Transcriptional Specificity of RpoN1 and RpoN2 Involves Differential Recognition of the Promoter Sequences and Specific Interaction with the Cognate Activator Proteins

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The four RpoN factors of Rhodobacter sphaeroides are functionally specialized. In this bacterium, RpoN1 and RpoN2 are specifically required for the transcription of the nitrogen fixation and flagellar genes, respectively. Analysis of the promoter sequences recognized by each of these RpoN proteins revealed some significant differences. To investigate the functional relevance of these differences, the flagellar promoter fliOp was sequentially mutagenized to resemble the nitrogen fixation promoter nifUp. Our results indicate that the promoter sequences recognized by these sigma factors have diverged enough so that particular positions of the promoter sequence are differentially recognized. In this regard, we demonstrate that the identity of the −11-position is critical for promoter discrimination by RpoN1 and RpoN2. Accordingly, purified RpoN proteins with a deletion of Region I, which has been involved in the recognition of the −11-position, did not show differential binding of fliOp and nifUp promoters. Substitution of the flagellar enhancer region located upstream fliOp by the enhancer region of nifUp allowed us to demonstrate that RpoN1 and RpoN2 interact specifically with their respective activator protein. In conclusion, two different molecular mechanisms underlie the transcriptional specialization of these sigma factors.

In eubacteria, the sigma subunit of the RNA polymerase is responsible for recognizing the promoter sequence to initiate transcription (1, 2). A large number of sigma factors have been described in eubacteria (1, 3, 4). In general, all of them can be grouped in two different families: the family of sigma-70 that includes σ70, σ32, σ24, σ5, and σ28 and the family of sigma-54, of which σ54 is the only member (5–8). The RNA polymerase core (E) associated with a sigma factor of the σ70 family is capable of binding to a promoter and initiating transcription without any ancillary factor. In contrast, Es54 holoenzyme is unable to form open complex in the absence of an activator protein (9–11). Es54 binds to highly conserved promoters with the consensus sequence TGGCAGN₅TTGCW, of which the most conserved positions are the GG and GC located at −24 and −12 nucleotides upstream from the transcription initiation site (12, 13). Mutation of the GG or GC dinucleotides in any σ54 promoter strongly reduces transcription, confirming its relevance in the transcriptional initiation process (14). The high degree of conservation of the σ54-dependent promoters in many bacteria allows the σ54 factor from Aquifex aeolicus to successfully recognize the σ54-dependent promoter glnH2p2 from E. coli (15).

As mentioned above, Es54 strictly requires the presence of an activator protein to carry out the transition from closed to open complex. Experimental evidence obtained in in vitro studies using heteroduplex DNA fragments containing a σ54 promoter sequence mimicking a fork junction suggests that the inability of Es54 holoenzyme to spontaneously melt DNA is based on the interaction between the N-terminal region of σ54 and a repulsive DNA fork formed transitorily by melted DNA at the −11- and −10-positions, which are adjacent to the GC dinucleotide. This interaction inhibits the ability of the holoenzyme to spread melting and keeps it inactive to initiate transcription. Therefore, it has been proposed that the bases in the −12 box have a regulatory role in the isomerization process (16–20).

Transcriptional activators of Es54 usually bind to sequence motifs located 100 bp upstream of the promoter sequence and for this reason are commonly known as enhancer-binding proteins (EBPs). These proteins belong to the family of AAA+ ATPases and couple the energy derived from ATP hydrolysis to remodel the nucleoprotein complex formed by the N terminus of σ54 bound to the DNA fork structure, relieving the inhibitory interactions and allowing open complex formation (21–23).

By sequence alignment, σ54 has been divided in three regions. Region I is located at the N terminus of the protein and contains determinants for nucleating DNA melting, for inhibiting open complex formation, and to mediate the response to the EBPs (24, 25). Recently, it has been established that Region I makes a major contribution to bind the fork junction structure located at the −11-position in heteroduplex probes (16–20). Region II is variable, and although its role in transcription is still unclear,

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2 The promoter numbering given in this work considers the dinucleotide GC as the −13- and −12-positions, respectively.

3 The abbreviations used are: EBP, enhancer-binding protein; WT, wild type; UAS, upstream activation site.
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TABLE 1
Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference/Source |
|-------------------|-------------------------|------------------|
| **R. sphaeroides** | Wild-type strain, Na' | Ref. 61 |
| WS8              | Wild-type strain, Na' | Ref. 61 |
| SP7              | Wild-type strain, Na' | Ref. 61 |
| SP13             | Wild-type strain, Na' | Ref. 61 |
| SP16             | Wild-type strain, Na' | Ref. 61 |
| SP17             | Wild-type strain, Na' | Ref. 61 |
| **E. coli**      | Wild-type strain, Na' | Ref. 61 |
| JM103            | Wild-type strain, Na' | Ref. 61 |
| S17-1            | Wild-type strain, Na' | Ref. 61 |
| LMG174           | Wild-type strain, Na' | Ref. 61 |
| SX8              | Wild-type strain, Na' | Ref. 61 |
| LMG174-1         | Wild-type strain, Na' | Ref. 61 |

Plasmids

| Plasmid | Description | Reference/Source |
|---------|-------------|------------------|
| pTZ19R  | Cloning vector, Ap'; pUC derivative | Fermentas |
| pJQ200mp18 | Vector used for gene replacement | Ref. 65 |
| pUC4K   | Source of the Kan cassette | Amersham Biosciences |
| pRK415  | pRK404 used for expression in R. sphaeroides | Ref. 34 |
| pBBMCS53| Transcriptionalai/udA fusion vector, Gm' | Ref. 67 |
| pBAD/HisC| Expression vector | Invitrogen |
| pBOp    | pBBMCS53 derivative, flOp-uidA gene fusion | Invitrogen |
| pRS210  | pRK415 carrying flc' | Ref. 34 |

