JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Universal Aptamer-Based Real-Time Monitoring of Enzymatic RNA Synthesis

Katharina Höfer, Lisa V. Langejürgen, and Andres Jäschke*

Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, 69120 Heidelberg, Germany

Supporting Information

ABSTRACT: *In vitro* transcription is an essential laboratory technique for enzymatic RNA synthesis. Unfortunately, no methods exist for analyzing quality and quantity of the synthesized RNA while the transcription proceeds. Here we describe a simple, robust, and universal system for monitoring and quantifying the synthesis of any RNA in real time without interference from abortive transcription byproducts. The distinguishing feature is a universal fluorescence module (UFM), consisting of the eGFP-like Spinach aptamer and a highly active hammerhead ribozyme, which is appended to the RNA of interest (ROI). In the transcription mixture, the primary transcript is cleaved rapidly behind the ROI, thereby releasing always the same UFM, independent of the ROI sequence, polymerase, or promoter used. The UFM binds to the target of the Spinach aptamer, the fluorogenic dye DFHBI, and thereby induces a strong fluorescence signal. This design allows real-time quantification, standardization, parallelization, and high-throughput screening.

Aptamers are single-stranded oligonucleotides that bind targets with high affinity and selectivity.[1] A wide variety of aptamers have been developed by combinatorial chemistry approaches (*in vitro* selection, SELEX) and applied for the dissection of biological mechanisms,[2] the diagnosis and therapy of diseases,[3] the detection of target molecules in biological samples,[3b,4] for materials science and nanotechnology applications,[5] and recently also in preparative organic synthesis.[6] Many attempts were also made to utilize RNA aptamers for imaging purposes, e.g., by developing binders for fluorogenic targets that have no intrinsic fluorescence but that light up upon binding to RNA.[7] Recently, Paige et al. described an RNA aptamer termed "Spinach" that fluoresces when binding to a fluorinated derivative of the GFP chromophore, namely 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). This dye-aptamer pair displays fluorescence properties similar to those of eGFP and is—unlike most fluorescent proteins—resistant to photobleaching.[8]

While for *in vivo* imaging of rare RNAs still severe hurdles have to be overcome, there is a strong demand for novel tools in molecular and synthetic biology.[9] Run-off transcription of RNA is a most common laboratory technique used routinely in thousands of laboratories. It is, however, generally performed in a black-box manner, with no process analytics at all, and RNA synthesis yields and purities are generally registered afterward by gel electrophoresis. Optimizations are therefore laborious and time-consuming. Likewise, numerous laboratories study mechanistic aspects of transcription, analyze promoter strength and its modulation, or attempt to find drugs that inhibit transcription. All these investigations would benefit tremendously from a robust real-time assay that reports immediately the quantity of full-length RNA synthesized, that is completely independent from the polymerase, RNA, or promoter under investigation and that can be operated parallelized in a high-throughput manner using standard laboratory equipment. None of the known aptamer-dye pairs has so far been shown to fulfill these criteria.[7] Alternative approaches for real-time transcription monitoring, based on labeled complementary probes,[10] either require a specific probe for each transcript or fail to distinguish between full-length and abortive products.[2a,10a,11] Here we report the development of a highly specific and robust system for *in vitro* transcription monitoring and quantification, based on the Spinach aptamer, which we termed Spinach TART (Spinach aptamer-based monitoring of Transcriptional Activity in Real Time).

The design of the assay is shown in Figure 1: The spinach aptamer is appended to the RNA of interest (ROI). As preliminary data indicated large variation in fluorescence

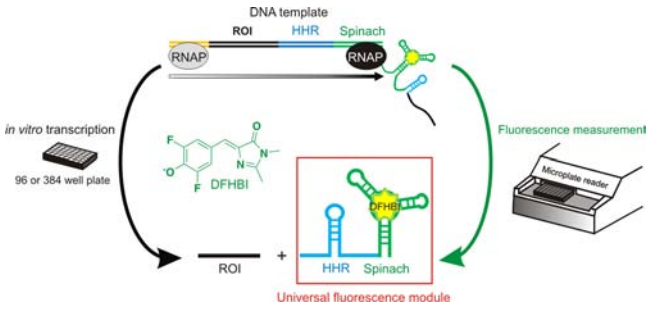

Figure 1. Principle of the Spinach TART assay: The DNA template consists of a promoter (T7, T3, SP6 or *E. coli* RNAP), an ROI, a HHR, and the Spinach aptamer. Transcription reactions are performed in a 384-well plate in the presence of DFHBI dye. During enzymatic RNA synthesis, fluorescence is continuously measured using a thermostatted microplate fluorescence spectrometer (37 °C). Transcribed RNA is posttranscriptionally cleaved by the HHR, resulting in the non-fluorescent ROI and the UFM which interacts with DFHBI. Only the full-length transcript gives rise to a functional UFM, ensuring that the fluorescence output (relative fluorescence units [RFU]) is proportional to the concentration of ROI.

