

Chemical roadblocking of DNA transcription for nascent RNA display

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Abstract

Site-specific arrest of RNA polymerases (RNAPs) is fundamental to several technologies that assess RNA structure and function. Current *in vitro* transcription “roadblocking” approaches inhibit transcription elongation by blocking RNAP with a protein bound to the DNA template. One limitation of protein-mediated transcription roadblocking is that it requires the inclusion of a protein factor that is extrinsic to the minimal *in vitro* transcription reaction. In this work, we developed a chemical approach for halting transcription by *Escherichia coli* RNAP. We first established a sequence-independent method for the site-specific incorporation of chemical lesions into double-stranded DNA templates by sequential PCR and translesion synthesis. We then show that interrupting the transcribed DNA strand with either an internal desthiobiotin-triethylene glycol modification or 1,N⁶-etheno-2'-deoxyadenosine base efficiently and stably halts *Escherichia coli* RNAP transcription. By encoding an intrinsic stall site within the template DNA, our chemical transcription roadblocking approach enables the display of nascent RNA molecules from RNAP in a minimal *in vitro* transcription reaction.

Introduction

The *in vitro* display of nascent RNA molecules from halted transcription elongation complexes (TECs) is a powerful tool with proven applications in nascent RNA folding (1-4) and systematic RNA aptamer characterization (5,6). Historically, transcription arrest at a defined DNA coordinate has been achieved by attaching a protein roadblock to a DNA template to halt the progression of RNA polymerases (RNAPs). Sequence-specific protein roadblocks, such as the catalytically inactive EcoRI_{E111Q} mutant (7) and the *Escherichia coli* (*E. coli*) DNA replication terminator protein Tus (5), can be directed to a precise DNA location by encoding a binding sequence in the template DNA. Alternatively, the biotin-streptavidin complex is capable of halting some RNAPs if positioned either at the downstream DNA template terminus (8) or internally within the transcribed DNA strand (3). Recently, a reversible dCas9 roadblock was

developed to enable time-dependent control of transcription arrest sequentially at multiple DNA positions (9). While these approaches have proven effective in diverse applications, their utility is limited to experimental contexts that tolerate the inclusion of extrinsic protein factors in the *in vitro* transcription reaction.

In contrast to protein roadblocks, chemical DNA lesions have not typically been used for nascent RNA display experiments despite their well-established inhibitory effect on transcription elongation (10). The lack of chemical transcription roadblocking approaches can be attributed to two challenges: First, chemical lesions that efficiently stall RNAPs are also likely to stall DNA polymerases so that preparation of DNA templates for *in vitro* transcription yields a truncated dsDNA product during PCR amplification. Preparation of internally modified double-stranded DNA (dsDNA) has been achieved for short DNA molecules by annealing and ligating modified oligonucleotides (11) and for long DNA molecules by enzymatically generating single-strand DNA gaps that can be filled with modified oligonucleotides (12-14). However, these approaches are sequence-dependent and therefore not suitable for internally modifying the complex DNA sequence libraries that are frequently used in nascent RNA display experiments (4-6). Second, some RNAPs have been shown to bypass non-bulky DNA lesions such as abasic sites (15-19); consequently, RNAP stalling at some chemical lesions is time-dependent. In this work, we address both of the above challenges to develop a simple and versatile chemical approach for halting *E. coli* RNAP transcription elongation *in vitro*.

We have determined that an internal desthiobiotin-triethylene glycol (desthiobiotin-TEG) modification positioned in the transcribed strand of *in vitro* transcription DNA templates efficiently and stably halts *E. coli* RNAP. We first show that internally modified dsDNA can be prepared by sequential PCR amplification and translesion synthesis. We then show that desthiobiotin-TEG alone efficiently halts *E. coli* RNAP one nucleotide upstream of the modification site, that bypass of the desthiobiotin-TEG modification by RNAP is sufficiently slow to enable manipulation of stalled TECs, and that stalled TECs are stable for at least

two hours at ambient temperature. Lastly, we demonstrate that our translesion synthesis approach can be used to enzymatically prepare DNA templates containing an internal 1,N⁶-etheno-2'-deoxyadenosine (etheno-dA) nucleotide, which was recently shown to halt *E. coli* RNAP transcription (19). Our findings establish a method for chemically encoding a transcription stall site within a dsDNA molecule to enable efficient RNAP roadblocking in a minimal *in vitro* transcription reaction.

Results

Rationale for DNA modifier selection

We observed that an internal desthiobiotin-TEG modification by itself (Figure 1A) in the transcribed DNA strand causes *E. coli* RNAP to stall when attempting to develop a reversible desthiobiotin-streptavidin transcription roadblock. Although the desthiobiotin-streptavidin complex was capable of halting RNAP, RNAP also appeared to stall at the desthiobiotin-TEG modification site following streptavidin elution. Given that *E. coli* RNAP has been previously reported to bypass abasic sites in the transcribed DNA strand (15), we envisioned that the desthiobiotin-TEG modification might enable protein-free transcription roadblocking at a defined DNA template position. Notably, the desthiobiotin-TEG modification is distinct from abasic lesions both due to the presence of the desthiobiotin moiety and because it introduces unnatural spacing (two vs. three carbon) in the transcribed DNA strand (Figure S1). We therefore performed our initial assessment of desthiobiotin-TEG as a transcription roadblock in comparison to an internal amino-linker modification, which preserves the natural three carbon spacing of the DNA phosphodiester backbone and contains a less perturbative functional moiety (Figure 1B, Figure S1). In the sections below, we first describe our characterization of desthiobiotin-TEG as a chemical transcription roadblock. We then describe a similar characterization of the etheno-dA DNA modification separately in the final section of the Results.

Translesion synthesis enables preparation of internally modified dsDNA

To assess the transcription roadblocking properties of the desthiobiotin-TEG and internal amino-linker modifications, we prepared *in vitro* transcription DNA templates using synthetic oligonucleotide primers that contain an internal modification site 29 bp upstream of the template end (Figure 1C, Table S1). Preparation of internally modified DNA templates by PCR amplification yielded a dsDNA product with one truncated strand, suggesting that Vent Exo- DNA polymerase cannot efficiently bypass the modification site (Figure 1D, E). To complete the truncated DNA strand, we performed translesion synthesis using the thermostable Y-family lesion bypass polymerase *Sulfolobus islandicus* DNA polymerase IV (Dpo4) (Figure 1D). The related *Sulfolobus solfataricus* Dpo4 was shown to discriminate between correct and incorrect nucleotides with reported misincorporation frequencies between 8×10^{-3} and 3×10^{-4} (20) and Dpo4 from several *Sulfolobus* species has been used in combination with *Taq* DNA polymerase to PCR amplify damaged and ancient DNA (21). In our application, we separate PCR amplification and translesion synthesis into independent steps so that the DNA template promoter and transcribed region are synthesized first using a DNA polymerase with fidelity suitable for downstream applications. Translesion synthesis is then used to bypass the modification site and complete the truncated DNA strand. In our initial protocol, we purified the truncated dsDNA PCR product before performing translesion synthesis. We later determined that, because Vent Exo- and Dpo4 are active in the same buffer and nucleotide conditions, efficient translesion synthesis could be achieved by simply adding Dpo4 directly to the reaction after PCR cycling without an intermediate clean-up step. Both translesion synthesis protocols yielded internally modified dsDNA templates that were indistinguishable from unmodified templates by denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 1E, Figure S2A). DNA templates containing an internal desthiobiotin-TEG modification were also analyzed by non-denaturing PAGE and showed a slight mobility shift relative to unmodified DNA (Figure S2B). DNA templates in which translesion synthesis was performed using the thermostability-enhancing dNTPs 2-amino-

dATP and 5-propynyl-dCTP showed an identical mobility shift, suggesting that the shift is a consequence of the desthiobiotin-TEG modification and not instability in the DNA downstream of the modification site (Figure S2C).

