Versatile T7 RNA polymerases that generate less immunostimulatory mRNA

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Introduction

In vitro transcription (IVT) utilizing T7 RNA polymerase (T7 RNAP) for the synthesis of messenger RNA (mRNA) is efficient and well-established for the production of large quantities of synthetic RNA. However, T7 RNAP can generate undesirable, immunostimulatory mRNA byproducts such as double-stranded RNA (dsRNA). To reduce these concerns, we rationally engineered a series of T7 RNAP mutants and compared their functional properties to those of wild-type and commercially available T7 RNAP. Among these characteristics are dsRNA, circular RNA, processivity, and yield.

RNA contaminants: 3'-extension and duplex RNA

3'-extensions and duplex RNA are ubiquitous. There is a general tendency for longer transcripts to be produced by all T7 RNAPs, regardless of the DNA template that is being used. In addition, all T7 RNAPs have a propensity to generate long duplex dsRNA transcripts from any and all DNA templates that have been examined. Several strategies, including post-synthesis purification methods and optimization of reaction constituents, have been proposed to reduce such byproducts. Purification techniques require the use of special resins and complex purification processes, while the optimization of reaction constituents necessitates customized conditions but is not 100% effective.

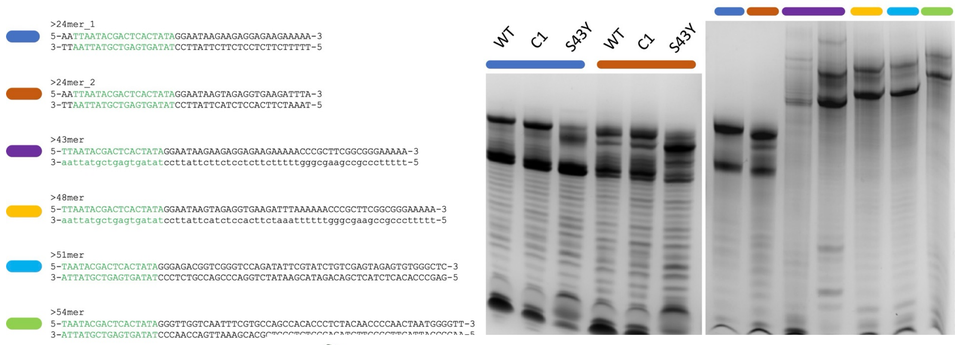


Figure 1. Various DNA templates transcribed using WT, commercial, and mutant T7 RNAPs. T7 RNAP tends to produce longer transcripts, irrespective of the DNA template.

Rational design of T7 RNAP

To develop T7 RNAP mutants with fewer immune-stimulatory byproducts, we rationally mutated based on functional structures.

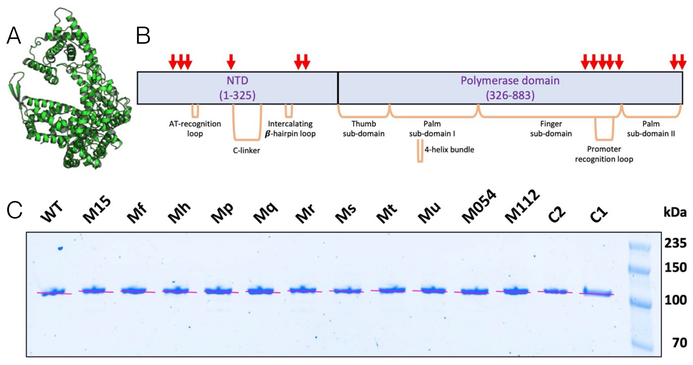


Figure 2. T7 RNAP subunit (A) and its sequence with relevant functional regions (B) engineered to create our T7 RNAP mutants (C).

CircRNA analysis of engineered T7 RNA polymerase

Long duplex RNA (dsRNA) cannot become circular RNA (circRNA). Hence, more circRNA would most likely be formed if the transcript sample contained less dsRNA. To analyze our T7 RNAP mutants for circRNA formation, we developed a Urea-PAGE method for analyzing circRNA.

