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Evolution of C-terminal modification tolerance in full-length and split T7 RNA Polymerase biosensors

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Abstract

T7 RNA polymerase (RNAP) is a powerful protein scaffold for the construction of synthetic biology tools and biosensors. However, both T7 RNAP and its split variants are intolerant to C-terminal modifications or fusions, placing a key limitation on its engineering and deployment. Here, we use rapid continuous evolution approaches to evolve both full-length and split T7 RNAP variants that tolerate modified C-termini and fusions, including fusions to entire other proteins. Moreover, we show that the evolved split C-terminal RNAP variants can function as small molecule biosensors, even in the context of large C-terminal fusions. Together, this work provides a panel of modified RNAP variants with robust activity and tolerance to C-terminal fusions, and provides insights into the biophysical requirements of the C-terminal carboxylic acid functional group of T7 RNAP.

Graphical Abstract

Evolving better biosensors: Rapid continuous evolution approaches were used to evolve away the dependency of a free C-terminus in full-length and split T7 RNA polymerase, increasing the versatility of engineering strategies for creating T7 RNA polymerase-based biosensors.

Keywords

biosensors; synthetic biology; directed evolution; transcription; RNA

Introduction

The field of synthetic biology relies on biosensors, molecular entities that can transduce chemical, biochemical, and physical inputs into changes in cellular signals.^[1] For example, genome engineering tools that are activated upon defined internal or external cues could increase efficacy and decrease side effects when used to treat disease.^[2] Engineered organisms integrated into a host's microbiome that measure gut health and trigger either the production of a marker or the production of a therapeutic could be used to develop "smart" therapies.^[3] Finally, while *in vivo-based* directed evolution platforms can provide powerful methods to reprogram biomolecules,^[4] controlling and accurately focusing selection pressures requires robust biosensor systems for each desired property or activity.^[5]

Recently, a variety of new methods for the creation of robust biosensors have been developed.^[6] Each biosensor platform tends to have very specific organismal and context requirements, and a truly versatile strategy for biosensor creation remains elusive. Most critically, developing a biosensor for a new target activity tends to be the rate-limiting step in projects aimed at leveraging rapid *in vivo* evolution tools to solve problems in chemistry and biology.

To address the limitations with current biosensor platforms, our group recently developed engineered RNA polymerases (RNAPs) as a new approach for biosensor creation. The concept was inspired by green fluorescent protein (GFP), which provided the chemical biology community with a robust protein scaffold to engineer fluorescent probes.^[7] We reasoned that if we could devise methods to engineer activity-responsiveness in RNAPs that transcribe from specific DNA promoters, this would provide a facile method for biosensor creation. First, we validated the concept by engineering T7 RNAP biosensors for protease activities, which we demonstrated could perform multidimensional protease detection in *E. coli* and mammalian cells.^[8]

To expand the generality of the engineered RNAP biosensor approach, we next sought to develop a proximity-dependent split RNAP. Richardson et al. first discovered T7 RNAP can be proteolytically cleaved at site 172/173, and the cleaved protein still retains RNAP activity.^[9] Next, Bennet et al. discovered that T7 RNAP can be split at site 179, and the two halves of the split protein can spontaneously reassemble to form a functional protein when coexpressed.^[10] Finally, Voigt et al. systematically screened every possible split site in the 883 amino acid T7 RNAP protein, revealing 36 sites that are still functional as a split protein.^[11] The assembly at some of the sites, as measured by RNA production, could be enhanced by fusing the split proteins to interacting zipper peptides, indicating a potential strategy for engineering biosensors using the split RNAP system.

