In vitro characterization of the ORF1-*ntrBC* promoter of *Rhizobium etli*

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Abstract ***Rhizobium*** **sigma vegetative-dependent promoters are different from those of enteric bacteria and have never been characterized before. We report here the biochemical characterization of the ORF1-*ntrBC* promoter of *Rhizobium etli*. The minimal promoter region was located by means of a transcriptional fusion and further characterized by in vitro transcription and gel retardation experiments. Oligonucleotides used as DNA competitors in runoff transcription experiments allowed the precise localisation of the promoter region. Protein extracts from an *ntrC*$^+$, but not from an *ntrC*$^-$ strain, inhibited in vitro transcription. The NtrC protein was found to bind specifically to the promoter, where an NtrC binding site overlapping the transcription initiation site, is present.**

Key words: Two-component system; *ntr* system; Runoff transcription; Gel retardation

1. Introduction

The response to nitrogen starvation is the most extensively investigated signal transduction pathway of prokaryotes. This signal is sensed and transmitted through the protein kinase NtrB and the transcriptional regulator NtrC, encoded by the *ntrB* and *ntrC* genes, respectively [1].

In enteric bacteria the *ntrBC* genes are part of the *glnA-ntrBC* operon, in which *glnA* codes for glutamine synthetase. Under nitrogen-excess conditions *ntrBC* expression is predominantly initiated from the *ntrBC* promoter. Under nitrogen-limiting conditions NtrC binds to a site overlapping the *ntrBC* promoter, thus repressing transcription initiation [2,3]. Simultaneously, NtrC activates transcription at the strong *glnA*p2 promoter and the transcript reads through the *ntrBC* genes. The consequence of this complex organization is to provide a low level of NtrC protein (5 dimers per cell) under nitrogen-excess conditions, as compared to a 14-fold higher level observed when nitrogen is limiting. A high intracellular concentration of NtrC is essential for expression of several unlinked nitrogen-controlled genes [1].

In *Rhizobium etli* NtrC is essential for expression of *glnII*, the structural gene for a glutamine synthetase [4], and the *ntrBC* genes are part of an ORF1-*ntrBC* operon, where ORF1 is an open reading frame of unknown function [5]. Unlike the case of enteric bacteria, the intracellular concentration of the NtrC protein is 2–3-fold higher under growth in nitrogen-excess conditions as compared to growth in a nitrogen-limiting source. Moreover, a 20-fold higher ORF1-*ntrBC* mRNA concentration was found in a *R. etli ntrC* mutant strain (*ntrC*$^-$) as compared to the wild-type strain, thus suggesting either negative autoregulation of ORF1-*ntrBC* expression by NtrC (or by another *trans*-acting factor(s), absent or inactive in the *ntrC*$^-$ strain), or a difference in mRNA stability [5]. Upstream of ORF1 there are two RNA 5′ ends, t_1 and t_2, each preceded by a sequence with some homology to the −10/−35 *consensus* promoter sequence of enteric bacteria [5]. A transcriptional ORF1-*ntrBC-lac* fusion, highly expressed in *R. etli*, is inactive in *Escherichia coli* (Patriarca E.J., unpublished), thus suggesting that the transcriptional apparatus of *R. etli* may be different from that of *E. coli*, as also indicated by the sequences of the *Rhizobium meliloti* [6] and *R. etli* (Luka S., unpublished) genes coding for the vegetative sigma factor. While the *Rhizobium* sigma 54-dependent promoters, such as those of genes involved in nitrogen fixation, are well studied [7], the sigma vegetative-dependent promoters are still poorly characterized [8]. The intracellular concentration of the ORF1-*ntrBC* mRNA is growth-phase regulated in free-living *R. etli* and it is down-regulated in the symbiosis, in coincidence with the arrest of bacterial division occurring in the nodule [9]. The down-regulation of this promoter might be part of a developmental mechanism required to reduce bacteroid metabolic activities and for this reason its characterization is particularly interesting.

In this paper we report in vitro transcription and gel retardation experiments on the ORF1-*ntrBC* promoter, showing that the NtrC protein binds to it and inhibits transcription initiation. These results are obtained with a crude system, which is the necessary starting point for fractionation of key components of this complex regulatory system and for testing the action of novel regulatory factors. Part of this work has been briefly reported previously [10].

