Efficient inhibition of RNA self-primed extension by addition of competing 3′-capture DNA-improved RNA synthesis by T7 RNA polymerase

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ABSTRACT

In vitro synthesized RNA is used widely in studies of RNA biology, biotechnology and RNA therapeutics. However, *in vitro* synthesized RNA often contains impurities, such as RNAs with lengths shorter and longer than the expected runoff RNA. We have recently confirmed that longer RNA products are formed predominantly via *cis* self-primed extension, in which released runoff RNA folds back on itself to prime its own RNA-templated extension. In the current work, we demonstrate that addition of a DNA oligonucleotide (capture DNA) that is complementary to the 3′ end of the expected runoff RNA effectively prevents self-primed extension, even under conditions commonly used for high RNA yields. Moreover, the presence of this competing capture DNA during 'high yield' transcription, leads to an increase in the yield of expected runoff RNA by suppressing the formation of undesired longer RNA byproducts.

INTRODUCTION

T7 RNA polymerase is a single subunit DNA-dependent RNA polymerase that efficiently synthesizes RNA *in vitro* with high fidelity from linear DNA templates containing the T7 promoter consensus sequence (1–5). This system (and systems using related phage polymerases) is used widely to synthesize RNA for a range of applications, including basic studies of RNA biology (such as ribosomal and spliceosomal RNAs, riboswitches and other noncoding RNAs (6–9)), and in biotechnology applications including siRNA, mRNA therapeutics, aptamers, riboswitch-based sensors and CRISPR guide RNAs (gRNAs) (10–14). However, it is well known that in addition to the expected runoff RNA, this system produces both short, abortive products (1,15) and products longer than encoded by the DNA (1,6,16–18),

often called 'nontemplated' additions, but known to be templated (19–23). Indeed, it has been shown previously that T7 RNA polymerase can act as an RNA-dependent RNA polymerase, taking correct length RNA and extending it to longer RNA products by priming on itself in *cis* or on a second RNA in *trans* (19–28).

Recently, we have confirmed that *cis* self-primed extension of product RNA is the predominant mechanism in the generation of longer RNA byproducts *in vitro* (22). In *cis* self-primed extension, T7 RNA polymerase first transcribes the expected runoff RNA through transcription initiation and elongation. Released runoff RNA then rebinds to the enzyme, folds back on itself and primes the extension of the RNA to longer products, using upstream RNA as a template, and leading to the formation of double stranded RNA byproducts (19,22,23).

It is well known that double stranded RNAs can stimulate the innate immune response by activating receptors such as Toll-like receptor 3 (TLR3), melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), which are natural receptors for responding to potential viral RNA threats (29–31). Additionally, double stranded RNAs inhibit protein synthesis by activating enzymes like protein kinase RNA-activated (PKR), oligoadenylate synthetase (OAS) and RNA-specific adenosine deaminase (ADAR) (32,33). As a result, in therapeutic applications of RNA, it is necessary to remove these long double stranded RNA byproducts after *in vitro* transcription by T7 RNA polymerase. A common approach to remove double stranded byproducts is gel electrophoretic or chromatographic purification post-synthesis (34–38). Recently, a new method for removal of dsRNA based on selective binding to cellulose in an ethanol-containing buffer, has been developed (39). These approaches, however, are imperfect and costly, as downstream yields of RNA can be very low and even trace amounts of double stranded RNA impurities may still trigger an immune response.

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Rather than attempt to purify the correct RNA post-synthesis (or as a key step prior to subsequent purification), we introduce here a novel method to inhibit the formation of double stranded RNA contaminants during transcription by including in the reaction a short capture DNA oligonucleotide complementary to the (correct) 3′ end of the RNA. Binding of this short DNA to 3′ end of the RNA prevents the RNA from folding back on itself and so inhibits RNA self-primed extension. In addition to yielding much higher purity, the elimination of primer extended RNA byproducts during transcription leads to dramatically improved yields of correct length runoff RNA.

MATERIALS AND METHODS

Reagents

DNA oligonucleotides used as transcription templates and as capture DNAs, and synthetic RNA for self-primed extension reactions, were purchased from Integrated DNA Technologies (IDT); and sequences are shown in Supplementary Table S1. All 'high yield' transcription reactions were conducted using HiScribe™ T7 High Yield RNA Synthesis Kit (New England BioLabs). For self-primed extension reactions, T7 RNA polymerase was prepared and purified in our lab (40).

