

# Recombinant *Thermus aquaticus* RNA Polymerase, a New Tool for Structure-Based Analysis of Transcription

LEONID MINAKHIN,<sup>1</sup> SERGEI NECHAEV,<sup>1</sup> ELIZABETH A. CAMPBELL,<sup>2</sup>  
AND KONSTANTIN SEVERINOV<sup>1\*</sup>

*Waksman Institute for Microbiology and Department of Genetics, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854,<sup>1</sup> and The Rockefeller University, New York, New York 10021<sup>2</sup>*

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**The three-dimensional structure of DNA-dependent RNA polymerase (RNAP) from thermophilic *Thermus aquaticus* has recently been determined at 3.3 Å resolution. Currently, very little is known about *T. aquaticus* transcription and no genetic system to study *T. aquaticus* RNAP genes is available. To overcome these limitations, we cloned and overexpressed *T. aquaticus* RNAP genes in *Escherichia coli*. Overproduced *T. aquaticus* RNAP subunits assembled into functional RNAP in vitro and in vivo when coexpressed in *E. coli*. We used the recombinant *T. aquaticus* enzyme to demonstrate that transcription initiation, transcription termination, and transcription cleavage assays developed for *E. coli* RNAP can be adapted to study *T. aquaticus* transcription. However, *T. aquaticus* RNAP differs from the prototypical *E. coli* enzyme in several important ways: it terminates transcription less efficiently, has exceptionally high rate of intrinsic transcript cleavage, and is highly resistant to rifampin. Our results, together with the high-resolution structural information, should now allow a rational analysis of transcription mechanism by mutation.**

Most bacterial RNA polymerase (RNAP) core enzymes consist of four core subunits ( $\beta'$ ,  $\beta$ , and a dimer of identical  $\alpha$  subunits). Binding of one of the several  $\sigma$  factors converts the core into the holoenzyme, which is able to initiate transcription from promoters. In vitro activity of several bacterial RNAPs can be recovered after separation of individual subunits in the presence of denaturing agents and subsequent mixing of the subunits and dialysis under controlled conditions (19). In vitro reconstitution of *Escherichia coli* RNAP from cloned and individually overexpressed and purified subunits provides a means of obtaining RNAP harboring lethal mutations in quantities sufficient for biochemical analyses (16). Structure-function studies of reconstituted recombinant *E. coli* RNAP mutants have provided crucial insights into the transcription mechanism and regulation and are primarily responsible for our increased understanding of this enzyme (see, for example, references 6, 8, and 17).

Recent structural analysis of RNAP from *Thermus aquaticus* provided the first tantalizing view of the bacterial RNAP core enzyme structure at 3.3 Å resolution (18). The availability of a relatively high-resolution structure qualitatively raises the importance of a rational structure-function analysis of RNAP. Despite obvious homology between RNAPs from *T. aquaticus* and *E. coli*, it would be highly desirable to perform mutational and structural studies using *T. aquaticus* RNAP rather than the better-studied *E. coli* counterpart. The important advantages of studying *T. aquaticus* RNAP include the ability to reduce assembly effects of mutations to a minimum and the ability to perform structural analysis of mutants. The disadvantages of the *T. aquaticus* system are the lack of functional assays, the general absence of data on gene transcription in this organism,

and, more importantly, the inability to manipulate *T. aquaticus* RNAP subunit (*rpo*) genes. To overcome these limitations and to make full use of the available structural information, we cloned each of the *T. aquaticus rpo* genes in *E. coli* expression vectors and overproduced recombinant subunits. We show that recombinant *T. aquaticus* RNAP can be assembled in vitro and can be studied using assays developed for *E. coli* system.

## MATERIALS AND METHODS

**Cloning and expression of *T. aquaticus rpo* genes.** Fragments of genes coding for the core subunits of *T. aquaticus* RNAP were cloned previously (18). We used conventional cloning and PCR to assemble entire *rpo* genes in plasmid pT7Blue (Novagene), and we used site-directed mutagenesis to introduce an *Eco*RI site after the termination codon of every *rpo* gene and an *Nde*I site (CATATG) overlapping with the initiation ATG codon of every *rpo* gene (note that *T. aquaticus rpo* genes contain no *Nde*I or *Eco*RI sites 18). *T. aquaticus rpo* genes were recovered by *Nde*I-*Eco*RI treatment of pT7Blue-based recombinant plasmids and were subcloned into appropriately treated pET28 and/or pET21 T7 RNAP expression vectors. The resultant plasmids, pET28*TaA*, pET28*TaB*, pET28*TaC*, and pET28*TaZ*, express *T. aquaticus* RNAP  $\alpha$ ,  $\beta$ ,  $\beta'$ , and  $\omega$ , respectively, with N-terminal hexahistidine tags. Similar plasmids of the pET21 series express untagged subunits. *E. coli* BL21(DE3) cells transformed with the resulting plasmids overproduced individual *T. aquaticus* core RNAP subunits at high level on induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), and the overexpressed proteins segregated into inclusion bodies.

