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Identification of the σ^{70}-Dependent Promoter Controlling Expression of the *ansPAB* Operon of the Nitrogen-Fixing Bacterium *Rhizobium etli*

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The aim of the present work was to examine the putative promoter region of the operon *ansPAB* and to determine the general elements required for the regulation of transcriptional activity. The transcriptional start site of the *ansPAB* promoter was determined by using high-resolution S1-nuclease mapping. Sequence analysis of this region showed -10 and -35 elements, which were consistent with consensus sequences for *R. etli* promoters that are recognized by the major form of RNA polymerase containing the σ^{70} transcription factor. Mutation studies affecting several regions located upstream of the transcriptional start site confirmed the importance of these elements on transcriptional expression.

Keywords: Asparagine operon, σ^{70} transcription factor, *Rhizobium etli*, S1-nuclease mapping

The symbiotic nitrogen-fixing bacterium *Rhizobium etli* is a gram-negative soil α-proteobacterium with the ability to colonize the roots of bean plants, specifically *Phaseolus vulgaris*, and substantially increase plant productivity [6, 12]. The bacteria have the ability to reduce atmospheric nitrogen to ammonium when some carbon compounds are exchanged with its plant host [5, 17]. This association enables legumes to grow in nitrogen-poor soil. Amino acid metabolism has been implicated as an important factor in the symbiotic interaction between rhizobia and leguminous plants [13]. In a general model, the plant supplies carbon sources and glutamate to the rhizobial bacteroid, while the bacteroids in turn provide the plant with ammonium, aspartate, and alanine [18].

In general, microorganisms can utilize L-asparagine as a carbon and nitrogen source *via* the activities of two enzymes [24]. L-Asparaginase (E.C. 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartate and ammonium, while L-aspartase (E.C. 4.3.1.1) catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonium. Previous study on the catabolism of asparagine by *R. etli* showed that the degradation involves the activity of L-asparaginase and L-aspartase; the two enzymes were positively regulated by asparagine and negatively regulated by the carbon source. Cultures grown on asparagine as a carbon and nitrogen source showed the highest asparaginase activity, but the activity diminished when cultures were grown on asparagine supplemented with glycerol, glucose, and succinate, or when grown on a rich PY medium. Additionally, the expression of both enzymes was not regulated by the amount of oxygen dissolved in the growth medium or by nitrogen catabolite repression, and some

asparaginase activity was also detected when grown on ammonium and succinate [8]. Further studies with a mutant strain (AHZ7) affected in L-asparaginase and L-aspartase activities showed low levels of asparaginase and aspartase activities when grown on asparagine, asparagine-succinate, and ammonium-succinate in comparison with the wild-type strain. The levels of asparaginase and aspartase activity of mutant AHZ7 were restored with pAHZ11 cosmid construction to wild-type levels [9].

Sequence analysis of cosmid pAHZ11 revealed four open reading frames named *ansR*, *ansP*, *ansA*, and *ansB;* the last three genes are organized as the *ansPAB* operon [15]. Further details obtained from *in silico* analysis of the sequence between the *ansR* and *ansP* showed the presence of three putative conserved elements; two resembled the -24 and -12 consensus sequence of σ^{54}-dependent promoters, and the third conserved element appeared to be a component of the recognition-binding site of the AnsR regulator, which showed an identity to recognition sequences for GntR repressors of the FadR subfamily [20]. Based on the above, the aim of this study was to identify the promoter elements required for transcription of the *ansPAB* operon by mapping the transcriptional start site (TSS) of the promoter, and show if the putative σ^{54} binding site for RNA polymerase is the functional regulatory element for transcription.

Bacterial strains, plasmids, oligonucleotides, growth, and transformation conditions used through the study are described in Table S1. Plasmids used to determine the promoter elements were constructed as follows: fragments containing different lengths of the *ansPAB* upstream region were amplified by PCR using Platinum Taq DNA polymerase High-Fidelity (Invitrogen) and the plasmid pAHZ11 as the template (Fig. S1).

To determine the β-glucuronidase activity of the mutants, overnight cultures of the *R. etli* strains carrying the plasmid construct were grown in PY medium supplemented with gentamicin at 15 μg/ml. An aliquot of these overnight cultures was used to inoculate 30 ml of MM supplemented with 10 mM L-asparagine with the appropriate antibiotic to an initial OD_{450} of 0.05, and grown for 24 h at 29°C with shaking to a final OD_{450} of 0.4. The cultures were then processed as described by Ramirez-Romero *et al.* [19]. Quantitative β-glucuronidase assays were performed using *p*-nitrophenyl glucuronide as previously described [25]. The results presented are the mean of three independent experiments. Data were analyzed using an analysis of variance at $p = 0.05$ with the statistical software package Statgraphics Centurion XVI (ver. 10).

