A σ^{54} -dependent promoter in the regulatory region of the *Escherichia coli rpoH* gene

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The *Escherichia coli rpoH* gene is transcribed from four known and differently regulated promoters: P1, P3, P4 and P5. This study demonstrates that the conserved consensus sequence of the σ^{54} promoter in the regulatory region of the *rpoH* gene, described previously, is a functional promoter, P6. The evidence for this conclusion is: (i) the specific binding of the σ^{54} -RNAP holoenzyme to P6, (ii) the location of the transcription start site at the predicted position (C, 30 nt upstream of ATG) and (iii) the dependence of transcription on σ^{54} and on an ATP-dependent activator. Nitrogen starvation, heat shock, ethanol and CCCP treatment did not activate transcription from P6 under the conditions examined. Two activators of σ^{54} promoters, PspF and NtrC, were tested but neither of them acted specifically. Therefore, PspF Δ HTH, a derivative of PspF, devoid of DNA binding capability but retaining its ATPase activity, was used for transcription *in vitro*, taking advantage of the relaxed specificity of ATP-dependent activators acting in solution. In experiments *in vivo* overexpression of PspF Δ HTH from a plasmid was employed. Thus, the σ^{54} -dependent transcription capability of the P6 promoter was demonstrated both *in vivo* and *in vitro*, although the specific conditions inducing initiation of the transcription remain to be elucidated. The results clearly indicate that the closed σ^{54} -RNAP-promoter initiation complex was formed *in vitro* and *in vivo* and needed only an ATP-dependent activator to start transcription.

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INTRODUCTION

The *Escherichia coli* heat shock stimulon consists of regulons controlled by the σ^{32} (Arsène *et al.*, 2000; Zhao *et al.*, 2005) and σ^{24} RNA polymerase (RNAP) subunits (Erickson & Gross, 1989; Connolly *et al.*, 1999), the *rpoH* and *rpoE* gene products, respectively. A third regulon of the stress response has been identified from recent data. It is under the control of the σ^{54} -RNAP subunit (the *rpoN* gene product). The first σ^{54} -dependent heat shock operon, *pspA-E*, was described by Model's group (Brissette *et al.*, 1991; Model *et al.*, 1997). Later, it was found that the *ibpB* gene is transcribed not only from the σ^{32} promoter upstream of the *ibpAB* operon (Allen *et al.*, 1992), but also from the internal σ^{54} promoter situated in the *ibpA-ibpB* intergenic space (Kuczyńska-Wiśnik *et al.*, 2001). Moreover, a canonical sequence of a σ^{54} promoter, proximal to the *rpoH* coding sequence, was found by sequence analysis (Pallen, 1999). Information as to whether the sequence represents a functional promoter is currently lacking.

Transcription of the *rpoH* gene can be initiated from four previously described promoters (Fig. 1). Three of them, P1,

P4 and P5, are recognized by σ^{70} -RNAP (Erickson *et al.*, 1987; Nagai *et al.*, 1990; Ramirez-Santos *et al.*, 2001; Solis-Guzman *et al.*, 2001) and P3 is recognized by σ^{24} -RNAP (Erickson & Gross, 1989; Wang & Kaguni, 1989a; Missiakas & Raina, 1998). The P2 promoter was found only in one *E. coli* strain, SC122 (Erickson *et al.*, 1987), and was absent from MG1655. Transcription of the *rpoH* gene from these promoters is regulated by CRP-cAMP, CytR (Ramirez-Santos *et al.*, 2001; Kallipolitis & Valentin-Hansen, 1998) and DnaA (Wang & Kaguni, 1989b; Messer & Weigel, 1997) proteins.

