T7 RNA POLYMERASE VARIANTS WITH EXPANDED SUBSTRATE RANGE AND ENHANCED TRANSCRIPTIONAL YIELD

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Provisional Application No.: 61/968,231 filed March 20, 2014. The entire text the above-referenced disclosure is specifically incorporated herein by reference without disclaimer.

BACKGROUND OF THE INVENTION

A. Field of the Invention

[0002] The invention generally concerns the field of protein engineering. More particularly, disclosed herein are variants of T7 RNA polymerase with the ability to incorporate modified nucleotides and with enhanced transcriptional activity.

B. Description of Related Art

[0003] RNA is widely versatile and useful, but its chemical instability can render it unsuitable for many therapeutic and biotechnology functions. Oligonucleotides with altered chemistry, especially modifications of 2' position of the (deoxy)ribose have proven to be of great value (Wilson & Keefe, 2006). 2'-O-methyl RNA has a greater Tm, faster kinetics, and greater stability as antisense probes (Majlessi, et al., 1998) and siRNA with 2'F and 2'-O-methyl RNA have also proven to be more stable and target-specific (Layzer, 2004; Kraynack & baker, 2006; Jackson, et al., 2006; Dean & Bennet, 2003. Additionally, in vitro selection with 2' modified NTPs has yielded aptamers and ribozymes with greater stability and enhanced chemical potential (Healy, et al., 2004; Waters, et al., 2011; Lupold, et al., 2002; Keefe & Cload, 2008; Burmeister, et al., 2005; Beaudry, et al., 2000).

[0004] While modified RNA can be chemically synthesized it is often preferable to enzymatically produce it (especially for in vitro selection) (Ellington & Szostak, 1990). T7 RNA polymerase has long been utilized for the generation of RNA in vitro, and has previously been engineered and evolved to have an expanded substrate range. Most famously, the Y639F mutant allows for the polymerization of RNA transcripts contain nucleotides with 2'-Fluoro and 2'-amino modified ribose (Kostyuk, et al., 1995; Sousa & Padilla, 1995; Huang, et al., 1997). A further mutation, H784A, is thought to eliminate premature termination following the incorporation of a modified nucleotide, and the Y639F,

H784A ("FA") double mutant can incorporate nucleotides with bulky modifications at the 2' position (e.g. 2'-O-methyl)(Padilla & Sousa, 2002; Brieba & Sousa, 2004).

[0005] A directed evolution approach, in which the aforementioned Y639 and H784 residues, as well as the important R425 and G542 were randomized, has been previously employed to create further T7 RNA polymerase variants with expanded substrate specificity (Chelliserrykattil & Ellington, 2004). The resulting library was enriched for T7 RNA polymerase variants that retained the ability to transcribe RNA in vivo (with natural ribose) and the screened for altered substrate specificities in vitro. A mutant, termed "RGVG," (R425, G542, Y639V, H784G plus additional E593G and V685A mutations that arose organically during the selection) showed strong activity with 2'-O-methyl UTP. A second mutant, termed "VRS," (G542V and H784S as well as the additional H772R mutation) was able to incorporate 2'-Fluoro modified pyrimidines. More recent works have also uncovered the "2P16" mutant (a version of RGVG with seven additional mutations (Siegmund, et al., 2012)) and the R425C mutant (Ibach, et al., 2013). Each of these mutants is reported to enable the creation of 2'-O-methyl RNA.

[0006] While the unique catalytic properties of these enzymes make them useful tools, several of them suffer from low activity, even with normal ribonucleotides. It has been proposed that mutations that confer new activity in an enzyme also destabilize the protein, rendering it less active overall, with low transcriptional yields (Wang, et al., 2002; Romero, et al., 2009).

SUMMARY OF THE INVENTION

[0007] The present application offers a solution to the current low activity problems associated with T7 RNA polymerase variants that are able to incorporate modified nucleotides. In certain aspects, disclosed are T7 RNA polymerase variants with mutations that can increase the activity of mutants that have expanded substrate range. The resulting polymerase mutants can be used to generate 2'-O-methyl modified RNA with yields much higher than enzymes currently employed.

[0008] Disclosed is a T7 RNA polymerase variant comprising: one or more substrate-broadening amino acid substitutions that confer an enhanced ability to incorporate 2'-modified mononucleotides compared to a wild-type T7 RNA polymerase; and one or more activity-enhancing amino acid substitutions that increase the transcriptional activity of the T7 polymerase variant relative to T7 polymerase variants without the activity-enhancing amino

