Overexpression and purification of T7 RNA Polymerase (T7RNAP) wild-type

The purification of T7RNAP wild-type (WT) exploited immobilized metal affinity-chromatography and was performed essentially as described earlier,[1] albeit with minor changes as outlined below. *Escherichia coli* BL21(DE3) cells were transformed with two plasmids: pQE9T7 (amp^r) and pREP4 (kan^r) which encode $(His)_6$-T7RNAP WT and *lac* repressor, respectively, and grown on a lysogeny broth (LB) agar plate containing 100 µg/mL carbenicillin and 35 µg/mL kanamycin at 37°C for 16 h. A single colony was picked to inoculate 5 mL of LB containing 100 µg/mL carbenicillin and 35 µg/mL kanamycin, and grown at 37°C for 16 h with shaking at 200 rpm. The 5-mL culture was then used to inoculate 500 mL of LB containing 100 µg/mL carbenicillin and 35 µg/mL of kanamycin, and grown at 37°C with shaking at 200 rpm until OD_{600} ~0.6. The culture was induced by addition of isopropyl-ß-D-thiogalactoside (IPTG) to a final concentration 1 mM, and then grown at 37°C for an additional 2.5 h. Cells were harvested by centrifugation at 6,000 g for 20 min, and the pellets stored at -80°C until further use.

A 500-mL cell pellet was thawed on ice and then resuspended in buffer A [100 mM sodium phosphate (pH 7.7), 300 mM NaCl, 0.5 mM DTT, 1 mM PMSF, 2 mini EDTA-free protease inhibitor tablets (Roche)]. Cells were lysed by sonication (60% amplitude, 12-min of alternating cycles of 4 s ON and 10 s OFF; Cole Parmer 130-Watt Ultrasonic Processor model GEX130) and the crude lysate was subjected to centrifugation at 20,000 g for 1 h at 4°C. The supernatant obtained was filtered through a 0.45 µm syringe filter. After addition of 20 mM imidazole to the filtered supernatant, it was loaded onto a pre-equilibrated 5-mL HisTrap HP (GE Healthcare) column. An ATKA FPLC (GE Healthcare) was used for the subsequent elution. The column was washed with 50 mL buffer B [100 mM sodium phosphate (pH 7.7), 300 mM NaCl, 0.5 mM DTT, 1 mM PMSF, 20 mM imidazole], and T7RNAP WT was eluted using a 100-ml linear gradient from 20-300 mM imidazole at a flow rate of 3 mL/min. T7RNAP WT typically eluted between 170-210 mM imidazole. Peak fractions, as judged by SDS-PAGE analysis, were pooled and subsequently dialyzed against 500 mL of buffer C [50 mM sodium phosphate (pH 7.7), 100 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 1 mM EDTA and 50% (v/v) glycerol] at 4°C overnight after one buffer exchange after 2 h. Aliquots of the purified T7RNAP (dialysate) were stored at -80°C until further use in in vitro transcriptions (IVTs).