2. Materials and methods

2.1. Bacterial strains and media

R. etli wild-type strain CE3 and strain CFN2012, an *ntrC*::Tn*5* derivative [11], were grown at 30°C in TYR rich medium [12] or in RMM minimal medium [13] with 1 g l^{-1} NH_4Cl or KNO_3 as sole nitrogen source. When NH_4Cl was used, 100 mM 3-{*N*-morpholino} propanesulfonic acid pH 7.2 was also added. Antibiotics used were (μg ml^{-1}): nalidixic acid (20); kanamycin (30).

2.2. In vitro transcription assay

DNA fragments were obtained by *Eco*RI or *Hin*dII linearization of plasmid pAR55 (Fig. 1). This was constructed with *Bam*HI-linearized DNA of plasmid pAR50 [5] digested with *Exo*III and then with *Eco*RI. A 0.8 kb fragment was purified by agarose gel electrophoresis, eluted and inserted into the *Nsi*I/*Eco*RI sites of pGEM7Zf(+) (Promega Inc.). To verify that this plasmid contains the promoter region, a *Cla*I/*Nar*I 156 bp fragment was isolated and inserted into the *Cla*I site of pGEM7Zf(+), followed by digestion with *Nsi*I/*Eco*RI and recloning of the fragment into the *Pst*I/*Eco*RI sites of the low copy

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′[-ethanesulfonic acid]; EDTA, [ethylenedinitrilo]tetraacetic acid

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number, promoter-probing vector pMP220 [14]. The resulting plasmid gave, as expected, 900–1000 units of β-galactosidase specific activity in *R. etli* CE3.

Protein extracts for in vitro transcription experiments (transcription extract) were prepared, with minor modifications of a protocol previously described for *E. coli* [15]. The bacterial pellet from a 200 ml *R. etli* culture (OD_{590} 0.2 in rich medium) was resuspended in 1 ml of cold extraction buffer (25% sucrose, 10 mM Tris-HCl pH 8 and 100 mM NaCl) and incubated for 15 min on ice. After addition of a solution (250 μl) containing 300 mM Tris-HCl pH 8, 100 mM EDTA and 4 mg/ml lysozyme, the samples were incubated for 5 min on ice and 1.25 ml of 1 M NaCl, 20 mM EDTA and 0.08% deoxycholate were added, followed by 10 min incubation at 10°C. After centrifugation (20 min at 10000×*g*) an equal volume of 40% glycerol (20% final concentration) was added to the supernatant and aliquots were stored at −80°C. The concentrations of NaCl and EDTA were 270 mM and 10 mM respectively. Protein concentration was determined by the Bio-Rad assay using bovine serum albumin as a standard. The extracts contained about 5 μg DNA per mg of protein, as determined by analysis on agarose gel before and after DNase treatment.

Runoff experiments were carried out in 20 μl of a solution containing 40 mM Tris-HCl pH 8, 6 mM $MgCl_2$, 50 mM NaCl, 10 mM DTT, 1 mM each of ATP, CTP and GTP, 50 μM UTP, 10 μCi [α-^{32}P]UTP and 50 ng of plasmid DNA. Reactions were started by addition of 9 μg (3 μl) of transcription extract to prewarmed (30°C) mixtures and, after 10 min, were stopped with 20 μl of phenol/chloroform (1:1). After ethanol precipitation, samples were run on denaturing 6% polyacrylamide–7 M urea gels. Markers were *Hae*III fragments of pBR322 labelled at their 5′ ends with [γ-^{32}P]ATP and T4-Polynucleotide Kinase (Marker V; Boehringer Mannheim GmbH). In competition experiments oligonucleotides were added to the reaction mixture just before addition of the extract. They were purchased from Genset and purified on a 20% polyacrylamide–7 M urea gel. Double-stranded oligonucleotides were obtained by mixing equal amounts of complementary oligonucleotides at 85°C for 5 min, followed by slow cooling to 30°C.