acid substitutions. In some embodiments, the one or more substrate-broadening amino acid substitutions comprise one or more of the following amino acid substitutions relative to the wild-type T7 RNA polymerase sequence of SEQ ID NO:1: G542V, E593G, Y639V, Y639F, V685A, H772R, H784A, H784S, and H784G. In some embodiments, the one or more substrate-broadening amino acid substitutions comprise G542V, H772R, and H784S. In some embodiments, the one or more substrate-broadening amino acid substitutions comprise Y639F. In some embodiments, the one or more substrate-broadening amino acid substitutions comprise Y639F and H784A. In some embodiments, the one or more substrate-broadening amino acid substitutions comprise E593G, Y639V, V685A, and H784G. In some embodiments, the 2'-modified mononucleotides that the T7 RNA polymerase variant is capable of incorporating into a growing RNA strand comprise one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, 2'-fluoro GTP, 2'-amino CTP, 2'-amino UTP, 2'-amino ATP, 2'-O-methyl UTP, 2'-O-methyl ATP, 2'-O-methyl CTP, and 2'-O-methyl GTP. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise one or more of the following amino acid substitutions: P266L, S430P, N433T, S633P, F849I, and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise two or more of the following amino acid substitutions: P266L, S430P, N433T, S633P, F849I, and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise S430P, N433T, S633P, F849I, and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise P266L, S430P, N433T, S633P, F849I, and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise P266L. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise S633P and F849I. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise S633P and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise F849I and F880Y. In some embodiments, the one or more activity-enhancing amino acid substitutions comprise S633P, F849I, and F880Y.

[0009] Also disclosed is a T7 RNA polymerase variant comprising the following amino acid substitutions: N433T, E593G, Y639V, V685A, H784G, S430P, S633P, F849I, and F880Y.

[0010] Also disclosed is a nucleic acid molecule encoding any of the T7 RNA polymerase variants described above. Also disclosed is an expression vector comprising a nucleic acid sequence encoding any of the T7 RNA polymerase variants described above.

Also disclosed is an isolated cell transformed with such an expression vector, wherein the transformed cell is capable of expressing any of the T7 RNA polymerase variants described above.

[0011] Also disclosed is a reaction mixture comprising any of the T7 RNA polymerase variants described above, a DNA template comprising a T7 RNA polymerase promoter, and one or more 2'-modified mononucleotides. In some embodiments, the one or more 2'-modified mononucleotides comprise one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, 2'-fluoro GTP, 2'-amino CTP, 2'-amino UTP, 2'-amino ATP, 2'-O-methyl UTP, 2'-O-methyl ATP, 2'-O-methyl CTP, and 2'-O-methyl GTP. Also disclosed is a method of making an RNA polynucleotide comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture described above at 37° C. In some embodiments, the RNA polynucleotide is an aptamer. In some embodiments, the RNA polynucleotide is nuclease resistant. Also disclosed is a method of making a therapeutic RNA polynucleotide comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture described above at 37° C, wherein the DNA template further comprises a template sequence complementary to the therapeutic RNA polynucleotide. In some embodiments, the therapeutic RNA polynucleotide is an miRNA or pre-miRNA. In some embodiments, the therapeutic RNA polynucleotide is an aptamer. In some embodiments, the one or more 2'-modified mononucleotides comprises one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, and 2'-fluoro GTP. In some embodiments, the nucleotide sequence of the therapeutic RNA polynucleotide is complementary to a portion of the sequence of a target gene mRNA. In some embodiments, the one or more 2' modified mononucleotides comprises one or more of 2'-O-methyl UTP, 2'-O-methyl ATP, 2'-O-methyl GTP, and 2'-O-methyl CTP. In some embodiments, the therapeutic RNA polynucleotide is nuclease resistant. Also disclosed is a method of making an RNA polynucleotide probe comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture described above at 37° C, wherein the DNA template further comprises a template sequence complementary to the RNA polynucleotide probe. In some embodiments, the one or more 2' modified mononucleotides comprises one or more of 2'-O-methyl UTP, 2'-O-methyl ATP, 2'-O-methyl GTP, and 2'-O-methyl CTP.

[0012] The terms "comprise" (and any form of comprise, such as "comprises" and "comprising"), "have" (and any form of have, such as "has" and "having"), "include" (and any form of include, such as "includes" and "including") and "contain" (and any form of

contain, such as "contains" and "containing") are open-ended linking verbs. As a result, the methods and systems of the present invention that "comprises," "has," "includes" or "contains" one or more elements possesses those one or more elements, but is not limited to possessing only those one or more elements. Likewise, an element of a method or system of the present invention that "comprises," "has," "includes" or "contains" one or more features possesses those one or more features, but is not limited to possessing only those one or more features.

[0013] The feature or features of one embodiment may be applied to other embodiments, even though not described or illustrated, unless expressly prohibited by this disclosure or the nature of the embodiments.

[0014] Any method or system of the present invention can consist of or consist essentially of—rather than comprise/include/contain/have—any of the described elements and/or features and/or steps. Thus, in any of the claims, the term "consisting of" or "consisting essentially of" can be substituted for any of the open-ended linking verbs recited above, in order to change the scope of a given claim from what it would otherwise be using the open-ended linking verb.

