

COMMUNICATION

Suppression Analysis of Positive Control Mutants of NifA Reveals Two Overlapping Promoters for *Klebsiella pneumoniae* *rpoN*

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Activation of gene expression relies on direct molecular interactions between the RNA polymerase and transcription factors. Eubacterial enhancer-binding proteins (EBPs) activate transcription by binding to distant sites and, simultaneously, contacting the σ^{54} -holoenzyme form of the RNA polymerase ($E\sigma^{54}$). The interaction between the EBP and $E\sigma^{54}$ is transient, such that it has been difficult to be studied biochemically. Therefore, the details of this molecular recognition event are not known. Genetic and physical evidences suggest that the highly conserved C3 region in the activation domain of the EBP has major determinants for positive control and for the interaction with $E\sigma^{54}$. To further investigate the target of this region we searched for extragenic suppressors of some C3 region mutant derivatives of NifA. As a first step we mutagenized *Klebsiella pneumoniae rpoN*, the gene that codes for σ^{54} . A mutant allele, *rpoN1320*, that suppressed two different NifA derivatives was obtained. Immunodetection of σ^{54} and transcriptional initiation studies demonstrated that the cause of the suppression was an enhanced expression of *rpoN*. A single point mutation was responsible for the phenotype. It mapped at the -10 region of an unidentified promoter, here denominated *rpoNp1*, and increased its similarity to the consensus. A second upstream promoter, denominated *rpoNp2*, was also identified. Its -10 region partially overlaps with the -35 region of *rpoNp1*. Interestingly, the promoter-up -10 mutation in *rpoNp1* caused a reduction in the expression from *rpoNp2*, likely reflecting a stronger occupancy of the former promoter by the RNA polymerase at the expense of the latter. The presence of two overlapping promoters competing for the RNA polymerase implies a complex regulatory pattern that needs elucidation. The fact that increasing the concentration of σ^{54} in the cell can suppress positive control mutants of NifA adds further evidence for their direct interaction in the activation process.

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Introduction

Activation of transcription in eubacteria results from the recruitment of the RNA polymerase holoenzyme to a promoter and/or by increasing the rate of the subsequent steps until the polymerase is engaged in elongation (reviewed by Ptashne

& Gann, 1997). The function of transcription factors is to stabilize, by a direct and productive interaction with the RNA polymerase holoenzyme, one or more of the intermediate states (Roy *et al.*, 1998). RNA polymerases are complex enzymes composed of at least five subunits, with a stoichiometry of $\alpha 2\beta\beta'\sigma$ (von Hippel *et al.*, 1984). The well-characterized *Escherichia coli* housekeeping RNA polymerase holoenzyme ($E\sigma^{70}$) provides a paradigm for understanding how transcription factors interact with different subunits to activate transcription.

Abbreviations used: EBP, enhancer-binding protein.

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Depending on the class of transcription factor and type of promoter, direct contacts between the activator protein and each of the RNA polymerase subunits, including two different domains of the α subunit, have been detected (reviewed by Busby & Ebright, 1997; Heyduk *et al.*, 1996; Greiner *et al.*, 1996).

In contrast to $E\sigma^{70}$, activation of promoters recognized by the σ^{54} -containing holoenzyme ($E\sigma^{54}$) is independent of recruitment of the RNA polymerase to a promoter. In the absence of transcription factors $E\sigma^{54}$ binds to its cognate promoters and forms stable, but inactive, closed complexes (Buck & Cannon, 1992; Popham *et al.*, 1989). These complexes are only isomerized to the initiation competent open promoter complexes by direct interaction with activators of the enhancer-binding protein (EBP) family (Popham *et al.*, 1989; Merrick, 1993; Lee *et al.*, 1993). The conformational changes leading to transcription initiation are dependent on nucleoside triphosphate hydrolysis by the EBP itself (Weiss *et al.*, 1991; Berger *et al.*, 1994).

The interaction determinants of $E\sigma^{54}$ and the EBP are largely unknown. It has been suggested that the EBP contact the polymerase holoenzyme through σ^{54} (Morett & Segovia, 1993), and chemical protein cross-linking studies indicated that the activator lies in close proximity to σ^{54} and to the β subunit (Lee & Hoover, 1995). However, these studies are not sensitive enough to determine the actual regions involved in protein-protein interactions.

