FEBS Letters 580 (2006) 3439–3444

The cyanobacterial principal σ factor region 1.1 is involved in DNA-binding in the free form and in transcription activity as holoenzyme

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Received 7 March 2006; revised 7 May 2006; accepted 8 May 2006

Available online 12 May 2006

Edited by Richard Cogdell

Abstract **Cyanobacterial principal σ factor, σ^A, includes a specifically conserved cluster of basic amino acids in the amino-terminal extension called region 1.1. We found that the σ^A in a thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 binds DNA in the absence of the core RNA polymerase and that σ^A lacking region 1.1 is not able to bind DNA. This indicates that, in the cyanobacterium, region 1.1 participates in DNA-binding, rather than inhibiting the interaction between free σ and DNA, as found in other principal σ factors of eubacteria. The results of in vitro transcription assays with the reconstituted RNA polymerase showed that region 1.1 reduces transcription activity from the *cpc* promoter.**
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Keywords: Cyanobacteria; Region 1.1; RNA polymerase; σ factor

1. Introduction

Cyanobacteria and chloroplasts of algae and higher plants are characterized by oxygen-evolving photosynthesis and are phylogenetically closely related. The transcription apparatus of their genomes is composed of a eubacteria-type core RNA polymerase (RNAP) and the transcription initiation factor, σ [1,2]. One of the unique features of their RNAPs is that the β′ subunit is split into two subunits, RpoC1 (γ in cyanobacteria or β′ in chloroplasts) and RpoC2 (β′ or β″). The carboxy-terminal portion of RpoC2 has an inherent insertion domain spanning about half of its length [2–4]. Another unique feature is that the cyanobacterial principal σ factor (σ^A) contains a cluster of basic amino acids in the carboxy-terminal end of region 1.1 (R1.1), which is not found in principal σ factors in other eubacterial branches. In chloroplasts, some kinds of σ factors also have a basic amino acids cluster in R1.1 [1].

R1.1 is an amino-terminal specific subregion in region 1 of principal (group 1) σ factors and is characterized by the conserved acidity [5]. Two functions of R1.1 have been proposed; first is autoinhibition of free-σ factor's interaction with promoter DNA, and second is to accelerate open complex formation at some promoters [6–8].

Previously, σ^A of a cyanobacterium *Anabaena* sp. PCC 7120 was unexpectedly indicated to bind to some promoters in the absence of the core RNAP [9]. It was still obscure which part of the σ^A participates in the DNA-binding and whether the binding is promoter specific. In this study, we found that R1.1 in a thermophilic cyanobacterium, *Thermosynechococcus elongatus* BP-1 (hereafter *Tel*), is involved in binding to double-stranded DNA non-specifically. Moreover, the results of in vitro transcription suggested that R1.1 repressed the transcription initiation from the *cpc* promoter of *Tel* without affecting the open complex stability. These findings provide insight into the novel role of R1.1 in cyanobacteria and chloroplasts, which are distinct from its hitherto known roles in other principal σ factors.

2. Material and methods

2.1. Protein purification

The holoenzyme and the core RNAP of *Escherichia coli* were purified according to the method of Hager et al. [10] and the proper activity of each enzyme used in the transcription reaction was confirmed previously [11]. The core RNAP in *Tel* was purified essentially according to the method of Goto-Seki et al. [11], with modification. *Tel* cells were cultivated in BG-11 medium [12] at 50 °C and bubbled with air containing 3–5% CO_2 under continuous illumination at about 100 μE m^{-2} s^{-1}. The cells at the stationary phase were harvested by centrifugation and frozen at −80 °C until use. About 30 g of frozen cells were suspended in TGED buffer (10 mM Tris–HCl (pH 8.0), 5% (v/v) glycerol, 0.1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol), containing 10 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, 0.5 M NaCl and 0.2% (v/v) Na-deoxychorate. The cells were lysed by French press (20000 p.s.i.) and the soluble fraction was separated from the membrane fraction by ultracentrifuging for 90 min at 120000 × *g*. Polyethyleneimine (Polymin P, Sigma) was added to the soluble fraction to a final concentration of 0.05% (v/v), and the RNAP was extracted from the Polymin P precipitant with TGED buffer containing 0.6 M NH_4Cl. The RNAP was precipitated by 60% (w/v) saturated $(NH_4)_2SO_4$ and resuspended in TGED buffer containing 0.6 M NH_4Cl, followed by purification using gel filtration chromatography (Sephacryl S-300R, Amersham Biosciences) equilibrated with the same buffer. The fraction including both core RNAP and holoenzyme mixture was dialyzed against TGED buffer containing 0.1 M NaCl and purified by heparin affinity chromatography (5 ml Hi-trap Heparin, Amersham Biosciences). The elution peak of the core RNAP (0.45 M NaCl) was distinct from that of the holoenzyme (0.55 M NaCl) in a linear gradient of 0.1–1 M NaCl in TGED buffer. The core RNAP fraction was dialyzed against TGED buffer containing 0.1 M NaCl and further purified by ion exchange chromatography