[0015] Details associated with the embodiments described above and others are presented below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] **FIGS. 1A – 1D. Stabilizing mutations increase the activity of the VRS mutant**. **A)** Real time measurement of ribonucleotide (rN) transcriptional output. **B)** Real time measurement of 2'-fluoropyrimidine (rRfY) transcriptional output. **C)** Measurement of ribonucleotide (rN) transcriptional output after three hours. **D)** Measurement of 2'-fluoropyrimidine (rRfY) transcriptional output after three hours. Fluorescent readings (in Relative Fluorescent Units, RFU) indicate the presence of the fluorescent aptamer, spinach. Error bars represent standard error resulting from 3 independently assembled reactions.

[0017] **FIGS. 2A – 2B. Structure of the transcribing thermostable "M5" RNA polymerase initiation complex. A)** The M5 T7 RNA polymerase (white) overlayed with the wildtype T7 RNA polymerase (dark gray, PDB accession number 1QLN (Cheetham, 1999)). **B)** The added hydroxyl group resulting from the F880Y mutation forms a hydrogen bond (dashed line) with the peptide backbone between P474 and F475.

[0018] **FIGS. 3A – 3C. Stabilizing mutations increase the activity of the several T7 RNA polymerase substrate specificity mutants. A)** Measurement of ribonucleotide (rN) transcriptional output after one hours. **B)** Measurement of 2'-fluoropyrimidine (rRfY) transcriptional output after two hours. Fluorescent readings (in Relative Fluorescent Units, RFU) indicate the presence of the fluorescent aptamer, spinach. Error bars represent standard error resulting from 3 independently assembled reactions. **C)** Transcription assay for incorporation of 2'-O-methyluridine (rVmU). Transcripts were labelled by inclusion of (α^{32}P)ATP and analyzed by denaturing PAGE. A reaction of WT T7 RNA polymerase with ribonucleotides (rN) is included for comparison. Transcriptions ran four hours, two distinct gels are shown.

[0019] **FIGS. 4A – 4C. Stabilized T7 RNA polymerase mutants have increased yield of heavily modified RNAs.** Transcription assay for incorporation of 2'-O-methyluridine (rVmU, **A**), 2'-O-methylpyrimidines (rRmY, **B**), or 2'-O-methyladenosine and 2'-O-methylpyrimidines (rGmH, **C**). Transcripts were labelled by inclusion of (α^{32}P)ATP (rVmU and rRmY) or (α^{32}P)GTP (rGmH) and analyzed by denaturing PAGE. All values are normalized to 100, representing the yield of WT T7 RNA polymerase with ribonucleotides (rN). Transcriptions ran four hours (rVmU and rRmY) or 20 hours (rGmH).

[0020] **FIG. 5. RGVG M6 can transcribe fully-modified RNA.** Transcription assay for RGVG-M6 catalyzed incorporation of ribonucleotides (rN); 2'-O-methyluridine (rVmU); 2'-O-methylpyrimidines (rRmY); 2'-O-methyladenosine and 2'-O-methylpyrimidines (rGmH); 2'-O-methylnucleotides (mN); 2'-fluoro-purines and 2'-O-methylpyrimidines (fRmY); and 2'-fluoro-guanosine, 2'-O-methyladenosine, and 2'-O-methylpyrimidines (fGmH). Transcripts were analyzed by denaturing PAGE and imaged after staining in SYBR-Gold. Transcriptions ran 20 hours. A reaction (10-fold diluted) containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

[0021] **FIG. 6. Transcription assay for incorporation of 2'-O-methylnucleotides (mN) in a permissive buffer.** Transcripts were analyzed by denaturing PAGE and imaged after staining in SYBR-Gold. Transcriptions ran 20 hours. A reaction (diluted 10-fold) containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

[0022] **FIG. 7. Transcription assay for incorporation of 2'-O-methyluridine (rVmU).** Transcripts were labelled by inclusion of (α^{32}P)ATP and analyzed by denaturing PAGE. Transcriptions ran four hours. A reaction containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

[0023] **FIG. 8. Transcription assay for incorporation of 2'-O-methylpyrimidines (rRmY).** Transcripts were labelled by inclusion of (α^{32}P)ATP and analyzed by denaturing PAGE. Transcriptions ran four hours. A reaction containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

[0024] **FIG. 9. Transcription assay for incorporation of 2'-O-methyladenosine and 2'-O-methylpyrimidines (rGmH).** Transcripts were labelled by inclusion of (α^{32}P)GTP and analyzed by denaturing PAGE. Transcriptions ran 20 hours. A reaction (diluted 50-fold) containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

[0025] **FIG. 10. RGVG-M6 transcription of 2'-O-methylnucleotides (mN) is various buffers.** Transcripts were analyzed by denaturing PAGE and imaged after staining in SYBR-Gold. Transcriptions ran 20 hours. A reaction containing RGVG-M6 with ribonucleotides (rN) is shown for comparison. The composition of each reaction is shown below.