Mutation and deletion analyses have allowed functions to be assigned to different regions of σ^{54} . The central part of the protein is involved in RNA polymerase binding, while mutations in the COOH terminus specifically affect promoter-DNA interactions (Coppard & Merrick, 1991; Guo & Gralla, 1997; Cannon *et al.*, 1995). The first 50 highly conserved residues at the NH_2 -terminal part play a central role in the response to transcription factors, and some mutations that "bypass" the requirement of activator proteins to initiate transcription have been mapped to this region (Syed & Gralla, 1997). Thus, this region is the most likely target of the activator proteins.

The EBPs are modular regulators consisting of three different functional domains, where their central and COOH-terminal domains have a common origin for the whole family, whereas the NH_2 -terminal domain is specific for each functional subgroup (Morett & Segovia, 1993). Mutation and deletion analyses have demonstrated that the activation determinants are located within the conserved central domain while the DNA-binding determinants are located at the COOH-terminal domain (Huala *et al.*, 1992; Morett *et al.*, 1988; Huala & Ausubel, 1989).

Positive control mutants of the EBP NtrC, DctD and NifA have been isolated (North *et al.*, 1996; Wang & Hoover, 1997; González *et al.*, 1998). In all cases the mutations map at the C3 region of the

central domain. This region is highly conserved among the members of the EBP family (Morett & Segovia, 1993), and in a structural model of the central domain it has been proposed to be a surface-exposed loop (Osuna *et al.*, 1997). Thus, the C3 region fulfills the requirements for protein-protein interaction.

Here we describe the isolation of a *rpoN* mutant able to partially suppress the positive control phenotype of two different C3 region mutants of NifA by enhancing the intracellular concentration of σ^{54} . Analysis of this mutant uncovered the existence of two overlapping promoters for the expression of *rpoN* in *K. pneumoniae*.

Results and Discussion

In this work we followed a classical second-site or extragenic suppressor search strategy in an attempt to elucidate transient protein-protein interactions in the activation of $E\sigma^{54}$ by the EBP. This strategy is based on the premise that within a macromolecular assembly a reduced function caused by mutation in one member can be compensated by modification of a second member. This compensation can be allele-specific, by restoring critical contacts required for the complex assembly, thus revealing an intimate protein-protein interaction. Alternatively, non-allele-specific suppressors may compensate indirectly the original defect by enhancing the assembly efficiency or the overall stability of the complex (Hartman & Roth, 1973).

Isolation of a NifA positive control suppressor allele of *rpoN*

As a first step to identify the component(s) of the RNA polymerase holoenzyme that respond to the activator protein, we searched for alleles of σ^{54} able to suppress positive control mutants of NifA. We previously reported two NifA C3 region mutants, NifA^{E298D} and NifA^{T308S}, that are specifically affected in positive control (González *et al.*, 1998). The conservative nature of the amino acid substitutions and the residual activity they presented made them suitable candidates for the search of extragenic suppressors. Samples of chemically mutagenized pMM70 plasmid, carrying the *K. pneumoniae rpoN* gene, were electroporated into the *E. coli* $\Delta rpoN$ TH1 strain harbouring a *K. pneumoniae nifH-lacZ* fusion in plasmid pRT22 and either the NifA^{E298D} or the NifA^{T308S} mutant proteins (for strains and plasmids, see Table 1). Transformants were spread onto LB plates supplemented with the chromophore X-gal. Putative suppressors showing an enhanced blue color phenotype were isolated, subcultured under microaerobic conditions and their β -galactosidase activity was determined. Only one clone, isolated as partial suppressors of the NifA^{T308S} mutant, showed a consistent enhanced *nifH-lacZ* expression (Figure 1).