Modification bypass exhibits distinct nucleotide incorporation preferences

Translesion DNA synthesis can involve the incorporation of a nucleotide opposite the traversed lesion (22). To further characterize our internally modified DNA templates, we determined the nucleotide incorporation preference of Dpo4-mediated lesion bypass for the internal desthiobiotin-TEG, amino-linker, and etheno-dA DNA modifications by primer extension (Figure 2A). On an unmodified DNA template, primer extension with all four dNTPs primarily yielded run-off products with one, and to a lesser extent, two more nucleotides than the expected full length template-encoded product (Figure 2B, C). This is consistent with observations that *Sulfolobus solfataricus* Dpo4 can extend a blunt DNA terminus by 1-2 nucleotides with a preference for dATP incorporation (23). The internal desthiobiotin-TEG modified template yielded a run-off product distribution comparable to that of the unmodified template, suggesting that desthiobiotin-TEG bypass frequently occurs without incorporation of a nucleotide opposite the modification site (Figure 2B, C, F). In contrast, the enrichment of full length+2 products from amino-linker and etheno-dA modified templates suggests that bypass of these lesions can involve incorporation of a nucleotide opposite the modification site (Figure 2B, C, F). Notably, the etheno-dA template essentially shifted the product distribution of the unmodified template by one additional nucleotide (Figure 2C). We confirmed these interpretations by primer extension in the presence of dideoxynucleoside triphosphates (ddNTPs) to determine how bypass of each modification distorts the observed sequencing ladder. In agreement with our analysis above, we observed that: (i) the desthiobiotin-TEG template yielded a sequencing ladder similar to that of the unmodified control, (ii) the amino-linker template yielded both the expected +0 sequencing ladder and a ladder with a +1 offset, and (iii) the etheno-dA

template yielded a mixed +0/+1 sequencing ladder with enrichment for +1 products (Figure 2D).

We further validated these observations by performing primer extension in the presence of one base at a time to determine the efficiency of lesion bypass using each dNTP. Under the conditions of our assay (incubation at 55°C for 5 minutes), starving Dpo4 of all but one dNTP appears to yield homopolymer primer extension products due to misincorporation (Figure 2E), however, the observation of a readily discernable sequencing ladder when all four dNTPs are present (Figure 2D) indicates that misincorporation to this degree is specific to nucleotide starvation conditions. Consistent with the observations above, we find that (i) desthiobiotin-TEG bypass is most efficient if dNTP incorporation is templated by the nucleotide immediately downstream of the modification site, (ii) amino-linker bypass is efficiently mediated both by templated dGTP incorporation and by dATP incorporation, and (iii) etheno-dA bypass is most efficient with dATP (Figure 2E).

The observation that Dpo4 efficiently bypasses the amino-linker and etheno-dA modifications in the presence of dATP is consistent with a previous finding that *Sulfolobus solfataricus* Dpo4 follows the ‘A rule’ (22) and typically incorporates dATP opposite an abasic site (20). One explanation for why desthiobiotin-TEG bypass preferentially occurs without incorporation of a nucleotide opposite the modification site is that its branched triethylene glycol scaffold interrupts the DNA backbone by only two carbons whereas the amino-linker and etheno-dA modifications preserve the natural three carbon spacing of DNA nucleotides (Figure S1). Importantly, the preparation of DNA templates containing these modifications was efficient despite the difference in Dpo4-mediated lesion bypass, suggesting that our translesion synthesis approach for enzymatic production of internally modified dsDNA will be generalizable to other modifications.

Desthiobiotin-TEG efficiently blocks *E. coli* RNAP transcription

We evaluated the transcription roadblocking properties of the internal desthiobiotin-TEG and

amino-linker modifications by single-round *in vitro* transcription with *E. coli* RNAP. Transcription was initiated in the absence of CTP to walk RNAP to U15, one nucleotide upstream of the first C in the transcript, before addition of NTPs to 500 μ M. Both internal modifications produced a transcription stall-site at C42, one nucleotide upstream of the modification position (Figure 1F,G). Importantly, an unmodified DNA template control showed no evidence of modification-independent transcription stalling at C42, indicating that the C42 stall site is entirely modification-dependent (Figure 3A). We next compared the desthiobiotin-TEG and amino-linker roadblocks to the ‘gold standard’ terminal biotin-streptavidin roadblock using a 32 minute time course in the presence of 500 μ M NTPs. To halt transcription at C42, the terminal biotin-streptavidin roadblock was placed 10 nucleotides downstream of C42 at the transcribed DNA strand 5’ end to account for collision of RNAP with streptavidin (3). After 32 minutes there was no indication that RNAP had bypassed the terminal biotin-streptavidin roadblock to produce run-off transcripts (Figure 3A). In contrast to the terminal biotin-streptavidin complex, retention of TECs at the desthiobiotin-TEG stall site was not absolute: after 32 minutes ~87% of TECs remained at the stall site (Figure 3A, B). Nonetheless, desthiobiotin-TEG outperformed the amino-linker modification, which retained only ~40% of TECs at the stall site after 32 minutes (Figure 3A, B). Lastly, we measured the rate at which RNAP bypasses the desthiobiotin-TEG modification site following promoter escape. In our standard reaction conditions for internal RNA labeling (200 μ M ATP, GTP, and CTP; 50 μ M UTP), we observed an initial decay of $t_{1/2}$ =592 minutes ($n=2$, $R^2 = 0.96$, 95% CI [541, 655]) (Figure 4). In our first replicate, which included time points to 256 minutes, desthiobiotin-TEG bypass slowed after the 64 minute time point. In our second replicate, which included time points to 64 minutes, modification bypass slowed after the 48 minute time point (Figure 4). While the origin of this effect is unclear, it suggests that stalled TECs may be heterogeneous. We conclude that virtually all TECs initially stall upon encountering the desthiobiotin-TEG modification and that the vast majority of TECs persist at the stall site well beyond the reaction time of typical nascent RNA display experiments.

The *E. coli* RNAP footprint blocks desthiobiotin-TEG from binding streptavidin

The primary caveat to using the desthiobiotin-TEG modification as a transcription stall site is that it is not functionally inert. Many applications of chemical transcription roadblocking will also depend on DNA template immobilization, which is typically achieved by attachment of a 5’-biotin modified DNA template to streptavidin-coated magnetic beads. It is therefore desirable that the DNA template contain only one attachment point. One solution to this limitation is to sequester the desthiobiotin-TEG modification within the RNAP footprint before DNA immobilization. To test this approach, we first positioned RNAP at the U15 walk site such that the desthiobiotin-TEG modification is exposed (Figure 5A). After incubation with streptavidin-coated magnetic beads, ~89% of U15 complexes partitioned into the bead pellet (Figure 5B). In contrast, when RNAP was positioned at the desthiobiotin-TEG stall site, only ~4% of stall site complexes were recovered in the bead pellet fraction (Figure 5A, B). Because the ability of desthiobiotin-TEG to bind streptavidin can be controlled by RNAP position, stall site-proximal elongation complexes can be enriched by exclusion from streptavidin-coated magnetic beads. To determine how the *E. coli* RNAP footprint interferes with desthiobiotin-streptavidin binding at nucleotide resolution, we distributed TECs using 3’-deoxynucleoside triphosphate (3’-dNTP) chain terminators before incubating the arrested complexes with streptavidin-coated magnetic beads and separating pellet and supernatant fractions. In agreement with the established downstream border of *E. coli* RNAP on dsDNA (24), elongation complexes ≤ 15 nucleotides upstream of the desthiobiotin-TEG modification were predominantly excluded from the bead pellet, whereas complexes upstream of this boundary were retained (Figure 5C). Thus, the use of desthiobiotin-TEG as a chemical transcription roadblock additionally provides a straightforward means of enrichment for stall site-proximal TECs by exclusion from streptavidin-coated magnetic beads.

Desthiobiotin-halted elongation complexes are stably bound to DNA

The observation that the RNAP footprint sequesters desthiobiotin function enabled us to assess the stability of desthiobiotin-TEG-stalled TECs over long incubation times. We prepared a DNA template containing both an internal desthiobiotin-TEG modification to stall RNAP and a 5'-biotin-TEG modification upstream of the promoter for DNA immobilization by attachment to streptavidin-coated magnetic beads (Figure 6A). After TECs were positioned at the desthiobiotin-TEG stall site, the transcription reaction was incubated with streptavidin-coated magnetic beads for 10 minutes, washed twice with transcription buffer supplemented with 1 mM MgCl₂ to remove free NTPs, and resuspended in this same buffer. Separation of bead pellet and supernatant fractions indicated that ~98% of stalled TECs remained attached to the beads after two hours of incubation at room temperature (Figure 6B, C). Thus, elongation complexes that are stalled at a desthiobiotin-TEG modification site retain the exceptional stability that is expected for ternary elongation complexes.