[0026] **FIG. 11. The relative thermal stability of each T7 RNA polymerase mutant.** Thermal melt assays were performed for several mutants T7 RNA polymerase. First derivatives of the change in fluorescence as a function of time were used to approximate the relative T_m. Data shown are the average of three independently assembled reactions with error bars representing standard error.

[0027] **FIG. 12. Comparison of RGVG-M5 and RGVG-M6 to Y639L H784A in the transcription of 2'-*O*-methylnucleotides (mN) in permissive buffer.** Transcripts were analyzed by denaturing PAGE and imaged after staining in SYBR-Gold. Transcriptions ran 20 hours. A reaction containing WT T7 RNA polymerase with ribonucleotides (rN) is shown for comparison.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Various features and advantageous details are explained more fully with reference to the non-limiting embodiments that are illustrated in the accompanying drawings

and detailed in the following description. It should be understood, however, that the detailed description and the specific examples, while indicating embodiments of the invention, are given by way of illustration only, and not by way of limitation. Various substitutions, modifications, additions, and/or rearrangements will become apparent to those of ordinary skill in the art from this disclosure.

[0029] In the following description, numerous specific details are provided to provide a thorough understanding of the disclosed embodiments. One of ordinary skill in the relevant art will recognize, however, that the invention may be practiced without one or more of the specific details, or with other methods, components, materials, and so forth. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the invention.

A. T7 RNA Polymerase

[0030] The wild type T7 RNA polymerase has the following sequence (SEQ ID NO: 1):

MNTINIAKNDFSDIELAAIPFNTLADHYGERLAREQLALEHESYEMGEARFRKMFER
QLKAGEVADNAAAKPLITTLLPKMIARINDWFEEVKAKRGKRPTAFQFLQEIKPEAV
AYITIKTTLACLTSADNTTVQAVASAIGRAIEDEARFGRIRDLEAKHFKKNVEEQLNK
RVGHVYKKAFMQVVEADMLSKGLLGGEAWSSWHKEDSIHVGVRCIEMLIESTGMV
SLHRQNAGVVGQDSETIELAPEYAEAIATRAGALAGISPMFQPCVVPPKPWTGITGG
GYWANGRRPLALVRTHSKKALMRYEDVYMPEVYKAINIAQNTAWKINKKVLAVA
NVITKWKHCPVEDIPAIEREELPMKPEDIDMNPEALTAWKRAAAAVYRKTRLASLA
VSALSSCLSKPISLLTIRPSGSLTTWTGAVRVYAVSMFNPQGNDMTKGRLTLAKGKPI
GKEGYYWLKIHGANCAGVDKVSFPERIKFIEENHENIMACAKSPLENTWWAEQDSP
FCFLAFCFEYAGVQHHGLSYNCSLPLAFDGSCSGIQHFSAMLRDEVGGRAVNLLPSE
TVQDIYGIVAKKVNEILQADAINGTDNEVVTVTDENTGEISEKVKLGTKALAGQWLA
YGVTRSVTKRSVMTLAYGSKEFGFRQQVLEDTIQPAIDSGKGLMFTQPNQAAGYMA
KLIWESVSVTVVAAVEAMNWLKSAAKLLAAEVKDKKTGEILRKRCAVHWVTPDGF
PVWQEYKKPIQTRLNLMFLGQFRLQPTINTNKDSEIDAHKQESGIAPNFVHSQDGSHL
RKTVVWAHEKYGIESFALIHDSFGTIPADAANLFKAVRETMVDTYESCDVLADFYD
QFADQLHESQLDKMPALPAKGNLNLRDILESDFAFA

B. EXAMPLES

[0031] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes only, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

EXAMPLE 1
MATERIALS AND METHODS

Preparation of T7 RNA polymerase variants

[0032] The T7 RNA polymerase ORF was cloned into pQE-80L (Qiagen). All T7 RNA polymerase variants were derived from this plasmid either by Mega-primer PCR (Bryksin & Matsumura, 2010) or Isothermal assembly (Gibson, 2011). Plasmids were transformed into BL21-gold (Agilent) E. coli cells. Cells were grown in 2xYT media at 37°C overnight. Subcultures were grown at 37°C until reaching OD600 ~0.7-0.8 at which point 1 mM IPTG was added. Cells were grown four hours at 37°C, pelleted, and frozen at -80'C. Pellets were resuspended in binding buffer (50 mM Tris-Hcl, pH8.0, 0.5 M NaCl, 5 mM imidazole). Resuspended cells were lysed via sonication on ice using 50% probe amplitude for 3 minutes (1s ON, 1s OFF). Cell debris was pelleted by centrifugation (30min: 10,000g). His-tagged T7 RNA polymerase was purified by immobilized metal affinity chromatography (IMAC). The lysate was run over 1 ml (bead volume) Ni-NTA (Fisher) gravity column pre-equilibrated with binding buffer. The column was washed with 10 column volumes of wash buffer (50 mM Tris-Hcl, pH 8.0, 0.5 M NaCl, 20 mM imidazole). T7 RNA polymerase was eluted off the column by the addition of 3 column volumes of elution buffer (50 mM Tris-Hcl, pH 8.0, 0.5 M NaCl, 250 mM imidazole). Dialysis was performed in final storage buffer (50 mM Tris-Hcl, pH 8.0, 100 mM NaCl, 1 mM DDT, 1 mM EDTA). Dialates were adjusted to 1 mg/ml and added to an equal volume of glycerol (final concentration 0.5 mg/ml).