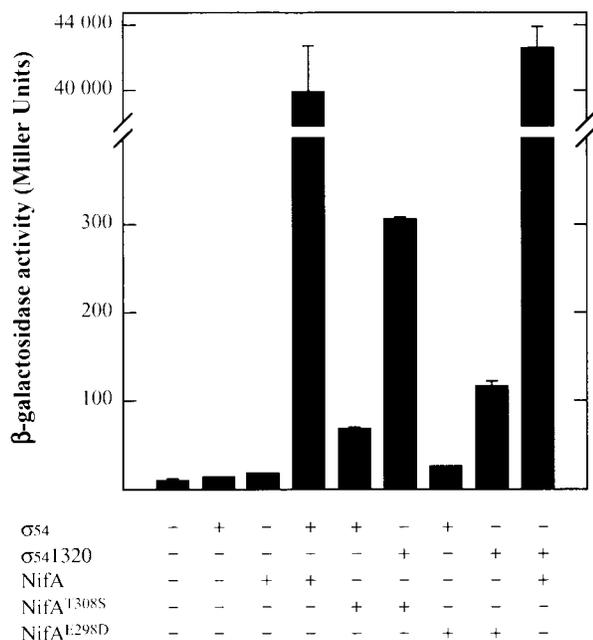


Figure 1. Transcriptional activity of the *rpoN* and *rpoN*1320 alleles. β -Galactosidase activity was determined in the TH1 strain harbouring the *K. pneumoniae* *nifH-lacZ* reporter plasmid pRT22 plus plasmids coding for the wild-type or mutant NifA proteins, as indicated. Values are the mean of three independent experiments with standard deviation shown. For the activation assays microaerobic cultures were inoculated with a 1:100 dilution of a saturated aerobic culture in 1.5 ml of fresh LB media plus glutamine and antibiotics in a 1.5 ml Eppendorf tube and shaken overnight at 30 °C. Samples of the microaerobic cultures were assayed for their β -galactosidase activity as reported (Miller, 1972). To generate the *K. pneumoniae* *rpoN* mutant library 1 μ g of plasmid pMM70 was treated with 100 mM hydroxylamine chloride in 100 mM phosphate buffer (pH 6.0) for 18 hours at 37 °C. After removing the hydroxylamine chloride by dialysis against 10 mM Tris-HCl (pH 7.5), the DNA was concentrated by precipitation, and samples of 50 ng were electroporated into strain TH1 harbouring plasmids pRT22 and pM1S or pM1D. Transformed cells were spread on LB plus antibiotics and X-gal. After overnight incubation at 30 °C, plates were stored at 4 °C until a blue colour developed. A library of about 50,000 colonies was screened for dark-blue colour.

To determine if the enhanced *nifH-lacZ* expression was due to a mutation in *rpoN*, plasmid pMM70 of clone 1320 (hereinafter called p1320) was segregated into strain JM109 and transformed back into the $\Delta rpoN$ strain TH1 harbouring the pRT22 plasmid and either the wild-type NifA, the NifA^{E298D} or the NifA^{T308S} proteins. As shown in Figure 1, activation of *nifH-lacZ* expression by the NifA^{E298D} and NifA^{T308S} proteins was increased four- and fivefold, respectively, in the presence of the p1320 plasmid, compared to the wild-type pMM70. Since both the NifA^{E298D} and NifA^{T308S} mutants were partially suppressed, the effect was not allele-specific. The wild-type NifA protein acti-

vated expression of the *nifH-lacZ* fusion at the same level with the p1320 as with the pMM70 plasmids, indicating that the mutation(s) in plasmid p1320 is/are not deleterious to the activity of the wild-type NifA (Figure 1).

Nucleotide sequence and product characterisation of plasmid p1320

In order to identify the mutation(s) responsible for the phenotype of plasmid p1320, the nucleotide sequence of the entire *rpoN* gene and 150 nucleotides of its regulatory region was determined. Only two G to A transitions were found, one was a silent substitution in the fifth codon of the protein, while the other was located 43 nucleotides upstream from the initiation codon (Figure 4(a), below). The location of the latter mutation suggested that a differential regulation of *rpoN* might be responsible for the phenotype. To assess this, we determined the relative concentration of σ^{54} protein in the cell by immunodetection. Clearly, the TH1/p1320 strain contained at least twice the amount of the σ^{54} protein than the TH1/pMM70 strain (Figure 2(a)).

