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RNA polymerase molecular beacon as tool for studies of RNA polymerase - promoter interactions

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Abstract

The molecular details of formation of transcription initiation complex upon the interaction of bacterial RNA polymerase (RNAP) with promoters are not completely understood. One way to address this problem is to understand how RNAP interacts with different parts of promoter DNA. A recently developed fluorometric RNAP molecular beacon assay allows one to monitor the RNAP interactions with various unlabeled DNA probes and quantitatively characterize partial RNAP-promoter interactions. This paper focuses on methodological aspects of application of this powerful assay to study the mechanism of transcription initiation complex formation by *Escherichia coli* RNA polymerase σ^{70} holoenzyme and its regulation by bacterial and phage encoded factors.

Keywords

RNA polymerase; transcription initiation; transcription regulation; fluorescence spectroscopy; RNA polymerase-promoter interactions

1. Introduction

The bacterial RNA polymerase (RNAP) initiates transcription in the form of the holoenzyme (subunit composition $\alpha|\alpha||\beta\beta'\omega\sigma$). The RNAP core enzyme ($\alpha|\alpha||\beta\beta'\omega$) is catalytically competent but does not recognize promoters. The specificity subunit σ is required for promoter recognition [1]. One σ (primary) is usually present at the highest level and is responsible for most transcription in a bacterial cell [2]. In model prokaryote *Escherichia coli* the primary σ factor is σ^{70} . Interactions between the σ^{70} RNAP holoenzyme and promoter DNA in the transcription initiation complexes involve about 80 DNA base pairs

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(from -60 to +20, relative to the transcription start site at +1) and vast regions of the RNA polymerase surface. In a catalytically-competent open promoter complex (RP_o), a stretch of ~13 base pairs of promoter DNA is unwound and melted (forming the so-called transcription bubble) and one strand of DNA is loaded into the active site, providing a template for RNA synthesis [1]. In the context of the RNAP holoenzyme, σ^{70} makes direct contacts with two conserved promoter elements located at positions -10 and -35 relative to the transcription start (consensus sequences TATAAT and TTGACA, respectively). The optimal length of the spacer separating the -10 and -35 promoter elements is 16–18 bp; an increase or decrease of optimal spacer length weakens promoter strength. Additional contacts between σ^{70} and the TG dinucleotide of an extended -10 element (consensus TGnTATAAT) can compensate for the lack of interactions with the -35 element absent from some promoters [1,3]. The non-template strand of the DNA region immediately downstream of the -10 element interacts with conserved region 1.2 of σ^{70} and with the β subunit [4–6]. The strong interaction between σ^{70} and non-template strand nucleotides of the -10 element and adjacent downstream nucleotides is the main source of energy driving the promoter DNA melting [4–9]. An A+T-rich promoter region between positions -40 and -60 (the UP element) is an additional important determinant of promoter activity [10,11]; it is contacted by the C-terminal domains of the α subunits. A zinc-binding element of the largest subunit β' can make specific contacts with promoter spacer around position -20, strengthening RNAP binding to some promoters [12]. RNAP also makes extensive sequence-nonspecific contacts with promoter DNA. While these contacts are not responsible for promoter recognition, they contribute to stabilization of transcription complexes and may facilitate promoter DNA melting [1, 13–16]. Thus, the rate of formation and stability of transcription initiation complexes and, ultimately, the level of transcription is determined by the numerous partial interactions between distinct structural elements of RNAP and segments of promoter DNA. Changing the strength of any one of these partial interactions can lead to drastic changes in transcription. It is therefore of no surprise that transcription initiation is a heavily regulated step of bacterial gene expression. One way to understand how a transcription factor affects transcription initiation by RNAP is to dissect its effects on RNAP interactions with different parts of promoter.