Ethno-dA is a functionally inert chemical transcription roadblock

For some applications, it may be desirable to block transcription using a smaller and less disruptive DNA modification such as ethno-dA, which was recently shown to halt *E. coli* RNAP efficiently in the presence of 100 μM NTPs (19) (Figure 7A). To confirm the suitability of ethno-dA as a chemical transcription roadblock, we first asked whether dsDNA transcription templates could be prepared by PCR with an internally ethno-dA modified primer followed by translesion synthesis using *Sulfolobus* Dpo4. Ethno-dA bypass by *Sulfolobus* Dpo4 is efficient (Figures 7B and S3) and appears to be predominantly mediated by incorporation of a dA nucleotide opposite the modification site (Figure 2). Transcription roadblocking by ethno-dA was virtually identical to desthiobiotin-TEG: ~84% of TECs were retained at the ethno-dA stall site after 32 minutes in the presence of 500 μM NTPs (Figure 7C, D). Lastly, nearly all TECs that were arrested at the ethno-dA stall site remained stably associated with the DNA template (Figure

7E). We therefore conclude that ethno-dA is an ideal functionally inert chemical transcription roadblock due to both its compatibility with sequence-independent modified DNA template preparation and its ability to efficiently stall *E. coli* RNAP.

Discussion

Nascent RNA display is a powerful tool with proven applications in mapping RNA structure and folding (1-4) and systematic characterization of RNA function (5,6). Established approaches for halting RNAP have typically relied on protein roadblocks that are not easily navigable by transcribing polymerases. While these approaches have proven effective for placing RNAP at a defined DNA template position (7-9) and for systematically distributing RNAP across every DNA template position (3), each depends on including an extrinsic protein component in the transcription reaction. Although this requirement is compatible with many applications, the chemical transcription roadblocking approach described here provides a straightforward alternative that expands the repertoire of nascent RNA display tools.

The primary advantage of chemical transcription roadblocking is that no extrinsic factors are needed to efficiently halt RNAP. The cost of this advantage is that, in contrast to the 'gold standard' terminal biotin-streptavidin roadblock, transcription arrest at chemical lesions is time-dependent: in the presence of NTPs some TECs can eventually bypass the modification site (Figures 3, 4, and 7). Nonetheless, the slow rate at which RNAP bypasses the desthiobiotin-TEG and ethno-dA modifications allows ample time for either making biochemical measurements or depleting NTPs, after which the halted ternary elongation complex persists for hours (Figure 6). Furthermore, the persistence of most stalled TECs for at least 32 minutes in the presence of 500 μM NTPs suggests that our approach is generalizable to many experimental contexts. Nonetheless, use of a chemical transcription roadblock should begin with kinetic characterization of lesion bypass in the conditions of the intended application to confirm satisfactory performance.

Our chemical approach for transcription roadblocking is enabled by the facile preparation of internally modified DNA templates of arbitrary length using translesion synthesis (Figures 1, 2 and 7). Because this approach is sequence-independent, it is suitable both for the preparation of dsDNA templates with a defined sequence and for the preparation of complex sequence libraries. Importantly, the lesion bypass reaction is simple and efficient: in our reaction conditions translesion synthesis by *Sulfolobus* Dpo4 resulted in DNA template preparations that were indistinguishable from an unmodified template by denaturing gel electrophoresis and remained fully intact after several freeze-thaw cycles throughout data collection (Figures 1E, 7B, and S2A, D, E, S3). Furthermore, our approach is likely to be generalizable to many DNA modifications: in this work we successfully applied a general translesion synthesis protocol to three chemically distinct DNA modifications. Critically, the preparation and detailed characterization of the resulting DNA templates uses common biochemical techniques and reagents and will therefore be readily accessible to many laboratories.

Our study has established the desthiobiotin-TEG and etheno-dA DNA modifications as potent transcription blockades that are compatible with enzymatic dsDNA template preparation. These DNA modifications have complementary properties that are likely to be advantageous for specific applications. In experiments where enrichment of TECs that have transcribed to the stall site is crucial, the ability of RNAP to sequester desthiobiotin from binding streptavidin provides a means for purification of stall site-proximal TECs by exclusion from streptavidin-coated magnetic beads (Figure 5). In contrast, etheno-dA has no inherent conflicts with immobilization of 5'-biotinylated DNA templates and its small size may prove useful in applications that benefit from a minimally disruptive transcription stall site. It is likely that other readily available oligonucleotide modifications are compatible with chemical transcription roadblocking and may have functional properties that complement those of desthiobiotin-TEG and etheno-dA. We suggest that a DNA modification is suitable for chemical transcription roadblocking if it satisfies four criteria: 1) the modification should efficiently halt the target

RNAP; 2) the modification should be efficiently bypassed by a translesion DNA polymerase to facilitate DNA template preparation; 3) the modification should be chemically stable; and 4) the modification should not interfere with downstream applications. We envision that the chemical transcription roadblocking approach presented in this work will facilitate increasingly demanding nascent RNA display applications by enabling RNA synthesis in a minimal transcription reaction.

Experimental procedures

Oligonucleotides

All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and are described in Table S1. All modified oligonucleotides were HPLC-purified to ensure efficient modification. The oligonucleotide used as a template for PCR amplification was PAGE-purified to enrich for complete product.

Unmodified DNA template preparation

Linear DNA templates without internal modifications were prepared by PCR amplification essentially as described previously (3). Briefly, five 100 μ l reactions containing 81.5 μ l of nuclease-free water, 10 μ l Thermo Pol Buffer (New England Biolabs, Ipswich, MA), 2 μ l of 10 mM dNTPs (New England Biolabs), 2.5 μ l of 10 μ M oligonucleotide A (unmodified forward primer; Table S1), 2.5 μ l of 10 μ M oligonucleotide C (unmodified reverse primer; Table S1) or oligonucleotide G (5'-biotinylated reverse primer, Table S1), 1 μ l of Vent Exo- DNA polymerase (New England Biolabs), and 0.5 μ l of 0.1 nM oligonucleotide H (template oligonucleotide, Table S1) were amplified for 30 PCR cycles. Following amplification, 100 μ l reactions were combined into two 250 μ l pools and precipitated by adding 25 μ l of 3M sodium acetate (NaOAc) pH 5.5 and 750 μ l of cold 100% ethanol (EtOH), chilling at -80°C for 15 minutes, and centrifugation at 20,000 x g for 15 minutes. DNA pellets were washed with 70% EtOH (v/v), dried using a SpeedVac, dissolved in 30 μ l of

nuclease-free water, run on a 1% (wt/v) agarose gel, and extracted using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was determined by a Qubit 1.0 Fluorometer (Life Technologies, Carlsbad, CA). The fully assembled DNA template sequence is shown in Table S1.

Internally modified DNA template preparation

Internally modified linear DNA templates were PCR amplified as above except that oligonucleotides A or B (unmodified and 5'-biotinylated forward primer, respectively, Table S1) were used as a forward primer and oligonucleotides D, E, or F (internal desthiobiotin-TEG, internal amino-linker, and internal etheno-dA reverse primers, respectively, Table S1) were used as a reverse primer. Two protocols were used for translesion synthesis: In our initial protocol (used for experiments in Figures 1, 3, 4, 5, and 6), ten 100 μ l PCRs were individually purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol, eluted in 30 μ l of nuclease-free water each, and combined with 100 μ l of Thermo Pol Buffer, 20 μ l of 10 mM dNTPs, 10 μ l of *Sulfolobus* DNA polymerase IV (New England Biolabs) and nuclease-free water to 1 ml. The 1 ml master mix was split into 100 μ l aliquots and incubated at 55°C for 1 hour. We later determined that, because Vent Exo- and Dpo4 use the same buffer and dNTP conditions, DNA purification after PCR was unnecessary and efficient translesion synthesis could be achieved by pooling the initial 100 μ l PCRs, adding 1 μ l of *Sulfolobus* DNA polymerase IV per 100 μ l reaction volume, splitting the reaction master mix into 100 μ l aliquots, and incubating at 55°C for 1 hour; this protocol was used to prepare DNA templates for Figure 7 and Figure S2A. For both protocols, the 1 mL translesion synthesis master mix contained ~4.5 μ g of the truncated PCR product. Translesion synthesis with thermostability enhancing dNTPs was performed using our original protocol, but with a dNTP mixture in which dATP and dCTP were completely substituted with 2-amino-dATP and 5-propynyl-dCTP (TriLink Biotechnologies, San Diego, CA). Following translesion synthesis, template DNA was precipitated, gel extracted, and

quantified as described for unmodified DNA templates.