2.3. DNA binding reactions

The DNA fragment used in band shift experiments was obtained by *ClaI*/*Nar*I digestion of pAR55 (Fig. 1), elution and labelling at its 3′ or 5′ end with [α-^{32}P]dCTP, [α-^{32}P]dGTP and Klenow fragment. The labelled DNA fragment was purified by agarose gel electrophoresis, electroeluted and ethanol precipitated. DNA binding proteins containing the NtrC protein with high DNA binding activity were prepared as previously described [16]. The incubation mixture (20 μl) for DNA–protein binding contained 20 mM HEPES pH 7.6, 2 mM EDTA, 0.1 M NaCl, 0.1 mM PMSF, 0.7 mM β-mercaptoethanol, 10% glycerol, 2 μg bovine serum albumin, 0.25–5 μg of DNA binding proteins, 1–2 ng of end-labelled DNA fragment and 0.5 μg poly(dI-dC). The binding reactions were allowed to proceed for 10 min at 25°C. Samples were loaded immediately on non-denaturing 5% polyacrylamide gels (acrylamide-bisacrylamide 29:1) in 45 mM Tris-borate pH 8.4, 1 mM EDTA and 10% glycerol. After electrophoresis (5 mA for about 12 h at room temperature), gels were fixed in 10% acetic acid, dried on a filter paper and autoradiographed.

3. Results

3.1. Exogenous template-dependent transcription using R. etli extracts

Fig. 1 shows in vitro transcription assays performed with a transcription extract prepared from the wild-type strain (CE3) of *R. etli* and DNA of plasmid pAR55, linearized with either *Hin*dII or *Eco*RI (see Fig. 1C) as a template, in order to obtain runoff transcripts of different lengths. Plasmid

