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A Panel of Protease-Responsive RNA Polymerases Respond to Biochemical Signals by Production of Defined RNA Outputs in Live Cells

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Supporting Information

ABSTRACT: RNA is an attractive biomolecule for biosensing and engineering applications due to its information storage capacity and ability to drive gene expression or knockdown. However, methods to link chemical signals to the production of specific RNAs are lacking. Here, we develop protease-responsive RNA polymerases (PRs) as a strategy to encode multiple specific proteolytic events in defined sequences of RNA in live mammalian cells. This work demonstrates that RNAP-based molecular recording devices can be deployed for multimodal analyses of biochemical activities or to trigger gene circuits using measured signaling events.

Synthetic biology-based methods are promising strategies for the manipulation of biological systems to both interrogate and control biological regulatory systems.[1] Elegant methods have been developed to sense and respond to RNA patterns and to create RNA and DNA-based computation systems.[2] RNA is an attractive biomolecule for engineering because it can easily be programmed to interact with other nucleic acids based on specific nucleotide binding. However, RNA parts are not well suited for integration with protein-based chemical modifications that regulate cellular systems. Therefore, a general strategy is needed to link protein-based chemical and biochemical events, such as protease activities, to programmable RNA output signals.[3]

Proteases regulate diverse processes such as viral infection, cell death, inflammation, differentiation, and cancer.[4] Current approaches to monitor protease activities in living systems include substrate-based[5] or activity-based small molecule probes[6] and genetically encoded fluorescent sensors.[7] While powerful for analysis, these approaches are not suitable for synthetic biology applications. Moreover, due to the information storage capacity of nucleotides and the amplification of polymerase chain reaction (PCR), molecular sensors that store endogenous protease information in RNA should have advantages in terms of multidimensionality and sensitivity.

While technologies to interrogate and compute RNA information have quickly progressed,[8] there are comparably few methods to integrate non-nucleic acid information into nucleic acid signals. Riboswitches are RNA-based elements that control transcription or translation, which can be used to detect the presence of small molecule metabolites or environmental factors.[9] Aptamers have been engineered to create RNAs that respond to small molecules with a fluorescent output or to engineer riboswitches.[10] Aptamers that respond to proteins and drive translation have been successfully deployed to control responses in mammalian cells.[11] While aptamers provide a powerful method to sense protein concentrations, integrating protein-based enzymatic activities into RNA-based devices presents significant obstacles. Recently, a calcium-sensitive DNA polymerase was proposed as a method to "record" neural firing events in DNA, thereby permitting analysis of neural connectivity by sequencing.[12] Although this concept illustrates the potential of nucleic acids serving as an endogenous biochemical information storage medium, it is not amenable to sensing properties outside calcium concentrations. Therefore, a general strategy to transduce protein-based chemical information into nucleic acids would permit downstream analysis or integration with nucleic acid based synthetic circuitry.

In this report, we developed protease-responsive RNA polymerases (PRs), molecular sensors that "record" specific protease activities in defined sequences of RNA, as an enabling technology to simultaneously monitor and respond to biochemical events in living cells. We deployed continuous directed evolution to create a panel of three T7 RNAP variants with orthogonal DNA promoter specificity. We then engineered protease-responsiveness into each RNAP variant using an approach we recently developed[13] that involves tethering catalytically inactive T7 lysozyme, which inhibits T7 RNAP, through a flexible linker containing a target protease substrate. The effective concentration forces the complex into the lysozyme-bound, RNAP-inactive state. Proteolysis of the target sequence releases an active RNAP that transcribes from a specific DNA promoter (Figure 1). We demonstrate that PRs function in live mammalian cells and respond to specific protease activities by driving programmed gene expression outputs. Our results establish RNAP-based molecular recording devices as a new strategy for the detection of or response to endogenous signaling events for both interrogating and engineering biological systems.

Multidimensional encoding of protease activities in RNA requires a panel of orthogonal PRs that transcribe from a unique DNA promoter when activated. A variety of methods have been developed to reprogram the DNA binding specificity of RNAPs.[14] We chose to implement Phage-Assisted Continuous Evolution (PACE), which can rapidly reprogram

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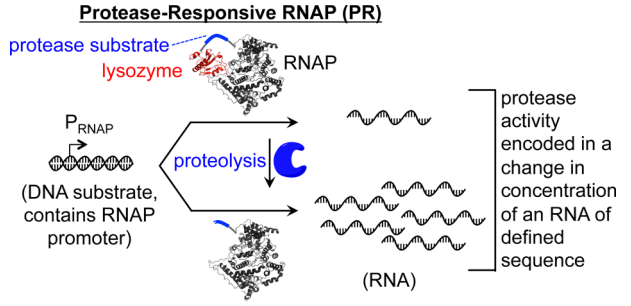

Figure 1. Design and mechanism of activation of PRs.

