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Factor-dependent archaeal transcription termination

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RNA polymerase activity is regulated by nascent RNA sequences, DNA template sequences, and conserved transcription factors. Transcription factors promoting initiation and elongation have been characterized in each domain, but transcription termination factors have been identified only in bacteria and eukarya. Here we describe euryarchaeal termination activity (Eta), the first archaeal termination factor capable of disrupting the transcription elongation complex (TEC), detail the rate of and requirements for Eta-mediated transcription termination, and describe a role for Eta in transcription termination in vivo. Eta-mediated transcription termination is energy-dependent, requires upstream DNA sequences, and disrupts TECs to release the nascent RNA to solution. Deletion of TK0566 (encoding Eta) is possible, but results in slow growth and renders cells sensitive to DNA damaging agents. Our results suggest that the mechanisms used by termination factors in archaea, eukarya, and bacteria to disrupt the TEC may be conserved, and that Eta stimulates release of stalled or arrested TECs.

RNA polymerase | factor-dependent transcription termination | Archaea | Eta | transcription

Each stage of transcription offers regulatory potential, and increasing evidence supports the idea that postinitiation transcription regulation may dominate in many instances (1–5). Although processive, transcription elongation is not uniform, and regulated pausing through interactions with DNA or nascent RNA sequences or through the action of conserved global and gene-specific regulators influences the elongation of RNA polymerase (RNAP) (5–11). Archaeal transcription is reliant on initiation factors with eukaryotic homology, but their access to promoter sequences is often limited or facilitated by bacterial-like transcription factors. In contrast, archaeal transcription elongation is seemingly regulated by universal or archaea-eukaryotic specific homologous factors (2, 12–18).

The ultimate control of transcription elongation is provided by factors and sequences that can disrupt the normally extremely stable transcription elongation complex (TEC) to terminate transcription and release the nascent transcript and RNAP from the DNA template (3, 19–27). The archaeal RNAP is sensitive to intrinsic transcription termination. DNA sequences encoding poly-U–rich sequences are sufficient to disrupt the archaeal TEC both in vivo and in vitro, and although sequence context can influence intrinsic transcription termination efficiency, there is no requirement for RNA structure for intrinsic termination (22, 26, 28). Bioinformatic analyses of archaeal genomes reveals that many genes are organized into operons. and that approximately one-half of genes and operons have sequences near their 3′ ends that are consistent with intrinsic termination signals (26, 29–31). The genome of *Thermococcus kodakarensis* is >92% coding, and the average intergenic space (after accounting for genes in operons) is only ∼50 bp (32). In the absence of an intrinsic termination sequence, the stability of the TEC would be predicted to easily permit continued elongation from one gene to the next and thereby remove the normal regulation imposed on expression of downstream sequences.

To date, protein factors that can disrupt the TEC have been characterized only for bacteria and eukarya. Insufficient intrinsic termination sequences for each gene and operon, polar repression of gene expression in the absence of coupled transcription and translation (i.e., polarity), and the recent description of transcription-coupled DNA repair in euryarchaea argue strongly that factors capable of disrupting TECs are encoded in archaeal genomes (32, 33). Bioinformatic analyses of archaeal genomes have identified some genes with limited homology to eukaryotic factors involved in RNA 3′-end formation (e.g., cleavage and polyadenylation specificity factor subunits), but to date, no biochemical activities have been described from archaeal cells that can disrupt the archaeal TEC. Importantly, these analyses have not identified any obvious homologs of the well-characterized bacterial termination factors rho (34) or Mfd (35).

We used a robust in vitro transcription system dependent on purified RNAP and basal initiation factors from the model archaea *T. kodakarensis* to purify the first archaeal-encoded activity that can disrupt the TEC (22, 36). Our assay is dependent on the disruption of stalled archaeal TECs that are normally extremely stable and remain intact even when challenged with the strong replicative minichromosome maintenance (MCM) helicase (37). The factor so purified, in native or recombinant form, requires access to upstream DNA and ATP hydrolysis to disrupt TECs. The encoding gene, TK0566, is universally conserved in known euryarchaea and thus was named euryarchaeal termination activity (Eta) (38, 39).

Eta was annotated as a DEAD-box RNA helicase (38, 40), but our analyses demonstrate that Eta does not require access to the nascent transcript to disrupt the archaeal TEC. Eta-mediated termination is not competitive with standard elongation rates, arguing that Eta targets stalled or arrested TECs. Although conserved, deletion of TK0566 (encoding Eta) is possible. Deletion of Eta does not influence polarity, suggestive of at least one additional termination factor in *T. kodakarensis*; however, deletion of Eta does render cells sensitive to DNA-damaging agents. The nucleic acid requirements, slow rate of termination, and sensitivity of strains lacking Eta to

Significance

Proper transcription regulation is necessary for timely and accurate gene expression underlying growth and development. Transcription is regulated at each stage of the transcription cycle—initiation, elongation, and termination—and it is critical to define the factors and sequences regulating RNA polymerase activity. Many studies have investigated the mechanisms used by transcription factors involved in regulation of transcription initiation and elongation, but a mechanistic understanding of transcription termination has been slower to emerge. Here we characterize the first archaeal transcription termination factor, termed euryarchaeal termination activity (Eta). The mechanisms of Eta-mediated termination provide the first understanding of archaeal factor-dependent termination and provide insight into and contrast with the mechanisms used for factor-dependent termination in extant life.

