# Purification and Initial Characterization of RNA Polymerase from *Thermus thermophilus* Strain HB8[†]

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ABSTRACT: Utilizing a novel and rapid two-column purification procedure, the DNA-dependent RNA polymerase (RNAP) from the thermophile, *Thermus thermophilus* HB8, was purified to electrophoretic homogeneity with a recovery of 65% (as determined by RNAP activity) in less than 2 days. The purified enzyme was characterized using DNA containing the $\lambda P_R$ promoter. $KMnO_4$ footprinting, abortive initiation assays, and the formation of the specific stalled elongation complex provide compelling evidence that *T. thermophilus* RNA polymerase can bind to DNA containing the $\lambda P_R$ promoter, form an open complex, and initiate transcription in a temperature-dependent manner. This evidence suggests that *T. thermophilus* RNAP possesses less intrinsic binding energy than *E. coli* RNAP. Instead, *T. thermophilus* relies on the high temperatures of its environment to provide the thermal energy required to stimulate open promoter complex formation, initiate transcription, and facilitate the conformational changes in RNA polymerase that result in nucleotide incorporation.

Transcription is the first step in the chain of events by which the genetic information encoded in double-stranded DNA is expressed as proteins. The process of transcription involves several steps that are accompanied by large, rate-limiting, conformational changes in the RNA polymerase and template DNA (*1*). For example, the initiation of transcription requires that approximately 10 base pairs of DNA be melted to form an open complex. While such conformational changes have been well characterized, the relative contributions of the conformational changes in the RNA polymerase and in DNA are not well understood. Investigation of thermostable homologues of mesophilic RNA polymerases should provide insight into the role of conformational transitions in the regulation of transcription because thermostable proteins appear to possess a high degree of global conformational stability (*2*). It is believed that the thermostability of enzymes derived from thermophilic sources comes at the cost of reduced conformational flexibility in the native state. This view dictates that a greater amount of thermal energy would be required for these enzymes to achieve the same degree of conformational dynamics as their mesophilic counterparts (*2*). Consistent with this idea, it has been shown that the maximal catalytic rates of homologous enzymes from mesophilic and thermophilic organisms are similar when the proteins are incubated at their respective temperature optima (*3*−*5*). These results suggest that ambient temperature provides a portion of the energy required to facilitate the conformational changes necessary for catalysis. Finally, the broad range of temperatures (∼20−100 °C) over which thermostable enzymes are active makes them ideal for studying the role of temperature in driving conformational transitions.

To elucidate the individual contributions of protein and nucleic acid conformational changes to transcription, it is essential to compare the enzymatic properties of RNA polymerase on the same template DNA. We have chosen to study the DNA-dependent RNA polymerase from the extreme thermophile, *Thermus thermophilus*. We reasoned that it should be possible to compare the properties of *Escherichia coli* and *T. thermophilus* RNAP on the same DNA template because they have the same highly conserved subunit composition ($\alpha_2\beta\beta'\sigma$) and similar consensus sequences in their promoters (*6*, *7*). Because transcription from the $\lambda P_R$ promoter has been well characterized with *E. coli* RNAP, we set out to investigate the properties of *T. thermophilus* RNAP on this promoter. By characterizing *T. thermophilus* RNAP on the $\lambda P_R$ promoter, we are able to directly compare the mechanistic similarities that exist between these two evolutionarily divergent polymerases. We describe a novel and rapid method of purification of the DNA-dependent RNA polymerase from *T. thermophilus* and characterize the temperature requirements that govern transcription from the $\lambda P_R$ promoter.

## MATERIALS AND METHODS

*Cells and Reagents. Thermus thermophilus* HB8 dry cells were purchased from the University of Wisconsin Biotechnology center. Cells were grown at 70−75 °C in medium D (*8*), supplemented with 5 g of tryptone, 4 g of yeast extract, 2 g of NaCl, and 1 g of glucose/L. The cells were stored at −70 °C prior to use in the RNAP preparation. *E. coli* XL1blue cells harboring the plasmid pDE13, which carries the $\lambda P_R$ promoter, were grown at 37 °C in Luria broth in the

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presence of ampicillin (50 $\mu$g/mL). Electrophoresis grade acrylamide, *N*,*N*′-methylenebisacrylamide, TEMED,[1] and ammonium persulfate were obtained from Bio-Rad. Mid-range molecular weight markers were purchased from Promega and Gibco. Calf thymus DNA and glycerin were purchased from Fisher. Coomassie Brilliant Blue R-250, enzyme grade ammonium sulfate, Polymin P, 2-mercaptoethanol, Tris-aminomethane, and EDTA were obtained from Sigma. Nucleotides were purchased from Boehringer Mannheim Biochemicals. [$\gamma$-$^{32}$P]ATP and [$\alpha$-$^{32}$P]CTP were purchased from New England Nuclear. Bacteriophage T7 DNA template was prepared as described previously (*9*). Scintisafe Econo1 scintillation fluid was purchased from Fisher.

