Deletion of Switch 3 Results in an Archaeal RNA Polymerase That Is Defective in Transcript Elongation

Thomas J. Santangelo and John N. Reeve
From the Department of Microbiology, Ohio State University, Columbus, Ohio 43210

Switch 3 is a polypeptide loop conserved in all multisubunit DNA-dependent RNA polymerases (RNAPs) that extends into the main cleft of the RNAP and contacts each base in a nascent transcript as that base is released from the internal DNA-RNA hybrid. Plasmids have been constructed and transformed into Thermococcus kodakaraensis, which direct the constitutive synthesis of the archaeal RNAP subunit RpoB with an N-terminal His6 tag and the Switch 3 loop either intact (wild-type) or deleted (ΔS3). RNAPs containing these plasmid-encoded RpoB subunits were purified, and, in vitro, the absence of Switch 3 had no negative effects on transcription initiation or elongation complex stability but reduced the rate of transcript elongation. The defect in elongation occurred at every template position and increased the sensitivity of the archaeal RNAP to intrinsic termination. Comparing these properties and those reported for a bacterial RNAP lacking Switch 3 argues that this loop functions differently in the RNAPs from the two prokaryotic domains. The close structural homology of archaeal and eukaryotic RNAPs would predict that eukaryotic Switch 3 loops likely conform to the archaeal rather than bacterial functional paradigm.

Atomic resolution structures of multisubunit RNA polymerases (RNAPs) from all biological domains reveal a highly conserved nucleic acid binding channel and catalytic core (1–9). In all of these RNAPs, a series of protein loops, designated the lid, rudder, zipper, switches 1 to 4, and fork loops 1 and 2, protrude into the channel, and make direct contact with the encapsulated nucleic acids. Although the mechanisms of ribonucleotide polymerization and RNA-DNA duplex translocation are conserved universally (8, 10, 11), RNAPs differ in promoter recognition, initiation, and termination requirements (12–22), and some of the polypeptide loop interactions may be RNAP-specific during initiation and termination. Consistent with this, although the loop structures generally are conserved, they have sequence differences, and there are loop interactions within a eukaryotic polymerase II plus transcription factor TFIIIB initiation complex (19) that are analogous but not homologous to loop interactions within a bacterial RNAP-sigma factor complex (7, 20). Similarly, when considering termination, bacterial RNAPs pause and terminate in response to inverted-repeat sequences (18), but this is not the case for archaeal (13–16) or eukaryotic RNAPs (23). Folding of the nascent transcript into a hairpin loop destabilizes bacterial elongation complexes, but either this does not occur, or the transcription-RNAP interactions do not destabilize archaeal and eukaryotic elongation complexes.

The presence of the RNA-DNA hybrid contributes substantially to the stability of an elongation complex (24–27), and interactions between the RNAP and hybrid help maintain this duplex and thus help stabilize the elongation complex. The length of the DNA-RNA hybrid is limited (8–10 bp) by space and topological constraints, and each rNTP addition requires the release of one RNA base from the hybrid (24, 25, 28, 29). The released base is contacted and immediately bound by a hydrophobic polypeptide loop, designated Switch 3, that is conserved within the second largest subunit of multisubunit RNAPs (see Fig. 1A) (6, 8, 30). Deletion of the Switch 3 loop from a bacterial RNAP resulted in an enzyme that was incapable of forming stable complexes with nucleic acid scaffolds (28). Bacterial Switch 3-transcript interactions apparently therefore stabilize elongation complexes and so likely contribute to bacterial transcription pausing and termination decisions (31). Although the Switch 3 loop is a universally conserved structure, there are sequence differences that predict that archaeal and eukaryotic Switch 3 loops differ from bacterial Switch 3 loops in charge and flexibility (see Fig. 1B). Given these differences, and that archaeal and bacterial RNAPs respond to different intrinsic termination signals (13–15, 18), it was of interest to determine whether the Switch 3 loop in an archaeal RNAP contributed similarly to elongation complex stability and transcription termination.

