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Analyses of *in vivo* interactions between transcription factors and the archaeal RNA polymerase

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Abstract

Transcription factors regulate the activities of RNA polymerase (RNAP) at each stage of the transcription cycle. Many basal transcription factors with common ancestry are employed in eukaryotic and archaeal systems that directly bind to RNAP and influence intramolecular movements of RNAP and modulate DNA or RNA interactions. We describe and employ a flexible methodology to directly probe and quantify the binding of transcription factors to RNAP *in vivo*. We demonstrate that binding of the conserved and essential archaeal transcription factor TFE to the archaeal RNAP is directed, in part, by interactions with the RpoE subunit of RNAP. As the surfaces involved are conserved in many eukaryotic and archaeal systems, the identified TFE-RNAP interactions are likely conserved in archaeal-eukaryal systems and represent an important point of contact that can influence the efficiency of transcription initiation.

Keywords

archaea; transcription; RNA polymerase; transcription factor; TFE

1. Introduction

Transcription is a highly regulated and orchestrated process in each domain. Chemical and structural transitions of RNA polymerase are often directed by conserved and essential transcription factors [1,2]. The transcription systems employed in Archaea mimic those employed in eukaryotes, and although component complexity is reduced, the archaeal RNA polymerase is highly conserved both in overall structure and regulatory capacity to eukaryotic RNA polymerase II (Pol II) [3-7]. All currently available archaeal genomes encode a single but multi-subunit RNA polymerase (RNAP) [4,8] that is regulated by conserved transcription factors to ensure proper transcription initiation, elongation, and termination [7,9-12]. Details of transcription mechanisms and regulation in the archaeal domain have lagged behind the knowledge of eukaryotic and bacterial systems, at least in

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part, due to the previous lack of genetic techniques. However, in recent years, several recombinant archaeal systems have been established, and genetic techniques for archaeal systems have evolved to permit rational manipulation of transcription systems *in vivo*. Tractable transcription systems from both of the dominantly studied and heavily populated archaeal phylums (the crenarcheota and euryarcheota) are available [13-25]. The hyperthermophilic marine euryarchaeon *Thermococcus kodakarensis* (*T. kodakarensis*) has both a robust genetic system and a highly purified *in vitro* transcription system [15,21,24,26,27]. The atomic resolution structure of 11-subunit RNAP from *T. kodakarensis* was recently solved at 3.5 Å (Figure 1) [5]. This is the first structural analysis of the euryarchaeal RNAP and the first structure wherein the RNAP clamp domain (shown in Figure 1) is newly solved crystal structure provides a wealth of information that can be in an open configuration. The used to probe RNAP activities and structural-based hypotheses.

Three transcription initiation factors are seemingly essential in archaeal organisms: TATA-binding protein (TBP; homologous to the eukaryotic TBP), transcription factor B (TFB; homologous to eukaryotic transcription factor IIB), and TFE (homologous to eukaryotic TFIIIE α) [10,28-30]. TFE is dispensable in *in vitro* transcription assays; however, all attempts to genetically remove TFE from the genome have, to date, been unsuccessful arguing for essential *in vivo* activities [21,22,31]. TFE is known to bind to the non-template strand of DNA and promote DNA melting to assist formation of the open-promoter complex [1,32]. Previous studies have also revealed that TFE interacts directly with both RNAP and TBP [33].

The mechanistic activities of the conserved transcription factors are only partially known. Identification of binding sites and defined protein-protein contact surfaces is critical to further establish the mechanistic details of transcription factors. There are several clues to sites of important molecular interactions between TFE and RNAP. Biochemical assays suggest that TFE binds both the RpoE subunit (also termed Rpo7, although conventional nomenclature for archaeal RNAP subunits will be used hereafter) and the clamp domain of RNAP (Figure 1) [1,31,34]. More specifically, the N-terminal winged-helix (WH) domain of TFE binds the coiled-coil domain of RNAP while the zinc-ribbon (ZR) C-terminal domain of TFE is predicted to bind the stalk domain comprised of RpoE and RpoF [1,35].