In vitro transcription assays

[0033] Real-time transcription reactions (FIG. 1, FIGS. 3A–3B) contained 40 mM Tris-HCl pH 8.0, 30 mM MgCl2, 6 mM spermadine, 6 mM each NTP (or modified NTP), 10 mM DTT, 500 mM T7 RNA polymerase, 500 mM DNA template, and 0.17 mg/ml DFHBI (in DMSO). Reactions were incubated for up to 4 hours at 37°C with spinach fluorescence (Excitation/ Emission 469/501) reading taken one to four minutes in a Safire monochromator (Tecan). Spinach templates were made by thermal cycling overlapping primers (5'-AATATAATACGACTCACTATAGAGGAGACTGAAATGGTGAAGGACGGGTCCAGT GCTTCG (SEQ ID NO: 2) and 5'-GAAAAGACTAGTTACGGAGCTCACACTCTACTCAACAGTGCCGAAGCACTGGAC CCG (SEQ ID NO: 3)) with Accuprime Pfx in its standard buffer (94°C:2 min, 12 cycles [94°C:15 s, 50°C:30 s, 68°C:30 s], 68°C:1 min). Templates were purified by QIAquick Gel Extraction Kit (Qiagen).

[0034] End point transcription reactions contained 40 mM Tris-HCl pH 8.0, 30 mM MgCl2, 6 mM spermadine, 6 mM each NTP (or modified NTP), 10 mM DTT, 500 mM T7 RNA polymerase, 500 mM DNA template. Reactions were incubated for up to 4 or 20 hours at 37°C. DNA templates were made as above. rVmU reactions (FIG. 3C, FIG. 4A, and FIG. 7) and rRmY reaction (FIG. 4B and FIG. 8) were run four hours, labelled by inclusion of 0.17 μM (α32P)ATP (3000 Ci/mMol,) and analyzed by denaturing PAGE. rGmH reactions (FIG. 4C and FIG. 9) were run twenty hours, labelled by inclusion of 0.17 μM (α32P)GTP (3000 Ci/mMol,) and analyzed by denaturing PAGE. RGVG-M6 reactions (FIG. 5) were run twenty hours, incubated for 1 hour 37°C with 0.03 U/ul Baseline-ZERO DNase in its supplied buffer, analyzed by denaturing PAGE and imaged after staining in SYBR-Gold. The buffer comparison (FIG. 10) used the buffers listed in the figure, was run twenty hours, incubated for 1 hour 37°C with 0.03 U/ul Baseline-ZERO DNase in its supplied buffer, analyzed by denaturing PAGE and imaged after staining in SYBR-Gold.

[0035] mN in the permissive buffer (FIG. 6) contained 200mM HEPES pH 7.5, 5.5 mM MgCl2, 2 mM spermidine, 0.5 mM each 2'-O-methyl-NTP, 40 mM DTT, 0.01% Triton, 10% PEG8000, 1.5 mM MnCl2, 10 U/ml YIPP, 200 nM RNA polymerase, and 200 nM DNA. Reactions were run twenty hours, incubated for 1 hour 37°C with 0.03 U/ul Baseline-ZERO DNase in its supplied buffer, analyzed by denaturing PAGE and imaged after staining in SYBR-Gold.

[0036] 32P gels were exposed to a storage phosphor screen (Molecular Dynamics) before imaging on a STORM 840 Phospoimager (GE Healthcare). Autoradiographs were analyzed using ImageQuant (GE Healthcare).

Thermal melt measurements

[0037] The relative thermal stability of each T7 RNA polymerase was assessed by incubating 0.5 mg/ml enzyme in PBS buffer with TexasRed dye (Invitrogen). Enzyme/dye mixtures were equilibrated at 37 °C for 10 minutes and heated at a rate of 0.07 °C/s to 97 °C using a LightCycler 96 thermocycler, while fluorescence was monitored (Excitation 577 nm / Emission 620 nm). The first derivatives of the change in fluorescence as a function of time were used to approximate the relative T_m. Data were analysed using Roche thermocycler software.