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Abbreviations: bp, base pair(s); EMSA, electrophoreticmobility-shift assay; R1.1, region 1.1; RNAP, RNA polymerase; *Tel*, *Thermosynechococcus elongatus* BP-1

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doi:10.1016/j.febslet.2006.05.017

(Mono Q, Amersham Biosciences). About 1 mg of core RNAP, with more than 90% purity in Coomassie brilliant blue stain, was dialyzed against TGED buffer containing 0.5 M NaCl and 50% glycerol and stored at −80 °C.

The σ^A and $\Delta1–67\sigma^A$ (beginning with the sequences of MTQA and MKHY, respectively) proteins of *Tel* were cloned between *Nde*I and *Bam*HI sites of the pET15b expression vector (Novagen) using standard PCR techniques. N-terminal His_6-tag fusions of σ^A and $\Delta1–67\sigma^A$ proteins were overexpressed from the resulting vectors in *E. coli* BL21 (DE3) carrying pLysS. The cells were lysed by sonication and the soluble fractions were purified by Ni-NTA agarose column chromatography (Qiagen). The N-terminal His_6-tags of both proteins were cleaved by thrombin (Amersham Biosciences), giving an additional sequence of GSH in its N-terminus, and the cleavage products were purified again by the same chromatography. Both of the first products purified by the Ni-affinity chromatography were cloudy because of non-precipitating partial denaturation, but the second products purified with the same chromatography after thrombin cleavage were recovered or removed from the denatured products, as judged from the clear solutions. The products were further purified by batch-mode POROS 20E heparin affinity chromatography (Perseptive Biosystems) and were dialyzed against TGED buffer containing 0.5 M NaCl and 50% glycerol and stored at −80 °C.

2.2. EMSA analysis

A 306 bp DNA fragment containing the *cpc* promoter of *Tel* corresponding to –161 to –467 (relative to the translation start site) of the *cpcB* gene was amplified by PCR with primers 5′-AGCTAAATTGCAACGGCTC-3′ and 5′-AAACGTGCAACGCTCTTGG-3′. The product was purified by polyacrylamide gel electrophoresis (PAGE). The five longest fragments of a pBR322-*Hae*III digest (587, 540, 504, 458, and 434 bp) were also purified by PAGE. The 5′ ends of DNA fragments were labeled by polynucleotide kinase (TaKaRa) and [γ-^{32}P]ATP (Amersham Biosciences). The ^{32}P-labeled DNA fragment of the *cpc* promoter (1 nM) or the five longest DNA fragments of the pBR322-*Hae*III digest (5 nM) (calculated as mono-fragment of 2523 bp in length) were mixed with purified σ^A or $\Delta1–67\sigma^A$ protein in 20 μl of a buffer containing 50 mM HEPES–KOH, pH 8.0, 3 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, 100 mM KCl, and 25 μg/ml bovine serum albumin. After incubation for 20 min at 30 °C, the mixtures were subjected to PAGE on a native 4% gel and detected with a BAS1000 image analyzer (Fuji). The experiments in Figs. 3 and 4 were performed at least twice to confirm the reproducibility.

2.3. In vitro transcription

Transcription reactions were performed as described previously [11]. The native core RNAP was mixed with a 3-fold molar excess of the overproduced σ^A or $\Delta1–67\sigma^A$ protein and the mixture was incubated for 20 min at 37 °C to allow holoenzyme formation. A transcription reaction mixture (35 μl) comprised of 4 nM template DNA and 60 nM holoenzyme in T buffer [13] was incubated for 20 min at 37 °C. Then, RNA synthesis was initiated by the addition of a prewarmed substrate mixture (15 μl) containing 160 μM each of ATP, GTP, and CTP as well as 50 μM UTP, 100 μg/ml of heparin (in the analysis of open complex stability, heparin was added at the indicated time as mentioned in the legend of Fig. 5) and 2 μCi of [α-^{32}P]UTP (Amersham Biosciences) in T buffer. After incubation for 5 min at 37 °C, the reaction was terminated by the addition of 50 μl ice-cold stop solution [11]. Transcripts were fractionated by PAGE on a 5% gel containing 8 M urea and then analyzed with a BAS1000 instrument. The lengths of transcripts were estimated as described previously [11]. The 306 bp DNA templates were prepared as described in electrophoreticmobility-shift assay (EMSA) analysis. The experiments in Figs. 5 and 6 were performed at least twice to confirm the reproducibility.