The results shown above suggest that an increased concentration of σ^{54} can partially suppress the positive control phenotype of the NifA C3 region mutants. To evaluate the suppression potential of accumulated σ^{54} , we overexpressed the wild-type *rpoN* gene. Plasmid pMM38 harbours an *rpoN* gene cloned under the control of a *ptac* promoter. As shown in Figure 2(b), induction of *rpoN* in pMM38 by addition of 1 mM IPTG yielded approximately the same amount of σ^{54} than plasmid p1320. Accordingly, when induced, activation of the *nifH-lacZ* fusion by either the NifA^{E298D} or the NifA^{T308S} proteins reached similar levels than with the p1320 plasmid (Figure 2(c)).

In conclusion, the partial suppression of the positive control phenotype of the NifA mutants resulted from an increased amount of σ^{54} protein in the cell. This is likely caused by a transcriptional regulatory effect of the 1320 mutation. This observation is the first genetic evidence in support of a direct interaction between an EBP and σ^{54} .

Mapping of the *rpoN* transcriptional start site

Merrick and co-workers proposed the existence of a putative σ^{70} -dependent promoter from 69 to 100 nucleotides upstream of the translational initiation codon of the *K. pneumoniae* *rpoN* gene (Merrick & Gibbins, 1985). Mutation 1320 is located 26 nucleotides downstream from the putative promoter, far apart to directly influence promoter function. Thus, the mutation could alter the mRNA stability or another expression-enhancing mechanism at the transcriptional level. To test these hypotheses we carried out a comparative analysis of the mRNA levels and the transcriptional start sites of the wild-type and 1320 *rpoN* alleles.