We sought to leverage the identified split T7 RNAP variants to create a proximity-dependent split reporter system with a large dynamic range. To accomplish this, we developed a new

Phage-Assisted Continuous Evolution (PACE) system^[12] specifically designed to evolve proximity-dependence in the N-terminal half of T7 RNAP split at site 180 (RNAP_N). The resultant evolved RNAP_N variants displayed >300-fold dynamic range based on fused interaction partners displayed on the C-terminal half of the RNAP (RNAP_C) after PACE. Moreover, we were able to easily adopt the system to small molecule and light-activated biosensors by simply swapping new fusion proteins into the evolved split system, indicating the evolved system is versatile and can function with interaction partners of varied orientations and affinities. In follow-up work, we adopted the split RNAP biosensors into multidimensional mammalian PPI network detectors^[13] as well as a new way to control genome engineered by CRISPR/Cas9 systems.^[14] Additionally, we were also able to further evolve the RNAP_N variants to expand the *in vivo* measured dynamic range of the platform to over 2000-fold.

Our evolved split RNAP biosensor platform is quite versatile. For one, we found minimal-to-no linker length dependence of the fusion interaction partners in terms of RNA output.^[12] Additional N- and C-terminal fusions on RNAP_N are both tolerated, allowing the orientation of interaction partners to be optimized based on fusion orientation selection. However, C-terminal fusions on RNAP_C are forbidden, as any modification to the C-terminus of T7 RNAP or variants thereof results in a complete inactivation of the enzyme.^[15] This is due to the carboxy group of the C-terminus of T7 RNAP is buried in the core of the protein structure^[16] where it forms important contacts.^[17] The inability to utilize C-terminal fusions on either full-length T7 RNAP variants on RNAP_C in our evolved split RNAP biosensor system limits the utility of engineered T7 RNAP systems. For example, fusion proteins that would benefit from C-terminal fusions on RNAP_C or immobilizing biosensors on surfaces through C-terminal fusions, are hampered by this biophysical restraint inherent to the protein.

Here, we evolve away the dependency of having an unmodified C-terminal carboxylate in both full-length and split T7 RNAP biosensors. First, we used PACE with a C-terminal modification library to evolve full-length T7 RNAP variants that are still functional with C-terminal modifications. Then, taking the mutations from that evolution as a starting point, we used PACE again to evolve split RNAP_C variants that are still functional with C-terminal modifications. We go on to show that the evolved RNAP_C variants, even with C-terminal modifications, can still act cooperatively with our previously-evolved proximity-dependent RNAP_N variants to create robust biosensors. Moreover, we show that the evolved RNAP_C variants can still function without substantial loss in dynamic range as a biosensor with a large C-terminal fusion protein. Together, this work expands the utility of our split RNAP biosensor platform and provides a clear demonstration of how key biophysical limitations of natural proteins can be removed through continuous evolution.

Results and Discussion

According to previous reports and our own experiences, C-terminal fusions to T7 RNAP abrogates its enzymatic activity. To directly test this, we used a T7 promoter-driven luciferase reporter system that we previously developed^[8] to measure T7 RNAP transcription from a C-terminal peptide modified RNAP fusion (Figure 1A). We appended a

GGSGGGS fusion onto either full-length wild type T7 RNAP (T7-C) or to the terminus of our previously developed split C-terminal T7 RNAP fragment (RNAPc-C). We selected a Gly/Ser-based unstructured linker to avoid any structural dependence on the assayed proteins. For the full-length protein, while T7 RNAP provides robust signal in the reporter assay, the modification on T7-C completely abrogates the transcriptional response (Figure 1B). Similarly, while the RNAPc fragment from split RNAP reporter system shows robust transcription when expressed with a corresponding RNAP_N evolved fragment in the presence of a zipper peptide interaction pair, no transcription is detected with the C-terminal modified RNAPc fragment, RNAPc-C (Figure 1C). Therefore, we first sought to evolve full-length T7 RNAP to tolerate C-terminal fusions.