If the potential σ^{54} promoter of the *rpoH* gene is functional, it should mean that stress responses encompass as yet unidentified processes. Functional σ^{54} promoters are rare in the *E. coli* genome: their number was estimated to be about 30, though sequences resembling canonical σ^{54} promoters are abundant (Reitzer & Schneider, 2001). The σ^{54} subunit is not essential for *E. coli* growth under laboratory conditions; however, it is necessary for expression of genes involved in diverse processes like nitrogen metabolism, transport of dicarboxylic acids, pilus formation, formate dehydrogenase synthesis, metabolism of aromatic compounds (xylene, toluene), and expression of the *pspA-E* operon and of the *ibpB* gene encoding a small heat shock protein.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazonate; EM, electron microscopy; EMSA, electrophoretic mobility shift assay; IHF, integration host factor; RNAP, RNA polymerase; wt, wild-type.

Fig. 1. Organization of the *rpoH* regulatory region. (a) The regulatory region of the *rpoH* gene [-524 to +183 bp relative to ATG (+1)]. The transcription start sites from the P1, P3, P4, P5 and P6 promoters are marked by vertical arrows. Binding sites for regulatory proteins CRP, CytR and DnaA are indicated. Horizontal arrows represent primers used in this study (listed in Table 1). The PCR products obtained with these primers are shown schematically under the figure. (b) DNA sequence of the *rpoH* regulatory region. The first base of the *rpoH* coding region is numbered +1. The IHF-binding site, the RBS sequence and the ATG codon are underlined. Vertical arrows represent transcription start sites from the P1 and P3–P6 promoters. The sequence of the P6 (σ^{54} -dependent) promoter is boxed. The putative binding sites for PspF (UAS II) and NtrC are overlined (continuous line and dotted lines respectively). These sequences are compared to the consensus sequences of PspF (Dworkin *et al.*, 1997) and NtrC (Weiss *et al.*, 1992) at the bottom of the figure.

E. coli cells contain seven different σ subunits (Arsène *et al.*, 2000; Wösten, 1998) that form two classes characterized by sequence homology and specificity of promoter recognition. Six of them belong to class I, represented by σ^{70} . The σ^{54} subunit is a unique member of class II (Merrick, 1993; Shingler, 1996; Wösten, 1998), sharing no homology with class I. The σ^{54} subunit recognizes the conserved sequence YTGGCACG-N₄-TTGCWNN of the promoters located at -12 and -24 bp from the transcription start site (Barrios *et al.*, 1999). Initiation of σ^{54} -RNAP-dependent transcription resembles eukaryotic transcription initiation by RNAP II. (Guo *et al.*, 1999, 2000; Fu *et al.*, 2000). A closed complex of $\sigma^{\text{class I}}$ -RNAP-promoter isomerizes spontaneously to the active, open complex. In contrast, isomerization of the σ^{54} -RNAP-promoter closed complex requires interaction with the specific enhancer-bound, ATP-dependent activator (reviewed by Merrick, 1993; Shingler, 1996). Contact between the activator and the σ^{54} -RNAP-promoter complex is achieved by DNA looping, facilitated either by the integration host factor (IHF) protein or by intrinsic DNA topology (Pérez-Martin *et al.*, 1994; Carmona *et al.*, 1997). Control, imposed on the DNA melting step, requires ATP hydrolysis and involves the promoter -12/-11 element (Guo *et al.*, 1999, 2000) bound inside the RNAP channel (Polyakov *et al.*, 1995; Zhang *et al.*, 1999; Severinov, 2000; Foster *et al.*, 2001).

Activators of σ^{54} -dependent promoters have a modular structure and share conserved sequence motifs with the AAA+ protein family (Shingler, 1996; Zhang *et al.*, 2002; Cannon *et al.*, 2003; Studholme & Dixon, 2003). The N-terminal domains of the majority of these activators act as specific sensors of inducing signals. NtrC may be regarded as a representative of this group. In response to nitrogen limitation, the ATPase activity of NtrC is positively regulated by phosphorylation of its N-terminal domain by the NtrB kinase and stimulated by DNA binding (Weiss *et al.*, 1991, 1992).

