HPLC. Mutations were verified by dideoxy sequencing of a region in the immediate vicinity of the target triplet using modified T7 DNA polymerase (Tabor and Richardson 1987).

Polymerase purification

Mutant T7 RNAPs were purified from heat-induced *Escherichia coli* cells harboring mutant derivatives of pACT7 following the procedure of Tunitskaya et al. (1988) for the wild-type enzyme.

In vitro transcription

Transcription reactions were carried out as described previously (Tunitskaya et al. 1988; Lyakhov et al. 1992) using a linearized plasmid pGEM-2 (Promega) as the template for assaying T7 promoter-dependent activity and poly (dC) for assaying non-specific

Communicated by G. P. Georgiev

V. O. Rechinsky · B. K. Chermov · S. M. Dragan · D. A. Kostyuk
V. L. Tunitskaya · S. N. Kochetkov (✉)
Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32, Vavilova St., Moscow 117984, Russia

catalytic activity. The mean rate of chain elongation (v_e) and the parameter t_x, which gives an estimate of the overall rate of initiation and termination, were evaluated using a set of templates of different lengths, as described by Lyakhov et al. (1992). Calculation of the kinetic constants was done using the computer program ENZFITTER (Elsevier Biosoft). Promoter binding of mutant T7 RNAPs was analyzed as previously described (Rechinsky et al. 1993b).

Other methods

DNA and protein preparations were analyzed and manipulated according to conventional protocols (Sambrook et al. 1989).

Results

Figure 1 presents the T7RNAP mutants that were generated in this study by oligonucleotide-directed mutagenesis. Based on the spatial arrangement of the mutated residues (located at the very N-terminus of helix W and in the adjacent loop (Sousa et al. 1993) and the character of the side chain modifications introduced, only local alterations in the native structure of the protein are expected, allowing the affected function to be assigned to a particular amino acid residue.

Mutant T7 RNAPs were purified by the procedure developed previously for the wild-type enzyme (Tunitskaya et al. 1988). When protein preparations were analyzed by SDS polyacrylamide gel electrophoresis, a single major band at the position expected for T7RNAP was observed. Both mutants altered at position 569 and, to a lesser extent, Q568A were produced in relatively low amounts, probably because of their increased protease sensitivity.

Purified mutant RNAPs were assayed *in vitro* for their ability to accomplish basic steps in DNA transcription as well as to carry out non-specific polyG synthesis with poly(dC) as template. Table 1 summarizes the data obtained. As seen in the left column, mutants S564A, Q568A, and Y571F retained much of the wild-type activity on a promoter-containing plasmid. More significantly, when plasmids, containing either the T7 promoter or both promoter and terminator and linearized with different restriction endonucleases were used as templates, and transcripts were examined by polyacrylamide gel electrophoresis as described previously (Lyakhov et al. 1992), it was found that the bulk of radioactivity incorporated into RNA was associated with transcripts of distinct lengths identical to those produced by the wild-type T7 RNAP. This clearly demonstrated that these mutants retain the ability to recognize the specific T7 promoter and terminator sequences. Further characterization of partly active mutant RNAPs is presented in Table 2. The K_m values for NTP for these mutants are within a factor of 1.5 of the wild-type level, suggesting that substrate binding by the enzyme is not markedly affected by these mutations.

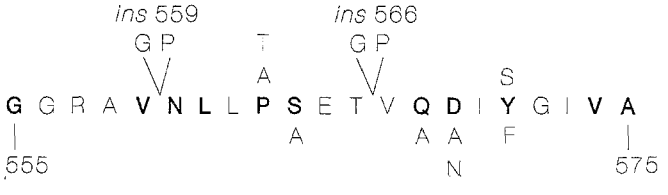

Fig. 1 Amino acid sequence of the T7 RNAP region under study. Residues that are invariant in all single-subunit RNAPs are in *bold*. Above the sequence the substitutions that cause inactivation of the enzyme are indicated (Rechinsky et al. 1993a, b); also shown are the null mutations *ins559* and *ins566* resulting from the insertion of two amino acids immediately after the residues indicated (Gross et al. 1992). Below the T7 RNAP sequence the mutants generated and characterized in this study are presented.