A ~600-bp fragment of *T. aquaticus rpoD* was amplified from *T. aquaticus* genomic DNA using degenerate primers specific for highly conserved regions 2 and 4, and its sequence was determined. The entire *rpoD* gene sequence was then obtained by applying inverse PCR to *Sac*I- and *Bgl*II-digested *T. aquaticus* DNA. The entire *rpoD* gene was next subcloned into pET system vectors. Expressed RpoD was found in inclusion bodies.

Plasmid pET28*TaABCZ*, coexpressing *T. aquaticus rpoA*, *rpoB*, *rpoC*, and *rpoZ* genes, was constructed from pET28*TaC*, which was treated with *Hind*III and Klenow enzyme followed by *Eco*RI and ligated to a *T. aquaticus rpoB*-containing fragment prepared from pET21*TaB* by treating with *Bgl*II and Klenow followed by *Eco*RI. The resultant plasmid, pET28*TaBC*, has two genes, *rpoB* and *rpoC*, in opposite orientations, each being preceded by the T7 RNAP promoter. pET28*TaBC* was treated with *Eco*RI and ligated with the *Eco*RI-treated PCR fragment of pET21*TaA*. Oligonucleotides used for PCR were designed to anneal upstream of the T7 RNAP promoter of pET21*TaA* and downstream of the termination codon of *rpoA*, and each contained an *Eco*RI site. The resultant

\* Corresponding author. Mailing address: Waksman Institute, 190 Frelinghuysen Rd., Piscataway, NJ 08854. Phone: (732) 445-6095. Fax: (732) 445-5735. E-mail: severik@waksman.rutgers.edu.

plasmid, pET28TaABC, has *T. aquaticus* *rpoA* and an upstream T7 RNAP promoter inserted between the *rpoB* and *rpoC* genes, with *rpoA* having the same orientation as *rpoC*. Plasmid pET28TaABCZ was constructed by ligating the *SgrAI*-treated pET28TaABC with the *SgrAI*-treated PCR fragment of pET21TaZ. Oligonucleotides used for PCR were designed to anneal upstream of the T7 RNAP promoter of pET21TaZ and downstream of the termination codon of *rpoZ*, and each contained an *SgrAI* site. The resultant plasmid, pET28TaABCZ, has three untagged genes, *rpoZ*, *rpoB*, and *rpoA*, in the same orientation, and C-terminally hexahistidine-tagged *rpoC* in the opposite orientation.

**RNAP assembly and purification.** RNAP was reconstituted by a published procedure (2, 16). The molar ratio of  $\alpha$ ,  $\beta$ , and mutant  $\beta'$  in the reconstitution reaction mixtures was 1:4:8. After reconstitution, RNAP preparations were either used directly or further purified by fast protein liquid chromatography and gel filtration on Superose-6 and Resource Q columns (Pharmacia-LKB Inc., Piscataway, N.J.) as described previously (2), concentrated by filtration through a C-100 concentrator (Amicon, Lexington, Mass.) to  $\sim 1$  mg/ml, and stored in 50% glycerol storage buffer at  $-20^\circ\text{C}$ .

To purify the *T. aquaticus* core from *E. coli* cells coexpressing *T. aquaticus* *rpo* genes, BL21(DE3) cells harboring pET28TaABCZ were induced with 1 mM IPTG for 3 h. Cells were collected, and the cell pellet was resuspended in 20 volumes of buffer containing 50 mM Tris-HCl, 100 mM KCl, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA (pH 7.9). The cells were lysed by sonication, and the cleared lysate was incubated at  $65^\circ\text{C}$  for 60 min. After centrifugation, the proteins in the supernatant were precipitated with ammonium sulfate (75% saturation), redissolved in IMAC starting buffer (10 mM Tris-HCl, 5% glycerol, 500 mM NaCl [pH 7.9]) and loaded on a 1-ml chelating HiTrap column (Pharmacia) loaded with  $\text{Ni}^{2+}$  and equilibrated in the same buffer. The column was washed with buffer containing 20 mM imidazole, and RNAP was eluted with 100 mM imidazole in the buffer. The fraction was diluted fivefold with TGE buffer (50 mM Tris-HCl, 5% glycerol, 50 mM NaCl, 1 mM  $\beta$ -mercaptoethanol) and loaded onto a Resource Q column equilibrated with the same buffer. The column was developed with a linear gradient of NaCl concentrations in TGE buffer. Chromatographically pure RNAP eluted at ca. 400 mM NaCl and was concentrated and stored as above.

**Affinity labeling.** Reaction volumes of 10  $\mu\text{l}$  contained 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM  $\text{MgCl}_2$ ,  $\sim 1.0$   $\mu\text{g}$  of RNAP core, 0.5 to 1.0 mM initiating AMP derivatized with an aldehyde group (3), and 100 ng of poly(dA-dT). The reaction mixtures were supplemented with 10 mM  $\text{BH}_4$  and incubated at  $37^\circ\text{C}$  for 10 min. [ $\alpha$ - $^{32}\text{P}$ ]UTP (3,000 Ci/mmol) was added to 0.3 mM (final concentration), and incubation was continued for 30 min at 37 or  $65^\circ\text{C}$ . Control experiments demonstrated that the resulting  $^{32}\text{P}$  labeling of RNAP depended on the addition of the template DNA (data not shown).