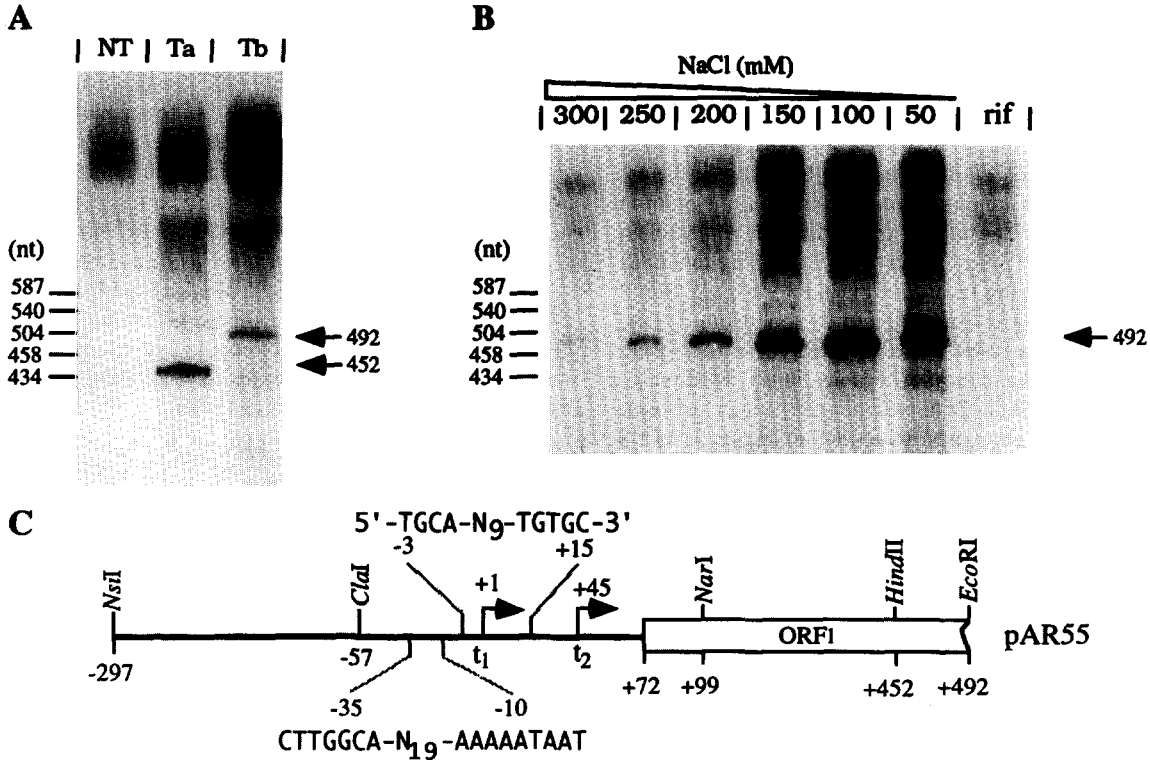

Fig. 1. In vitro transcription of the ORF1-*ntrBC* promoter (panels A and B). Physical map (panel C, not to scale) of pAR55 DNA (3.8 kb) containing the promoter region of the ORF1-*ntrBC* promoter. The putative start codon of ORF1 (indicated at +72) is at nt 209 of the published sequence (EMBL/Genbank X71436 [5]). A putative NtrC binding site, spanning nt −3 to +15, and the −10/−35 sequence are shown. Panels A and B show autoradiograms of RNA products after in vitro transcription experiments. Size markers (nt) are indicated on the left, arrows on the right indicate approximate transcript size. Panel A: No template (lane NT), pAR55 DNA linearized with *Hin*dII (Ta) or with *Eco*RI (Tb). Panel B: Effect of NaCl or 0.5 μg/ml rifampicin (rif). The latter was tested after 5 min preincubation of the extract and template, followed by addition of nucleotides and rifampicin.

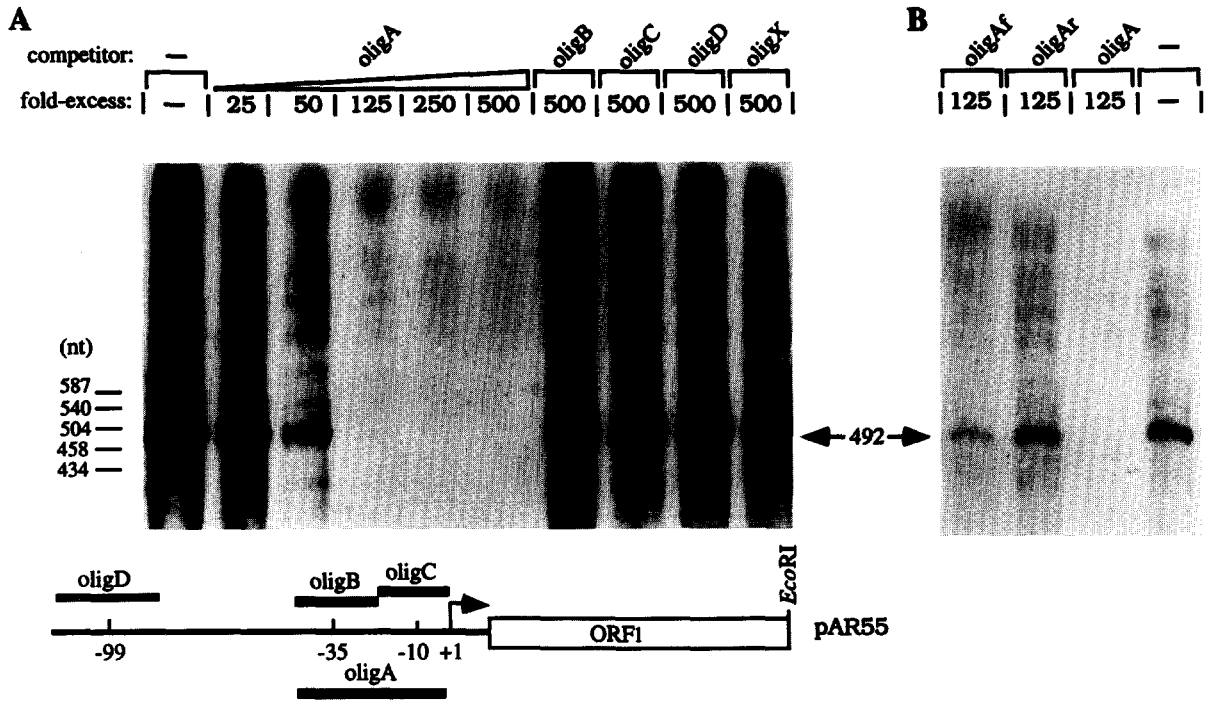

Fig. 2. (A) Competition experiments with different double-stranded oligonucleotides. In vitro transcription assays were carried out in the presence of excess oligonucleotides, as indicated. At the bottom the oligonucleotides used are positioned on the DNA of pAR55, except for oligX, a 47-mer containing a sequence unrelated to the ORF1-*ntrBC* operon (5′-CAAAGACTCTTGGACTGTTAACGACATCCAGAAACTGGTTG-GAC-3′). (B) Comparison of transcription competition (125-fold excess) of double-stranded oligA and its single strands (oligAf: lower; oligAr: upper).