Table 1 Effect of mutations on properties of T7 RNA polymerase

Mutation	Catalytic activity[a]		Promoter binding[b]	
	pGEM-2	Poly(dC)	K_d (μM)	K_i (μM)
Wild type	100	100	22	—
S564A	130	100	20	—
Q568A	50	100	90	—
D569A	<1	<1	100	20
D569N	<1	5	100	22
Y571S	<1	70	> 1000	> 1000
Y571F	40	50	45	—

[a] Catalytic activities are expressed as percentages of wild-type activity

[b] K_d values are apparent dissociation constants determined by the conventional nitrocellulose binding technique. Binding reactions (20 μl) containing 0.7–2.0 pmole T7 RNAP (wild-type or mutant), 0.05 pmole ^{32}P-labeled promoter-containing DNA fragment, and a variable (0.05–1.0) amount of nonlabeled fragment were incubated at 37° C for 5 min and were passed through nitrocellulose filters, which were then processed as described (Lyakhov et al. 1992). K_i values are inhibition constants deduced from competition for the promoter. To this end, preparations of mutant and wild-type T7 RNAPs equalized in concentration were subjected to serial double dilutions and used in standard activity assays. The acid-insoluble radioactivity was plotted against the molar ratio of mutant/wild-type enzyme to evaluate the relative promoter affinity (Rechinsky et al. 1993b). The mean values of 4 experiments are presented; standard deviation was less than 20%

Of special interest is a comparison between the impacts of the same substitution (phenylalanine for tyrosine) at two distinct positions in T7 RNAP, 571 and 639 [The effects of the latter mutation have already been reported (Osumi-Davis et al. 1992; Bonner et al. 1992; Tunitskaya et al. 1994)]. In both cases some increase (which is larger for Y639F) in the K_m values for NTP is observed, i.e. substrate binding is slightly impaired by each mutation. However, for the Y639F mutant this is accompanied by a rise in the catalytic activity, whereas the Phe substitution for Tyr-571 reduces activity markedly (see Table 1). This dramatic difference strongly indicates that the tyrosine residues at positions 571 and 639 have dissimilar functions.

With a set of templates of different lengths one can evaluate the mean rate of RNA chain elongation v_e,

Table 2 Kinetic constants of partly active mutant T7 RNA polymerases

Enzyme	K_m (μM)[a]				v_e[b]	t_x[b]
	GTP	ATP	CTP	UTP		
Wild type	160	40	75	40	100	1–3
S564A	190	50	70	40	105	1–3
Q568A	125	40	75	40	35	35–40
Y571F	200	60	100	60	60	20

[a] For K_m determinations, three NTPs were held at 0.5 mM while the concentration of the fourth one was varied by serial double dilutions from 0.5 mM to 10 μM. Standard assays (Tunitskaya et al. 1988; Lyakhov et al. 1992) were started by adding the enzyme for 15 min at 37° C

[b] To determine the mean rate of RNA chain elongation v_e (in nucleotides per sec) we followed the procedure of Lyakhov et al. (1992) which is based on the kinetic analysis of the reaction. Assuming that transcription *in vitro* shows steady-state kinetics, that is, at every instant there are equal numbers of initiated and terminated chains, we arrive at a linear function of τ (the time it takes an enzyme molecule to synthesize a single transcript) on a template of length L: $\tau = L/v_e + t_x$, where t_x is the time (in sec) needed for recycling (i.e., termination and reinitiation). This allows one to evaluate both v_e and t_x using a set of templates of different lengths, which can be obtained by digestion of a promoter-containing plasmid with appropriately chosen restriction endonucleases

and estimate the overall rate of its initiation and termination, which is characterized by the value of the parameter t_x (Lyakhov et al. 1992). As seen in Table 2, both Q568A and Y571F mutations affect chain initiation and/or termination more significantly than elongation: for both mutants t_x is an order of magnitude longer than for the wild-type T7 RNAP, whereas v_e is reduced by only half.