EXAMPLE 2
STABILIZING MUTATIONS INCREASE THE ACTIVITY OF THE T7 RNA POLYMERASE MUTANT G542V H784S

[0038] Previous experiments selecting for RNA polymerases with altered substrate specificity (Chelliserrykattil & Ellington, 2004) focused on the four amino acids that are proximal to the incoming nucleotide (Cheetham, 1999; Temiakov, et al., 2004), and thus likely played a role in substrate recognition. One of the resulting mutants, called "VRS," could incorporate 2'F-modified pyrimidines. VRS had mutations at two of the randomized residues (ie G542V and H784S). Interestingly, an H772R mutation also arose during the selection, despite H772 not being randomized. H772R is not near the substrate recognition domain, but has been seen in other selections for T7 RNA polymerase activity (Ellefson, et al., 2013; Dickinson, et al., 2013). To test whether H772R is a general stabilizing mutation, a derivative of VRS without H772R, termed "VS," was constructed. Purified enzymes were tested for their ability to polymerase RNA composed either of natural NTPs (rN) or of ribo-purines and 2'-F-pyrimidines (rRfY; FIG. 1). Real-time polymerase activity was assayed using the fluorescent aptamer spinach in the presence of DFHBI (Van Nies *et al.*, 2013). Spinach will bind DFHBI and fluoresce irrespective of whether it is transcribed as a purely ribo-aptamer or when substituted with 2′-F-pyrimidines, although the 2′-F-pyrimidine version is only about 30% as fluorescent as the purely ribonucleotide version. 2′-*O*-methyl substituted spinach is not detectably fluorescent.

[0039] Notably, VS showed a decrease in activity for each substrate composition. This suggests that H772R contributes to the overall activity of VRS, apart from any substrate preference considerations. Several more derivatives of VRS with additional mutations were created and tested for their ability to increase the activity of VRS. The so-called "M5" (S430P, N433T, S633P, F849I, and F880Y; (U.S. Patent No. 7,507,567) and "M6" (M5 with the additional P266L mutation, associated with promoter clearance (Guillerez, et al., 2005) sets of mutations increased activity of the VRS mutant, both for rN and rRfY incorporation.

EXAMPLE 3
THE "M5" MUTATIONS INCREASE THE ACTIVITY OF SEVERAL T7 RNA POLYMERASE SUBSTRATE SPECIFICITY MUTANTS

[0040] The "M5" mutations arose in a T7 RNA polymerase selection for transcriptional activity at higher temperatures. In a wild type background, these mutations increase the half-life of enzyme at 50'C and allow for transcription at that temperature. The M5 protein was crystalized, and few gross morphological differences to the wild-type T7

RNA polymerase crystal (Cheetham, 1999) are apparent (FIG. 2A). There is, however, an added hydrogen bond made by F880Y, which may stabilize the two halves of the palm domain (FIG. 2B). It should be noted that the F880Y mutation is not sufficient to increase VRS activity (see VRSY in FIGS. 1C–1D).

[0041] It was then tested whether the M5 and M6 mutations could increase the activity of other T7 RNA polymerase mutants. Several known polymerases with altered ribose specificity namely WT, Y639F, FA, RGVG, VRS, and R425C (Table 1) were tested. To each of these specificity mutants was added a set of stability mutations, namely "L" (P266L), M5, and M6. Also included was a recently described mutant, 2P16, which is likely a stabilized version of RGVG. These 25 polymerases were purified and assayed for transcriptional activity in vitro (FIG. 3).

Table 1. List of T7 RNA polymerase mutants

Enzyme	Sequence
WT	WT T7 RNAP
VS	G542V, H784S
VRS	G542V, H772R, H784S
VRS-L	P266L, G542V, H772R, H784S
VLRS	G542V, V625L, H772R, H784S
VRIS	G542V, H772R, V783I, H784S
VLRIS	G542V, V625L, H772R, V783I, H784S
VRSY	G542V, H772R, H784S, F880Y
VRS-M5	S430P, N433T, G542V, S633P, H772R, H784S, F849I, F880Y
VRS-M6	P266L, S430P, N433T, G542V, S633P, H772R, H784S, F849I, F880Y
M5	S430P, N433T, S633P, F849I, F880Y
L	P266L
M6	P266L, S430P, N433T, S633P, F849I, F880Y
Y639F	Y639F
Y639F-M5	S430P, N433T, S633P, Y639F, F849I, F880Y
Y639F-L	P266L, Y639F
Y639F-M6	P266L, S430P, N433T, S633P, Y639F, F849I, F880Y
FA	Y639F, H784A
FA-M5	S430P, N433T, S633P, Y639F, H784A, F849I, F880Y
FA-L	P266L, Y639F, H784A
FA-M6	P266L, S430P, N433T, S633P, Y639F, H784A, F849I, F880Y
R425C	R425C
R425C-M5	R425C, S430P, N433T, S633P, F849I, F880Y
R425C-L	P266L, R425C
R425C-M6	P266L, R425C, S430P, N433T, S633P, F849I, F880Y
RGVG	E593G, Y639V, V685A, H784G
RGVG-M5	S430P, N433T, E593G, S633P, Y639V, V685A, H784G, F849I, F880Y
RGVG-L	P266L, E593G, Y639V, V685A, H784G
RGVG-M6	P266L, S430P, N433T, E593G, S633P, Y639V, V685A, H784G, F849I, F880Y
2P16	I119V, G225S, K333N, D366N, F400L, E593G, Y639V, S661G, V685A, H784G, F880Y