We used PACE to evolve C-terminal modification tolerance in T7 RNAP. To link T7 RNAP transcription to phage replication for the purpose of continuous evolution, we used an accessory plasmid (AP) with T7 promoter-driven gIII, a system that has performed well in several previous evolutions (Figure 2A).^[8,12–1318] We then cloned phage encoding T7 RNAP variants with C-terminal fusions. Since the starting modified protein has no detectable activity, we reasoned that we should initiate PACE with a library of variants. Therefore, we cloned phage libraries of T7 RNAP with a modified C-terminus, including three sites of randomization (Figure 2B). Our hypothesis was that mutants from the C-terminal modification library could act cooperatively with the GGSGGGS fusion to allow mutations to accumulate and regain function in the protein. We validated the selection pressure by performing activity-dependent plaque assays. While phage that encode wild type T7 RNAP show activity-dependent plaques on the T7 promoter-driven AP (p1–1), the library of T7 RNAP phage with modified C-termini showed no plaques (Figure 2C). These results indicate the T7 RNAP variants produced by the phage cannot transcribe from the T7 promoter, as expected based on the luciferase results. The combination of *in vivo* transcriptional assays using the luciferase reporter system and activity-dependent plaque assays demonstrate there is robust selection pressure, which in turn motivated us to try to use PACE to evolve back RNAP activity in the modified variants.

We initiated a PACE experiment with approximately 10⁴ phage variants from the randomized library and then allowed the phage to evolve for three days (Figure 3A). We allowed some neutral drift for the first two days of PACE by mixing in 1059 cells, which contain a plasmid that encodes activity-independent, “free” gIII,^[20] allowing the proteins more capacity to evolve for the desired function. At the end of the three days of PACE, the phage populations showed activity-dependent plaques on the T7 promoter-driven AP cells (Figure 3B), indicating the phage had evolved. We then cloned out the T7 RNAP gene variants from each of the phage libraries to independently test whether they had indeed regained enzymatic activity. Using the T7 promoter-driven luciferase reporter assay, we found that five of the six variants tested from the PACE experiment showed robust T7 RNAP activity (Figure 3C), confirming that the evolution was successful. Moreover, in this *in vivo* reporter assay, the active variants all displayed comparable transcriptional activity levels as wild type, unmodified T7 RNAP, indicating the high efficiency of the C terminal modification tolerance evolution through our adjusted PACE system.

We then sequenced the genes in each of the assay vectors to see what mutations permitted the robust gains in activity, as well as to confirm that the C-terminal modifications are still present after PACE (Figure 4A). Gratifyingly, all of the variants still contained the fusion peptide on their C-termini. Five-of-six variants contained the same sequence of amino acids at the randomized region (ILEPTLE), while the sixth used a slightly different sequence (ILESSLFE). All of the T7-C variants contained mutations at G538E. Interestingly, this mutational position aligns directly adjacent to the C-terminal carboxylate in T7 RNAP crystal structures, strongly suggesting the G538E carboxylate substitution accounts for the loss of the C-terminus in the protein core when its modified (Figure 4B). However, variant T7-C-D has G538E, but is not active (Figure 4A), indicating that G538E is important but not sufficient to regain robust activity. The other five active variants also have V685A, and four of the five have A724S, suggesting additional mutations work synergistically with G538E to regain activity in the presence of the modified C-terminus.

We then assayed whether the function of evolved T7-C variants was dependent on the specific modified C-terminus that emerged during PACE. We fused a 26 kDa DsRed fluorescent protein to the C-terminus of each variants as a model protein fusion and repeated the luciferase transcriptional assay. Gratifyingly, all of the active variants retained their robust RNAP activity, even in the presence of the larger C-terminal fusion (Figure S1). Although the split RNAPc-C with these mutations resulted in some enhanced tolerance to the C-terminal fusions, the activity was relatively weak (Figure S2). Therefore, we next sought to evolve the split RNAPc fragment with these initial mutations to better accommodate C-term fusion.

With the evolved full-length T7 RNAPs in hand, we next aimed to evolve robust function of RNAPc-C with our previously-evolved RNAP_N biosensor component. To develop a PACE system to evolve RNAPc, we again used a T7 promoter-driven gIII AP along with a helper plasmid (HP) that expresses RNAP_N fused to ZA, a zipper peptide (Figure 5A). We then cloned phage encoding RNAPc, with the key mutations identified in the full-length evolution (G538E, V685A, A724S), fused to ZB, a zipper peptide that forms a tight interaction with ZA. After validating all of the system components, we initiated two replicate PACE experiments with the phage and allowed the evolution to proceed for seven days (Figure 5B). For the first half of the evolution, we used wild type RNAP_N fused to the ZA peptide, because the wild type split protein has the highest overall level of activity and therefore the lowest selection pressure. Then, during the evolution we increased the selection pressure for proximity-dependent assembly by switching the RNAP_N variant to N-29-1, our previously evolved proximity-dependent RNAP_N.^[12]

