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Isolation of the *Anabaena* sp. Strain PCC 7120 *sigA* Gene in a Transcriptional-Interference Selection

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A transcriptional-interference selection was performed to identify genes of *Anabaena* sp. strain PCC 7120 that encode DNA-binding proteins able to bind to the *rbcL* promoter. Unexpectedly, the selection yielded the previously identified *sigA* gene, which encodes the principal sigma factor. Protein extracts from *Escherichia coli* containing the *sigA* gene bound the *rbcL* promoter fragment in mobility shift assays, and competition experiments indicated binding to *rbcL* and *glnA* but not *xisA* or *nifH* upstream regions.

An in vivo transcription interference method designed to clone DNA-binding proteins (8) has been previously used to clone the *ntcA* gene from *Anabaena* sp. strain PCC 7120 (16). We sought to use a similar approach to clone the gene encoding a second DNA-binding protein, factor 2, from strain PCC 7120. Partially purified factor 2 protein binds to the upstream region of the *rbcL* gene, which encodes the ribulose-1,5-bisphosphate carboxylase large subunit (5, 13). Factor 2 also binds upstream of *xisA*, which encodes a site-specific recombinase involved in the excision of an 11-kb DNA element from the chromosome during heterocyst development (4, 10, 12). The factor 2 binding site on *rbcL* has been localized by DNase I footprinting to a region 15 to 77 bp upstream of the transcription start site (13). We used the factor 2 binding site on *rbcL* to construct an assay plasmid that could be used in an in vivo genetic selection for cloning the factor 2 gene. In our efforts to do this, we serendipitously cloned the PCC 7120 *sigA* gene, which encodes the principal vegetative cell sigma factor. We demonstrate that protein extracts from *Escherichia coli* containing the *sigA* gene bind the *rbcL* promoter fragment in mobility shift assays. Our results show that a combination of in vivo genetic selection and in vitro screening with mobility shift assays can be used to clone genes encoding DNA-binding proteins such as sigma factors even when it is hard to detect the proteins by mobility shift assays alone.

In vivo transcriptional-interference selection. A 94-bp *Kpn*I-*Eco*RI fragment from pAM942 (AM496Δ104) (13), which contains the *rbcL* factor 2 binding site, was cloned into the corresponding sites of pNN396 (7). A *Not*I-*Hin*dIII fragment containing the upstream *con*II promoter and the binding site was then subcloned into the same sites of pNN388 (7) to produce pAM972 (Fig. 1).

For selection of clones producing *rbcL*-binding proteins, *E. coli* JM107 containing pAM972, or pNN388 as a control, was transformed with a plasmid expression library of PCC 7120 DNA (16) or with pSE380, the expression library vector (2). Transformants were plated on Luria-Bertani agar plates supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mM) and the antibiotics chloramphenicol (34 μg/ml; for the selection plasmid), ampicillin (50 μg/ml; for the library vector), and spectinomycin and streptomycin (Sp+Sm; 1:1) at total concentrations of 0, 20, 30, 40, 45, and 50 μg/ml to determine the activity of the *aadA* gene. pNN388, which does not contain an antisense promoter, allowed growth on Sp+Sm at 100 μg/ml. When JM107 containing the selection plasmid pAM972 was transformed with the pSE380 vector, a thin lawn of minute colonies grew on Sp+Sm plates, decreasing in density with increasing concentrations of Sp+Sm. When the same strain was transformed with the expression library, distinct larger colonies grew on selection plates. About 15,000 transformants were screened for growth on Sp+Sm. A marked decrease in the number of larger colonies occurred at the higher Sp+Sm concentrations. For example, in one typical experiment only 17 colonies grew on Sp+Sm at 50 μg/ml.

Since strain PCC 7120 is normally grown at 30 rather than 37°C, which is used for *E. coli* growth, we did the in vivo selection at both temperatures with the assumption that the binding of a PCC 7120 DNA-binding protein might be more favorable at 30°C. Whereas we did enrich for clones containing the *sigA* gene (described below) at 30°C, a parallel selection done at 37°C failed to identify *sigA*-containing clones.