Members of another smaller, group of activators of σ^{54} -dependent promoters, which lack the N-terminal regulatory domain, include PspF of *E. coli* (Jovanovic *et al.*, 1996; Model *et al.*, 1997) and HrpR of *Pseudomonas syringae* (Grimm *et al.*, 1995). PspF, the activator of the *pspA-E* operon, occurs in cells at low concentrations and is constitutively active. Modulation of its activity, unlike that of NtrC, depends on protein regulators: PspA (negative) or PspB and PspC (positive) (Shingler, 1996; Elderkin *et al.*, 2002; Hankamer *et al.*, 2004). PspF responds to heat shock and ethanol treatment, but the strongest reaction is evoked by bacterial membrane impairments coincident with the dissipation of charge as by the gene IV protein of filamentous phages (e.g. f1, M13) or by carbonyl cyanide *m*-chlorophenylhydrazide (CCCP; Model *et al.*, 1997). The molecular mechanisms of signal reception and transduction in this system are not yet understood.

The purpose of this work was to establish whether the σ^{54} -RNAP holoenzyme binds the putative σ^{54} -dependent

promoter (P6) in the regulatory region of the *rpoH* gene, and whether it is able to initiate transcription from P6 *in vitro* and *in vivo*. We present evidence of the holoenzyme binding and initiation of transcription *in vitro* and *in vivo*, based on gratuitous activation by PspFAHTh. The identity of the specific activator and inducing conditions remain to be elucidated.

METHODS

Bacterial strains, plasmids, culture conditions, primers and DNA fragments. The *E. coli* strains, plasmids and primers used in this study are listed in Table 1. The bacteria were grown in Luria-Bertani (LB) medium at 30 °C (unless otherwise stated). When appropriate the following antibiotics were added: ampicillin (100 µg ml⁻¹), tetracycline (15 µg ml⁻¹), kanamycin (30 µg ml⁻¹) and chloramphenicol (34 µg ml⁻¹). Transformation of *E. coli* cells was performed according to Sambrook *et al.* (1989) by using the CaCl₂ procedure.

DNA fragments W1-W2, W8-W9, W2-W5 and W3-W7 (Fig. 1a) were amplified by PCR with the corresponding primers.

Proteins. The His-tagged proteins listed below were purified by Ni-affinity chromatography using the BioLogic LP chromatography system (Bio-Rad). His- σ^{54} and His-NtrC proteins were overproduced from strains BL21(DE3)(pS54-2) and BL21(DE3)(pNTRC-3), respectively and purified as described by Rippe *et al.* (1997, 1998). His-PspF and His-PspFAHTh were obtained by overproduction from SG13009(pREP,pMJ16) and K1527 (pMJ15) respectively and purified according to Jovanovic *et al.* (1999). The IHF protein was purified as described by Filutowicz *et al.* (1994) after overexpression from strain DH5 α (pHN β α). σ^{70} was overproduced from CF1690 Δ lac(pVI690) and purified as described by Fujita & Ishihama (1996). *E. coli* core RNAP was purchased from Epicentre Technologies. Reconstituted RNAP holoenzyme was obtained by incubating the core RNAP with an appropriate σ subunit at a 1:4 molar ratio for 15 min at 30 °C in STA buffer (25 mM Tris/acetate pH 8.0, 8 mM magnesium acetate, 10 mM KCl, 1 mM DTT, 3.5 % PEG). NtrC was phosphorylated to NtrC-P before use by the addition of carbamyl phosphate (10 mM) to the reaction mixture.

Electrophoretic mobility shift assays (EMSAs). The DNA fragments W1-W2, W2-W5 and W8-W9 were end-labelled with [γ -³²P]ATP (166.5 TBq mmol⁻¹) by using T4 polynucleotide kinase (Promega). DNA-protein binding was carried out in STA buffer supplemented with BSA (100 µg ml⁻¹) and poly[dI-dC] (30 µg ml⁻¹) for 20 min at 30 °C and was stopped by the addition of 2 µl loading dye (0.05 % bromophenol blue, 50 %, v/v, glycerol). The amounts of DNA and holoenzyme are given in the legends for Figs 2–5. Electrophoresis was performed in 4.5 % polyacrylamide gel in Tris/glycine buffer (25 mM Tris, 200 mM glycine) at 8 V cm⁻¹ for 2 h. The gels were dried and submitted to autoradiography or analysed with a Molecular Imager FX (Bio-Rad).