While several studies have suggested that TFE binds directly to RpoE, the molecular surfaces involved have not been identified. It was of interest to further investigate these molecular interactions, and this article provides details of a structure-function analysis of the role of RpoE in retention of TFE. The principles outlined within can easily be adapted to permit *in vivo* analysis of any predicted or known molecular interactions with the archaeal RNAP.

2. Genetic techniques in *T. kodakarensis*

Genetic techniques available to probe and explore the physiology, metabolism, and regulation of several archaeal lineages have exploded over the past ten years [15-17,19,24,27,36-38]. The natural competence and facile genetic system available for *T. kodakarensis* is consistently employed to probe the composition, activities, and interplay of

the information processing systems [15,23,24,27,37]. Any gene in the *T. kodakarensis* genome can be markerlessly affinity tagged, deleted (only non-essential genes), and/or modified to encode mutational protein variants permitting detailed dissections of protein activities *in vivo*. Furthermore, autonomously replicating expression plasmids can be retained in *T. kodakarensis* allowing for ectopic expression of a WT or a mutational-variant protein [24].

2.1. Genomic manipulations to RNAP subunits

T. kodakarensis strains have been constructed wherein the gene encoding for a single subunit of RNAP (*rpoL*) was modified to encode a protein with a His₆ or His₆-HA affinity tag [28,31]. Through tagging a single subunit of RNAP, the entire, *in vivo* assembled RNAP complex can be purified from cells using a single column purification. Interestingly, when purifying *T. kodakarensis* RNAP TFE co-purifies in a sub-stoichiometric manner [24,31]. Co-purification is independent of the affinity-tag employed, and co-purification of TFE continues after several chromatography steps [31].

2.2. Plasmid expressed RNAP subunits

T. kodakarensis does not contain endogenous plasmids. This limitation hindered progress in many arenas, and thus an expression vector was constructed by combining sequences from several origins to generate a shuttle vector that could autonomously replicate in both *T. kodakarensis* and *E. coli*. Briefly, the full sequence of a naturally occurring plasmid (pTN1) from a related Thermococcale species, *Thermococcus nautilus* (*Tn*), was fused with a common *E. coli* cloning vector (pCR2.1-Topo). The resultant fusion vector was further modified by inclusion of sequences encoding selectable markers that could be employed to force retention of the plasmid in select *T. kodakarensis* strains (Figure 2A) [24,39].

The flexible movement of this shuttle vector between species permits researchers to clone and modify plasmid-encoded sequences in *E. coli*, then immediately transfer the desired plasmid to *T. kodakarensis*. The transformed plasmid often encodes a modified *T. kodakarensis* gene resulting in the establishment of a *T. kodakarensis* strain that is merodiploid; that is, the cells will contain both an endogenous genomic and ectopically-expressed copy of the gene of choice. Construction of merodiploid strains is a convenient methodology that allows for manipulation of essential genes in *T. kodakarensis* without the need to alter the genomic copy of the essential factor. Each of the 11-subunits of *T. kodakarensis* RNAP has been individually cloned into a pLC70-based vector under a constitutive promoter, *P_{hmtB}*, that drives high level expression of the subunit-encoding gene.

2.2.1. Construction of plasmids encoding modified RpoE subunits—Our

immediate goal was to identify and mutationally alter the proposed surface(s) of RpoE that directly interact with TFE. We predicted that any TFE-RpoE interactions would be conserved across many species, and thus we modeled conserved and surface exposed residues of many RNAPs focusing on the solvent exposed surfaces of RpoE. This modeling revealed two immediate targets that were conserved and solvent exposed. A hydrophobic solvent exposed patch on RpoE (residues Y95, F98, and L107) was identified, and we predicted that this surface might represent an important point of contact between RNAP and

TFE. A second conserved loop region was identified on RpoE and these residues (S150-R157) were similarly predicted to contribute to the TFE-RpoE interaction surface (Figure 2B). We rationalized that mutation of these surfaces may affect the stability of the TFE-RpoE interaction, and thus the overall affinity and co-purification of TFE with RNAP. We therefore generated a plasmid (pJW1) expressing full-length *rpoE* modified to encode a protein with a C-terminal His₆-tag (Figure 2A). Site-directed mutagenesis was employed to delete the nucleic acid sequences encoding for S150-R157 of RpoE, resulting in pJW2. A third vector, pJW3, was constructed wherein the sequences encoding for the solvent exposed hydrophobic patch of RpoE were altered to encode for glutamic acids (Y95E, F98E, and L107E).