*Columns*. HQ column: Protein separation was performed at room temperature utilizing a perfusion chromatography system (PerSeptive Biosystems, Inc) and employing a 4.6 mm diameter/100 mm length HQ anion exchange column (also purchased from PerSeptive Biosystems, Inc). The column was prepared using flow packing techniques as per the manufacturer's specifications. Phosphocellulose column (purchased from Bio-Rad): A 50-mL column of phosphocellulose was prepared as described previously (*10*).

*Buffers*. All buffers and stock solutions used for *T. thermophilus* RNAP purification were the same as those used for the purification of the *E. coli* holoenzyme (*11*). TGED buffer (0.01 M Tris-HCl, pH 7.9, at 20 °C, 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT) was used as the base buffer throughout the purification, with the ionic strength varied by the addition of NaCl. Cells were resuspended in grinding buffer (TGED + 23 $\mu$g/mL PMSF and 0.2 g/100 mL lysozyme) prior to lysis. All buffers, solutions, and Nanopure water used in the chromatography were filtered through a 0.22-$\mu$m filter. The reaction mix for the enzyme activity assays contained 40 mM Tris-HCl, pH 7.6, 4 mM $MgCl_2$, 120 mM potassium acetate (KAc), 1 mM DTT, 4 mM spermidine, 2.7 mM ATP, 1.4 mM UTP, 1.1 mM GTP, 0.7 mM [$\alpha$-$^{32}$P]CTP, and 50 $\mu$g/mL T7 DNA. The reactions were quenched with YEP solution (0.5 mg/mL yeast tRNA, 50 mM EDTA, and 50 mM sodium pyrophosphate). The phosphocellulose column was equilibrated with P5 buffer (40 mM potassium phosphate, pH 8.0, 1 mM DTT, 0.1 mM EDTA, and 5% glycerol) before the addition of the protein sample. P50 buffer (40 mM potassium phosphate, pH 8.0, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol) served as running buffer during phosphocellulose column chromatography.

*DNA Templates*. T7 DNA was used for all activity assays. All other reactions were run on a 542 nucleotide (nt) PCR fragment, which is derived from pDE13 (*12*). The first 30 nucleotides of the transcript are as follows: pppAUGUAGUAAG GAGGUUGUAU GGAACAACGC.

*Protein Determination*. Protein concentrations were determined by UV absorbance using extinction coefficient data (*13*). The purity of the RNAP preparation was estimated by SDS−PAGE.

*Enzyme Activity Assay*. RNA polymerase activity assays were performed as described previously with slight modification (*14*). Specifically, 2 $\mu$L of protein sample were added to 98 $\mu$L of reaction mix and incubated at 55 °C for 3 min. The reaction was quenched with 200 $\mu$L of ice-cold YEP. A total of 3 mL of ice-cold 5% trichloroacetic acid was added to each reaction and incubated on ice for 30 min. The precipitated nucleic acids were collected on Whatman GF/A filters and washed with 30 mL of HCl−$PP_i$ buffer (1 M HCl and 0.1 M sodium pyrophosphate). Filters were dried and immersed in Scintisafe Econo1 scintillation fluid, and the radioactivity was measured in a Tri-Carb 2000 liquid scintillation counter.

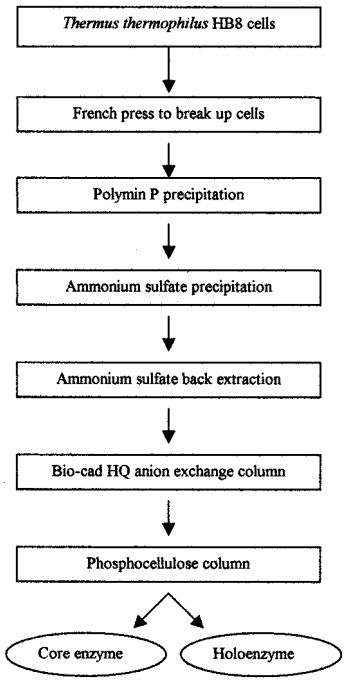


FIGURE 1: Schematic for the purification and fractionation of RNAP core enzyme and holoenzyme (*17*).

*Gel and Phosphorimager Analysis.* Gel image data were obtained from a Molecular Dynamics phosphorimager and analyzed by Image Quant software (Molecular Dynamics, version 4.2).

*Purification Procedure.* A schematic for the purification procedure is shown in Figure 1.

(*i*) *Polymin P Trial*. Trials were run to determine the concentration of Polymin P required to precipitate *T. thermophilus* RNAP and the concentration of NaCl required to elute it. Polymin P precipitation trial: To a 200-$\mu$L aliquot of the 8 K supernatant, 10% (v/v) Polymin P was added in increasing amounts. The tubes were mixed, incubated 20 min at 4 °C, and centrifuged at 13 000 rpm for 5 min. The supernatant was assayed for RNAP activity. NaCl elution trial: To a 2-mL aliquot of 8 K supernatant, 100 $\mu$L of 10% Polymin P was added as described above. After centrifugation, the pellets were resuspended in 200 $\mu$L of TGED buffer containing various NaCl concentrations. The tubes were centrifuged, and the supernatants were assayed for polymerase activity.