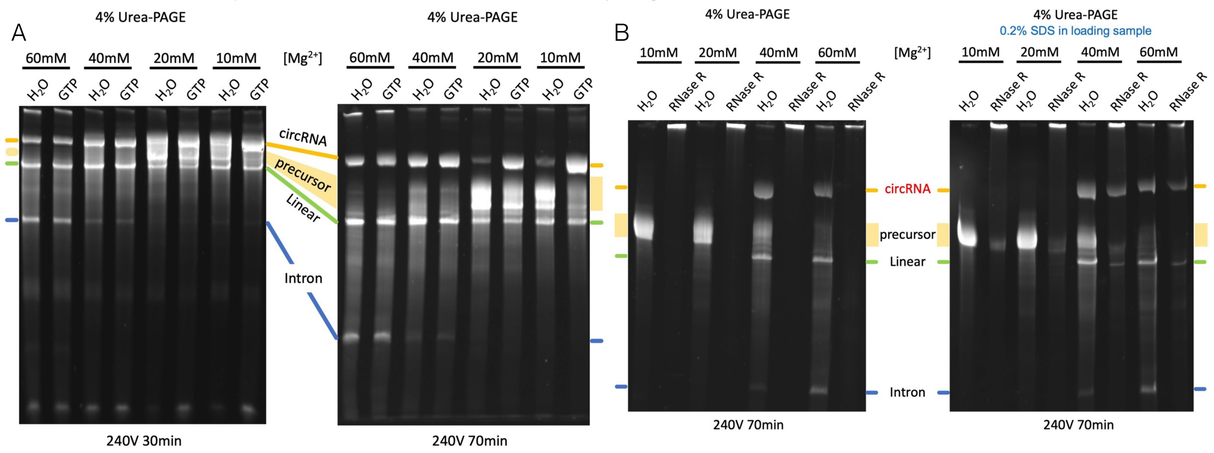


Figure 3. (A) We optimized a denaturing Urea-PAGE to analyze circRNA formation. (B) Using RNase R, we identify the location of circRNA and its byproducts.

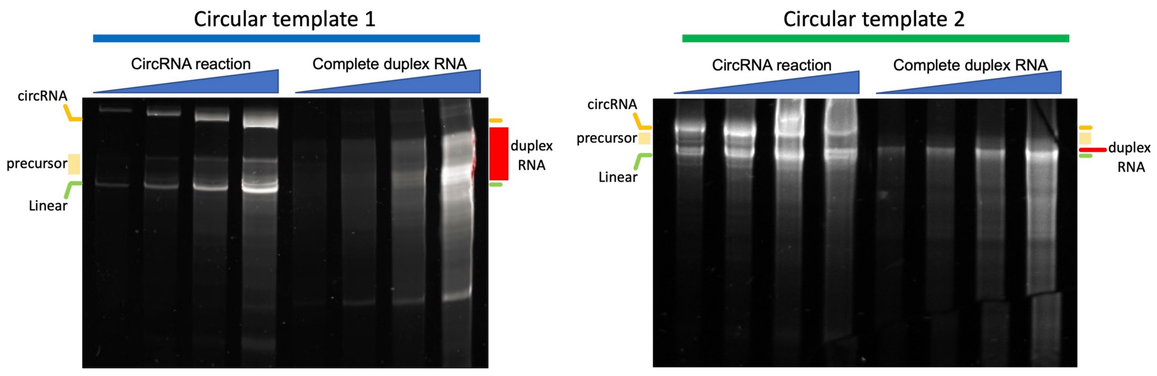


Figure 4. Templates analyzed for circRNA and duplex RNA using our Urea-PAGE analysis method.

Engineered T7 RNA polymerase can reduce duplex RNA

Fewer long, sense-antisense duplex RNAs (aka dsRNAs) are generated by some of our T7 RNAP mutants (Figure 5). Additionally, we tested the immunostimulatory activity of our T7 RNAP mutants using MDA5 ATPase activities (Figure 6). Some of our mutants stimulate far less MDA5 filament formation or ATPase activity than the wild type or C1.