At the conclusion of the evolution, we cloned out four variants from each replicate PACE experiment and assayed their RNAP activities. Aside from measuring overall activity with N-29-1 RNAP_N, we also aimed to assess whether the evolved RNAPc variants maintained their “proximity dependent” activity, meaning that the transcriptional activity is dependent on interactions between ZA and ZB fusions on RNAP_N and the evolved RNAPc-C variants, respectively. To test for this, we performed luciferase-based transcriptional assays with a RNAP_N variant fused to either ZA or a control, non-interacting peptide. In the context of the ZA/ZB interaction partners, all of the evolved variants showed robust activity (Figure 4C),

confirming the evolution was successful. Moreover, without the interaction partners, all of the variants displayed very low background transcription, additionally confirming that the variants retained their proximity-dependent synergistic function with our previously-evolved RNAP_N.

To assess the mutations that evolved during PACE and endowed the RNAPc-C variants with robust activity, we next sequenced the eight variants from the two lagoons (Figure 6A). All eight variants retained the core initial mutations, G538E, V685A, and A724S. Additionally, all of the variants from each lagoon also acquired S430P and F536Y, indicating these mutations may also be critical for activity. F536Y is in close proximity to the C-terminus of T7 RNAP in crystal structures (Figure 6B). In addition to the mutations that were set in both replicate PACE populations, each replicate also acquired several set mutations within the replicate. Replicate one contained S228R, A247V, L446M, E683K, and V687A set in all four variants, while replicate two contained V185A, M369T, E498G and L562I set in all four assayed variants. Each variant also contained additional unique mutations. Mapping the mutations onto T7 RNAP crystal structures provides some clues as to the roles of the mutations. In addition, the mutations are present throughout the protein (Figure S3), further highlighting the strength of our approach as these positions would likely have been challenging to predict ahead of time.

Finally, we tested whether the evolved RNAPc-C variants could still function as biosensors in the presence of C-terminal modifications. To test their performance, we targeted the generation of rapamycin-inducible split RNAP biosensors, which can be accomplished by fusing the rapamycin-inducible binding domains, FRB and FKBP, to the evolved split RNAP partners. We cloned each of the evolved RNAPc variants, along with their C-terminal modifications, with an N-terminal FKBP fusion protein. We then assayed each vector for RNAP activity *in vivo* in the absence and presence of rapamycin with an evolved RNAP_N partner fused to FRB. Gratifyingly, each of the evolved RNAPc biosensors still functioned in a rapamycin-dependent manner (Figure S4). Especially encouraging is that the overall RNAP activity of the RNAPc variants with modified C-termini in the presence of rapamycin is quite similar to the non-modified RNAPc variant we originally generated.^[12] As a final validation of the generality and tolerance of the evolved RNAPc variants, we assayed whether they could still function as small molecule-inducible biosensors with larger C-terminal fusions. We again utilized a C-terminal DsRed fusion as a model larger fusion protein in the split RNAP reporter assay (Figure 7A). As anticipated, all of the RNAPc variants still functioned in a rapamycin-dependent manner when fused to DsRed (Figure 7B), displaying between a 40- and 623-fold dynamic range of RNA production in the presence of rapamycin.

