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Strategies to Reduce Promoter-Independent Transcription of DNA Nanostructures and Strand Displacement Complexes

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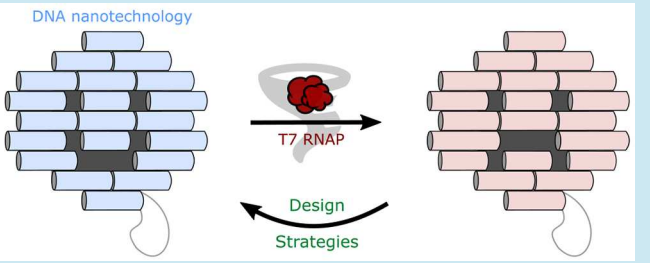
ABSTRACT: Bacteriophage RNA polymerases, in particular T7 RNA polymerase (RNAP), are well-characterized and popular enzymes for many RNA applications in biotechnology both *in vitro* and in cellular settings. These monomeric polymerases are relatively inexpensive and have high transcription rates and processivity to quickly produce large quantities of RNA. T7 RNAP also has high promoter-specificity on double-stranded DNA (dsDNA) such that it only initiates transcription downstream of its 17-base promoter site on dsDNA templates. However, there are many promoter-independent T7 RNAP transcription reactions involving transcription initiation in regions of single-stranded DNA (ssDNA) that have been reported and characterized. These promoter-independent transcription reactions are important to consider when using T7 RNAP transcriptional systems for DNA nanotechnology and DNA computing applications, in which ssDNA domains often stabilize, organize, and functionalize DNA nanostructures and facilitate strand displacement reactions. Here we review the existing literature on promoter-independent transcription by bacteriophage RNA polymerases with a specific focus on T7 RNAP, and provide examples of how promoter-independent reactions can disrupt the functionality of DNA strand displacement circuit components and alter the stability and functionality of DNA-based materials. We then highlight design strategies for DNA nanotechnology applications that can mitigate the effects of promoter-independent T7 RNAP transcription. The design strategies we present should have an immediate impact by increasing the rate of success of using T7 RNAP for applications in DNA nanotechnology and DNA computing.



KEYWORDS: *DNA nanotechnology, promoter-independent transcription, RNA nanotechnology, nucleic acid circuits, T7 RNA polymerase*

INTRODUCTION

Bacteriophage RNA polymerases, in particular T7 RNA polymerase (RNAP), are popular enzymes for RNA production in biotechnology *in vitro*, in cell lysates, and inside cells.[1−7] Bacteriophage polymerases are monomeric and only require magnesium (Mg^{2+}) for efficient transcription, making them relatively inexpensive to produce and easy to use. Bacteriophage polymerases also have high transcription rates and processivity, enabling quick production of large quantities of RNA.[3,8,9] T7 RNAP, perhaps the most used and characterized polymerase of any type, catalyzes RNA transcription from a double-stranded DNA (dsDNA) complex that contains its 17-base promoter site. The dsDNA transcription complex is composed of a nontemplate (also known as a coding, or sense, strand) and a template strand. The polymerase initiates transcription downstream of the promoter site and synthesizes an RNA copy of the nontemplate strand in the 5′ to 3′ direction by reading the template strand of the transcription complex in the 3′ to 5′ direction[8] (Figure 1A).

T7 RNAP also has high promoter specificity; on dsDNA templates, it initiates transcription downstream of its promoter site.[8,9] However, there are several promoter-independent T7 RNAP transcription reactions in which the polymerase initiates transcription or extends transcripts using single-stranded DNA (ssDNA) (Figure 1B−D). While in many applications, such promoter-independent transcription reactions can be avoided by using purely dsDNA transcription templates, there is growing interest in coupling T7 RNAP transcription to other processes in which ssDNA domains are required, including DNA computing[10−13] and structural DNA nanotechnology.[14−19]

In these applications, the specificity of nucleic acid base pairing, the well-characterized thermodynamics of DNA hybridization, and the sequence-invariant structure of the DNA double helix are used to program networks of reacting DNA species that execute computational operations or to form DNA structures with precise nanoscale features. In DNA computing, DNA strand displacement reaction networks rely on ssDNA toeholds that facilitate strand displacement

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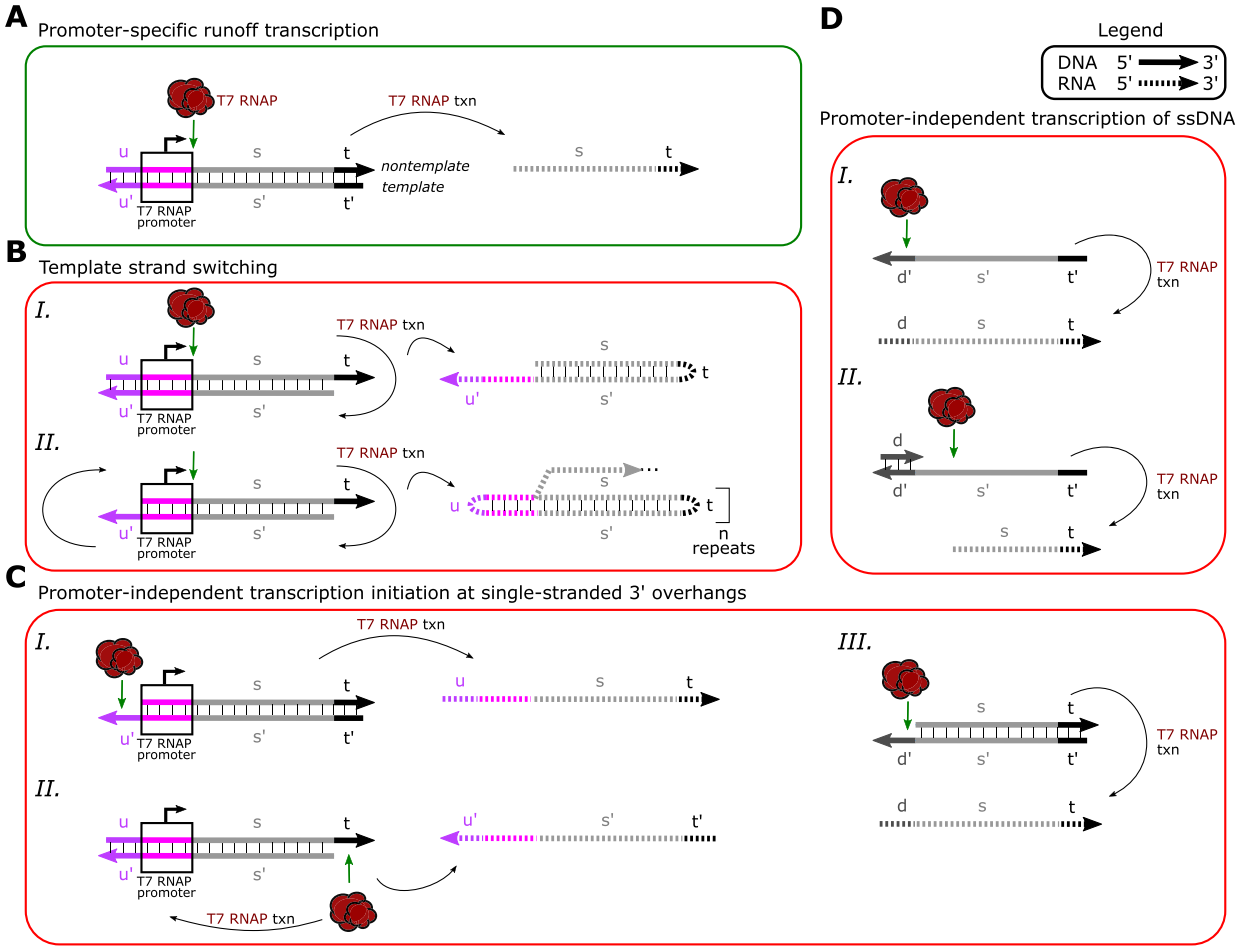