(*ii*) *Large-Scale Purification.* A total of 100 g of *T. thermophilus* HB8 cells was resuspended in 400 mL of grinding buffer and lysed using a French press. The cell extract centrifuged for 45 min at 8000 rpm, and the supernatant was collected (390 mL). A 10% (v/v) solution of Polymin P (pH 7.9) was added to the 8 K supernatant to

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RNAP, RNA polymerase; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

a final concentration of 0.5%. The mixture was centrifuged for 15 min at 6000 rpm. The pellet was washed with 400 mL of TGED, and the RNAP was subsequently eluted with 400 mL of TGED + 0.5 M NaCl. Solid ammonium sulfate was added to the 0.5 M NaCl eluate to a final saturation of 50%. After being stirred for 1 h at 4 °C, the sample was centrifuged for 45 min at 8000 rpm, and the supernatant was discarded. The nucleic acids were extracted from the pellet by washing with 2 M ammonium sulfate (*15*). The ratio of $A_{280}/A_{260}$ was 1.8 after this step, indicating that the majority of the nucleic acids was removed. The pellet was resuspended in TGED and diluted or dialyzed with TGED until the conductivity was equal to that of TGED + 0.1 M NaCl.

(*iii*) *HQ Chromotography*. Prior to loading the sample, the HQ column was washed with 30 column volumes (CV) of deionized water and then equilibrated with 30 CV of TGED + 0.1 M NaCl. The protein sample was loaded onto the column in TGED + 0.1 M NaCl. Subsequently, the column was washed two times to remove the contaminating proteins: once with 40 CV of TGED + 0.1 M NaCl and then once with 40 CV of TGED + 0.3 M NaCl. A shallow gradient from 0.3 M NaCl to 0.6 M NaCl over 100 CV was used to elute RNAP. SDS−PAGE and RNAP activity assays were performed on every second fraction. Fractions containing the highest polymerase activity were pooled. Approximately 60 mg of total protein were loaded onto the HQ column for each run, which approaches the binding capacity of the HQ resin (60 mg/mL).

(*iv*) *Phosphocellulose Column*. The purification on the phosphocellulose column was performed as described for *E. coli* RNA polymerase (*10*). The pooled fractions from the HQ column containing the highest RNAP activity were dialyzed overnight against a solution containing 50 mM Tris-HCl, pH 8, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 50% glycerol. The dialyzed sample was adjusted to a final protein concentration of 1 mg/mL with the same buffer. A phosphocellulose column (1.2 cm × 12 cm) was prepared and washed with 150 mL of P5 buffer. A total of 5 mg of pooled protein from the HQ column was loaded onto the phosphocellulose column and equilibrated with P50 buffer until the absorbance at 280 nm was below 0.01. RNAP holoenzyme was then eluted with P50 buffer containing 0.2 M KCl. After equilibrating the column with P50 buffer until $A_{280}$ was below 0.01, the core RNAP was eluted with P50 buffer containing 0.5 M KCl. Fractions containing RNAP holoenzyme were adjusted to 10 mM $MgCl_2$ and stored at protein concentrations of 100 $\mu$g/mL at −20 or −80 °C.

*Enzyme Characterization.* (*i*) *$KMnO_4$ Footprinting*. The nontemplate strand of the 542-nt PCR product containing the $\lambda P_R$ promoter was labeled by performing PCR using a $^{32}$P-labeled primer. Open complexes were formed by incubating the purified RNAP holoenzyme with 10 nM DNA at the indicated temperature (37 or 55 °C). Formation of open complexes was assayed by $KMnO_4$ footprinting (*16*). To monitor the time dependence of open complexes formation, RNAP was incubated with DNA for increasing lengths of time. At the time points indicated in Figure 7 (1′, 2′, 5′, 10′, 15′, 20′, and 25′), 4 mM $KMnO_4$ was added to each reaction, and the complexes were incubated for an additional 5 min at 55 or 37 °C. $KMnO_4$ modification reactions were then quenched with $\beta$-mercaptoethanol and EDTA at final concentrations of 0.6 M and 20 $\mu$M, respectively. The DNA was ethanol precipitated two times and resuspended in 63 $\mu$L of water. A total of 7 $\mu$L of redistilled piperidine was added to the samples, and they were incubated at 90 °C for 30 min. Samples were precipitated two times, resuspended in 3 $\mu$L of TE (10 mM Tris-HCl, pH 8.0, and 10 mM EDTA) and 7 $\mu$L of formamide, and run on a 8 M urea and 8% acrylamide gel.

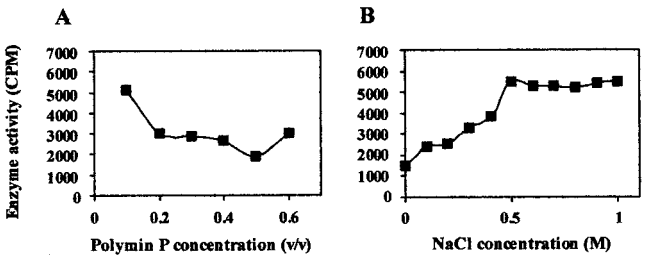


FIGURE 2: Polymin P trials. The RNAP activity of the supernatant is plotted as a function of the polymin P concentration (A) and of the NaCl concentration (B).