**KMnO<sub>4</sub> footprinting.** The 106-bp *EcoRI* DNA fragment containing the T7 A2 promoter (positions  $-84$  to  $+32$ ) was prepared as described previously (14). The fragment was  $^{32}\text{P}$ -end labeled by filling in *EcoRI* sticky ends with Klenow enzyme in the presence of [ $\alpha$ - $^{32}\text{P}$ ]dATP. The fragment was then treated with *HincII* (which cuts at position  $+22$ ) to obtain bottom-strand-labeled fragment. Promoter complexes were formed in 20- $\mu\text{l}$  reaction mixtures containing 200 nM RNAP holoenzyme, 100 nM  $^{32}\text{P}$ -end-labeled DNA fragment, 40 mM Tris-HCl (pH 7.9), 40 mM KCl, and 10 mM  $\text{MgCl}_2$ . The reaction mixtures were preincubated for 15 min at  $37^\circ\text{C}$  (*E. coli* RNAP) or  $65^\circ\text{C}$  (*T. aquaticus* RNAP). Promoter complexes were then treated with 1 mM  $\text{KMnO}_4$  for 15 s at  $37^\circ\text{C}$ . The reactions were terminated by the addition of  $\beta$ -mercaptoethanol to 300 mM followed by phenol extraction, ethanol precipitation, and 10% piperidine treatment. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE) (6% polyacrylamide).

**In vitro transcription and transcript cleavage reactions.** Abortive initiation reaction mixtures (10  $\mu\text{l}$ ) contained 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM  $\text{MgCl}_2$ , 50 nM RNAP core enzymes, and 100 nM recombinant *E. coli*  $\sigma^{70}$  or *T. aquaticus* RpoD. The reaction mixtures were preincubated for 10 min at  $45^\circ\text{C}$ , and transcription was initiated by the addition of 100 mM CpA and 0.5 mM [ $\alpha$ - $^{32}\text{P}$ ]UTP (3,000 Ci/mmol) and allowed to proceed for an additional 10 min at  $45^\circ\text{C}$ . The reactions were terminated by the addition of an equal volume of urea-containing loading buffer, and the products were analyzed by denaturing gel electrophoresis (7 M urea, 20% polyacrylamide) and autoradiography.

To determine transcription elongation rates, elongation complexes stalled at position  $+20$  (EC<sup>20</sup>) were prepared in 50- $\mu\text{l}$  reaction mixtures containing 40 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM  $\text{MgCl}_2$ , 20 nM DNA fragment containing the T7 A1 promoter fused to the  $\lambda$  trR2 terminator (15), 50 nM RNAP, 0.05 mM CpApUpC, 50  $\mu\text{M}$  (each) ATP and GTP, and 2.5  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ]CTP (3,000 Ci/mmol). The reaction mixtures were incubated for 15 min at  $23^\circ\text{C}$  (*E. coli*) or  $45^\circ\text{C}$  (*T. aquaticus*). EC<sup>20</sup> were synchronously restarted by the addition of nucleoside

triphosphates (NTPs). Reactions proceeded for 0 to 600 s at  $45^\circ\text{C}$ . The reactions were terminated by the addition of formamide-containing loading buffer. Products were analyzed by urea-PAGE (7 M urea, 6% polyacrylamide), followed by autoradiography and PhosphorImager analysis.

To monitor transcript cleavage, EC<sup>21</sup> was prepared by incubating immobilized purified EC<sup>20</sup> with 25  $\mu\text{M}$  UTP for 5 min at room temperature (*E. coli* RNAP) or  $42^\circ\text{C}$  (for *T. aquaticus* RNAP). Immobilized complexes were washed and left in  $\sim 20$   $\mu\text{l}$ , in the presence or absence of recombinant *E. coli* GreA, for 10 min at  $37^\circ\text{C}$  (*E. coli* RNAP complexes) or  $65^\circ\text{C}$  (*T. aquaticus* RNAP complexes). Reactions were terminated by the addition of an equal volume of formamide-containing loading buffer and analyzed by denaturing gel electrophoresis (7 M urea, 20% polyacrylamide) and autoradiography.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *rpoA* gene (encoding the  $\alpha$  subunit of RNAP), the *rpoB* and *rpoC* genes (encoding the  $\beta$  and  $\beta'$  subunits of RNAP, respectively), and the *rpoZ* gene (encoding the  $\omega$  subunit of RNAP) have been deposited in GenBank under accession nos. Y19222, Y19223, and AJ295839, respectively.

## RESULTS

***T. aquaticus* RNAP assembles in vitro.** As a preliminary feasibility experiment, we wished to establish that *T. aquaticus* RNAP assembles in vitro after denaturation. A highly pure *T. aquaticus* RNAP core sample prepared as described by Zhang et al. (18) was incubated in a denaturing buffer containing 7 M guanidine and then dialyzed in a low-salt buffer under conditions favoring RNAP reconstitution. The resulting preparation was assayed for nonspecific transcription on synthetic template poly(dA-dT). Transcription activity was recovered with high yield (data not shown), suggesting that *T. aquaticus* RNAP withstands the in vitro denaturation-renaturation cycle.