Received: July 16, 2013
Published: August 30, 2013

dx.doi.org/10.1021/ja407142f | J. Am. Chem. Soc. 2013, 135, 13692−13694

properties when ROI and Spinach are covalently attached to each other (data not shown), a highly active hammerhead ribozyme[12] was inserted in between. This results in the posttranscriptional cleavage of the synthesized RNA strand, ultimately leading to the formation of two products: the ROI and a universal fluorescence module (UFM, consisting of the hammerhead ribozyme and the Spinach RNA). The UFM interacts with the 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) dye added to the transcription mixture and thereby induces a fluorescence signal that can be easily detected either in a standard cuvette-type fluorescence spectrometer or in a fluorescence microtiterplate reader. This design could have the following advantages: (1) As the sequence of the UFM is always the same and completely independent from the ROI, the fluorescence properties should become independent from the ROI as well, allowing standardization and comparison of different ROIs. (2) Abortive RNA synthesis products should not give rise to a signal, allowing the exclusive quantification of full-length ROI. (3) The use of the a hammerhead ribozyme (HHR) will ensure the absence of undesired tags and appendages in the ROI as well as a homogeneous 3′-end.[13] (4) The modular nature of the Spinach TART system should allow the use of a simple cloning vector containing the UFM sequence, into which any ROI together with its promoter can be inserted in one step. (5) The high quantum yield of the DFHBI–Spinach complex[8b] should ensure high sensitivity for the RNA of interest. (6) The excitation wavelength of DFHBI at 469 nm will not affect RNA integrity.

To investigate whether these assumptions hold true, we constructed a set of transcription templates encoding for two different RNAs (one for a highly structured 295mer riboswitch and a second for a short 32mer RNA) in combination with promoters for four commonly used polymerases (bacteriophage polymerases T7,[13] T3,[14] or Sp6[15] and *E. coli* RNAP[16] (Figure S1, for sequences see Table S1) and monitored the increase in DFHBI fluorescence during transcription in a thermostatted 384-well fluorescence microtiterplate reader. The fluorescence vs time plots yielded typical enzyme kinetics progress curves for all four polymerases (Figures 2a,b and S2). The increase in signal was found to be proportional to the template concentration. The progress curves revealed (known) differences between the polymerases in their stabilities, relative transcription rates, and dependence on other parameters. Experimental problems could be spotted immediately (e.g., complete consumption of nucleoside triphosphates (NTPs) after 1.5 h in the green curve in Figure 2b).

To ensure that the measured fluorescence intensities are consistent with the synthesized RNA levels, PAGE analysis was performed for each transcription mix after recording the fluorescence vs time trace (Figures 2c and S3). In all cases, the primary transcript was cleaved completely into ROI and UFM (Figure S4). Moreover, the intensities of the product bands on the gel were found to be consistent with the measured fluorescence intensities (Figure S5). DFHBI did not affect transcription efficiency (Figure S6).

Inclusion of a dilution series of purified UFM in the presence of DFHBI, ideally on the same microtiterplate, and construction of a calibration curve furthermore allows absolute quantification of the desired full-length RNA transcript in real time (Figures 2d and S7). The results corresponded very well with those from densitometric analysis of PAGE gels (Figure S7c). The dye-based Qubit measurements, however, gave slightly higher values (Figure 2d), which is to be expected, as

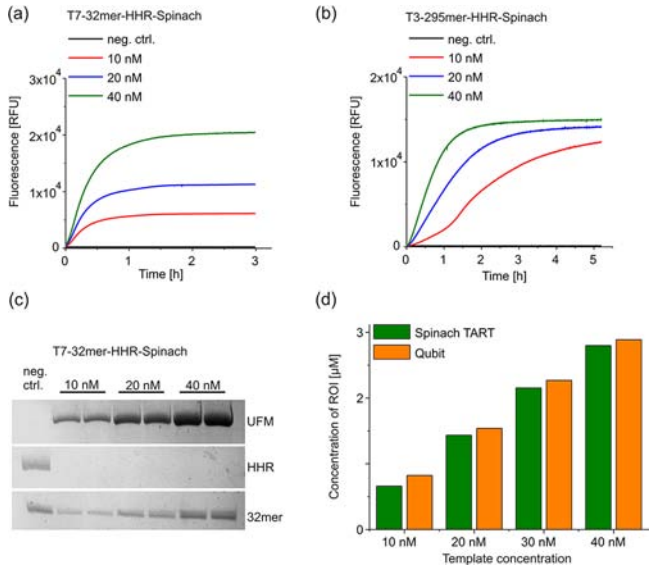

Figure 2. Spinach TART analysis of *in vitro* RNA synthesis by T7 and T3 RNAP using two different templates, encoding for a 32mer (a) and a 295mer ROI (b), at different template concentrations. As negative control, a DNA template (40 nM) was applied, that is lacking the Spinach motive at the 3′ end. *In vitro* transcriptions were performed in duplicate in a 384-well format. Reported values represent the mean of two measurements with a standard deviation <2%. (c) PAGE analysis of the T7 transcription mixture (32mer, panel a), performed in duplicate. (d) Comparison of RNA yields calculated by Spinach TART fluorescence measurements or by Qubit fluorometer. The 295mer template was applied in combination with T7 RNAP.