Overexpression and purification of T7RNAP P266L

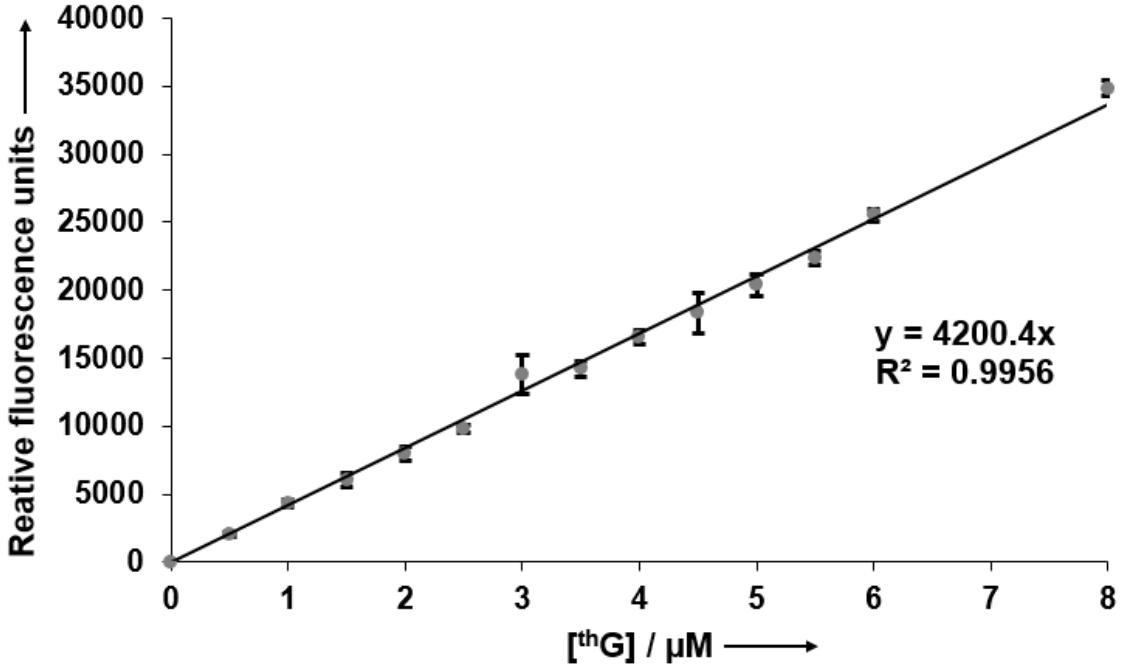
The overexpression and purification of $(His)_6$-T7RNAP P266L was based on a protocol kindly provided by the laboratory of Prof. Thomas A. Steitz, Yale University,[2] with some notable modifications (exclusion of a polishing step as well as dialysis and final preparative steps). *E. coli* BL21(DE3) cells were transformed with pBH161 (a gift from the Steitz laboratory), which encodes $(His)_6$-T7RNAP P266L, and grown on an LB-agar plate containing 50 µg/mL carbenicillin at 37°C for 16 h. A single colony was picked to inoculate 5 mL of Terrific Broth (TB) containing 50 µg/mL carbenicillin and the culture was grown at 37°C for 16 h with shaking at 200 rpm. This 5-mL seed culture was added to 500 mL of TB containing 50 µg/mL carbenicillin and grown at 37°C with shaking at 200 rpm until OD_{600} ~1. Next, carbenicillin was supplemented to a final concentration of 100 µg/mL and the culture induced with 1 mM IPTG at 37°C for 4 h. Cells were harvested (as described above for WT) and the pellets stored at -80°C.

For purification of T7RNAP P266L, a 500-mL cell pellet was thawed on ice and then resuspended in buffer D [10 mM Tris-HCl (pH 8.0), 375 mM NaCl, 0.5 mM DTT, 1 mM PMSF, 2 mini EDTA-free protease inhibitor tablets (Roche)]. Subsequent steps mirrored the T7RNAP WT procedure described above with the exception of use of a 1-mL HisTrap (GE Healthcare) column. After washing with 10 mL of buffer E [10 mM Tris-HCl (pH 8.0), 375 mM NaCl, 0.5 mM DTT, 1 mM PMSF, 20 mM imidazole], T7RNAP P266L was eluted using a 12-ml linear gradient from 20-200 mM imidazole. T7RNAP P266L typically eluted between 140-180 mM imidazole. Peak fractions, as assessed by SDS-PAGE analysis, were pooled and subsequently dialyzed in 500 mL of buffer F [100 mM sodium phosphate (pH 7.7), 200 mM NaCl, 2 mM DTT, 0.2 mM PMSF, 2 mM EDTA] at 4°C for 3 h with buffer exchanges every hour. An equal volume of glycerol was added to the dialysate and the solution was mixed at 4°C using a nutator. Aliquots of the purified T7RNAP P266L [in 50% (v/v) glycerol)] were stored at -80°C until further use.