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mutagens suggest that Eta may function analogously to the bacterial transcription-repair coupling factor Mfd. The combined in vitro and in vivo characterization of Eta demonstrates that factor-dependent transcription termination is used in all extant life and reveals similarities in TEC stability and susceptibility to factor-dependent termination.

Results

Identification of Eta. DNA templates attached to a solid support permit the generation of stable TECs at defined template positions by nucleotide deprivation (36, 41) (Fig. 1; experimental details in *SI Materials and Methods*). Stalled TECs are resistant to repeated washing, and we made use of the ability of washed TECs to resume elongation to assess TEC stability. Stalled TECs were incubated with partially purified *T. kodakarensis* lysates to provide any termination factors present with an opportunity to act on the stalled TECs. Reactions were then supplemented with NTPs to determine whether the TECs could resume transcription and generate a full-length transcript of 450 nt (Fig. 1*B*). Fractionated lysates were defined as "active" if the TECs incubated with these fractions were unable to resume elongation upon NTP addition (Fig. 1*C*). Known termination factors are energy-dependent; thus, a constraint on ATP dependence of presumptive termination activity was added to define active fractions.

Several active fractions were chromatographically identified from *T. kodakarensis* lysates, implying the presence of multiple termination factors or the differential association of a single termination factor in separate complexes. The complexity of one active fraction was refined by repeated chromatographic separations until only a few proteins were present. The dominant proteins present in this purified fraction were identified by mass spectrometry (MS) (Table S1). Of the four abundant proteins, the ~96-kDa product of TK0566 was deemed likely responsible for the ATP-dependent transcription termination activity.

Eta Is an Energy-Dependent Transcription Termination Factor. The failure of TECs to resume elongation on NTP addition (Fig. 1*C*) is suggestive of, but not definitive evidence of, transcription termination activity. To eliminate concerns of any unidentified factor being responsible for disrupting TECs, the protein product of TK0566 (832 aa; termed Eta) was recombinantly expressed and purified (Fig. S1). To demonstrate that Eta is a bona fide

