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Real-time monitoring of a stepwise transcription reaction on a quartz-crystal microbalance

Shuntaro Takahashi, Kazuya Hisanaga, Aya Yoshida, Yoshio Okahata *

Department of Biomolecular Engineering and Global Centre of Excellence, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

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ABSTRACT

We monitored real-time DNA transcription by T7 RNAP using a 27-MHz DNA-immobilized quartz-crystal microbalance (QCM) in buffer solution to investigate the stepwise reaction of transcription. We designed a template double-stranded DNA that consisted of a T7 promoter, a stall position (15 bp downstream from the promoter), and a 73-bp transcription region. Based on the frequency (mass) changes of the template-immobilized QCM in response to the addition of T7 RNAP and monomers of NTP, we obtained the kinetic parameters of each step of the T7 RNAP reactions: the enzyme-binding rate (k_{on}) to and the dissociation rate (k_{off}) from the promoter, the proceeding rate (k_{for}) from the promoter to the forward stall position, the polymerization rate (k_{cat}) of RNA along DNA, and the release rate (k_r) from the end of the template DNA. We found that k_{cat} (120 s^{-1}) was extremely large compared with k_{off} (0.014 s^{-1}), k_{for} (0.062 s^{-1}), and k_r (0.014 s^{-1}), revealing that the rate-limiting steps of T7 RNAP involve the binding to the promoter, the movement to the stall position, and the release from DNA. These kinetic parameters were compared with values for other DNA-binding enzymes.

DNA-dependent RNA polymerase (RNAP)[1] transcribes DNA sequences into RNA, which mainly regulates gene expression [1]. This transcription reaction includes many important processes. First, RNAP binds to the promoter sequence on a template DNA, and melts the DNA to form an open complex (OC). Second, RNAP begins to polymerize several RNAs—and an elongation complex (EC) quickly elongates the nascent RNA chain. Finally, RNAP releases the synthesized RNA chain, and dissociates from the template DNA. The regulation of gene expression can occur in each step of the transcription reaction. In biological systems, transcription factors (TFs) usually play a variety of roles to tightly control all phases of transcription [1–8]. Therefore, to fully understand the transcription regulation, it is necessary not only to quantify the RNA amount transcribed but also to evaluate the kinetics and dynamics of each process in transcription comprehensively. To date, many sophisticated methods have been established to investigate the precise mechanism of transcription using single molecule techniques [9–17]. However, most of them only focused on the binding to DNA, the initiation process, or the elongation catalysis of RNA, and the release of RNAP from the DNA has not been well characterized before. Of course, there are only a few existing approaches for observing all steps of transcription sequentially on a single device.

In this study, we employed a template double-stranded DNA (dsDNA)-immobilized 27-MHz quartz-crystal microbalance (QCM) to analyze the stepwise reaction of a single-turnover transcription by RNAP (see Fig. 1). The QCM has previously been shown to measure increases in mass on its surface at the nanogram level by detecting decreases in frequency in real time [18–20]. Therefore, the binding and dissociation of RNAP from the template DNA, the RNA transcription along the template DNA, and the release of RNAP from the template are revealed as simple mass changes on the QCM. Herein, we focused on T7 RNAP as a model enzyme of the transcription reaction, which is a monomeric protein that shows a high productivity of transcripts without the involvement of any transcription factors [21–26]. We designed the template DNA to include a T7 promoter, a stall position (15 bp downstream from the promoter after a sequence containing only C and T bases), and 58 bp of the transcription region (106-bp total length, see Fig. 1B). Based on the frequency changes (mass changes) of the template-immobilized QCM, we successfully obtained the kinetic parameters of the transcription reaction, including the RNAP-binding rate (k_{on}) of T7 RNAP to and the dissociation rate (k_{off}) from the T7 promoter region, the proceeding rate (k_{for}) to the forward stall position, the polymerization rate of RNA (k_{cat}), and the release rate (k_r) of RNAP from the template (see Fig. 1C). We also studied the effects of site-directed mutations of T7 RNAP (P266L, I810S, and P2166L/I810S double mutants) on the kinetic parameters obtained at each step. Thus,

* Corresponding author. Fax: +81 45 924 5836.
E-mail address: yokahata@bio.titech.ac.jp (Y. Okahata).

[1] *Abbreviations used:* CC, closed complex; EC, elongation complex; OC, open complex; PBD, promoter-binding domain; PCR, polymerase chain reaction; QCM, quartz-crystal microbalance; RNAP, RNA polymerase; TFs, transcription factors; WT, wild type.