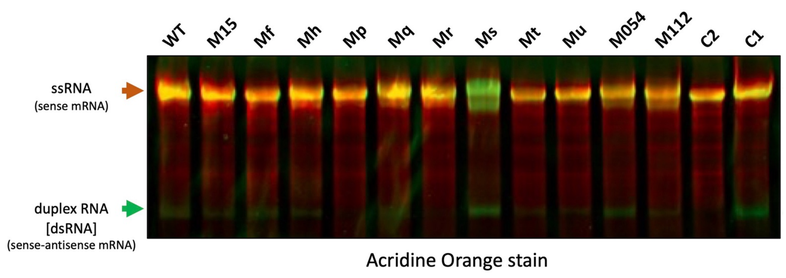


Figure 5. dsRNA byproducts analyzed on RNAs transcribed using our T7RNAP mutants via acridine orange. Acridine orange intercalates into dsRNA and produces green fluorescence at 530 nm and electrostatically binds to the phosphate groups of single-stranded mRNA and produces red fluorescence at 640 nm.

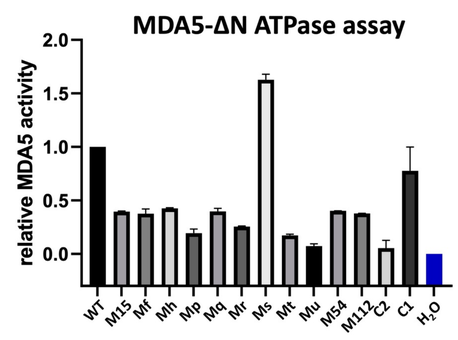


Figure 6. MDA5 ATPase activity measured on RNAs transcribed using our T7 RNAP mutants. The amount of dsRNA byproducts observed in the transcripts correlates with cellular MDA5 signaling activity and MDA5 ATPase activity.

T7 RNAP mutants produce more circRNA

We demonstrate via our Urea-PAGE analysis that some our T7 RNAP mutants produce more circRNA.

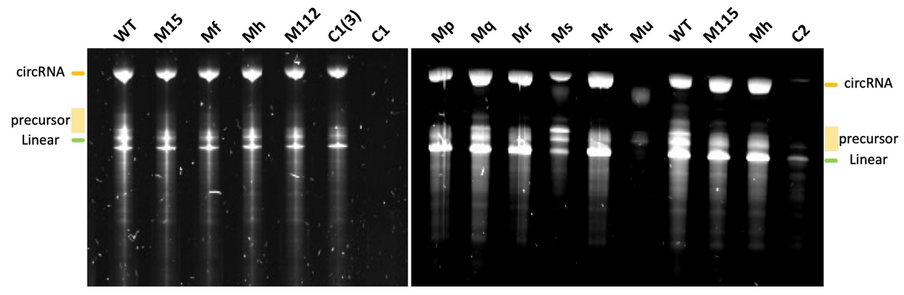


Figure 7. Wild type, commercial, and mutant T7 RNAPs were analyzed for circRNA, precursor RNA, and linear RNA using Urea-PAGE analysis. Some mutants produce less circRNA.