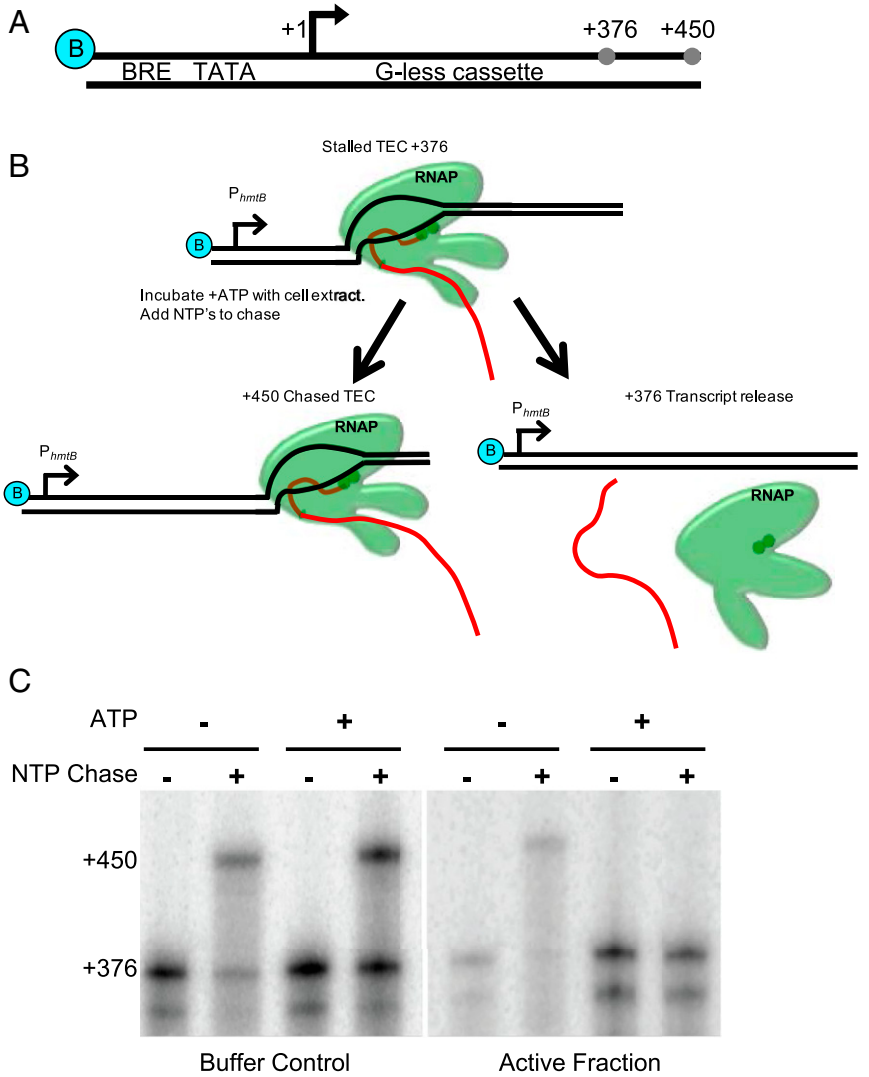

Fig. 1. Identification of an archaeal termination factor. (*A*) DNA templates contain a biotin moiety (blue B), a strong promoter, P_{hmtB}, a 376bp G-less cassette, and permit elongation to produce a full-length transcript of +450. (*B*) Stalled TECs at the end of a G-less cassette (TEC_{+376}) were incubated with cell lysate to identify termination factors. (*C*) Active fractions were identified as those that did not produce +450 transcripts when supplemented with lysate and ATP.

termination factor, we added Eta to in vitro transcription assays containing stalled TEC_{+58}. TECs were stalled on a solid support that permits washing and separation of pellet and supernatant fractions to monitor dissociation of the TEC. Eta was capable of disrupting stalled TECs to release transcripts to solution (Fig. 2*A*). In the absence of Eta, or in the presence of Eta but in the absence of an energy source, just 4–8% of TEC_{+58} dissociate and release transcripts to solution (Fig. 2*A*). In contrast, the addition of Eta resulted in ATP-dependent dissociation of nearly one-half of all TECs. Both ATP and dATP support Eta activity, and dATP is used to avoid supplying RNAP with ATP.

Eta-Mediated Termination Is Dependent on ATP Hydrolysis. Eta is a superfamily II helicase (39), and conserved Walker A and B motifs are readily identified in a central P-loop NTPase domain (Fig. S1*A*). The Walker B consensus sequence hhhhDE contains an aspartate that coordinates Mg^{2+} and a glutamate that is essential for NTP hydrolysis (42, 43). The N terminus of Eta (residues 1–193) is less conserved and appears to contain a Zn-finger motif.

When ATP was replaced with ADP or the nonhydrolyzable analog AMP-PNP, Eta could not stimulate RNA release (Fig. 2*B*). An Eta variant, in which two Walker B residues were replaced by alanine (D344A + E345A), could not stimulate transcription termination above background levels (Fig. 2*B*). Near-homogenous preparations of Eta and $Eta^{D344A+E345A}$ were possible, but laborious. Deletion of the N terminus (amino acids 1–193) resulted in a protein that chromatographed more uniformly and retained full termination activity in vitro (Fig. S1*C*).

Eta-Mediated Termination Is Not Competitive with Elongation. The addition of Eta to stalled TECs results in a slow and near-linear dissociation rate (Fig. 3 *A* and *B*). Transcription elongation and known mechanisms of factor-dependent transcription termination are in competition. Eta was added to stalled or slowly elongating TECs to monitor the ability of Eta to release transcripts from active TECs (Fig. 3*C*). When TECs were stalled without NTP, most remained intact but exhibited shortened transcripts, presumably by endonucleolytic cleavage and/or reverse catalysis (Fig. 3*C*, lanes 3 and 4). TEC_{+58} could be maintained by supplementing buffers with low concentrations of ATP, GTP, and

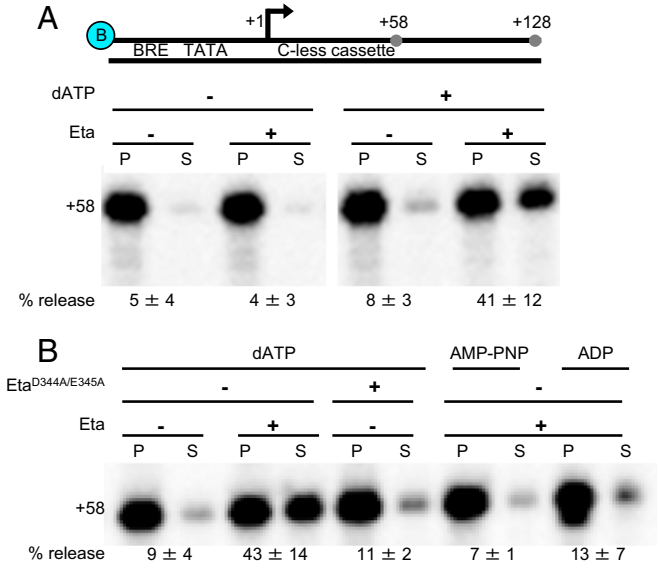

Fig. 2. Eta is an energy-dependent transcription termination factor. (*A*) DNA templates contain a biotin moiety, P_{hmtB}, a 58 bp C-less cassette, and permit elongation to produce full-length +128 transcripts. (*B*) Eta requires ATP or dATP hydrolysis to mediate transcription termination.