An examination of the binding of mutant T7 RNAPs to its promoter (Columns 3 and 4 in Table 1) provided information that is compatible with the speculation that the block of invariant amino acids QDIY (see Fig. 1) is involved in promoter binding (Rechinsky et al. 1993b): Q568A, D569A, D569N, and Y571F all have a promoter affinity 2–5 times lower than that of the wild-type enzyme. However, the catalytic properties of D569A and D569N proved to be quite unexpected: neither of the two mutants showed any detectable promoter-driven or non-specific activity (Columns 1 and 2 in Table 1), which clearly indicates that the acidic group of Asp-569 is of prime importance for a function other than promoter binding.

Discussion

Using random mutagenesis coupled with an appropriate selection procedure, we have previously identified two residues in T7 RNA polymerase, Pro-563 and Tyr-571, which are essential for its function (Rechinsky et al. 1993a). The latter was then shown to be involved in promoter binding (Rechinsky et al. 1993b) together with the asparagine residue at position 748, which had been found to interact directly with base pairs −10 and −11 in the T7 promoter (McAllister and Raskin 1993). It is remarkable that both Tyr-571 and Asn-748 are located within a putative DNA-binding cleft, the first lying at the N-terminus of helix W in the immediate vicinity of the putative "catalytic pocket" formed by residues Asp-537, Lys-631, Tyr-639, Gly-640, and Asp-812 (Sousa et al. 1993). Other mutations that cause loss of promoter binding with little, if any, effect on catalytic activity map near codons 159, 222, 240, and 242 (Gross et al. 1992; Patra et al. 1992).

In an attempt to obtain further information on the functional role of the T7 RNAP region in Fig. 1, we used targeted mutagenesis to change the residues within it which are invariant in all single-subunit RNAPs and characterized the derived mutant enzymes *in vitro*.

The results obtained show that substitution of alanine for Ser-564 does not markedly affect the properties of T7 RNAP (Table 1). This argues in favor of the proposal that the neighboring Pro-563 serves primarily to arrange some parts of the enzyme molecule properly in space. Substitutions at this position, as well as mutations *ins*559 and *ins*566 which give rise to additional proline residues in the immediate vicinity, disrupt the tertiary structure of the protein and inactivate it.

Both Q568A and Y571F T 7RNAPs retain much of the wild-type catalytic activity and exhibit properties that could be expected for enzymes with impaired promoter affinity. A completely different type of situation occurs in the case of mutations at position 569. Substitution of either Ala and Asn for Asp-569 has an effect on promoter binding almost identical to that of mutations Q568A and Y571F, but leads to a drastic decrease in catalytic activity (Table 1). On the strength of these data, one cannot but infer that D569A and D569N are blocked at some stage following promoter recognition and binding, with the negatively charged acidic group of Asp-569 playing a critical role in catalysis. In this connection it seems pertinent to call attention once again to the close spatial proximity of Asp-569 (located immediately ahead of helix W) to other residues identified as catalytically critical: Asp-537, Lys-631, and particularly those localized at the C-terminus of the T7 RNAP molecule (Sousa et al. 1993).

The detrimental effect of the Phe for Tyr571 substitution on the promoter affinity of T7 RNAP is two orders of magnitude less than that of the Ser substitution (Table 1), suggesting that it is the phenolic ring that is the essential functional group of the residue at this position. Assuming that Tyr 571 is directly implicated in recognition of the promoter by T7 RNAP, and considering that the Y571S mutation results in a complete loss of promoter binding, it is attractive to speculate that this residue interacts with bases at positions −7, −8, and −9 in the promoter. This is evident since only these bases and a G at −11 were found to be

a prime necessity for T7 promoter function (Ikeda et al. 1992), and the latter base contacts Asn748 in T7 RNAP (McAllister and Raskin 1993). However it is clear that the results are not yet sufficient to allow definite conclusions regarding the structure of the polymerase-promoter complex.

