A TEMPERATURE SENSITIVE MUTATION OF THE β′-SUBUNIT OF DNA-DEPENDENT RNA POLYMERASE FROM *E. COLI* T16

Susan R. PANNY*, Alfred HEIL, Barbara MAZUS**, Peter PALM and Wolfram ZILLIG

Max Planck Institut für Biochemie, 8033 Martinsried bei München, West-Germany

and

Sofia Z. MINDLIN, Tamilla S. ILYINA and R. B. KHESIN

Kurchatov Institute of Atomic Energy, Moscow D-182, USSR

Received 25 August 1974

1. Introduction

Functional changes of DNA-dependent RNA polymerase caused by mutations have so far exclusively been assigned to the β-subunit which determines sensitivity or resistance to streptolydigin, rifampicin and streptovaricin [1–4].

This has two main causes: first, selection procedures for polymerase mutants were commonly based on the use of rifamycin or other specific inhibitors of the enzyme [5–12]. Second, many mutants exhibiting significant changes in in vivo transcription yield an RNA polymerase not significantly different from the wildtype enzyme in the usual test system [13–17].

A close linkage of the β and β′ genes within one operon has been demonstrated both biochemically [18] and genetically [19]. As will be shown in this paper the tsX-mutation found in *E. coli* K12 strains Ts 19 [21] and T 16 [22] by a streptomycin method [23] is a genetic marker for the β′-subunit. An enzymological comparison of the T16 enzyme with wildtype RNA polymerase reveals differences which might be due to the lesion in β′.

Other possible mutations in RNA polymerase gene(s) have not yet been assigned to subunits [20].

* Present address: Mass. General Hospital, Children's Service, Fruit Street, Boston, Mass. 02114, USA.

** Present address, Instytut Biochemii I Biofizyki, Polska Akademia Nauk, 36, Rakowiecka, Warsawa 12, Polen.

2. Experimental

2.1. *Bacterial strains*

Wildtype *E. coli* K12 and the mutant T16 (F^-,thi, tsX,str-r) [22] were grown in a rich medium (2% meat extract, 3% peptone from casein, 2.4% yeast extract and 0.5% NaCl) in a 300 l fermenter (Bio-engineering) at 37°C and 32°C respectively and harvested during the late log phase.

2.2. *RNA polymerase purification*

Normal enzyme was prepared as previously described [24]. For the preparation of mutant T16 enzyme a modified method had to be used because of the marked lability of this enzyme. All preparations were done in magnesium-free TA buffer (0.05 M Tris, pH 7.5, 0.022 M NH_4Cl, 0.001 M EDTA, 0.02 M β-mercaptoethanol, 20% glycerol). The debris was removed from the homogenate by centrifugation in a Sorvall centrifuge. The supernatant was precipitated with polimin *P*. The precipitate was washed three times with 0.4 M NH_4Cl and then eluted three times with 1.0 M NH_4Cl in TA buffer. The eluate was subjected to precipitation at 50% $(NH_4)_2SO_4$ saturation. Since dialysis after this step invariably resulted in rapid, complete and irreversible loss of activity, the redissolved precipitate was desalted by chromatography over a Sephadex G25 column. The protein-containing fractions were applied to a DNA-agarose [25] column and eluted with a linear gradient ranging from 0.1 to

1.2 M NH_4Cl in TA buffer, the active enzyme appearing in a broad peak from 0.4 to 0.6 M NH_4Cl. The enzyme (fraction IV) was precipitated at 50% ammonium sulfate saturation and dissolved in 50% glycerol TA and stored in liquid nitrogen.

Fraction IV enzyme has a specific activity of 9.2 nM AMP/mg/min incorporated at 30°C (9.2 mU/mg) corresponding to a 90-fold enrichment from the crude extract. Further purification by centrifugation on a sucrose glycerol gradient yielded a maximal specific activity of 10 to 16 mU/mg and a 50% pure enzyme as shown by polyacrylamid-gel electrophoresis, but only a 20% recovery of enzyme activity. Most contaminants could be easily removed by electrophoresis on cellogel [2]. Subsequent reconstitution of full enzyme from isolated subunits [4] yielded a specific activity between 4 and 6 mU/mg.