DNA template quality control

To verify success of the translesion synthesis reaction, all internally modified DNA templates were subjected to quality control by denaturing urea polyacrylamide gel electrophoresis (PAGE) using the UreaGel System (National Diagnostics, Atlanta, GA) (Figure 1E, Figure 7B, S2A, D, E, and S3). Each quality control gel included a reaction aliquot that had not undergone translesion synthesis as a negative control and an unmodified positive control. In some quality control gels we observe a smear beneath both the full length unmodified positive control and the modified template that has undergone translesion synthesis. We believe this smear is due to renaturation of a small fraction of the 121 bp dsDNA which results in faster migration than ssDNA due to the increased charge of the dsDNA duplex. This effect was typically resolved in technical replicates that were run at higher voltage but remained somewhat variable. For this reason, it is critical to always include an unmodified positive control when performing translesion synthesis DNA template quality control and to ensure a sufficiently high gel running temperature. Quality control was performed as follows: a 10% polyacrylamide urea-PAGE gel was pre-run at 300 V (Figure 1E, Figure 7B and Figure S2A, E) or 250 V (Figure S2D) for 30 minutes on a Mini-PROTEAN Tetra Cell (Bio-Rad, Hercules, CA) that was assembled so that 1X Tris/Borate/EDTA (TBE) buffer in the outer chamber covered only the bottom ~1 centimeter of the gel plates to help maintain a hotter running temperature. Immediately prior to loading, 1 μ l of 50 nM DNA template (50 fmol) was mixed with 15 μ l of formamide loading dye (1X transcription buffer (described below under *Single-round in vitro transcription*), 80% (v/v) formamide, 0.025% (wt/v) bromophenol blue and xylene cyanol) and boiled for 5 minutes before placing on ice for 2 minutes. All non-denaturing PAGE quality control gels were 8% polyacrylamide in 1X TBE buffer. Gels were stained with SYBR Gold (Life Technologies) and imaged using an Amersham Biosciences Typhoon 9400 Variable Mode Imager. Following collection of all data, a second denaturing PAGE quality control gel was performed to confirm that the internal desthiobiotin

modified DNA template remained fully intact (Figure S2D).

End-labeling and primer extension reactions

³²P end-labeled primer was prepared by incubating 10 pmol of oligonucleotide I (Table S1) in a 50 µl reaction containing 1X polynucleotide kinase (PNK) buffer (New England Biolabs), 20 units of PNK (New England Biolabs), and 100 µCi of [γ -³²P]ATP (Perkin-Elmer, Waltham, MA) at 37°C for one hour followed by a 20 minute incubation at 65°C to heat-inactivate PNK. Free [γ -³²P]ATP was removed by applying the end-labeling reaction to a Tris Buffer Bio-Spin P-30 Gel Column (Bio-Rad) according to the manufacturer's protocol; final sample volume was raised to 100 µl for an approximate final primer concentration of 100 nM. End-labeled oligonucleotide I (Table S1) primer was annealed to oligonucleotides C, D, E, F, J, or K (Table S1) at a ratio 1:1.5 (0.05 pmol of primer, 0.075 pmol template) in 1X Thermo Pol Buffer by heating at 95°C for 3 minutes, cooling to 55°C at a rate of 0.1°C/s, incubating at 55°C for 5 minutes, and cooling to 4°C at a rate of 0.1°C/s. All 10 µl primer extension reactions contained 5 nM end-labeled primer, 7.5 nM template oligonucleotide (pre-annealed), 1X Thermo Pol Buffer, and 0.02 U/µl *Sulfolobus* DNA polymerase IV. For dNTP starvation experiments, the specified dNTP mixture (Invitrogen, Carlsbad, CA) was included at a final concentration of 100 µM and reactions were performed by placing all samples on a thermal cycler block that was pre-cooled to 4°C, raising the temperature to 55°C for 5 minutes, and cooling the block to 4°C. For ddNTP sequencing ladder experiments, dNTPs were included at a final concentration of 100 µM, the specified ddNTP was included at either 500 µM or 1 mM, and reactions were performed by placing all samples on a thermal cycler block that was pre-cooled to 4°C, raising the temperature to 55°C for 30 minutes, and cooling the block to 4°C. After primer extension, 90 µl of stop solution (0.6 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 12 mM ethylenediaminetetraacetic acid (EDTA)) was added to each reaction. Reactions were then extracted by adding 100 µL of phenol/chloroform/isoamyl alcohol (25:24:1),

vortexing, centrifugation, and collection of the aqueous phase and then ethanol precipitated by adding 300 µL of 100% ethanol, 10 µL 3M NaOAc pH 5.5, 1.2 µl of GlycoBlue Coprecipitant (Thermo Fisher Scientific, Waltham, MA) and storing at -20°C overnight. After centrifugation and removal of bulk and residual ethanol, precipitated RNA was resuspended in formamide loading dye and fractionated by Urea-PAGE using either a 15% (dNTP starvation experiments) or 10% (sequencing ladder experiments) polyacrylamide sequencing gel prepared with the UreaGel System.

Quantification of radiolabeled primer extension reactions

Reactive nucleotides were detected by an Amersham Biosciences Typhoon 9400 Variable Mode Imager and quantified using ImageQuant (GE Life Sciences). In Figure 2E, 'Fraction Extended' is calculated by dividing the band intensity of primers that were extended by at least one nucleotide by the total of band intensity of extended and unextended primer.

Single-round *in vitro* transcription

Single-round *in vitro* transcription reactions were performed essentially as previously described (4,25). All single-round transcription reactions contained 5 nM DNA template and 0.016 U/µl *E. coli* RNA polymerase holoenzyme (New England Biolabs) in transcription buffer (20 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 50 mM potassium chloride (KCl)), and 0.2 mg/ml bovine serum albumin (BSA) in a total volume of 25 µl. All NTP mixtures were prepared using High Purity ATP, GTP, CTP, and UTP (GE Life Sciences, Chicago, IL).

Two protocol variations were performed: For experiments in which transcription was initiated by walking RNAP to +15 relative to the transcription start site (Figure 1F,G, Figure 3, and Figure 7C), TECs were stalled at U15 by incubating reactions containing 2.5 µM ATP and GTP, 1.5 µM UTP, 0.2 µCi/µl [α -³²P]UTP (Perkin-Elmer), and 10 mM MgCl₂ at 37°C for 10 minutes before adding NTPs to 500 µM and rifampicin (Gold Biotechnology, St. Louis, MO) to 10 µg/ml; 25 µl aliquots were

removed at the specified time points and added to 125 μ l of stop solution. RNA sequencing ladders were generated by walking RNAP to +15 before adding NTPs supplemented with a chain terminating 3'-dNTP (TriLink Biotechnologies) to 100 μ M; reactions proceeded at 37°C for 5 minutes before addition of 125 μ L of stop solution. For Figure 3, the biotin-streptavidin roadblock was prepared by adding streptavidin monomer (Promega, Madison, WI) to 100 nM during reaction master mix preparation.

For Figure 4, open promoter complexes were formed by incubating reactions containing 200 μ M ATP, GTP, CTP, 50 μ M UTP and 0.2 μ Ci/ μ l [α -³²P]UTP (Perkin-Elmer) at 37°C for 10 minutes and initiated by adding magnesium chloride (MgCl₂) to 10 mM and rifampicin to 10 μ g/ml; 25 μ l aliquots were removed at the specified time points and added to 125 μ l of stop solution. A mineral oil overlay was applied to the reaction master mix after taking the 4 minute time point to prevent evaporation over the 256 minute and 64 minute time courses.