T7 RNAP to selectively recognize a DNA substrate of almost arbitrary composition (Figure 2A).[15]

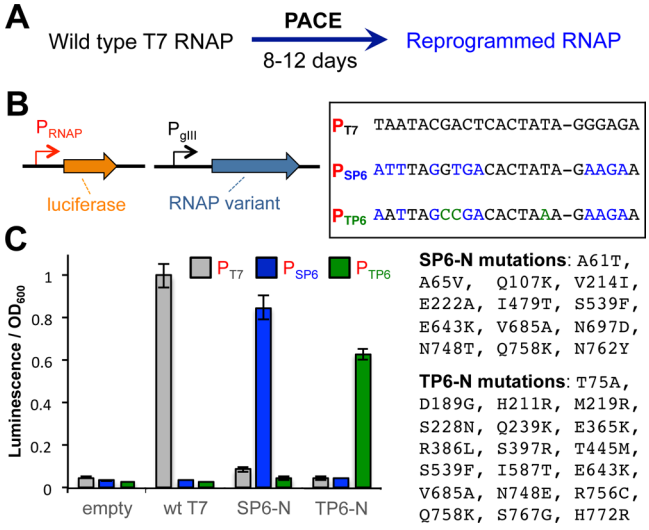

Figure 2. (A) PACE rapidly reprograms DNA specificity of RNAPs. (B) Vectors used to test promoter specificity of RNAP variants. (C) Characterization of the DNA promoter specificity of wild-type T7 RNAP, variant SP6-N, and variant TP6-N by measuring promoter driven luciferase activity in *E. coli* (error bars std. error, $n = 3$).

In previous evolutions, PACE yielded T7 RNAP variants that transcribe from either the SP6 promoter (P_{SP6}) or the "TP6 promoter" (P_{TP6}, Figure 2B).[16] However, these variants are not selective; the RNAPs transcribe from not only their target promoter but also promoters that they acted on in their previous evolutionary history. Therefore, we initiated PACE with libraries of the promiscuous RNAP variants and then deployed PACE negative selection strategies[17] against P_{T7} activity. Briefly, 4−9 days of PACE followed by 3−4 days of PACE with negative selection resulted in populations of RNAP variants with the desired levels of either SP6 or TP6 activity (evolutionary details provided in the Supporting Information). After a small screen from each population, we isolated variant "SP6-*N*", which achieved SP6 activity while losing all detectable T7 activity, and variant "TP6-*N*", which lost all detectable T7 and SP6 activities while maintaining TP6 activity (Figure 2C). These results demonstrate that PACE permits the rapid and selective reprogramming of RNAPs to generate panels of orthogonal variants for engineering RNAP-based sensors.

With the evolved RNAP variants in hand, we set out to engineer a panel of PRs. We cloned each RNAP variant into an *E. coli* expression vector in which the RNAP variant is N-terminally tethered to a catalytically inactivated T7 lysozyme by a flexible linker containing the HRV protease cutsite, to generate T7-PR-HRV, SP6-PR-HRV, and TP6-PR-HRV. In order to assay the protease activation and selectivity of each PR, we cotransformed *E. coli* cells with (1) a PR expression vector, (2) a reporter vector containing luciferase under the control of P_{T7}, P_{SP6}, or P_{TP6}, and (3) an HRV protease expression vector or control vector (Figure 3A). Expression of HRV protease,