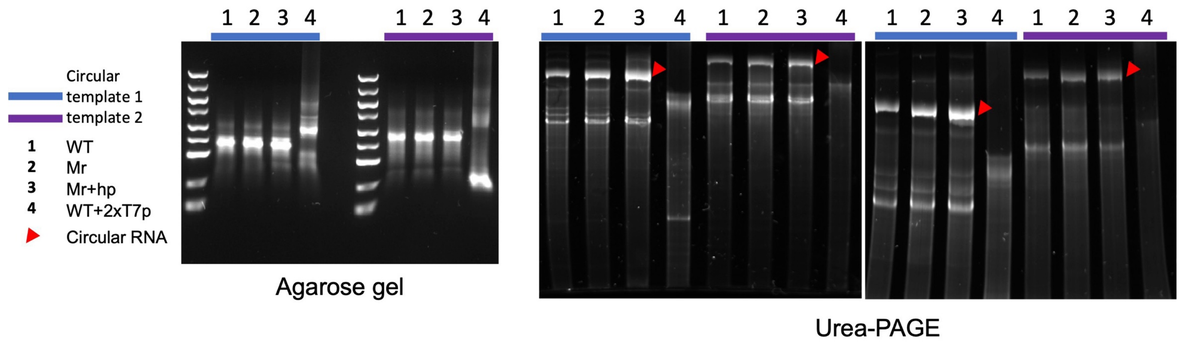
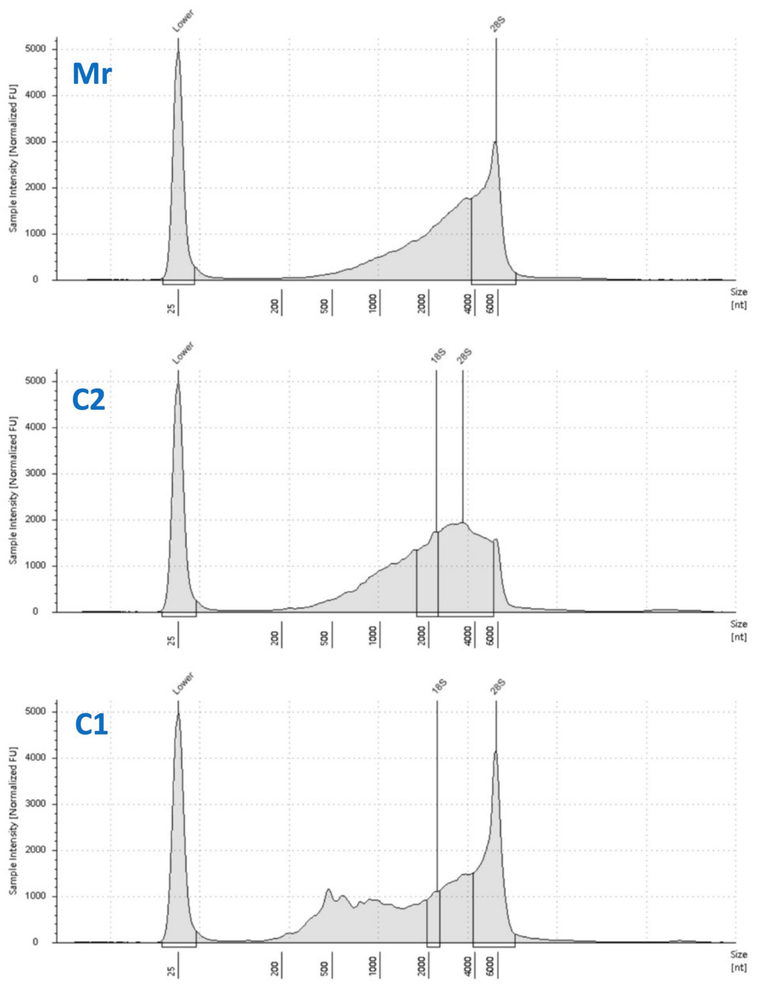


Figure 8. Using our T7 RNAP mutants in conjunction with template sequence optimization can result in more circRNA formation.



Processivity of T7RNAP Mutants

One of the T7RNAP mutants (Mr) can produce RNA transcripts that are longer than its competitors.

Figure 9. Commercial and mutant T7 RNAPs were used to generate 10k base mRNA and processivity was analyzed using capillary electrophoresis (Agilent 4150 Tapestation).

Conclusion

A series of rationally designed T7 RNAP mutants were produced to reduce the immunostimulatory content of mRNA therapeutics. Mutants displayed a range of behaviors, including reduced dsRNA, high circular RNA, and improved processivity. These improved T7 RNAP variants have the potential to facilitate the synthesis of immunostimulatory-free mRNA.

References

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