Certain promoter fragments, even though incapable of supporting transcription, can specifically interact with RNAP and resulting complexes may mimic the interactions of RNAP with corresponding segments of native promoters. Such promoter fragments can be used as DNA probes to dissect RNAP-promoter interactions and gain insights into transcription regulation [7, 17–19]. Quantitative measurements of RNAP interactions with promoter DNA or promoter fragments are frequently complicated by the non-specific DNA binding to non-cognate RNAP sites [19–21]. A recently developed fluorometric RNAP beacon assay allows one to monitor the RNAP interactions with various DNA probes and quantitatively characterize partial RNAP-promoter interactions [8]. The assay relies on the detection of a specific increase in fluorescence intensity upon DNA binding to the RNAP σ^{70} subunit. An important advantage of the RNAP beacon assay is that it allows selective detection of specific DNA-RNAP interactions in the presence of a relatively large background of non-specific interactions. Here, we explain the principle of the RNAP beacon assay, provide detailed protocols of its use, and give several examples of its application to

the mechanisms of *E. coli* RNAP σ^{70} holoenzyme transcription initiation complex formation and its regulation by RNAP-binding bacterial and phage-encoded factors.

2. Description of the method

Fluorescence quenching in fluorophore/quencher pairs incorporated in proteins can be used to investigate conformational dynamics and protein-ligand interactions [22]. Efficient fluorescence quenching through interactions of fluorescent dyes with tryptophan residues in proteins occurs via photoinduced electron transfer mechanism at fluorophore-quencher separation below 1 nm [23,24]. Some organic fluorophores can also be substantially quenched by tyrosine residues [24–26]. Other natural amino acids usually show only minor quenching capacity [24]. A schematic representation of a fluorometric assay to quantitatively monitor site-specific interactions of promoter DNA and various promoter fragments with bacterial RNAP holoenzyme is shown in Fig. 1. The assay is based on measuring fluorescence of a fluorescent label specifically attached to the RNAP σ^{70} subunit close to a cluster of σ^{70} aromatic residues directly participating in recognition of the –10 promoter element [6,27–29]. By design, the fluorophore of labeled σ^{70} is quenched by the nearby σ^{70} region 2.3 Trp and Tyr residues (Fig. 1A). Thus, the baseline fluorescence of labeled σ^{70} or labeled σ^{70} -containing RNAP holoenzyme is relatively low. The aromatic amino acids of σ^{70} 2.3 change their environment upon interaction with promoter DNA (1). The exact nature of this change is, however, unknown. It could be expected that the interaction between the fluorescent label and the aromatic amino acid quenchers will be hindered upon specific promoter DNA binding to RNAP, leading to enhancement of the fluorescent signal (Fig. 1B). Indeed, we found that upon addition of T5N25 promoter DNA or certain promoter fragments to RNAP holoenzymes containing mutant σ^{70} with tetramethylrhodamine (TMR) attached to residue 192 or TMR, BODIPY FL or ATTO-520 attached to residue 211, the fluorescent signal considerably increased (8). Large increases in fluorescence intensity were also observed in similar experiments with σ^{70} derivatives labeled with Alexa 555 at position 211 (28) and with TMR or BODIPY FL at position 422 (Mekler and Severinov, unpublished data). The short range of Trp-mediated quenching implies that the assay reports only on interactions confined to a small part of RNAP involved in specific promoter interactions. The assay is therefore “blind” to non-specific RNAP-DNA interactions that occur at extraneous sites (Fig. 1C). By analogy with previously described molecular and peptide beacons assays [30,31], we named this assay “an RNAP beacon assay”.

Site-specifically labeled derivatives of *E. coli* σ^{70} are prepared from functional single-cysteine mutants of σ^{70} [32]. The mutants are constructed by site-specific mutagenesis of a cloned *rpoD* (σ^{70}) gene, in which the three codons coding for natural cysteine residues been substituted by those for serine. The resulting no-cysteine σ^{70} is fully functional [32]. Unique cysteines are introduced based on available structural data [33]. It is essential that the fluorescent label does not impair the *in vitro* function of σ and the RNAP holoenzyme formed with it. Several RNAP beacons containing σ^{70} derivatives labeled with various fluorophores have been described [8, 29]. A derivative of σ^{70} labeled at position 211 with tetramethylrhodamine-5-maleimide ((211Cys-TMR) σ^{70}) is particularly well-suited for practical work [8]. A structural model of (211Cys-TMR) σ^{70} is shown in Fig. 2A.