All reactions were extracted by adding 150 μ L of phenol/chloroform/isoamyl alcohol (25:24:1), vortexing, centrifugation, and collection of the aqueous phase and then ethanol precipitated by adding 450 μ L of 100% ethanol, 1.2 μ l of GlycoBlue Coprecipitant (Thermo Fisher Scientific, Waltham, MA) and storing at -20C overnight. After centrifugation and removal of bulk and residual ethanol, precipitated RNA was resuspended in formamide loading dye and fractionated by Urea-PAGE using a 12% polyacrylamide sequencing gel prepared with the UreaGel System.

Equilibration of streptavidin-coated magnetic beads

All transcription reactions with magnetic separation were performed using 10 μ l of Dynabeads MyOne Streptavidin C1 beads (Invitrogen, Waltham, MA) per 25 μ l transcription volume. For each experiment, magnetic beads were prepared in bulk by removing storage buffer, incubating with 500 μ l hydrolysis buffer (100 mM sodium hydroxide, 50 mM sodium chloride (NaCl)) for 10 minutes at

room temperature with rotation, washing once with 1 ml of high salt wash buffer (50 mM Tris-HCl pH 7.5, 2 M NaCl, 0.5% Triton X-100), once with 1 ml of binding buffer (10 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Triton X-100), twice with 1 ml of 1X transcription buffer supplemented with Triton X-100 to 0.1%, and resuspended in 1X transcription buffer with 0.1% Triton X-100 to a volume equivalent to that of the total transcription master mix in the respective experiment for storage until use.

Desthiobiotin protection assay

For the desthiobiotin protection experiment in Figure 5B, transcription was initiated by walking RNAP to U15 as described above. To assess desthiobiotin-streptavidin binding when RNAP is at U15, one half of the reaction was mixed with rifampicin to 10 μ g/mL and immediately mixed with pre-equilibrated streptavidin-coated magnetic beads. To assess desthiobiotin-streptavidin binding when RNAP is at the desthiobiotin-TEG stall site, the second half of the reaction was mixed with NTPs to 500 μ M and rifampicin to 10 μ g/ml and incubated at 37°C for 5 minutes before mixing with pre-equilibrated streptavidin-coated magnetic beads. After 10 minutes of incubation with streptavidin-coated magnetic beads at room temperature, 25 μ l of the reaction was added to 125 μ l stop solution as a 'total' sample control and an additional 25 μ l was added to a new tube and placed on a magnetic stand for 1 minute to separate the bead pellet and supernatant. The supernatant was added to 125 μ l of stop solution and the pellet was resuspended in 25 μ l of 1X transcription buffer supplemented with 10 mM MgCl₂ before 125 μ l of stop solution was added. Samples were phenol/chloroform extracted, precipitated, and fractionated by Urea-PAGE as described above.

For the experiment in Figure 5C, transcription was initiated by walking RNAP to U15 as described above before adding NTPs to 100 μ M, 3'-dNTPs to 25 μ M, and rifampicin to 10 μ g/ml and incubating at 37°C for 5 minutes to distribute TECs across the DNA template; the 'total' reaction sample was added to 125 μ l of stop solution immediately after this incubation. Distributed TECs were then mixed with pre-equilibrated streptavidin-coated magnetic

beads. After 10 minutes of incubation at room temperature, 25 μ l of the reaction was added to a new tube and placed on a magnetic stand for 1 minute to separate the bead pellet and supernatant, which were then processed as described above.

Elongation complex stability assays

The elongation complex stability assay in Figure 6 was performed using a DNA template containing both an internal desthiobiotin-TEG modification and a 5'-biotin-TEG modification upstream of the promoter. Stalled TECs were prepared by first walking RNAP to U15 as described above and then chasing RNAP to the desthiobiotin-TEG stall site by adding NTPs to 500 μ M and rifampicin to 10 μ g/mL and incubating at 37°C for 5 minutes. The transcription reaction was then mixed with pre-equilibrated streptavidin-coated magnetic beads and incubated at room temperature for 10 minutes. Following DNA template immobilization, beads were washed twice with 1 ml of 1X transcription buffer supplemented with 1 mM MgCl₂ to remove free NTPs. After removing a 'zero' time point for fractionation, the transcription reaction was incubated at room temperature for 2 hours with rotation and time points were taken as indicated. At each time point, 25 μ l of the reaction was added to 125 μ l stop solution as a 'total' sample control and a second 25 μ l was added to a new tube and placed on a magnetic stand for 1 minute to separate the bead pellet and supernatant. The supernatant was added to 125 μ l of stop solution and the pellet was resuspended in 25 μ l of 1X transcription buffer supplemented with 1 mM MgCl₂ before 125 μ l of stop solution was added. Samples were phenol/chloroform extracted, precipitated, and fractionated by Urea-PAGE as described above.

For Figure 7E, a DNA template containing both an internal etheno-dA modification and a 5'-biotin-TEG modification upstream of the promoter was used. For each reaction volume, 0.125 pmol of DNA template was incubated with pre-equilibrated streptavidin-coated magnetic beads in 1X transcription buffer at room temperature for 10 minutes. After immobilizing the DNA template, the beads were washed once with 1X transcription buffer supplemented with 0.1% Triton-X100 and resuspended in the transcription master mix

described above for walking RNAP to U15. After walking RNAP to U15, TECs were chased to the desthiobiotin-TEG stall site by adding NTPs to 500 μ M and rifampicin to 10 μ g/mL and incubating at 37°C for 5 minutes. Reactions were then fractionated into bead pellet and supernatant and processed as described above for the desthiobiotin protection assay.

Quantification of radiolabeled *in vitro* transcription reactions

Reactive nucleotides were detected by an Amersham Biosciences Typhoon 9400 Variable Mode Imager and quantified using ImageQuant (GE Life Sciences). For experiments in which RNAP was walked to U15, transcripts were considered end-labeled due to the high probability of radiolabel incorporation during the initial walk (~4.25% per U nucleotide) and the low probability of radiolabel incorporation during the chase (~0.013% per U nucleotide) and no normalization was applied. For the experiments in Figure 4, band intensity was divided by transcript U content to normalize for the incorporation of [α -³²P]UTP. In Figure 3, the fraction of run-off, stall site, and U15 transcriptions was determined by dividing the band intensity of each transcript class by the total band intensity of all three classes. In Figure 4, fraction stalled was determined by dividing the normalized band intensity of desthiobiotin-TEG-stalled transcripts by sum of the normalized band-intensity of stalled and run-off transcripts; $t_{1/2}$ was determined by applying a one phase exponential decay fit to time points from 2 minutes (after new arrival of TECs at the stall site is negligible) to 48 minutes (before the semi-logarithmic plot deviates from a straight line) (26) using GraphPad Prism 8. In Figures 5, 6, and 7 the fraction of transcripts in the bead pellet was determined by dividing the band intensity of the indicated complex in the pellet fraction by the sum of the band intensities for the indicated complex in both the pellet and supernatant fractions.

Data Availability : All data are contained in the manuscript as either plotted values or representative gels. Source files in .gel format are available from the corresponding author (E.J.S.) on request.

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Author contributions

Conceptualization, E.J.S.; Methodology, E.J.S.; Investigation, E.J.S.; Formal Analysis, E.J.S.; Validation, E.J.S.; Writing – Original Draft, E.J.S.; Writing – Review & Editing, E.J.S., J.T.L., and J.B.L.; Funding Acquisition, E.J.S., J.T.L., and J.B.L.

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Conflict of interest : The authors declare no conflicts of interest in regard to this manuscript.

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Abbreviations : TEC, transcription elongation complex; RNAP, RNA polymerase; desthiobiotin-TEG, desthiobiotin-triethylene glycol; etheno-dA, 1,N⁶-etheno-2'-deoxyadenosine; ddNTP, dideoxynucleoside triphosphate; PNK, polynucleotide kinase.