Electron microscopy (EM). Buffer A (50 mM Tris/acetate pH 8.0, 100 mM potassium acetate, 8 mM magnesium acetate, 1 mM DTT, 27 mM ammonium acetate, 3.5 % PEG 8000) was used for protein-DNA binding reactions (Su *et al.*, 1990). The W1-W2 (707 bp) DNA fragment containing the putative P6 promoter (200 nM) and σ^{54} -RNAP (35 nM) were incubated for 15 min at 30 °C in a final volume of 20 µl.

For visualization of PspF or NtrC-P binding the W1-W2 fragment was prepared by PCR with biotinylated W2 primer (W2_{biot}). W1-W2_{biot} (620 ng) was incubated with streptavidin (Promega) in a total volume

Table 1. *E. coli* strains, plasmids and primers used in this study

Characteristics*		Source or reference
Bacterial strains		
K-12 MG1655 (wt)	F ⁻ λ^- <i>ilvG rfb-50 rph-1</i>	Blattner <i>et al.</i> (1997)
K1527	HfrC λ^+ <i>relA1spoT1 T₂^r (omp627 fad701) lacI^q pspF::mTn10-tet (pspF₈₇₇); Tc^r</i>	Jovanovic <i>et al.</i> (1996)
SG13009	F ⁻ <i>his pyrD Δlon-100 rpsL</i>	Qiagen
ET8000 (wt)	<i>rbs lacZ::IS1 gyrA hurC_K</i>	MacNeil (1981)
ET8045	ET8000 <i>rpoN208::Tn10; Tc^r</i>	MacNeil (1981)
DH5 α	<i>supE44 ΔlacU169 (ϕ80 <i>lacZ</i> ΔM15) <i>hsdR1 recA1 endA1 gyrA96 thi1 relA1</i></i>	Hanahan (1983)
BL21[DE3]	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) dcm gal (DE3)</i>	Studier & Moffat (1986)
CF1693 Δ lac	<i>ΔrelA251::kan ΔspoT207::cm Δlac, (p)ppGpp⁻</i>	Sze <i>et al.</i> (2002)
Plasmids		
pS54-2	Ap ^r ; pET15b derivative overproducing His-tagged σ^{54}	Rippe <i>et al.</i> (1997)
pNTRC-3	Ap ^r ; pET15b derivative overproducing His-tagged NtrC	Rippe <i>et al.</i> (1998)
pVI690	Ap ^r ; <i>E. coli rpoD</i> (σ^{70}) expressed from λ P _R P _L	Laurie <i>et al.</i> (2003)
PVI688	Sp ^r ; <i>E. coli rpoN</i> gene expressed from <i>P_{tac}</i>	Laurie <i>et al.</i> (2003)
pMJ13	Ap ^r ; pQE-70 derivative overproducing PspFAH TH	Jovanovic <i>et al.</i> (1999)
pMJ15	Ap ^r ; pQE-32 derivative overproducing His-tagged PspFAH TH	Jovanovic <i>et al.</i> (1999)
pMJ16	Ap ^r ; pQE-32 derivative overproducing His-tagged PspF	Jovanovic <i>et al.</i> (1999)
pREP	Km ^r , expressing LacI repressor	Qiagen
pHN $\beta\alpha$	Ap ^r ; pHX3-8 derivative expressing <i>ihfA</i> and <i>ihfB</i> under control of the <i>tac</i> promoter	Granston & Nash (1993)
pTE103	Ap ^r ; contains multicloning site from pUC8 upstream of a bacteriophage T7 transcriptional terminator	Elliott & Geiduschek (1984)
pTE103-W1-W2	Ap ^r , pTE103 derivative with W1-W2 fragment cloned into <i>Bam</i> HI and <i>Eco</i> RI sites	This study
Primers		
	Sequence†	
W1	5'-atgaattccgacagatggattcatcctgcg-3'	This study
W2	5'-taggatccgcaggtgagacaggatcagcg-3'	This study
W3	5'-cgcggtgcaccctgaataaaaagcggtgtatactc-3'	This study
W5	5'-gcgctgcagcagtgatgataacctcgttg-3'	This study
W7	5'-cgtagctgcccggatgtaggaatccagg-3'	This study
W8	5'-gattggtgcgacagatggattcatcctgc-3'	This study
W9	5'-tcaggcgtaaatggcgtaaatgtgtaccg-3'	This study

*Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Sp, spectinomycin.