Upon saturation binding of RNAP holoenzyme containing (211Cys-TMR) σ^{70} to DNA probes interacting with the σ^{70} region 2.3 or with adjacent regions 2.4 and 3.0 the fluorescence intensity increases 2 to 5-fold. The generation of this beacon signal is a robust effect and experiments can be performed at various buffer solutions. We observed high fluorescence signals upon RNAP binding to DNA probes at pH in the range of 6.5–8.5, various salt compositions and in the presence of mild non-denaturing detergents. We found that (211Cys-TMR) σ^{70} holoenzyme carrying a double substitution of Trp-433 and Trp-444 to Ala exhibited less than 10% increased fluorescence in the presence of promoter DNA, suggesting that Trp-433 and Trp-444 play major role in the 211Cys-TMR fluorescence quenching (8). Precise quantification of partial contributions of region 2.3 aromatic residues to fluorescence quenching is complicated by their involvement in the –10 element binding (25–27). It should be noted that fluorescence quantum yield of a free or non-specifically attached TMR label can be significantly higher than that of (211Cys-TMR) σ^{70} , whose fluorescence is intrinsically quenched. Therefore, the (211Cys-TMR) σ^{70} protein needs to be carefully purified from contaminating proteins and excess unreacted label to diminish background fluorescence which can hamper the measurements.

Since σ^{70} region 2.3 tryptophan residues are evolutionary conserved, beacons based on σ subunits other than *E. coli* σ^{70} can be constructed. Indeed, *Thermus aquaticus* σ^A -based beacon has been described [34]. In this case, the natural σ^A protein lacked cysteines, simplifying the task. A structural model of a fragment of single-cysteine σ^A derivative used as a beacon in this study complexed with –10 promoter element ss DNA oligo is shown in Fig. 2B. The structure suggests that specifically bound ss DNA may sterically hinder the interaction between a fluorophore attached to the introduced at 245 position Cys and σ^{70} region 2.3 Trp residues. When designing a new beacon, it is advisable to modify, by trial and error, several residues located close to the quenching tryptophan with fluorophores, until a pair of quenching tryptophan/modified residue that gives a desirable beacon behavior is found.

3. Materials and equipment

Preparation of σ^{70} Derivative with Single Cys Residue at 211 Position

Plasmid pGEMD(211Cys) expressing a σ^{70} derivative with single Cys residue at position 211 under control of the T7 RNAP is described in [35]. The plasmid is transformed into *E. coli* BL21(DE3)pLysS cells and transformants are grown on TYE agar plates containing 100 μ g/mL ampicillin for 16 h at 37 °C. A single colony is inoculated into 5 mL of LB containing 100 μ g/mL ampicillin and cells are allowed to grow for 12 h at 37 °C with vigorous shaking. The culture is next transferred to a 15-mL polypropylene centrifuge tube and centrifuged for 5 min at 3,000 g at room temperature. The supernatant is discarded, the cell pellet is resuspended in 5 mL LB and transferred into 1 L of LB containing 100 μ g/mL ampicillin. The culture is grown at 37 °C with vigorous agitation until OD600 reaches 0.6 (usually 2.5–4 h). At this point, IPTG is added to 1 mM final concentration to induce expression and growth is continued for additional 3 hours. Cells are harvested by centrifugation for 20 min at 4,000 g at 4 °C. Cell pellet is resuspended in 30 mL of buffer A (20 mM Tris-HCl pH 7.9, 5% glycerol, 1 mM DTT, 0.1mM EDTA, 1 mM PMSF, 0.2%