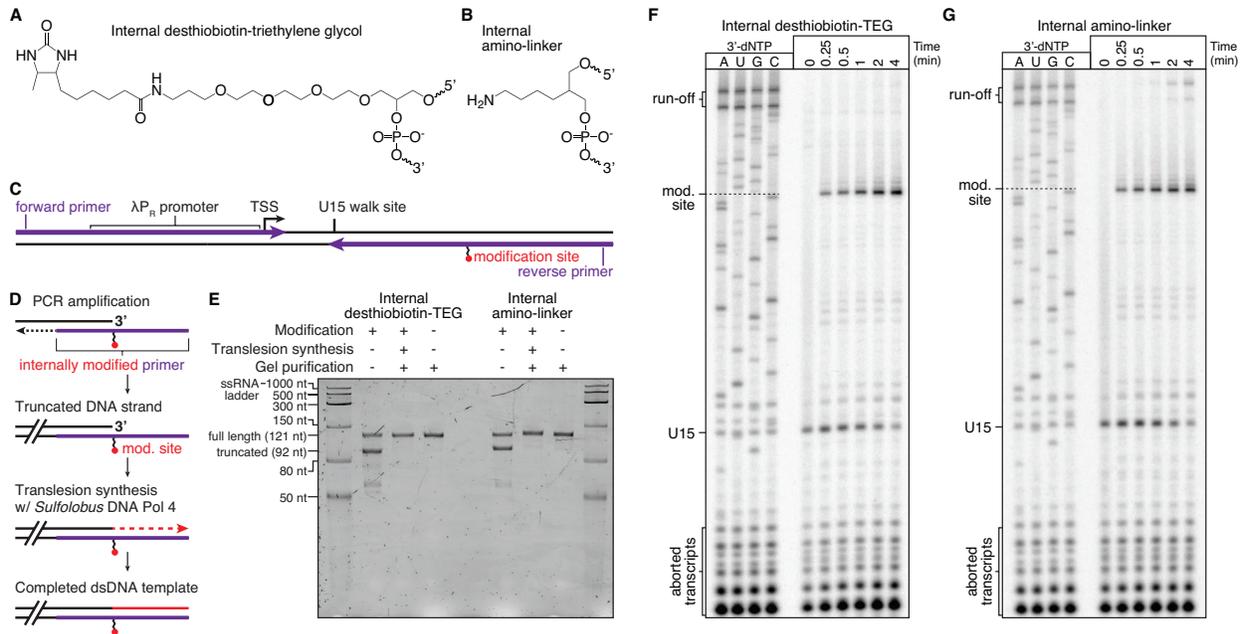


Figure 1. Preparation and characterization of chemical roadblocking DNA templates. **(A-B)** Chemical structures of internal desthiobiotin-TEG (A) and amino-linker (B) modifications. **(C)** Layout of internally modified DNA templates. The positions of the forward primer and the internally modified reverse primer are shown. The transcription start site (TSS) and U15 walk site are indicated. **(D)** Overview of internally modified DNA template preparation. The position of the internally modified reverse primer is shown in purple. **(E)** Denaturing PAGE quality analysis of internally modified DNA template preparations (Modification +) alongside an unmodified (Modification -) positive control. The size marker is the Low Range ssRNA Ladder (New England Biolabs). **(F-G)** Single-round *in vitro* transcription of internal desthiobiotin-TEG (F) and amino-linker (G) modified DNA templates. In both cases, RNAP stalls one nucleotide upstream of the modification (mod.) site. Panel E is representative of several DNA template quality control gels performed throughout this study. The experiments in panels F and G were performed once to precisely map critical bands for all subsequent experiments.

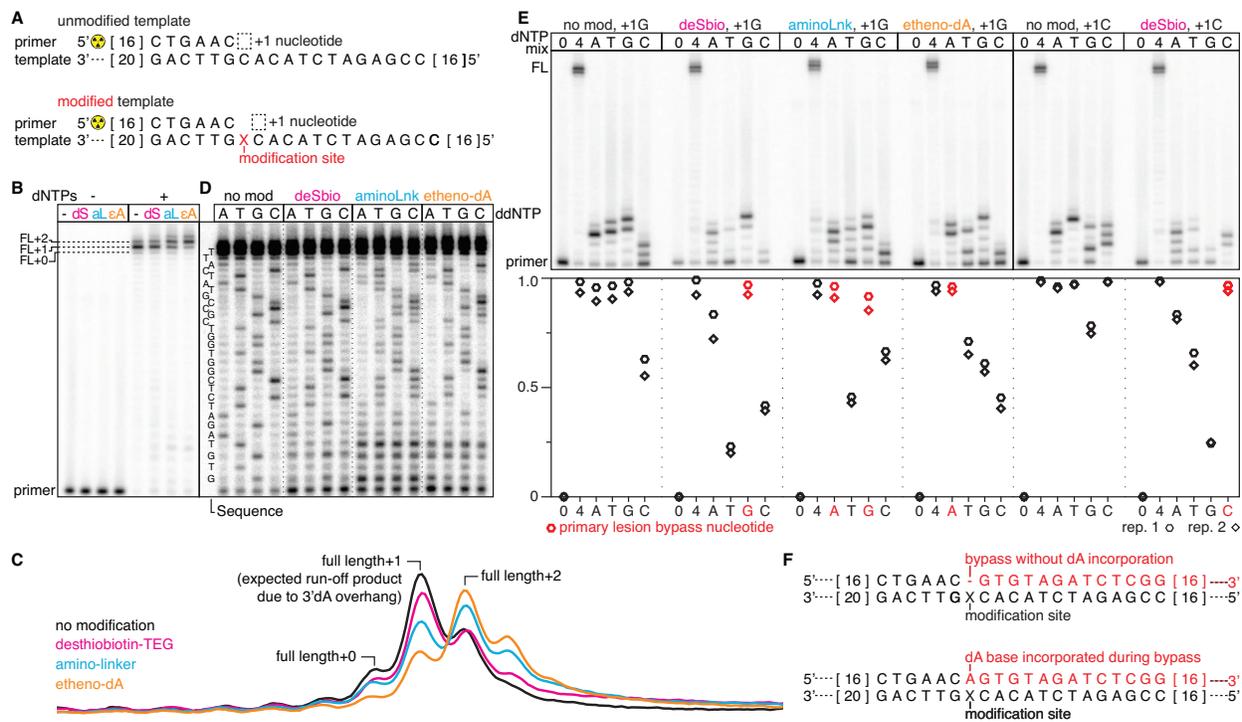


Figure 2. Characterization of *Sulfolobus* Dpo4-mediated DNA modification bypass. **(A)** Overview of the primer extension reaction on an unmodified and internally modified template. The length of DNA nucleotides that are not shown is indicated by the numbers in brackets at the ends of the sequence. **(B)** Characterization of full-length (FL) primer extension products for unmodified (-) and desthiobiotin-TEG (dS), amino-linker (aL), and etheno-dA (εA) modified templates. The primary run-off product is FL+1 due to the untemplated incorporation of a 3'dA overhang; some run-off products contain a second overhang nucleotide (23). The amino-linker and etheno-dA modifications increase the occurrence of FL+2, suggesting the incorporation of a dNTP opposite the modification site upon lesion bypass. **(C)** Raw intensity traces of the full length products from (B). **(D)** ddNTP sequencing ladders for unmodified and desthiobiotin-TEG (deSbio), amino-linker (aminoLnk), and etheno-dA modified templates. In agreement with (B), bypass of the desthiobiotin-TEG modification yields a sequencing ladder that resembles the unmodified control. In contrast, the amino-linker and etheno-dA templates yield a mixed sequencing ladder that suggests incorporation of an additional base during modification bypass. For each modified oligonucleotide, ddNTP truncation products within 4-5 nucleotides of the modification site were obscured by short products that were not fully extended. **(E)** Primer extension reactions for unmodified and modified DNA templates performed in the absence of dNTPs (0), with all dNTPs (4), or with only one dNTP (A, T, G, C). The unmodified control and desthiobiotin-TEG reactions were performed both with the sequences shown in (A) (+1G) and with a template in which the +1 nucleotide is a C (+1C). Quantification of primer extension is shown in the plot below; red points indicate individual dNTP conditions that enable high efficiency modification bypass. **(F)** Schematic of primer extension products resulting from bypass without dA incorporation (top) and bypass with dA incorporation opposite the modification site (bottom). Panels B, C, and D are representative of two independent replicates. The data in (B) are controls that were performed alongside the reactions in (D); the solid line between these two panels indicates a gel splice site used to facilitate clear visualization of the sequencing ladders in (D). Panel E is n=2.