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Plasmids, and Growth Conditions—Plasmids and strains of **R. sphaeroides** and **E. coli** used in this work are listed in Table 1. **R. sphaeroides** was grown in Sistrom’s minimal medium (36) or in minimal medium with malate (0.4%) and glutamate as sole carbon and nitrogen sources, respectively (MG medium) (34). For the N$^+$-aerobic growth conditions, a saturated culture was diluted 1:10 with fresh Sistrom’s medium in a 250-ml Erlenmayer flask. The cultures were incubated in the dark with strong shaking (200 rpm). To achieve nitrogen fixing conditions, **R. sphaeroides** was grown as described before (34). Briefly, a culture grown under heterotrophic conditions was collected when it reached an A$^{600}$ of 0.4. After two washes with minimal medium, the cells were suspended in the original volume of MG medium. This culture was used to completely fill screw cap tubes that were incubated under constant illumination for a period of 12 h. Antibiotics were used at the following concentrations: for **R. sphaeroides**, spectinomycin (50 $\mu$g/ml), kanamycin (25 $\mu$g/ml), tetracycline (1 $\mu$g/ml), and gentamycin (5 $\mu$g/ml); for **E. coli**, spectinomycin (100 $\mu$g/ml), kanamycin (50 $\mu$g/ml), tetracycline (15 $\mu$g/ml), gentamycin (30 $\mu$g/ml), and ampicillin (100 $\mu$g/ml). **E. coli** strains were grown in LB medium at 37 °C with shaking at 200 rpm.

Recombinant DNA Techniques—Plasmid DNA preparations were carried out with a minicolumn plasmid purification kit (Qiagen Inc., Valencia, CA). Restriction enzymes were used according to the recommendations from the manufacturer. Standard methods were used for transformation, ligation, and other related techniques. To isolate SP16 and SP17 strains, the wild-type nifA gene was obtained by PCR using total DNA from WS8 cells and the oligonucleotides nifA1 (5'-GCTTACAGCATCGCTGCCTCCCTCGTGCG-3') and nifA1 (5'-ACCGATTTACACCTCCACCAAGC-3'). These oligonucleotides were designed in accordance with the **R. sphaeroides** 2.4.1 genomic sequence. The PCR product was cloned in pTZ19R, generating pRS310. From this plasmid, most of the coding region of nifA...
was deleted by inverse PCR, using the oligonucleotides nifAreV1 (5'-GGTACCCCGAGGTCCGCTGTC-3') and nifAreV3 (5'-GGTACCCCGAGGTCCGCTGTC-3'), which included a KpnI restriction site. The PCR product was gel-purified and digested with KpnI. The resultant product was ligated with the Kan' cassette obtained from pUC4K. The fragment carrying the nifAΔ::Kan allele was then subcloned in pQ200mp18 and introduced to *R. sphaeroides* WS8 wild type or into SP8 strain (34). Allelic exchange was confirmed by Southern blot or PCR. The upstream region of *fliOp* wild type or that of the mutant versions of *fliOp* was substituted with the upstream region of *nifUp* following the next steps. First, the region upstream of *nifU* was amplified by PCR using chromosomal DNA from WS8 cells and the oligonucleotides nif3Eco 5'-GGAATTCGCTCCGGAGGGGCGGC-3' and nifBgl 5'-GGAATTCCACTGCAGGGAGTTCG-3'. The amplification product was gel-purified and digested with EcoRI and BglII. The plasmids carrying *fliOp* wild type and the mutant versions of *fliOp* were amplified by PCR using the oligonucleotides fliOBgl 5'-GGAATTCGCTCCGGAGGGGCGGC-3' and fliOP 5'-GAATTCACTGCAGGGAGTTGTCG-3'. The amplification product was gel-purified and digested with EcoRI and BglII. The plasmids carrying *fliOp* wild type and the mutant versions of *fliOp* were amplified by PCR using the oligonucleotides fliOBgl 5'-GGAATTCGCTCCGGAGGGGCGGC-3' and fliOP 5'-GAATTCACTGCAGGGAGTTGTCG-3'. The products were gel-purified and digested with EcoRI and BglII, and each of these products was ligated with the PCR product carrying the upstream region of *nifUp*. The presence of the insert as well as the correct mutation in *fliOp* was confirmed by DNA sequencing. Finally, the complete fragment was transferred into pBBRCS53, and the appropriate orientation was confirmed.

**Site-directed Mutagenesis**—Site-directed mutagenesis was performed according to the method of Kunkel (37) with a uracil-containing single-stranded DNA as template. The plasmid carrying the *fliOp* promoter consists of a 391-bp DNA fragment, cloned in pTZ19R. This DNA fragment carries 254 bp upstream of the transcriptional start site (38). The oligonucleotides used for mutagenesis were as follows: 5'-GTCCTGACACATCTGTGCGC-3', 5'-CAACATCCGTGCCGTTTACAGGC-3', and 5'-GTCCCTCTCCGACGACATCTGTGCGC-3'. The presence of the desired mutation in the resultant plasmid was confirmed by sequencing. Finally, the fragments carrying the wild-type promoter or its derivatives were subcloned into pBBRCS53 in the appropriate orientation.