Figure 1. Overview of T7 RNAP promoter-specific and promoter-independent transcription. (A) Promoter-specific transcription of a dsDNA complex by T7 RNAP. The boxed pink domain represents the T7 RNAP promoter site. Transcription begins downstream of the promoter site and produces an RNA copy of the sequence of the nontemplate strand (also known as the coding or sense strand) of the dsDNA complex that is downstream of the promoter.[8] (B) Template strand switching.[39,40] (*I.*) T7 RNAP initiates transcription at its promoter site and then switches to copying the nontemplate strand upon encountering a single-stranded 3′ overhang (domain t). (*II.*) Template strand switching can occur multiple times if there are single-stranded 3′ overhangs on both strands of the dsDNA transcription complex (domains t and u′). (C) Promoter-independent transcription of dsDNA complexes initiated at single-stranded 3′ overhangs.[39,40] Transcription can initiate on either (*I.*) the template strand (domain u′) or (*II.*) the nontemplate strand (domain t) of dsDNA transcription complexes depending on where the single-strand 3′ overhang is located. (*III.*) dsDNA complexes that lack the T7 RNAP promoter site can also be transcribed if a single-stranded 3′ overhang is present (domain d). (D) Promoter-independent transcription of ssDNA.[41,42] Transcription can initiate anywhere along a region of ssDNA both (*I.*) with and (*II.*) without a free 3′ end. Green arrows indicate where transcription is initiated for each depicted reaction. Letters indicate unique sequence domains to illustrate the mapping from DNA to RNA sequences and apostrophes denote sequence complementarity.

reactions[20] and in DNA nanotechnology ssDNA domains are often used to stabilize, organize, or functionalize structures.[21−23] Integrating transcription with these DNA circuits or structures can expand their functionality. For example, T7 RNAP transcription has been used to engineer out-of-equilibrium chemical reaction networks for DNA computing applications[10−12,24−29] and to amplify chemical signals that can drive downstream assembly, and alter the function, of DNA nanostructures.[14,30−32] Transcription-based sensors that use T7 RNAP have also been integrated with DNA logic circuits to make smart biosensors.[13] DNA nanostructures have been used to control and measure the transcriptional activity of T7 RNAP,[17,18,33,34] and gene-encoding DNA origami are being developed as therapeutics.[35,36] Given the growing interest in using T7 RNAP transcription in reactions involving, or environments with, DNA circuit elements and nanostructures, it is important to understand design strategies for coupling these two technologies; in particular, how to avoid unintended promoter-independent transcription reactions induced by ssDNA domains.

Here we review the existing literature on promoter-independent transcription reactions by bacteriophage RNA polymerases (primarily T7 RNAP) that occur on DNA substrates. Although there are many known promoter-independent transcription reactions whereby T7 RNAP uses RNA as a substrate,[37,38] we restrict the scope of this review to DNA-based promoter-independent transcription reactions due to their implications for DNA nanotechnology. We highlight specific case studies, both previously published and unpublished, that demonstrate how promoter-independent transcription of DNA can disrupt DNA computing reactions (presented in the second section below) or alter the function of DNA nanostructures (presented in the third section below). Further, we describe design strategies that can mitigate the

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effects of promoter-independent transcription on DNA computing processes and on DNA nanostructure function with the goal of accelerating the development of dynamic DNA circuits and materials that use T7 RNAP transcription to create new functions.

RESULTS

T7 RNA Polymerase Promoter-Independent Transcription of DNA Templates. We first review different mechanisms by which T7 RNAP can initiate promoter-independent DNA transcription. One of the initially identified promoter-independent T7 RNAP transcription reactions involves transcription from dsDNA transcription complexes that have been cut from plasmids by restriction enzymes. Unusually long transcripts were produced from dsDNA transcription complexes prepared with restriction enzymes that left behind single-stranded 3′ overhangs while the transcripts produced from complexes with single-stranded 5′ overhangs were of the desired length.[39] These observations led to the discovery of a mechanism by which T7 RNAP, upon encountering a single-stranded 3′ overhang on the nontemplate strand of a DNA complex, could "flip around" and begin copying the nontemplate strand instead of dissociating after reaching the 5′ end of a template strand[40] (Figure 1B, *I.*). Further, if a single-stranded 3′ overhang was also present on the template strand of the DNA complex, the polymerase could repeat this template strand switching process multiple times, producing high molecular weight RNA products[39,40] (Figure 1B, *II.*). Template strand switching is exclusive to single-stranded 3′ overhangs because T7 RNAP reads a template strand in the 3′ to 5′ direction, so once the polymerase reaches the end of a transcription complex with a 3′ overhang on the nontemplate strand it can insert the free 3′ end into its active site and flip around to copy the nontemplate strand in the 3′ to 5′ direction.[40]

During template strand switching as described above, T7 RNAP initiates transcription at its promoter within the dsDNA complex. However, it was also found that single-stranded 3′ overhangs could serve as sites where T7 RNAP could initiate transcription in a promoter-independent manner.[39,40] Interestingly, this behavior was observed for dsDNA complexes that also contain the T7 RNAP promoter site (Figure 1C, *I.* and *II.*), indicating that promoter-independent transcription can be prevalent even when promoter-specific transcription can also occur.[39,40] Importantly, promoter-independent initiation can occur on the 3′ single-stranded overhang of duplexes of DNA that do not contain a T7 RNAP promoter (Figure 1C, *III.*). Because the polymerase reads a template strand in the 3′ to 5′ direction, promoter-independent initiation of transcription at a single-stranded 3′ overhang allows the polymerase to transcribe along the entire length of the dsDNA complex.

T7 RNAP can also continue to transcribe single-stranded regions of DNA once it has initiated transcription.[3] This fact, in combination with the observations of transcription initiation at 3′ ssDNA overhangs, suggests that T7 RNAP should also be able to initiate transcription on, and transcribe, ssDNA. Indeed, T7 RNAP[41−43] and SP6 RNAP[44] have been reported to transcribe single-stranded oligodeoxynucleotides (Figure 1D, *I.*). While transcription can begin anywhere along a ssDNA, creating a myriad of transcripts, many transcripts are produced through transcription of the full length of a ssDNA oligonucleotide.[41,44] Additionally, SP6 RNAP, and presumably T7 RNAP, can catalyze multiple rounds of promoter-independent transcription from ssDNA, in some cases producing over 30 transcripts per molecule of ssDNA.[44] This result has been attributed in part to the fact that bacteriophage polymerases can actively separate RNA transcripts from DNA templates during transcription.[45] Importantly, the 3′ end of an oligonucleotide need not be single-stranded for it to serve as a template for promoter-independent transcription. In principle, the polymerase can initiate transcription anywhere along ssDNA and then read the strand from 3′ to 5′ to synthesize a complementary RNA (Figure 1D, *II.*), although the rates of transcription initiation likely depend on many factors, including sequence, template secondary structure, and the length of the ssDNA.

Once T7 RNAP has successfully initiated transcription, it is highly processive unless a specific termination sequence is encountered.[46,47] After initiating transcription, T7 RNAP can continue transcription through a myriad of different nucleic acid structures including ssDNA regions,[3] nicks, gaps, and abasic sites in both the nontemplate and template strands,[40,48,49] and through noncanonical structures such as immobile holiday junctions.[50] Templates with dsRNA and ssRNA,[51] locked nucleic acids,[52] and phosphorothioate modifications[53] downstream of a dsDNA T7 RNAP promoter are also suitable substrates. Consistent with these observations, promoter-independent initiation of transcription on ssDNA domains can facilitate transcription of complex DNA nanostructures, such as double-crossover motifs,[54] DNA nanotubes,[55] and DNA origami.[56]

Promoter-independent initiation of transcription has also been observed for RNA polymerases other than T7 RNAP. As mentioned above, other bacteriophage RNA polymerases (such as T3 and SP6 RNAP) can nonspecifically transcribe single-stranded DNA[34,44] and initiate transcription at single-stranded 3′ overhangs on DNA templates.[39] *E. coli* RNA polymerase and other bacterial polymerases are able to initiate transcription on ssDNA templates,[57] single-stranded 3′ overhangs,[58] and within single-stranded regions of mismatched bases on DNA complexes.[34,59] And some eukaryotic RNA polymerases can initiate promoter-independent transcription at single-stranded 3′ overhangs[58] and at nicks and/or single-stranded gaps in DNA templates.[60]

Promoter-Independent Transcription Can Disrupt DNA Strand Displacement Reactions. The promoter-independent T7 RNAP transcription reactions depicted in Figure 1 have implications for the design of DNA strand displacement circuits that operate in the presence of T7 RNAP. T7 RNAP transcription and DNA strand displacement circuits have been used in tandem for purposes such as measuring the rate of transcription,[34] making smart biosensors for molecules that regulate transcription,[13] and for information processing tasks.[10]

DNA strand displacement circuits operate via a process of toehold-mediated strand displacement in which an input oligonucleotide binds to a complementary single-stranded region, termed a toehold, of a dsDNA complex and undergoes a branch migration process to displace a strand from the dsDNA complex.[61−63] The single-stranded toehold domains of DNA strand displacement complexes could serve as sites for promoter-independent T7 RNAP transcription, especially if the toeholds have 3′ ends. The RNA transcripts produced in these reactions can participate in strand displacement and in doing so could disrupt the function of the strand displacement networks. For example, a transcript produced when tran-