To obtain an archaeal RNAP lacking the Switch 3 loop, a plasmid was constructed and transformed into Thermococcus kodakaraensis, which directs the synthesis of the RpoB subunit, with the Switch 3 loop deleted. A control plasmid directed the synthesis of an RpoB subunit with the wild-type Switch 3 loop, and both plasmid-encoded RpoB subunits had an N-terminal hexahistidine (His6) tag sequence. The His6 tag facilitated the rapid and selective purification of RNAP molecules from T. kodakaraensis cell lysates that contained the plasmid-encoded RpoB subunit. The reported results document that the absence of Switch 3 had no negative effects on promoter-dependent initiation or elongation complex stability in vitro but...
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did reduce the rate of transcript elongation and resulted in increased sensitivity to intrinsic termination. As discussed, the results argue that the Switch 3 loop contributes differently to archaeal and bacterial transcription, and, as archaeal and eukaryotic RNAPs have very similar structures, eukaryotic Switch 3 loops likely have archaeal rather than bacterial Switch 3 functions.

EXPERIMENTAL PROCEDURES

Plasmid and Strains—Plasmids pTS522 and pTS523 (see Fig. 1C) were constructed from pLC70 (32) by standard molecular biology techniques. The P<sub>hmb</sub> promoter and RpoB-encoding gene (TK1083; 33) cloned into these plasmids were sequenced to confirm the accuracy of the constructions. These plasmids allow growth in the absence of tryptophan, confer resistance to mevinolin, and result in the constitutive synthesis of His<sub>6</sub>-tagged RpoB. Plasmid pTS523 is identical to pTS522 except for a sequence change in the gene (TK1083) that encodes RpoB that replaced the Switch 3 loop with Gly-Gly.

Cells were harvested by centrifugation, resuspended at 0.33 g/ml (wet weight) in lysis buffer (25 mM Tris-HCl (pH 8), 1 M NaCl, 10% (v/v) glycerol), lysed, and RNAP preparations were purified at room temperature using equipment housed within a Coy anaerobic chamber with a 5% H<sub>2</sub>, 95% N<sub>2</sub> atmosphere. Briefly, 30% polyethylene glycol in 2 M KCl was added slowly to a clarified lysate to a final concentration of 6% polyethylene glycol. Precipitated nucleic acids were removed by centrifugation, and the resulting supernatant was loaded onto a 5-ml HiTrap chelating column charged with Ni<sup>2+</sup>. The column was washed with 30 column volumes of lysis buffer and eluted using a linear gradient (12 column volumes) from lysis buffer to 25 mM Tris-HCl (pH 8), 10% glycerol, 0.55 M NaCl, and 125 mM imidazole. The fractions that contained RNAP, identified by Coomassie blue staining after SDS-PAGE, were pooled, diluted with 25 mM Tris-HCl (pH 8), 10% glycerol to a conductivity below that of 0.2 M NaCl, and loaded on to a 1-ml MonoQ column. Fractions were eluted using a 50-column volume linear gradient of 0.2 to 0.4 M NaCl in 25 mM Tris-HCl (pH 8), 10% glycerol. Fractions that contained RNAP were identified, pooled, and loaded on to a 5-ml HiTrap, Ni<sup>2+</sup>-charged chelating column. Fractions were eluted using a 25-column volume linear gradient from 25 mM Tris-HCl (pH 8), 1 M NaCl, 10% glycerol to 25 mM Tris-HCl (pH 8), 0.55 M NaCl, 10% glycerol, 125 mM imidazole. Fractions containing RNAP were pooled, concentrated using Millipore MWCO centrifugal concentrators (100-kDa cut-off), dialyzed, and stored in 25 mM Tris-HCl (pH 8), 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 200 mM KCl, 20 mM β-mercaptoethanol, and 50% glycerol.

RNAP preparations were also purified using the same Ni<sup>2+</sup> affinity and MonoQ chromatography protocol from <i>T. kodakaraensis</i> KW128 (pTS474). This RNAP has a C-terminal His<sub>10</sub>-tagged RpoL subunit and has activity in vitro indistinguishable from that of wild-type RNAP purified from <i>T. kodakaraensis</i> KW128 cells by standard multicomponent chromatography (16). In reaction mixtures that contained equimolar concentrations...
of purified RNAP and template DNA, the RNAPs containing His<sub>10</sub>-RpoL, His<sub>6</sub>-RpoB (WT), or His<sub>6</sub>-RpoB (∆S3) synthesized transcripts, under single round assay conditions, from >90, 70, and 70% of the templates provided, respectively. The recombinaent versions of T. kodakaraensis TBP, TFB1, or TFB2 (T. kodakaraensis transcription factors TFB1 and TFB2) used to support transcription initiation were prepared as described previously (16).