2.2.4. Protocol for transformation and selection of plasmids into *T.*

kodakarensis—Transformation of *T. kodakarensis* differs significantly from more routine transformations of aerobic bacteria and yeasts. As such, care must be taken to ensure that anaerobic conditions are maintained at all times. We typically perform all manipulations within an anaerobic chamber maintained with an atmosphere of 95% nitrogen and 5% hydrogen. The metabolism of *T. kodakarensis* also produces large quantities of pressured gases, typically H₂ and H₂S, and these explosive and toxic gases should always be properly and safely vented.

1. Anaerobically and sterilely inoculate 100 ml of anaerobic medium, typically an artificial sea water base supplemented with tryptone, yeast extract, and sulfur (ASW-YT-S) (see section 3.3.) with 1 ml of a saturated overnight culture of TS517. Incubate the inoculated culture at 85°C for ~12 hours until cells have reached and remained in stationary phase for approximately 2 hours.
2. Anaerobically harvest the 100 ml culture and resuspend the cells in 3 ml of 0.8× ASW (see section 3.3.). Allow cells to rest, anaerobically, at 4°C for ~30 minutes
3. Split the cell suspension into ~200 µl aliquots and add ~3 µg of plasmid DNA for each transformation. Chill the cells at 4°C for ~60 minutes. Heat shock the cell-DNA mixture for 45 seconds at 85°C, then rapidly reduce the temperature of the transformation by immediately chilling the tube to 4°C for minimally 5 minutes.
4. Carefully spread the resultant cell mixture onto gel-rite solidified solid media containing only a 19 amino acid mixture (-trp) (see section 3.3.) as a nutrient source. The lack of tryptophan in the media inhibits the growth of all tryptophan auxotrophic cells. However, the reintroduction of a complete tryptophan biosynthetic pathway by means of plasmid-encoded proteins capable of catalyzing the terminal steps in tryptophan biosynthesis restores prototrophic cells. Place the dry, inverted plates in a sealed anaerobic vessel fitted with a GasPak (BD; 260678) at 85°C for ~4 days. The anaerobic vessel should not be artificially pressured with gases before incubation, and care should be taken to safely vent temperature- and microbiological-induced pressurized gases when opening the anaerobic vessel.
5. Identify initial colonies resultant from transformation. *T. kodakarensis* colonies are nearly colorless, rise only slightly above the plane of the surface of the medium, and typically reach a maximum diameter of only ~2 mm, although colony

morphology is often irregular. Proper identification of colonies is non-trivial given the propensity of defects in the solid-medium to mimic small colonies. *T. kodakarensis* colonies are often identifiable by halos of reduced opacity in the solid medium, and occasionally by dark centers. Presumptive colonies are lifted from the medium (typically with a sterile pipette tip), resuspended, and serially diluted in 0.8× ASW. Several dilutions are necessary to yield single colonies in step 6 (below), as a typical *T. kodakarensis* colony contains ~10⁶ cells.

6. Aliquots of serially diluted colonies are applied as small spots to gel-rite solidified media containing 13 µM mevinolin. This second round of selective pressure eliminates all cells not carrying the desired plasmid construct and ensures clonal populations of cells for subsequent studies. Colonies should be allowed to form (as above) on ASW-YT-S based medium containing 13 µM mevinolin for ~48 hours at 85°C in a sealed anaerobic vessel fitted with a GasPak.
7. Discrete single colonies resultant after two days growth on gel-rite solidified ASW-YT-S + 13µM mevinolin media can be lifted and used to inoculate ~10 ml liquid cultures of ASW-YT-S + 13µM mevinolin medium. It is critical to ensure that the desired plasmid is retained in the clonal culture by physically purifying the plasmid to verify its presence as a plasmid (see section 2.2.5. below). At a frequency of approximately just 1%, plasmid sequences can be randomly incorporated into the *T. kodakarensis* genome. After anaerobically inoculating and sealing the culture, incubate the culture at 85°C for ~24 hours to permit growth to stationary phase.