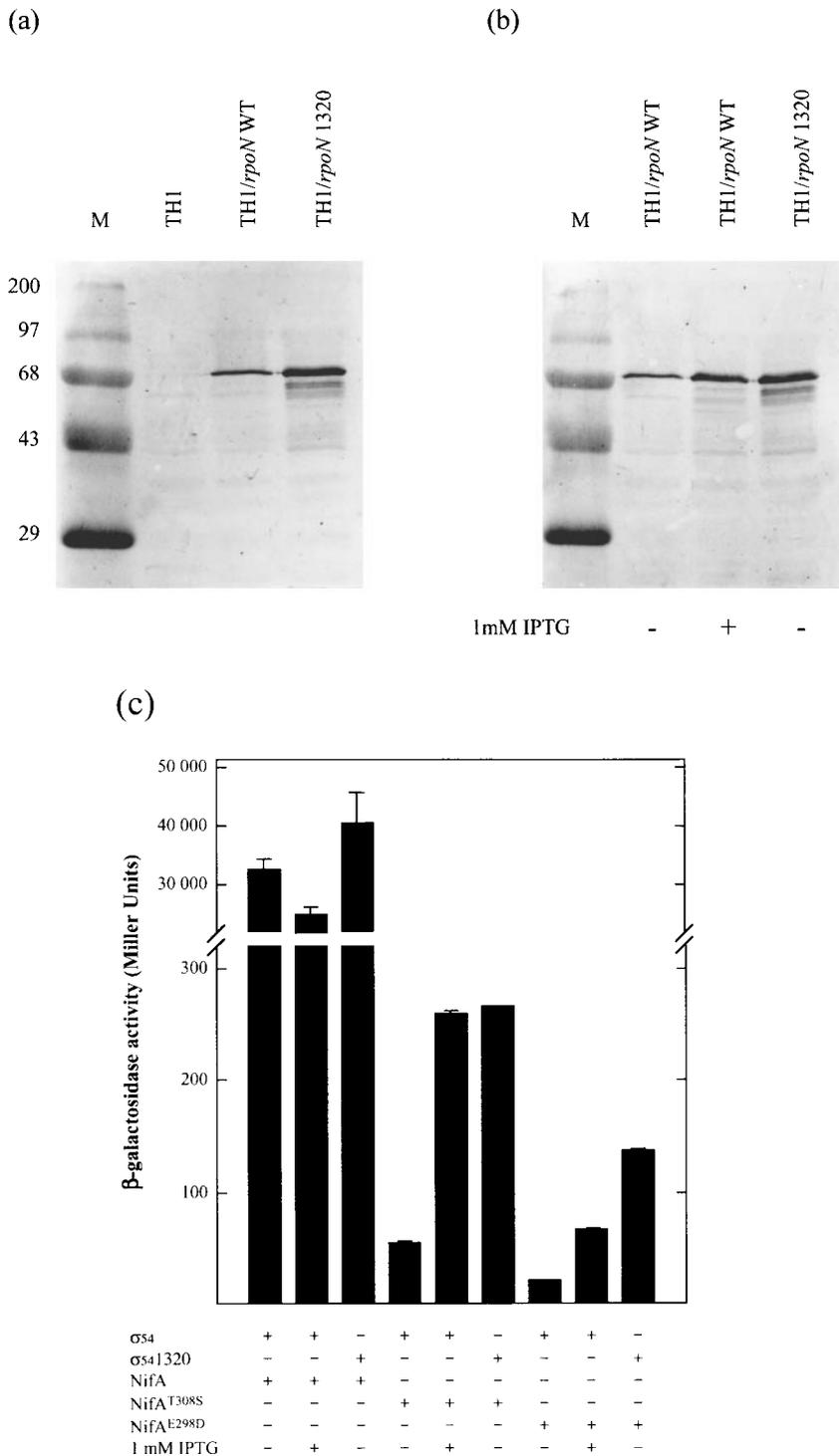


Figure 2. Immunodetection and activity of accumulated *K. pneumoniae* σ^{54} . (a) Lane 1, TH1 ($\Delta rpoN$); lane 2, TH1 harbouring pMM70 (*rpoN*); lane 3, TH1 harbouring p1320 (*rpoN1320*). (b) Immunodetection of induced wild-type *K. pneumoniae* σ^{54} . Lane 1, TH1 with uninduced pMM38 (σ^{54}); lane 2, TH1 with 1 mM IPTG induced pMM38; lane 3, TH1 with uninduced p1320. (c) Transcriptional activity of *rpoN*, *rpoN1320* and induced *rpoN* alleles. β -Galactosidase activity was measured as described for Figure 1. Samples of microaerobic cultures (0.5 ml) were concentrated by centrifugation, resuspended in 0.1 ml of distilled water, diluted with 0.1 ml of 2 \times SDS-loading buffer (González *et al.*, 1998) and denatured for ten minutes at 95 °C. Aliquots of 20 μ l were loaded on SDS-7.5% PAGE gels and blotted onto Hybond C membranes (Amersham Life Sciences). For the immunodetection of σ^{54} a specific anti- σ^{54} antibody (ab72), raised in rabbits by inoculation of pure $\Delta\sigma^{54}$ (residues 1-424), was used as described (Gonzalez *et al.*, 1998).

As shown in Figure 3 (lane 2), two different transcripts were detected by primer extension experiments from the wild-type *rpoN* gene. The first transcript started 62 nucleotides upstream from the translational start site, seven nucleotides downstream from the promoter element proposed by Merrick & Gibbins (1985). We named this promoter *rpoNp2*. The second transcript started at two contiguous positions 34 and 35 nucleotides upstream

from the translational start site. This transcript starts 27/28 nucleotides downstream from the first transcriptional start and we named this promoter *rpoNp1*. None of these transcripts were observed when total RNA from plasmid-less $\Delta rpoN$ strain TH1 was extended (Figure 3, lane 3). Hence, the transcriptional start mapping experiments revealed the existence of two promoters for the expression of *K. pneumoniae* *rpoN* in *E. coli*.