pAR55 contains the ORF1-*ntrBC* promoter region, as demonstrated by means of a transcriptional fusion (see section 2). No bands are observed in the absence of exogenous template (Fig. 1A, lane NT), the smear being due to transcripts generated from endogenous template, namely chromosomal DNA. Upon addition of pAR55 DNA digested with *Hin*dII (Fig. 1A, lane Ta) a band of approximately 452 nt is observed, while upon addition of pAR55 DNA digested with *Eco*RI (Fig. 1A, lane Tb) a transcript of approximately 492 nt is observed. The same results are obtained with a transcription extract prepared from the *ntrC*$^-$ strain (CFN2012) of *R. etli* (not shown in Figure A). Based on the size of the runoff transcript we can infer that the transcriptional start site is at about 72 nt upstream of the putative start codon of ORF1, thus corresponding to the previously recognized t_1 5′ RNA end of the in vivo transcript [5]. In fact, a primer extension analysis using a runoff transcript synthesized with cold ribonucleotides, demonstrated that its 5′ end coincides with t_1 (data not shown). Data of Fig. 1B demonstrate that the RNA polymerase holoenzyme of *R. etli* is sensitive to NaCl and rifampicin.

In Fig. 2A it is shown that a specific double-stranded oligonucleotide (oligA, a 44-mer covering nt −45 to −2 with respect to t_1) inhibits transcription initiation from both the exogenous (Tb) and the endogenous template very efficiently (the gel was overexposed as compared to Fig. 1). The double-stranded oligonucleotides oligB (a 20-mer spanning nt −45 to −26), oligC (a 20-mer spanning nt −21 to −2), oligD (a 20-mer spanning nt −91 to −72) and oligX (a 47-mer representing an unrelated sequence, see legend to Fig. 2) do not cause inhibition, even at a 500-fold excess. In panel B it is shown that the inhibition exerted by the single strands of oligA is less pronounced than that achieved with double-stranded oligA, the lower strand (oligAf) being more efficient than the upper one (oligAr).

In conclusion, these experiments show that using a crude transcription extract as a source of RNA polymerase holoenzyme and the promoter region of the ORF1-*ntrBC* operon as a template it is possible to obtain precise transcription initiation in vitro. Moreover, these experiments also show that the runoff transcript starts at t_1 (but not at t_2, see Section 4 and that the DNA spanning from nt −45 to −2 with respect to t_1 contains at least part of the sequence essential to interact with the transcriptional apparatus of *R. etli*.

3.2. *The NtrC protein binds the ORF1-*ntrBC *promoter in vitro*

Fig. 3A shows the interaction of *trans*-acting factors, present in the extract containing DNA binding proteins, with a ^{32}P-labelled DNA fragment (probe 'C') spanning nt −57 to +99 with respect to the transcription initiation site t_1 of the ORF1-*ntrBC* promoter. The protein extract (2.5 μg) prepared from *R. etli* wild-type strain incubated with probe 'C' causes the appearance of two retardation complexes (indicated as C_I and C_{II}). No significant retardation of this probe is observed with extracts (2.5 μg) prepared from the *ntrC*$^-$ strain. When the amount of non-specific competitor poly(dI-dC) is increased from 0.5 to 2 μg (not shown in Fig. 3), complex C_I is competed out, whereas complex C_{II} is unaffected. Moreover, when the NtrC-containing extract is preincubated with different dilutions of a polyclonal antiserum raised against the *R. etli* NtrC protein [5], complex C_{II} disappears and new slowest-migrating complexes are observed (Fig. 3A). No supershift is observed when the preimmune serum (40-fold diluted) is added to the binding mixture. The antiserum against NtrC,