Qubit registers both full-lengths and abortive transcripts, while in Spinach TART only full-lengths products contribute to the signal.

After demonstrating the general feasibility of the Spinach TART assay, we tested its application to three time-consuming real-world applications (Figure 3a–d). The first is the optimization of reaction conditions for run-off transcription that normally involves PAGE analysis after completion of transcription and often requires several iterations. Small differences in the composition of the transcription mixture and, in particular, in the concentrations of magnesium ions and NTPs are known to cause large variations in transcription yields.[17] Figure 3c shows the optimization of the Mg^{2+}-ion concentration, recorded in parallel in a microtiterplate reader. Already during the first hour of the reaction it becomes evident that 19 mM Mg^{2+} is ideal for this transcript (Figure S8c). The fluorescence intensity of the UFM–DFHBI complex shows only low sensitivity to Mg^{2+} ions in the relevant concentration range[8b] (Figure S9).

Next, the Spinach TART system was applied to investigate the effect of potent polymerase inhibitors on transcriptions in real time (Figure 3a). Heparin is a well-established competitive inhibitor of T7 RNAP, occupying DNA binding sites on the RNA polymerase.[18] The time traces indicate almost full inhibition of transcription by 2 mM heparin, in agreement with previous reports[18] (Figures 3b and S8a).

Finally we used the Spinach TART system to compare T7 RNAP transcription initiation by different well-characterized promoters.[19] DNA templates were designed that contain either a ϕ2.5 or a ϕ6.5 promoter and vary in adenosine or guanosine initiation. The kinetic curves in Figure 3d reveal that T7ϕ2.5 in combination with G-initiation results in low RNA yields, while

dx.doi.org/10.1021/ja407142f | J. Am. Chem. Soc. 2013, 135, 13692–13694

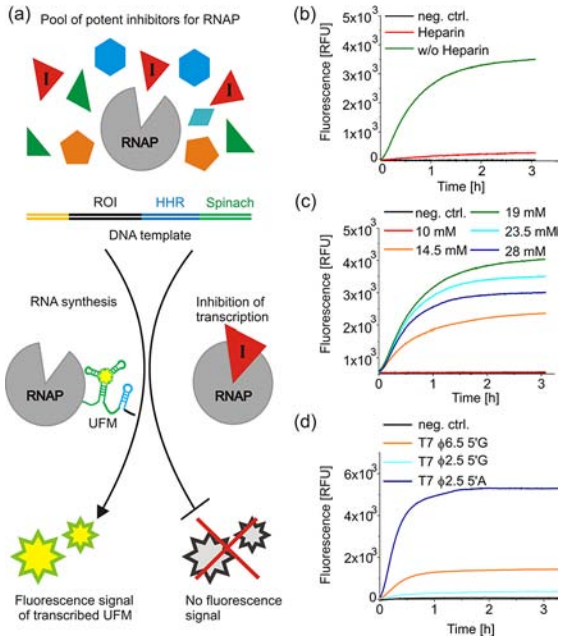

Figure 3. Applications of the Spinach TART assay. (a) Cartoon depicting Spinach TART screening for small-molecule inhibitors of transcription. (b) Inhibition of enzymatic RNA synthesis by heparin. As template, the 295mer-HHR-Spinach (20 nM) was used in combination with T7 RNAP. (c) Screening of $MgCl_2$ concentrations to optimize transcription conditions of the 295mer-HHR-Spinach (40 nM) by T7 RNAP. (d) Characterization of the T7ϕ2.5 and T7ϕ6.5 promoter strength and influence of the initiator nucleotide on the transcription of the 295mer-HHR-Spinach (40 nM).

with adenosine initiation, transcriptions yields are 60 times higher (Figures 3d and S8b). On the other hand, the strength of a T7ϕ6.5 promoter with G-initiation is higher than T7ϕ2.5 with G-initiation, in agreement with the literature.[20]

In conclusion, we describe here a robust and universal real-time fluorescent assay that reports the quantity of synthesized full-length RNA of interest during *in vitro* transcription. This assay does not require additional steps, compared to a standard transcription, and costs are comparable. We expect the Spinach TART method to greatly facilitate research in RNA biology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Spinach TART protocols and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

jaeschke@uni-hd.de

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Marlene Tesch for technical assistance.

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