**In Vitro Transcription**—The construction and sequences of the DNAs used as templates (designated 372, 442, 443, 452, 468, 552, 553, and 556) for in vitro transcription assays have been published (14). The template DNA (10 nM) was incubated with 40 nM RNAP, 80 nM TBP, 80 nM TFB1 or TFB2, 75 μM [32P]ApC in transcription buffer (20 mM Tris–HCl (pH 8), 250 mM KCl, 5 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, and 7% glycerol) for 10 min at 85 °C to allow open complex formation. Transcription was then initiated by adding 1 mM ATP, GTP, CTP, and/or UTP, and the 5′-[32P]ApC-labeled transcripts synthesized were purified, separated by denaturing polyacrylamide gel electrophoresis, visualized, and quantified using a Storm 840 PhosphorImager. Using this protocol, in most experiments, only transcripts synthesized in the first round of transcription were effectively [32P]-labeled. The [32P]ApC dinucleotide used to initiate the first transcript was outcompeted for initiation in subsequent rounds of transcription by the presence of 1 mM ATP (data not shown). When only GTP was present, the [32P]ApC dinucleotide repeatedly was incorporated during multiple rounds of abortive transcription resulting in the accumulation of 5′-[32P]ACGG abortive transcripts (see Fig. 3). 5′-Biotinylated templates were used to separate transcripts that remained attached to the template DNA from those that were terminated and released from the template DNA. Following transcription, streptavidin-coated paramagnetic beads were added, and the mixture was placed on ice for 10 min. The beads and attached complexes were then removed by centrifugation. Terminated transcripts remained in the supernatant (S fraction) and transcripts that remained in elongation complexes were pelleted (P fraction). These transcripts were separated by electrophoresis in adjacent gel lanes, visualized, and quantified using a PhosphorImager.

**Scaffold Transcript Elongation**—Equimolar aliquots (10 nM) of 5′-[32P]GAGUCUCGGCGGAUA (see Fig. 4 and supplemental Fig. S2) or 5′-[32P]GAGUCUCGGCGGAU (see Fig. 5 and supplemental Fig. S3) were incubated with a partially complementary 24-nt single stranded template DNA in transcription buffer for 10 min at 85 °C. The reaction mixture was slowly cooled to room temperature, RNAP (40 nM) added, and the reaction mixture returned to 85 °C for 10 min. For most scaffolds, a single-stranded DNA (40 nM) was then added with a sequence complementary to part or the entire template DNA (see Figs. 4 and 5 and supplemental Figs. S2 and S3), and incubation was continued at 85 °C for 10 min. As stated in the figure legends, transcript elongation was initiated by the addition of one or more rNTPs, α-thio-ATP, or pyrophosphate (PPi). The [32P]-labeled transcripts synthesized at 85 °C were separated by electrophoresis through 22.5% (w/v) denaturing polyacrylamide gels, visualized, and quantified.

**Purification of Elongation Complexes and Assay of NaCl and Heparin Sensitivity**—In vitro transcription reaction mixtures that contained biotinylated template 442 (14), RNAP, TBP, TFB2, 75 μM [32P]ApC, 1 mM GTP, 1 mM UTP, and 50 μM ATP were incubated for 10 min at 85 °C. This allowed initiation and accumulation of elongation complexes stalled at +7 (see Fig. 6). Streptavidin-coated paramagnetic beads were added, and after incubation on ice for 10 min, the beads and attached elongation complexes were removed and washed three times with transcription buffer that contained 50 μM ATP. They were then resuspended and incubated at room temperature for 3 min in transcription buffer that contained 100 nM ATP, GTP, and CTP. Aliquots were removed, 3 or 4 M NaCl with or without 1 mg heparin/ml was added, and the mixtures were incubated for 2 min at room temperature. [32P]-labeled transcripts, which remained in elongation complexes and attached to the beads, were separated by centrifugation from transcripts that were released from the beads during incubation with NaCl and heparin. The transcripts were separated by electrophoresis in adjacent gel lanes through 22.5% (w/v) denaturing polyacrylamide gels, visualized, and quantified.