**Conjugation**—Plasmid DNA was mobilized into *R. sphaeroides* by conjugation according to previously reported procedures (39).

**β-Glucuronidase Assay**—β-Glucuronidase was determined from sonicated cell-free extracts using 4-methylumbelliferyl-β-D-glucuronide as substrate and following a previously reported protocol (40). Briefly, cell-free extracts were incubated at 37 °C in reaction buffer. Samples of 100 μl were taken at three different time points and mixed with 0.9 ml of stop buffer (0.2 M Na₂CO₃). Fluorometric determinations were made in a PerkinElmer Life Sciences LA-5 apparatus (excitation wavelength, 360 nm; emission wavelength, 446 nm). The fluorometer was calibrated using 4-methylumbelliferone standards. Specific activities are expressed as nmol of 4-methylumbelliferone formed/min/mg of protein.

**DNA and Proteins**—Synthetic PAGE-purified oligonucleotides, corresponding to the −59 to +22 sequence of the *fliOp* promoter (38) or the −58 to +23 sequence of the predicted *nifUp* promoter were used to construct the heteroduplex molecules used in this work (see Fig. 4). DNA probes were prepared as follows. The top strand was labeled with [γ-³²P]ATP and mixed with the complementary strand. The mixture containing 4 pmol of ³²P-end-labeled DNA and 6 pmol of complementary strand in 20 mM Tris-HCl, pH 7.5, and 80 mM NaCl was boiled briefly and cooled slowly to room temperature. The resulting annealed probes were then diluted in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 80 mM NaCl.

To obtain RpoN1 and RpoN2 proteins, the coding regions of *rpoN1* and *rpoN2* were amplified by PCR and cloned into pBADHis-C vector. The resultant plasmids were introduced to the LMG174-1 strain, and different growth temperatures and arabinose concentrations were tested in order to determine the optimal condition to overproduce these polypeptides. Both proteins remained in the insoluble fraction in all tested conditions, and for this reason, the proteins were purified from the inclusion bodies. The procedure for protein purification is based on previously reported methods (41) with minor modifications. Briefly, after 3 h of induction at 30 °C with 0.02% arabinose, the cells were lysed by sonication at 4 °C. The insoluble fraction was extracted with TGED buffer containing 1 mM NaCl and 1 mM phenylmethylsulfonyl fluoride or a mixture of protease inhibitors manufactured by Roche Applied Science, and a second extraction with 1% Triton X-100 was carried out. Detergent was removed by washing three times with TGED. Finally, the pellet was carefully resuspended in 8 M urea buffer with TGED. The protein was dialyzed overnight at 4 °C against TGED buffer containing 250 mM NaCl, followed by a further 6-h dialysis with fresh TGED buffer containing 100 mM NaCl. Insoluble material was removed by centrifugation. The oligonucleotides 5'-AGATCTGGTGCCCCGGAGGGGGCGC-3' and 5'-AGATCTGGTGCCCCGGAGGGGGCGC-3' were used as forward primers to obtain the PCR product coding for RpoN1ΔI and RpoN2ΔI. These products were cloned into pBAD/His-C and introduced into LMG174-1 strain. To obtain the polypeptides corresponding to Region I of RpoN1 and RpoN2, the DNA region encoding the first 57 residues of these proteins was amplified by PCR using as reverse primers the 57Nif or 57Fli oligonucleotide (5'-AGATCTGGTGCCCCGGAGGGGGCGC-3' and 5'-AGATCTGGTGCCCCGGAGGGGGCGC-3'). The presence of the desired mutation in the resultant plasmid was confirmed by sequencing. Finally, the fragments carrying the wild-type promoter or its derivatives were subcloned into pBBRCS53 in the appropriate orientation.

**Circular Dichroism**—Previous to CD analysis, the proteins were dialyzed against 10 mM sodium phosphate buffer, pH 8.0, 10 mM NaCl, and 20% glycerol, with three changes of the dialysis buffer, each of 100 times the sample volume. Dialysis was carried out over a period of 24 h at 4 °C. Molar ellipticity values were obtained using an Aviv Biomedical CD spectrophotometer model 202-01. The resulting spectra were analyzed using CDPro (available on the World Wide Web at lamar.colostate.edu/~sreeam/CDPro/main.html).

**Gel Mobility Shift Assay**—Binding reactions were carried out at 30 °C in STA buffer (24) in a total volume of 20 μl. The reactions included 1 μg of poly(dI-dC) as nonspecific competet...
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**Figure 1. Logo Sequences of the \( \alpha^{54} \)-Dependent Promoters from R. sphaeroides.** The promoter sequences were obtained from the analysis of the complete genome of *R. sphaeroides* (59) (available on the World Wide Web at mmg.uth.tmc.edu/sphaeroides/). The nif consensus includes six sequences located upstream of genes involved in nitrogen fixation (nifB, nifH, nifE, orf-nifU, nifA, and fprA). The fli consensus includes eight promoters located upstream of genes involved in flagellar biogenesis (fleT, fleK, fleO, fliA, orf-figA, figB, figG, and motA). The initiation site for four of the fli promoters has been identified previously (35, 38). The logo was created using WebLogo (available on the World Wide Web at weblogo.berkeley.edu/) (60). The promoter numbering given in this work is taking the dinucleotide GC as the −13- and −12 positions, respectively.