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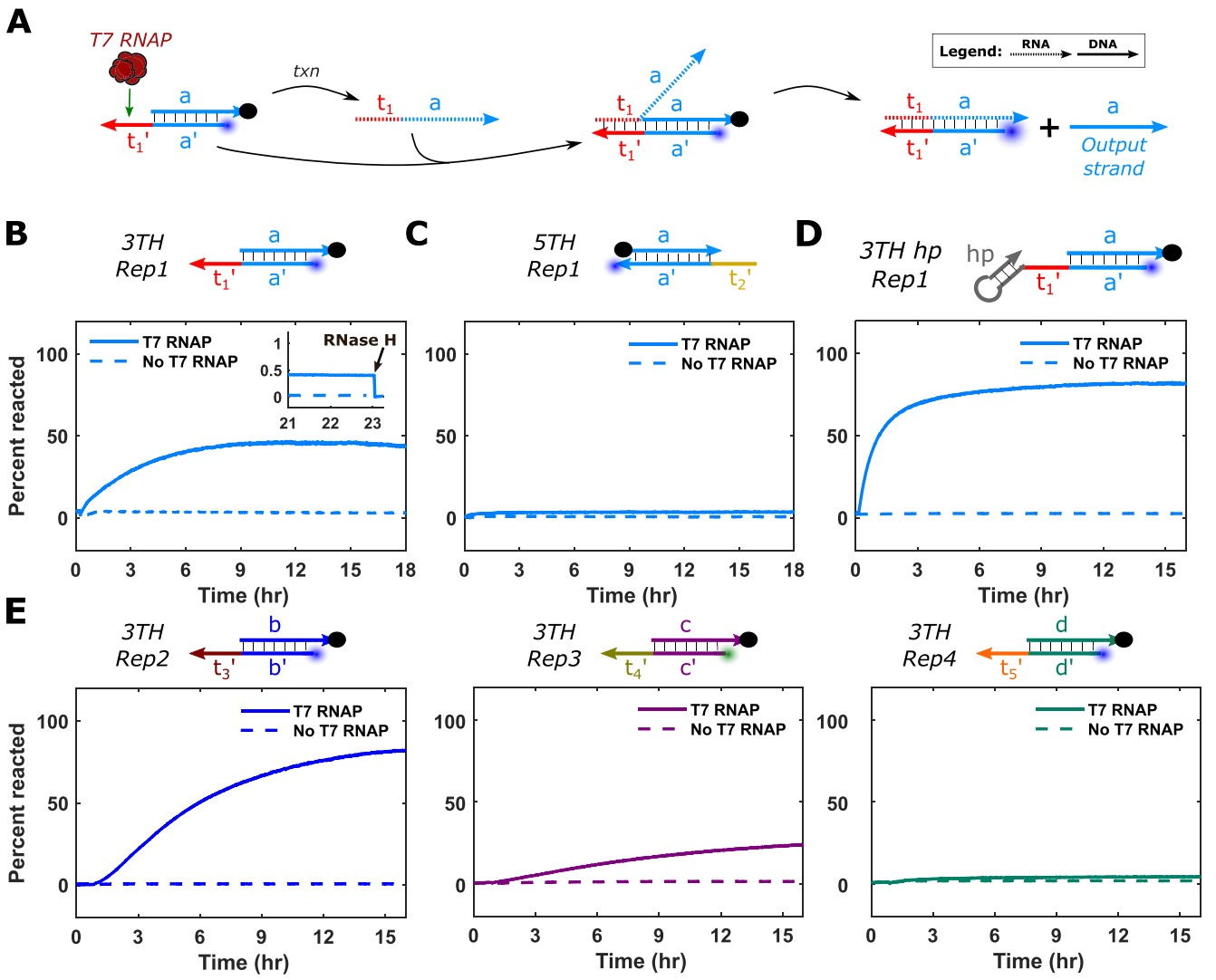

Figure 2. Single-stranded 3′ toeholds can cause DNA strand displacement complexes to actuate when incubated with T7 RNAP. (A) Schematic of T7 RNAP-induced actuation of a DNA strand displacement reporter complex. Strand separation is measured by a fluorescence increase. The green arrow indicates where transcription could initiate. Colored labels above and below complexes indicate sequence identity and apostrophes denote sequence complementarity. Gradient circles represent fluorophore modifications and black circles represent quencher modifications. (B–E) Reacted reporter kinetics of different reporter complexes during incubation with (solid lines) and without (dashed lines) T7 RNAP in transcription conditions. The structure and sequence domains of each reporter complex are depicted above the plots. 3TH and 5TH indicate that complexes have 3′ and 5′ toeholds, respectively. All toeholds, i.e., t_i', are 5 bases long. In all reactions, reporter complexes were at 150 nM and [T7 RNAP] = 3.57 U/μL. For the inset of (B), [RNase H] (an enzyme that only degrades RNA in an RNA:DNA duplex) was added to 0.071 U/μL to demonstrate that the reporter reaction was due to RNA binding to one of the DNA strands of the reporter. Maximum fluorescence values were obtained for normalization by adding excess of the DNA strand complementary to the fluorescently tagged strand of each reporter complex at the end of the experiment. Reactions were otherwise conducted as described in the Materials and Methods. Sequences are in Supporting File S1.

scription is initiated at a 3′ toehold of a DNA strand displacement complex could displace the output strand from the complex (Figure 2A). The spuriously released output strand could initiate a downstream cascade if the complex is part of a larger strand displacement circuit. Indeed, we found that the fluorescence of an initially quenched dsDNA reporter complex with a 3′ toehold increased in fluorescence to an extent consistent with the release of 50% of its output strand within a few hours when incubated with T7 RNAP in transcription conditions (Figure 2B). This observed reaction was caused by RNA molecules binding to the reporter complex, as the addition of RNase H, an enzyme that degrades only RNA in an RNA:DNA complex,[64] caused the fluorescence to return to its minimum value, indicating RNA degradation allows the quenched DNA complex to reassemble (Figure 2B, inset). Moving the toehold to the 5′ end of the reporter complex prevented fluorescence increases upon the addition of T7 RNAP, further suggesting that the spurious reaction observed in Figure 2B was due to promoter-independent transcription initiation at the 3′ toehold of the reporter complex (Figure 2C). Others have reported spurious separation of DNA strand displacement complexes in the presence of T7 RNAP transcription.[13,34]

The 3′ end of the reporter's toehold does not need to be unpaired for spurious reactions with T7 RNAP: a double-stranded hairpin preceding the 3′ end of the single-stranded toehold also reacted when incubated with T7 RNAP (Figure 2D). Similar results were observed for reporter complexes with

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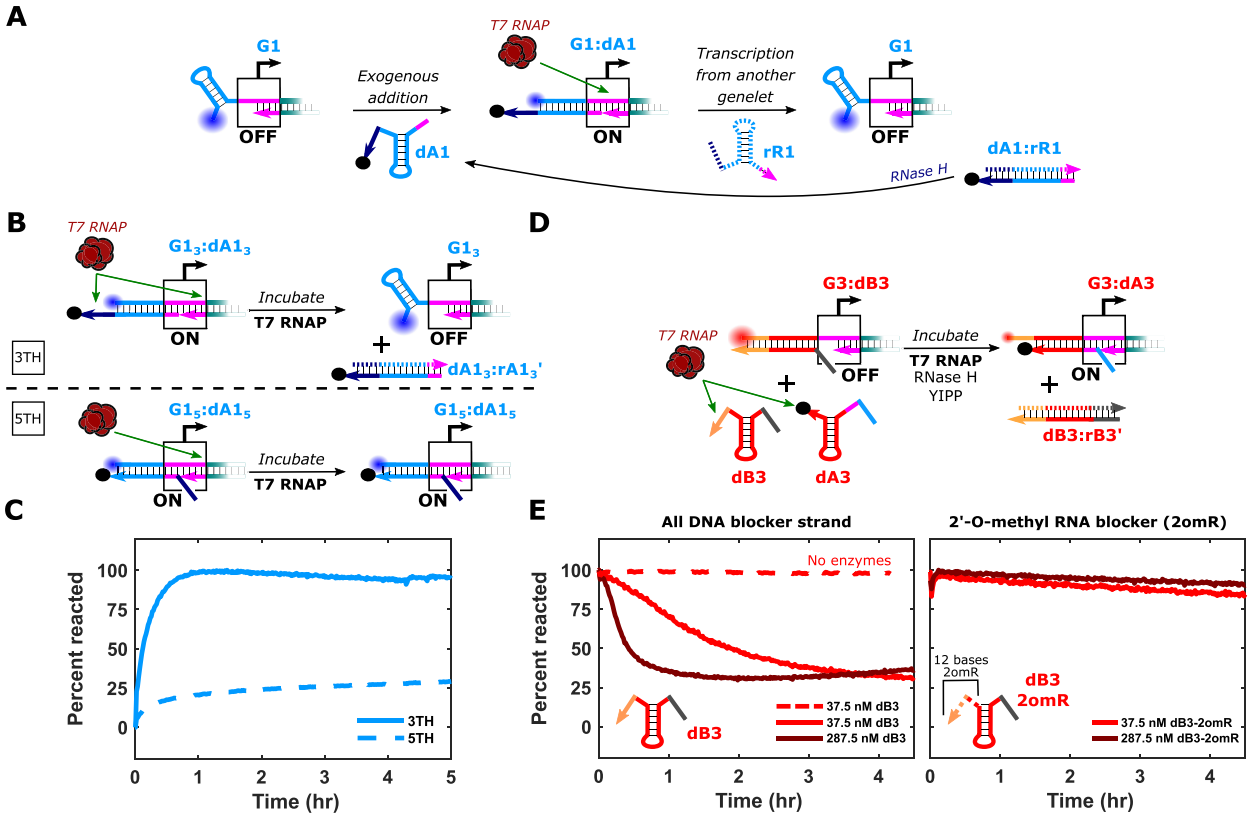