**RESULTS**

Loss of Switch 3 Does Not Affect Initiation but Reduces Elongation—For convenience, the RNAPs purified by Ni<sup>2+</sup> affinity chromatography from lysates of T. kodakaraensis KW128 (pTS522) and KW128 (pTS523) are designated WT and ∆S3, respectively. Their ability to initiate and transcribe template DNAs in vitro was assayed using templates constructed previously (14) designated 443, 452, and 468 (Fig. 2) and 372, 552, 553, and 556 (supplemental Fig. S1) in reaction mixtures that contained recombinant T. kodakaraensis TBP and either TFB1 or TFB2, one of the two versions of the TFB initiation factor present in T. kodakaraensis. These templates all had the same constitutive promoter (pTS522) and sequence from the site of transcription initiation (+1) to +10. The sequence immediately downstream from +10 in 452 originated from within an archaeal open reading frame, whereas the sequences at this location in 443, 468, and 556 were from archaeal intergenic regions and contained oligoT tracts. These natural oligoT-rich sequences and a synthetic oligoT<sub>9</sub> tract positioned at this location in template 552 stimulate archaeal intrinsic termination in vitro (14). As shown for template 452 (Fig. 2), there was essentially no difference in the pattern of transcripts synthesized in vitro by WT or by ∆S3 when initiation was supported by TFB1 or by TFB2. The patterns of the transcripts synthesized by WT and ∆S3 also were very similar, but WT synthesized substantially larger amounts of transcripts longer than abortive transcripts. For example, on 452 with initiation supported by TFB1 or TFB2, WT synthesized ~5- and ~8-fold more run-off transcripts than did ∆S3. The reduced ability of ∆S3 to elongate transcripts through the full length of a template was exacerbated by the presence of an intrinsic terminator sequence. For example, WT elongated ~10-fold more transcripts through the oligoT region and downstream sequence on 443 to produce run-off transcripts than did ∆S3 (Fig. 2). At each template position, the RNAP may add a nucleotide, and thus elongate the transcript, or it may terminate. As documented
previously in detail (14), WT terminates in vitro to different extents at multiple sites on all of the templates used in this study, with termination predominating at oligoT-rich sequences. For ΔS3, termination rather than elongation was amplified on all templates, and, with a reduced ability to elongate, the effectiveness of intrinsic terminators was increased on all templates and under all reaction conditions tested (Fig. 2 and supplemental Fig. S1).

**WT and ΔS3 Abortive Transcription**—In single round transcription assays, very similar patterns of abortive transcripts were synthesized by WT and ΔS3 (Fig. 2 and supplemental Fig. S1). To obtain a more detailed comparison of the abortive transcription by WT and ΔS3, initiation was allowed on 442 in the presence of [32P]ApC but in reaction mixtures that lacked one or more rNTP (Fig. 3). The transcripts synthesized were then separated into those that remained bound to RNAP in elongation complexes and those that were terminated and released. Under identical conditions, the total amounts of abortive transcripts synthesized by ΔS3 were 70% of the amounts synthesized by WT. The deletion of Switch 3 did not therefore dramatically reduce initiation and had only a modest effect on abortive transcript synthesis. Under these reaction conditions, with all four NTPs present, WT transcription resulted in detectable amounts of a full-length (116 nt) run-off transcript, but, consistent with a reduced ability to elongate transcripts following promoter escape, this was not the case for ΔS3.

**WT and ΔS3 Elongation of Scaffold RNAs**—To evaluate elongation in the absence of promoter initiation and escape, elongation.
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![Diagram of RNA elongation by WT and ΔS3](image)

FIGURE 4. Scaffold RNA elongation by WT and ΔS3. The sequence and structure of the nucleic scaffold used is shown below the electrophoretic separation of the 5′-[32P]-labeled RNAs elongated at 85 °C by WT and ΔS3 in reaction mixtures that contained the rNTP(s) (200 μM concentrations) listed above the gel lane. Based on intensities and anomalous migration, similar low levels of misincorporation occurred with WT and ΔS3 when supplied with only GTP or ATP. Individual elongation products are identified to the right of the gel by their 3′-nucleotide.

The complexes contained WT or ΔS3 and a [32P]RNA hybridized to the template DNA. A complementary non-template DNA strand was present in most (Figs. 4 and 5 and supplemental Figs. S2B and S3) but not all scaffolds (supplemental Fig. S2A). With all four rNTPs present, the scaffold RNA was elongated by both WT and ΔS3 into run-off transcripts but, under identical conditions, ΔS3 synthesized only ~30% of the run-off transcripts synthesized by WT (Fig. 4). When elongation of the scaffold RNA was limited by omission of one or more rNTPs, it was apparent that ΔS3 exhibited reduced ability to add a nucleotide at every elongation step along the template DNA. In reaction mixtures that contained only GTP or ATP, base misincorporation occurred at detectable levels, but this occurred to the same extent with WT and ΔS3 (Fig. 4). Comparisons of scaffold RNA elongations in the presence or absence of the noncoding DNA strand revealed that the presence of the non-template strand reduced the amount of RNA elongated, consistent with a reduction in scaffold assembly. The presence of the complementary strand, however, had no discernible positive or negative effect on the different elongation abilities of WT and ΔS3 (supplemental Fig. S2).