Promoter versions with the reporter gene *uidA* (encoding \( \beta \)-glucuronidase) were constructed using pBBMCS53. The amount of \( \beta \)-glucuronidase expressed from these plasmids was determined for WS8 cells grown under N\(^+\)-aerobic conditions, which would maintain a low level of RpoN1 (43). Compared with the level of \( \beta \)-glucuronidase produced by the wild-type *fliOp* promoter, a 5-fold reduction was observed when changes were introduced at positions −28, −27, and −26, and a 9-fold reduction was observed when changes were introduced at positions −15 and −16. When these changeswere combined in a single construction, the promoter activity was reduced by only 5-fold (Fig. 2). It is noteworthy that when the wild-type A at the −11-position was substituted by any other nucleotide, a reduction of 50–100-fold in the promoter activity was observed, suggesting a major contribution of this particular nucleotide in the activity of the *fliOp* promoter. For simplicity, the changes at −28, −27, and −26 will be referred to hereafter as up-24, and the changes at −16 and −15 will be referred to as up-12.

All previous constructions were introduced into the SP7/pRS210 strain, which carries a mutation in *rpoN2* but properly expresses the *fleT* and *fleQ* genes encoding the flagellar EBPs (35). As shown in Fig. 2, in this strain, the activities of all of these promoters were strongly reduced, indicating that RpoN2 was responsible for the activity detected in WS8 cells. As mentioned above, the expression of RpoN1 would be at a low level under the growth conditions used in the previous experiments (43). Therefore, we correctly evaluate if RpoN1 could promote transcription from these promoters, we cultured these strains under nitrogen-fixing conditions, as described under “Experimental Procedures.” As an induction control, the plasmid carrying the fusion *nifUp-uidA* was included in the assay. All of the *fliOp* promoter versions tested in the WS8 strain showed a similar amount of \( \beta \)-glucuronidase activity to that observed under N\(^+\) -aerobic conditions (Fig. 3), indicating that these promoters are not significantly expressed by RpoN1. A slight increase in the activity level could be detected in SP7/pRS210 cells grown under nitrogen-fixing conditions, when the *fliOp* promoter more closely resembled *nifUp* (Fig. 3D), although the \( \beta \)-glucuronidase activity is only 5% of that observed for *nifUp* under the same condition. From these results, we hypothesized that the RpoN1 holoenzyme was either not able to efficiently recognize any of these promoters or that the flagellar EBP bound to the enhancer region (UAS) was not able to remodel the regulatory center created by the RpoN1 holoenzyme and the promoter. To investigate these possibilities, we replaced the enhancer region.
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FIGURE 2. Activities promoted by the wild-type and mutant versions of fliOp. Specific $\beta$-glucuronidase activities were determined from different strains carrying the mutant versions or the wild-type fliOp promoter, fused to uidA. The conserved dinucleotides GG and GC located in the $-24$ and $-12$ promoter boxes are shaded. The asterisk indicates the $-11$-position. The sequence of the nifUp promoter is shown for comparison. The nucleotides mutagenized to resemble nifUp promoter are underlined in each case. Values are the mean of at least three independent culture determinations that showed <20% variation. The activities are expressed as nmol of 4-methylumbelliferone/min/mg of protein. The changes made at positions $-28$, $-27$, $-26$ or at positions $-16$ and $-15$ are referred as up-24 and up-12, respectively.

FIGURE 3. Activity of the wild-type and mutant versions of fliOp under nitrogen-fixing conditions. Relative $\beta$-glucuronidase activities determined from cells grown in nitrogen-fixing or N$^-$-aerobic conditions. Values are given as a percentage of the activity promoted by fliOp in WS8 cells grown in N$^-$-aerobic conditions (466 nmol of 4-methylumbelliferone/min/mg of protein). Values are the mean of at least three independent culture determinations that showed <20% variation.

The fliOp-Nif transcriptional fusion in the SP7/pRS210 cells grown under N$^-$-aerobic conditions expressed a very low amount of $\beta$-glucuronidase (Fig. 4C) (~0.1% as compared with that detected for nifUp in nitrogen-fixing conditions), confirming the notion that in this strain RpoN1 and NifA are responsible for the activity detected under nitrogen-fixing conditions. In contrast, in WS8 cells grown under N$^-$-aerobic conditions, only fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12) showed a moderate activity level (Fig. 4A); given that no activity was detected from any of these promoters in SP7/pRS210 cells in this growth condition (Fig. 4C), we conclude that RpoN2 is responsible for the activity detected in WS8 strain grown in N$^-$-aerobic conditions. Since the enhancer region upstream of the fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12) should not be recognized by FleQ/FleT, we infer that RpoN2 is activated by the flagellar EBP from solution in these promoters. Additional evidence obtained in our laboratory supports the idea that RpoN2 can be activated from solution by the flagellar activator. $^4$ An alternative explanation would be that the flagellar EBPs are nonspecifically binding to the nifUp enhancer region. Under nitrogen-fixing conditions, in WS8 cells (Fig. 4B), the accumulation of RpoN1 and NifA allows the expression of fliOp-Nif (up-24, up-12, and $-11$T) and nifUp, whereas fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12) show activity values similar to those observed under N$^+$-aerobic conditions (Fig. 4,

$^4$ S. Poggio, A. Osorio, G. Dreyfus, and L. Camarena, unpublished results.
compare A and B), further supporting the idea that these last two promoters are mainly transcribed by RpoN2 and probably activated from solution by the flagellar EBPs. Activation from solution has previously been reported to occur for some EBPs (44) and, although less efficiently, when the activator is overexpressed from solution by the flagellar EBPs. Activation from two promoters are mainly transcribed by RpoN2 and probably (NifA, respectively. To clearly determine the preference of RpoN2 by the wild-type and mutant versions of fliOp-Nif, relative β-glucuronidase activities determined from cells grown in nitrogen-fixing or N⁺-aerobic conditions. Values are given as percentage of the activity promoted by fliOp in SP17 cells grown in N⁺-aerobic conditions (569.8 nmol of 4-methylumbelliferone/min/mg of protein). Values are the mean of at least three independent culture determinations that showed <20% variation.