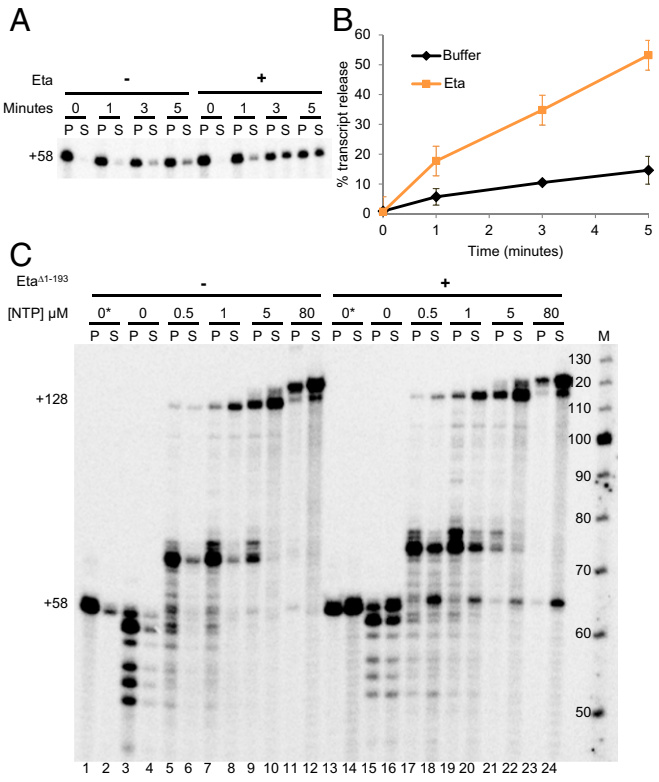

Fig. 3. Eta mediates termination of stalled or slowly elongating TECs. (*A*) Eta mediates the slow release of transcripts from stalled TEC_{+58}. (*B*) Quantification from *A*. (*C*) Eta-mediated termination limits backtracking and is only competitive with transcription elongation at low NTP concentrations. M, labeled ssDNA maker to provide an approximation of RNA lengths.

UTP (Fig. 3*C*, lanes 1 and 2). The addition of all NTPs released TEC_{+58} to generate full-length +128-nt transcripts. Elongation was limited by sequences that direct pausing near ~+70 nt at low NTP concentrations, but almost all TECs generated +128-nt transcripts without spontaneously dissociating. In contrast, the addition of Eta to stalled or slowly elongating TECs resulted in substantial transcript release to solution (Fig. 3*C*, lanes 13–20). Eta released most stalled TECs to solution (Fig. 3*C*, lanes 13 and 14), and Eta limited the percentage of TECs with shortened transcripts (Fig. 3*C*, lanes 15 and 16), suggesting that Eta keeps TECs in the forward configuration and/or pushes forward backtracked complexes. Eta disrupted TECs at the NTP-dependent pause at ~+70 (Fig. 3*C*, lanes 17–22), but as the average duration of the pause was shortened by increasing NTP concentrations, Eta directed less termination. At NTP concentrations well below physiological concentrations (i.e., just 5 or 80 μM), Eta-mediated termination was noncompetitive with elongation, with the exception of a small percentage of TEC_{+58} that likely failed to elongate quickly (Fig. 3*C*, lanes 21–24).

Eta Interacts with RNAP in Vivo. A strain of *T. kodakarensis* (Table S2) in which sequences encoding affinity and epitope tags were appended to the N terminus of TK0566 permitted purification of Eta via single-step chromatography under gentle conditions (44). The identity of copurifying partners was revealed by MS (Table S3), and several subunits of RNAP were identified, supporting Eta–RNAP interactions in vivo. Several large helicases, an ATPase, and NusA also were identified as Eta partners, suggesting that Eta may participate in activities besides termination.

Eta-Mediated Termination Requires Access to Upstream DNA Sequences. Despite the ability to identify RNAP in purifications of Eta from *T. kodakarensis* lysates, Eta does not retain long-lived associations with RNAP in solution (Fig. S2). Eta likely targets TECs through association with upstream or downstream DNA sequences or through the nascent transcript. To address the requirements for upstream DNA sequences, stalled TEC_{+58} were generated on templates that could be cleaved with Ssp1 to remove some or most upstream sequences (Fig. 4*A*). TECs were stable during the Ssp1 digestions, and monitoring radiolabeled DNA before and after cleavage demonstrated digestion of each template and release of the TEC_{+58} to solution (Fig. 4*B*). Digestion of templates containing the +37 Ssp1 site was less efficient at releasing TECs to solution compared with digestion at the +18 site in the presence of TEC_{+58} (Fig. 4*B*, lanes 7, 8, 15, and 16). The decrease in digestion efficiency is likely due to RNAP obstructing access of Ssp1 to this RNAP-proximal site.