2.3. *Reconstitution of isolated subunits*

Preparation of subunits was performed as previously described [2,29] by electrophoresis on cellogel strips (Chemetron, Milano) and subsequent excision and centrifugation of the strips containing the subunit bands [2]. The procedure for mixed reconstitution is reported elsewhere [4,26].

2.4. *Assay*

Enzymatic activity was tested by standard test conditions [27] with modifications described in the legends. T_5-phage DNA was used as a template.

2.5. *Analytical procedures*

Polyacrylamid-gel electrophoresis was run in logarithmic gradient gels from 7.5% to 20% [28]. Protein was determined as described [2].

3. Results and discussion

3.1. *Enzymological differences between wildtype and mutant enzymes*

As shown in fig.1, the broad pH-optimum for T16 enzyme ranges between pH 6.8 and 7.4, whereas the optimum for normal enzyme is pH 7.9 [27] where the mutant enzyme is inhibited by 50%.

The salt requirements for optimal activity are also different for T16 and for wildtype enzyme. At constant Mg^{2+} concentration of 0.03 M, the dependence of wild-

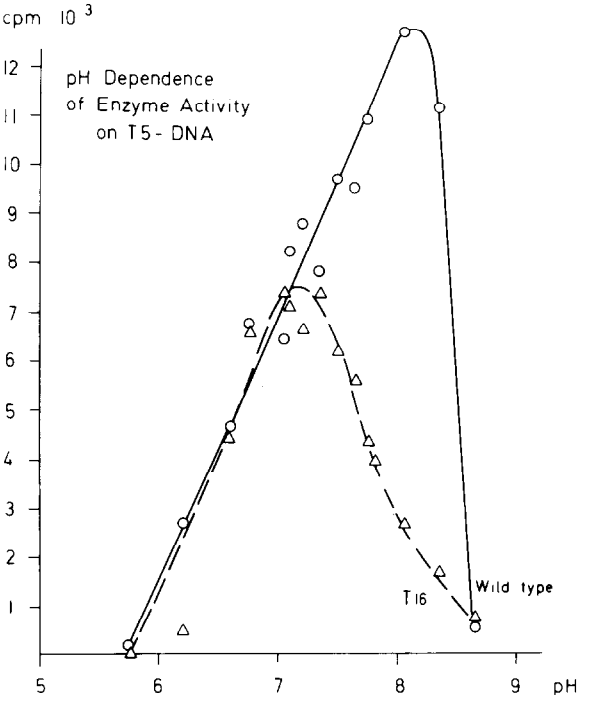

Fig. 1. pH-Dependence of T16 enzyme as compared with that of normal enzyme. pH was adjusted at 30°C by mixing Tris acetate buffers of varying pH with unbuffered reagents. The actual mixture lacking only enzyme contained in a total volume of 1 ml: 0.1 ml 0.3 M Tris buffer, 1 mM CTP, GTP, UTP, 0.03 mg T_5-DNA, 0.12 M NH_4Cl, 0.03 M Mg acetate, 200 μg BSA, 0.2 mM [^{14}C] ATP, 1 Ci/mole, pH was measured again at 30°C before addition of 18.1 μg T 16 or 2.35 μg normal enzyme respectively to aliquots of 0.25 ml. The time of incubation was 20 min, the temperature 30°C.

type enzyme activity on NH_4Cl concentration at pH 7.0 is similar to that found by Fuchs et al. [27] at pH 7.9 (fig.2) with two activity peaks at 0.1 M and around 0.23 M NH_4Cl. T16 enzyme has only one activity maximum at 0.1–0.12 M NH_4Cl under these conditions.

On the other hand, an increase of Mg^{2+} concentration from 0.03 to 0.07 M at optimal NH_4Cl concentration (0.12 M) and pH (7.0) increases the specific activity of mutant enzyme 2.5-fold, whereas that of wildtype enzyme remains almost unchanged.

The specific activity of mutant enzyme is several-fold lower than that of wildtype enzyme, even under conditions optimal for T16 RNA polymerase.