Plasmid DNA was prepared from representative colonies from different antibiotic concentrations, digested with *Hin*dIII, and analyzed by electrophoresis on 0.6% agarose gels. The percentage of clones that contained an insert increased at higher concentrations of Sp+Sm. Five of six analyzed clones from colonies on 50-μg/ml Sp+Sm and one colony from the 45-μg/ml Sp+Sm plate contained an apparently identical 4-kb insert. We suspected that these clones were expressing a DNA-binding protein that interacted with the *rbcL* upstream region.

Mobility shift assays. To check for the presence of DNA-binding activity, protein extracts were made from individual clones and analyzed by mobility shift assays using an *rbcL* probe (Fig. 2). One of the apparently identical clones (strain AM1080), another clone that contained a different insert, and a clone without any insert were further analyzed. Five-milliliter cultures of each strain were grown to mid-log phase and induced with IPTG (1 mM). Cells were harvested and protein extracts were made as described previously (16). A *Kpn*I-*Eco*RI fragment from pAM942 containing 94 bp of the *rbcL* upstream region (nucleotides −96 to −3) was labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. The labeled DNA fragment was incubated with 2 μl of crude protein extract, and mobility shift assays were done as described previously (13).

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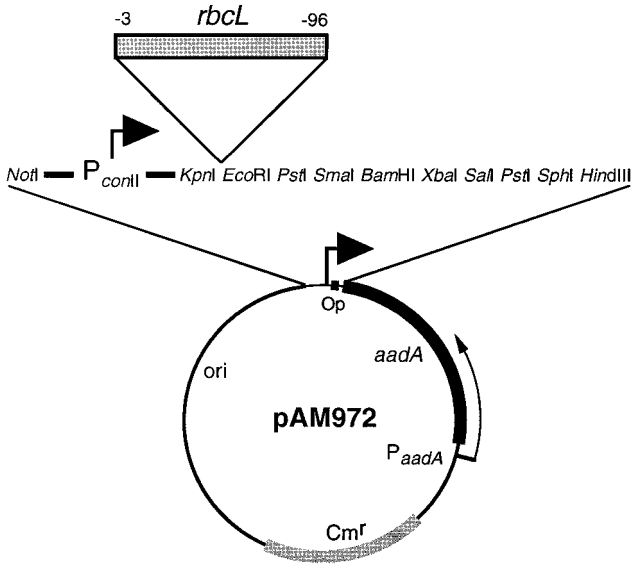

FIG. 1. Map of selection plasmid pAM972. The *rbcL* upstream region, −3 to −96 with respect to the transcription start site, acts as an artificial operator (Op) when cloned downstream of the *con*II promoter in pNN396. The *Not*I-*Hin*dIII fragment was cloned into the single-copy-number vector pNN388 downstream of and antisense to the *aadA* gene, which is driven by its weak native promoter (P_{aadA}). The *aadA* gene provides resistance to spectinomycin and streptomycin. Cm^r, chloramphenicol resistance; ori, origin of replication. Arrows indicate the direction of transcription.

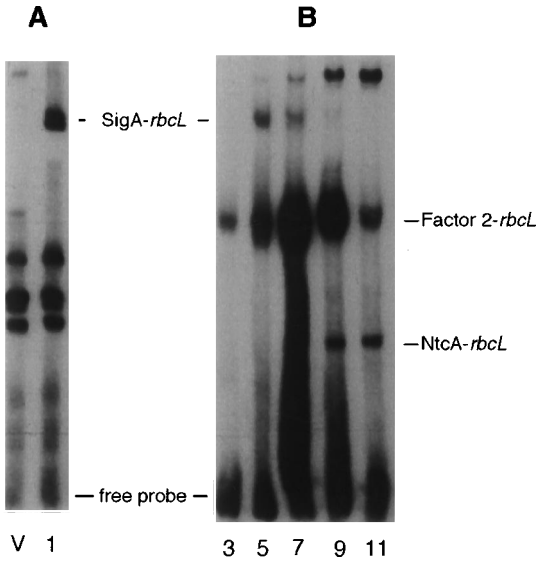

FIG. 3. Detection of SigA-*rbcL* DNA-binding activity from *Anabaena* sp. strain PCC 7120 vegetative cells. (A) Protein extracts from DH10B containing the vector pSE380 (lane V) or the *sigA* clone pAM1080 (lane 1). (B) Vegetative cell protein extract fractionated by heparin-Sepharose chromatography. Lane numbers refer to column fractions. Samples of *E. coli* crude protein extracts (2 μl) or of column fractions (5 μl) collected during elution with a linear 0.1 to 0.5 M ammonium sulfate gradient were equilibrated with 10,000 cpm of a labeled *rbcL* fragment and analyzed on a 5% polyacrylamide gel. The positions of the free DNA probe, the SigA-*rbcL* complex, the factor 2-DNA complex, and the NtcA-DNA complex are indicated.