As mentioned previously, based on the fact that fliOp (up-24, up-12, and −11T) was efficiently transcribed only when it carried the enhancer region recognized by NifA, we hypothesized that the EBPs are also specific for a particular RpoN. To further test this idea, we introduced the plasmids carrying the fliOp-Nif promoters into SP13 and SP16 strains, which have a lesion preventing the expression of the flagellar EBP, FleQ (and hence also of FleT), and of the nitrogen fixation EBP NifA, respectively.

All of the promoters were practically inactive in SP13 strain (fleQ mutant) grown under N⁺-aerobic conditions (Fig. 4E), confirming that the activity observed in the WS8 strain grown under the same conditions was dependent on RpoN2 and FleQ/FleT. In nitrogen-fixing conditions, only the promoters recognized by RpoN2 showed a high level of reporter gene activity (Fig. 4F), although RpoN2 is present and must be bound to the fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12) promoters. This result implies that NifA is not able to activate RpoN2 efficiently.

The inability of FleQ/FleT to activate RpoN1 has been previously observed, since fliOp (up-24, up-12, and −11T), which is efficiently recognized by RpoN1, promoted a very high level of activity only when the UAS Fli was present (compare Fig. 4, B, D, and F with Fig. 3, B and D). Moreover, expression of RpoN1 from a plasmid did not support transcription from fliOp (up-24, up-12, and −11T) with the UAS Fli in the WS8 strain (data not shown). As expected, in the SP16 strain, only the promoters recognized by RpoN2 (i.e. fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12)) are active (Fig. 4, G and H), whereas the fliOp-Nif (up-24, up-12, and −11T) and nifUp promoters did not show significant activity, indicating that, in contrast to what occurs with RpoN2, FleQ/FleT are unable to activate RpoN1 from solution.

The idea that fliOp-Nif (WT) and fliOp-Nif (up-24 and up-12) are activated from solution by FleQ/FleT is confirmed by the lack of activity of these promoters in the SP13 strain, whereas in SP16 these promoters show a level of activity similar to that in the WS8 strain.

To clearly determine the preference of RpoN2 by the wild-type and mutant versions of fliOp, we introduced these constructions into SP17 (carrying a mutation in nifA and rpoN1); in this strain, RpoN2 should be activated only by the flagellar EBP and would not compete with RpoN1 for promoter binding. As shown in Fig. 5, fliOp (−11T) and fliOp (up-24, up-12, and −11T) showed 54 Transcriptional Specificity
(up-24 and up-12) showed a 5-fold reduction regarding the activity promoted by fliOp. These results are in agreement with those observed in WS8 cells under N\textsuperscript{−}\textsuperscript{-}-aerobic conditions (Fig. 3A).

Although the GG and GC nucleotides are central for promoter recognition by the σ\textsuperscript{54} factors (21, 38), our results allow us to conclude that additional information in the promoter sequence contributes to the observed specificity of the RpoN proteins of \textit{R. sphaeroides}. In particular, RpoN2 shows a strong dependence on the identity of the −11-position and a minor dependence on the identity of the nucleotides located upstream of the −24- and −12-positions. In contrast, RpoN1 depends equally on the bases adjacent to the −24- and −12-positions and on the identity of the −11-position, although the −11-position seems to be by itself more relevant than any of the −24- and −12-positions independently. Transcription by the proper RpoN protein is further assured by the strict interaction of the activator proteins with their cognate sigma factor.

**Purification of the RpoN1 and RpoN2 Proteins**—To better understand the molecular mechanism of promoter discrimination by RpoN1 and RpoN2, we decided to study the in vitro properties of these proteins. For this purpose, we purified RpoN1 and RpoN2 fused to a His\textsubscript{6} tag. The coding region of each gene without the initiator methionine was cloned into pBADHisC plasmid producing a fusion of six residues of histidine at the N terminus of each protein. Both constructions were introduced in an \textit{E. coli} strain carrying a lesion in \textit{rpoN} to avoid any contamination with endogenous RpoN protein. RpoN1 and RpoN2 were induced, adding arabinose to exponentially growing cultures; both proteins remained in the insoluble fraction of the cell extract, even when the amount of arabinose was reduced (data not shown). The proteins were obtained from inclusion bodies and refolded as described previously for some RpoN mutant versions from \textit{Klebsiella pneumoniae} (41). The same procedure was followed to obtain RpoN proteins lacking Region I. The purity of all of the proteins was verified by SDS-PAGE (supplemental Fig. 1).

**Binding of RpoN1, RpoN2, RpoN1ΔI, and RpoN2ΔI to Early Melted Probes**—We were not able to observe binding of RpoN1 and RpoN2 to fliOp as a homoduplex in electrophoretic mobility shift assays (data not shown). A similar situation has been reported for RpoN from enteric bacteria, where, depending on the promoter, RpoN binds with a low affinity or does not bind at all (48–50). From this, we conclude that RpoN1 and RpoN2 are not particularly deficient in the binding of homoduplex DNA promoters.