sodium deoxycholate) and lysed by passing through a French Press (Avestin). Overproduced σ^{70} is segregated in inclusion bodies, which are collected, along with cell debris, by centrifugation (10,000 g, 15 min at 4 °C). The pellet is washed first with 10 mL of buffer B (20 mM Tris-HCl pH 7.9, 100 mM NaCl, 5% glycerol, 1 mM DTT, 0.1 mM EDTA) containing 0.1% n-octyl- β -D-glucopyranoside and then two more times with 10 mL of buffer B. The washes are performed by sonication with four 40-s sonication pulses at 25% maximum sonicator output (with 1-min pauses between each pulse) followed by centrifugation for 15 min at 13,000 g at 4 °C. Washed inclusion bodies are solubilized in buffer C (50 mM Tris-HCl pH 7.9, 6 M guanidine chloride, 1 mM EDTA, 1 mM DTT, 10% glycerol) to reach the final protein concentration of about 1 mg/mL and dialyzed against two 1-L changes of buffer D (20 mM Tris-HCl pH 7.9, 200 mM KCl, 0.1 mM EDTA, 5 mM β -mercaptoethanol, 20% glycerol) for 16 h at 4 °C. The dialysed solution is centrifuged for 15 min at 15,000 g at 4 °C. The pellet that contains misfolded protein is discarded. The σ^{70} derivative from the supernatant is further purified using anion exchange chromatography on a MonoQ HR column (GE Healthcare Life Sciences). We use Buffer E (20 mM Tris-HCl pH 7.9, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA) +0.1 M NaCl and Buffer E +0.6 M NaCl as chromatographic buffers A1 and A2, respectively, and apply a linear gradient (from 100% A1 to 100% A2 for 1 hour). The σ^{70} derivative elutes at 0.37–0.42 M NaCl. The yield of more than 90% pure σ^{70} typically is 3–10 mg from 1 L of induced cell culture. This amount is sufficient for hundreds of experiments and will last a very long time.

Labeling of 211Cys- σ^{70} with TMR-maleimide

To ensure that the modified 211Cys residue is in a reduced form, Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP) is added to a final concentration of 1 mM to 1 ml of the freshly purified 211Cys- σ^{70} solution (2–4 mg/ml) and incubated on ice for 30 min. After reduction, the protein sample is buffer-exchanged into labeling buffer (50 mM phosphate buffer, pH 7.0) using Econo-Pac 10DG desalting column (BIO-RAD) and chilled on ice. A five-fold molar excess of TMR-maleimide is rapidly added to the protein solution and the sample is incubated on ice for 1 hour protected from direct light. Low temperature during the labeling step significantly improves the selectivity of labeling. The labeling reaction is terminated by the addition of DTT to 1 mM final concentration and excess unreacted dye is removed using an Econo-Pac 10DG desalting column. The TMR concentration in the labeled sample can be measured spectrophotometrically using an extinction coefficient $\epsilon_{560} = 8 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in a pH 8.0 buffer containing 0.1% SDS. The efficiency of labeling (TMR/protein ratio) usually is about 0.8. For storage, add glycerol to 50% and keep the sample at –20 °C if used within 6 months or at –70 °C for long-term storage.

DNA probes

We prepare DNA probes from DNA oligonucleotides synthesized by Integrated DNA Technologies. Unmodified oligonucleotides shorter than 20 bases were ordered without additional purification; oligonucleotides with lengths between 20 and 40 nt or longer than 40 nt were purified by HPLC or PAGE, respectively. The fork junction and double-stranded (ds) DNA probes are formed by mixing equimolar amounts of synthetic complementary strands (final concentrations were within low μM range) in a buffer containing 40 mM Tris,

pH 7.9, 100 mM NaCl; heating for 2 min at 95 °C and cooling the reactions to 20 °C in a PCR machine at a rate of 0.01° C/s. The probes can be further purified by non-denaturing gel electrophoresis, if necessary.

Fluorometric measurements

We measure fluorescence using a QuantaMaster QM4 spectrofluorometer (PTI) in transcription buffer (40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5% glycerol, 1 mM DTT and 10 mM MgCl₂) containing 0.02% Tween 20 at 25° C. Final assay mixtures (800 µl) contain 1 nM labeled (211Cys-TMR) σ^{70} , 1–1.5 nM core RNAP and DNA probes at various concentrations. We use commercial *E. coli* RNAP core enzyme purchased from Epicentre Biotechnologies. The assay mixtures can be placed into either quartz or plastic cuvettes. Quartz cuvettes have much better optical characteristics. However, a drawback of using quartz cuvettes is that RNAP can significantly adsorb on cuvette walls upon stirring of the cuvette content, leading to decreased RNAP concentration in solution and, consequently, decreased fluorescence intensity. Therefore, for quantitative binding titrations we use disposable methacrylate cuvettes (Fisher Scientific, # 14-955-128), which have low affinity for RNAP. Mild nonionic surfactant Tween 20 helps further diminish RNAP adsorption.