RNA self-primed extension reactions

Reactions with synthetic RNA, in the absence of promoter DNA, were conducted with 25 μM synthetic RNA in the presence of 0.5 μM T7 RNA polymerase and 0.4 mM each of guanosine triphosphate, cytidine triphosphate, adenosine triphosphate (ATP) and uridine triphosphate. Reactions were carried out at 37°C for both 5 min and 4 h in a transcription buffer containing 15 mM magnesium acetate, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM potassium glutamate, 0.25 mM ethylenediaminetetraacetic acid and 0.05% Tween-20. For self-primed extension reactions in the presence of capture DNA, DNA oligonucleotides, complementary to the 3′ end of RNA and containing 3′ amino modification were added to reaction mixtures to a final concentration of 25 μM.

Transcription reactions

All reactions were performed using partially single-stranded DNA constructs, in which the nontemplate DNA oligonucleotide extends downstream only to position +2 (1,41). All 'high yield' transcription reactions were carried out in the presence of 2 μM each of nontemplate and template DNA oligonucleotides, 7.5 mM of each NTP, and 1.5 μl T7 RNA polymerase Mix™ (New England BioLabs) in an overall 20 μl reaction volume at 37°C for 4 h (unless noted otherwise in the manuscript). High yield transcription reactions in the presence of capture DNA additionally contained 400 μM (unless noted otherwise) capture DNA. RNase Inhibitor Murine (New England BioLabs) was added to a final concentration of 1 U/μl in all reaction mixtures. Both self-primed extension and transcription reactions were heat inactivated at 70°C for 5 min.
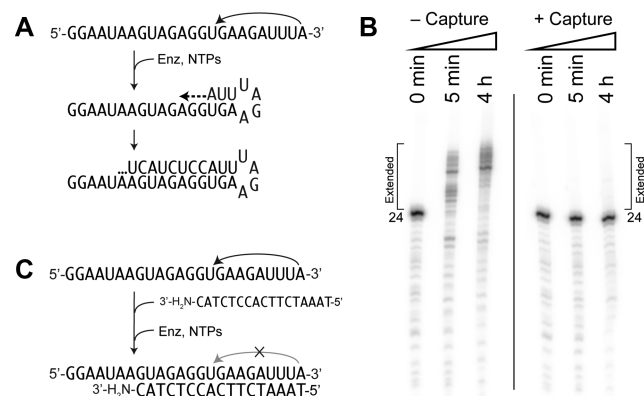
Figure 1. Inhibition of self-primed extension of synthetic RNA. (**A**) Self-primed extension reaction of synthetic RNA. (**B**) Denaturing (20% urea) gel electrophoretic analysis: radiolabeled 24-base synthetic RNA was reacted with T7 RNA polymerase for 0 min, 5 min and 4 h, in the absence (−) or presence (+) of capture DNA. See Supplementary Figure S1 for quantification. (**C**) DNA captures the 3′ end of the RNA, competing with self-primed extension.

Gel electrophoretic analyses

Reaction products were analyzed with 20% polyacrylamide, denaturing (7 M urea) gel electrophoresis. For self-primed extension reactions, the 5′ end of the synthetic RNA was labeled by incubating 50 μM synthetic RNA with $[\gamma\text{-}^{32}P]$ ATP (PerkinElmer) and 1 μl (10 Units) of T4 polynucleotide kinase (New England BioLabs) in a 10 μl reaction volume at 37°C for 30 min. Labeling was inactivated by heating at 65°C for 20 min. Transcribed RNAs were labeled by including $[\alpha\text{-}^{32}P]$ ATP (PerkinElmer) in the reaction mixture (without reducing the concentration of ATP). All gel experiments were repeated at least twice and showed no significant differences. Quantifications of gel data, using ImageJ v1.52a (42,43; https//imagej.nih.gov/ij/) on unprocessed and uncompressed TIF output, are presented in Supplementary Material.

RESULTS

The determination that 3′ end additions arise from (transient, active site bound) RNA structures in which the 3′ end of the nascent RNA folds back on itself to prime extension (22), points to a potential intervention to competitively inhibit this process. The addition to the reaction of a short capture DNA complementary to the 3′ end of the expected runoff RNA should drive formation of an RNA–DNA hybrid, as shown in Figure 1, inhibiting self-primed extension. However, the possibility exists that RNA polymerase might in turn carry out primed synthesis from the 3′ end of the capture DNA (44), generating partially chimeric double stranded impurities. To prevent such 3′ extension of the DNA, for all capture DNAs used here we replace the 3′ sugar hydroxyl of the 3′ terminal base with an amino group. While other 3′ sugar modifications should similarly prevent nucleotide addition (phosphoryl transfer), 3′ amino modification of DNA is commercially available during synthesis, at minimal added expense.