Figure 3. 3′ toeholds and ssDNA can cause DNA strand displacement complexes with T7 RNAP promoter sites to actuate with T7 RNAP. (A) Overview of designed reactions of a type of transcriptional circuit termed genelets. In an OFF genelet, part of the T7 promoter domain (pink) is missing, preventing promoter-specific transcription. A complementary DNA activator (dA1) can bind the genelet to complete the promoter domain to produce an ON genelet, from which promoter-specific transcription can occur. The 3′ toehold on the activator in the genelet:activator complex allows an RNA repressor (rR1) to remove the activator to return the genelet to the OFF state. RNA repressors are typically produced by other genelets in a network. The d and r prefixes indicate DNA or RNA species, respectively. (B) Schematics of a genelet (G1) bound to a DNA activator (dA1) with either a 3′ ($dA1_3$) or 5′ ($dA1_5$) toehold (3TH or 5TH, respectively) and the possible transcription reactions for each. The green arrows indicate where transcription could initiate. Here the activator bound genelet serves as a fluorescent reporter, with gradient circles representing fluorescent modifications and black circles representing quencher modifications. (C) Reacted reporter kinetics of the two genelet:activator complexes in (A) during incubation with T7 RNAP in transcription conditions. Genelets and activators were both present at 100 nM and [T7 RNAP] = 3.57 U/μL. (D) Schematic of a genelet bound to its DNA blocker (dB3) and a possible reaction in which the unbound blocker strand is transcribed, allowing the activator strand (dA3) to bind to genelet G3. (E) Reacted reporter kinetics of the sample depicted in (D) during incubation with T7 RNAP in transcription conditions with either excess ssDNA blocker (left) or excess blocker consisting of 2′-*O*-methyl RNA at its 3′ end (right). G3 and dA3 were at 25 nM and 250 nM, respectively and [T7 RNAP] = 3.57 U/μL. The dashed line in the left plot is a control experiment in which no enzymes were added. In (E), RNase H and yeast inorganic pyrophosphatase (YIPP) were also present. Figures adapted from ref 26.

a double-stranded duplex directly upstream of the 3′ end of a toehold.[29] In these cases, the polymerase likely initiates transcription within the single-stranded toehold domain and transcribes through the dsDNA region of the reporter complex to make a transcript that can displace the output strand. It is important to note that the degree of spurious reaction in the presence of T7 RNAP depends on the sequence of the DNA complexes. For example, three reporter complexes with 5-base single-stranded 3′ toeholds with different sequences exhibited differing degrees of spurious output release when incubated with T7 RNAP in transcription conditions (Figure 2E). These differences may be due to preferences for initiating transcription at certain nucleotides[43,65] or sequence-specific differences in the thermodynamics or kinetics of RNA:DNA interactions compared to DNA:DNA interactions that favor or disfavor RNA strand displacement.[66]

None of the reporter complexes tested in Figure 2 contained a T7 RNAP promoter, so the rate of promoter-independent transcription may be relatively high because the polymerase is not occupied with promoter-specific transcription. However, we found that the same promoter-independent transcription occurs even on complexes with a T7 RNAP promoter. Specifically, we have observed promoter-independent transcription with genelets, elements of *in vitro* transcriptional circuits that have been used to build synthetic networks to explore the properties of genetic regulatory networks.[11,24,25,27−29] Genelets, when bound to a ssDNA activator, have a complete T7 RNAP promoter and can undergo transcription. Genelet-bound DNA activators present a single-stranded 3′ toehold, which is present to allow an RNA repressor produced from other genelets to remove a bound activator, turning the genelet off (Figure 3A). But this 3′ toehold can serve as a site for T7 RNAP to initiate promoter-independent transcription. The resulting transcript is complementary to the DNA activator and can remove the activator from the genelet:activator complex. We successfully mitigated this issue by moving the toehold to the 5′ end of the DNA activator (Figure 3B,C). The results in Figure 2 and Figure 3C

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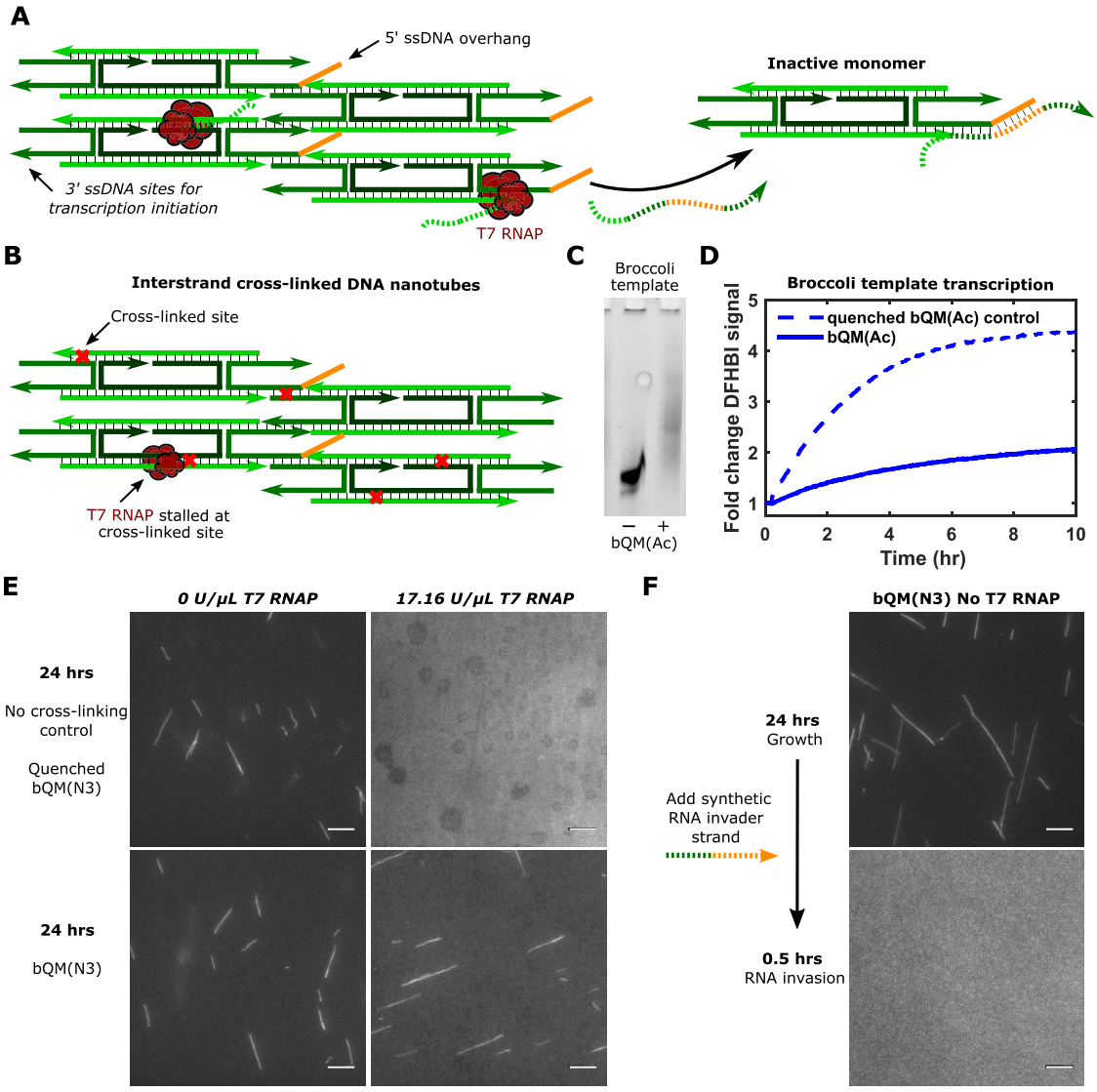