(*ii*) *Abortive Initiation Assay*. RNAP and 10 nM template DNA (542-nt PCR fragment) were mixed in reaction buffer (40 mM Tris-HCl, pH 8.0, 80 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT) and kept on ice. Assays were initiated by the addition of 20 $\mu$M ATP ([$\gamma$-$^{32}$P]ATP) and 20 $\mu$M UTP. Reactions were incubated at either 55 or 37 °C, depending on the polymerase. Aliquots were periodically removed over a 30-min time period and quenched with 0.2 M EDTA. The formation of the abortive product pppApU was assayed by spotting the sample onto a PEI−cellulose plate and running it in 4 M urea/0.75 M phosphate buffer at pH 3.5.

(*iii*) *Stalled Elongation Complex Formation*. Reactions were performed in 30 mM Hepes buffer (pH 7.8), 200 mM potassium glutamate, acylated bovine serum albumin (25 $\mu$g/mL), 10 mM magnesium glutamate, and 1 mM DTT. *T. thermophilus* open promoter complexes were formed by incubating RNAP and template DNA at 55 °C for 15 min. *E. coli* open promoter complexes were prepared as previously described (*12*). Complexes stalled at position 24, prior to the first cytosine to be incorporated, were formed at either 55 or 24 °C by adding 10 $\mu$M ATP, 10 $\mu$M UTP, 2.5 $\mu$M GTP, and 2.5 $\mu$M [$\alpha$-$^{32}$P]GTP. Aliquots were removed over a 20-min time period and quenched with 80% formamide. Transcription products were analyzed by electrophoresis on a 8 M urea and 20% acrylamide gel.

## RESULTS AND DISCUSSION

*Early Steps of the RNAP Purification*. Trials were run to determine the concentration of Polymin P required to precipitate *T. thermophilus* RNAP as well as the concentration of NaCl required to elute RNAP from the Polymin P pellet (Figure 2) (*17*). Inspection of Figure 2A reveals that at 0.5% Polymin P, only a small amount of RNAP activity remained in the supernatant, indicating that the majority of the RNAP had been precipitated. Figure 2B shows that although 0.5 M NaCl was required to elute all of the RNAP from the pellet, some of the precipitated RNAP eluted below 0.5 M NaCl. This condition is significantly different from that used in the purification of *E. coli* RNAP in which 1 M NaCl is required to elute RNAP from the Polymin P pellet, and no RNAP is eluted below 0.5 M NaCl. Accordingly, in

Table 1: Summary of Purification[a]

| stage of purification | 8K supernatant | 0.5 M NaCl elutant | HQ column pooled fractions | phosphocellulose pooled fractions |
|---|---|---|---|---|
| total protein (mg) | 8 840 | 850 | 50 | 15 |
| total activity (units) | 247 500 | 226 000 | 220 000 | 168 200 |
| specific activity (units/mg) | 28 | 270 | 4 400 | 11 220 |
| purification fold | 1 | 9.5 | 160 | 400 |
| total polymerase (mg)[b] | 80 | 70 | 45 | 15 |
| yield (%)[c] | 95 | 87 | 84 | 65 |

[a] From 100 g of *Thermus thermophilus* HB8 cells. [b] Determined by quantitation of $\beta'\beta$-subunits on gels. [c] Calculated from total activity, the yield for cell extract is 100%.

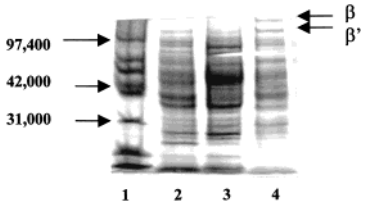


FIGURE 3: SDS−PAGE of Polymin P precipitation and NaCl elution profiles. Lane 1, protein molecular mass marker (size indicated by arrows on the left side); lane 2, 0 M NaCl wash; lane 3, 0.5% Polymin P supernatant; lane 4, 0.5 M NaCl elution. The $\beta'$- and $\beta$-subunits are identified on the right side of the gel.

the large-scale purification, 0.5% Polymin P was used to precipitate *T. thermophilus* RNAP, the pellet was washed with TGED + 0 M NaCl, and RNAP was subsequently eluted with TGED + 0.5 M NaCl. These two steps resulted in a 9.5-fold purification of RNAP (Table 1 and Figure 3). Finally, prior to loading the partially purified RNAP sample onto the anion exchange column, the 0.5 M NaCl eluate was concentrated by ammonium sulfate precipitation, and the nucleic acids were removed by back-extraction with ammonium sulfate according to methods described previously (*15*). The removal of nucleic acids was critical to this purification scheme because it allowed for better protein fractionation in the subsequent HQ chromatography steps. If the ammonium sulfate back-extraction step was omitted, a significant proportion of the RNAP did not bind to the HQ column and was eluted in the void volume.