2.2.5. *T. kodakarensis* plasmid prep protocol

1. Anaerobically and sterilely remove ~5 ml of culture medium from the serum vial, and aerobically harvest the cell mass at room temperature (RT) via centrifugation (~10,000 rpm in a table top unit is overly sufficient). Discard supernatant and resuspend the cell pellet in 500 µl 100 mM Tris-HCl pH 7.5, 50 mM EDTA, and 4 M NaCl.
2. Add 25 µL 20% SDS and mix well by vortexing to solubilize the cellular membranes.
4. Add 20 µL freshly prepared 5 M NaOH and mix well by vortexing to fully lyse the cells. Incubate ~3 minutes at RT.
5. Add 400 µL 3 M potassium acetate pH 4.8, mix gently by inverting tube, and allow lysate to coagulate at RT for 1-2 minutes.
6. Centrifuge at 15,000 rpm for ~10 minutes at RT to clear the cellular debris from the solution.
7. Carefully remove the resultant clarified cellular lysate to new tube. Add one-tenth volume 3 M sodium-acetate pH 5.2, then seven-tenths volume 100% isopropanol to the solution. Mix thoroughly, then spin at ~15,000 rpm in a bench top centrifuge to pellet the nucleic acids. Discard the supernatant and air dry the resulting pellet. Resuspend the pellet of nucleic acids in ~20 µl 10 mM Tris-HCl pH 8.0.

8. Transform ~3 µg of recovered plasmid DNA into a 50 µL aliquot of XL1-blue competent cells. Plate on LB-AMP and incubate at 37°C overnight. Recovery of Amp^R-colonies from the transformation is used to verify plasmid recovery and maintenance in the original *T. kodakarensis* culture. Plasmid recovery from *E. coli* using established techniques followed by sequencing is used to verify retention of the desired mutational variants in the original *T. kodakarensis* cultures.

2.2.5. Preparation of gel-rite solidified media—The optimal growth temperature of *T. kodakarensis* is 85°C. These temperatures demand use of glass petri-plates and specialized agars that retain solid-form at elevated temperatures. The use of gel-rite, which activates and solidifies within ~30 seconds upon exposure to divalent cations, in an artificial sea water based medium necessitates an accurate and rapidly deployed mechanism to yield anaerobic, solid media for genetic manipulations.

Preparation of solid-medium with only a pure amino-acid based nutrient source: Combine 50 ml 2× ASW and 500 µl of 200× trace minerals in a 100 ml glass serum bottle and seal bottle (bottle #1) with a temperature resistant septum and crimped aluminum ring. Also combine 50 ml 18 mega-ohm purified water and 1.0 g gelrite in a 100 ml glass serum bottle and seal bottle (bottle #2) with a temperature resistant septum and crimped aluminum ring. Autoclave both bottles at 121°C for 20 minutes and slowly exhaust the chamber using a liquid cycle. When internal autoclave temperature reaches ~95°C, remove the contents and rapidly transfer the heated materials inside an anaerobic chamber. Carefully open bottle #1, add 5.0 ml 20× 19-AA mixture, 100 µl 1000× vitamin mixture, and 200 µL 500× polysulfides solution. Carefully open bottle #2, add the entirety of bottle #1 into bottle #2, gently swirl, and quickly dispense the contents into four glass plates (~25 ml/plate). The media will solidify fully in ~30 seconds following the mixture of bottles #1 and #2.

Preparation of solid-medium with a rich nutrient source: Combine 50 ml 2× ASW, 500µL 2000× trace minerals, 0.5 g yeast extract, and 0.5 g tryptone in a 100 ml glass serum bottle and seal bottle (bottle 1) with a temperature resistant septum and crimped aluminum ring. Also combine 50 ml 18 mega-ohm purified water and 1.0 g gelrite in a 100 ml glass serum bottle and seal bottle (bottle #2) with a temperature resistant septum and crimped aluminum ring. Autoclave both bottles at 121°C for 20 minutes and slowly exhaust the chamber using a liquid cycle. When internal autoclave temperature reaches ~95°C, remove the contents and rapidly transfer the heated materials inside an anaerobic chamber. Carefully open bottle #1, and add 100 µl 1000× vitamin mixture and 200 µL 500× polysulfides solution. When desired, mevinolin should be added here. Carefully open bottle #2, add the entirety of bottle #1 into bottle #2, gently swirl, and quickly dispense the contents into four glass plates (~25 ml/plate).