The TMR fluorescence intensities are recorded in time-based mode with an excitation wavelength of 550 nm and an emission wavelength of 578 nm. Excitation and emission slit widths are set to 5 nm each. To eliminate background signal caused by the scattered excitation light, fluorescence emission is passed through a 10 nm bandpass interference filter. Time-dependent fluorescence changes are monitored after manual-mixing of 800 µl of RNAP beacon solution and a 20 µl or less of DNA probe directly in cuvette; the mixing dead-time is about 15 s.

4. Examples of measuring of RNAP interactions with model promoter fragment probes using the RNAP beacon assay

The RNAP beacon assay allows one to rapidly investigate RNAP interaction with a large number of DNA probes since no DNA labeling is required. Schematic structure of a consensus *E. coli* σ^{70} RNAP holoenzyme promoter along with structures of several promoter fragments that are known to specifically interact with RNAP are shown in Fig. 3. Probes 1 and 2 represent ds fragments of a strong N25cons promoter [36]. RNAP specifically binds short DNA oligos containing sequences corresponding to the non-template strand of the –10 promoter element (probes 3, 4) [7,8,29]. Upon the RP₀ formation, two DNA fork junction structures are created on the opposing sides of the transcription bubble, at about positions –11 and +2. Probes 5 and 6 represent upstream fork junction promoter fragments mimicking upstream part of native RP₀ complexes [17]. It was recently shown that downstream fork junction promoter fragments (probes 7 and 8) recapitulate functional properties of the transcription bubble and downstream DNA of RP₀ and thus can be used to investigate downstream promoter interactions [37]. The downstream fork junction probes are also useful for structural studies of bacterial transcription initiation [6,38,39]. Typical time-dependent changes in RNAP beacon fluorescence intensity obtained with promoter DNA and promoter fragments are shown in Fig. 4. It is noteworthy that upstream fork junctions

bearing the –35 element bind to RNAP much faster than truncated fork junctions lacking the –35 promoter element under similar conditions (compare kinetic curves for probes 5 and 6 in Fig. 4). Equilibrium RNAP binding to short oligo probes is usually reached for very short time (see curve for probe 3 in Fig. 4).

Certain probes, such as probes 9–11 in Fig. 3, generate negligible signals upon binding to the RNAP beacon. A very low fluorescence intensity increase observed upon RNAP binding to probes 9 and 10 is a consequence of the substitution of a consensus A for a non-consensus T at the –8 position, which abolishes the beacon effect produced by the –10 element in the context of oligonucleotide and downstream fork junction probes [8,37]. Negligible beacon signal generated by ds probe 11 can be explained by strong quenching of fluorescence of TMR attached at the 211 position by the G/C base pair at the 3' terminus of this probe. This explanation is supported by the fact that DNA similar to probe 10 but bearing the A/T base pair at 3' terminus generates a moderate beacon signal (Mekler and Severinov, unpublished data). Guanine is known to be the only base that efficiently quenches rhodamine fluorophores through photoinduced electron transfer mechanism when the dye is attached close to the 3'-terminal G/C base pair, while G in a ss oligonucleotide only weakly quenches the fluorescence of nearby dye [23,40]. Such “dark” probes can be conveniently used as reference competitors in competition binding experiments (see below).

Titration of RNAP beacon with a DNA probe (shown in Fig. 5) and fitting the dependence of the fluorescent signal amplitude (F) on DNA probe concentration to a chemical equilibrium Equation 1 allows calculation of the RNAP/probe complex equilibrium dissociation constant (K_d)

$$(1-X)([DNA]-[RNAP]X)=K_dX \quad (1)$$

where $X=(F-F_0)/(F_{max}-F_0)$, F_0 and F_{max} are the amplitudes of free RNAP and RNAP-DNA complex (e.g. the amplitudes at $[DNA]=0$ and $[DNA]=\infty$, respectively) [8]. The data were analyzed using SigmaPlot software (SPSS, Inc.). Since the signal amplitude is temperature-dependent, unintended changes in sample temperature during measurements should be avoided. The values of baseline fluorescence intensity (F_0 in Eq. 1) may somewhat differ between RNAP beacon preparations. This variation likely reflects differences in the level of non-specific labeling and presence of some quantities of partially denaturated proteins. The changes in baseline beacon fluorescence intensity, however, do not affect determination of K_d from the titration curves.