To investigate whether ΔS3 deficiency reflected reduced ability to add nucleotides and/or translocate, RNA elongation was compared in scaffolds designated Sc1 and Sc2 (nucleic acid scaffolds 1 and 2). In Sc1, an unpaired T was present at the site of nucleotide addition, whereas in Sc2, the RNA, non-template strand, and template DNA were fully base-paired (Fig. 5, A and B and supplemental Fig. S3). Incubation with 10 μM and 100 μM PPi, stimulated WT and ΔS3 phosphorolysis of the RNA in Sc1, consistent with the RNAP oscillating readily between pre- and post-translocated states (supplemental Fig. S3). Adding one nucleotide to the scaffold RNA in Sc1 therefore would be dependent on both nucleotide addition (polymerization) and translocation events. In contrast, the RNA in Sc2 largely was resistant to PPi-stimulated phosphorolysis (supplemental Fig. S3), arguing for a stable post-translocated complex and that polymerization would dominate as the event required to elongate the scaffold RNA by one nucleotide. Sc1 and Sc2 contained the same 5′-[32P]-labeled RNA, and, as dictated by the template sequence, elongation of this 14-mer in the presence of only ATP or α-thio-ATP resulted in the synthesis of a [32P]-labeled 15-mer (Fig. 5). Misincorporation of ATP for GTP in Sc1 also resulted in low but detectable synthesis of a [32P]-labeled 17-mer. Further misincorporation of ATP for the UTP required at position 18 of Sc1 or for the UTP required at position 16 of Sc2 did not occur. To measure the rates of RNA elongation, the [32P]-labeled transcripts present after increasing periods of incubation of the scaffold complexes at 85 °C in the presence of ATP or α-thio-ATP were separated by electrophoresis and quantified. Transcription elongation by ΔS3 was clearly slower than by WT with both ATP and α-thio-ATP but, for practical reasons, to obtain reproducible measurements of the initial rates of transcript elongation, most experiments used α-thio-ATP. During the first minute of incubation, the scaffold RNA in Sc1 and in Sc2 was elongated ~3 times faster by WT than by ΔS3. There was, however, no difference in the initial rates of RNA elongation in Sc1 versus Sc2 by either enzyme (Fig. 5C) indicating that the additional translocation requirement for RNA elongation in Sc1 was not rate-limiting. The reduced rate of elongation by ΔS3 most likely therefore reflected a deficiency in nucleotide polymerization rather than in translocation.

ΔS3 Forms Salt-stable Elongation Complexes—Deletion of the Switch 3 loop from a bacterial RNAP resulted in an enzyme that formed unstable elongation complexes that were readily disrupted by exposure to physiological salt concentrations (~300 mM KCl (28)). To determine whether elongation complexes formed by ΔS3 were similarly salt-sensitive, complexes containing either WT or ΔS3 stalled at +7 on template 442 were isolated, washed, and then incubated with 100 μM ATP, GTP, and CTP. Under these conditions, only limited elongation of the transcript occurred resulting in an ensemble of elongation complexes with the RNAP stalled and distributed at every template position from +7 to +20 (Fig. 6). When NaCl was added to these elongation complexes at concentrations approaching the
limits of salt solubility only complexes containing very short 7-, 8-, and 9-mer transcripts were disrupted. All elongation complexes formed by ΔS3 that contained 10-mer or longer transcripts were almost totally resistant to 4M and 3 M salt plus heparin challenges (Fig. 6). In these assays, there was no discernible difference in the stability of the WT and ΔS3 complexes stalled at any template position.