To analyze binding to early melted promoters, we initially used two different probes corresponding to the sequence of fliOp and nifUp. In both cases, the −11- and −10-positions of the nontemplate strand were changed to simulate the early melted state of these promoters (Fig. 6). It should be noticed that this would mean changing the bases that until now have been implicated in the specificity of the RpoN proteins. However, as previously mentioned, it has been shown that in the closed complex, RpoN binds tightly to the −11- and −10-positions of the template strand, exposed transitorily before activation (16, 17), which in these probes remain unchanged. However, according to the nomenclature for the identification of sequences in promoters, we refer to these bases by their identity at the nontemplate strand.

RpoN1 bound to nifUp early melted probe in a concentration-dependent manner. In contrast, RpoN2 did not bind significantly to it (Fig. 7A). The reverse was true using the early melted fliOp probe (i.e. RpoN2 generated a clear retarded complex, whereas RpoN1 did not) (Fig. 7B).

Since Region I of RpoN has been involved in the recognition of the −11-position, we evaluated if this region was involved in the differential binding. For this, we tested our probes with both RpoN proteins lacking Region I. As shown in Fig. 8, A and B, RpoN1ΔI was able to bind to both probes to a comparable extent, suggesting that Region I of RpoN1 inhibits binding to fliOp. The retarded complex formed by this protein with both probes showed a faster migration than that formed with RpoN1.
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wild type or between RpoN2ΔI and the fliOp probe (compare Fig. 8B, lanes 3 and 7), suggesting that the fast migrating complex formed by RpoN1ΔI can be ascribed to a different conformation of the protein. Supporting this idea, we observed that RpoN1 forms a fast migration complex when it interacts with the Nif-Fli probe (see below; Fig. 10A). In contrast, RpoN2ΔI was barely able to bind to fliOp, and no binding was detected with nifUp (Fig. 8, A and B). These results suggest that binding of RpoN2 is strongly dependent on the interactions made by Region I.

When RpoN1 and RpoN1ΔI competed for binding to the nifUp probe, the complex formed by RpoN1ΔI could only be detected by reducing the amount of wild-type RpoN1. This result indicates that Region I of RpoN1 also has a positive role in the binding of its specific probe nifUp (Fig. 9).

It has been reported that $\sigma^{54}\Delta I$ from K. pneumoniae does not bind efficiently to early melted DNA; however, binding is restored when Region I is added in the assay, indicating that Region I is able to complement binding of RpoNΔI in trans (19). The addition of the N-terminal region of RpoN1 or RpoN2 did not produce any change in the migration of the complexes previously detected, and no interaction could be detected between the N-terminal peptides and the fliOp or nifUp probes. Since the N-terminal peptides were purified from inclusion bodies, we measured their far-UV CD spectra to verify that they had folded appropriately. Both polypeptides had considerable amounts of secondary structure (data not shown). Nevertheless, in the absence of the solved structure of the RpoN protein, the possibility remains that the structure adopted by these proteins does not correspond to that found in the native protein.

FIGURE 8. Binding of heteroduplex probes by RpoN1ΔI or RpoN2ΔI. The probes used in the gel mobility shift assay are indicated above each panel. RpoN1 and RpoN2 were included as controls. The amount of protein used in each reaction is as indicated in the legend to Fig. 7. The numbers above each lane represent the percentage of bound probe, calculated as the relation between total radioactivity in each lane and the radioactivity of the bound complexes. nd, not detected.

FIGURE 9. Binding competition between RpoN1 and RpoN1ΔI. Gel mobility shift assay showing the complexes formed by RpoN1 and RpoN1ΔI with the nifUp heteroduplex probe. The amount of protein used in the reaction is as indicated in the legend to Fig. 7. The numbers above each lane represent the percentage of bound probe, calculated as the relation between total radioactivity in each lane and the radioactivity of the bound complexes.

The binding of RpoN1 and RpoN2 to Early Melted Probes with Different Sequences in the Mismatch Region—To determine if the absence of binding of the RpoN proteins to their non-specific probes was related to the identity of the bases at the unpaired region, we tested the Nif-Fli and Fli-Nif probes depicted in Fig. 6. In these probes, the sequence at the homoduplex region corresponds to the fliOp or nifUp, but the $-11$ and $-10$-positions were switched between these promoters. For this reason, the templates were called Fli-Nif and Nif-Fli.

Our first observation was that all of the proteins tested were less able to interact with these probes (Fig. 10), suggesting that the proper combination of promoter sequence and the identity of the bases at the fork structure is important for binding of RpoN1 and RpoN2. Nevertheless, we could still detect the interaction of RpoN1 or RpoN2 with these probes. Interestingly, binding of RpoN1 to the Nif-Fli probe yielded two complexes (Fig. 10A); one of them showed a similar migration to the complex formed with RpoN1ΔI in the nifUp template (fast migration complex), whereas the other showed the usual migration of the complex formed by RpoN1 and the nifUp probe. The presence of the fast migration complex could indicate a defective interaction of Region I of RpoN1 with the $fli$ bases at the fork. RpoN1ΔI also binds to the Nif-Fli probe, but it only forms the fast migration complex (Fig. 10A). Remarkably,
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RpoN2 was able to form a complex with the Nif-Fli probe (Fig. 10A); since RpoN2 did not bind nifLp probe (see Fig. 7), this result supports the notion that recognition of the $-11$ and $-10$ nucleotides in the unpaired region makes an important contribution to the binding of RpoN2. As observed previously with the fliOp and nifLp probes, RpoN2ΔI also binds poorly to the Nif-Fli and Fli-Nif probes, regardless of using a higher protein concentration (Fig. 10, A and B). As shown in Fig. 10B, neither RpoN1 nor RpoN2 bound efficiently to the Fli-Nif probe.