Conclusions

In conclusion, we developed a strategy for the evolution of tolerance for C-terminal modifications in full-length and split T7 RNAP. Although we evolved both the full-length and split RNAPs in the context of short model C-terminal fusions, we showed the proteins also function robustly in the context of large, protein C-terminal fusions. Moreover, the evolved split RNAP system can still be used to easily generate biosensors when coupled to

our previously-evolved proximity-dependent split RNAP biosensor platform. Other T7 RNAP-based engineered systems currently in development will likely also benefit from this expanded engineering requirement.^[21]

From an application perspective, the tools evolved in this work open up new opportunities for engineering T7 RNAP-based detection systems and other synthetic biology tools, as a key restriction to T7 RNAP biosensor design has now been removed. From a basic science perspective, this work demonstrates the potential of using rapid directed evolution approaches to reprogram key biophysical constraints of natural proteins, even if those properties appear to be integrally linked to protein function. Indeed, although structural and classical biochemical data indicated that the C-terminus of T7 RNAP was absolutely required for its activity,^[22] our work here shows that this biophysical constraint could be lifted after a few days of continuous evolution. Here, we chose to evolve the protein using a “stepping stone” approach, in which we first evolved the full-length protein, and then evolved the split protein from that starting point. In principle, we may have been able to directly evolve the split protein, but given the numbers of mutations that emerged during PACE, this may have been challenging. Using evolution as a method to assess structure-function relationships and to test purported mechanisms of proteins and other biomolecules will continue to help shed light on how complex molecular machines function and how to better design them.

Experimental Section

Cloning.

All plasmids and phage were constructed by Gibson assembly from PCR products generated using Q5 DNA Polymerase (NEB) or Phusion Polymerase. All plasmids were sequenced at the University of Chicago Comprehensive Cancer Center DNA Sequencing and Genotyping Facility and are listed in Table S1, with links to online vector maps included. All materials are available upon request.

Phage-Assisted Continuous Evolution (PACE).

PACE was carried out using previously described methods^[21] with slight modifications. All vectors for APs and HPs are shown in Table S1. During the evolution, phage samples were collected every 24 h, then boiled for 10 min to lyse the phage and release the genomes. PCR was then used to amplify the DNA library containing the full-length or RNAPc variants, which were then cloned into the T7 RNAP-expression vectors. Single colonies were picked from the transformation and subjected to analysis by Sanger sequencing.

Transcriptional reporter assays.

Transcriptional reporter assays on the T7 promoter were conducted as previously described.^[8, 12] Briefly, for full-length T7 RNAP assays, S1030 cells were transformed with two plasmids: (i) an arabinose induced T7 RNAP-expression plasmid (2–41, Jin 491 or Jinn 493, Table S1) which encodes control T7 RNAP or variant to be tested, and (ii) a T7 promoter driven luciferase expression plasmid (2–22, Table S1). For split RNAP assays, S1030 cells were transformed with three plasmids: (i) a constitutive RNAPN-expression plasmid (5–74,

9–2 or 7–69, Table S1), (ii) an arabinose induced RNAPc-expression plasmid (2–39, 2– 55, Jin490, Jin492 or 7–68, Table S1), and (iii) a T7 promoter driven luciferase expression plasmid (2–22). Single colonies were then grown in a 96-deep-well plate overnight at 37 °C, and 40 µL of the culture was transferred to a new 96-deep-well plate containing 460 µL of LB with antibiotics and 10 mM arabinose. After growth with shaking at 37 °C for 3 h, 150 µL of each culture was transferred to a 96-well black wall, clear bottom plate (Nunc), and luminescence and OD₆₀₀ were measured on a Synergy Neo2 Hybrid Multi-Mode Reader (BioTek). The data were analyzed by dividing the luminescence values by the background-corrected OD₆₀₀ value.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