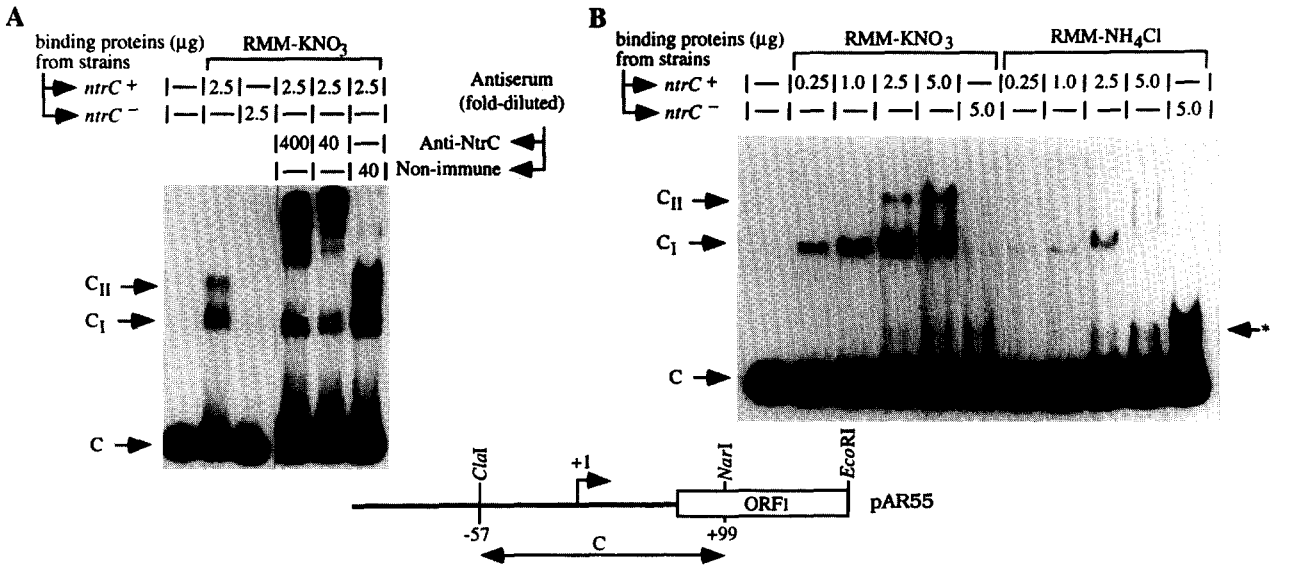

Fig. 3. (A) Complex formation between the *ClaI/NarI* 156 bp DNA fragment carrying the ORF1-*ntrBC* promoter region and DNA binding proteins (2.5 μg) from *R. etli* CE3 (wild-type) and CFN2012 (*ntrC*$^-$) strains grown in minimal medium (RMM) with KNO_3 (1 g l^{-1}) as a sole nitrogen source. The effect of an anti-NtrC serum on the complex formation at the dilution indicated is shown. (B) Comparison of the complex formation between the ORF1-*ntrBC* promoter region and DNA binding proteins from *R. etli* CE3 (*ntrC*$^+$) and CFN2012 (*ntrC*$^-$) strains previously grown in minimal medium (RMM) with 1 g l^{-1} of KNO_3, or NH_4Cl as sole nitrogen source.

even at the highest concentration used (40-fold diluted), has only a weak influence on complex C_I, thus indicating that it does not contain NtrC. We conclude that complex C_{II}, but not complex C_I, contains the NtrC protein.

In Fig. 3B it is shown that, using increasing amounts (0.25–5 μg) of DNA binding proteins prepared from KNO_3-grown cells, complexes C_I and C_{II} are more evident than when DNA binding proteins are prepared from NH_4Cl-grown cells.

Complex C_I is competed out by poly(dI-dC), is not affected by the anti-NtrC serum, is absent in DNA binding proteins prepared from the *ntrC*$^-$ strain and it is regulated by the nitrogen source. Therefore, this non-specific complex is due to the presence of a DNA binding protein(s) whose activity or synthesis is regulated by NtrC. In contrast, the binding capacity of the protein responsible for the second non-specific complex, indicated with an asterisk in Fig. 3B, is independent of the nitrogen source or of the strain used to obtain the DNA binding proteins. When 5 μg of extract from the *ntrC*$^-$ strain was used, this complex (asterisk) was predominant.