Figure 4. Covalently cross-linking DNA complexes with reversible cross-linkers stabilizes DNA nanostructures in the presence of T7 RNAP. (A) Schematic of promoter-independent nanotube transcription and RNA hybridization-directed nanotube disassembly. Nanotube-derived RNA transcripts, potentially from promoter-independent transcription initiated at 3′ sticky ends, bind to a single-stranded 5′ overhang and invade adjacent sticky ends to detach monomers. (B) Schematic of interstrand cross-linked DNA nanotubes. (C) 10% denaturing PAGE results for the bQM(Ac) cross-linking of a dsDNA complex containing the T7 RNAP promoter and encoding the Broccoli aptamer transcript (Broccoli template). Smearing of the +bQM(Ac) band indicates covalent cross-linking of the dsDNA complex. (D) Fold change in DFHBI fluorescence during transcription of the dsDNA Broccoli template with and without bQM(Ac) cross-linking. (E) Fluorescence micrographs of DNA nanotubes assembled from 1 μM monomers that had not (top) and had (bottom) undergone bQM(N3) cross-linking after 24 h of incubation with T7 RNAP in transcription conditions. Scale bars: 10 μm. (F) Fluorescence micrographs of cross-linked DNA nanotubes before (top) and after (bottom) invasion with addition of an RNA invader strand (final concentration: 2 μM). Sequences are in Supporting File S1.

thus highlight a key design strategy for successfully using T7 RNAP with DNA strand displacement networks: (Strategy 1) Avoid single-stranded 3′ overhangs and design DNA strand displacement circuits that use 5′ toeholds.

T7 RNAP can also disrupt the operation of strand displacement networks by transcribing single-stranded DNA.[42] For example, the ssDNA activators in genelet circuits are typically used in excess of their target genelets, and the free ssDNA can serve as a site for promoter-independent transcription. We found when a ssDNA activator was present in 9-fold excess over its target genelet, the rate at which the DNA activator was removed from the genelet was much higher than in a 1:1 mixture of activator and genelet,[29] presumably because increasing the concentration of ssDNA activator increases the rate of promoter-independent transcription of activator. Transcription of single-stranded DNA can also be problematic for the operation of DNA strand displacement cascades, as such strands are often present at significant concentrations: it can be common to prepare complexes with a slight excess of certain strands to minimize the presence of single strands that could instigate downstream reactions or cascades. These results highlight another key design strategy for successfully using T7 RNAP with DNA strand displacement networks: (Strategy 2) Avoid ssDNA species or long regions of ssDNA.

Removing unbound strands and/or single-stranded domains from a system is not always possible. One way to prevent transcription of these domains is to use nucleic acids that

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polymerase's will not initiate transcription on. For example, T7 RNAP has not been reported to initiate transcription on ssRNA[43] so using ssRNA (or modified RNA to prevent degradation) in place of ssDNA could mitigate promoter-independent transcription. We found such an approach worked in a genelet system in which a DNA blocker and DNA activator, both containing 5′ ssDNA toeholds, compete to bind a genelet to regulate the genelet's transcriptional activity[26] (Figure 3D). With a blocker composed entirely of DNA, we found transcription of blocker not bound to the genelet likely caused the blocker that was bound to the genelet to be removed, resulting in higher levels of genelet activation. Higher concentrations of free blocker led to more activation (Figure 3E). Changing the 12 single-stranded bases at the 3′ end of the blocker to 2′-*O*-methyl RNA bases (so that the domain could not serve as a site for T7 RNAP to initiate transcription), almost completely eliminated spurious genelet activation[26] (Figure 3E). Others have reported similar success using 2′-*O*-methyl RNA to prevent promoter-independent transcription of DNA strand displacement reporters similar to those shown in Figure 2.[13,34] While 2′-*O*-methyl RNA was used to prevent the domain from being degraded by RNase H, which was also present in our experiments, we expect that substituting unmethylated RNA (or other modified nucleic acids that are resistant to promoter-independent transcription) would have the same effect. These results highlight another key design strategy for successfully using T7 RNAP with DNA strand displacement networks: (Strategy 3) Use RNA in lieu of DNA for necessary single-stranded DNA or 3′ regions.

There are also other examples in the DNA computing and synthetic biology literature where promoter-independent T7 RNAP transcription may have led to undesired results. For example, initiation of transcription at single-stranded 3′ overhangs or on ssDNA regions could explain a number of leak reactions that have been reported in other transcriptional circuits[10] and transcription-based sensors.[13,67]

Promoter-Independent Transcription Can Destabilize DNA Nanostructures and Materials. Promoter-independent T7 RNAP transcription can also occur from DNA nanostructures and DNA-based materials, potentially altering their function and stability. Many DNA nanostructures have ssDNA regions that serve as sites for binding DNA strands that link components together.[23,68] If promoter-independent transcription occurs at these sites, the resulting RNA transcripts can bind to the DNA structures and potentially displace functional components or cause the DNA nanostructures to disassemble.

We have found that promoter-independent T7 RNAP transcription can cause DNA nanotubes to disassemble.[69] Transcription, potentially initiated at the single-stranded 3′ sticky ends of the monomers that make up the DNA nanotubes, produced RNA that was complementary to portions of these monomers. On assembled nanotubes, the RNA transcripts can bind to a ssDNA overhang[14,70] adjacent to one of the sticky ends binding the monomer to the nanotube lattice and disrupt this sticky end through strand displacement (Figure 4A). Many such displacement events result in nanotube disassembly.[69] As the RNA produced from nanotubes is likely complementary to other portions of the monomers, monomer structure could be altered in other ways.

This process of T7 RNAP-induced nanotube disassembly required the single-stranded 5′ overhang on the monomers: removing this domain or sequestering it in a dsDNA complex prevented nanostructure melting.[69] However, these design changes did not prevent the transcription of portions of the nanotubes or monomers; they simply slowed RNA invasion of the nanotubes. Because transcripts complementary to portions of nanotubes and monomers were still produced, we found it was difficult to grow nanotubes with T7 RNAP present, likely because the transcripts bind to sticky ends and prevent assembly. It is possible to grow DNA nanotubes when T7 RNAP is present, but RNases are usually necessary at relatively high concentrations for growth to occur, likely because the RNases clean up the monomer-derived transcripts. Even in these conditions, far fewer nanotubes form when T7 RNAP is present than when it is absent.[14,30]

Preventing promoter-independent transcription of DNA nanostructures (rather than just stabilizing them in the presence of T7 RNAP) would make it easier to build reliable systems containing both DNA nanostructures and RNAP. The ability to stabilize structures with single-stranded overhangs would also be desirable for many applications. To achieve these goals, we tried using monomers with single-stranded 3′ sticky ends composed of RNA rather than DNA to prevent transcription initiation at these domains. However, we found these monomers did not form nanotubes large enough to visualize with epifluorescence.

We also considered chemically modifying bases within the DNA monomers to arrest transcription. Unfortunately, there are not many simple nucleic acid modifications that arrest T7 RNAP once it has begun transcription—T7 RNAP has been reported to transcribe through gaps and nicks,[48,49] abasic sites,[49] regions of ssDNA,[3,51] ssRNA, dsRNA,[51] locked nucleic acids,[52] and phosphorothioate modifications.[53] One modification that has been shown to stop T7 RNAP transcription is the introduction of a covalent interstrand DNA cross-link that prevents the DNA duplex from separating during transcription.[71,72] Reversible intrastrand covalent cross-links have also been shown to stall T7 RNAP.[73,74] To see if we could reduce promoter-independent transcription of nanotubes with covalent cross-links (Figure 4B), we adopted a previously described reversible interstrand DNA cross-linking chemistry based on bisquinone methides (bQM).[75] bQMs sequentially generate two reactive quinone methide intermediates that react with DNA to create intra/interstrand cross-links. The bQM cross-linking molecules also contain functional groups (R groups) that facilitate their association with DNA, which helps promote high cross-linking efficiencies (>85% with respect to number of DNA molecules modified).[75] Both an aromatic acridine[75] (Ac) and a trimer of positively charged quaternary amines[76] (N3) R groups have been studied. Here we will refer to these molecules generically as bQM(R) where R can be either the Ac or N3 group.