See section 3.3. for recipes. Note that our procedures necessitate that sealed vessels be autoclaved. Extreme care should be taken to ensure the vessels employed are rated for such temperatures and pressures, that all vessels are housed within explosion proof secondary containers, and that proper protective equipment is employed at all times.

3. Preparation of RNAP variants

3.1. Biomass of *T. kodakarensis* for protein purification of RNAP variants

T. kodakarensis is able to use elemental sulfur (S^0) and/or protons as a terminal electron acceptor, thereby generating H_2S or H_2 respectively during growth [40]. A recent transcriptomics study revealed that the abundance of transcripts encoding *T. kodakarensis* TFE (TK2024) was most abundant when the cultures were supplied with both S^0 and grown glycolytically in the presence of pyruvate [41]. Liquid cultures of TS517 carrying pLC70, pJW1, pJW2, and pJW3 were therefore grown in ASW-YT- S^0 -pyruvate supplemented with 13 μM mevinolin and harvested at mid-exponential growth phase at an OD_{600} of ~ 0.4 .

3.1.2. Protocol for growth of liquid *T. kodakarensis* cultures and collection of biomass

1. Liquid medium is composed of 1 $\times$ artificial sea water (ASW) supplemented with 0.5% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) pyruvate and 1 $\times$ trace mineral solution (recipe below). Media is prepared using boiled, 18 mega-ohm water to reduce overall O_2 content. 1.2 L of media is autoclaved (20 minutes, 121°C, with a liquid exhaust cycle) in septum sealed 2 L pyrex vessels. Care should be taken not to exceed this ratio to ensure vessel integrity under high internal pressures. Always autoclave media in a secondary explosion proof container and employ necessary safety equipment.
2. Transfer vessels containing sterilized medium to an anaerobic chamber, sterily add 2.0 g/L of finely pulverized elemental sulfur (flowers of sulfur). Mevinolin is added to 13 μM [final]. Add 1 ml/L of 1000 $\times$ vitamin mixture (recipe available in 3.1.3. below). Inoculate media with an appropriate amount of *T. kodakarensis* culture (1:100).
3. Incubate sealed vessel at 85°C until an $OD_{600} \sim 0.4$ is achieved.
4. Harvest biomass (aerobically, 5000 $\times g$) and discard supernatant. Caution should be taken when harvesting biomass as H_2S and H_2 have been produced and the bottles are under extreme pressure (vent all headspace gases properly in a fume hood).
5. Store pellets at $-80^\circ C$.

3.2. Protein purification and co-isolation of TFE with RNAP

Purification of *in vivo* assembled RNAP containing ectopically expressed and affinity tagged RpoE, in WT and mutationally varied forms from pJW1, pJW2, and pJW3, should permit co-purification of TFE. If the introduced variants disrupt TFE interaction with RpoE in the context of RNAP holoenzyme, a discrepancy in the yields of TFE co-purifying with RNAP is anticipated. Ni^{2+} -affinity chromatography is employed for an initial purification of RNAP away from crude cell lysate. *T. kodakarensis* encodes several protein complexes with a natural affinity for the Ni^{2+} -charged affinity matrix, only through more exhaustive chromatographic purifications is highly purified RNAP recovered.