3.3. *In vitro repression of ORF1*-ntrBC *transcription*

In Fig. 4 it is shown that addition of increasing amounts (0.5–2.5 μg) of DNA binding proteins, prepared from the *R. etli* wild-type strain (CE3), to an in vitro transcription assay causes a progressive decrease of the 492 nt specific transcript, while transcription from the endogenous template is unaffected. DNA binding proteins prepared from the *R. etli ntrC*$^-$ strain (CFN2012) are ineffective to inhibit transcription initiation. As a control it is shown that DNA binding proteins prepared from either the wild-type or the *ntrC*$^-$ strain are not able to initiate transcription from the exogenous template. On the contrary, as in the case of transcription extract (see Fig. 1A), transcripts generated from a contaminating endogenous template are observed. These experiments were performed in the presence of high concentration of poly(dI-dC) as a competitor to avoid non-specific binding (see above). We conclude that the NtrC protein is required for the repression of transcription initiation at position t_1 of the ORF1-*ntrBC* promoter.

4. Discussion

We report in this paper an in vitro transcriptional characterization of the ORF1-*ntrBC* promoter. This analysis is important because this promoter, highly active in *R. etli*, is inactive in *E. coli*, as demonstrated by the lack of β-galactosidase activity of an ORF1-*ntrBC-lac* fusion in the latter species (Patriarca, E.J., unpublished). For this reason we set up a protocol for runoff transcription experiments, never reported previously for *Rhizobiaceae*. The RNA polymerase activity of *R. etli* was found to be present in the supernatant of the extraction solution containing 0.54 M NaCl (see Section 2) and not in the DNA pellet as in the case of *E. coli*, indicating differences in their biochemical properties. The activity present in this extract initiates transcription at a specific starting site, coincident with the t_1 5′ end of ORF1-*ntrBC* mRNA, and it is sensitive to NaCl and rifampicin. We conclude that the RNA polymerase activity present in the extract contains the sigma factor specific for this promoter. This extract will be very useful to purify and characterize the RNA polymerase holoenzyme of *R. etli*.

Two 5′ ends, indicated as t_1 and t_2 and located at 72 and 29 nt upstream of the putative start codon of ORF1, are present in the in vivo mRNA [5], but only one transcription initiation site, corresponding to t_1, is found in the in vitro experiments reported here. One possibility to explain this difference is that a factor(s) essential to initiate transcription at t_2 is absent or inactive in the extract used. Another possibility is that the presence of the t_2 5′ end is the consequence of a specific processing in vivo of the ORF1-*ntrBC* mRNA. Indeed,