The crude extract from AM1080 showed an additional complex (labeled SigA-*rbcL*) that was not seen in the general background produced by clones carrying a different insert or the vector alone (Fig. 2A). The plasmid isolated from strain AM1080(pAM1080) was partially mapped with the restriction enzymes *Hin*dIII and *Eco*RI. A subclone, pAM1086, which lacked 1- and 0.8-kb *Hin*dIII fragments, still produced the additional complex. The 2.3-kb insert from pAM1086 was cloned into pBluescript KS(+) as a *Sma*I-*Hin*dIII fragment to make pAM1083. Mobility shift assays showed that pAM1083 produced the additional complex, confirming that the 2.3-kb PCC 7120 insert contained the gene encoding the protein responsible for the novel complex with *rbcL* (Fig. 2B).

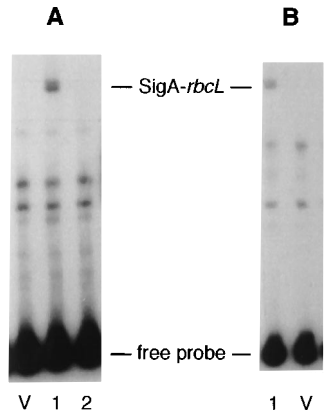

FIG. 2. SigA-dependent DNA-binding activity in *E. coli* extracts. (A) Mobility shift assays were done with crude protein extracts from *E. coli* JM107 containing the vector pSE380 alone (lane V), pAM1080, which contains *sigA* cloned in pSE380 (lane 1), or a random clone in pSE380 (lane 2). (B) Mobility shift assays of extracts from *E. coli* DH10B containing the *sigA* clone pAM1083 (lane 1) or the pBluescript KS(+) vector alone (lane V). Extracts (4 μg of protein) were equilibrated with 10,000 cpm (1.25 ng) of a labeled *rbcL* fragment and analyzed on a 5% polyacrylamide gel. The *Eco*RI-*Kpn*I fragment from pAM942 containing 94 bp of the *rbcL* upstream region was used as the labeled probe. The positions of the free DNA probe and the SigA-*rbcL* complex are indicated.

The insert in pAM1083 contains the *sigA* gene. Partial sequencing of the pAM1083 insert was done by the dideoxy-chain termination method and analyzed as previously described (16). The pAM1083 insert was found to contain the PCC 7120 *sigA* gene, which encodes the principal vegetative-cell sigma factor (1).

***Anabaena* extracts show binding to the *rbcL* upstream region.** We identified a weak protein-*rbcL* complex produced by PCC 7120 vegetative-cell extracts that had a mobility shift similar to that produced by *E. coli* containing the *sigA* gene (Fig. 3). PCC 7120 vegetative cell filaments were harvested from late exponential growth phase 2-liter cultures, and protein extracts were partially purified by heparin-Sepharose CL6B column chromatography as previously described (13). Samples (5 μl) of each column fraction were assayed for DNA-binding activity with the 94-bp *rbcL* upstream region. Mobility shift assays of protein extracts from *E. coli* containing pAM1080 (*sigA* in pSE380) and those from *E. coli* containing the pSE380 vector alone were run in parallel. SigA-dependent DNA-binding activity was seen in extracts from *E. coli* containing pAM1080, and a similar complex was detected in heparin-Sepharose fractions 5 and 7 from PCC 7120 (Fig. 3). The PCC 7120 extract also showed complexes of the *rbcL* probe with factor 2 and with NtcA (13).