**Cloning and overexpression of *T. aquaticus* RNAP core subunit genes, and in vitro assembly of the recombinant *T. aquaticus* RNAP core.** The sequences of *T. aquaticus* RNAP core subunit genes were determined previously (18). Each of the *T. aquaticus* *rpo* genes was cloned in *E. coli* pET expression vectors as described in Materials and Methods. Plasmids expressing *rpo* genes with or without affinity hexahistidine tags were constructed. *E. coli* cells harboring the expression plasmids overproduced high levels of recombinant *T. aquaticus* RNAP core subunits (Fig. 1A). Most of the overexpressed subunits were found in inclusion bodies and could be recovered by standard procedure (2).

A separate plasmid pET28TaABCZ, simultaneously expressing all four *T. aquaticus* *rpo* genes from T7 RNAP promoters, was also prepared. *E. coli* BL21(DE3) cells transformed with pET28TaABCZ expressed *T. aquaticus* RNAP subunits at low levels. The amount of overproduced *T. aquaticus*  $\beta$  and  $\beta'$  was only slightly larger than the amount of the endogenous *E. coli* RNAP largest subunits, which form a characteristic double band on sodium dodecyl sulfate (SDS)-containing gels of whole-cell lysates (Fig. 1B, lanes 1 and 2). Coexpressed *T. aquaticus* subunits formed a complex that remained soluble after high-temperature treatment of *E. coli* extract (lane 3) and, when purified to homogeneity by IMAC and Resource Q chromatography, appeared indistinguishable from RNAP core enzyme purified from *T. aquaticus* cells (lane 5).

Recombinant *T. aquaticus* RNAP was also assembled from individually expressed subunits in vitro, as judged by the appearance of characteristic chromatographic peaks in the course of purification that separates assembled enzyme from assembly intermediates and unassembled subunits (reference 2

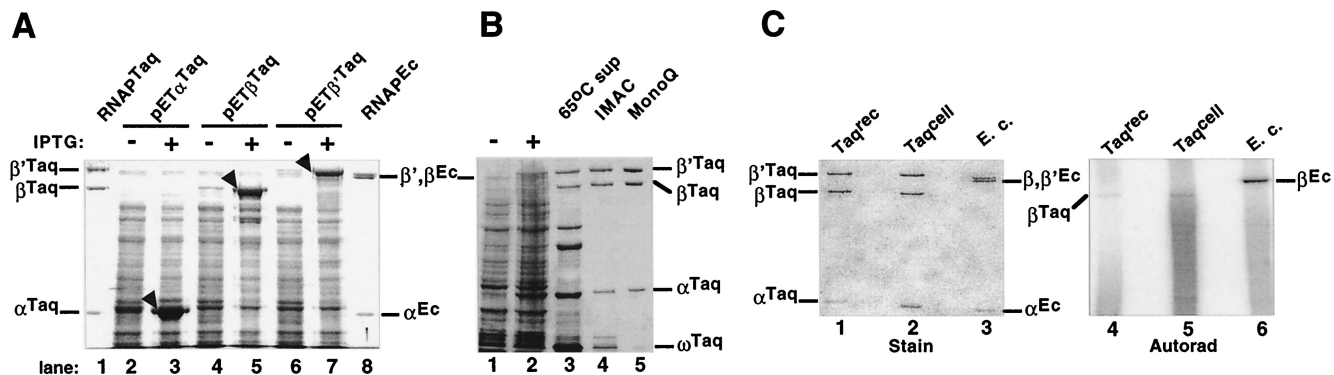


FIG. 1. Recombinant *T. aquaticus* RNAP. (A) Overexpression of *T. aquaticus* *rpo* genes in *E. coli*. *T. aquaticus* *rpoA*, *rpoB*, and *rpoC* genes coding for the  $\alpha$ ,  $\beta$ , and  $\beta'$  RNAP subunits, respectively, were cloned in pET (Novagen) expression vectors. The plasmids were transformed in *E. coli* BL21(DE3) cells, the cells were induced with 1 mM IPTG, and proteins were separated on an SDS–8% polyacrylamide gel and revealed by Coomassie blue staining. Lanes 1 and 8 are controls and contain purified *T. aquaticus* and *E. coli* RNAP core enzymes, respectively. (B) Coexpression of *T. aquaticus* *rpo* genes in *E. coli* leads to assembled *T. aquaticus* RNAP. *E. coli* BL21(DE3) cells were transformed with pET28*TaABCZ*, expressing the *T. aquaticus* *rpoA*, *rpoB*, *rpoC*<sub>His6</sub>, and *rpoZ* genes. Cells were induced with IPTG (lane 2) and lysed, and the extract was incubated at 65°C for 60 min. The supernatant (lane 3) was subjected to Ni<sup>2+</sup>-IMAC affinity chromatography, and material eluted from the Ni<sup>2+</sup> sorbent in the presence of imidazole (lane 4) was further purified on a Resource Q column (lane 5). A stained SDS–12% polyacrylamide gel is presented. (C) Affinity labeling of recombinant *T. aquaticus* RNAP with a derivatized AMP initiator. In vitro-assembled *T. aquaticus* RNAP (Taq<sup>rec</sup>), RNAP purified from *T. aquaticus* biomass (18) (Taq<sup>cell</sup>), and control RNAP from *E. coli* (E. c.) were incubated with derivatized AMP (3) under conditions favoring cross-linking to *E. coli*  $\beta$  Lys<sup>1065</sup>, which is strictly conserved. Poly(dA-dT) template and [ $\alpha$ -<sup>32</sup>P]UTP were added next, followed by additional incubations at 37°C (*E. coli* RNAP) or 65°C (*T. aquaticus* RNAPs). The reaction products were then resolved on an SDS-containing gel. The gel was stained (lanes 1 to 3) and autoradiographed (lanes 4 to 6). Radioactive labeling of RNAP  $\beta$ , which is template dependent (data not shown), testified that the enzymes are active.