Radiolabeling of the nascent transcripts in Ssp1-released TECs allowed the ability of Eta to disrupt the TECs to be inferred by the ability of the TECs to elongate on the addition of NTP. Templates permitting Ssp1 digestion at +18 resulted in the release of TECs with at least a full turn of accessible upstream DNA and, based on the inability of the majority of released TECs to resume elongation on NTP addition, Eta could disrupt these complexes (Fig. 4*C*, lanes 4 and 5). In contrast, templates permitting digestion at +37 resulted in the release of TECs with essentially all upstream sequences removed and generated TECs that were largely resistant to Eta activity and produced full-length transcripts on NTP addition (Fig. 4*C*, lanes 9 and 10). No such requirement for downstream DNA or nascent RNA sequences was found for Eta-mediated termination of TECs (Fig. S3).

Eta Is Nonessential, but Deletion Impacts Growth of *T. kodakarensis*. The entirety of the TK0566 coding sequence was markerlessly deleted from the *T. kodakarensis* genome (45, 46) (Fig. S4 and Table S2). Deletion or inactivation of Eta through changes to TK0566, such that a D344A + E345A variant Eta was encoded, slowed and limited growth (Fig. 5*A*). Western blot analysis using anti-Eta antibodies confirmed the loss of Eta in ΔTK0566 strains (Fig. S4*C*). Quantitative Western blot analysis suggested that Eta is normally present at low levels (~50 copies per cell) compared with RNAP levels (~2,000–3,000 copies per cell) (Fig. S4*D*).

Eta Is Not Responsible for Polarity. To determine whether Eta is the factor responsible for polarity in *T. kodakarensis*, we compared repression of a previously characterized polarity-influenced operon (32) in strains with and without Eta (Fig. 5*B* and Table S2). Activity from the downstream reporter gene in both strains was substantially reduced by the introduction of nonsense codons into the coding region of the upstream gene, but this reduction was not changed by the absence of Eta.

Deletion of Eta Increases Cellular Sensitivity to DNA-Damaging Agents. The requirements for Eta-mediated transcription termination—energy dependence, a static or slowly elongating TEC, access to upstream DNA sequences, and no requirement for downstream DNA or RNA sequences—are reminiscent of the only other prokaryotic factor capable of disrupting the TEC, namely Mfd (47–49). Mfd mediates RNAP removal and initiates transcription-coupled DNA repair (TCR) in bacteria, and cells deleted for *mfd* exhibit a mild phenotype to some DNA-damaging agents (50, 51). Although TCR has not yet been demonstrated in *T. kodakarensis*, the archaeal RNAP does arrest at template strand DNA lesions (52),

