DNA-DEPENDENT RNA POLYMERASE FROM THE THERMOPHILIC BACTERIUM *THERMUS AQUATICUS*

Gillian M. AIR and J. Ieuan HARRIS
Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, England

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1. Introduction

As part of a study of enzymes from the thermophilic bacterium *Thermus aquaticus,* which grows at 70–75°C [1], the DNA-dependent RNA polymerase (ribonucleoside triphosphate: RNA nucleotidyl transferase, EC 2.7.7.6) has been isolated from this source and, for comparison, from *Bacillus stearothermophilus* which grows at 60–65°C. The *T. aquaticus* enzyme as purified contains subunits of mol. wt. approx. 170 000, 160 000, 99 000 and 45 000, similar to the β', β, σ and α subunits of the RNA polymerase from *Escherichia coli* [2] and *B. stearothermophilus*, but other lower molecular weight polypeptides are carried through the preparation with the enzyme. The *T. aquaticus* RNA polymerase has similar metal ion and pH requirements to the *E. coli* and *B. stearothermophilus* enzymes. It is maximally active at 50–60°C and loses only 20% of the activity after incubation for 30 min at 70°C. The polymerase reaction is sensitive to salts, 0.15 M KCl or NH_4Cl causing a 50% loss in RNA synthesis.

2. Materials and methods

2.1. Assay of RNA polymerase

The assay medium was based on that of Burgess [2]. The final volume was 45 µl, containing 5 mM $MgCl_2$, 5 mM $MnCl_2$, 6 mM dithiothreitol, 0.45 mM phosphate, 0.5 mM each of UTP, ATP, CTP and GTP with 0.1 µCi of [^{14}C]UTP, 9 µg calf thymus DNA and 50 mM Tris–HCl of a pH calculated to be 8.0 at the assay temperature, routinely 50°C. The enzyme was added to start the reaction. After 10 min incubation the reaction was terminated by adding 3 ml cold 5% trichloroacetic acid and the precipitate collected on a Whatman GF/C filter, washed three times with 3 ml 2.5% trichloroacetic acid, dried with three washes of 3 ml absolute ethanol, and counted in a liquid scintillator counter. One unit of activity is defined as incorporation of 1 nmole UTP into acid-precipitable material in 10 min at 50°C.

2.2. Preparation of T. aquaticus RNA polymerase

The initial stages of the preparation were carried out by J.D. Hocking as described for the preparation of glyceraldehyde-3-phosphate dehydrogenase from *T. aquaticus* strain ATCC 25104 [3]. After disruption of the cells in the French press, treatment with DNAase and additiona of Whatman DE-23, most of the RNA polymerase activity remained in the supernatant fraction. Solid ammonium sulphate (40 g per 100 ml supernatant) was then added and the precipitate containing RNA polymerase sedimented firmly by centrifugation for 90 min at 23 000 *g*. The precipitate was suspended in 2 M ammonium sulphate in buffer 'B' (20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA and 5% glycerol), and centrifuged for 60 min at 23 000 *g*. Some RNA polymerase was in this supernatant; the rest was extracted by 1 M ammonium sulphate in buffer 'B'. The 2 M and 1 M ammonium sulphate supernatants were combined and 30 g ammonium sulphate added per 100 ml to re-precipitate the RNA polymerase. After centrifugation the precipitate was dissolved in and dialysed briefly against buffer 'B' until the specific conductivity was reduced

to 10 mmho. This solution was loaded on a column of Whatman DE-52 (3 × 30 cm) equilibrated in buffer 'B', and washed with buffer 'B' until the extinction at 280 nm due to unadsorbed material began to fall. A gradient was then started, containing 750 ml each of buffer 'B' plus 0.1 M KCl and buffer 'B' plus 0.6 M KCl. The RNA polymerase was eluted between 400 and 500 ml of this gradient. Fractions containing the activity were pooled and the enzyme precipitated with ammonium sulphate (45 g/100 ml).