We first tested whether bQM(R) cross-linking of a dsDNA transcription template containing the T7 RNAP promoter could prevent promoter-specific transcription of the Broccoli aptamer.[77] We verified cross-linking using denaturing PAGE analysis (Figure 4C), and kinetic assays measuring Broccoli aptamer production indicated cross-linking substantially reduced the rate of T7 RNAP transcription compared to a control in which a quenched bQM(R) was used in the cross-linking step[78] (Figure 4D). We then cross-linked DNA nanotubes grown from monomers with single-stranded 5′ overhangs. The cross-linked nanotubes were stable even at T7 RNAP concentrations much higher than necessary to disassemble a control group of nanotubes for which quenched

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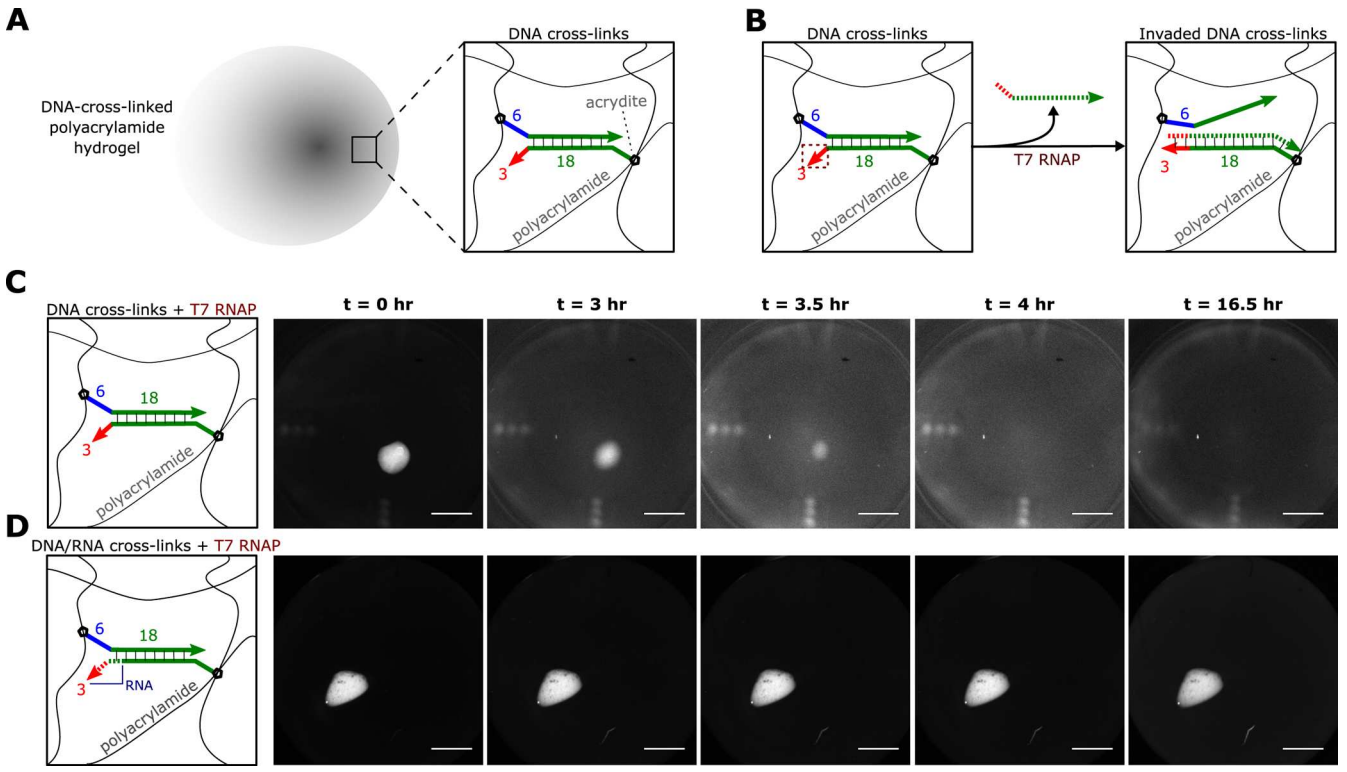

Figure 5. Promoter-independent transcription can alter the stability of DNA-based hydrogels. (A) Diagram of a DNA cross-linked polyacrylamide hydrogel.[82,83] Inset shows the acrydite-modified dsDNA complex that cross-links polyacrylamide strands within the hydrogel. Numbers indicate domain lengths. (B) Schematic of a hypothesized mechanism of hydrogel disintegration in the presence of T7 RNAP. Promoter-independent transcription could initiate at the single-stranded 3′ overhang (red dashed box) and produce an RNA transcript that is complementary to the bottom strand of the DNA cross-link allowing the cross-links to be invaded. (C, D) Fluorescence micrographs of polyacrylamide gels cross-linked with a complex of either entirely DNA strands (C) or hybrid DNA-RNA strands in which the last five bases of the 3′ end of the bottom strand of the cross-links are RNA (D) during incubation with T7 RNAP in transcription conditions. Scale bars: 1 mm. Reactions otherwise conducted as described in the Materials and Methods with [T7 RNAP] = 2.85 U/μL and yeast inorganic pyrophosphatase at 1.35 × 10^{-3} U/μL. A dsDNA complex containing the T7 RNAP promoter site (HG-D in Supporting File S1) was also included at 200 nM in the reactions in an unsuccessful attempt to enhance stability by reducing the rate of promoter-independent transcription. DNA-cross-linked hydrogels were prepared as previously described.[83] Sequences are in Supporting File S1.

bQM(R) was used during the cross-linking step[78] (Figure 4E). This increased stability suggested that reversible cross-linking reduced the rate at which the DNA monomers and nanotubes were being transcribed, as the resulting transcripts should still be able to disassemble the nanotubes. To confirm that nanotubes could still be disassembled, we added an RNA invader strand that was complementary to the single-stranded 5′ overhang and the adjacent sticky end to cross-linked nanotubes and found this induced disassembly[14,70] (Figure 4F). Together, these results highlight another potential approach to mitigating promoter-independent transcription: (Strategy 4) Covalently cross-link structures to halt transcription. However, there are drawbacks to this approach. For example, covalent cross-linking can alter DNA structure.[79,80] Further, if T7 RNAP is stalled for extended periods at cross-linked sites along the structures,[71] promoter-specific transcription will likely be reduced. Lastly, chemicals that interact with DNA are highly toxic so safety can be a concern when working with these cross-linking agents.

We have also found that promoter-independent T7 RNAP transcription can alter the stability of other DNA-based materials, for example, DNA-cross-linked polyacrylamide hydrogels. These hydrogels contain dsDNA complexes with 5′ acrydite modifications on both strands, which allow them to cross-link polyacrylamide chains to produce hydrogels (Figure 5A). If the dsDNA cross-links are designed with short, single-stranded domains, they can serve as initiators for a hybridization chain reaction[81] (HCR) process in which the insertion of DNA hairpins into the cross-links drives high-degree swelling.[82,83] However, the single-stranded 3′ domains on the DNA cross-links designed to facilitate hairpin insertion could also serve as sites where T7 RNAP could initiate promoter-independent transcription. The resulting transcripts would be able to displace the DNA cross-links, causing the hydrogels to disintegrate (Figure 5B). Indeed, we found that these DNA cross-linked hydrogels disappeared after 4 h of incubation with T7 RNAP in transcription conditions (Figure 5C). Given that the single-stranded 3′ domain on the DNA cross-links is crucial to the HCR process,[81] it was not possible to simply remove this domain from the cross-link design to prevent promoter-independent transcription. We therefore modified the cross-link design to have the single-stranded 3′ domain (and two additional bases 5′ upstream) be composed of RNA bases, which our previous results (Figure 3E) suggested should reduce transcription initiation at this 3′ overhang. We found hydrogels with these DNA-RNA hybrid cross-links no longer disintegrated in the presence of T7 RNAP (Figure 5D). These results again highlight how understanding the mechanisms of promoter-independent T7 RNAP transcription can lead to design modifications that allow T7 RNAP and DNA-based

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materials to be successfully coupled: (Strategy 3) Modify single-stranded 3′ overhangs to be RNA.