3.2.3. Protocol for RNAP purification

1. Resuspend biomass (3 ml per gram) in 25 mM Tris-HCl pH 8.0, 1 M NaCl, 10% (v/v) glycerol and lyse by repeated freeze/thawing (-80°C to 85°C). Sonicate until all viscosity is lost indicating complete shearing of the nucleic acids.
2. Clarify lysate by centrifugation ($15,000 \times g$). Discard pelleted debris
3. Slowly add sufficient 30% 8K PEG in 2 M KCl to achieve a final concentration of 6% 8K PEG. Stir for ~1 hour or overnight at 4°C to precipitate nucleic acids. Clarify lysate by centrifugation ($15,000 \times g$). Discard pelleted debris
4. Load the clarified supernatant over a 5 ml Ni^{2+} -charged Hi-Trap chelating column (pre-equilibrated with 25 mM Tris-HCl pH 8.0 containing 1 M NaCl and 10% (v/v) glycerol) attached to a chromatography system. Discard flowthrough.
5. Wash the column with minimally 20 column volumes (CV) 25 mM Tris-HCl pH 8.0 containing 1 M NaCl and 10% (v/v) glycerol. Continue to flush the column with 25 mM Tris-HCl pH 8.0 containing 1 M NaCl and 10% (v/v) glycerol if substantial protein elution can be detected by monitoring the UV absorbance of the eluant. Elute the bound proteins, including RNAP, with a ~30 CV linear gradient from 25 ml Tris-HCl pH 8.0 containing 1 M NaCl and 10% (v/v) glycerol to 25 ml Tris-HCl pH 8.0 containing 0.1M NaCl, 10% (v/v) glycerol, and 100 mM imidazole.
6. Collect 1-2 ml fractions during the elution. 10 μl of peak fractions (identified via UV absorbance) are combined with 2 μl 6 $\times$ SDS-loading buffer and boiled for 3 minutes then separated using 10% discontinuous SDS-PAGE.
7. Fractions containing RNAP (molecular weight ~380,000 kDa; 11 subunits) should be identified by SDS-PAGE and coomassie staining.
8. Fractions containing RNAP should be pooled, diluted with 25 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 10 mM MgCl_2 , 10% (v/v) glycerol until the conductivity of the sample is below the conductivity of 25 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 10 mM MgCl_2 , 200mM KCl, 10% (v/v) glycerol. The diluted sample should be loaded onto and resolved through a 1 ml Mono Q column. Discard flowthrough. The column should be washed with minimally 20 CV 25 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 10 mM MgCl_2 , 10% (v/v) glycerol containing 200 mM KCl, then bound proteins should be eluted with a linear gradient of 200 mM to 400 mM KCl in 25 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 10% (v/v) glycerol.
9. Collect 1-2 ml fractions during the elution. 10 μl of peak fractions (identified via UV absorbance) are combined with 2 μl 6 $\times$ SDS-loading buffer and boiled for 3 minutes.
11. Fractions containing RNAP should be identified by SDS-PAGE and coomassie staining.

12. Fractions containing RNAP should be pooled and concentrated to < 1 ml using 100 kDa molecular-weight cut-off centrifugal filter device. Concentrated material should be loaded onto and resolved through a Superdex 200 16/60 column equilibrated with 20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 100 mM NaCl at 0.2 ml/min.
13. Collect 1-2 ml fractions during the elution. 10 µl of peak fractions (identified via UV absorbance) are combined with 2 µl 6× SDS-loading buffer and boiled for 3 minutes.
14. Fractions containing RNAP should be identified by SDS-PAGE and coomassie staining. Pool appropriate fractions.
15. Fractions containing RNAP should be pooled and loaded onto a 1 ml heparin column equilibrated with 200 mM Tris-HCl pH 8.0, 100 mM NaCl. Bound proteins should be isocratically eluted with 200 mM Tris-HCl pH 8.0 and 1M NaCl.
16. Collect 1 ml fractions during the elution. 10 µl of peak fractions (identified via UV absorbance) are combined with 2 µl 6× SDS-loading buffer and boiled for 3 minutes.
17. Fractions containing RNAP should be identified by SDS-PAGE and coomassie staining.
18. Fractions containing RNAP should be identified by SDS-PAGE and coomassie staining. These fractions should be pooled. Secure samples in 100 kDa dialysis tubing and dialyze samples (twice, for at least 6 hours) against minimally 1000-volumes protein storage buffer (see section 3.3.). Recover purified protein from the dialysis tube and store at -20°C.

3.3. Recipes

1× ASW contains 20 g NaCl, 3 g MgCl₂ · 6H₂O, 6 g MgSO₄ · 7H₂O, 1 g (NH₄)₂SO₄, 200 mg NaHCO₃, 300 mg CaCl₂ · 2H₂O, 0.5 g KCl, 420 mg KH₂PO₄, 50 mg NaBr, 20 mg SrCl₂ · 6H₂O, and 10 mg Fe(NH₄)₂(SO₄)₂ · 6H₂O sulfate per L.