DISCUSSION

Transcription is a multistep, complex process catalyzed by complex enzymes. Given the wealth of established biochemistry, sophisticated genetics, and relative simplicity, studies of bacterial RNAPs are attractive and most appropriate for investigations of universally conserved features. However, there are also clear differences in bacterial, archaeal, and eukaryotic transcription that demand direct studies of RNAPs from each domain (1–3, 5, 11, 12, 31, 37, 38). Reconstitution of functional bacterial and archaeal RNAPs from individual subunits (39–41), a technology not yet established for eukaryotic RNAPs, is a powerful approach and, by taking advantage of bacterial genetics, the results obtained with variant bacterial RNAPs assembled in vitro can be tested and validated with enzymes assembled in vivo. To provide such in vivo validation of research with archaeal RNAPs, we have established the necessary genetics, in vivo expression and rapid variant RNAP purification technology from T. kodakaraensis (32, 42). Using these developments, we report here the first structure-function investigation of an archaeal RNAP variant assembled in vivo.

As a model, and as a structure not previously investigated in an archaeal RNAP, we chose to investigate Switch 3. This polypeptide loop is conserved in all multisubunit RNAPs and binds to each RNA base in a nascent transcript as it dissociates from the RNA-DNA hybrid (Fig. 1A) (8, 28). The results obtained reveal that deletion of Switch 3 does not effect initiation but results in an archaeal RNAP (ΔS3) that has reduced ability to elongate transcripts in both promoter-initiated elongation complexes and in scaffold complexes. The elongation complexes formed by ΔS3 are stable and not impaired in translocation or maintenance of the post-translocation state but catalyze nucleotide addition at a reduced rate.
(Fig. 5). It has been suggested that Switch 3 binding helps remove the nascent RNA from the template strand and so facilitates repairing of the two DNA strands at the upstream edge of the transcription bubble (28). If so, the loss of this function does not appear to be a significant factor in determining the ΔS3 phenotype. The ΔS3 RNAP exhibited reduced elongation ability in the presence and absence of a nontemplate DNA strand (supplemental Fig. S2). Switch 3 binding to the nascent RNA also limits the length and movement of the DNA-RNA hybrid within the RNAP. The loss of these constraints may well result in partial or temporary misalignments of the participants at the catalytic site that would reduce the rate of polymerization.

The results obtained with ΔS3 argue that Switch 3 does not function identically in archaeal and bacterial RNAPs. Initiation by the bacterial RNAP lacking Switch 3 was not investigated, but this enzyme was incapable of forming stable elongation complexes (28), whereas ΔS3 formed very stable elongation complexes. The Switch 3-transcript interactions that must stabilize a bacterial elongation complex therefore do not occur or are not necessary for archaeal elongation complex stability. This difference in Switch 3 contribution to elongation complex stability might correlate with the different sequence requirements for bacterial versus archaeal intrinsic transcription termination. With this in mind, it seems noteworthy that although transcript elongation by ΔS3 was reduced at all template positions, the presence of sequences known to function as archaeal intrinsic terminators exacerbated this defect (Fig. 2 and supplemental Fig. S1). Whatever archaeal RNAP interactions with these sequences promote termination, presumably by hindering elongation, are apparently retained by ΔS3.

As illustrated (Fig. 1), a Gly-Gly dipeptide was substituted for the Switch 3 loop in ΔS3. This replacement was based on high resolution RNAP crystal structures (1–9) and was designed to bridge the ends of the Switch 3 deletion with minimal distortion. Given that ΔS3 is fully proficient in initiation and only partially defective in elongation, it seems that the Gly-Gly substitution did not cause a radical structural change. However, it remains possible that it does cause a long range effect on the structure at the catalytic site that reduces polymerization ability. This would explain the ~30% reduction in abortive transcript synthesis by ΔS3 (Fig. 2 and supplemental Fig. S1) transcripts that are not long enough to make direct contact with Switch 3 in the main channel of an RNAP.

With the plasmids used here to investigate RpoB, we have to date constructed plasmids that direct the synthesis of Histagged versions of 11 of the 12 subunits of the T. kodakaraensis RNAP. Using Ni$^{2+}$-affinity purification from cell lysates, we have confirmed that the plasmid-encoded subunits are assembled into RNAP holoenzymes in vivo, and so the roles of each archaeal RNAP subunit in transcription can now be directly investigated. As exemplified by the ΔS3 experiments, each plasmid-expressed subunit-encoding gene can be mutated, and a battery of in vitro assays is available to evaluate the Ni$^{2+}$-affinity-purified variant archaeal RNAP. It also is possible to transform T. kodakaraensis and replace the wild-type chromosomal copy of a gene with a mutated allele (32, 42). Using the reporter assays available, the consequences of any nonlethal change in an archaeal RNAP subunit on transcription initiation, termination, and spontaneous mutation therefore also can be assayed in vivo in T. kodakaraensis (14, 16, 32, 42).

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