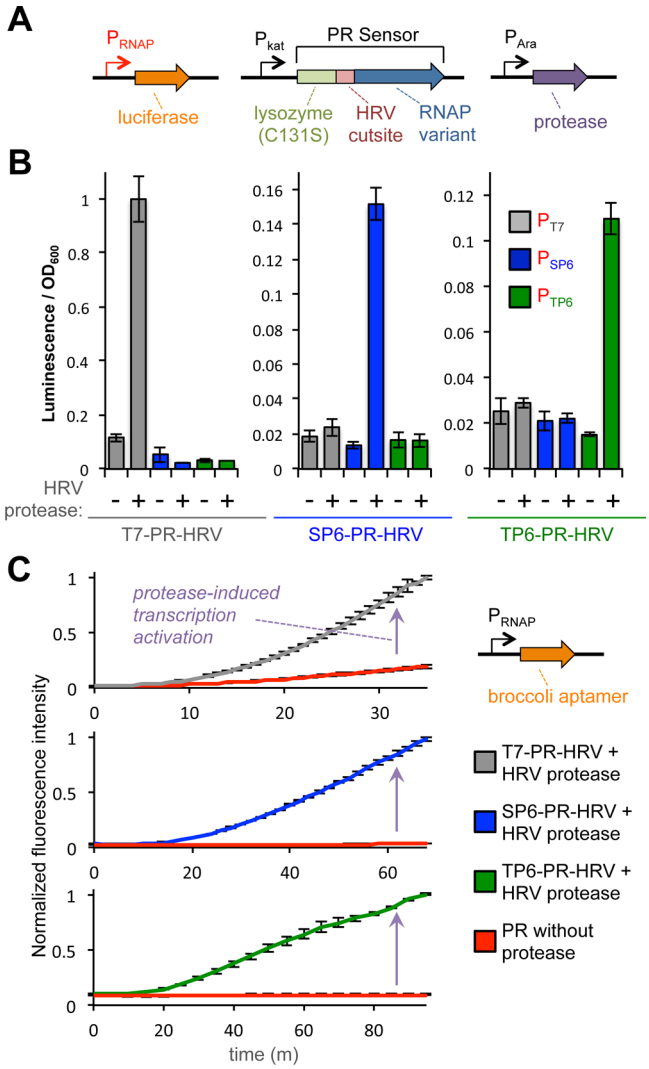

Figure 3. (A) Vectors used to assay PR activation. (B) *E. coli* cotransformed with an expression vector for a PR-RNAP, a protease expression vector, and a reporter vector with a promoter of interest driving luciferase and luminescence analyzed (error bars std. error, $n = 4$). (C) *In vitro* assay with purified PR sensors using the broccoli aptamer RNA transcriptional reporter (error bars std. dev., $n = 3$).

which cleaves the target recognition site, along with an on-target DNA promoter results in a significant boost in transcription (Figure 3B). If an inactive protease or a reporter with an off-target promoter is used, no enhancement in RNA synthesis is observed. To verify that the cell-based assays accurately reported differences in RNA output, we expressed and purified the three PR-HRVs and HRV protease. *In vitro* cleavage assays and transcription assays confirmed that proteolysis boosts transcriptional output from the sensors (Figures 3C, S9). Together, these data demonstrate that all

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three PRs respond to proteolysis by transcribing from their respective DNA promoters.

We next turned our attention to deploying PRs in mammalian cells. Inspired by work developing light-responsive RNAPs,[18] we cloned a vector containing a CMV promoter driven T7-PR sensor containing the HRV-3C, HCV, or TEV protease cutsite ("T7-PR-HRV", "T7-PR-HCV", or "T7-PR-TEV", respectively), along with P_{T7}-driven IRES-GFP (Figure 4A), so RNAP activity levels can be measured by GFP

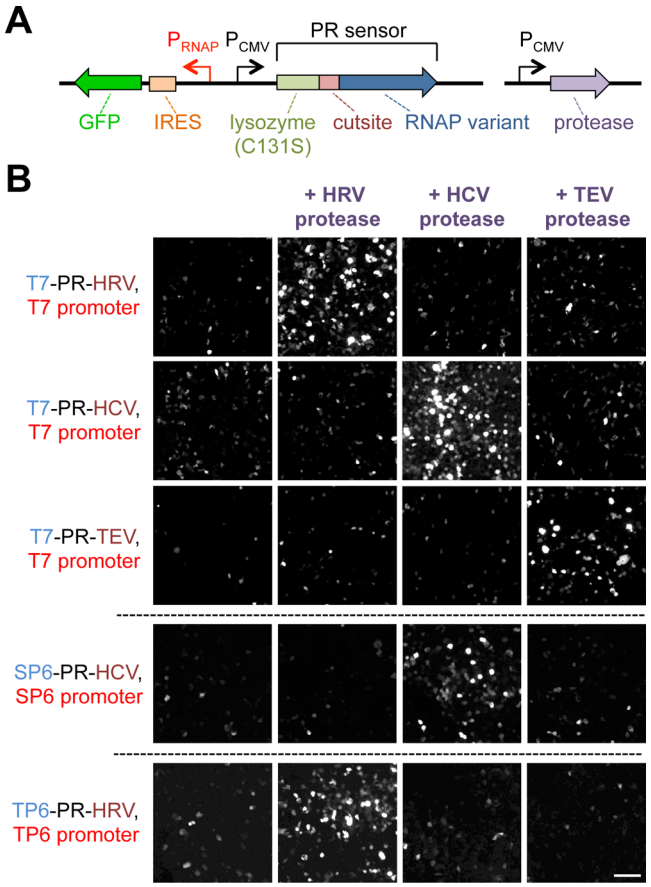

Figure 4. (A) Vectors used to deploy PRs in mammalian cells. (B) HEK293T cells cotransfected plasmids shown in (A). 40 h after transfection, the cells were analyzed by fluorescence microscopy. An example image of GFP fluorescence is shown for each set of conditions. Only if the cotransfected protease vector can activate the PR-RNAP is enhanced GFP fluorescence observed. 100 μm scale bar shown.

fluorescence microscopy. We also cloned mammalian expression vectors that express HRV-3C protease, HCV protease, or TEV protease. HEK293T cells transfected with a T7-PR contain low levels of GFP fluorescence, likely due to background transcriptional activity. However, when a protease that can cleave the target sequence of the T7-PR sensor is coexpressed, a significant boost in GFP fluorescence is observed (Figures 4B, S10–S12), demonstrating that the T7-PR sensor responds to proteases in mammalian cells. Additionally, the T7-PR sensor responds to different levels of protease activities, as validated with varied promoter strengths (Figure S13) and small molecule inhibitors (Figure S14).