†The underlined sequences indicate restriction sites introduced by primers (W1, *Eco*RI; W2, *Bam*HI; W3, *Alw*44I; W5, *Pst*I).

of 40 μ l in TM buffer for 1 h at 37 °C. The excess of streptavidin was removed by filtration through Amicon Microcon-PCR (Milipore) filters. The PspF or NtrC-P proteins (500 nM) and the W1-W2_{biot} DNA fragment (200 nM) were incubated at 30 °C for 15 min in buffer A.

The reaction products were cross-linked by the addition of 0.2% glutaraldehyde, and, after incubation at room temperature for 15 min, diluted 1:20 with TM buffer. Preparations for electron microscopy were made by adsorption to mica (Spiess & Lurz, 1988), stained with uranyl acetate, platinum-carbon coated and analysed with a Philips CM100 electron microscope. The lengths of the DNA fragments and the positions of complexes were measured by using an electronic digitizer and evaluated with a computer program as described previously (Weigel *et al.*, 1997).

DNase I footprinting. Primers were end-labelled with [γ -³²P]ATP (166.5 TBq mmol⁻¹) and T4 polynucleotide kinase (Promega). DNA fragments were PCR amplified using ³²P end-labelled W7 primer and unlabelled W3 primer for σ^{54} , σ^{54} -RNAP and σ^{70} -RNAP footprints. ³²P end-labelled W9 primer and unlabelled W8 primer were used for IHF footprints. DNase I footprinting assays were performed as described by Leblanc & Moss (2001) in a total

volume of 10 μ l of STA buffer. The labelled DNA fragments at a final concentration of 10 nM were incubated, for 15 min at 30 °C, with proteins in quantities given in the figure descriptions. DNase I (5×10^{-4} units) was added and after incubation for 1 min at 37 °C the reaction was stopped by the addition of EDTA to a final concentration of 50 mM followed by heating for 2 min at 95 °C in formamide loading dye. The reaction products were separated on an 8% PAGE/urea gel. In parallel, the products of DNA sequencing reactions, performed using the *fmol* DNA Cycle Sequencing System (Promega), were separated. Gels were subjected to autoradiography or exposed to Kodak screen and analysed using the Molecular Imager FX (Bio-Rad).

In vitro transcription. Multiple-round transcription assays were performed essentially as described by Wigneshweraraj *et al.* (2003) with minor modifications. Briefly, the supercoiled DNA template pTE103-W1-W2 for *in vitro* transcription was prepared by cloning the W1-W2 (707 bp) DNA fragment digested with *Bam*HI and *Eco*RI into the pTE103 transcription vector, purified by centrifugation (at 2×10^5 g, for 16 h) through a caesium chloride/ethidium bromide gradient and verified by sequencing (Macrogen). Reactions were conducted in STA buffer supplemented with BSA (100 μ g ml⁻¹), in a total volume of 20 μ l. First, 1 μ g of template

Fig. 2. σ^{54} -RNAP binding to the *rpoH* promoter region. (a) EMSA. The γ - 32 P end-labelled 277 bp DNA fragment W2-W5 (8 nM) was incubated with increasing amounts of σ^{54} -RNAP, as indicated in the figure. The position of the DNA fragment and the shifted DNA- σ^{54} -RNAP complex are marked with arrows. (b) Electron microscopy: photomicrographs show the position of the σ^{54} -RNAP on the W1-W2 DNA fragment (707 bp). The conditions for holoenzyme binding to DNA were as described in Methods. σ^{54} -RNAP (200 nM) was incubated with a W1-W2 DNA fragment (35 nM) for 15 min at 30 °C. Photomicrographs were digitized to measure the position of the holoenzyme. (c) Statistical analysis of about 100 EM images showing σ^{54} -RNAP bound to the putative P6 promoter, within the W1-W2 DNA fragment [-524 to +183 bp relative to ATG (+1)]. The scheme below the graph represents the DNA fragment containing the putative P6 promoter.