Self-primed extension of synthetic RNA is inhibited by 3′ complementary capture DNA

Our previous work demonstrated self-primed extension of a specific 24-base RNA, synthesized as runoff from a DNA template or synthesized chemically (22). In that earlier work, we demonstrated very efficient self-primed extension from synthetic RNA, in the absence of T7 promoter DNA. The (same) synthetic RNA construct shown in Figure 1A (25 μM) was incubated with T7 RNA polymerase and 0.4 mM of each NTP for 0 min, 5 min and 4 h at 37°C. The results shown in Figure 1B in the absence (−) of 25 μM capture DNA show that in a 5 min reaction the synthetic RNA readily extends to longer RNA products that are chased to still longer products over 4 h, as observed previously (22).

As predicted, the results in Figure 1B show that in the presence (+) of 25 μM of the 17-base capture DNA (Figure 1C), self-primed extension is dramatically inhibited. This demonstrates that the presence of 3′ complementary single stranded capture DNA is an effective way to competitively prevent 3′ end additions.

Effect of varying the length of capture DNA in self-primed extension

The above data confirm that 17 bases of complementarity provides sufficient target affinity for maximal competitive binding. How short can capture DNA be to still effectively compete with functional binding of the free RNA to RNA polymerase? To test the effect of the length of capture DNA, we conducted self-primed extension reactions with 25 μM synthetic RNA in the presence of 25 μM capture DNA oligonucleotides with lengths of 14, 11 and 9 bases and compared them with 17-base capture DNA. All capture DNAs were designed to hybridize to the RNA from its 3′ end, included a 3′ amino modification, and were added to the reaction in equal concentrations to the synthetic RNA.

As shown in Figure 2, a 9-base capture DNA (Capture-9) is unable to compete well with self-primed extension; the product profiles at both 5 min and 4 h are similar to those in the reaction containing no capture DNA. In contrast, the 11-base capture DNA (Capture-11) limits self-primed extension in a 5 min reaction, but shows significant self-primed extension in a 4 h reaction. To a first approximation, at 37°C, the 14-base capture DNA (Capture-14) functions about as well as the 17-base capture DNA (Capture -17) in preventing unwanted self-primed extension.

Capture DNA prevents self-primed extension during high yield transcription

We have previously shown that, as commonly carried out, when pushing transcription reactions to high yield conditions (for example, 7.5 mM each NTP and 4 h incubation at 37°C), the desired runoff RNA can be a minority of the product (22). The high concentration of runoff RNA drives its rebinding to the polymerase to effectively compete with *de novo* initiation, and through the mechanism described above, generates *n+1*, *n+2* and substantially longer RNA products. Just as a 3′ complementary capture DNA can block rebinding; we expect that it should also inhibit rebinding from competing with initiation during transcription. To
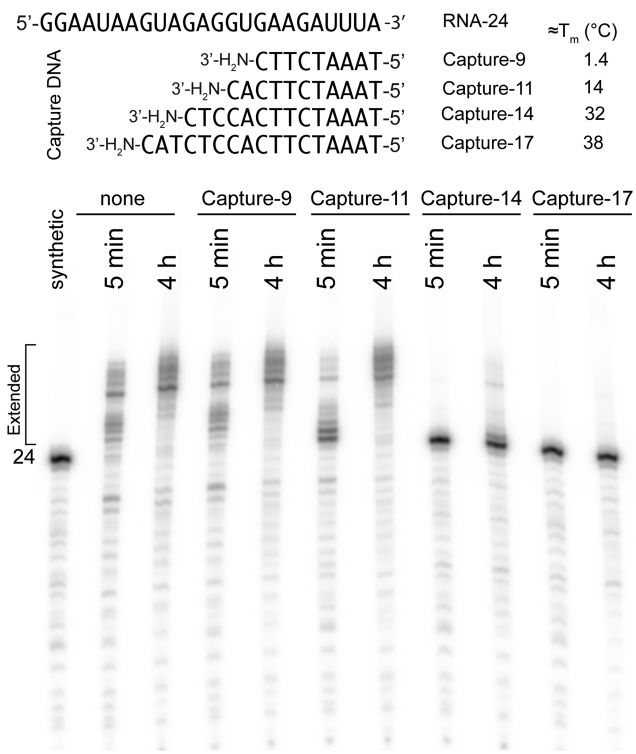

Figure 2. Length optimization of capture DNA. Denaturing gel analysis, as in Figure 1B, in the absence and presence of 3′-modified complementary capture DNA oligonucleotides of lengths 9, 11, 14 and 17-bases. Reactions were carried out at 37°C; approximate predicted melting temperatures (51) are shown for the RNA/DNA duplexes. The sequence of the RNA is presented for reference. See Supplementary Figure S2 for quantification.

test this expectation, we added excess capture DNA to an *in vitro* transcription reaction with template DNA encoding the above 24-base RNA (with the same 3′ end as above, and with only a slight difference in the sequence from position +4 to +6).