19 amino acid mixture (-trp) (20×) contains 5 g cysteine (or cystine), 5 g glutamic acid, 5 g glycine, 2.5 g arginine, 2.5 g proline, 2 g asparagine, 2 g histidine, 2 g isoleucine, 2 g leucine, 2 g lysine, 2 g threonine, 2 g tyrosine, 1.5 g alanine, 1.5 g methionine, 1.5 g phenylalanine, 2 g serine, 1 g aspartic acid, 1 g glutamine, 1 g valine per L.

0.8× ASW contains 16 g NaCl, 2.4 g MgCl₂ · 6H₂O, 4.8 g MgSO₄ · 7H₂O, 0.8 g (NH₄)₂SO₄, 160 mg NaHCO₃, 240 mg CaCl₂ · 2H₂O, 0.4 g KCl, 336 mg KH₂PO₄, 40 mg NaBr, 16 mg SrCl₂ · 6H₂O, and 8 mg Fe(NH₄)₂(SO₄)₂ · 6H₂O sulfate per L.

Trace mineral solution (1000×) contains 0.5 g MnSO₄ · H₂O, 0.1 g CoCl₂ · 6 H₂O, 0.1 g ZnSO₄ · 7 H₂O, 0.01 g CuSO₄ · 5 H₂O, 0.01 g AlK(SO₄)₂ · 12 H₂O, 0.01 g H₃BO₃, 0.01 g Na₂MoO₄ · 2 H₂O per L.

Vitamin mixture (200×) contains 0.2 g niacin, 0.08 g biotin, 0.2 g pantothenate, 0.2 g lipoic acid, 0.08 g folic acid, 0.2 g P-aminobenzoic acid, 0.2 g thiamine, 0.2 g riboflavin, 0.2 g pyridoxine, 0.2 g cobalmin per L.

Polysulfide solution (500×) contains 66.7 g sodium sulfide ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) and 3 g Sulfur per L.

Storage buffer contains 25 mM Tris-HCl pH 8.0, 10 mM MgCl_2 , 0.1 mM EDTA, 200 mM KCl, 2 mM β -mercaptoethanol, 50% glycerol.

4. Analysis of co-purification of TFE with the variant RpoE-RNAP variants

TS517 strains carrying pJW1, pJW2, and pJW3 all yield obvious large quantities of RNAP holoenzyme from clarified cell lysates and the resultant RNAP complex remained intact through many chromatographic separations. Excess and non-RNAP incorporated RpoE was not visualized under any circumstances and this was anticipated given that RpoE is unlikely to stably fold and remain soluble or intact in the absence of its binding partner, RpoF. TFE was visible as a co-purification partner in RNAP preparations from TS517 carrying pJW1-3 and was easily identified based on the migration of the protein during SDS-PAGE. In contrast, TS517 carrying the empty vector pLC70 did not yield identifiable quantities of RNAP following any chromatographic purification; TFE could also not be identified in any bound fractions from such purifications.

The relative stoichiometry of TFE and RNAP within each preparation was quantified following a combination of Ni^{2+} -affinity and MonoQ purification (steps 1-11 of protein purification protocol). To analyze the amount of TFE co-purifying with RNAP, 7.5 μg of each protein preparation was resolved via SDS-PAGE and SYPRO Ruby stained according to the manufacturer's instructions. The stained gel was digitized using a Typhoon scanner and the relative staining of protein bands was quantified using ImageJ software. Specifically, the staining intensity of TFE was quantified within each preparation and normalized to the staining intensity of the easily identified subunit of *T. kodakarensis* RNAP, RpoE. The amount of TFE co-purifying with WT RpoE-RNAP in *T. kodakarensis* TS517 with pJW1 was set as a value of 100%.