[0042] Whether transcribing natural ribotides (rN; FIG. 3A), 2'-F-pyrimidines (rRfY; FIG. 3B), or 2'-O-methyluridine (rVmU; FIG. 3C) the M5 and M6 mutations increased activity of the mutants FA, RGVG, and VRS. WT and Y639F activity on rN was slightly increased by the M5 mutations, but this trend did not hold up with rRfY or rVmU

incorporation. It is evident that the 2P16 is indeed more active than RGVG (as previously reported (Siegmund, et al., 2012)) but is not as active as either RGVG M5 or RGVG M6. No transcription was detected from the R425C family of polymerases.

[0043] A subset of the most active polymerases were assayed for the ability to incorporate 2'-O-methyluridine (rVmU), 2'-O-methylpyrimidines (rRmY), and 2'-O-methyladenosine and 2'-O-methylpyrimidines (rGmH) (FIG 4, FIGS. 7 – 9). As was case for rN and rRfY above, the M5 mutations enhanced the activity of the FA and RGVG enzymes for each set of substrates. RGVG-M6 was the most active enzyme in all conditions, yielding at least 25-fold more RNA than the FA mutant, which is the most commonly used enzyme for generating 2'-O-methyl RNA.

[0044] Thermal-melt assays confirmed that, for all T7 RNA polymerase variants tested, addition of the M5 mutations increased their thermal stability (**FIG. 11**). The weakly active RGVG mutant has a T_m almost 5 °C lower than that of WT T7 RNA polymerase, but this loss of stability and RGVG's activity are rescued by the M5 mutations. Contrary to expectations, however, the similarly weak VRS and FA mutants do not have low melting temperatures, and the H772R mutation did not have the expected effect on T_m. It seems that the increase in activity due to the addition of these mutations cannot be solely attributed to an increase of stability.

EXAMPLE 4
T7 RNA POLYMERASE R6 IS EFFECTIVE FOR HIGH-YIELD TRANSCRIPTION OF FULLY MODIFIED RNA

[0045] After demonstrating that RGVG-M6 could catalyse the formation of RNA containing three 2'-O-methylnucleotides, its ability to generate fully-modified RNA was assayed. RGVG-M6 was able to polymerase using a combination 2'-F-purines and 2'-O-methylpyrimidines (fRmY) as well as a combination of 2'-F-guanosine, 2'-O-methyladenosine, and 2'-O-methylpyrimidines (fGmH) (FIG. 5). Fully 2'-O-methyl RNA (mN) was not obtained.

[0046] Previous reports of mN incorporation have used more permissive buffer compositions, including manganese as well as rGMP and/or rGTP. RGVG-M6's ability to synthesize mN RNA in several such permissive buffers was tested (FIG. 10) and it was determined that effective mN polymerization was achieved in buffers that included rGMP or rGTP. A panel of enzymes for mN polymerization in this permissive buffer (200 mM HEPES pH 7.5, 5.5 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each 2′-*O*-methyl-NTP, 40 mM DTT,

0.01% Triton, 10% PEG8000, 1.5 mM $MnCl_2$, 10 U/ml yeast inorganic pyrophosphatase, 200 nM RNA polymerase, and 200 nM DNA) was tested. FA-M5 and FA-M6 show an increase in activity relative to the parental FA mutant. RGVG-M5, RGVG-M6, and 2P16 showed a marked improvement over the parental RGVG. In addition, RGVG-M5 and RGVG-M6 generate substantially more RNA in this buffer than the Y639L H784A mutant (U.S. Pat. No. 8,105,813).

[0047] Although certain embodiments have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those skilled in the art could make numerous alterations to the disclosed embodiments without departing from the scope of this invention. Further, where appropriate, aspects of any of the examples described above may be combined with aspects of any of the other examples described to form further examples having comparable or different properties and addressing the same or different problems. Similarly, it will be understood that the benefits and advantages described above may relate to one embodiment or may relate to several embodiments.

[0048] The claims are not to be interpreted as including means-plus- or step-plus-function limitations, unless such a limitation is explicitly recited in a given claim using the phrase(s) "means for" or "step for," respectively.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

1. A T7 RNA polymerase variant comprising:

one or more substrate-broadening amino acid substitutions that confer an enhanced ability to incorporate 2'-modified mononucleotides compared to a wild-type T7 RNA polymerase; and

one or more activity-enhancing amino acid substitutions that increase the transcriptional activity of the T7 polymerase variant relative to T7 polymerase variants without the activity-enhancing amino acid substitutions.