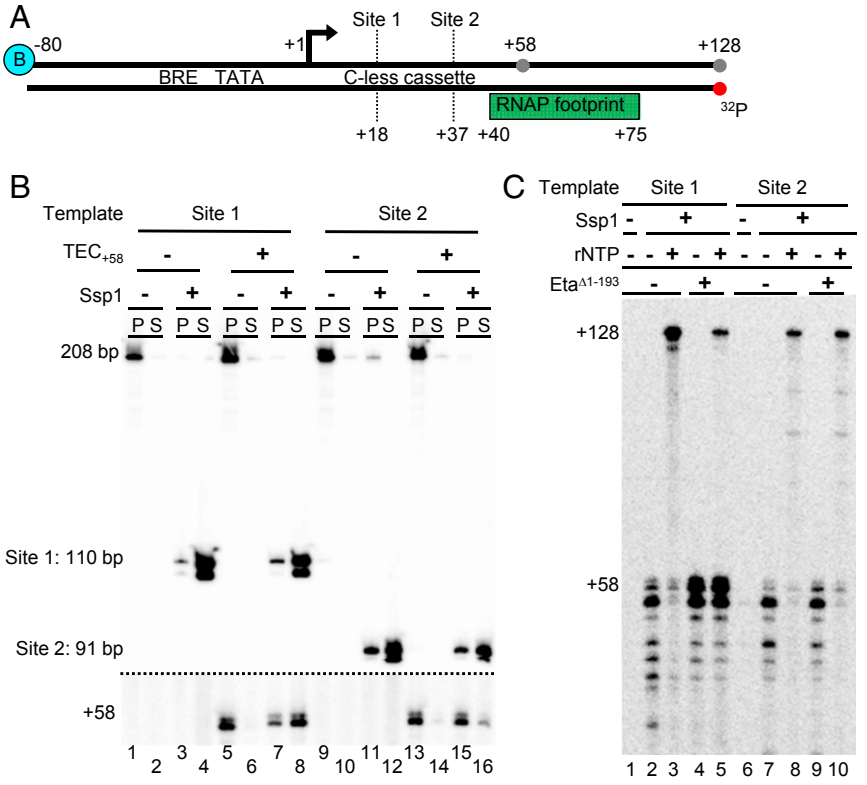

Fig. 4. Eta-mediated termination requires upstream DNA sequences. (*A*) DNA templates are identical except for the Ssp1-recognition sequences at positions +18 (site 1) and +37 (site 2) respectively. (*B*) Ssp1 digestion releases stalled TEC_{+58} to solution. DNA templates were radiolabeled at the 5′ position of the template strand (red dot). Radiolabeled DNA (above dashed line) and radiolabeled RNA (below dashed line) were visualized on a single gel, shown at two different contrast levels. (*C*) Upstream DNA is required for Eta-mediated transcription termination in vitro.

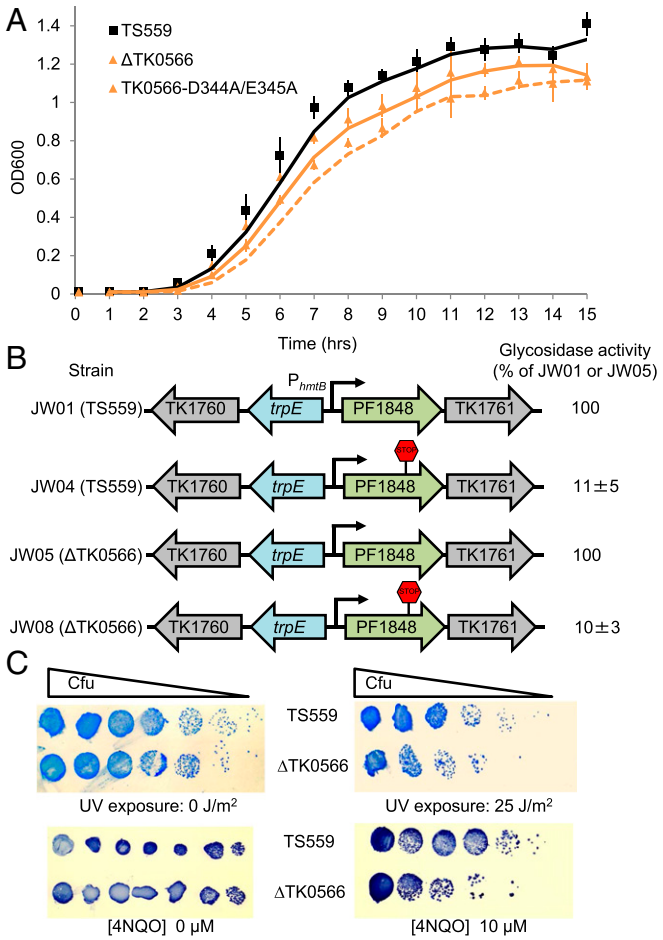

Fig. 5. Eta is nonessential, is not responsible for polarity in vivo, but loss of Eta limits growth and increases DNA damage susceptibility. (*A*) Deletion or inactivation of Eta hinders growth rate and final cell densities. The curves and errors shown represent means and standard averages of triplicate technical repeats of triplicate biological samples. (*B*) Eta is not responsible for polarity in vivo. The presence or absence of the nonsense codon in PF1848 is noted, and the percentage of β-glycosidase activity is reported as the mean and standard average of triplicate technical repeats of triplicate biological samples. (*C*) Deletion of Eta increases sensitivity to DNA damaging agents.

and recent support for TCR in related euryarchaea has been reported (33). We accessed the potential for Eta to influence DNA repair by challenging parental and Eta-deleted strains of *T. kodakarensis* to common DNA-damaging agents. Eta-deleted strains are at least an order of magnitude more sensitive to UV exposure than the parental strain (Fig. 5*C*, *Top*), and introduction of the heterocyclic mutagen 4-nitroquinoline 1-oxide (4NQO) (50) limits the growth of Eta-deleted strains more severely (Fig. 5*C*, *Bottom*).