Further purification of *T. aquaticus* RNA polymerase was based on the property of reversible aggregation at low ionic strength, as observed for the *E. coli* enzyme [2]. Repeated passage through a Bio-Gel A5m column (1.5 × 70 cm) alternately in buffer 'B' plus 1 M ammonium chloride and in buffer 'B' alone gave significant purification of the enzyme. In the high ionic strength buffer the RNA polymerase has an R_f of about 0.5, although fractions must be dialysed free of the salt before any activity can be detected. In the low ionic strength buffer the enzyme emerges in the breakthrough peak. After two to four cycles of the high and low ionic strength gel filtration, monitoring the purification by gel electrophoresis in sodium dodecyl sulphate [4], the enzyme was precipitated with ammonium sulphate (45 g/100 ml), dialysed against buffer 'B' in 50% glycerol, and stored at −20°C.

2.3. Preparation of B. stearothermophilus RNA polymerase

The preparation was similar to that for the *T. aquaticus* enzyme, but omitted the batch addition of DE-23.

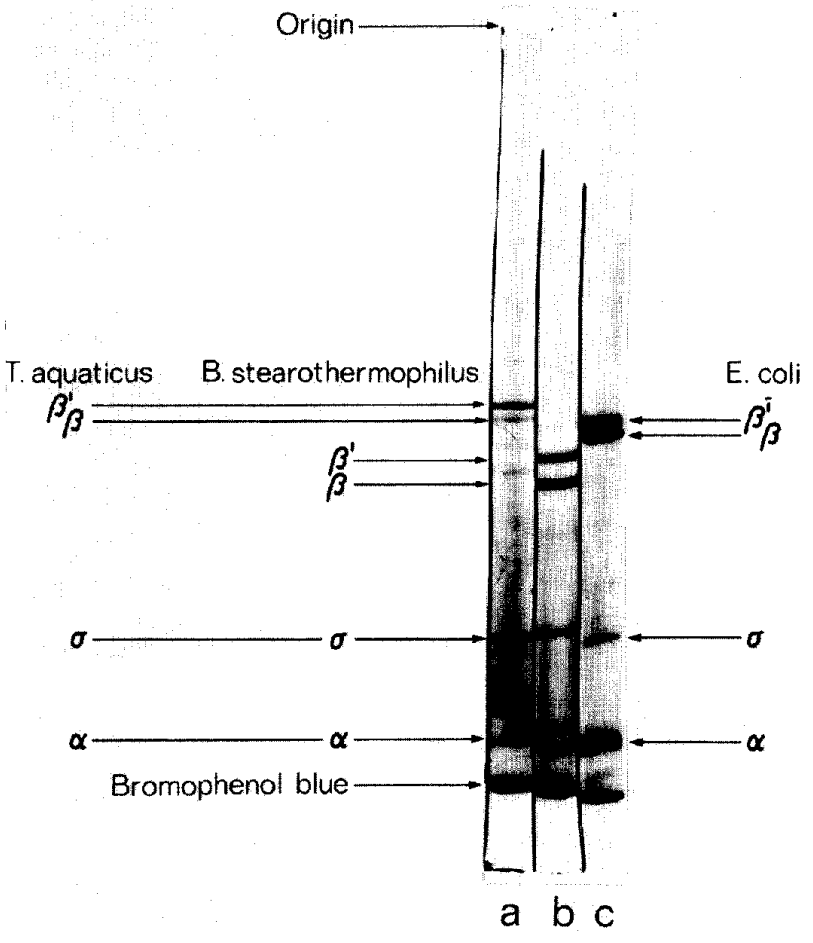

Fig. 1. SDS-gel electrophoresis on 5% polyacrylamide gels [4] of polymerases from (a) *T. aquaticus*, (b) *B. stearothermophilus* and (c) *E. coli*.

3. Properties of the RNA polymerase

Fig. 1 shows the results of electrophoresis on 5% polyacrylamide gels in 0.1% SDS [4] of the *T. aquaticus* RNA polymerase compared to that of *B. stearothermophilus* prepared by the same method, and *E. coli* RNA polymerase prepared by Dr. A. Travers. Bands corresponding to the *E. coli* β', β, α and σ, can be seen in both the *T. aquaticus* and *B. stearothermophilus* preparation, although in the *T. aquaticus* enzyme the supposed β band stains less intensively than the β'. This may be due to proteolysis during the preparation, since this band decreased even further storage of the enzyme for several months. On 10% polyacrylamide gels (fig. 2) many other bands, mostly of lower molecular weight, are seen. It has not been possible to remove these by further re-cycling through the Bio-Gel A5m column in high an low salt, or by chromatography on Bio-Rex 70, SP-Sephadex, phosphocellulose (Whatman P11), hydroxyapatite, DEAE-Sephadex or DNA-cellulose. In the first three cases the enzyme was not adsorbed to the ion-exchanger; in the other cases it was adsorbed to varying extents, but almost entirely inactivated on elution without signigicant changes in the SDS gel electrophoresis patterns.