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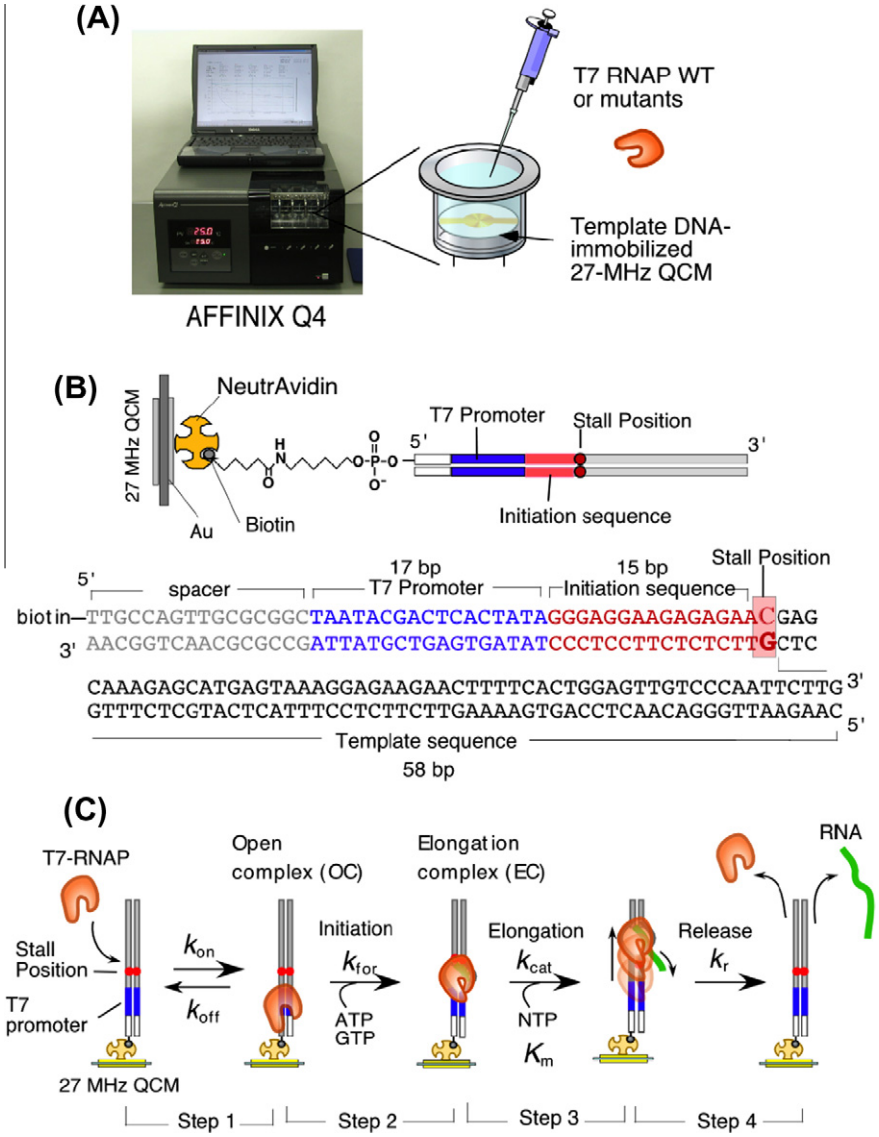

Fig.1. (A) Experimental setup of AFFINIX Q4 instrument for RNA transcription with T7 RNAP on a 27-MHz QCM. (B) DNA structures immobilized on a NeutrAvidin-QCM surface. (C) Reaction schemes of T7 RNAP on a QCM plate and the kinetic parameters obtained in this work.

the correlation between the kinetic parameters obtained using this QCM technique and the RNA amounts transcribed *in vitro* could essentially reveal how RNAP regulates RNA synthesis.

Materials and methods

Preparation of T7 RNAP wild type and mutants

The sequence encoding T7 RNAP wild type (WT) was amplified by polymerase chain reaction (PCR), and was inserted into the pQE-80L plasmid vector (Qiagen) between the BamHI and HindIII sites. Oligonucleotide-directed mutagenesis was carried out using the resultant plasmid with the QuickChange site-directed mutagenesis kit (Stratagene) for the construction of mutant T7 RNAP, P266L, I810S, and P266L/I810S double mutants. The expected mutations were verified by DNA sequencing. The *Escherichia coli* BL21 strain was transformed with each constructed vector, and was cultured in LB broth at 37 °C. The expressions of T7 RNAP WT and mutants were induced by the addition of 1 mM IPTG. The cells were harvested and sonicated to obtain their lysate. The cleared lysate was loaded onto a HisTrap HP column (GE Healthcare), and an imidazole gradient was applied to elute the His-tagged T7 RNAP WT and mutants. The eluent was collected and loaded onto a HisTrap heparin Sepharose HP column (GE Healthcare) for further purification with a NaCl gradient. The purified T7 RNAP WT and mutants were dialyzed against stock buffer (50 mM Tris-HCl with pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol) and stored at −20 °C until use.

27-MHz QCM setup and calibration in aqueous solutions

The design of DNA was as follows: the sequence of the retemplate chain was 5′- TTGCCAGTTGCGCGGCTAATACGACTCACTATAGGGAGGAAGAGAGAACGAGCAAAGAGCATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTG (the T7 promoter region and the stall position are shown underlined, respectively). Based on this design, when the reaction is started with GTP and ATP, T7 RNAP stalls at the +15 position. The dsDNA was prepared with oligonucleotides (Operon) and amplified by PCR with the primer 5′-CAAGAATTGGGACAACTCC and the 5′-biotinylated primer biotin-5′-TTGCCAGTTGCGCGGC (Operon). The total length of the biotinylated template dsDNA was 106 bp (see Fig. 1B).