*Bio-Cad HQ Chromatography*. HQ column chromatography was used in place of MonoQ (*18*) to improve flow rate and elution profile reproducibility. A typical elution profile from the HQ column is shown in Figure 4A. After multiple trials, we found that loading the sample in TGED + 0.1 M NaCl, washing with 40 CV of TGED + 0.1 M NaCl, and finally washing with TGED + 0.3 M NaCl effectively eluted 80% of the contaminant proteins from the column. In addition, employing a shallow linear elution gradient over the course of 100 CV enhanced the fractionation of RNAP from the HQ column. The maximum RNAP activity eluted between 0.35 and 0.38 M NaCl (Figure 4A: fractions 42−50). Consistent with this result, electrophoretic analysis of fractions 42−50 by SDS−PAGE indicated a large increase in RNAP purity (Figure 4B). A band corresponding to approximately 90 kDa can be seen in fractions 42−46. Because these fractions contain the highest level of enzyme activity (Figure 4A), we believe that this band represents the $\sigma$ subunit. This band is faint relative to the other subunits, which is not surprising because RNAP is generally only 30% saturated with the $\sigma$ subunit in vivo (*19*, *20*). In addition, $\sigma$ was found to be a similar size in an earlier purification (*7*).

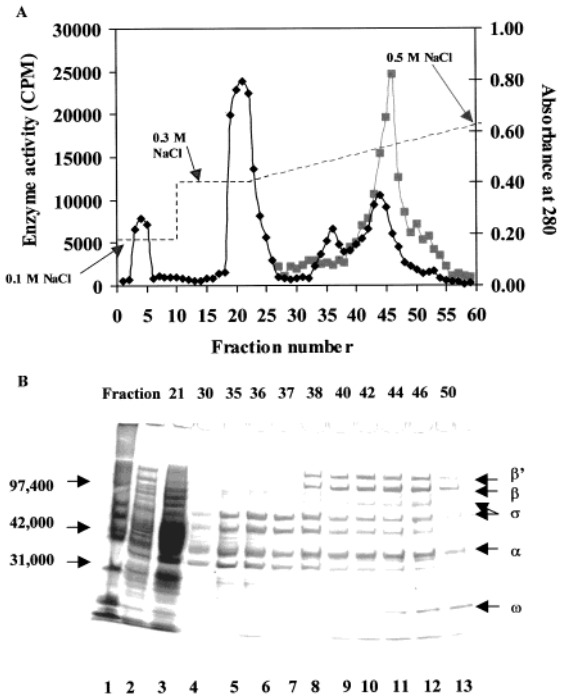


FIGURE 4: (A) Absorbances and activities of the fractions eluted during HQ chromatography. The absorbance (♦) and activity (■) are plotted as a function of fraction number. The salt gradient is shown as the dashed line. (B) SDS−PAGE analysis of the eluted fractions. Lane 1, mid-range protein molecular weight marker; lane 2, HQ column load; lane 3, TGED + 0 M NaCl wash (fraction 21); lanes 4−13: fractions eluted from the HQ column during the NaCl gradient. The fraction number of each lane is listed on the top of the gel. The gel was stained by Coomassie Blue R-250.

Fractions 40−50 were pooled, and the specific activity was measured (Table 1). Significantly, this step resulted in a 160-fold increase in the RNAP specific activity. It is important to note that the 0.3 M NaCl wash combined with the shallow salt gradient is essential to obtain this level of purity.

*Phosphocellulose Chromatography*. Phosphocellulose chromatography was used to remove remaining impurities and to separate core enzyme from holoenzyme. Two major absorbance peaks are seen in the elution profile shown in Figure 5. The first peak, which corresponds to the 0.2 M KCl elution, contains the majority of the enzymatic activity, while the second peak, which represents the 0.5 M KCl elution, has very low RNAP activity. Visualization by SDS−PAGE reveals that the first peak contains holoenzyme (Figure 6), while the second peak contains predominately core enzyme (data not shown). To examine the purity of the holoenzyme, phosphocellulose fractions 12−15 were pooled and run in increasing amounts on a SDS−PAGE gel (Figure 6). Inspection of the gel reveals five bands. The two highest

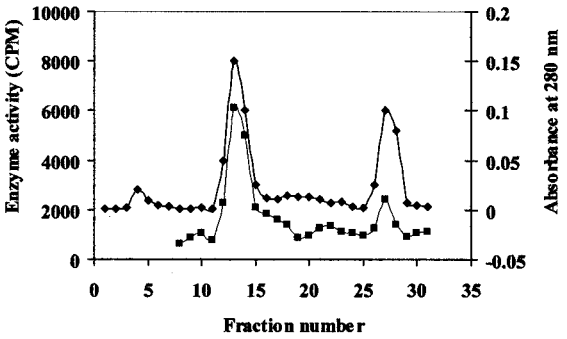


FIGURE 5: Absorbances and activities of the fractions eluted during phosphocellulose chromatography. The absorbance (♦) and activity (■) are plotted as a function of fraction number. Elution was carried out as described in Materials and Methods with 0.2 M KCl (beginning with fraction 11) and subsequently with 0.5 M KCl (beginning with fraction 25).