No DNAase or RNAase activity could be detected in the enzyme purified as described. The final specific activity of the preparations was routinely about 100 units/mg, but it is not possible to estimate the overall

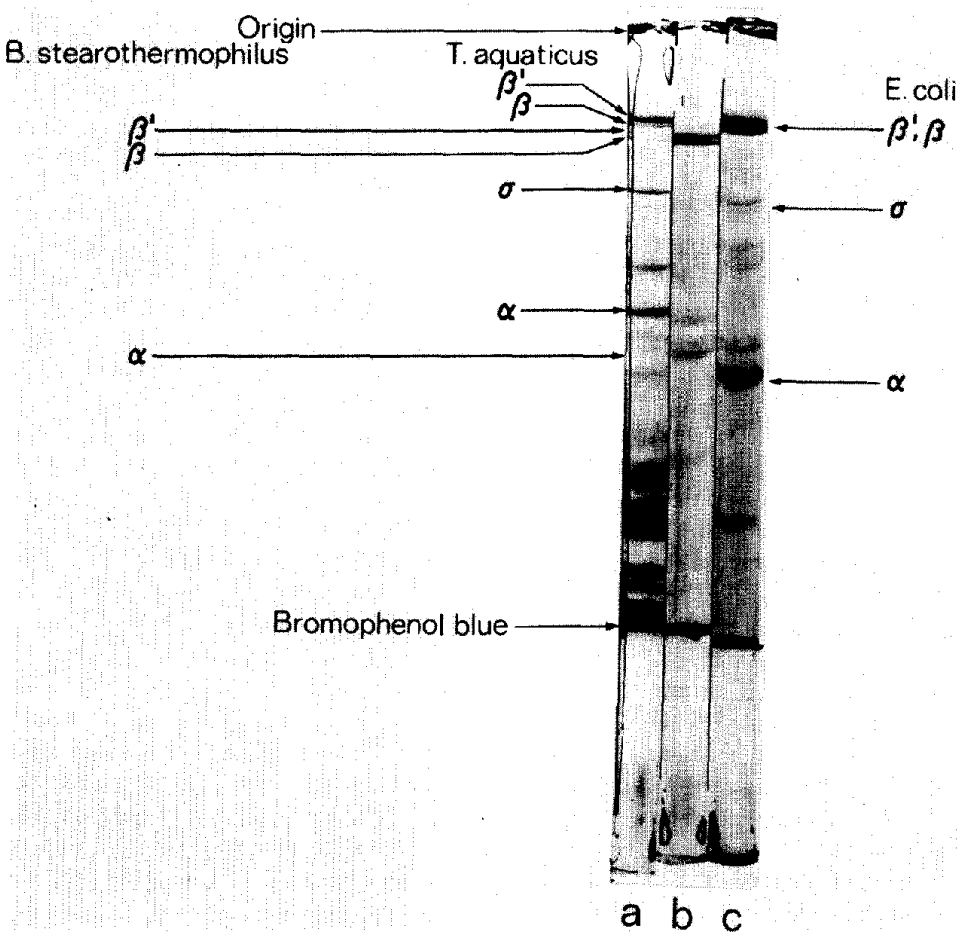

Fig. 2. SDS-gel electrophoresis on 10% polyacrylamide gels of RNA polymerases from (a) *T. aquaticus*, (b) *B. stearothermophilus* and (c) *E. coli*.

Table 1
Effect of salts on the activity of RNA polymerase from *T. aquaticus*.

Concentration of salt (mM)	Percentage activity of RNA polymerase		
	KCl	NH_4Cl	NH_4 acetate
0	100	100	100
50	87	92	105
100	67	68	83
250	5	13	31
500	1	4	2

In these experiments the assay mix was as described in the Materials and methods section but without DNA. The salts were added to the enzyme plus substrates, the mixture incubated 15 min at 50°C, and DNA added to start the reaction. The same results were obtained if the enzyme was added last.

yield of RNA polymerase activity or the extent of purification, since no activity can be detected in the initial cell extract from either *T. aquaticus* or *B. stearothermophilus*. Each step of the preparation gives an increase in total enzyme activity provided that salt above 0.1 M is dialysed out before the assay.