Other DNA-based materials like DNA origami, or self-assembled DNA-functionalized nanoparticles or colloidal particle clusters could be susceptible to disassembly by promoter-independent T7 RNAP transcription followed by RNA strand displacement through the mechanism described above. Consistent with this idea, T7 RNAP has been reported to disrupt the stability of DNA tweezers[31] and DNA origami structures.[33,84] We have found that DNA origami structures[85] that were tagged with fluorophore modified DNA oligonucleotides (Figure 6A) could no longer be detected with fluorescence imaging after 3.5 h of incubation with T7 RNAP in transcription conditions (Figure 6B). The decrease in fluorescence required T7 RNAP transcription as the fluorescence of the structures only diminished in the presence of T7 RNAP and all four ribonucleotides; incubation with T7 RNAP and ATP only did not cause the fluorescence of the structures to decrease (Figure 6C). Since the fluorescent tags are anchored to regions of single-stranded M13 bacteriophage DNA, we hypothesize that promoter-independent initiation of transcription on single-stranded M13 DNA could produce transcripts that displace the fluorescent complexes from the M13 DNA. It is also possible that the labeling strands bound to M13, or unbound excess of these strands in solution, are transcribed to produce RNAs that displace the bound strands. The use of fluorescent tags in these experiments made the loss of modified oligonucleotides straightforward to monitor, but similar issues could arise for DNA nanostructures functionalized with other modifications that are harder to directly observe.[22,86−89] These results again highlight Strategy 2: Avoid ssDNA near hybridization sites on structures.

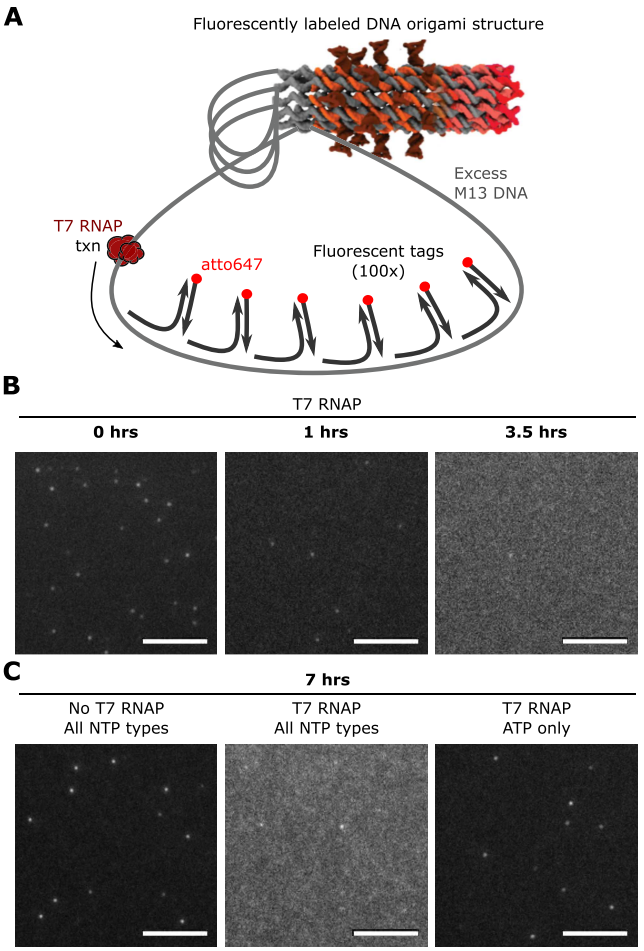

Figure 6. Promoter-independent transcription can alter the functionalization of DNA nanostructures. (A) Diagram of a DNA origami nanostructure that has been fluorescently tagged via strands bound to the excess single-stranded M13 DNA scaffold that was not used to fold the structure.[85] (B) Fluorescence micrographs of 5 pM DNA origami structures after different durations of incubation with T7 RNAP in transcription conditions. (C) Fluorescence micrographs of DNA origami structures after 7 h of incubation without (left) or with T7 RNAP in transcription conditions with all four NTP types (middle) or only ATP at 30 mM (right). Scale bars: 10 μm. Reactions otherwise conducted as described in the Materials and Methods with [T7 RNAP] = 3.57 U/μL. DNA origami nanostructures were prepared as previously described.[90] Sequences are in Supporting File S1.

Other Strategies for Using T7 RNAP Transcription in DNA Nanotechnology Applications. Based on the results presented above, there are numerous design strategies, presented in Figure 7, that could be applied to mitigate the effects of promoter-independent transcription by T7 RNAP of DNA strand displacement complexes, DNA nanostructures, and DNA-based materials. In addition to these design strategies, there are other previously reported mitigation strategies. For example, other DNA cross-linking strategies prevent disassembly of DNA origami in the presence of T7 RNAP.[84] Presumably, other chemical[91,92] or enzymatic ligation[93,94] techniques could stabilize DNA structures for use alongside T7 RNAP, but some of these modifications could preclude the programmed rearrangement we demonstrated with DNA nanotubes (Figure 4F). Additionally, DNA-T7 RNAP conjugates that localize the polymerase only on its desired transcription template can prevent promoter-independent transcription of other DNA-based materials in solution.[12,33,95] While this method is a feasible approach for reducing promoter-independent transcription, it does require the synthesis and purification of a DNA-T7 RNAP conjugate so other "off-the-shelf" approaches like modifying 3′ overhangs to be RNA might be more desirable when applicable. Further, including RNases alongside T7 RNAP can reduce the accumulation of transcripts derived from promoter-independent transcription;[14,30,69] depending on the system, it can be possible to find conditions in which promoter-specific transcripts can accumulate and promoter-independent transcripts remain low. Lastly, T7 RNAP can be repressed by incorporating transcription terminator sequences within DNA nanostructures[96,97] or by designing triplex-forming oligonucleotides that bind to nanostructure sequences and prevent T7 RNAP from initiating transcription.[98] These mechanisms do impose constraints on the DNA sequences used to make materials though.

CONCLUSION AND OUTLOOK

Here we reviewed many mechanisms of promoter-independent DNA transcription by T7 RNAP and illustrated how this undesired transcription can wreak havoc in many DNA nanotechnology applications. We present many design strategies for mitigating these undesired effects (Figure 7), but there are likely new avenues that can be investigated to further improve the coupling of transcription with self-assembly and reconfiguration of DNA nanostructures. We focused on swapping DNA domains for RNA domains to

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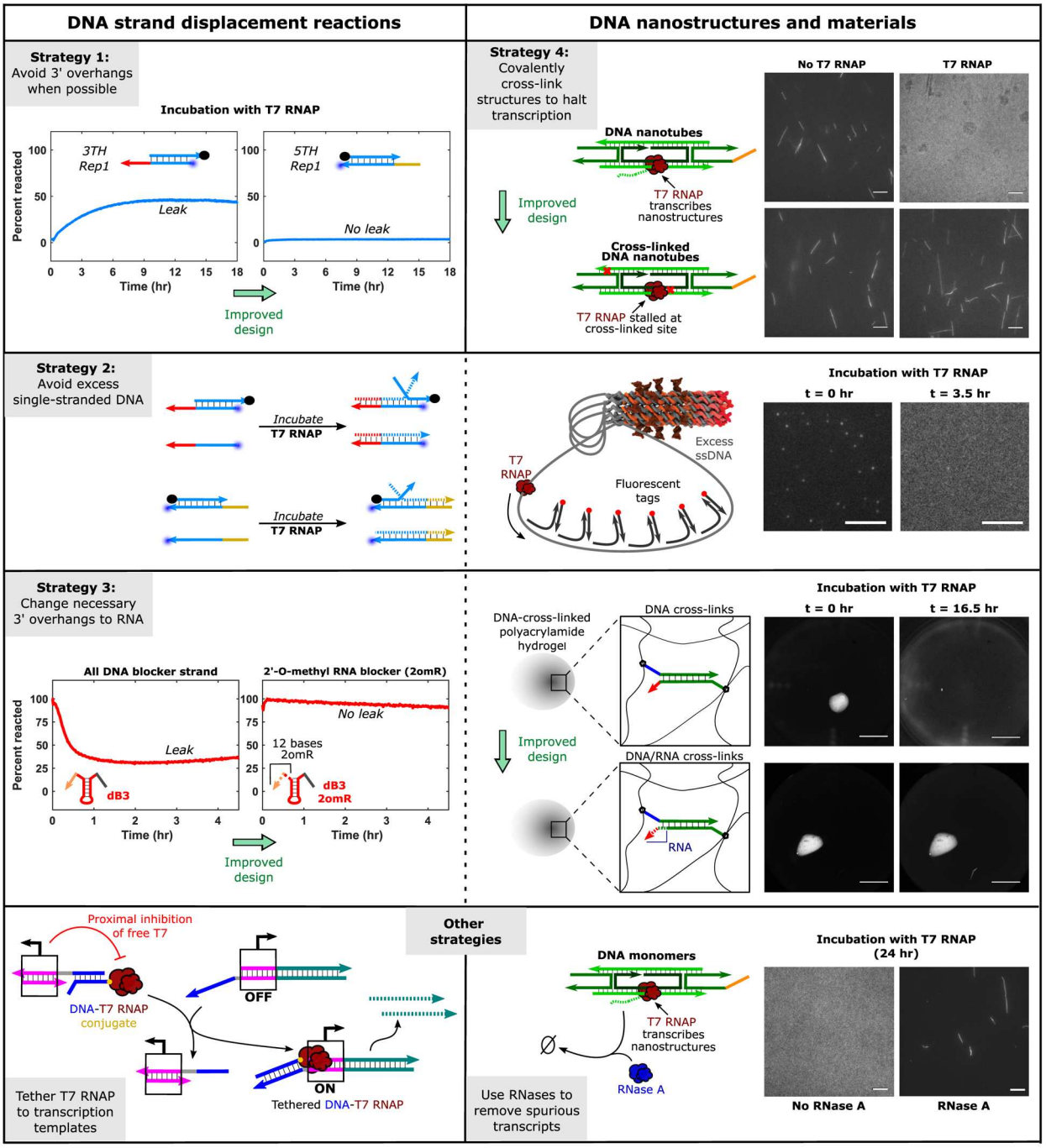