Determination of the transcriptional start site of the *ansPAB* promoter used total RNA from cultures purified using standard protocols according to Kieser *et al.* [10]. An overnight culture of the mutant strain *R. etli* AHZ7 harboring the plasmid pAHZ11 was grown in PY medium supplemented with gentamicin at 15 μg/ml. Next, the cultures were inoculated at an initial OD_{450} of 0.05 in PY medium, MM + 10 mM succinate + 10 mM ammonium, and MM + 10 mM L-asparagine, and incubated for 16 h at 29°C with shaking. The *R. etli* CE3 cultures grown under the same conditions were used as the control. A 371 nt probe for S1 mapping was prepared by PCR amplification using the oligonucleotides PROMANS1 and PROMANS2, and Platinum Taq DNA polymerase (Invitrogen) (Table S1 and Fig. S1). The PROMANS2 oligonucleotide was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Invitrogen) prior to the PCR, resulting in a probe that was uniquely labeled at one end. Further processing of the PCR fragment, and samples and denaturating gel electrophoresis of the protected fragments were performed as previously described [23]. The protected fragments were run in parallel to the sequence ladders obtained with the same labeled primer used for the probe preparation. DNA sequencing reactions were performed using a Thermosequenase Cycle Sequencing Kit (USB). Densitometry was performed using Image J analysis software (NIH) [22]

Results from previous studies have shown a single mRNA transcript covering the *ansP*, *ansA*, and *ansB* genes of the asparaginase operon in *R. etli*. Moreover, *in silico* analysis of the sequence upstream from gene *ansP* showed two putative conserved sequences that resembled the -24 and -12 consensus elements for a σ^{54} transcription factor binding site [15]. To determine whether this putative σ^{54} binding region is a functional element to direct transcription, it seemed important to locate the TSS of the *ansPAB* operon. Therefore, total RNA isolated from cultures of *R. etli* CE3 grown in the different media was subject to high-resolution S1 mapping experiments. The results showed that transcription of the *asnPAB* operon started from a single TSS when strain CE3 was grown in MM supplemented with L-asparagine (Fig. 1A, lane 4), and that transcription of the *ansPAB* operon was completely repressed when CE3 cultures were grown on either PY or MM supplemented with 10 mM of succinate and ammonium (Fig. 1A, lanes 2 and 3, respectively). Moreover, similar results were obtained for mapping assays with RNA from the mutant strain AHZ7 carrying pAHZ11; transcription of the *ansPAB* operon started from the same TSS when grown on L-asparagine as the sole carbon and nitrogen source