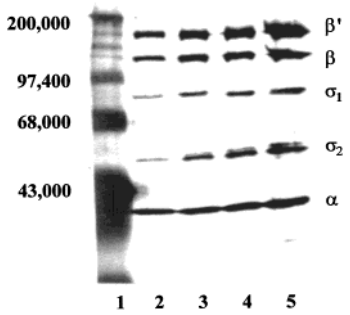


FIGURE 6: SDS−PAGE gel electrophoretic analysis of the final purified *T. thermophilus* RNAP. Gel analysis was carried out on a 10% polyacrylamide gel with 0.2 $\mu$g (lane2), 0.4 $\mu$g (lane3), 0.6 $\mu$g (lane 4), and 0.8 $\mu$g (lane 5) of the protein in each lane. Lane 1 is a molecular weight marker from Gibco. The gel was stained by silver staining.

molecular mass bands (170 and 140 kDa) are consistent with the expected molecular mass of $\beta'$ and $\beta$, respectively, and the band with the greatest electrophoretic mobility (40 kDa) is consistent with the expected molecular mass of the α-subunit. The two intermediate bands (90 and 60 kDa) are believed to be two different $\sigma$ subunits. The slower migrating band was identified in active fractions from the HQ column (Figure 4). This band as well as the one migrating as 60 kDa have been identified previously as $\sigma$-subunits (*7*, *6*). Furthermore, both of these subunits have been shown independently to be capable of recognizing *T. thermophilus* promoters and stimulating open complex formation (*7*, *21*).

*Yield and Purity*. The purification of *T. thermophilus* RNAP from 100 g of cells is summarized in Table 1 and Figure 6. A total of 15 mg of holoenzyme was obtained with an overall activity yield of 65% and a purity of greater than 99% (Figure 6).

*Characterization of T. thermophilus RNAP*. Because we wish to compare the properties of *T. thermophilus* RNAP and *E. coli* RNAP, we have characterized the properties of *T. thermophilus* RNAP on the $\lambda P_R$ promoter. Below, we compare the initiation and elongation properties of *T. thermophilus* and *E. coli* RNAP on DNA containing the $\lambda P_R$ promoter.

(*i*) *$KMnO_4$ Footprinting*. To test whether *T. thermophilus* RNAP can form open complexes on template DNA containing the $\lambda P_R$ promoter, we used $KMnO_4$ to footprint the putative complexes (*22*). There are two thymine residues at

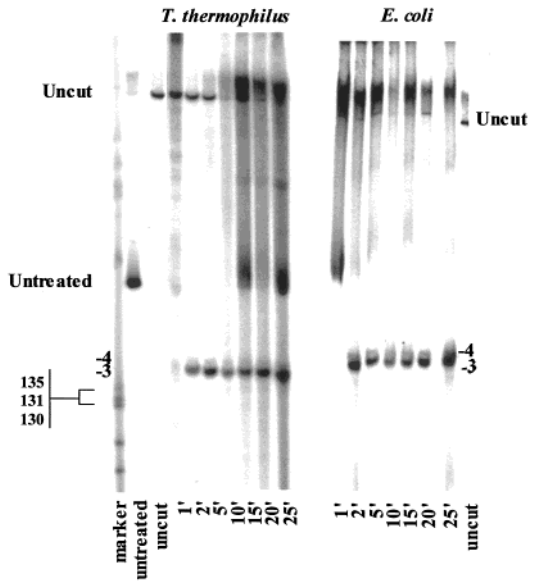


FIGURE 7: $KMnO_4$ probing of nontemplate strand with *E. coli* RNAP at 37 °C (right panel) and *T. thermophilus* RNAP at 55 °C (left panel). The lane labeled marker is a RNA molecular MW marker. The sizes are shown on the left side of the gel. The lane marked untreated contains DNA that has been footprinted with $KMnO_4$ in the presence of RNAP but has not been treated with piperidine. The lane marked uncut contains DNA that has not been treated with $KMnO_4$ or piperidine. The times below each lane correspond to the length of time DNA and RNAP were incubated together prior to the addition of $KMnO_4$. After addition of $KMnO_4$, each reaction was incubated for an additional 5 min and then quenched with $\beta$-mercaptoethanol and EDTA. The two thymine residues at positions −3 and −4 of the nontemplate strand of the $\lambda P_R$ promoter that should become accessible to $KMnO_4$ modification upon open complex formation are indicated on the left and right side of the gels.

positions −3 and −4 of the nontemplate strand of the $\lambda P_R$ promoter that should become accessible to $KMnO_4$ upon open complex formation. Figure 7 shows that these two residues are modified when $\lambda P_R$ containing DNA is incubated with *T. thermophilus* RNAP at 55 °C or *E. coli* RNAP at 37 °C. Incubating DNA alone with $KMnO_4$ did not result in any footprint at this or any other position (data not shown). These results demonstrate that *T. thermophilus* RNAP as well as *E. coli* RNAP can recognize and form open complexes on the $\lambda P_R$ promoter. Interestingly, no modification was observed with *T. thermophilus* RNAP at 37 °C (data not shown), indicating that *T. thermophilus* RNAP cannot form an open complex at this temperature. This result suggests that *T. thermophilus* RNAP requires more thermal energy than *E. coli* RNAP to melt the double-stranded DNA. Additional thermal energy could contribute to the open complex formation by destabilizing the double-stranded DNA and/or by facilitating the conformational changes in *T. thermophilus* RNAP that are required for open complex formation.