The beacon assay makes it possible to quantitatively measure very weak but specific binding of short oligonucleotides bearing the consensus –10 element non-template strand sequence to holo RNAP and even to free (211Cys-TMR) σ^{70} . This was used to address the effect of core RNAP interaction with σ^{70} on recognition of the non-template strand segment of the transcription bubble [8]. The results showed that 14-nt long oligonucleotide corresponding to the whole non-template strand segment of the transcription bubble (probe 3 in Fig. 3) bound to RNAP holoenzyme ~300-fold stronger than to free σ^{70} . However, the affinity of RNAP holoenzyme for a shorter oligonucleotide corresponding to the –10 promoter element alone was only slightly higher than that of free σ^{70} . These data suggest that the large

difference between the holo RNAP and free σ^{70} affinities to the non-template strand of the transcription bubble is mainly a consequence of holo RNAP higher affinity for bases outside of the -10 element.

Studies on RNAP-promoter interactions often require quantitative characterization of specific RNAP–DNA complexes with widely different stabilities, which is a technically challenging task because of RNAP propensity for non-specific DNA binding. With its high sensitivity and low intensity of non-specific background signal, the RNAP beacon assay is well suited for performing such measurements, as it allows to quantitatively measure both weak and strong interactions. Feklistov and Darst compared *E. coli* RNAP holoenzyme binding of dsDNA probes with and without -10 element sequences under conditions favoring closed promoter complex formation (4°C) [29]. The results of this study support a model where the recognition of the -10 element sequence drives promoter opening as the bases of the non-template strand are extruded from the DNA double-helix and captured by σ . In another work, interdependencies of RNAP interactions with the -10 promoter element nucleotides were investigated [41]. This study revealed the existence of a strong cooperation between RNAP interactions with individual consensus -10 element non-template strand nucleotides, which previously could not have been detected with less sensitive methods [17,19].

K_d for very tight RNAP-DNA complexes ($K_d < 0.1\text{ nM}$) can not be calculated from the titration data since fluorescence intensity reaches saturation level at minimal DNA probe concentrations used. This case is exemplified in Fig. 5 by data for probe 3. Affinities of *E. coli* RNAP to many DNA probes that form such tight complexes can be measured using RNAP beacon method-based equilibrium competition binding assays [8, 34]. It should be taken into account that reaching the equilibrium binding of DNA probes to RNAP when performing the competition experiments with tight complexes can take very long time. Control competition experiments with altered order of addition of a probe of interest and competitor DNA allow one to monitor whether reaction equilibrium has been reached (Fig. 6).

A highly sulfated glycosaminoglycan heparin is known to compete with DNA for binding to RNAP. Heparin resistance is the hallmark of stable intermediates in the formation of the open complex [17,42]. Thus, to assess relative affinities of DNA probes to RNAP, stabilities of RNAP complexes in the presence of heparin can be compared. The data in Fig. 7 demonstrate RNAP beacon assay measurements of heparin-mediated dissociation of RNAP complexes with downstream fork junctions having upstream duplex edges (junction points) at different positions. Lengths of the ss segments in the probes with junction points at positions from +1 to +4 (11–14 nt) correspond to length of melted DNA regions in many σ^{70} -dependent promoters [1]. For reasons unknown, heparin causes some increase in fluorescence intensity of the downstream fork junction complexes immediately after its addition (Fig. 7). This effect indicates that RNAP can simultaneously bind heparin and downstream fork junctions, but the resulting complex is short-lived. The data in Fig. 7 show that the rate of dissociation of probes with junctions at +2, +3, and +4 are very similar and comparatively slow. Fork junction with junction point at +1 dissociates ~2-fold faster, while moving the edge of the duplex upstream of +4 or downstream of +1 positions results in

considerable destabilization of complexes. This result indicates that RNAP interaction with properly positioned downstream fork junction structure that arises upon RP_o formation is one of the factors that determines the downstream boundary of the transcription bubble.

5. Use of RNAP beacon assay to map RNAP-promoter interactions affected by transcription factors

While the majority of studied transcription factors interact with specific target DNA sequences, many important regulators of transcription initiation bind directly to RNAP [43]. The RNAP beacon assay can be conveniently used to identify RNAP-promoter interactions affected by regulators by measuring the affinity of various promoter DNA probes in the presence or absence of regulators. This approach is demonstrated below by applications to phage- and host-encoded transcription factors.