**DISCUSSION**

The RpoN proteins of *R. sphaeroides* are the first $\sigma^{54}$ factors described that show specificity to transcribe a particular set of genes. In this work, we present evidence suggesting that two different mechanisms bring about the transcriptional specificity shown by these sigma factors. One involves the differential recognition of the flagellar and nitrogen fixation promoters by RpoN1 and RpoN2, whereas the other acts at some point in the interaction between the sigma factor and its cognate activator protein.

A closer inspection of the $\sigma^{54}$ promoters revealed differences between the fli and nif promoters, which consisted in an extended consensus nif promoter and a marked differential preference at the $-11$-position (T for the nif and A for the fli promoters). The relevance of these variations for RpoN2-dependent transcription was tested *in vivo* by site-directed mutagenesis of the fliOp promoter. From these experiments, it was observed that substitution of the $-11$-position exerted the strongest negative effect, whereas substitution of the $-28$, $-27$, and $-26$-positions or $-16$- and $-15$-positions reduced RpoN2-dependent transcription less severely. The contribution of the bases upstream of the $-24$ and $-12$ boxes to the binding of RpoN2 was expected, since variations around the conserved dinucleotides GG and GC have been reported to affect RpoN-dependent transcription in a moderate degree (13, 51). In contrast, the remarkable dependence on the identity of the $-11$-position was surprising, since changes at this position were previously reported to affect RpoN-dependent transcription between 10 and 50%, depending on the identity of the substitution (51). In our case, the identity of the $-11$-position seems to be critical to allow RpoN2-dependent transcription, since any base different from A reduced the fliOp activity in more than 90% (Fig. 2).

To detect RpoN1-dependent transcription from the different fliOp promoter versions, it was necessary to substitute the upstream region with the one that is recognized by NifA. From these chimeric promoters, transcription dependent on RpoN1 and NifA was observed under nitrogen fixation conditions. The strongest activity (~90% of that promoted by nifLp) was detected for the promoter carrying all of the substitutions (i.e. fliOp-Nif (up-24, up-12, and $-11$T), whereas only 10% of activity was detected for fliOp-Nif (up-24 and up-12) and fliOp-Nif (−11T), indicating that the identity of the $-11$-position has a strong influence on the activity promoted by RpoN1, but, in contrast to RpoN2, the nucleotides upstream of the $-12$ and $-24$-positions are together equally relevant. Interestingly, all nif promoters carry a T at the $-11$-position, whereas none of the flagellar promoters has this nucleotide at this position (Fig. 1). Therefore, the inference made from the logo sequences suggesting that RpoN1 recognizes an extended promoter sequence in comparison with that recognized by RpoN2 seems to be confirmed.

The second conclusion obtained from these results is that the flagellar EBP (FleQ/FleT) interacts specifically with RpoN2 to achieve transcription and that the same occurs between NifA and RpoN1. Consequently, the specific interaction between these sigma factors and its cognate activator protein is a second and equally effective mechanism that favors transcriptional specificity. It is interesting that two mechanisms that by themselves would be enough to achieve transcriptional specificity have evolved simultaneously. It is tempting to propose that, given the close functional relationship between the region I of RpoN with both the $-11$ promoter position and the activator protein, changes in either one of these interactions would affect the other, forcing them to evolve in conjunction.

It was recently reported that the identity of the $-11$-position in the fork structure is relevant, since, depending on it, either negative or positive interactions will be established with Region I of RpoN, which are then modulated by the EBP to achieve transcriptional control (52). In this regard, it could be hypothesized that the specificity of RpoN1 and RpoN2 is in part accomplished by using these interactions differentially.

Consistent with this idea, the gel mobility shift assays showed a strong preference of RpoN2 to bind the early melted fliOp probe, whereas RpoN1 was able to bind only the nifLp probe. In the absence of Region I, both sigma factors interact less efficiently with all of the probes, but whereas RpoN1ΔI gained the ability to bind the fliOp probe, indicating an inhibitory effect of Region I on the binding of this probe, RpoN2ΔI did not bind to nifLp and bound poorly to fliOp, suggesting that the strongest binding contacts of this sigma factor are made by Region I. These results are in agreement with the hypothesis of a differential interaction of RpoN1 and RpoN2 with the sequence in the fork, which is mediated by Region I.

In a competition experiment using the nifLp probe,
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RpoN1ΔI was completely displaced by RpoN1, indicating that Region I of this protein, besides inhibiting binding to the early melted $fliOp$ probe, contributes with positive interactions with its specific probe. This property would allow the binding to be favored or destabilized, depending on the identity of the −11-position.

In the case of RpoN2, Region I appears to be only a positive binding element, since RpoN2ΔI did not bind efficiently even to its specific probe. This situation is similar to that reported for $\alpha^{54}$I from K. pneumoniae, which does not bind efficiently to some sequence variants of early melted probes (19, 52).

Although we tested if Region I from any of the two RpoN proteins was capable of binding and discriminating between the $fli$ and $nif$ promoters, none of the polypeptides could bind to these probes by itself nor affect binding of the complete or truncated proteins. However, it remains to be analyzed if the purified Regions I of these two proteins are folded in the proper conformation, since, in contrast to the situation found in K. pneumoniae, these polypeptides were obtained from inclusion bodies.