(*ii*) *Abortive Initiation Assay*. To determine the rate of open complex formation, we measured the abortive synthesis of pppApU from the $\lambda P_R$ promoter (*23*). Figure 8 shows the results of the abortive initiation assays for *T. thermophilus* RNAP at 37 (Figure 8C) and 55 °C (Figure 8B) and for *E. coli* RNAP at 37 °C (Figure 8A). Consistent with the footprinting studies, no abortive initiation was observed at 37 °C with *T. thermophilus* RNAP. Notably, both *E. coli* RNAP and *T. thermophilus* RNAP exhibit a lag phase before

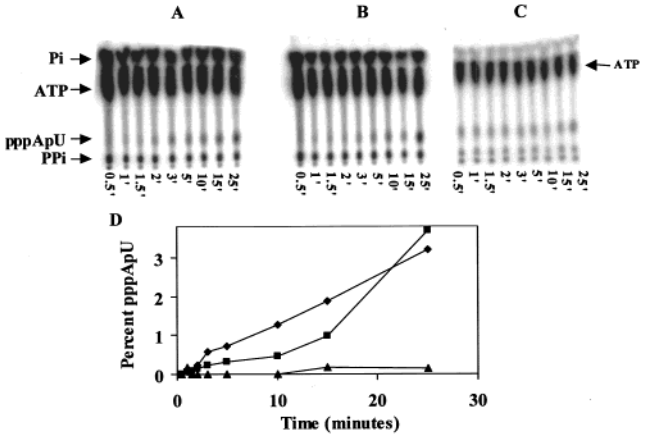


FIGURE 8: Abortive initiation assays. Abortive products produced with *E. coli* RNAP at 37 °C (A), with *T. thermophilus* RNAP at 55 °C (B), and with *T. thermophilus* RNAP at 37 °C (C). (D) Kinetics of abortive initiation on $\lambda P_R$ promoter at 55 or 37 °C. The amount of radioactivity in each pppApU spot on the PEI plate in Figure 7 was measured on a radioanalytic imaging system. The percent of pppApU in each fraction was calculated by dividing the amount of radioactivity in the indicated spot pppApU by the total amount of radioactivity in all spots of each lane. Closed diamonds (♦) indicate pppApU formed by *E. coli* RNAP at 37 °C; closed squares (■) indicate pppApU formed by *T. thermophilus* RNAP at 55 °C; closed triangles (▲) indicate pppApU formed by *T. thermophilus* RNAP at 37 °C.

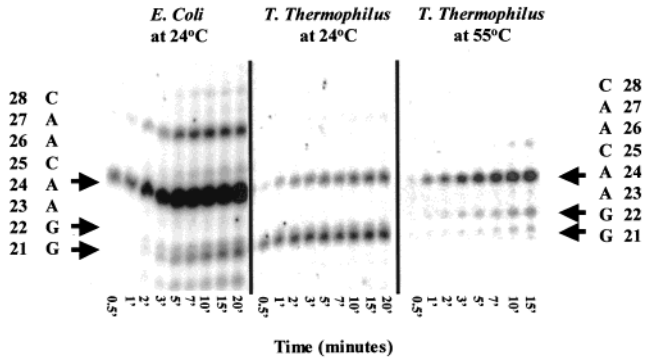


FIGURE 9: Stalled elongation complex formation from the $\lambda P_R$ promoter. Distribution of the transcription products on a 20% acrylamide and 8 M urea gel as a function of time after the addition of 10 $\mu$M ATP, 10 $\mu$M UTP, 2.5 $\mu$M GTP, and 2.5 $\mu$M [$\alpha$-$^{32}$P]-GTP (300 Ci/mmol). *E. coli* RNAP was incubated with $\lambda P_R$ containing DNA at 37 °C for 10 min to initiate open complex formation. Elongation to the 24-nt transcript occurred at 24 °C (left). *T. thermophilus* stalled elongation complexes were generated by initiating open complex formation at 55 °C and elongating at either 24 or 55 °C (middle and right, respectively). The lengths and sequences of the transcripts are indicated on the left and right side of the gels. Note that the reactions were performed in the absence of CTP; therefore, transcripts greater than 24 nt in length represent a misincorporation event where U has been incorporated in place of C (*12*).

a steady-state rate of synthesis is observed. This lag corresponds to the rate of open complex formation (*24*−*26*). The lag time for *E. coli* RNAP is about 3 min (Figure 8D), which is consistent with previous studies (*24*, *25*). The lag time for *T. thermophilus* RNAP is significantly longer (10−15 min) (Figure 8D); however, the steady-state turnover rate of pppApU is greater than that for *E. coli* RNAP. The slower rate of open complex formation is probably a result of the relatively low temperature of this assay (55 °C) as compared to the normal growth temperature (~80 °C) of *T. thermophilus*. The observed rate could be the result of a slower conformational change in *T. thermophilus* RNAP or fewer DNA binding interactions that cause the DNA to melt. The faster steady-state is likely the result of the higher temperature which provides more energy to overcome the activation barrier for single nucleotide incorporation.