T4 phage AsiA protein binds to σ^{70} region 4 and inhibits transcription from -35 element dependent promoters by *E. coli* RNAP σ^{70} holoenzyme [44,45]. Another potent inhibitor of transcription initiation, the T7 phage gp2 protein, binds to the β' jaw domain and prevents RNAP interaction with dsDNA downstream of the transcription start point [46,47]. Upstream and downstream fork junction probes were used to characterize the effects of AsiA and gp2 on the strengths of corresponding upstream and downstream RNAP-promoter contacts [36,37]. As expected, gp2 inhibits RNAP binding to downstream probe but has no effect on upstream probe binding. The reverse is true for AsiA (Figs. 8A and B). We also found that AsiA does not influence RNAP interaction with oligonucleotide probes, which mimic the central part of promoter (the -10 element and sequences immediately downstream). In contrast, gp2 considerably decreases RNAP affinity for oligonucleotide probe 3 (shown in Fig. 3), suggesting that in addition to direct interference with downstream DNA binding it may also exert a long-range allosteric effect. Indeed, subsequent experiments indicated that gp2 strongly inhibits RNAP interaction with the discriminator segment of promoter [36].

Recently the beacon assay was applied to RNAP interaction with transcription factor DksA and alarmone ppGpp, which synergistically regulate bacterial transcription initiation in response to various cell stresses. DksA belongs to a class of transcription regulators that bind in the RNAP secondary channel, whereas the ppGpp binding site is located between the β' and ω subunits on the outer surface of RNAP [48–50]. Neither of the two regulators interacts directly with DNA. DksA/ppGpp inhibit transcription by destabilizing short-lived RP_o complexes formed on sensitive promoters [43,51]. Beacon assay measurements revealed that DksA/ppGpp decrease RNAP affinity for downstream fork junction probes by 16-fold, while slightly improving RNAP binding to an upstream fork junction and exerting no effect on RNAP binding to oligo probes similar to those shown in Figure 3 [16]. These experiments indicate that DksA and ppGpp considerably weaken the RNAP interaction with the downstream DNA duplex. Together with biochemical data, the results suggested that weakening of downstream RNAP⁺ promoter contacts by DksA/ppGpp may be the cause of specific inhibition of transcription initiation from promoters on which the RP_o formation is relatively energetically unfavorable [16].

6. Conclusion

The fluorometric RNAP beacon method allows one to measure various RNAP promoter interactions spanning a dissociation constant range from picomolar to nearly millimolar. The examples presented above demonstrate the use of the assay for quantitative dissection of fine details of *E. coli* RNAP σ^{70} holoenzyme interactions with promoters and for identification of promoter recognition steps affected by several transcription regulators. We believe that similar protocols can be used for preparation of *E. coli* RNAP beacons containing alternative σ subunits and beacons based on RNAPs from other bacteria, thus further extending the usefulness of the assay and allowing quantitative comparative analysis of transcription initiation in different bacteria. The main limitation of the current version of the assay is that it depends on promoter interactions with σ region 2.3. An important direction for future research will be development of similar assays based on fluorescence quenching through interactions of Trp and organic fluorophores located in other parts of RNAP, which should allow selective detection and quantitative measurements of RNAP-DNA interactions throughout the entire DNA binding site of the RNAP holo and core enzymes.

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Abbreviations

RNAP	RNA polymerase
RP_o	open promoter complex
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
TMR	tetramethylrhodamine

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Highlights

RNA polymerase fluorescent beacon assay applied to promoter interactions.

Protocol for preparation of a beacon based on *E. coli* RNA polymerase σ^{70} holoenzyme.

Illustrating the method with data on dissecting RNA polymerase-promoter interactions.

Mapping promoter interactions affected by inhibitors of transcription initiation.

Figure 1.

Schematic representation of the RNAP beacon assay. (A) The red circle labeled (F) indicates the fluorophore; aromatic residues that quench F fluorescence in the absence of bound DNA are shown as yellow circles. (B) On specific RNAP binding to promoter DNA or promoter fragments, the amino acid quencher lose contact with the fluorescent probe, decreasing the quenching efficiency and leading to increased fluorescence. (C) Non-specific DNA binding at extraneous sites does not alter the fluorescence intensity.