DNA was incubated for 10 min at 37 °C with 100 nM σ^{54} -RNAP and each of the activators (PspF, PspFAHTh or NtrC-P) at concentrations indicated in the figure legends. Then ATP was added to the final concentration of 5 mM, and the reaction was continued for 5 min to allow for isomerization of the closed complex into an active, open complex. Transcription was initiated by the addition of ribonucleotide mixture (0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.06 mM UTP, 0.11–0.185 MBq [γ - 32 P]UTP) and incubation for 10 min at 37 °C. Then heparin (100 μ g ml $^{-1}$) was added and incubation was continued for 5 min. The reaction was stopped by the addition of a formamide loading dye. Samples were heated (2 min at 95 °C) and subjected to electrophoresis in 4.5 % PAGE/urea gel at 250 V in TBE buffer. Gels were subjected to autoradiography or exposed to Kodak screen and analysed using the Molecular Imager FX (Bio-Rad).

Primer extension analysis. Cells were grown in LB medium at 30 °C, to an OD $_{575}$ of 0.4 and harvested by centrifugation at 2×10^3 g for 10 min. Total RNA was isolated using the RNA Prep Plus kit (A & A Biotechnology). For PspFAHTh or σ^{54} overexpression from pMJ13 or pVI688, respectively, cultures at an OD $_{575}$ of 0.4 were induced by the addition of IPTG (1 mM) and incubation was continued for 0.5–1 h for protein synthesis. The W7 primer complementary to nucleotides +73 to +44 of the *rpoH* gene was 5'-end labelled using T4 polynucleotide kinase (Promega) and [γ - 32 P]ATP (166.5 TBq mmol $^{-1}$). RNA (50 μ g) was mixed with the labelled W7 primer (1 pmol) and heated to 85 °C for 20 min. Then M-MuLV reverse transcriptase buffer was added and the W7 primer was hybridized to the RNA at 62 °C for 1 h. Then, RevertAid H Minus M-MuLV reverse transcriptase (Fermentas), ribonuclease inhibitor (1 unit μ l $^{-1}$) and dNTPs (2.5 mM each) were added. Primer extension reactions were carried out at 42 °C for 1 h and stopped by adding loading dye containing formamide. DNA sequencing reactions were carried out with the same primer using the *fmoI* DNA Cycle Sequencing System (Promega). Reaction products were heated for 2 min at 95 °C and loaded on a 8 % PAGE/urea gel together with products of the DNA sequencing reactions carried out with the *fmoI* DNA Cycle Sequencing System. Gels were subjected to autoradiography or exposed to Kodak screen and analysed using a Molecular Imager FX (Bio-Rad).

RESULTS

Analysis of the promoter region of the *rpoH* gene

At the start of this work only the presence of canonical sequence for a σ^{54} promoter in the promoter region of the *rpoH* gene was known (Pallen, 1999), raising the question whether it might be an active promoter. Analysis of the upstream sequence of the *rpoH* gene by a BLAST search showed that the σ^{54} promoter was correctly situated according to the rules proposed by Reitzer & Schneider (2001) for active σ^{54} promoters. Moreover, an IHF-binding site homologous to the consensus sequence was found at the position -349 to -362 (Fig. 1b) (Craig & Nash, 1984; Goodrich *et al.*, 1990). The search for a possible enhancer was focused on sequences resembling those of PspF- and NtrC-binding sites, since PspF is the activator of the σ^{54} -dependent stress-induced *pspA–E* operon, and NtrC activates a few σ^{54} -dependent promoters. It seemed probable that one of these activators could participate in transcription of the *rpoH* gene encoding σ^{32} , the main