2.4. Density gradient flotation assay

Free σ proteins and core RNAP-binding σ proteins were separated by the flotation of 10–35% (v/v) glycerol gradients prepared with GRADIENT MATE (TOWAKAGAKU). Reconstitutions of holoenzymes were performed in the same condition as in vitro transcription assay. 3 pmol core RNAP and 9 pmol σ^A or $\Delta1–67\sigma^A$ mixture was incubated for 20 min at 37 °C. After separation of each density gradient fraction of glycerol using a Piston Gradient Fractionator (Biocomp); σ^A, $\Delta1–67\sigma^A$, β, and β′ subunit proteins were subjected to immunoblot analysis as described previously [14]. σ^A and $\Delta1–67\sigma^A$ proteins were detected by *E. coli* anti-σ^{70} antibody [15], and β and β′ subunit proteins were detected by *Synechocystis* sp. PCC 6803 anti-β and anti-β′ antibodies [16,17], respectively.

3. Results and discussion

3.1. DNA-binding ability of σ^A and $\Delta1–67\sigma^A$

It has been proposed that the autoinhibition of DNA-binding by σ^A is due to high acidity of its R1.1 [6]. However, cyanobacterial σ^A and some kinds of σ factors of chloroplasts, mostly annotated as *SIG2*, possess a conserved cluster of basic amino acids, lysine and arginine, on the carboxyl-end of R1.1, including *Tel* σ^A which was used in this study (Fig. 1A). In the amino-terminal fragment of R1.1 beyond the basic region, an acidic region is conserved in the majority of cyanobacteria, but there is little sequence similarity. To examine the effect of the R1.1 on DNA-binding, we performed the EMSA using *Tel* wild-type σ^A or the σ^A lacking R1.1 ($\Delta1–67\sigma^A$) (truncated region; see Fig. 1B), and the DNA fragment containing the *cpc* promoter of *Tel* (Fig. 2). The *cpc* promoter is known as a strong cyanobacterial promoter and is recognized by σ^A-holoenzyme in vitro [3]. The mobility shifts corresponding to DNA–protein complexes were detected in the addition of σ^A but not in that of $\Delta1–67\sigma^A$ when more than 50 nM of the protein was added. The considerably faint shift was detected when the concentration of $\Delta1–67\sigma^A$ was increased to 150 nM. This suggests that the R1.1 of *Tel* σ^A positively participates in DNA-binding in the absence of core RNAP. However, it was still obscure whether the DNA-binding is sequence-specific or not. To investigate the DNA-binding sequence specificity, we used the five major fragments of the pBR322-*Hae*III digests as DNA probes for EMSA. As shown in Fig. 3A, all of the five DNA fragments showed mobility-shifts by the incubation with 40–80 nM σ^A protein, which indicates that the DNA-binding of σ^A is not promoter DNA specific. When the DNA fragment B was used as a control to compare to the other fragments, the relative amount of each fragment shifted by binding to 40 nM σ^A was significantly different (Fig. 3B). In addition, an increase in σ^A concentration from 40 to 80 nM raised the variation of the shifted amount for each fragment. If σ^A binds only to the ends of the double-stranded DNA or binds to DNA in a completely random-manner, the relative amount of each shifted fragment should be the same or be dependent on the fragment length, respectively. Thus, this data suggests that the σ^A binds DNA without strict sequence specificity but with some sequence preference.

In *E. coli* σ^{70}, R1.1 acts to inhibit DNA-binding in the absence of the core RNAP [18,19], but the mechanism for inhibition remains controversial [6]. In this study, we found an opposite role of R1.1 in cyanobacterial σ^{70}-like protein σ^A, i.e., based on the EMSA analysis, the σ^A binds DNA and R1.1 is required for the binding. One simple explanation derived from a comparison of the primary structures between *E. coli* σ^{70} and *Tel* σ^A in R1.1 (Fig. 1A), is that the basic region of *Tel* σ^A R1.1 participates in electrostatic interactions with DNA. Our data suggests that R1.1 may be variable in bacterial lineages, and that free σ^A in a cyanobacterial cell might affect some pathways of the transcription system by binding to the chromosomal DNA in vivo.

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