A two-fold decrease in co-purification of TFE with RNAP was observed for TS517 pJW2 (S150-R157) as well as TS517 pJW3 (Y95E, F98E, and L107E) when compared to TS517 pJW1 (WT) (Figure 3B). This decrease was repeatedly observed in multiple RNAP preparations from each strain, and the results obtained argue that both mutational variants of RpoE hinder stable interactions of TFE and RpoE in the context of the fully formed RNAP complex. Further purification of RNAP (steps 12-17 of protein purification protocol) was employed to determine if TFE would continue to co-purify with WT RNAP and the variant RNAPs as conditions became more selective. Size exclusion chromatography followed by heparin affinity chromatography separated the remaining non-specifically bound proteins from RNAP. To analyze the amount of TFE co-purifying with this more highly purified form of RNAP, 7.5 μg of each RNAP variant was resolved and quantified as before (Figure 3). A two-fold decrease was observed in co-purification of TFE in TS517 pJW3. An unanticipated loss of the stalk domain of RNAP (along with TFE) from the bulk of the

RNAP purified from TS517 pJW2 following size exclusion and heparin affinity chromatography hindered accurate measurements of TFE-RNAP ratios (Figure 3B). This loss reflects both an abolished co-purification of TFE with RNAP, as well as an unexpected decrease in the abundance of the stalk domain with respect to the remaining RNAP subunits. RpoE residues S150-R157 were not anticipated to directly interact with the core subunits of RNAP, but it is possible that misfolding or altered interactions between RpoE and RpoF in this variant could lower the affinity of the stalk domain in general for the core of RNAP. This result is supported with studies done by other groups where the RNAP stalk domain is critical for association and/or activity of TFE [1,31,34,42].

5. Concluding Remarks

In this article, we describe a method to directly probe and quantify the binding of a conserved and essential transcription factor, TFE, to RNAP *in vivo*. *In vivo* structure-function analyses of transcription factor binding provide insight into mechanistic details that underlie regulation of RNAP. Structure-function analyses are often limited to *in vitro* assays; however, we describe a protocol that facilitates the investigation of transcription factor binding to RNAP *in vivo*. Furthermore, considerations specific to obtaining *T. kodakarensis* biomass, purifying RNAP, and final analysis of data were addressed.

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Highlights

- Quantifications of *in vivo* interactions under native conditions are possible.
- Use of merodiploid strains permits rapid evaluation of RNA polymerase variants.
- RNA polymerase (*i.e.* RpoE) variants disrupt the binding of TFE to the archaeal RNAP.

Figure 2. Plasmid expressed RNAP variants. **A.** A shuttle vector that can autonomously replicate in both *E. coli* and *T. kodakarensis* has been constructed with *rpoE* under control of a constitutive promoter, P_{hmtB} . **B.** *T. kodakarensis* RNAP as a surface model with the exception of the coiled-coil domain shown in a ribbon model. The core of RNAP is shown in gray, RpoF is shown in green, and RpoE is shown in blue. The hydrophobic patch of RpoE is highlighted in yellow (Y95, F98, L107). Residues S150-R157 of RpoE are highlighted in red. PDB ID: 4QIW. **C.** Description of the plasmids employed and the mutational variants encoded.

Figure 3.

RpoE variants reduce co-purification of TFE with RNAP. **A.** SYPRO Ruby staining after SDS-PAGE of aliquots of RNAP (7.5 μ g) purified from *T. kodakarensis* strain TS517 containing the vectors shown above the gel. Lane 1 contains size standards in kDa. Lanes 2-4 contain final purifications of RNAP from strains containing pJW1-3 respectively. An identical purification scheme from TS517 with the empty vector pLC70 is shown in lane 5 to demonstrate that RNAP purification is only possible based on the His₆-tag on RpoE. RNAP subunits are labeled to the right of the gel. **B.** Quantification of the normalized amount of TFE co-purifying with RNAP in the initial purification, denoted as a solid bar to the left, and final purification, denoted as a lined bar to the right. TFE was normalized to 100% in the *T. kodakarensis* strain encoding for the WT RNAP variant (pJW1), the amount of TFE co-purifying with RNAP in the mutant RNAP variants was normalized to WT RNAP. pJW2 denotes the RpoE variant wherein S150-R157 were deleted. pJW3 denotes the RpoE variant wherein the hydrophobic patch was disrupted.