(*iii*) *Stalled Elongation Complex Formation*. The data in Figure 9 demonstrate that *T. thermophilus* RNAP can form a specific 24-nt stalled complex from the $\lambda P_R$ promoter at both 24 and 55 °C, provided that open complexes are formed at 55 °C. Consistent with the $KMnO_4$ footprinting and abortive initiation data, no stalled elongation products were observed if template DNA and *T. thermophilus* RNAP were preincubated at 37 °C prior to NTP addition (data not shown). While stalled complexes can be formed at 55 and 24 °C, the properties of elongation are highly temperature dependent. A smaller percentage of elongation complexes reach position 24 when the elongation reaction is performed at 24 °C as compared at 55 °C. At the lower temperature, approximately 50% of the complexes formed with *T. thermophilus* RNAP pause at positions 21 and 22, apparently due to the low concentration of GTP used in the reaction. This same pause at positions 21 and 22 is observed with *E. coli* RNAP at 24 °C and *T. thermophilus* RNAP at 55 °C, but the effect is much less dramatic, with the majority of transcripts reaching position 24 within 5 min (Figure 9). To determine if the paused transcripts represented ternary elongation complexes trapped in an arrested state, aliquots were periodically removed, and high concentrations of all four NTPs (100 $\mu$M) were added. Stalled complexes formed with *E. coli* RNAP at 24 °C and *T. thermophilus* RNAP at 55 °C could be extended to full-length transcripts (chased) under these conditions, indicating that these complexes were not trapped in an arrested state (*12*). In contrast, at 24 °C, the percent of *T. thermophilus* complexes at positions 21 and 22 that could be chased decreased with time of incubation (data not shown), indicating that they were becoming trapped in the arrested state. This result suggests that thermal energy is required to keep *T. thermophilus* RNAP in the activated state and to stimulate the necessary conformational changes in RNAP that result in nucleotide incorporation.

## CONCLUSION

We have presented a rapid and reproducible procedure for the purification of RNAP from *T. thermophilus* HB8 cells. We have also found this method to be applicable to the purification of RNAP from the closely related organism *T. aquaticus* (data not shown). Compared with the method of Wnendt and Hartmannn (*7*), the crucial step in our purification procedure is the HQ chromatography. Although HQ columns are expensive, the cost for each run is relatively low because the columns themselves are very durable and can be reused many times. In addition to their durability, the binding capacity of the HQ column is very high, which enables us to fractionate high concentrations of protein extract at one time. We found that by directly increasing the ionic strength of the wash buffer to 0.3 M NaCl and maintaining this concentration for many column volumes, we were able to effectively eliminate greater than 80% of the contaminant proteins from the prep. If this wash is omitted and a linear NaCl gradient from 0.1 to 0.6 M is

employed, the percent recovery and overall purity of the RNAP is greatly decreased. Finally, it is likely that this procedure, which is significantly less laborious than earlier methods, can be adapted to purify RNAP from many different and evolutionarily diverse organisms.

Our work represents the first direct biochemical characterization of a thermostable RNA polymerase on the $\lambda P_R$ promoter. The fact that *T. thermophilus* RNA polymerase is capable of binding the $\lambda P_R$ promoter and forming an open complex is not surprising, given the evolutionary conservation of prokaryotic promoter consensus sequences and the structural similarity of eubacterial RNA polymerases. Of greater significance is the role that ambient temperature plays in contributing thermal energy for open complex formation, initiation of transcription, and elongation. As demonstrated by both the $KMnO_4$ footprinting and abortive initiation assays, *T. thermophilus* RNAP is incapable of forming a stable open promoter complex and initiating transcription on the $\lambda P_R$ promoter at 37 °C. It is not clear whether ambient temperature contributes directly to melting template DNA or whether it facilitates the enthalpy-driven conformational changes in RNAP that subsequently provide energy for DNA melting. Further thermodynamic studies will be required to clearly elucidate which possibility or combination of possibilities predominates during open complex formation. Temperature also appears to have a significant effect on the polymerase post initiation. As demonstrated by stalled elongation complex formation, elongation with *T. thermophilus* from the $\lambda P_R$ promoter is possible at 24 °C; however, the efficiency of catalysis is greatly reduced. These results suggest that ambient temperature facilitates the conformational changes that must occur for specific nucleotide incorporation.

Taken together, these data show that, although their respective optimal temperatures are very different, *T. thermophilus* and *E. coli* RNAP are mechanistically very similar in their ability to stimulate transcription from the $\lambda P_R$ promoter. The similarities that exist between the two polymerases suggest that the events governing the first critical steps in gene expression are highly conserved throughout evolution.

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