- [1]. a) Gautier A, Gauron C, Volovitch M, Bensimon D, Jullien L, Vriza S, Nat Chem Biol 2014, 10, 533–541 [PubMed: 24937071] b) Smanski MJ, Zhou H, Claesen J, Shen B, Fischbach MA, Voigt CA, Nat Rev Microbiol 2016, 14, 135–149 [PubMed: 26876034] c) Lienert F, Lohmueller JJ, Garg A, Silver PA, Nat Rev Mol Cell Biol 2014, 15, 95–107 [PubMed: 24434884] d) Packer MS, Liu DR, Nat Rev Genet 2015, 16, 379–394 [PubMed: 26055155] e) Ruder WC, Lu T, Collins JJ, Science 2011, 333, 1248–1252 [PubMed: 21885773] f) Zhang J, Jensen MK, Keasling JD, Curr Opin Chem Biol 2015, 28, 1–8. [PubMed: 26056948]
- [2]. Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, Kuhn R, Nat Biotechnol 2015, 33, 543–548. [PubMed: 25803306]
- [3]. a) Courbet A, Endy D, Renard E, Molina F, Bonnet J, Sci Transl Med 2015, 7, 289ra283b) Pinero-Lambeck C, Ruano-Gallego D, Fernandez LA, Curr Opin Biotechnol 2015, 35, 94–102 [PubMed: 26070111] c) Riglar DT, Silver PA, Nat Rev Microbiol 2018, 16, 214–225. [PubMed: 29398705]
- [4]. a) Arnold FH, Angew Chem Int Ed Engl 2018, 57, 4143–4148 [PubMed: 29064156] b) Turner NJ, Nat Chem Biol 2009, 5, 567–573. [PubMed: 19620998]
- [5]. a) Conroy PJ, Hearty S, Leonard P, O’Kennedy RJ, Semin Cell Dev Biol 2009, 20, 10–26 [PubMed: 19429487] b) Pavoov TV, Cho YK, Shusta EV, Proc Natl Acad Sci U S A 2009, 106, 11895–11900 [PubMed: 19574456] c) Davis AM, Plowright AT, Valeur E, Nat Rev Drug Discov 2017, 16, 681–698. [PubMed: 28935911]
- [6]. a) Cabantous S, Nguyen HB, Pedelacq JD, Koraichi F, Chaudhary A, Ganguly K, Lockard MA, Favre G, Terwilliger TC, Waldo GS, Sci Rep 2013, 3, 2854 [PubMed: 24092409] b) Filonov GS, Moon JD, Svensen N, Jaffrey SR, J Am Chem Soc 2014, 136, 16299–16308 [PubMed: 25337688] c) Martell JD, Yamagata M, Deerinck TJ, Phan S, Kwa CG, Ellisman MH, Sanes JR, Ting AY, Nat Biotechnol 2016, 34, 774–780 [PubMed: 27240195] d) Win MN, Smolke CD, Science 2008, 322, 456–460 [PubMed: 18927397] e) Jester BW, Tinberg CE, Rich MS, Baker D, Fields S, ACS Synth Biol 2018, 7, 2457–2467 [PubMed: 30204430] f) Villa JK, Su Y, Contreras LM, Hammond MC, Microbiol Spectr 2018, 6g) Skjoedt ML, Snoek T, Kildegaard KR, Arsovska D, Eichenberger M, Goedecke TJ, Rajkumar AS, Zhang J, Kristensen M, Lehka BJ, Siedler S, Borodina I, Jensen MK, Keasling JD, Nat Chem Biol 2016, 12, 951–958 [PubMed: 27642864] h) Green AA, Silver PA, Collins JJ, Yin P, Cell 2014, 159, 925–939. [PubMed: 25417166]