Discussion

Processive transcription requires extremely stable TECs (53). Biochemical and structural studies demonstrate that the overall stability of the TECs is composite, with inputs from RNAP–DNA, DNA–RNA, and RNAP–RNA interactions collectively stabilizing the TECs (10). DNA sequences, the encoded RNA sequences, and structures that form within or adjacent to RNAP can disrupt these contacts and destabilize the TECs, driving transcription termination (54). Intrinsic termination sequences often suffice to separate independent genes and operons by blocking continued downstream transcription, but not all termination events can be initiated via DNA sequences alone. Scenarios arise in all life where the stable TECs may halt transcription at any position, most likely in response to protein roadblocks or DNA damage, and these arrested TECs must be removed to maintain genome stability (55). In bacteria and archaea, transcription and translation are normally coupled (56, 57), and the uncoupling of these apparatuses offers regulatory potential that is exploited by factors (e.g., rho in bacteria), that disrupt the TECs and thus limit downstream expression.

We have demonstrated that factor-dependent transcription termination occurs in archaea and have characterized a bona fide archaeal transcription termination factor in vivo and in vitro. We purified this biochemical activity directly from cell lysates and then demonstrated that a single protein, Eta, drives TEC disassembly and release of RNA to solution. Our results confirm that factor-dependent transcription termination is conserved in all life, and that the factors capable of disrupting the TECs are all energy-dependent (4, 47, 58–60).

Factor-mediated disruption of the TECs in bacteria and eukarya is discriminatory to ensure that functional TECs generally are not terminated prematurely. Eta mediates transcript release from stalled or slow elongating TECs, but does so slowly. Eta-mediated termination is not competitive with standard RNAP elongation rates, and thus functional archaeal TECs are unlikely to be targeted for disruption. The rate of Eta-mediated termination is not supportive of Eta directing the 3′-end formation of many genes or operons, or of Eta governing polarity; direct assays of polarity have confirmed this.

What then is the biological role of Eta? Eta likely targets RNAPs that are translocationally blocked at sites of DNA damage or are arrested owing to chromatin or other protein roadblocks. In support of such a role, deletion of TK0566 (encoding Eta) or introduction of mutations that encode inactive variants of Eta is possible, resulting in strains with slow growth and DNA-damage–sensitive phenotypes. Our results suggest that Eta follows a similar mechanism of termination as Mfd (Fig. 6) (47, 48). Eta likely binds to the DNA upstream of stalled TECs and, through ATP hydrolysis, translocates along the DNA, pushing RNAP forward. In the absence of continued synthesis, RNAP is hypertranslocated and/or the

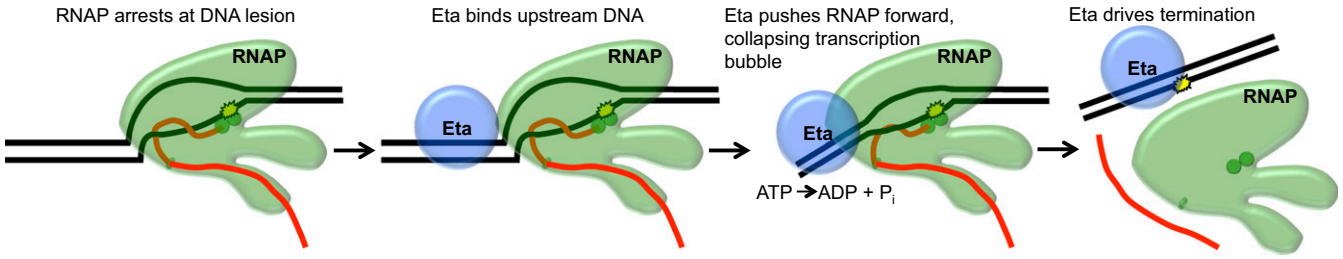

Fig. 6. Model of Eta-mediated transcription termination. Eta recognizes RNAP arrested at the site of a DNA lesion (yellow). Eta binds to the upstream DNA and uses ATP hydrolysis to push RNAP forward, causing transcription bubble collapse and promoting transcription termination.

transcription bubble collapses, resulting in disassociation of the TEC and transcription termination. In addition, the increased sensitivity to DNA damage of strains lacking Eta is suggestive of a potential role for Eta in the recognition and removal of TECs stalled at DNA lesions. The presence of TCR in *T. kodakarensis* and any role for Eta in archaeal transcription-coupled DNA repair remains to be determined.

The conservation of Eta in most archaeal lineages (38, 39) argues that factor-mediated termination is commonplace in archaeal regulatory strategies. Continued probing of the mechanism of Eta-mediated transcription termination should provide insight into shared aspects of TEC stability and highlight susceptibilities of the TECs that can be exploited for regulatory control.

Materials and Methods

Strains, Plasmids, and Oligonucleotides. Strains and plasmids are listed in Table S2, and oligonucleotides are listed in Table S4. All *T. kodakarensis* strains were constructed as described previously (45).

Protein Purifications. RNAP, TBP, and TFB were purified as described previously (36). rEta, rEta$^{D344A/E345A}$, and rEta$^{\Delta 1-193}$ were purified from Rosetta2 (DE3) cells carrying pQE-80L-Eta, pQE-80L-Eta$^{D344A/E345A}$, and pQE-80L-Eta$^{\Delta 1-193}$, respectively, as described in *SI Materials and Methods*.