The SigA-dependent mobility shift with *rbcL* is consistent with our identification of the *sigA* clone in the transcriptional-interference selection. However, we do not know whether the binding of SigA to *rbcL* involves core RNA polymerase or other factors. It is likely that our protein extracts and ammo-

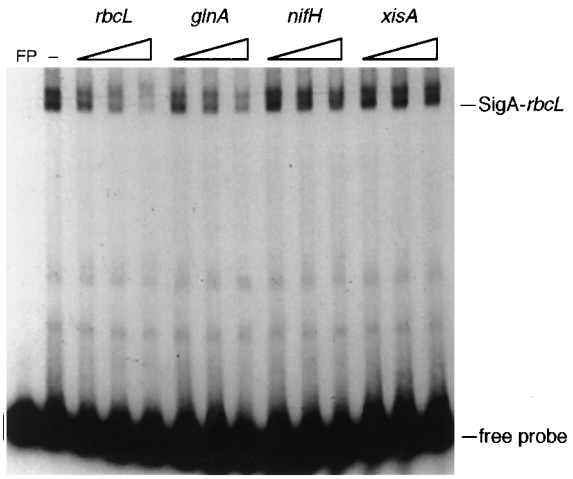

FIG. 4. Competition of *rbcL*, *glnA*, *nifH*, and *xisA* upstream regions for binding to a labeled *rbcL* probe. Mobility shift assays were carried out with 20 μg of total protein from a 50 to 75% ammonium sulfate saturation fraction containing SigA produced by pAM1080 in *E. coli* DH10B. The assays contained 5,000 cpm (0.44 ng) of the labeled 94-bp *rbcL* upstream region fragment with or without unlabeled competitor DNA. The competitor fragments used were the unlabeled *rbcL* fragment, a 249-bp *glnA* fragment from pAM658, a 191-bp *nifH* fragment from pAM657, and a 235-bp *xisA* fragment from pAM709. No competitor (lane −) or a molar excess (25-, 50-, and 100-fold) of an unlabeled competitor fragment was added to the samples. Lane FP, no protein. The protein-DNA complex was run on a 5% polyacrylamide gel at 30 mA for 2.5 h. The positions of the free DNA probe and the SigA-*rbcL* complex are indicated.

nium sulfate fractions from *E. coli* and PCC 7120 contain RNA polymerase.

SigA-containing extracts bind *rbcL* and *glnA* but not *nifH* or *xisA*. Competition experiments were done with the labeled 94-bp *rbcL* promoter fragment and unlabeled *rbcL* fragment, an *Xba*I-*Hin*dIII fragment from pAM658 containing 249 bp of the *glnA* promoter region (13), an *Xba*I-*Hin*dIII fragment from pAM657 containing 191 bp of the *nifH* promoter region (13), or an *Xba*I-*Hin*dIII fragment from pAM709 containing 235 bp of the *xisA* upstream region (16) (Fig. 4). The DNA fragments were incubated with partially purified *E. coli* extracts containing SigA and then analyzed by mobility shift assay (13). Autoradiographs were scanned and the amount of binding complex formed was quantitated with MacBAS 2.0 (Fujix) software. The SigA-dependent complex with *rbcL* was reduced by *rbcL* and *glnA* promoter fragments but not by *nifH* and *xisA* upstream fragments (Fig. 4). A 100-fold excess of the unlabeled *rbcL* competitor reduced the binding to 22%, and a 100-fold excess of the *glnA* competitor reduced binding to 35%. The *nifH* and *xisA* competitors did not significantly reduce the complex formed with labeled *rbcL*.

The in vitro DNA-binding results correlate well with the expression pattern of the tested genes. *rbcL* is expressed in vegetative cells but not in heterocysts (6, 9), and it is transcribed in vitro by RNA polymerase purified from PCC 7120 vegetative cells (14). *glnA* is transcribed from multiple promoters that are differentially expressed in response to changes in nitrogen availability (15). During growth on ammonia, transcription is mainly from RNA_{II}, a promoter that resembles the typical *E. coli* σ^{70} promoter (15). In vitro transcription assays using a purified RNA polymerase holoenzyme showed that this promoter is indeed recognized by the principal vegetative-cell sigma factor (14). In contrast, *nifH* is transcribed exclusively in heterocysts (6, 9). Although *xisA* transcripts have not been detected, *xisA* is thought to be expressed only in heterocysts because the activity of its product is heterocyst specific (3, 11, 12).

The in vivo selection worked surprisingly well for SigA, considering the modest in vitro interaction in *E. coli* and PCC 7120 extracts. Our results show that a combination of selection by transcription interference, which relies on the ability of a DNA-binding protein to bind target DNA in vivo, followed by screening with mobility shift assays can be used to identify clones that express DNA-binding proteins even when it is difficult to detect the proteins by mobility shift assays alone.

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