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Evaluation of small ligand–protein interactions by using T7 RNA polymerase with DNA-modified ligand

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ABSTRACT

The interaction between proteins and ligands was evaluated by T7 RNA polymerase transcription with a DNA-modified ligand. The principle of this method is suppression of T7 RNA polymerase transcription by binding of a protein to small ligand modified by conjugation with a T7 RNA polymerase promoter. To demonstrate proof of principle, biotin or antifolate methotrexate was modified by covalent attachment of a T7 RNA promoter. Using these T7 RNA promoter-modified ligands, T7 RNA polymerase transcriptions were performed in the presence or absence of an anti-biotin antibody or recombinant human dihydrofolate reductase, respectively. Transcription was suppressed in the presence of each binding protein plus its modified ligand, but not in the absence of the binding protein.

Assessment of the binding capacity of small molecules as drug candidates directed against specific receptors is essential for drug screening. Recent development of improved synthetic techniques has increased the number of small molecule libraries. From these libraries, molecules that bind to target proteins are isolated during the drug discovery process. One of the drug discovery approaches, DNA-encoded chemical libraries, uses collections of small molecules that are covalently conjugated to DNA tags [1]. This makes it possible to identify each small molecule based on the DNA sequence of its tag. Recently, using this strategy, various molecules were selected from DNA-encoded chemical libraries [2–5].

DNA tags are useful not only for identification but also for signal enhancement of small molecules. Evaluation of interactions between small ligands and proteins can be difficult due to the low molecular weight of the ligands. Thus, techniques with signal enhancement are required for the measurement of small ligand interactions with proteins. Previously, we developed techniques to assess small ligand interactions with proteins using DNA tags [6]. The interactions between DNA-modified small ligands and proteins were detected as ligation reactions of the DNA tags; the ligation reactions were inhibited by binding of the protein to the small ligand. Ligated probes could be visualized on electrophoretic gels. However, to detect small amounts of ligated DNA tags, polymerase chain reaction (PCR)[1] amplification was required following the ligation reaction.

T7 RNA polymerase (T7RNAP) catalyzes the formation of RNA after binding to the T7RNAP promoter DNA sequence. T7RNAP may be used not only for transcription followed by translation but also for signal amplification by combination of the polymerase with a DNA tag consisting of the T7RNAP sequence [7,8]. Similar to immuno-PCR, which is a well-known sensing method that uses DNA tags to generate signals following binding of target molecules to DNA-modified antibodies, signal amplification by T7RNAP transcription was detected with high sensitivity. Transcription involves three phases: initiation, elongation, and termination. Conformational changes accompanying these processes have been well characterized [9–12]. To initiate transcription, T7RNAP binds to a specific promoter, separates the two strands of the DNA, and encloses the template strand in its active site. Elongation then begins with the coding strand of the downstream DNA as a template.

Based on this information, we developed a new method to evaluate interactions between small ligands and proteins by T7RNAP transcription with DNA-modified small ligands. The basic principle of this method is suppression of T7RNAP transcription by binding of a protein to a small ligand conjugated to the T7RNAP promoter (Fig. 1). In the absence of the small ligand binding protein,

[1] *Abbreviations used:* PCR, polymerase chain reaction;T7RNAP, T7 RNA polymerase;MTX, methotrexate;IgG, immunoglobulin G;hDHFR, human dihydrofolate reductase;EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide;NHS, *N*-hydroxysuccinimide; HPLC, high-performance liquid chromatography;PBS, phosphate-buffered saline;EDTA, ethylenediaminetetraacetic acid;IPTG, isopropyl β-d-thiogalactopyranoside;NTA, nitrilotriacetic acid.

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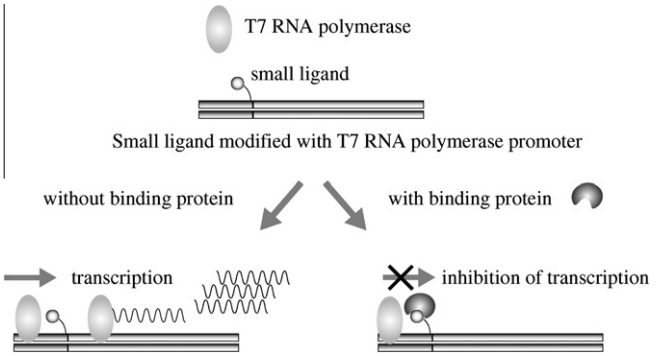

Fig. 1. Principle of this method. A small ligand is modified with the T7 RNA polymerase promoter. Using these DNA-modified small ligands, T7 RNA polymerase transcriptions are performed in the presence of a protein that binds to the small ligand. Transcription occurs normally when the protein is not present but is suppressed when the protein binds to the small ligand.

transcription occurs. However, in the presence of this protein, transcription should be suppressed. To demonstrate proof of principle, biotin and antifolate methotrexate (MTX, 4-amino-4-deoxy-N10-methylpteroylglutamic acid) were modified with DNA tags that incorporated the T7RNAP promoter, and then T7RNAP transcription was evaluated in the presence or absence of anti-biotin immunoglobulin G (IgG), anti-MTX IgG, or recombinant human dihydrofolate reductase (hDHFR).

Materials and methods

Materials

All synthetic oligonucleotides were synthesized by Operon Biotechnologies (Japan). Reagents for transcription were obtained from the MEGAshortscript Kit (Ambion, USA). For RNA staining, SYBR Gold was purchased from Molecular Probes (USA). Goat anti-biotin IgG (cat. no. SP-3000) was purchased from Vector Laboratories (USA). Rabbit anti-MTX IgG (cat. no. 915-056) was purchased from Assay Designs (USA). All other chemicals were of analytical grade.