7. a) Kamiyama D, Sekine S, Barsi-Rhyne B, Hu J, Chen B, Gilbert LA, Ishikawa H, Leonetti MD, Marshall WF, Weissman JS, Huang B, Nat Commun 2016, 7, 11046 [PubMed: 26988139] b) Platasa J, Vasan G, Yang A, Pieribone VA, ACS Chem Neurosci 2017, 8, 513–523. [PubMed: 28045247]
8. Pu J, Chronis I, Ahn D, Dickinson BC, J Am Chem Soc 2015, 137, 15996–15999. [PubMed: 26652972]
9. alKeda RA, Richardson CC, J Biol Chem 1987, 262, 3790–3799 [PubMed: 3546319] b) Tabor S, Richardson CC, Proc Natl Acad Sci U S A 1985, 82, 1074–1078. [PubMed: 3156376]
10. Shis DL, Bennett MR, Proc Natl Acad Sci U S A 2013, 110, 5028–5033. [PubMed: 23479654]
11. Segall-Shapiro TH, Meyer AJ, Ellington AD, Sontag ED, Voigt CA, Mol Syst Biol 2014, 10, 742. [PubMed: 25080493]
12. Pu J, Zinkus-Boltz J, Dickinson BC, Nat Chem Biol 2017, 13, 432–438. [PubMed: 28192413]
13. Pu J, Dewey JA, Hadji A, LaBelle JL, Dickinson BC, J Am Chem Soc 2017, 139, 11964–11972. [PubMed: 28767232]
14. Pu J, Kentala K, Dickinson BC, ACS Chem Biol 2018, 13, 431–437. [PubMed: 28809467]
15. Gardner LP, Mookhtiar KA, Coleman JE, Biochemistry 1997, 36, 2908–2918. [PubMed: 9062120]
16. Cheetham GM, Steitz TA, Science 1999, 286, 2305–2309. [PubMed: 10600732]
17. Lykke-Andersen J, Christiansen J, Nucleic Acids Res 1998, 26, 5630–5635. [PubMed: 9837993]
18. Dickinson BC, Leconte AM, Allen B, Esvelt KM, Liu DR, Proc Natl Acad Sci U S A 2013, 110, 9007–9012. [PubMed: 23674678]
19. Badran AH, Liu DR, Nat Commun 2015, 6, 8425. [PubMed: 26443021]
20. Hubbard BP, Badran AH, Zuris JA, Guilinger JP, Davis KM, Chen L, Tsai SQ, Sander JD, Joung JK, Liu DR, Nat Methods 2015, 12, 939–942. [PubMed: 26258293]
21. Hussey BJ, McMillen DR, Nucleic Acids Res 2018, 46,
22. Patra D, Lafer EM, Sousa R, J Mol Biol 1992, 224, 307–318. [PubMed: 1560454]

Figure 1.

T7 RNAP C-terminal fusions abolish activity. A) Schematic of C-terminal fusions tested on full-length T7 RNAP (T7-C) and split T7 RNAP (RNAP_C-C). B) Transcriptional reporter assay in *E. coli* using a T7 promoter-driven luciferase gene and expression of either an empty vector (ctrl.), wild type full-length T7 RNAP (T7 RNAP), which shows strong activity, or full-length T7 RNAP with the modified C-terminal (T7-C), which shows no activity. C) Transcriptional reporter assay of the split T7 RNAP in *E. coli* using a T7 promoter driven luciferase gene. Vector background (ctrl.), expression of the evolved N-29–1 RNAP_N variant (RNAP_N), or expression of the RNAP_C variant (RNAP_C) does not induce transcription, but coexpression of RNAP_C and RNAP_N, each fused to interacting zipper peptides, produces robust T7 promoter-driven RNA. However, when RNAP_C is modified on the C-terminus (RNAP_C-C), there is no detectable signal produced when coexpressed with RNAP_N, indicating the C-terminal fusion also abolishes activity in the split protein context. Error bars are $\pm$ s.e.m. ($n = 4$ biological replicates).

Figure 2.

Design and validation of PACE system to evolve activity back in C-terminal modified full-length T7 RNAP. A) Schematic of PACE system: Selection phage (SP) carry an evolving C-terminal modified T7 RNAP in their engineered genome, in place of gIII. Phage infect *E. coli* cells that house a T7 promoter-driven source of gIII (p1-1), as well as a mutagenesis plasmid (MP)^[19] that induces mutations during PACE. B) Design of phage libraries encoding a randomized region on the C-terminus of a truncated T7 RNAP, followed by a modified GGSGGGS linker fusion. C) Phage replication in activity-dependent plaque assays. *E. Coli* cells housing p1-1 were plated with full-length unmodified T7 RNAP SP phage (left) or the C-terminal modified phage library (right).

Figure 3.