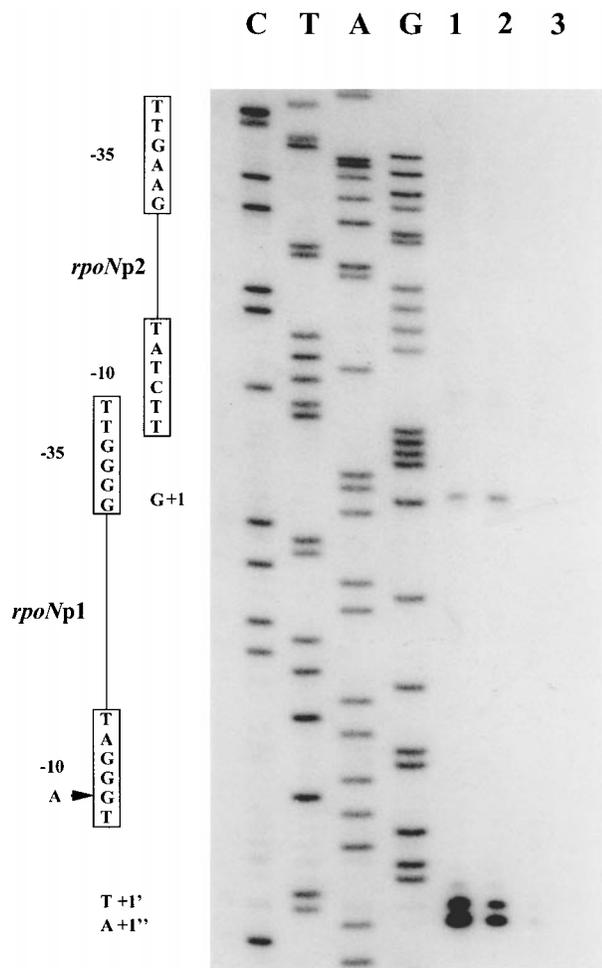


Figure 3. Transcriptional start site mapping of *rpoN* and *rpoN1320* mRNA. The DNA sequence ladder (from p1320) is shown in lanes C, T, A, and G. Oligonucleotide B2 (see below) was used for dideoxy-sequencing and for the primer extension analyses (lanes 1 to 3). Primer extension of total RNA isolated from TH1 harbouring p1320 (lane 1), pMM70 (lane 2) or without plasmids (lane 3). DNA sequence was performed manually by the dideoxy method using labelled [32 P]terminators and Thermosequenase following the suppliers instructions (Amersham Life Sciences). Total RNA was isolated from 5 ml of microaerobic cultures of strain TH1 harbouring different plasmids as described, using the DNA/RNA Isolation Kit from Amersham Life Sciences. Primer extension was performed using 32 P end-labelled oligonucleotide B2 (GGCTTAGCCTTAAGTGCAAACCTTGC) as described elsewhere (Barrios *et al.*, 1995). Reaction products were separated by PAGE and visualized on a Molecular Dynamics PhosphorImager. Quantification of the transcripts was carried out by using Molecular Dynamics ImageQuant software.

When total RNA from strain TH1 harbouring plasmid p1320 was extended, we observed the same transcripts as with the wild-type gene (Figure 3, lane 1). Interestingly, the level of expression from *rpoNp1* was significantly higher (60%, see the legend to Figure 3) with the *rpoN1320* allele than with the wild-type allele,

while expression from *rpoNp2* was slightly lower (24%). Thus, the intracellular accumulation of σ^{54} caused by the *rpoN1320* mutation was due to an overexpression from *rpoNp1*. It is relevant to notice that the G to A transition in the *rpoN1320* allele lies within *rpoNp1* (Figure 4(b)), increasing its similarity to the consensus -10 sequence (TATAAT) (Hawley & McClure, 1983; Kumar *et al.*, 1993). Thus, *rpoN1320* behaves as a typical promoter-up mutation.

Two overlapping promoters for *rpoN* in *Klebsiella pneumoniae*

The two *rpoN* promoters described here have low conservation, for *rpoNp2* only four positions at each of the -10 and -35 regions match the consensus promoter sequence (Figure 4(b)). For *rpoNp1* only three positions at each region match the consensus although this promoter presented the "extended -10 element", a TG dinucleotide upstream of the -10 hexamer (Figure 4(a)) (Keilty & Rosenberg, 1987). Likely, the presence of this extended element might contribute to the fourfold higher level of expression observed from this promoter compared to *rpoNp2* (Figure 3, lane 2). Promoter *rpoNp2* is comprised of sequences TTGAAG-N20-TATCTT (Figure 4(b)). The TT dinucleotide at the beginning of the -10 region of this promoter also forms part of the TTGGGG -35 hexanucleotide of *rpoNp1*. Thus, both promoters partially overlap each other.

The fact that the promoters share at least two residues implies that they must compete for the binding of the RNA polymerase. This may account for the reduced expression from *rpoNp2* observed with the *rpoN1320* allele (Figure 3, lane 1). We interpret this as a result of a stronger RNA polymerase occupancy of *rpoN1320p1*, at the expense of *rpoNp2*, caused by the 1320 mutation that increased the similarity of this promoter to the consensus. We have previously reported a similar competition effect for the overlapping *fixRnifA* promoters of *Bradyrhizobium japonicum* (Barrios *et al.*, 1995, 1998).