Conclusion

The data presented in this work make it evident that two closely adjacent amino acid residues of a protein (D569 and Y571 in T7 RNAP) may be involved in distinct biochemical functions. Consequently, a given region of an enzyme molecule may take part in more than one function. This seems likely to be true for the conserved structural motif B in T7 RNAP, for example. On the one hand, is believed to be associated with the template, and on the other to participate in NTP binding (Delarue et al. 1990; Maksimova et al. 1991; Gross et al. 1992; Osumi–Davis et al. 1992; Bonner et al. 1992; Patra et al. 1992; Sousa et al. 1993; Tunitskaya et al. 1994). This is not to say that a single function cannot involve several amino acids that are widely separated in the sequence, as is the case in the T7 RNAP-promoter binding interaction (see above). Hence one should be very careful when assigning a function to a particular region of a protein on the basis of deletion/insertion mutagenesis.

Acknowledgement We are grateful to Dr. A. Galkin from Moscow State University for critical reading of the manuscript.

References

Bonner G, Patra D, Lafer EM, Sousa R (1992) Mutations in T7 RNA polymerase that support the proposal for a common polymerase active site structure. EMBO J 11:3767–3775

Delarue M, Poch O, Tordo N, Moras D, Argos P (1990) An attempt to unify the structure of polymerases. Prot Engin 3:461–467

Gross L, Chen W-J, McAllister WT (1992) Characterization of bacteriophage T7 RNA polymerase by linker insertion mutagenesis. J Mol Biol 228:488–505

Ikeda RA, Ligman CM, Warshamana S (1992) T7 promoter contacts essential for promoter activity *in vivo*. Nucleic Acids Res 20:2517–2524

Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Meth Enzymol 154:367–382

Lyakhov DL, Ilgenfritz H, Chernov BK, Dragan SM, Rechinsky VO, Pokholok DK, Tunitskaya VL, Kochetkov SN (1992) Site-directed mutagenesis of Lys-172 residue of T7 RNA polymerase: characterization of the transcription properties of mutant proteins. Mol Biol (Moscow) 26:1022–1035

Maksimova TG, Mustaev AA, Zaychikov EF, Lyakhov DL, Tunitskaya VL, Akbarov AKh, Luchin SV, Rechinsky VO, Chernov BK, Kochetkov SN (1991) Lys631 residue in the active site of the bacteriophage T7 RNA polymerase. Affinity labeling and site-directed mutagenesis. Eur J Biochem 195:841–847

McAllister WT, Raskin CA (1993) The phage RNA polymerases are related to DNA polymerases and reverse transcriptases. Mol Microbiol 10:1–6

Osumi-Davis PA, de Aguilera MC, Woody RW, Woody A-YM. (1992) Asp537, Asp812 are essential and Lys631, His811 are catalytically significant in T7 RNA polymerase activity. J Mol Biol 226:37–45

Patra D, Lafer EM, Sousa R (1992) Isolation and characterization of mutant bacteriophage T7 RNA polymerase. J Mol Biol 224: 307–318

Rechinsky VO, Kostyuk DA, Lyakhov DL, Chernov BK, Kochetkov SN (1993a) Random mutagenesis of the gene for bacteriophage T7 RNA polymerase. Mol Gen Genet 238:455–458

Rechinsky VO, Tunitskaya VL, Dragan SM, Kostyuk DA, Kochetkov SN (1993b) Tyr-571 is involved in the T7 RNA polymerase binding to its promoter. FEBS Lett 320:9–12

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sousa R, Chang YJ, Rose JP, Wang B-C (1993) Crystal structure of bacteriophage RNA polymerase at 3.3 Å resolution. Nature 364: 593–599

Tabor R, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA 84:4767–4771

Tunitskaya VL, Mishin AA, Tyurkin VV, Lyakhov DL, Rechinsky VO, Kochetkov SN (1988) Affinity modification of bacteriophage T7 DNA-dependent RNA polymerase by 5′-p-fluorosulfonylbenzoyl-adenosine. Mol Biol (Moscow) 22:1642–1649

Tunitskaya VL, Dragan SM, Kostyuk DA, Lyakhov DL, Memelova LV, Rechinsky VO, Kochetkov SN (1994) Functional studies of bacteriophage T7 RNA polymerase point mutants containing amino acid substitutions in motif B of the enzyme active site. Biokhimiya (Moscow) 59:494–502