Evolution of activity recovery in C-terminal modified full-length T7 RNAP. A) Schematic of PACE experiment: 1059 cells, which provide “free” gIII and therefore neutral drift, were used to mix into the activity-dependent p1-1 cells over three days of PACE. B) Activity-dependent plaque assays showing that the library of phage was unable to show plaques in *E. coli* housing p1-1 cells at the start of the evolution, but evolved robust plaque formation during the three days of PACE. C) Transcriptional reporter assays of T7-C variants that emerged from the evolution. *E. coli* cells were co-transformed with a vector expressing a T7 promoter-driven luciferase and either a vector expressing full-length T7 RNAP (T7 RNAP), C-terminal modified T7 RNAP (T7-C) or variants (T7-C-variant) from the evolution. Error bars are $\pm$ s.e.m. ($n = 4$ biological replicates).

A

T7-C-A	Y44C, G538E , V685A , A724S , C-term: ILEPTLE-GSGGGGS
T7-C-B	G538E , V685A , A724S , C-term: ILEPTLE-GSGGGGS
T7-C-C	A409S, G538E , A617S, V685A , A724S , C-term: ILEPTLE-GSGGGGS
T7-C-D	Y44C, G538E , C-term: ILEPTLE-GSGGGGS
T7-C-E	G538E , V685A , R829H, C-term: ILESSLFE-GSGGGGS
T7-C-F	G538E , M677L, V685A , A724S , A827T, C-term: ILEPTLE-GSGGGGS

B **Figure 4.**

Genotypes and structural analysis of evolved T7-C variants. A) Genotypes and C-terminal modifications for the six variants assayed in Figure 3C. B) Crystal structure of the T7 RNAP initiation complex (PDB 1QLN) showing the three common mutations evolved during PACE. Position G538, which is directly adjacent to the C-terminus carboxylate and was mutated to a glutamic acid during PACE, is highlighted.

Figure 5.

Design and evolution of split RNAP C-terminal fusion tolerance. A) Schematic of PACE to evolve RNAPC to tolerate C-terminal fusions: Phage carry an evolving C-terminal modified RNAPC split protein on their engineered genome in place of gIII (SP), which infect *E. coli* cells that house a T7 promoter-driven source of gIII (p1-1), as well as an MP that induces mutagenesis, plus a helper plasmid (HP) that provides a source of RNAP_N. ZA/ZB zipper peptide interactions on the RNAP_N and RNAP_C-C variants drive the assembly. B) Schematic of PACE protocol: PACE was initiated with phage encoding RNAP_C-C with G538E, V685A, and A724S and evolved on wild type RNAP_N-ZA for the first part of the evolution. The selection pressure was then increased by gradually switching to N-29-1 RNAP_N, which was previously evolved to be proximity dependent. C) Transcriptional reporter assay of the evolved variants with N-29-1 RNAP_N fused with either a non-interacting peptide (no fusion, grey bars) or the ZA interaction zipper peptides (with zipper peptides, blue bars). “Ctrl.” is

vector background. RNAPc-C is inactive, whereas all eight evolved variants are active in the context of a proximity-dependent driving interaction. Error bars are $\pm$ s.e.m. ($n = 4$ biological replicates).

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Figure 6.

Genotypes and structural analysis of evolved RNAP_C variants. A Genotypes and C-terminal modifications for the six variants assayed in Figure 5C. All of the variants have the same C-terminal fusion: ILEPTLE-GSGGGS. B Crystal structure of the T7 RNAP initiation complex (PDB 1QLN) showing the common mutations evolved during PACE, as well as the split site.

Figure 7.

Evolved RNAP_C-C variants function as small molecule biosensors with evolved proximity-dependent RNAP_N fusions. A) Vector design to create a rapamycin-inducible biosensor system, using 1) T7 promoter driven luciferase to measure transcription, 2) constitutively expressed evolved proximity-dependent RNAP_N fused to FRB (29-1 RNAP_N), and 3) P_{BAD}-inducible RNAP_C variants with an N-terminal FKBP fusion and a C-terminal DsRed fusion. B) Transcriptional reporter assay of the evolved variants with either DMSO (control, grey bars) or induced by 20 μ M rapamycin for 3 hours (+ rapamycin, blue bars). Variant RNAP_C-L1C seems unstable during outgrowth, resulting in large variation in endpoints assays. Error bars are $\pm$ s.e.m. ($n = 4$ biological replicates).