The results shown in the lanes labeled '24' in Figure 3A and B demonstrate that, as expected, the presence (+) of 400 μM 3′ complementary capture DNA during a 4 h, high yield transcription reaction dramatically reduces formation of primer-extended products relative to the reaction in the absence of capture DNA (−). Encoded expected length RNA product increases substantially, while primer-extended products are reduced dramatically.

We previously observed that not all RNAs efficiently prime self-extension (22). To confirm that the presence of capture DNA does not interfere with synthesis of RNAs with low propensity to form primer extended products, we carried out identical transcription reactions on a template encoding RNA-24Alt that has been shown not to generate large amounts of longer RNA products. The results presented in Figure 3 in the lanes labeled '24Alt' confirm similar transcription profiles in both the presence (+) and absence (−) of capture DNA complementary to 24Alt RNA, confirming that capture DNA has no negative effect on transcription efficiency.
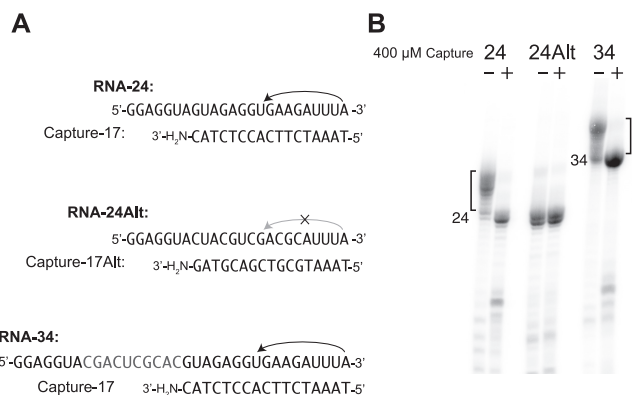
Figure 3. Capture DNA eliminates self-primed extension during RNA synthesis. Presence of 3′-complementary capture DNA sequesters product RNA, inhibiting self-primed extension during transcription. (**A**) Encoded RNAs and corresponding capture DNAs. (**B**) Denaturing (20% urea) gel analysis of 4 h high yield transcription reactions. Transcripts were labeled by [α-^{32}P] ATP. Lanes '24' above demonstrate clearly that Capture-17 inhibits formation of primer extended products. Lanes '24Alt' show that for an RNA sequence that does not promote self-primed extension, the presence of Capture-17 has no effect. At last, lanes '34' show that the effect is not dependent on the length of the RNA and that self-primed extension proceeds farther on longer RNAs. See Supplementary Figure S3A for quantification.

Generalizability of the 3′ capture DNA—length and sequence of the RNA

The RNAs synthesized above are short, allowing high resolution in the electrophoretic analysis of primer extended RNA products. The capture DNA at 17 bases, however, is only a bit shorter than the 24-base RNA itself. In order to extend the observation to longer length RNA, we used the same 17-base 3′ complementary capture DNA to sequester a RNA with the same 3′ terminal sequence, but with a 10 base insertion in the upstream, uncaptured region of the RNA, yielding a 34-base RNA, labeled '34' in Figure 3. The results shown in Figure 3 are essentially identical to the results on the shorter 24-base RNA, indicating the expected inhibition of self-primed extension in the presence of the complementary capture DNA.

Moreover, to confirm that this approach can be used as a general method to prevent self-primed extension we tested the effect of capture DNA for transcription on a template encoding a 24-base RNA with a different sequence, labeled '24B' in Supplementary Figure S3B. The results presented in Figure S3B show that addition of capture DNA to the transcription mixture can be used as a general method to inhibit self-primed extension for templates encoding different RNA sequences.