Fig. 3. DNase I footprints of σ^{54} -RNAP and σ^{70} -RNAP in the *rpoH* promoter region. (a) The γ - 32 P end-labelled DNA fragment W3-W7 was incubated with increasing quantities of σ^{70} -RNAP, σ^{54} or σ^{54} -RNAP. Lane 1, DNA control, no protein. Lanes 2–5, σ^{70} -RNAP: 100, 200, 500 and 700 nM, respectively. Lanes 6–9, σ^{54} : 500, 1000, 2000 and 4000 nM, respectively. Lanes 10–12, σ^{54} -RNAP: 100, 200 and 500 nM, respectively. Lane 13, control, core RNAP (100 nM). After a brief digestion with DNase I, the reaction products were subjected to electrophoresis alongside the products of the sequencing reactions (G, A, T, C) performed with the W7 primer. Regions protected by σ^{54} -RNAP and σ^{70} -RNAP are indicated with brackets. (b) The DNA sequence containing the overlapping P5 and P4 promoters and protected from DNase I digestion by σ^{70} -RNAP is shaded. The DNA sequence encompassing the putative P6 promoter (bold letters) protected by σ^{54} -RNAP from the DNase I digestion extends from –37 to –68 bp relative to the *rpoH* start codon (white box).

stress-response factor. Sequence analysis of the *rpoH* regulatory region indicated the presence of some imperfect NtrC- binding sites and PspF-binding sites spread over the whole region of interest (Fig. 1b).

σ^{54} -RNAP binding to the putative P6 promoter

σ^{54} -RNAP binding to P6 was tested by electrophoretic mobility-shift assay (EMSA), electron microscopy (EM) and DNase I footprinting. For EMSA the 277 bp DNA fragment containing the putative P6 promoter was prepared by PCR using primers W2 and W5 (Fig. 1a) and end-labelled with [γ - 32 P]ATP. We observed a DNA shift at a σ^{54} -RNAP : DNA molar ratio of 5 : 1 or higher (Fig. 2a), a preliminary indication of σ^{54} holoenzyme binding to this DNA region. EM gave strong evidence for specific binding of the holoenzyme to the putative σ^{54} promoter, P6 (Fig. 2b). The 707 bp DNA fragment W1-W2 (encompassing W2-W5) was used. Statistical analysis of approximately 100 EM images revealed that σ^{54} -RNAP binding was centred on the postulated promoter (Fig. 2c). The precise site of σ^{54} -RNAP binding was determined by DNase I footprinting (Fig. 3a). The W3-W7 (326 bp) γ - 32 P end-labelled DNA fragment was prepared by PCR, as detailed in Methods. In agreement with the EM results (Fig. 2b, c) σ^{54} -RNAP bound exactly at the

postulated P6 promoter, covering the sequence –37 to –68 (Fig. 3). As a reference, DNase I footprinting with σ^{70} -RNAP was performed. The protected region from –72 to –117 corresponded to the P5 and P4 promoters. The nucleotide sequence of the protected area is shown below the autoradiogram (Fig. 3b). There was no binding of the σ^{54} protein alone, which is observed only with σ^{54} promoters containing a stretch of four Ts immediately upstream of the conserved GC of the promoter sequence (Buck & Cannon, 1992). P6 contains a TTGTT sequence instead. A DNA fragment with no protein added (Fig. 3a, lane 1) and a DNA fragment incubated with core RNAP only (lane 13) were used as controls and showed negative results as expected.

IHF, NtrC and PspF binding upstream of the P6 promoter

Analysis of the regulatory region of the *rpoH* gene revealed the presence of a putative IHF-binding site, with remarkable homology to the consensus site (Craig & Nash, 1984; Goodrich *et al.*, 1990). To our knowledge this has never been detected before. To determine whether this site actually binds IHF, EMSA and DNase I footprinting were performed. EMSA demonstrated IHF binding to the 277 bp DNA fragment W8-W9. Binding occurred at a