In contrast to wildtype, T16 enzyme is quite unstable

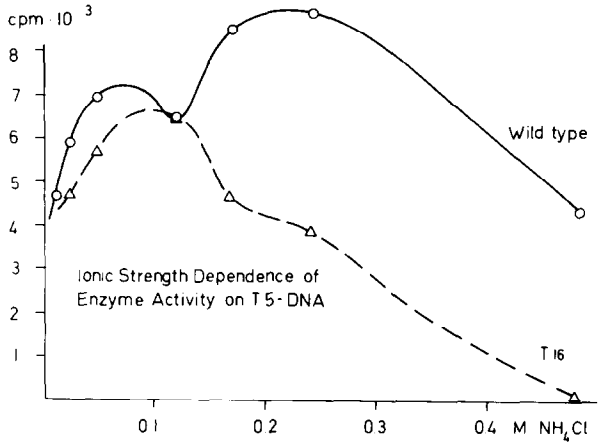

Fig. 2. Dependence of T16 and wildtype enzyme on NH_4Cl concentration. The amounts of T16 and normal enzyme and the test conditions were as described in the legend of fig.1 at pH 6.8, except for varying concentrations of NH_4Cl as indicated in fig.2.

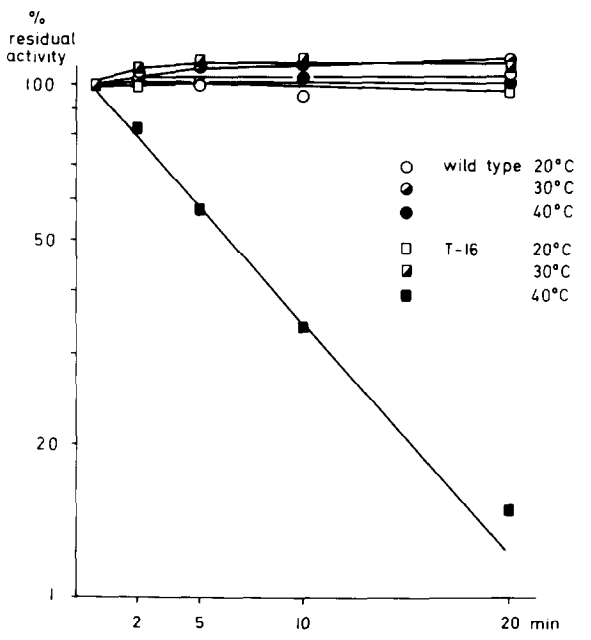

Fig. 3. Stability of T16 and wildtype enzyme at 30°C and 40°C. Wildtype enzyme (0.235 mg) and T16 enzyme (0.09 mg) were diluted in reconstitution buffer [2] containing BSA in a concentration of 0.5 mg/ml to a total volume of 0.035 ml each. A first sample was taken for determination of the zero point. Then the mixtures were preincubated at 30°C or 40°C. 0.005 ml aliquots were taken at 2,5,10 and 20 min and tested at pH 7.1 for 20 min at 30°C. Specific activity of [^{14}C] ATP was 5 Ci/mol. Specific activity of normal enzyme: 53.5, of T 16 enzyme: 9.2 mU/mg.

even at 0°C. As demonstrated by gradient centrifugation it shows a great tendency to dissociate into subunits or complexes of subunits and also to form aggregates even at 0.5 M NH_4Cl. On the other hand it may be reconstituted from isolated subunits with an activity yield comparable to that of wildtype enzyme.

There is some evidence that the tsX mutation interferes with the interaction of enzyme and DNA [21]. Preliminary results indicate that the heparin stable complex [29] of T 16 enzyme with DNA is formed more slowly than with normal enzyme. Nevertheless, the decay of a complex formed with T16 enzyme follows the same kinetics as that of a complex formed with wildtype enzyme not only at 30°C but even at 40°C [29]. This indicates that a step prior to formation of the heparin stable complex is influenced by the mutation.

The marked temperature sensitivity of T16 enzyme comprises two different effects. 1) At 40°C T16 enzyme is subject to a first order inactivation with a half life of 7 min, which is not observed at 30°C. Wildtype enzyme is stable at both temperatures (fig.3). 2) No RNA-synthesis can be observed at 40°C. Temperature shift to 30°C restores the activity of the surviving fraction of the enzyme.

This thermosensitivity is also exhibited by enzymes from several other strains bearing the tsX mutation (ts19, R8, R3) [21].