Titration of capture DNA oligonucleotide

The above experiments were carried out with 400 μM capture DNA in solution on the assumption that the RNA would not accumulate to levels higher than 400 μM. To examine the effect of the concentration (total amount) of capture DNA, we conducted transcription reactions under high yield condition in the presence of concentrations of capture DNA ranging from 400 μM down to 100 μM. The results presented in Figure 4A, show that while 400 μM cap-
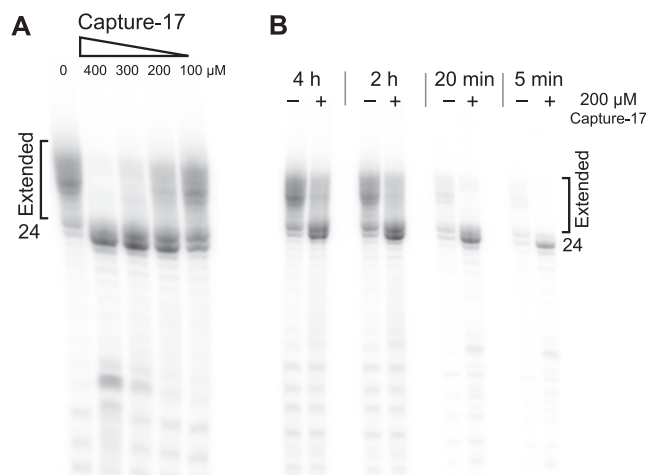
Figure 4. Effect of concentration/stoichiometry of capture DNA. Denaturing (20% urea) gel electrophoreses analysis. All transcripts were labeled by [α-^{32}P] ATP. (**A**) High yield transcription reaction (4 h) for RNA-24 in the presence of varying concentrations of complementary capture DNA (Capture-17). (**B**) Time dependence of transcription reaction products in the presence (+) or absence (−) of 200 μM capture DNA. See Supplementary Figure S4 for quantification.

ture DNA dramatically reduces RNA 3′ extension, reducing capture DNA concentration to 300, 200 and 100 μM allows for increasing self-primed extension. These results are consistent with the expected stoichiometric titration of capture DNA by increasing product RNA. As product RNA concentrations exceed that of capture DNA, residual free RNA now rebinds to RNA polymerase and primes extension.

A more subtle interpretation might be that lower concentrations of capture DNA yield lower fractional formation of RNA–DNA capture complex, due to incomplete binding. To test this, we carried out transcription reactions in the presence of 200 μM capture DNA as a function of time. Consistent with the limiting stoichiometry model, at the lower reaction times of 5 and 20 min (and therefore lower RNA concentrations), this lower amount of capture DNA nevertheless effectively inhibits self-primed extension (Figure 4B). The results confirm that the concentration of capture DNA (of tight binding length and sequence) needs only to exceed the final concentration of RNA synthesized in the reaction.

DISCUSSION

Formation of longer RNA products during the synthesis of expected runoff RNA has been reported by various research groups over the years (1,17–23,45,46). The origin of these undesired longer RNA products synthesis has been attributed to different mechanisms, including turn-around synthesis, in which RNA polymerase switches to the non-template strand at the 5′ end of the DNA template (46), non-templated addition (1,17,18), and primer extension (19–23). In recent work using RNA-Seq to characterize products of transcription, we have shown that *cis* self-primed extension is the main mechanism leading to synthesis of these unexpected RNA products (22). In *cis* self-primed extension, as the runoff RNA accumulates, it can rebind to the RNA

polymerase in a mode wherein it folds back on itself, priming transcription from its own upstream sequence as the template. Self-primed extension of the runoff RNA leads to a reduction in the yield of the expected RNA product, but more importantly, yields partially double stranded RNA impurities that may interfere with its applications (e.g., triggering the innate immune response in therapeutic applications).

In order to eliminate self-primed extension during high yield synthesis, we have introduced a new approach involving the addition to the reaction of a short capture DNA that is complementary to the 3′ end of the expected runoff RNA. Hybrid duplex formation effectively sequesters the RNA 3′ end, preventing it from looping back on itself (or binding in *trans* to another RNA) to prime RNA-templated extension. Furthermore, in order to exclude the possibility that RNA polymerase uses this capture DNA as a primer, we introduced a minor and inexpensive modification to capture DNA: replacement of the 3′ hydroxyl group by an amino group in the current study, but other modifications should readily serve the same function.

Model studies with synthetic RNA

As reported previously, incubation of synthetic RNA, RNA polymerase and substrate NTPs yields substantial self-primed extension (22). In contrast, the results presented in Figure 1 show that the presence of a 1:1 ratio of 17-base capture DNA leads to a dramatic reduction in the formation of primer extended products.