References

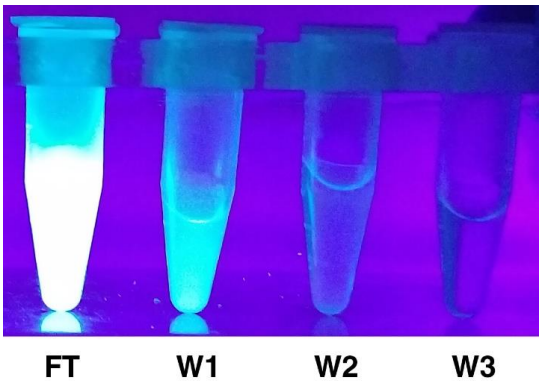
[1] T. Ellinger, R. Ehricht, *BioTechniques* **1998**, *24*, 718-720.

[2] K. J. Durniak, S. Bailey, T. A. Steitz, *Science* **2008**, *322*, 553-557.

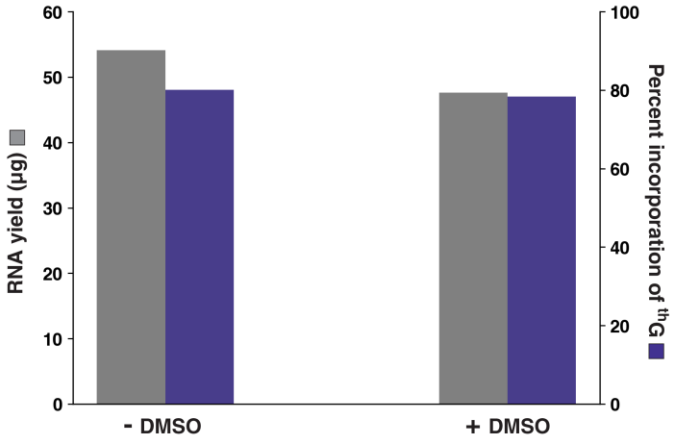


Supplementary Figure S1. Standard curve used to determine percent incorporation of thG into pre-tRNA^{Cys}. Mean and standard deviation values were calculated from three independent measurements.

Supplementary Figure S2. T7 RNAP P266L yields fewer abortive transcripts than WT when IVTs are performed with thG. IVTs to generate pre-tRNA^{Cys} were performed using either T7 RNAP WT or P266L as described in the main text, except each reaction also contained 10 µCi of α-^{32}P-GTP (Perkin Elmer, Shelton, CT). Transcription was terminated by addition of a loading dye containing 7 M urea and 20% (v/v) phenol. One-tenth of the 20 µL IVT reaction was loaded on a 7% (w/v) polyacrylamide/7 M urea gel (40 cm length x 20 cm width), and electrophoresed for 75 min at 30 mA. The gel was then exposed for 2 h to a phosphorimager screen, and IVT products were visualized using an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare). Based on quantitation performed using ImageQuant 5.1 software, there is a 1.3-fold increase in the full-length pre-tRNA^{Cys} and a corresponding decrease in aborted transcripts.



Supplementary Figure S3. Efficacy of the Zymo Clean and Concentrator™-25 approach to remove unincorporated thG. Tubes were illuminated at 312 nm using a Fotodyne UV transilluminator to visualize the removal of free thG. FT: flow-through; W1: wash using the RNA prep buffer (400 µl); and W2: wash using the RNA wash buffer (700 µl). W3: wash using the RNA wash buffer (400 µl). Short RNAs <18 nt are expected to be removed by this desalting approach using the recommended volumes (according to the manufacturer).



Supplementary Figure S4. RNA yield and percent incorporation of thG into pre-tRNA^{Cys} using T7RNAP P266L in the absence or presence of 5% (v/v) DMSO. The data reported here are from a single IVT trial.