In Vitro Transcription. Assembly of preinitiation complexes and elongation via NTP deprivation were generally carried out as described previously (36). TEC$_{+376}$ were captured with streptavidin-coated magnetic beads (SCMBs) and washed three times in wash buffer (WB) (20 mM Tris·HCl pH 8.0, 0.1 mM EDTA, 250 mM KCl, 4 mM $MgCl_2$, and 20 μg/mL BSA). TEC$_{+376}$ were resuspended in transcription buffer (TB) (250 mM KCl, 20 mM Tris·HCl pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT), then incubated at 85 °C ± 5 mM ATP in the presence or absence of partially purified lysates from *T. kodakarensis*. After 5 min, 200 μM rNTPs were added, and incubation at 85 °C was continued for 10 min. Then 5 volumes of 1.2× STOP buffer (0.6 M Tris·HCl pH 8.0 and 12 mM EDTA) containing 7 μg of tRNA (total) were added, the reaction was subjected to an equal volume phenol/chloroform/isoamyl alcohol (25:24:1, by vol) extraction, and radiolabeled RNA transcripts were precipitated from the aqueous phase with alcohol. Templates and RNA transcripts were separated on denaturing polyacrylamide gels, and radiolabeled RNA/DNA was detected using phosphorimaging (GE Healthcare) and analyzed using GE Imagequant 5.2.

TEC$_{+58}$ were captured with Ni^{2+}-coated magnetic beads (NCMBs), washed three times in WB, then resuspended in TB with 10 μM ATP, 10 μM GTP, and 10 μM UTP. Then 10-μL aliquots were combined with equal-volume reactions containing 15 mM Tris·HCl pH 8, 5 mM $MgCl_2$, and 2 mM DTT, ± 4 mM energy source (dATP, AMP-PNP, or ADP) and ± 500 nM purified Eta or Eta variant. Reactions were incubated at 85 °C for 5 min, and then SCMBs were used to separate reactions into pellet and supernatant fractions. The pellet and supernatant fractions were incubated with STOP buffer and extracted, and RNA transcripts were purified as above. Release was calculated by quantifying transcripts in the S divided by transcripts quantified in the pellet and supernatant.

Washed TECs$_{+58}$ were generated as above, then 10-μL aliquots were combined with equal-volume reactions containing 15 mM Tris·HCl pH 8, 5 mM $MgCl_2$, 2 mM DTT, and 4 mM dATP, ± 500 nM Eta. Reactions in lanes 1 and 2 and lanes 13 and 14 were supplemented with 10 μM each of UTP, GTP, and ATP (indicated by 0*) (Fig. 3*C*). The concentrations of NTPs that allowed elongation to +128 during 7 min of incubation at 85 °C are listed in the figure. SCMBs were used to separate reactions into pellet and supernatant fractions.

Washed TECs$_{+58}$ were generated on ^{32}P-labeled, biotinylated templates as above, then TEC$_{+58}$ were resuspended in digestion buffer (1× NEB Buffer 2.1 ± 8 U Ssp1-HF) and incubated at 37 °C for 30 min. SCMBs were used to separate reactions into pellet and supernatant fractions, and the pellet fraction was discarded. TEC$_{+58}$ retained in the supernatant were captured with NCMBs and washed twice in WB then resuspended in TB with 10 μM ATP, 10 μM GTP, and 10 μM UTP, and 4 mM dATP ± 500 nM Eta. Reactions were incubated at 85 °C for 7 min, followed by the addition of 200 μM rNTPs, and incubation was extended at 85 °C for 5 min. Then 5 volumes of 1.2× STOP buffer containing 7 μg tRNA (total) were added, the reactions were extracted, and RNA transcripts purified and analyzed as above.

Polarity Assay. Plasmids and strains (Table S2) generated for the polarity assay were constructed and β-glycosidase activity was measured as described previously, except that the parental strains were TS559 or ΔTK0566 (32). Percent activity was calculated by comparing activities of identical strains (TS559 or ΔTK0566) with and without stop codons in PF1848.

DNA Damage Assays. UV irradiation assays were carried out as described previously (61). For 4NQO sensitivity assays, *T. kodakarensis* strains TS559 (parental) and ΔTK0566 were grown to an $OD_{600\ nm}$ = 0.6 in ASW-YT-pyruvate media containing agmatine (45). ~1 × 10^8 cells were anaerobically harvested, collected via centrifugation, and resuspended in 100 μL of 0.8× ASW. Cells were serially diluted with 0.8× ASW and spotted (in duplicate) onto ASW-YT plates lacking or containing 10 μM 4NQO. Plates were incubated at 85 °C for 18 h, after which the cells were transferred to a PVDF membrane, followed by staining with Coomassie Brilliant Blue.

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