and data not shown). The catalytic proficiency of recombinant *T. aquaticus* RNAP was demonstrated by promoter-independent transcription of synthetic template poly(dA-dT) at the high temperature of 65°C (data not shown) and template-dependent affinity labeling with an initiating substrate derivative (Fig. 1C). In this reaction, recombinant, in vitro-reconstituted *T. aquaticus* RNAP was cross-linked to derivatized initiating AMP (3). The reaction mixtures were then supplemented with poly(dA-dT) template and [ $\alpha$ -<sup>32</sup>P]UTP. As controls, labeling reactions were also performed with RNAP purified from *T. aquaticus*, as well as with *E. coli* RNAP. As explained elsewhere, in *E. coli* the affinity-labeling protocol results in covalent attachment of the radioactive pApU dinucleotide tag to the  $\beta$  subunit Lys<sup>1065</sup> (3, 8). Previous studies demonstrated that the cross-linkable AMP derivative modifies  $\beta$ -like subunits in RNAP from bacterial, archaeal, and eucaryal systems and that residues homologous to the universally conserved *E. coli*  $\beta$  Lys<sup>1065</sup> are cross-linked (3, 4, 12). Therefore, we expected that in *T. aquaticus* RNAP, the 1,119-amino-acid  $\beta$  subunit would be labeled. This expectation was fulfilled; as can be seen on the autoradiogram of the SDS-gel in Fig. 1C, the  $\beta$  subunit of in vitro-assembled *T. aquaticus* RNAP was labeled as efficiently as in the control enzyme purified from *T. aquaticus* cells and the larger, 1,342-amino-acid *E. coli*  $\beta$  subunit was absent from the recombinant *T. aquaticus* RNAP lane, establishing that little or no contaminating *E. coli* RNAP was present. On the basis of these results, we conclude that recombinant *T. aquaticus* RNAP assembled in vitro is active and free of contaminating *E. coli* RNAP. Similarly, *T. aquaticus* RNAP purified from *E. coli* cells coexpressing *T. aquaticus* *rpo* genes was also active and free of contaminating *E. coli* enzyme (data not shown). Below, we present an initial characterization of the recombinant *T. aquaticus* enzyme. Since the enzyme prepared

by in vitro reconstitution was indistinguishable from that purified from *E. coli* cells (data not shown), all the data below are presented for in vitro-reconstituted *T. aquaticus* RNAP.

**Promoter-specific transcription by recombinant *T. aquaticus* RNAP.** When recombinant *T. aquaticus* RNAP core was combined with *E. coli*  $\sigma$ <sup>70</sup> at 45°C, a condition under which both *T. aquaticus* and *E. coli* RNAP core enzymes are active, no promoter-specific initiation from strong,  $\sigma$ <sup>70</sup>-dependent T7 A1 promoter was observed (Fig. 2A, lane 5). Control experiments demonstrated that  $\sigma$ <sup>70</sup> imparted promoter specificity to the *E. coli* core, as expected (lane 2). The result implies that *E. coli*  $\sigma$ <sup>70</sup> is unable to form a productive complex with the *T. aquaticus* core. To overcome this problem, we cloned the homologue of the *E. coli* *rpoD* ( $\sigma$ <sup>70</sup>) gene from *T. aquaticus* (see Materials and Methods). Alignment of the deduced amino acid sequence revealed that the product of the *T. aquaticus* *rpoD* gene is very similar to the recently published sequence from *T. thermophilus* (11), as expected. *T. aquaticus*  $\sigma$  is also very similar to  $\sigma$ <sup>70</sup> in conserved regions 2 and 4, responsible for promoter recognition. In striking contrast, *T. aquaticus*  $\sigma$  completely lacks the N-terminal conserved region 1. Instead, it contains a ~100-amino-acid segment without homology to any of the published sequences.