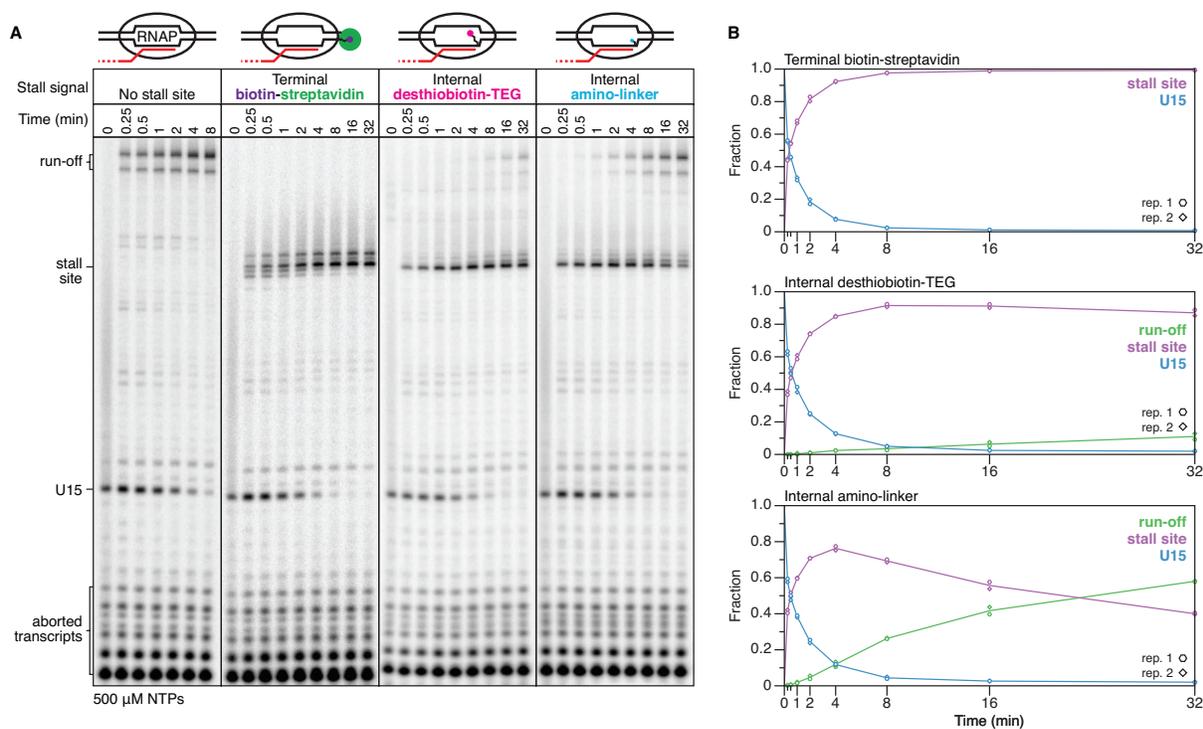


Figure 3. Comparison of biotin-streptavidin and chemical roadblock efficiency. **(A)** Single-round *in vitro* transcription of DNA templates without and with terminal biotin-streptavidin, internal desthiobiotin-TEG, or internal amino-linker stall sites in the presence of 500 μ M NTPs. Solid lines between gel images denote gel splices. **(B)** Quantification of gels shown in (A). All data are $n=2$ independent replicates; representative gels are shown. Desthiobiotin-TEG and amino-linker experiments were performed together, unmodified and biotin-streptavidin controls were performed separately.

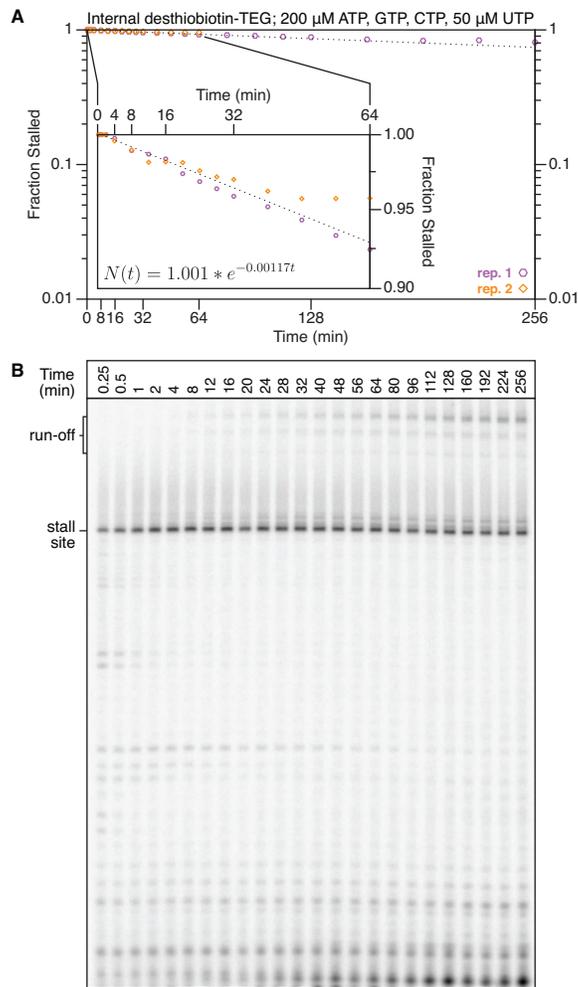


Figure 4. Time-dependent desthiobiotin-TEG bypass by *E. coli* RNA polymerase. **(A)** Plot of the fraction of TECs that reached but did not bypass the desthiobiotin-TEG stall site over 256 minute (replicate 1) and 64 minute (replicate 2) initiation-synchronized time courses in the presence of 200 μ M ATP, GTP, and CTP and 50 μ M UTP. The exponential decay curve was fit using time points from 2 to 48 minutes. The inset plot shows time points to 64 minutes with a truncated y-axis to facilitate clear data visualization and shows the decay equation. **(B)** Gel showing the 256 minute time course from (A). Time points from 0.25 minutes to 64 minutes are n=2; time points from 80 minutes to 256 minutes are n=1.