3.2. *tsX is a mutation in the β′ gene*

A first assignment of β′ to the tsX mutation could be achieved by complementation of inactivated enzyme preparations or crude extracts with pure wildtype β′ yielding reactivated and temperature independent enzyme. Normal subunits α and β were unable to complement T16 extracts. Final proof was obtained by mixed reconstitution of mutant with wildtype subunits. The products were preincubated either at 30°C or at 40°C and assayed for remaining enzyme activity at 30°C (fig.4).

The wildtype reconstituate and the mixed reconstituates containing mutant α and β retained essentially

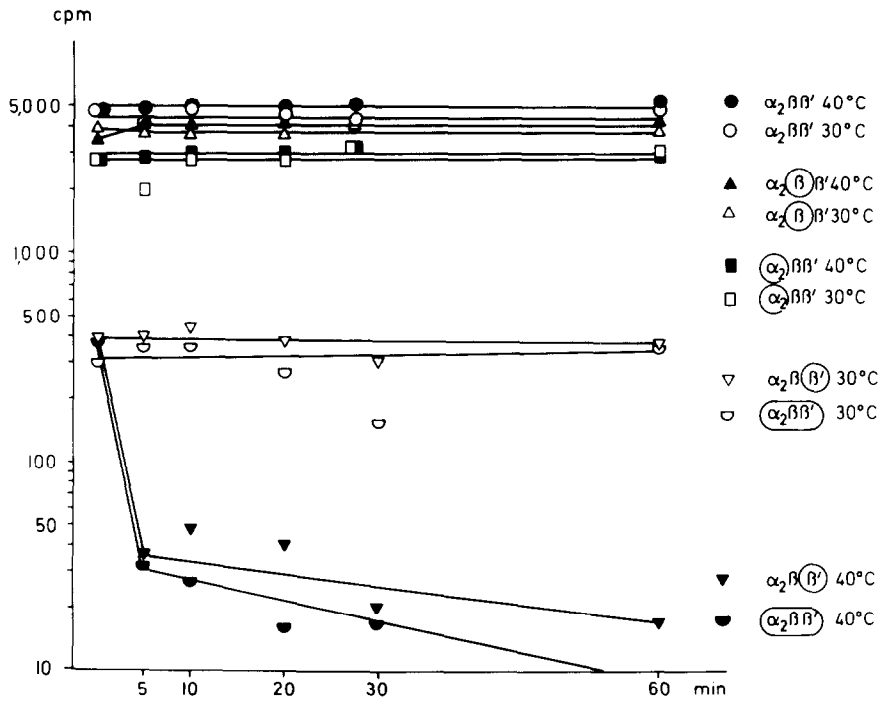

Fig. 4. Activity and temperature sensitivity of reconstituted RNA polymerase. After mixing subunits in stoichiometric amounts (0.2 mg/ml each) reconstituates were kept at 20°C for 20 min, then gradually warmed to 30°C for another 20 min. One half of each sample was further incubated at 30°C, the other at 40°C. Aliquots were removed at the indicated times and assayed for residual activity at 30°C. 10 µl of enzyme, containing 2 µg, were added to prewarmed assay mixture (240 µl) cf. fig.3. 30 sec later, 10 µl heparin (20 mg/ml) was added, to prevent initiation by enzyme reactivated during the assay. Specific activity of [^{14}C] ATP was 5 Ci/mol. Encircled symbols designate subunits derived from T16 enzyme.

100% of their zero time activity after 60 min of incubation at either 30°C or 40°C. The mutant reconstituate and the mixed reconstituate containing mutant β' also retained their zero time activity at 30°C for 60 min, but lost all their activity after 5 min of incubation at 40°C. In addition, at 30°C all reconstituates with β' derived from T16 enzyme had only 10% of the specific activity of reconstituates containing wildtype β' regardless of the origin of α and β.

The properties of the T16 enzyme such as low specific activity, even at permissive temperatures, and thermosensitivity can be transferred only by the β'-subunit, giving clear evidence that the tsX mutation is located in the β' gene. It was shown [21] that the rif and the tsX locus are closely linked. This is consistent with evidence that β and β' are in one operon [19,20]. Since enzymes with the tsX mutation are inhibited in the binding to DNA [21] this finding supports evidence for the involvement of β' in DNA binding [29]. The genetic finding that two rif-r-mutations are incompatible with tsX [22] points to a structural incompatibility of the mutant β and β'-subunits. Other double mutations rif-r-tsX are viable, indicating a mutual influence of β- and β'-subunits, as the ability of the enzyme to bind rifampicin is partially restored in these strains [22].