Design of T7RNAP promoter-modified biotin

The DNA tags incorporating the T7RNAP promoter sequence were generated with or without the addition of an internal biotin. The oligonucleotides with biotin were synthesized by Operon Biotechnologies as Bio-ON modifications. The biotin moieties were located in the synthesized oligonucleotides of template strands at different positions, as shown in Table 1. Equal amounts of each non-template and template strand were mixed and incubated for 1 min at 95 °C. The resulting double-stranded DNA probes were named *probe–T7* (without biotin), *probe–bind* (biotin located in the binding region of the T7RNAP promoter), *probe–int* (biotin located in the initiation region of the T7RNAP promoter), and *probe–elo* (biotin located in the elongation region of the T7RNAP promoter).

Design of T7RNAP promoter-modified MTX

For construction of MTX modified with the T7RNAP promoter, an amino-modified oligonucleotide was synthesized by Operon Biotechnologies using the uni-link amino modifier. First, 20 μl of MTX (10 mg/ml, Sigma) was incubated with 10 μl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 62.5 mg/ml, Pierce) and 10 μl of *N*-hydroxysuccinimide (NHS, 30 mg/ml, Pierce) for 15 min at 37 °C. Next, the mixture was incubated with 60 μl of amino-modified oligonucleotide (100 pmol/μl in carbonate buffer) for 3 h at 37 °C. After the reaction, the unmodified MTX and coupling reagents were removed using a PD-10 column (GE Healthcare). The MTX-modified oligonucleotide was then separated on a Wakosil 5C18 column (Wako, Japan) by high-performance liquid chromatography (HPLC). Finally, the MTX-modified oligonucleotide was incubated with the template strand oligonucleotide for 1 min at 95 °C. The resulting double-stranded T7RNAP promoter-modified MTX was named *probe–MTX*.

Table 1
Biotin-modified oligonucleotides.

Non-template strand	5′-GGGTAATACGACTCACTATAGGGATACC AGACACGTGTAGCTCATTAGCTCCGAGTGAGATAT-3′
Template strand for probe–T7	3′-CCCATTATGCTGAGTGATATCCCTATGG TCTGTGCACATCGAGTAATCGAGGCTCACTCTATA-5′
Template strand for probe–bind	3′-CCCATTATG [*bio*] CTGAGTGATATCCCTATGG TCTGTGCACATCGAGTAATCGAGGCTCACTCTATA-5′
Template strand for probe–int	3′-CCCATTATGCTGAGTGATA [*bio*] TCCCTATGG TCTGTGCACATCGAGTAATCGAGGCTCACTCTATA-5′
Template strand for probe–elo	3′-CCCATTATGCTGAGTGATATCCC [*bio*] TATGG TCTGTGCACATCGAGTAATCGAGGCTCACTCTATA-5′

Evaluation of transcription efficiency with T7RNAP promoter-modified biotin by T7RNAP in the presence of anti-biotin IgG

To evaluate the transcription efficiency using probes in the presence or absence of a binding protein, probes were incubated with the reagents included in the MEGAshortscript Kit. At first, 3 μl of probes (3 μM) was incubated with 1 μl of T7 10× reaction buffer, 6 μl of MilliQ water, and 1 μl of anti-biotin IgG or phosphate-buffered saline (PBS) for 1 h at 37 °C. Next, the mixture was incubated with 1 μl of 10× reaction buffer, 2 μl each of a four-nucleotide solution (T7 ATP, CTP, GTP, and UTP solution), and 2 μl of T7 enzyme mix for 5 h at 37 °C.

For anti-biotin IgG concentration-dependent transcription reactions, probe–int was used. First, 3 μl of probe–int (20 μM) was incubated with 2 μl of T7 10× reaction buffer and 15 μl of various concentrations of anti-biotin IgG or MilliQ water (control) for 1 h at 37 °C. Next, the mixture was incubated with 2 μl of 10× reaction buffer, 3 μl each of the four-nucleotide solution, 5 μl of MilliQ water, and 1 μl of T7 enzyme mix for 5 h at 37 °C.

To evaluate the transcription efficiency with free biotin as a competitor in the presence of anti-biotin IgG, 1 μl of probe–int (20 μM) was incubated with 1 μl of T7 10× reaction buffer, 3 μl of various concentrations of biotin or MilliQ water, and 4 μl of anti-biotin IgG (6.7 μM) for 1 h at 37 °C. Next, the mixture was incubated with 1 μl of 10× reaction buffer, 2 μl each of the four-nucleotide solution, 5 μl of MilliQ water, and 2 μl of T7 enzyme mix for 5 h at 37 °C.

After allowing the transcription reaction to proceed for 5 h, to remove the probe, 2 μl of TURBO DNase (2 U/μl) was added to the mixture, followed by incubation for 30 min at 37 °C. Finally, 5 μl of ammonium acetate stop solution was added to stop the reaction. Transcripts were evaluated with SYBR Gold solution by a fluorometer or separated on a polyacrylamide gel using TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) with urea and then visualized by staining with SYBR Gold.

Evaluation of transcription efficiency with T7RNAP promoter-modified MTX in the presence of anti-MTX IgG

For the anti-MTX IgG concentration-dependent transcription reaction, 1 μl of probe–MTX (2 μM) was incubated with 1 μl of T7 10× reaction buffer, 6 μl of MilliQ water, and 2 μl of