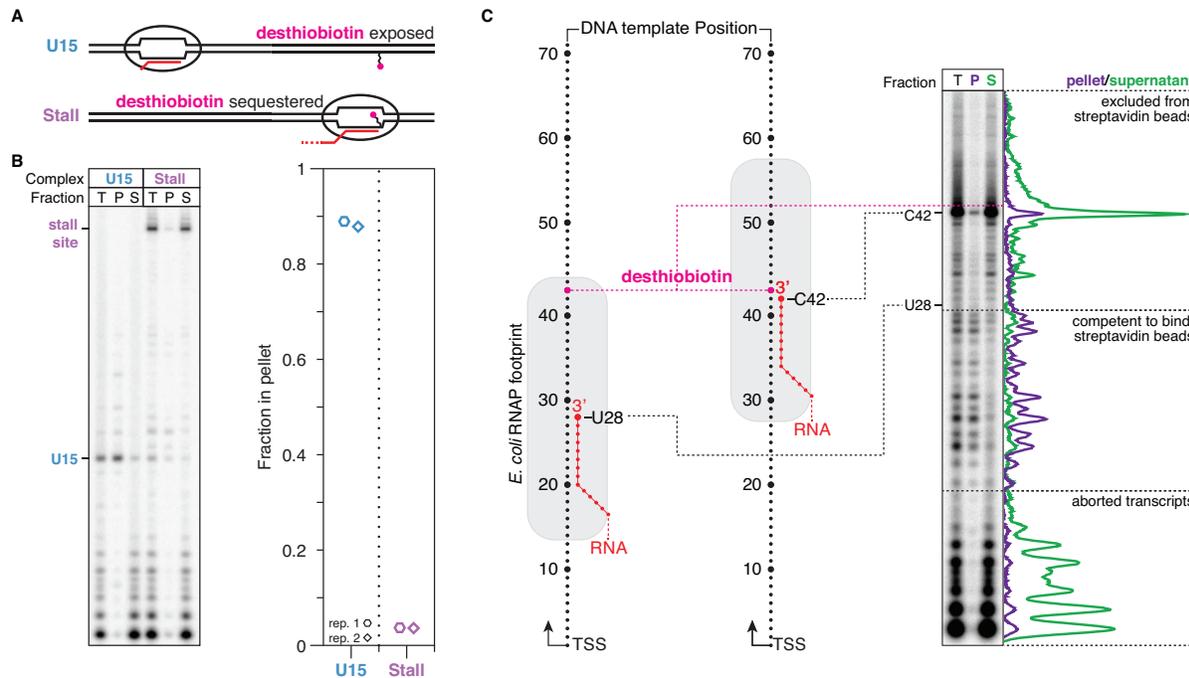


Figure 5. Characterization of desthiobiotin sequestration by *E. coli* RNAP. **(A)** Overview of the desthiobiotin sequestration experiment in **(B)**. If RNAP is positioned at U15, the desthiobiotin-TEG modification is predicted to be exposed and capable of binding streptavidin-coated magnetic beads. If RNAP is positioned at the stall site, the desthiobiotin-TEG modification is predicted to be sequestered and incapable of binding streptavidin-coated magnetic beads. **(B)** Fractionation of internal desthiobiotin-TEG modified DNA templates with streptavidin-coated magnetic beads in the presence of the TECs described in **(A)**. Total reaction (T), pellet (P), and supernatant (S) fractions are shown. The plot shows the fraction of TECs retained in the bead pellet. **(C)** Fractionation of internal desthiobiotin-TEG modified DNA templates with streptavidin-coated magnetic beads in the presence of intermediate TECs. Intermediate TECs were generated by transcription in the presence of chain terminating 3'-dNTPs. Comparison of P and S fractions indicates that the *E. coli* RNAP footprint blocks desthiobiotin from binding streptavidin when the nascent RNA 3' end is ≤ 15 nts upstream of the modification site. Background subtracted intensity traces are shown for the pellet and supernatant fractions alongside the gel. All data are from $n=2$ independent replicates.

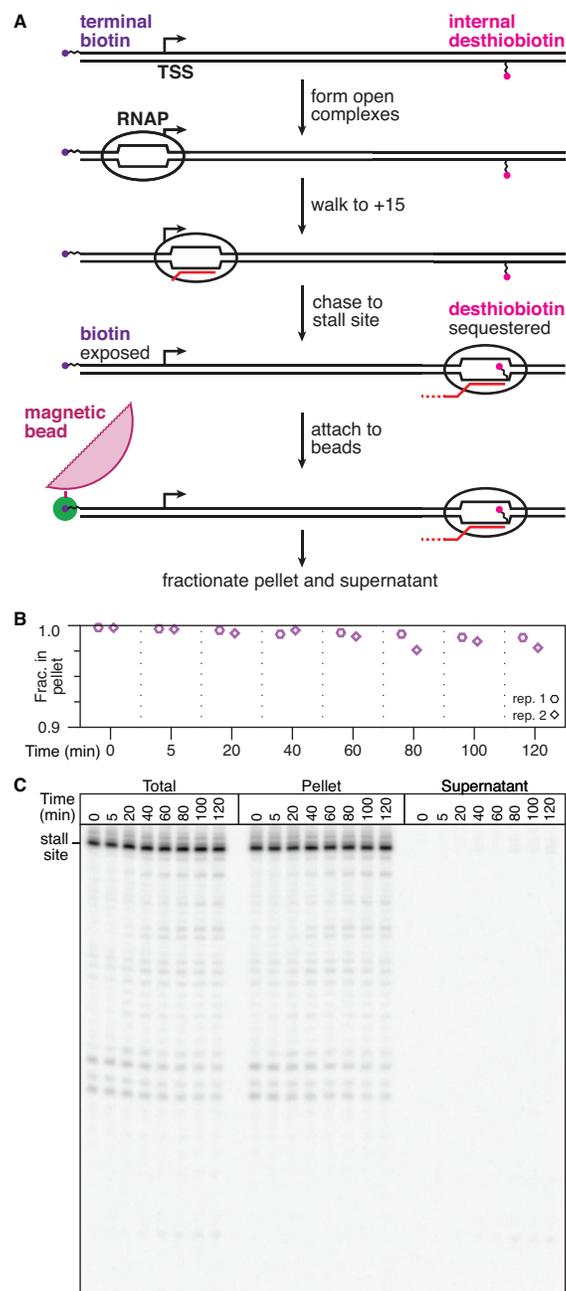


Figure 6. Quantification of elongation complex stability at a desthiobiotin-TEG stall site. **(A)** Overview of the experiment shown in **(B)** and **(C)**. Using a DNA template with an internal desthiobiotin-TEG stall site and a 5'-terminal biotin, RNAP is positioned at the stall site to sequester desthiobiotin, but the 5'-biotin remains exposed. These complexes are immobilized on streptavidin-coated magnetic beads, washed to remove free NTPs and incubated at room temperature before separation into bead pellet and supernatant fractions. **(B)** Quantification of the fraction of desthiobiotin-TEG-stalled TECs retained in the bead pellet over time. Note that the y-axis range is 0.9 to 1.0 to facilitate clear data visualization. **(C)** Gel showing replicate 1 from **(B)**. Pellet and supernatant data points in Panels **B** and **C** are from $n=2$ independent replicates; the total reaction control was performed once.

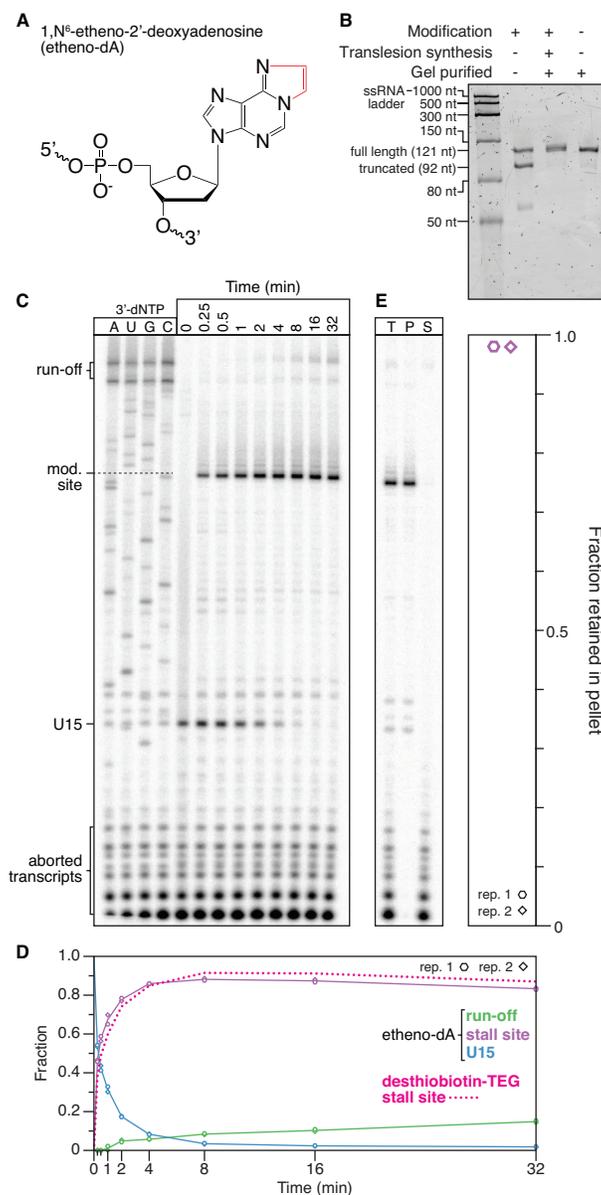


Figure 7. Transcription roadblocking by etheno-dA. **(A)** Chemical structure of etheno-dA. The etheno bridge that occludes the adenine Watson-Crick face is highlighted in red. **(B)** Denaturing PAGE quality analysis of an internal etheno-dA modified DNA template preparation (Modification +) alongside an unmodified (Modification -) positive control. The size marker is the Low Range ssRNA Ladder (New England Biolabs). **(C)** Single-round *in vitro* transcription of an internal etheno-dA modified DNA template in the presence of 500 μ M NTPs. RNAP stalls one nucleotide upstream of the modification (mod.) site. **(D)** Quantification of the gel shown in (C). The decay of TECs stalled at a desthiobiotin-TEG modification is shown for comparison and is from Figure 3. **(E)** Fractionation of TECs stalled at an etheno-dA modification site using streptavidin-coated magnetic beads. DNA templates were attached to streptavidin-coated magnetic beads by a 5'-biotin-TEG modification upstream of the promoter. Total (T), pellet (P), and supernatant (S) fractions are shown. The plot shows the fraction of TECs retained in the bead pellet. All data are from $n=2$ independent replicates. A comparison of DNA template preparation replicates is shown in Figure S3.

Chemical roadblocking of DNA transcription for nascent RNA display

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