*T. aquaticus* *rpoD* was cloned in the *E. coli* expression vector, and recombinant *T. aquaticus*  $\sigma$  was purified to homogeneity. The addition of *T. aquaticus*  $\sigma$  stimulated the synthesis of the abortive trinucleotide CpApU from the CpA initiator and UTP by the recombinant *T. aquaticus* core in the presence of a DNA fragment containing the T7 A1 promoter (Fig. 2A, lane 6). In contrast, the addition of *T. aquaticus*  $\sigma$  failed to stimulate transcription by the *E. coli* core enzyme (lane 3). The patterns of abortive products synthesized by *E. coli* and *T. aquaticus* holoenzymes on the T7 A1 promoter fragment in the presence of limiting sets of NTP substrates were identical, thus proving



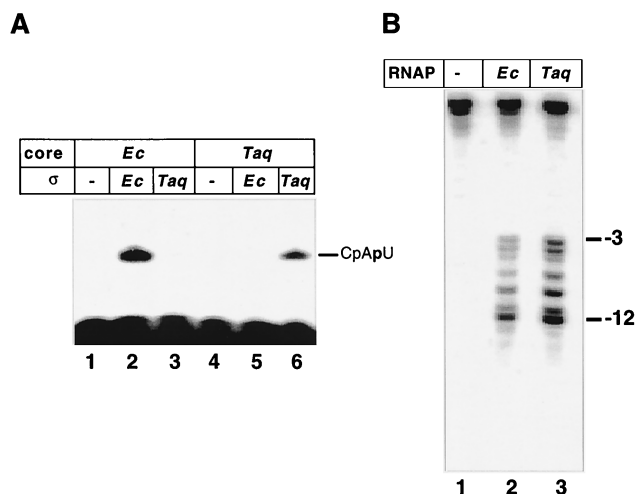


FIG. 2. Transcription initiation by recombinant *T. aquaticus* RNAP. (A) The indicated RNAP core enzymes were combined with recombinant *E. coli* (*Ec*)  $\sigma^{70}$  or *T. aquaticus* (*Taq*) RpoD in the presence of the T7 A1 promoter-containing DNA fragment, CpA primer, and [ $\alpha$ - $^{32}$ P]UTP substrate. The reaction mixtures were incubated at 45°C for 15 min (lanes 1 to 3 and 4 to 6, respectively), and the products were resolved by denaturing PAGE and revealed by autoradiography. (B) Transcription complexes were formed at the indicated temperatures on the T7 A2 promoter-containing DNA fragment radioactively end labeled on the bottom strand, using recombinant *E. coli* (*Ec*) or *T. aquaticus* (*Taq*) RNAP holoenzymes, and probed with  $\text{KMnO}_4$ . Reaction products were resolved on a 6% denaturing polyacrylamide gel and revealed by autoradiography. The permanganate-sensitive bands were assigned using G/A markers (not shown) (14).

that the observed transcription initiation was promoter specific (data not shown). In addition,  $\text{KMnO}_4$  probing established that promoter complexes formed by the *T. aquaticus* holoenzyme on the well-studied T7 A2 promoter (14) are very similar or identical to the *E. coli* complexes (Fig. 2B).

**In vitro transcription by the *T. aquaticus* RNAP holoenzyme.** Having established conditions for promoter-specific transcription initiation by *T. aquaticus* RNAP, we wished to compare the transcription elongation, transcription termination, and transcript cleavage properties of *T. aquaticus* RNAP with those of the prototypic *E. coli* enzyme. Immobilized RNAP was used to obtain stalled elongation complexes containing 20-mer RNA on the T7 A1 promoter-containing DNA fragment (15). Purified elongation complexes were incubated in the presence of different concentrations of NTP, to monitor the transcription elongation rate and transcription termination on the rho-independent  $\lambda$  tR2 terminator located downstream. Alternatively, complexes containing 20-mer RNA were walked to position 21, purified, and incubated in the absence of NTP with or without *E. coli* GreA protein to monitor transcript cleavage. The most important conclusions from these experiments are summarized below. (i) *T. aquaticus* RNAP elongated RNA chains less efficiently than the *E. coli* enzyme did; in general a 10-fold-higher nucleotide concentration was required to achieve comparable elongation rates. Both enzymes exhibited transcription pausing, evident at shorter times (Fig. 3A, lanes 2 and 5); however, the pattern appeared to be distinct. (ii) *T.*