This sensitivity to salt was further investigated with the purified enzyme. Table 1 shows the effect of increasing concentrations of KCl, NH_4Cl and NH_4 acetate on *T. aquaticus* RNA polymerase. The results with *B. stearothermophilus* RNA polymerase, adding NaCl instead of NH_4 Cl, were almost identical. Both are 50% inhibited by about 0.15 M KCl, NH_4Cl or NaCl. Ammonium acetate is less inhibitory. Removal of salts by rapid dialysis gave complete recovery of activity. The same inhibition was observed if the enzyme was preincubated with salt for 15 min at 50°C and the reaction started by adding DNA, or if the enzyme was added last as in the standard assay.

The *T. aquaticus* enzyme is maximally active at pH 8.0, although there is little variation in activity between pH 7.5 and 8.4. This is similar to the pH requirements of the *E. coli* [2] and *B. stearothermophilus* [5] enzymes. A requirement for Mg^{2+} or Mn^{2+} is the same for the RNA polymerases from the three sources, and all are dependent on a DNA template being present.

Studies on the thermostability of the *T. aquaticus* RNA polymerase were complicated by the large variation of the acid dissociation constant of Tris buffer with temperature. The enzyme is inhibited by phosphate (25 mM giving 90% inhibition) or borate, hence the temperature studies were carried out in Tris buffers, made up at room temperature to a pH calculated to be pH 8.0 at the required temperature. The *T. aquaticus* enzyme was found to be maximally active between 50°C and 60°C. The activity is half this maximum at 30°C and 75°C, while at 85°C most activity was lost. The *B. stearothermophilus* enzyme was found to be most active at 45–50°C as reported by Remold-O'Donnell and Zillig [5].

To study the thermostability of the enzymes, the *T. aquaticus* and *B. stearothermophilus* RNA polymerases were incubated in buffer 'B' for varying times at 70°C, cooled in ice, then substrates added and the enzymes assayed at 50°C. after 10 min at 70°C the *B. stearothermophilus* enzyme is over half inactivated, while the *T. aquaticus* enzyme is still 65% active after 1 hr. at 60°C the *T. aquaticus* RNA polymerase was fully active after 1 hr incubation. The enzyme from *E. coli* has been reported as half inactivated after 10 min at 45–50°C [6].

4. Discussion

The RNA polymerase from *T. aquaticus* appears to have the same basic subunit structure as the *E. coli* enzyme. The molecular weights estimated from SDS-gel electrophoresis (figs. 1 and 2) were ~170 000, ~160 000, ~99 000 and ~45 000 for the β', β, σ and α components respectively. The values obtained for the same subunits of *B. stearothermophilus* RNA polymerase were ~148 000, ~140 000, ~95 000 and ~40 000. These estimates were calculated from the mobilities of the *E. coli* subunits of molecular weights of 165 000, 155 000, 96 000 and 39 000 [2]. However, several other polypeptides are present in the *T. aquaticus* preparation which we have been unable to remove We assume these are contaminants which absorb to the RNA polymerase, or perhaps to residual acidic carbohydrates of the *T. aquaticus* 'slime' remaining in the preparation, but we cannot yet dismiss the possibility that some or all of them are involved in the enzyme reaction. The final specific activity of the preparation at 50°C was about 25% of the value given for *E. coli* RNA polymerase at 37°C [2]. Both the *T. aquaticus* and *B. stearothermophilus* enzymes are sensitive to salt, at least when assayed with calf thymus DNA, resulting in apparent loss in activity at several stages of

the purification unless the fractions are dialysed before the assay.

As in the case of the smaller and less complex glycolytic enzymes [3, 7], the difference in growth temperatures of *B. stearothermophilus* (60–65°C) and *T. aquaticus* (70–75°C) is reflected in the increased thermostability of the RNA polymerases, particularly when compared with *E. coli* RNA polymerase. This marked thermostability of RNA polymerase from *T. aquaticus* may be useful for studies on transcription control, since the polymerase is active at temperatures approaching the melting temperatures of many DNA's.

Acknowledgements

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