Figure 2.

The positions of modified with fluorophore Cys and Trp quenchers on structural models of σ derivatives used to assemble RNAP beacons. (A) A structural model of a fragment of σ^{70} showing the positions of side chains of Trp and Tyr residues in region 2.3 and the modeled position of TMR fluorophor attached to Cys211 [8]. The structure is based on Protein Data Bank ID 1SIG [33]. (B) A structural model of *Thermus aquaticus* σ^A domain 2 bound to -10 promoter element ssDNA oligo (in green) showing the positions of SH group in 245Cys σ^A derivative (yellow sphere). The structure is based on Protein Data Bank ID 3UGO [29].

Figure 3. Schematic representation of a consensus *E coli* σ^{70} RNAP holoenzyme promoter and structure of representative promoter fragment probes. Conserved promoter elements are highlighted in bold. Probe 8 contains a non-complementary extension of the template strand (highlighted in italic). The DNA probes are loosely based on T5 N25 promoter sequence.

Figure 4.

Representative experimental data on time dependence of the fluorescence intensity upon the addition of –65+35 T5 N25 promoter fragment or promoter fragment probes shown in Fig. 3 to 1 nM RNAP beacon. The number of probes in Fig. 3 and their concentrations are indicated near the curves.

-38 -11
1. TAT**TTGACA**TCAGGAAAATTTTCTGT**TA**
ATA**AACTGT**AGTCCTTTTAAAAAGACA

-38 -9

2. TAT**TTGACA**TCAGGAAATTTTCTGT**TATA**
ATA**AACTGT**AGTCCTTTTAAAAAGACA

-38 -7

3. TAT**TTGACA**TCAGGAAAATTTTCTGT**TATAT**
ATA**AACTGT**AGTCCTTTTAAAAAGACA

4. TAT**TTGACA**TCAGGAAAATTTTCTG**TTTAA**
ATA**AACTGT**AGTCCTTTTAAAAAGACA

Figure 5. Representative experimental data on titration of the RNAP beacon with upstream fork junction probes [41]. Structures of the probes are shown in the top part of the figure. Continuous lines correspond to nonlinear regression fit of the data.

-59
 CGCTAAAATTTTTTTTAAAAGTAT**TTGACAT**CAGGAAAATTTTTCTG**TA**
 GCGATTTTAAAAAAATTTTCATA**AACTGT**AGTCCTTTTAAAAAGACA
 - 11

Figure 6.

Competition binding assay for *E. coli* RNAP binding to upstream fork junction probe (shown above the panel). Sequence of reference competitor probe is shown in Fig. 3 (probe 11). The panel shows the kinetics of decrease of beacon signal of the preformed RNAP beacon complex with fork junction probe upon the addition of competitor (black curve). A control order of addition experiment in which fork junction was added to the preformed RNAP-competitor complex (red curve) was carried out to verify that the competition assay reached equilibrium. The concentrations of fork junction and competitor were 2 nM, the RNAP beacon concentration was 1 nM.

-11   -1   +3   +6
                              * * * * *
 ATAATAGATTCAATAATTTGAGAGAGGAGTTTAAATA   +26
                              GTATTTAAACTCTCTCCTCAAATTTAT

Figure 7. The effect of junction point position on the strength of RNAP interaction with downstream fork junction probes [37]. Time-dependent changes of the fluorescence signal were measured upon the addition of 50 $\mu\text{g/ml}$ heparin to RNAP beacon complexes with fork junction probes whose junction points were located between positions -1 to $+6$. The concentrations of RNAP beacon and fork junctions were 1 and 2 nM, respectively.

Figure 8.

The effect of phage T7 gp2 and phage T4 AsiA transcription initiation inhibitors on RNAP binding to downstream and upstream fork junction probes. The 4 nM of upstream fork junction shown in panel A or 8 nM of downstream fork junction shown in panel B combined with RNAP beacon (1 nM) preincubated for 15 min with or without 0.2 μ M gp2 or AsiA and increase in fluorescence was monitored.