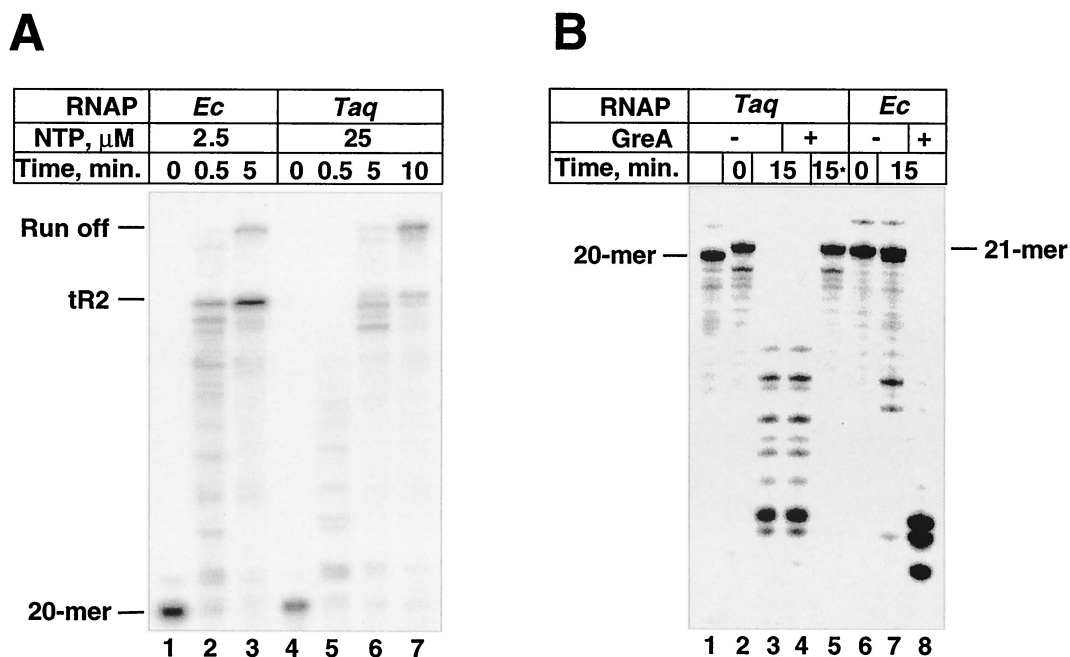


FIG. 3. Transcript elongation, termination, and cleavage by recombinant *T. aquaticus* RNAP. (A) Elongation complexes stalled at position 20 were prepared using DNA fragment containing the T7 A1 promoter and  $\lambda$  tR2 terminator as a template (lanes 1 and 4). Transcription was resumed at 45°C by the addition of the indicated concentrations of NTPs; at the times indicated, aliquots were withdrawn and reactions were terminated by the addition of formamide containing stop buffer. Reaction products were analyzed by 6% urea PAGE followed by autoradiography. (B) Immobilized elongation complexes stalled at position 20 (lane 1) were walked to position 21 (lane 2). The complexes were desorbed from  $\text{Ni}^{2+}$ -nitrilotriacetic acid agarose and supplemented with recombinant *E. coli* GreA (0.04  $\mu\text{g}$ ), reaction mixtures were incubated for 15 min at 37 and 65°C (for *E. coli* and *T. aquaticus* RNAP, correspondingly), and the products were resolved by denaturing PAGE in a 20% urea gel. In lane 5, RNA was extracted from the stalled *T. aquaticus* elongation complex with phenol and then incubated in the presence of *T. aquaticus* RNAP and *E. coli* GreA.

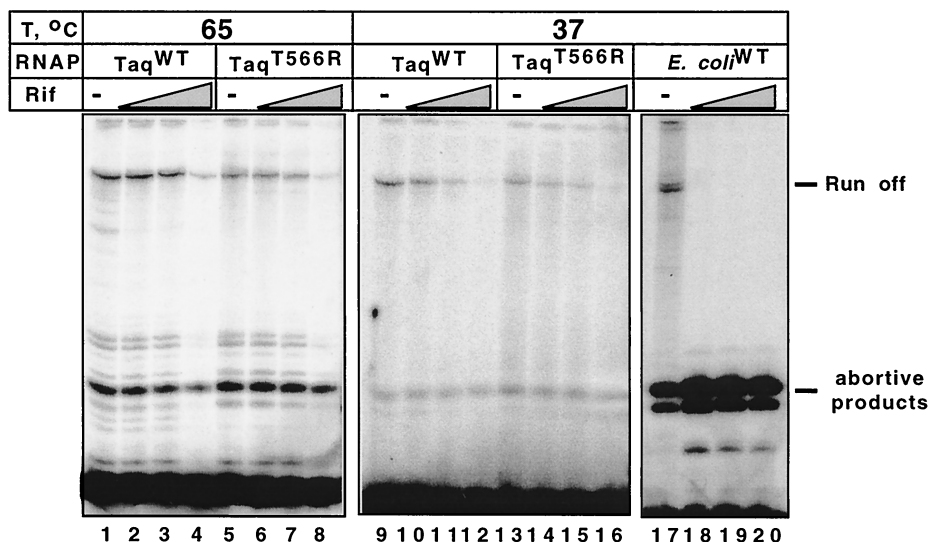


FIG. 4. The high level of rifampin resistance by *T. aquaticus* RNAP is not due to  $\beta$  Rif-cluster III Thr<sup>566</sup>. The indicated RNAPs were used to transcribe the T7 A1 promoter-containing DNA fragment (8) in a multiple-round transcription assay in the presence of three different rifampin (Rif) concentrations: 10, 100, and 1,000  $\mu\text{g/ml}$ . Reaction products were resolved on a 20% denaturing polyacrylamide gel and revealed by autoradiography.

*aquaticus* RNAP recognized the tR2 terminator less efficiently (transcription termination efficiency of  $\sim 20\%$ , compared to 86% with the *E. coli* enzyme). (iii) *T. aquaticus* RNAP actively cleaved 21-mer RNA in the stalled elongation complex in the absence of added cleavage factor. This activity was not further stimulated by *E. coli* GreA and was not due to RNase contamination, as shown by the control lane of Fig. 3B (lane 5) (in the control reaction, 21-mer RNA was prepared by phenol extraction of stalled complexes and pure RNA was then incubated with *T. aquaticus* RNAP before being loaded onto the gel).