Acknowledgements

Thanks are due to the Deutsche Forschungsgemeinschaft and SFB 51 for generous support of this work and to Miss H. Büger for valuable technical assistance.

References

[1] Rabussay, D. and Zillig, W. (1969) FEBS Lett. 5, 104–106.
[2] Heil, A. and Zillig, W. (1970) FEBS Lett. 11, 165–168.
[3] Iwakura, Y., Ishihama, A. and Yura, T. (1973) Molec. gen. Genet. 121, 181–196.
[4] Boyd, D. H., Zillig, W. and Scaife, F. J. G. (1974) Molec. gen. Genet. 130, 315–320.
[5] Tocchini-Valentini, G. P., Marino, P. and Colvill, A. J. (1968) Nature (London) 220, 275–276.
[6] Yura, T. and Igarashi, K. (1968) Proc. Natl. Acad. Sci. U.S. 61, 1313–1319.
[7] Schleif, R. (1969) Nature (London) 223, 1068–1069.
[8] Sokolova, E. V., Ovadis, M. J., Gorlenko, Zh. M. and Khesin, R. B. (1970) Biochem. Biophys. Res. Commun. 41, 870–876.
[9] Austin, S. J. Tittawella, J. P., Hayward, R. S. and Scaife, J. G. (1971) Nature (London) New Biol. 232, 133–136.
[10] Kalyaeva, E. S. (1971) Genetica 8, 96–104.
[11] Austin, S. J. and Scaife, J. G. (1970) J. Mol. Biol. 49, 263–267.
[12] Marshall, S. and Gillespie, A. (1972) J. Bacterio. 110, 782–784.
[13] Khesin, R. B., Mindlin, S. Z., Ilyina, T. S., Ovadis, M. I. and Gorlenko, Zh. M. (1971) Molekularnaya Biologiya 5, 862–877.
[14] Babinet, Ch. (1970) in: Lepetit Colloq. RNA Polymerase and Transcription (Silvestri, L. ed.) p. 37–45, North Holland Publ. Comp., Amsterdam.
[15] Jacobsen, A. and Gillespie, D. (1971) Biochem. Biophys. Res. Commun. 44, 1030–1040.
[16] Yura, T., Igarashi, K., Masukata, K. (1970) in: Lepetit Colloq. RNA Polymerase and Transcription (Silvestri, L. ed.) p. 71–89, North Holland Publ. Comp., Amsterdam.
[17] Palm, P. (1970), unpublished data.
[18] Matzura, H., Molin, S. and Maaløe, O. (1971) J. Mol. Biol. 59, 17–25.
[19] Errington, L., Glass, R. E., Hayward, R. S. and Scaife, J. G. (1974) Nature (London) 249, 519–522.
[20] Nikiforov, V. G., Kalyaeva, E. S. and Velkov, V. V. (1974) Molec. gen. Genet. 130, 1–7.
[21] Khesin, R. B., Gorlenko, Zh. M., Shemyakin, M. F., Stvolinsky, S. L., Mindlin, S. Z. and Ilyina, T. S. (1969) Molec. gen. Genet. 105, 243–261.
[22] Ilyina, T. S., Ovadis, M. I., Mindlin, S. Z., Gorlenko, Zh. M. and Khesin, R. B. (1971) Molec. gen. Genet. 110, 118–133.
[23] Khesin, R. B., Mindlin, S. Z., Gorlenko, Zh. M. and Ilyina, T. S. (1968) Molec. gen. Genet. 103, 194–208.
[24] Zillig, W., Zechel, K. and Halbwachs, H. J. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 221–224.
[25] Schaller, H., Nüsslein, C., Bonhoeffer, F. J., Kurz, C. and Nietzschmann, I. (1972) Eur. J. Biochem. 26, 474–481.
[26] Palm, P. et al. (1974), in preparation.
[27] Fuchs, E., Millette, R. L., Zillig, W. and Walter, G. (1967) Europ. J. Biochem. 3, 183–193.
[28] Laemmli, U. K. (1970) Nature (London) 227, 680–685.
[29] Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V. S., Palm, P, Heil, A. and Seifert, W. (1970) Cold Spring Harbor Symp. Quant. Biol. 35. 47–57.