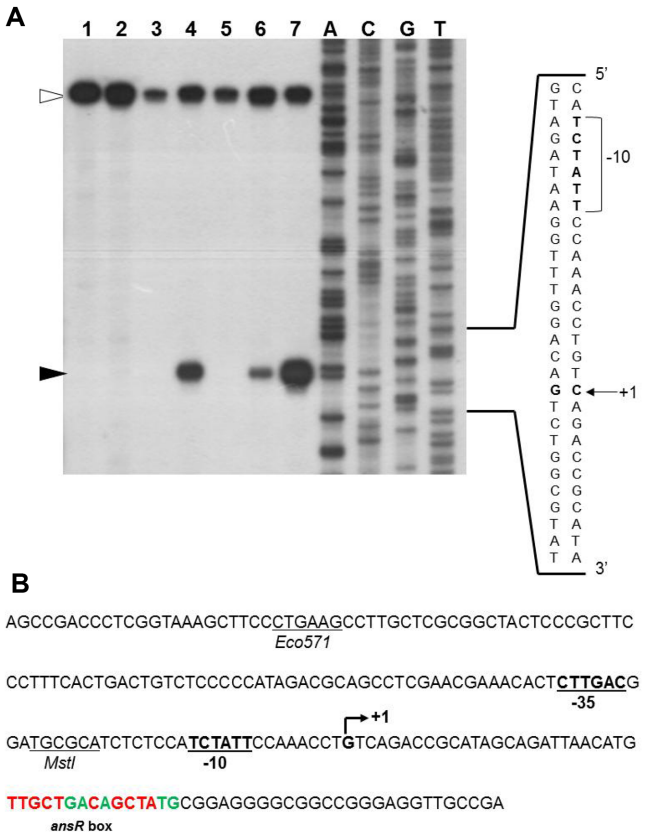

Fig. 1. Determination of the TSS of the *ansPAB* operon.
(**A**) High-resolution S1 nuclease mapping of the transcript from RNA obtained from cultures of *R. etli* CE3 (lanes 2 to 4) and *R. etli* AHZ7/pHZ11 (lanes 5 to 7) grown on different media. The filled arrowhead indicates the band corresponding to the transcript-protected probe, and the empty arrowhead indicates the position of undigested full-length probe. Lanes labeled A, C, G, and T represents the sequencing ladder generated using the same radiolabeled oligonucleotide that was used to make the S1 mapping probe. Lane 1, no RNA; lanes 2 and 5, RNA from PY media; lanes 3 and 6, RNA from MM + succinate + ammonium; lanes 4 and 7, RNA from MM + L-asparagine. (**B**) Sequence of the *ansPAB* operon promoter. The TSS (+1); -10 and -35 elements (bold and underlined); and downstream putative binding sites for AnsR (red) are shown. The restriction enzyme site for *Mst*I (underlined) is located between the -10 and -35 elements.

(Fig. 1A, lane 7), and operon expression was repressed when grown in PY medium or in MM supplemented with 10 mM of succinate and ammonium (Fig. 1A, lanes 5 and 6, respectively). Furthermore, an incomplete repression was observed in the mutant strain (lane 6), that could be due to the extra copies of the operon into the cells contained in the pAHZ11 plasmid in combination with a reduction of succinate from the medium. Rhizobia members (*Sinorhizobium*, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*) use the C4-dicarboxylic TCA cycle intermediates succinate, fumarate, and malate in preference to carbon sources, including glucose, fructose, galactose, lactose, and myo-inositol; for example, in *Sinorhizobium meliloti,* the production of β-galactosidase was repressed when succinate and lactose were present together, and it increased to higher levels after succinate had been exhausted from the medium for a mechanism called succinate-mediated catabolite repression [2]. Catabolite repression of phenol utilization by succinate appears to be a characteristic feature of many rhizobia [21]. As shown in Fig. 1A and according to the densitometry analysis, a 2-fold higher level of transcripts was accumulated in cultures of *R. etli* AHZ7/pAHZ11 compared with *R. etli* CE3 grown on L-asparagine, which may be due to the copy number of the pAHZ11 plasmid.

The above results clearly reveal that *ansPAB* operon transcription initiation is induced by asparagine and is negatively regulated by the carbon source. As noted in earlier studies, asparaginase activity in cell extracts of *R. etli* was high when cells were grown on asparagine, but this activity decreased when it was grown on asparagine plus glycerol, glucose, and succinate (ammonium did not have a synergic effect with the carbon source to reduce the enzymatic activity) [8]. Sequence analysis of the mapped promoter demonstrated that the TSS of the *ansPAB* operon corresponded to a G nucleotide (Fig. 1B; labeled as +1); two sequences (CTTGAC and TCTATT) were identified upstream this site, which correspond to the conserved -35 and -10 consensus sequences of the promoter regions identified in *R. etli*, respectively. Both elements were separated by 17 nt. Thus, the *ansPAB* promoter region has the molecular formula 5′-CTTGACN_{17}TCTATT-3′, exhibiting similarity to other promoters recognized by the σ^{70} transcriptional factor [11, 19]. Therefore, in spite of previous observations that suggested a σ^{54}-dependent promoter, our results clearly show that the *ansPAB* operon is transcribed from a σ^{70}-dependent promoter. A promoter dependent of a σ^{54} transcription factor is tightly regulated and requires for its expression key elements for RNA polymerase binding and access to the open complex for transcription initiation; it requires two short recognition sequences localized at nucleotides -12 and -24, an activator protein that binds to an upstream activator sequence to interact with the sigma factor and facilitates the interaction with the RNA polymerase holoenzyme by proteins IHF-like (integration host factor). Moreover, the use of σ^{54} may confer the capacity to vary the transcriptional efficiency without the use of a separate repressor [1, 4]. In contrast, an RNA polymerase that contains σ^{70} factor is competent for transcription initiation