Upon validation of T7-PR, we next assayed whether the SP6-PR and TP6-PR sensors also function in mammalian cells. We cloned SP6-N and TP6-N in place of T7 RNAP in the expression system and changed the promoter driving IRES-GFP to either P_{SP6} or P_{TP6}. Similar to the performance in *E. coli*, both the SP6-PR and the TP6-PR also responded to specific proteolysis with enhanced transcription as measured by GFP fluorescence (Figures 4B, S15–S16).

Finally, we attempted to deploy the SP6-PR and TP6-PR sensors simultaneously, due to their similar relative activity levels (Figure 3B). We cloned vectors expressing the SP6-PR-HCV sensor with P_{SP6}-driven RFP and the TP6-PR-HRV sensor with P_{TP6}-driven GFP, so that both RNAPs could be simultaneously monitored (Figure 5A). We cotransfected

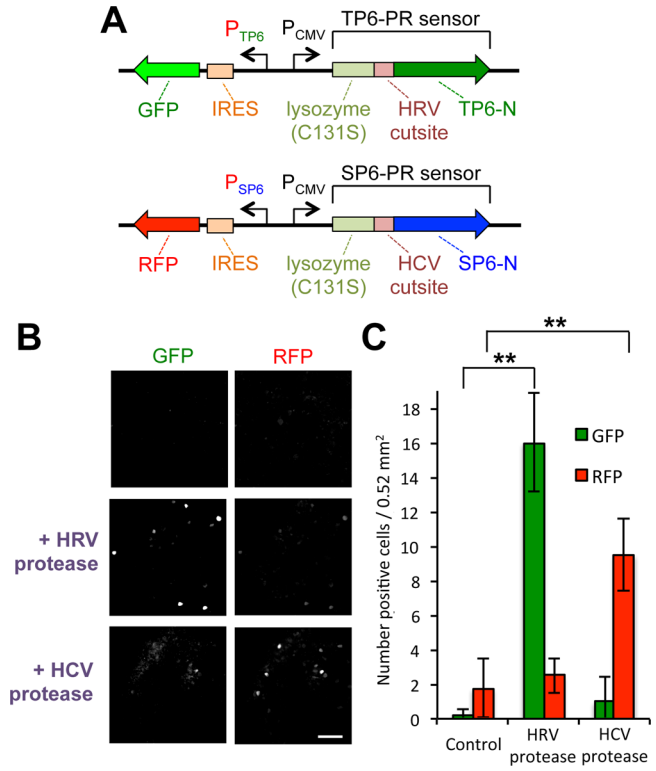

Figure 5. (A) Dual SP6-PR-HCV RFP reporter and TP6-PR-HRV GFP reporter vector system to monitor two proteases simultaneously. (B) HEK293T cells cotransfected with vectors shown in (A) with or without protease vector. 48 h after transfection, the cells were imaged for GFP and RFP fluorescence. (C) Quantification of (B) (error bars std. error, $n = 4$). Student's t-test; $^{**}P \leq 0.005$. 100 μm scale bar shown.

HEK293T cells with both sensor/reporter vectors and a protease expression vector and monitored GFP and RFP fluorescence. Selective activation of each PR sensor resulted in more fluorescent cells only from the reporter protein driven by the specific promoter (Figures 5B, 5C, S17). The number of fluorescent cells was low, likely due to transfection efficiency and DNA accessibility issues, which need further optimization. However, this experiment demonstrates that multiple PRs can be deployed simultaneously, each responding to a specific protease activity by transcribing a unique RNA output.

In this first demonstration of an RNAP-based biosensing strategy, we showed that PRs trigger proteolysis-induced gene expression using GFP and RFP as model proteins. Our results suggest that biochemical activities can be measured using nucleic acid analysis technologies, presaging a new approach to multimodal analysis. Further studies exploring whether PRs can trigger other output responses, such as gene knockdown by

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RNAi, as well as expanding the approach to detect other activities, are now ongoing to explore the potential of this approach.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b10290.

Figures, supporting data, and experimental methods (PDF)

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Notes

The authors declare no competing financial interest.

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