The nucleotide sequence of the upstream regulatory region of the *K. pneumoniae*, *Salmonella typhimurium*, *S. typhi* and *E. coli* *rpoN* genes is identical up to the -35 element of the *rpoNp1* promoter, after this position the sequence of *K. pneumoniae* starts to diverge (Figure 4(a)). Interestingly, *E. coli* and *S. typhi* do not have a recognizable *rpoNp2* -35 promoter region, thus, it is likely that this promoter only exists in *K. pneumoniae*. In support of this interpretation Kustu and collaborators reported a single transcriptional start site for *S. typhimurium* *rpoN* (Popham *et al.*, 1991). The proposed promoter corresponds to *K. pneumoniae* *rpoNp1* (Figure 4(a)).

Why *K. pneumoniae* has two promoters controlling the expression of *rpoN* while the other closely related enterobacterial species have only one? It is likely that the presence of the additional promoter

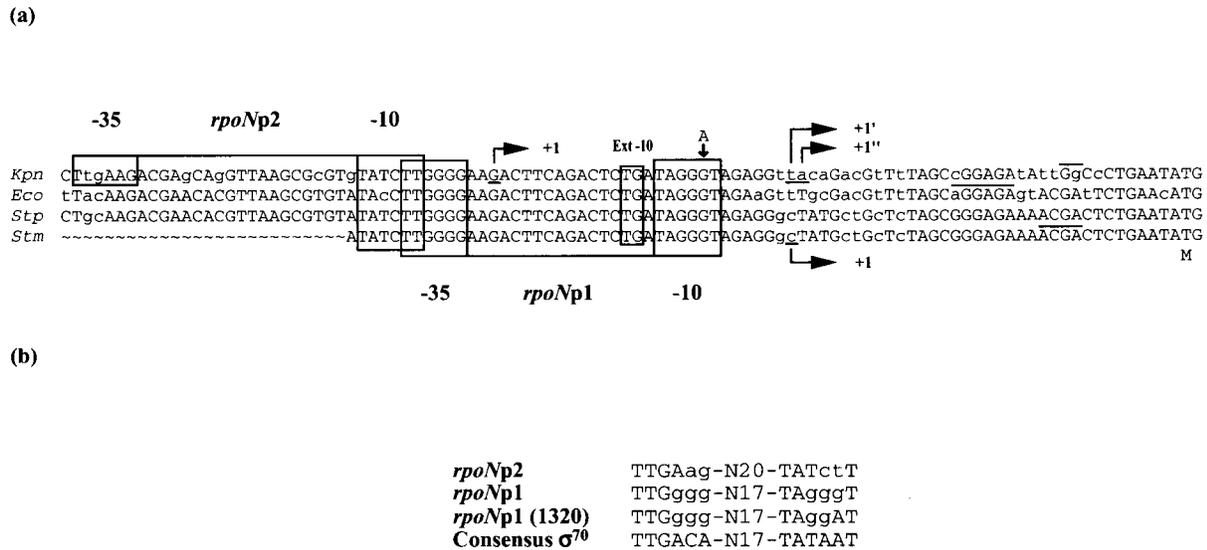


Figure 4. Comparison of the nucleotide sequence of *K. pneumoniae* (*Kpn*), *E. coli* (*Eco*), *S. typhi* (*Stp*) and *S. typhimurium* (*Stm*) *rpoN* promoter regions. (a) *rpoNp1* and *rpoNp2* promoters -35 , "extended -10 " and -10 elements are in open boxes. The start site for each *K. pneumoniae* *rpoN* transcript is marked with an arrow, being the longest transcript identified as $+1$ and the shorter transcript as $+1'$ and $+1''$. Mutation 1320 (G to A) is indicated within the *rpoN1320p1* promoter -10 element. Putative ribosome-binding sites are overlined. A single transcriptional start site has been reported for *S. typhimurium*, which coincides with transcript $+1'$ of *rpoNp1*, and is marked with an arrow as $+1$ (Popham *et al.*, 1991). This promoter has also been proposed for *E. coli* (Jones *et al.*, 1994). *rpoNp2* was previously proposed for *K. pneumoniae* (Merrick & Gibbins, 1985). (b) Comparison of the nucleotide sequences of *rpoNp2*, *rpoNp1*, and *rpoN1320p1* promoters with the consensus σ^{70} -dependent sequence (Hawley & McClure, 1983).