2. The T7 RNA polymerase variant of claim 1, wherein the one or more substrate-broadening amino acid substitutions comprise one or more of the following amino acid substitutions relative to the wild-type T7 RNA polymerase sequence of SEQ ID NO:1: G542V, E593G, Y639V, Y639F, V685A, H772R, H784A, H784S, and H784G.

3. The T7 RNA polymerase variant of claim 1 or 2, wherein the one or more substrate-broadening amino acid substitutions comprise G542V, H772R, and H784S.

4. The T7 RNA polymerase variant of claim 1 or 2, wherein the one or more substrate-broadening amino acid substitutions comprise Y639F.

5. The T7 RNA polymerase variant of claim 1 or 2, wherein the one or more substrate-broadening amino acid substitutions comprise Y639F and H784A.

6. The T7 RNA polymerase variant of claim 1 or 2, wherein the one or more substrate-broadening amino acid substitutions comprise E593G, Y639V, V685A, and H784G.

7. The T7 RNA polymerase variant of claim 1, wherein the 2'-modified mononucleotides comprise one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, 2'-fluoro GTP, 2'-amino CTP, 2'-amino UTP, 2'-amino ATP, 2'-*O*-methyl UTP, 2'-*O*-methyl ATP, 2'-*O*-methyl CTP, and 2'-*O*-methyl GTP.

8. The T7 RNA polymerase variant of any one of claims 1 to 7, wherein the one or more activity-enhancing amino acid substitutions comprise one or more of the following amino acid substitutions: P266L, S430P, N433T, S633P, F849I, and F880Y.

9. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise two or more of the following amino acid substitutions: P266L, S430P, N433T, S633P, F849I, and F880Y.

10. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise S430P, N433T, S633P, F849I, and F880Y.

11. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise P266L, S430P, N433T, S633P, F849I, and F880Y.

12. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise P266L.

13. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise S633P and F849I.

14. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise S633P and F880Y.

15. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise F849I and F880Y.

16. The T7 RNA polymerase variant of any one of claims 1 to 8, wherein the one or more activity-enhancing amino acid substitutions comprise S633P, F849I, and F880Y.

17. A T7 RNA polymerase variant comprising the following amino acid substitutions: N433T, E593G, Y639V, V685A, H784G, S430P, S633P, F849I, and F880Y.

18. A nucleic acid molecule encoding the T7 RNA polymerase variant of any of claims 1 to 17.

19. An expression vector comprising the nucleic acid molecule of claim 18.

20. An isolated cell transformed with the expression vector of claim 19, wherein the transformed cell is capable of expressing the T7 RNA polymerase variant.

21. A reaction mixture comprising the T7 RNA polymerase variant of any of claims 1 to 17, a DNA template comprising a T7 RNA polymerase promoter, and one or more 2'-modified mononucleotides.

22. The reaction mixture of claim 21, wherein the one or more 2'-modified mononucleotides comprise one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, 2'-fluoro GTP, 2'-amino CTP, 2'-amino UTP, 2'-amino ATP, 2'-*O*-methyl UTP, 2'-*O*-methyl ATP, 2'-*O*-methyl CTP, and 2'-*O*-methyl GTP.

23. A method of making an RNA polynucleotide comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture of claim 21 or 22 at 37° C.

24. The method claim 23, wherein the RNA polynucleotide is an aptamer.

25. The method of any of claims 23 to 24, wherein the RNA polynucleotide is nuclease resistant.

26. A method of making a therapeutic RNA polynucleotide comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture of claim 21 or 22 at 37° C, wherein the DNA template further comprises a template sequence complementary to the therapeutic RNA polynucleotide.

27. The method of claim 26, wherein the therapeutic RNA polynucleotide is an miRNA or pre-miRNA.

28. The method of claim 26, wherein the therapeutic RNA polynucleotide is an aptamer.

29. The method of claim 28, wherein the one or more 2'-modified mononucleotides comprises one or more of 2'-fluoro CTP, 2'-fluoro UTP, 2'-fluoro ATP, and 2'-fluoro GTP.

30. The method of claim 26, wherein the nucleotide sequence of the therapeutic RNA polynucleotide is complementary to a portion of the sequence of a target gene mRNA.

31. The method of claim 30, wherein the one or more 2' modified mononucleotides comprises one or more of 2'-*O*-methyl UTP, 2'-*O*-methyl ATP, 2'-*O*-methyl GTP, and 2'-*O*-methyl CTP.

32. The method of claim 30, wherein the therapeutic RNA polynucleotide is nuclease resistant.

33. A method of making an RNA polynucleotide probe comprising one or more 2'-modified mononucleotides, the method comprising incubating the reaction mixture of claim 21 or 22 at 37° C, wherein the DNA template further comprises a template sequence complementary to the RNA polynucleotide probe.

34. The method of claim 33, wherein the one or more 2' modified mononucleotides comprises one or more of 2'-*O*-methyl UTP, 2'-*O*-methyl ATP, 2'-*O*-methyl GTP, and 2'-*O*-methyl CTP.