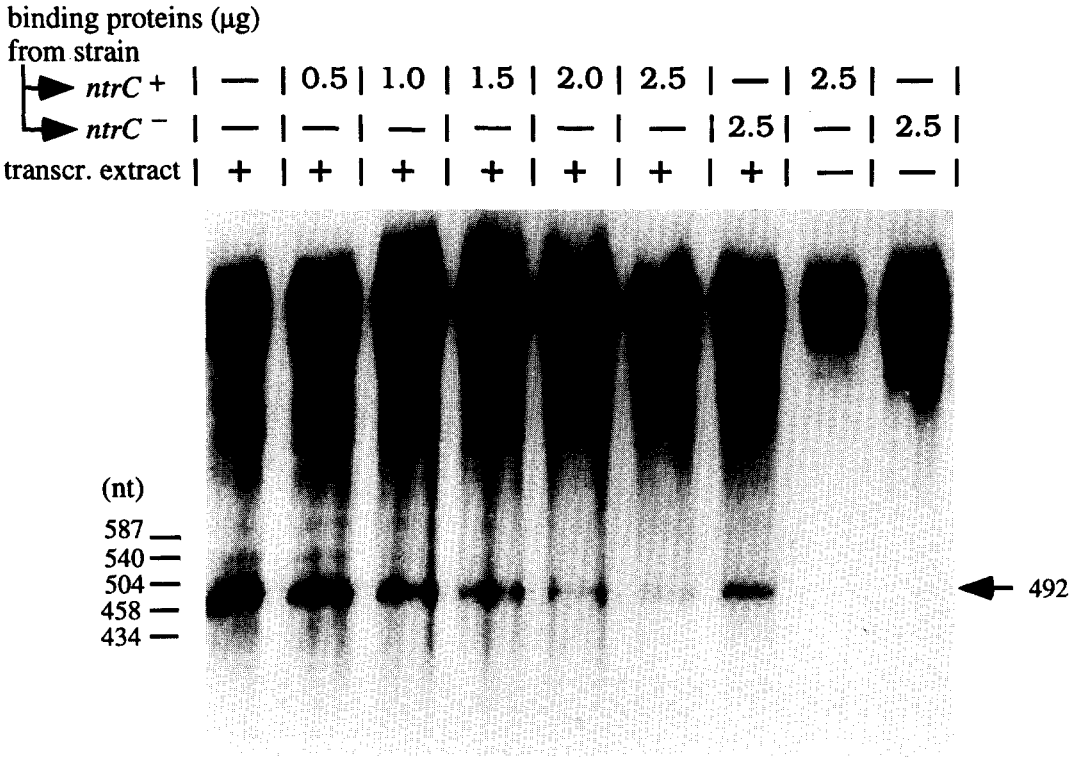

Fig. 4. Inhibition of in vitro transcription by DNA binding proteins from *R. etli* strains CE3 (*ntrC*$^+$) and CFN2012 (*ntrC*$^-$) grown in minimal medium with KNO_3 (1 g l^{-1}) as sole nitrogen source. DNA binding proteins from both strains were used at the same concentration. Different amounts of DNA binding proteins, as indicated, were preincubated at 25°C for 10 min with 50 ng of *Eco*RI-cut pAR55 template and 2 μg of poly(dI-dC). Transcription reactions were started by adding nucleotides and transcription extract.

Northern blot analysis shows that this mRNA is more unstable than other *R. etli* mRNAs (Riccio, A. and Patriarca, E.J., unpublished).

The inhibition of transcription initiation by oligonucleotide A shown in Fig. 2 suggests that the DNA region spanning from nt −45 to −2 with respect to t_1 interacts with the RNA polymerase holoenzyme, and that the polymerase recognizes analogous promoters present in the endogenous template. The latter statement is justified by the observation that oligA inhibits transcription initiation also from chromosomal DNA (Fig. 2A). We thus believe that this inhibition is due to the formation of a complex between oligA and the holoenzyme and therefore this oligonucleotide may be useful for the purification of transcriptional factors interacting at this promoter.

The intracellular concentration of ORF1-*ntrBC* mRNA was found to be more abundant in an *ntrC*$^-$ strain as compared to the wild type [5], thus suggesting that the NtrC protein is involved either in the stability of the mRNA or in autogenous regulation of transcription initiation. In this paper we show that DNA binding proteins from an *ntrC*$^+$ but not from an *ntrC*$^-$ strain inhibit in vitro transcription (Fig. 4) and form only one specific complex (C_{II}) with a 156 bp fragment containing the ORF1-*ntrBC* promoter (Fig. 3A). In this DNA region only one NtrC binding site overlapping t_1 was identified by sequence analysis (Fig. 1). Since the NtrC protein from KNO_3-grown bacteria is about 10-fold more efficient in binding the promoter than that from NH_4Cl-grown bacteria (Fig. 3B), even if it is about 3-fold less abundant, it is clear that the activity of the NtrC protein is regulated (possibly through phosphorylation) in a way related to the bacterial growth conditions. Thus, while a post-translation modification would probably be lost during purification, the extract used here keeps the NtrC protein in its active form. This experiment shows that the ORF1-*ntrBC* promoter of *R. etli* is subject to negative autoregulation by the NtrC protein. It also shows that the non-specific complex indicated as C_I does not contain NtrC but it requires it for its presence or binding activity and thus it is related to regulation of nitrogen metabolism. Furthermore, competition experiments with specific oligonucleotides show that their inhibitory effect is due to binding within a very short region of the promoter sequence. Oligonucleotide competition of in vitro transcription activity may be a powerful tool to identify the DNA sequences recognized by the *R. etli* RNA polymerase holoenzyme of this as well as of other promoters.

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