reflects distinct requirements of σ^{54} in the cell. The most striking physiological difference between, *E. coli*, *S. typhi*, *S. typhimurium* and *K. pneumoniae* is that the latter bacteria fixes nitrogen. For this process more than 20 gene products are required. The high level of expression of these genes, transcribed by $E\sigma^{54}$, certainly demands an enhanced amount of this holoenzyme. The presence of two different promoters may contribute to generate, in a tightly regulated fashion, the levels of $E\sigma^{54}$ required for nitrogen fixation. We are currently

investigating the possible differential regulation of *rpoN* expression under different growth conditions.

Conclusions

The experiments presented here show that increasing the amount of σ^{54} in the cell, by altering its expression either by promoter induction or by a promoter-up mutation, results in an enhanced ability of certain positive control mutants of NifA to

Table 1. Strains and plasmids

Strains	Relevant genotype and characteristics	References
A. <i>E. coli</i>		
JM109	F' <i>traD36 lacI^q Δ(lacZ)M15 proA⁺B⁺/e14⁻ (McrA⁻) Δ(lac-proAB) thi gyrA96 (Nal^r) endA1 hsdR17 (r_Km_K) relA1 SupE44 recA1</i>	Yanisch-Perron <i>et al.</i> (1985)
TH1	<i>thi endA hsr ΔlacU169 hutC_{klebs} rpoN</i>	T. Hunt
B. Plasmids		
pRT22	pACYC177 based <i>nifH-lacZ</i> translational fusion, Cm ^r	Tuli & Merrick (1988)
pRJ7511	pRJ7517 derived <i>pcat'-nifA</i> fusion of <i>B. japonicum</i> , Tc ^r	Fischer & Hennecke (1987)
PWKS130	Multipurpose low-copy number vector, Km ^r	Wang & Kushner (1991)
pM1-k	pWKS130 with <i>KpnI</i> site removed by filling in and <i>B. japonicum nifA</i> from pRJ7511 cloned as <i>BamHI-PstI</i> , Km ^r	This work
pM1D	pWKS130 derived with <i>B. japonicum nifA</i> ^{E298D} cloned as <i>BamHI-PstI</i> , Km ^r	This work
pM1S	pWKS130 derived with <i>B. japonicum nifA</i> ^{T308S} cloned as <i>BamHI-PstI</i> , Km ^r	This work
pMM38	pMM40 based <i>K. pneumoniae proN</i> expressed from <i>ptac</i> , Cb ^r	Austin <i>et al.</i> (1987)
pMM70	pTZ19R derivative with <i>K. pneumoniae rpoN</i> expressed from its own promoter	M. Merrick

Plasmids were maintained in strain JM109 and electroporated into strain TH1 for the expression assays. Strain TH1 was grown in Luria-Bertani (LB) medium plus 0.1 mM glutamine. Antibiotics were added when needed at the following final concentrations: 200 μ g/ml ampicillin (Am), 30 μ g/ml kanamycin (Km), 20 μ g/ml chloramphenicol (Cm) 15 μ g/ml tetracycline (Tc). X-gal was used at 20 μ g/ml.

activate transcription. We have previously proposed that these NifA mutants, which map in a region predicted to be surface-exposed (Osuna *et al.*, 1997), are most likely defective in interacting with σ^{54} (González *et al.*, 1998). A greater amount of σ^{54} in the cell likely increases the probability of interaction with the defective NifA proteins and, therefore, to form the macromolecular assembly required for gene expression. Although this observation favours a direct interaction between NifA and σ^{54} we cannot rule out that other critical contacts with the RNA polymerase can take place. Allele-specific suppressor mutants of the NifA^{T308S}, that mapped at the NH₂ terminus of σ^{54} , provided us with additional information in favour of a direct NifA- σ^{54} interaction (R.G., B.V. & E.M., unpublished results). Nevertheless, the search of the suppressor mutants reported here revealed the existence of a complex regulatory system consisting of two partially overlapping promoters for the expression of *rpoN* in *K. pneumoniae*.

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