Structural analysis of the *T. aquaticus* core indicates that sites in subunit  $\beta$  that correspond to sites of known rifampin resistance mutations in *E. coli* form a cluster in the ceiling of DNA binding channel of the enzyme (18). Previous reports indicated that *T. aquaticus* RNAP is highly resistant to rifampin (5). With the exception of *T. aquaticus*  $\beta$  Thr<sup>566</sup>, which corresponds to *E. coli*  $\beta$  Arg<sup>687</sup>, which defines Rif cluster III (6), all *T. aquaticus* amino acids that can be involved in rifampin binding are identical to the corresponding amino acids in the wild-type, rifampin-sensitive *E. coli*  $\beta$  subunit. To test whether Thr<sup>566</sup> is responsible for rifampin resistance, we substituted it for Arg, assembled the mutant enzyme *in vitro*, and determined its sensitivity to rifampin in a steady-state T7 A1 promoter transcription assay. The result, presented in Fig. 4, shows that in agreement with earlier data, *T. aquaticus* RNAP was highly resistant to rifampin and continued to synthesize full-sized transcripts even in the presence of 1,000  $\mu\text{g}$  of rifampin per ml in the reaction mixture. In contrast, synthesis of the full-sized transcripts by the *E. coli* enzyme was completely inhibited in the presence of 10  $\mu\text{g}$  of the drug per ml. *T. aquaticus* RNAP harboring the T566R substitution in subunit  $\beta$  was unaffected in its level of rifampin resistance. Therefore, the determinants of the resistance of *T. aquaticus* RNAP to rifampin must lie outside sites determined by genetic studies of resistance in *E. coli* and other sensitive bacteria.

## DISCUSSION

The recent advances in our understanding of the mechanism and regulation of bacterial transcription are largely due to recombinant *E. coli* RNAP technology that has allowed the preparation of sufficient quantities of RNAP mutants harboring lethal mutations for biochemical analysis. Until recently, this powerful approach had a significant limitation, since mutations that affected RNAP assembly could not be studied. The recent determination of the high-resolution structure of the *T. aquaticus* RNAP core allows one to perform a precise, structure-based analysis of bacterial RNAP function.

As our results demonstrate, recombinant *T. aquaticus* RNAP can be prepared in large amounts, and the discriminative transcription assays developed for the *E. coli* enzyme can be adapted to study this thermophilic enzyme. However, the *T. aquaticus* enzyme differs from the *E. coli* enzyme in several significant ways. Promoter complexes formed by the *T. aquaticus* holoenzyme are indistinguishable from *E. coli* RNAP complexes, suggesting that the promoter specificities of *T. aquaticus* RpoD and *E. coli*  $\sigma^{70}$  are the same. However, the two sigmas are not interchangeable. The functional specialization of sigmas is not due to the lack of formation of hybrid holoenzymes (data not shown). The unusual N-terminal extension present in *T. aquaticus* RpoD and/or the long (300-amino-acid) insertion in *T. aquaticus*  $\beta'$ , which is absent from *E. coli* counterpart and is located close to the conserved segment C involved in  $\sigma$  binding (1), may be responsible for the inability of hybrid holoenzymes to recognize promoters.

As with sigma, the *E. coli* transcript cleavage factor GreA did not alter the properties of *T. aquaticus* RNAP. In fact, *T. aquaticus* RNAP appears to be very active in factor-independent transcript cleavage even at low pH. High levels of intrinsic transcript cleavage were observed when *T. aquaticus* RNAP was purified from *E. coli* coexpressing *T. aquaticus* *rpo* genes or

when the enzyme was prepared by in vitro reconstitution from isolated subunits (data not shown). Thus, the cleavage activity probably reflects the true properties of *T. aquaticus* RNAP rather than contamination with the *E. coli* Gre factor(s). High levels of cleavage activity may be essential for an enzyme which transcribes DNA at high temperature, a condition known to stimulate transcription arrest in *E. coli* (9). Despite the relatively slow elongation, *T. aquaticus* RNAP terminates transcription less efficiently, lending further support to the idea that the relationship between the transcription elongation rate and transcription termination is more complex than was previously thought (10).

The unusually high resistance of *T. aquaticus* RNAP to rifampin is not due to *T. aquaticus*  $\beta$  Thr<sup>655</sup>, the only *T. aquaticus*  $\beta$  amino acid that is different from *E. coli* residues known to be involved in rifampin resistance. This result suggests that additional amino acids weaken rifampin binding to *T. aquaticus* RNAP. It is not clear why mutational changes in these amino acids were not detected during intensive screens for rifampin resistance in *E. coli* and other rifampin-sensitive bacteria (7, 13). One possible explanation is that such changes lead to a lethal phenotype. Nevertheless, this result suggests that details of rifampin interaction with *T. aquaticus* RNAP may be significantly different from those of the interaction in rifampin-sensitive enzymes.

To summarize, the availability of recombinant *T. aquaticus* RNAP and discriminative transcription assays for this enzyme should make it possible to test, by means of genetic engineering and biochemical analysis, many of the predictions of the structure-functional model of transcription put forward by Zhang et al. (18). The ability to purify mutant *T. aquaticus* RNAP directly from *E. coli* cells should also allow a structural analysis of RNAP mutants.

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