Figure 7. Design strategies for mitigating promoter-independent T7 RNAP transcription. 1: Avoid 3′ overhangs. For toehold-mediated DNA strand displacement reactions, use 5′ toeholds rather than 3′ toeholds. 2: Avoid ssDNA species or stretches of ssDNA. Free single-stranded species in DNA strand displacement circuits can be transcribed (left) and ssDNA regions of structures can be transcribed 3′ to 5′ to alter functionality (right). Additionally, 5′ overhangs can serve as binding sites for transcribed RNAs and facilitate RNA invasion; such sites should be avoided or hybridized.[69] 3: Change necessary 3′ overhangs to RNA or 2′-*O*-methyl RNA. RNA modifications prevent T7 RNAP from initiating transcription, but transcription through RNA is possible if transcription initiates elsewhere.[51] 4: Covalent dsDNA cross-links can halt transcription or stabilize structures.[84] These modifications could alter DNA helix structure. Others: Left: To reduce off-target behavior, tether T7 RNAP to the desired transcription template. Adapted from ref 12. Copyright 2019, American Chemical Society. Right: RNases degrade RNAs produced from promoter-independent transcription. Adapted from ref 69. Copyright 2018, The Author(s), published by Oxford University Press.

prevent initiation of transcription from ssDNA, but other DNA modifications could work and warrant further investigation. The rate of promoter-independent transcription also appears to depend sensitively on the sequences of the DNA substrates. For example, some fluorescent reporter complexes with 3′ ssDNA toeholds react almost completely when incubated with T7 RNAP while others do not react at all (Figure 2). Further investigation into which sequences exacerbate or mitigate promoter-independent transcription might provide new means of mitigating this problem through sequence design. Conversely, the community could compile a library of

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sequences that mitigate promoter-independent transcription for specific use as ssDNA domains.

We also observed widely variable time scales over which promoter-independent transcription became prevalent across applications. For some genelet constructs (Figure 3), promoter-independent transcription caused spurious transcription that competed with designed processes within minutes of T7 RNAP addition, while in other cases, spurious transcription only induced measurable leak reactions after hours (Figure 2). For the DNA nanostructures presented here, it took hours for T7 RNAP to induce observable disassembly. In these applications, a critical concentration of transcript may need to accumulate before disassembly will occur. Additionally, for structures like DNA nanotubes, the RNA transcripts produced may be long and they may have secondary structure that impedes their reactions with the DNA material. These potential effects make inferring rates of promoter-independent transcription from rates of disassembly difficult. The time scale over which promoter-independent transcription manifests is an important consideration for including T7 RNAP in a reaction system, as applications that require short time frames may not be affected.

Another route to reducing undesired reactions with T7 RNAP is through enzyme engineering or evolution. This has been employed to produce T7 RNAP variants that can recognize orthogonal promoter sequences,[99] have reduced RNA self-priming ability,[100] have higher thermal stability,[101] or have reduced termination efficiency.[102] Such efforts could possibly be used to create variants with lower propensity for promoter-independent transcription of ssDNA.

This review highlights an important lesson that applies generally when designing processes involving DNA nanostructures and circuits along with nucleic acid manipulating enzymes: care should be taken to thoroughly investigate the documented nonspecific reactions of any chosen enzyme. Unfortunately, it is often difficult to know what to look for beforehand, so often, as illustrated here, careful experiments may be necessary to elucidate the source of the undesired interactions. While T7 RNAP is extremely well-characterized, many enzymes that may be of use in DNA nanotechnology will not be. Since most enzymes will exhibit some level of nonspecific activity, careful elucidation of potential sources of nonspecific reactions is probably warranted and will likely be an important aspect of adopting enzymes for use in technological applications.

MATERIALS AND METHODS

DNA and Materials. All sequences were ordered from Integrated DNA Technologies (IDT) and are tabulated in Supporting File S1. 3,5-Difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) fluorescent dye was purchased from Lucerna, Inc. T7 RNAP was purchased in bulk (300,000 units) from Cellscript (200 U/μL). Yeast inorganic pyrophosphatase was purchased from NEB (0.1 U/μL). RNase H (5 U/μL), ribonucleotide triphosphates (ATP, UTP, CTP, GTP 100 mM solutions), and SYBR gold were purchased from Thermo Fisher Scientific. bQM(Ac) and bQM(N3) were synthesized in house. All other chemicals were purchased from MilliporeSigma.

Transcription Conditions. Unless otherwise stated, reactions were conducted at 37 °C in NEB RNAPol reaction buffer (40 mM Tris-HCl - pH 7.9, 6 mM $MgCl_2$, 1 mM DTT, 2 mM spermidine) supplemented with 24 mM $MgCl_2$; ribonucleotide triphosphates (ATP, UTP, CTP, GTP) at a final concentration of 7.5 mM each; and bovine serum albumin (at a final concentration of 0.1 mg/mL). The concentrations of enzymes used in the experiments were based on values used in previous studies.[14,26,29]

Interstrand Cross-Linking of dsDNA Complexes and DNA Nanotubes. Cross-linking of the dsDNA Broccoli transcription template was conducted in 10 mM 2-(N-morpholino)ethanesulfonic (MES) buffer, pH 7 with bQM-(Ac) at either 500 μM (Figure 6B) or 100 μM (Figure 6C) and NaF at 500 mM or 100 mM, respectively. The dsDNA Broccoli template was prepared by annealing 90 to 20 °C (−1 °C/min) 5 μM of the nontemplate and template strands in MES buffer. The dsDNA Broccoli template was present at 3 μM in the cross-linking reactions.

The 10% denaturing PAGE experiments were conducted with denaturing polyacrylamide gels (40 mM Tris-Acetate, 1 mM EDTA buffer supplemented with 12.5 mM magnesium acetate and 7 M urea). The denaturing gel was run at 69 °C and 100 V for 1 h, stained with SYBR Gold, and imaged using a Syngene EF2 G:Box gel imager equipped with a blue light transilluminator (emission max ∼450 nm) and a UV032 filter (bandpass 572−630 nm).

The Broccoli aptamer transcription assays were conducted in NEB RNAPol reaction buffer supplemented with 2 mM of each ribonucleotide triphosphate type, 100 nM of the Broccoli transcription template, 15 μM of the 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) dye, and T7 RNAP at 5 U/μL. Fluorescence measurements were obtained on a Agilent Mx3000P quantitative PCR machine as previously described.[29]

The DNA monomers used in Figure 6D and 6E were the var1_7 monomers from ref 69. DNA nanotube cross-linking was conducted in transcription buffer (40 mM Tris-HCl, 6 mM $MgCl_2$, 2 mM spermidine) without DTT, pH 7.9 with 100 μM bQM(N3) and 100 mM NaF. DNA nanotubes were prepared with 5 μM monomers as previously described[69] and were added to the cross-linking reactions to a final concentration of 3 μM. Nanotube stability assays were conducted with cross-linked nanotubes as previously described.[69]

For samples with quenched (inactive) bQM(R), the bQM(R) precursors were incubated with 500 μM DTT and 100 mM NaF for 24 h prior to the addition of DNA or DNA nanotubes.

ASSOCIATED CONTENT

Data Availability Statement

The data for the results presented in this manuscript is available in an online archive at: 10.7281/T1/UUDAWP. The archive includes raw fluorescence data, scripts to analyze fluorescence data, DNA sequences used, and uncropped image files from this manuscript.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00726.

DNA sequences used in this manuscript (XLSX)

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Author Contributions

S.W.S. and R.S. conceived the study. S.W.S. conducted most experiments. S.R.B. synthesized bQM(R) compounds and helped with DNA cross-linking experiments in Figure 4. J.F. conducted the experiments in Figure 5. S.W.S., E.K., and R.S. wrote the manuscript with input from all authors.

Notes

The authors declare no competing financial interest.

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