Effective competition by 3′ capture DNA should depend on the affinity of capture DNA for the 3′ end of the RNA. Very approximate predictions suggest that 17-base capture DNA should bind to its target RNA with a (free in solution) T_m of about 38°C. Shortening capture DNA to 14, 11, and 9 bases will weaken affinity, as reflected in the predicted melting temperatures. Not surprisingly, the results in Figure 2 demonstrate that capture DNA complementary to only the 3′ terminal 9 bases does not inhibit self-primed extension significantly. A 3′ complementary 11-base capture DNA shows evidence of competition but is not effective at long reaction times. However, a 3′ complementary 14-base capture DNA does provide effective competition. While it may seem surprising that oligonucleotides with relatively weak predicted (in solution) affinities compete well, it must be remembered that competition is relative to hairpins with only 2–3 base pair (internal) stems. We have proposed that the latter functions in self-primed extension due to interactions within the enzyme binding cleft that stabilize otherwise weak solution structures. It is possible that capture DNA benefits from similar stabilization, but of course, does not extend because of its 3′ amino modification.

Application to transcription reactions

Extending the above to *in vitro* transcription reactions, we observed a similar inhibition of self-primed extension in the presence of capture DNA, without substantial inhibition of promoter-directed initiation (Figure 3). While pre-

cise quantification of total RNA is complicated given the spread in product lengths, assays with a DNA template encoding RNA that does not undergo significant self-primed extension (24Alt in Figure 3), does not show inhibition of promoter-directed synthesis by the appropriate 17-base capture DNA. This suggests that RNA-DNA hybrid binding to RNA polymerase is not overly strong, relative to promoter binding and *de novo* initiation.

To generalize these results to longer RNAs, we inserted 10 additional bases into the template DNA sequence encoding RNA-24, yielding a new construct encoding a 34-base RNA (RNA-34). The results shown in Figure 3 yield identical behavior: in the absence of capture DNA, self-primed extension predominates and in the presence, it is dramatically reduced. We fully expect that any length RNA will benefit from this approach.

As an aside, this experiment also provides preliminary information on the possible lengths of self-primed extension. In our original study, self-primed extension showed a range of extended lengths, but rarely continued to the maximum theoretical length, corresponding to the 5′ end of the RNA (22). It could be that self-primed extension is fundamentally limited to short lengths, or alternatively, the functional complex may weaken as it nears the 5′ end of the templating RNA. The results with promoter DNA encoding a 34-base transcript strongly suggest the latter. Noting the compression of longer length RNAs inherent in gel electrophoresis (see, for example, the DNA standards in Figure 3), it appears that self-primed extension on 34-base RNA is extending to longer added lengths than on 24-base RNA. This predicts that still longer RNA–RNA duplex formation will occur on still longer RNAs, and indeed other studies have observed very long extensions (20,47).

To demonstrate the sequence generality of this approach, we tested the effect of capture DNA on templates encoding different RNA sequence with high abundance of self-primed extension products. The results in Supplementary Figure S3B indicate inhibition of self-primed extension in the presence of capture DNA complementary to the 3′ end of RNA.

It is expected in this competitive inhibition that when runoff RNA concentrations exceed that of capture DNA, the free residual RNA will now begin to prime self-templated extension. The results presented in Figure 4A are consistent with this prediction, as lower concentrations of capture DNA during transcription allow for some self-primed extension during preparative-scale synthesis. Even 200 μM capture DNA allows for essentially complete inhibition of self-primed extension at short times / low RNA levels, but as transcription proceeds and RNA concentrations increase, self-primed extension begins to increase, as capture DNA is consumed.

At last, most researchers will want to remove the capture DNA from the reaction and variety of approaches are common, as reviewed recently (48–50). The preferred solution will likely depend on the length of the RNA. While denaturing gel purification will readily separate product from capture DNA (and from enzyme, promoter DNA and abortive products), for RNAs significantly longer than the capture DNA, simpler commercial kits exist.

SUMMARY

We demonstrate here a simple and practical solution to unwanted RNA self-primed extension, allowing for dramatically improved RNA purity during *in vitro* transcription, prior to any subsequent purification. Since runoff RNAs are not extended to long products, overall yields will also typically increase. The results presented also broadly lay out some of the experimental design considerations necessary to optimally achieve this benefit. There is every reason to expect that this approach should be applicable to RNAs of any length or sequence. Although intrinsic RNA structure involving the target region may compete with capture DNA binding, the intrinsic structure alone may be sufficient to limit self-primed extension (20).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

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