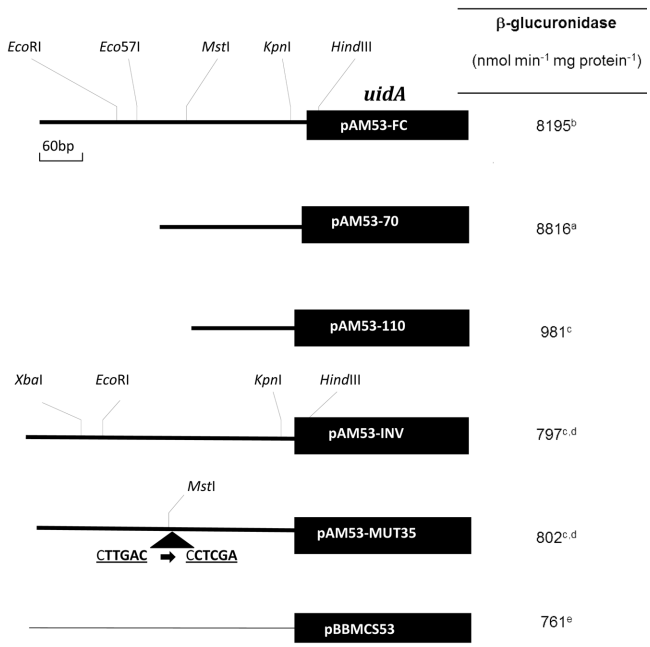

Fig. 2. Effect of mutations on the promoter fusion *ansPAB:uidA* on β-glucoronidase activity.

The fragments carried by the pBBMCS53 plasmid are shown, including the relevant restriction sites used for plasmid construction. The -10 and -35 elements of the *ansPAB* promoter are positioned one at each side of the *Mst*I restriction enzyme site. The pAM53-70 and pAM53-110 plasmids carry a deletion of -70 and -110 nt, respectively. pAM53-110 retains only the -10 element. The sequence in pAM53-INV corresponds to the complete promoter cloned into an inverted position. Mutation of the -35 element was produced, changing the sequence CTTGAC by the sequence CCTCGA, creating the *Xho*I site as indicated (pAM53-MUT35). The left column indicates the promoter construct and the right column indicates the β-glucoronidase activity. Results with the same latter are not significantly different (p = 0.05).

in the absence of an activator, although at some promoters, an activator is needed to recruit the RNA polymerase [16].

To confirm the functionality of the promoter elements previously described, we constructed mutant versions of the *ansPAB* operon promoter fused to the promoter-probe vector pBBMCS53 (Fig. 2). Cultures of *R. etli* carrying a plasmid construct with a fragment lacking the upstream 70 base pairs, but maintaining the -35 and -10 elements (pAM53-70), exhibited similar levels of β-glucoronidase activity to wild type (pAM53-FC). A longer deletion of 110 bp in the promoter (pAM53-110), which eliminates the -35 element and retains the -10 element, showed a strong reduction in β-glucoronidase activity to levels comparable to those from the vector alone. The same results were obtained with a plasmid construct with the wild-type promoter fragment cloned into the opposite direction (pAM53-INV). Finally, a promoter derivative was constructed to evaluate the importance and functionality of the -35 element for full activation (pAM53-MUT35). This promoter mutant retained the -10 element but the -35 sequence was altered by the introduction of the *Xho*I enzymatic restriction site. A reduction in β-glucuronidase activity to promoter-probe vector level was obtained. Therefore, the above results showed that the -35 conserved element and its integrity were essential for full activation of the *ansPAB* promoter. The position of the -35 region was consistent with its involvement in transcriptional activation, and the direction of the promoter was crucial for proper activation. Thus, it was clear that the operon expression was dependent on the σ^{70} instead of the σ^{54} transcription factor.

An important element of the promoter region for transcriptional activation is the putative AnsR binding site localized at the +26 to +41 downstream the transcriptional start site [3]. Further studies analyzing the molecular interactions of the transcriptional repressor (AnsR) to conserved elements localized upstream and downstream of the TSS of the *ansPAB* promoter are required. The present work is important, because most of the FadR-like proteins, including AnsR, are involved in the regulation of oxidized substrates related to amino acid metabolism or at the crossroads of various metabolic pathways such as pyruvate (PdhR), glycolate (GlcC), galactonate (DgoR), lactate (LldR), malonate (MatR), or gluconate (GntR) [20].

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