We further analyzed the importance of the bases at the −11- and −10-positions and of the rest of the promoter in the binding of RpoN1 and RpoN2 by changing the nucleotides of the fork in the $fliOp$ probe by those in the $nifUp$ probe and vice versa. The fact that RpoN2 was able to bind to the $nifUp$ probe with the $fli$ fork (Nif-Fli probe), whereas RpoN2 could not bind $nifUp$, brings additional support to the idea that binding of RpoN2 strongly depends on interactions with specific nucleotides at the −11-position. In contrast, RpoN1 could bind to the Nif-Fli probe regardless of the presence of the $fli$ −11, −10 fork. This result suggests that RpoN1 is able to bind this probe through its interaction with the promoter bases upstream of the −11-position (in agreement with the results obtained with RpoN1ΔI); however, the absence of the positive interaction with the $nif$ fork and the negative effect exerted by the $fli$ fork results in weak binding as compared with the binding of RpoN1 to the $nifUp$ probe and in the generation of a migration complex similar to the one produced by RpoN1ΔI (compare Figs. 7A and 10A). In contrast, both proteins bind very poorly to the Fli-Nif probe, producing almost no retarded complex (Fig. 10B). This result can be explained by the requirement of RpoN2 for the particular sequence at the $fli$ fork to bind stably, whereas RpoN1 requires making contact with the promoter sequence besides the sequence in the fork to form a stable complex.

We have also shown that each EBP is specific for the activation of a particular DNA-RpoN regulatory center; this differentiation could occur at any of the steps of the activation process. In agreement with this idea, it has been observed that the EBP, PspF, in agreement with this idea, it has been observed that the EBP, PspF, could not activate transcription from the $glnHp2$ promoter using the $\alpha^{54}$I factor from A. aeolicus, but an EBP from this bacterium was able to do it, suggesting that this $\alpha^{54}$I and its activator have coevolved (15).

The molecular interactions between the EBPs and RpoN proteins have been difficult to characterize, since the EBPs do not bind tightly to RpoN; however, in the simplest model, a first interaction between RpoN and the EBP takes place transitorily and independent of NTP hydrolysis. Subsequently, during hydrolysis, the central region of the EBP adopts a different conformation exposing the GAFTGA motif that interacts with Region I of RpoN. This step is central to bringing about an open complex formation. In this context, it will be interesting to determine if Region I is responsible for restricting the interaction to its cognate EBP in one of the steps of the activation process (53–57).

Promoter discrimination mediated by RpoN1 and RpoN2 seems to parallel the situation observed for the $\alpha^{70}$ and $\alpha^{5}$ factors, since $\alpha^{70}$ recognizes a particular subset of genes whose promoter sequence is practically identical to that recognized by $\alpha^{70}$. The resemblance of these promoters is such that in vitro a $\alpha^{5}$-dependent promoter can be transcribed by $\alpha^{70}$ and vice versa. Recently, it was reported that a C at the −13-position of the promoter sequence contributes importantly to $\alpha^{5}$-dependent transcription. However, in the absence of −13 C, selectivity can be generated by other sequence elements such as an A/T-rich region downstream of the −10 box, a distal UP-element combined with a well-conserved −35 box, etc. (for a recent review, see Ref. 58). In the case of RpoN1 and RpoN2, promoter discrimination must also have other sequence elements besides the −11-position, since at least two flagellar promoters (motA and fleT) have a C at this position. In these cases, other nucleotides could compensate for the absence of an A at the −11-position.

In conclusion, we propose that the specificity of the RpoN sigma factors of R. sphaeroides is accomplished by two mechanisms: (i) differential recognition of the promoter sequence involving relevant interactions between Region I of RpoN and the fork structure and (ii) the specific interaction of NifA with RpoN1 and of FleT/FleQ with RpoN2.

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REFERENCES

1. Burgess, R. R., and Travers, A. A. (1970) FASEB J. 29, 1164–1169
2. Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969) Nature 221, 43–46
3. Mittenhuber, G. (2002) J. Mol. Microbiol. Biotechnol. 4, 77–91
4. Wosten, M. M. S. M. (1998) FEMS Microbiol. Rev. 22, 127–150
5. Helmann, J. D., and Chamberlin, M. J. (1988) Annu. Rev. Biochem. 57, 839–872
6. Ishihama, A. (2000) Annu. Rev. Microbiol. 45, 499–518
7. Gross, C. A., Lonetto, M., and Losick, R. (1992) in Transcription Regulation (McKnight, S. L., and Yamamoto, K. R., eds) pp. 129–176, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Merrick, M. J. (1993) Mol. Microbiol. 10, 903–909
9. Popham, D. L., Szeto, D., Keener, J., and Kustu, S. (1989) Science 243, 629–635
10. Sasse-Dwight, S., and Gralla, J. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8934–8938
11. Morett, E., and Buck, M. (1989) J. Mol. Biol. 210, 65–77
12. Beynon, J., Cannon, M., Buchanan-Wollaston, V., and Cannon, F. (1983) Cell 34, 665–671
13. Barrios, H., Valderrama, B., and Morett, E. (1999) Nucleic Acids Res. 27, 4305–4313
14. Merrick, M., and Chambers, S. (1992) J. Bacteriol. 174, 7221–7226
15. Studholme, D. J., Wigneshweraraj, S. R., Gallegos, M. T., and Buck, M. (2000) J. Bacteriol. 182, 1616–1623
16. Guo, Y., Wang, L., and Gralla, J. D. (1999) EMBO J. 18, 3736–3745
17. Guo, Y., Lew, C. M., and Gralla, J. D. (2000) Genes Dev. 14, 2242–2255