Fig. 4. IHF binding to the *rpoH* gene regulatory region. (a) EMSA. The γ - 32 P end-labelled 277 bp DNA fragment W8-W9 (7.8 nM) was incubated with increasing quantities of IHF protein (as indicated in the figure). The positions of the W8-W9 DNA and the retarded DNA-IHF complex are indicated with arrows. (b) DNase I footprint. γ - 32 P end-labelled W8-W9 DNA was incubated with increasing amounts of IHF protein, as indicated. After brief digestion with DNase I, the reaction products were subjected to electrophoresis alongside the products of the sequencing reactions (G, A, T, C), performed with W9 primer. The region protected by IHF is bracketed. (c) The IHF-binding site. The consensus sequence of the IHF-binding site is shown in bold letters. The region protected from DNase I digestion is shaded. The footprint extends from -337 to -383, relative to the *rpoH* start codon.

molar ratio of IHF to DNA of 2:1 (Fig. 4a). DNase I footprinting showed IHF binding at the predicted site, i.e. at -337 to -383, corresponding to the extended IHF-binding site (Goodrich *et al.*, 1990) (Fig. 1 and Fig. 4b, c).

The search for NtrC- or PspF-binding sites by sequence analysis of the 707 bp W1-W2 DNA fragment resulted in a complicated picture of several imperfect binding sites for each of these proteins (Fig. 1b). Nevertheless, it seems possible that some of them act as enhancers, binding the activator protein.

EMSA, EM and footprinting experiments were used to check the possible binding of PspF and NtrC-P (NtrC phosphorylated *in vitro* by carbamyl phosphate) to the 707 bp DNA fragment W1-W2. A high molar ratio of either of the two proteins (200 nM PspF monomer or 250 nM NtrC-P monomer per 2 nM DNA) was needed to produce a mobility shift (Fig. 5a, d). These data suggested that both activators bind DNA nonspecifically. For EM the 707 bp DNA fragment was end-labelled with streptavidin (see Methods), which served as a reference point for measurements of the position of the nucleoprotein complex. It was

determined from the photomicrographs and by statistical analysis that both proteins, PspF and NtrC-P, bound all along the DNA fragment (Fig. 5b, c, e, f). Since the previously observed specific binding of the σ^{54} -RNAP detected by EM was limited to the narrow area of the P6 promoter, it was evident that neither of the activators bound specifically. Also, the results of NtrC-P and PspF DNase I footprinting were negative (data not shown). This evidence supports the hypothesis that PspF and NtrC-P are not the specific activators for the transcription from P6.

Transcription *in vitro* from the P6 promoter

The P6 promoter was tested for *in vitro* transcriptional activity in multiple-round transcription. The supercoiled template was prepared by cloning the W1-W2 DNA fragment containing P1, P3, P4, P5 and the putative P6 promoters in the pTE103 transcription vector (Elliott & Geiduschek, 1984). Each reaction mixture contained template DNA (pTE103-W1-W2), σ^{54} -RNAP and the activator protein at increasing concentration. Use was made of the relaxed specificity of activators on supercoiled templates (Dworkin *et al.*, 1998), and PspF, NtrC-P and

Fig. 5. PspF and NtrC-P binding to the *rpoH* regulatory region. (a, d) EMSA. The γ - ^{32}P end-labelled 707 bp DNA fragment W1-W2 (2 nM) was incubated with increasing amounts of PspF (a) or NtrC-P (d), as indicated. Arrows or brackets indicate the positions of the DNA fragment and the retarded DNA–protein complex. (b, e) Electron microscopy: photomicrographs show the binding of PspF (b) and NtrC-P (e) to the W1-W2 (707 bp) DNA fragment. The conditions for protein binding to DNA are described in Methods. The activator proteins (500 nM) were incubated with streptavidin end-labelled W1-W2_{biot} DNA (35 nM) for 15 min at 30 °C. Photomicrographs were digitized to measure the position of the activator proteins on the DNA fragment. (c, f) Statistical analysis of about 100 EM images showing PspF (c) and NtrC-P (f) bound within the 707 bp W1-W2 DNA fragment containing the *rpoH* regulatory region. The diagrams below the graphs represent the DNA fragment with the P6 promoter and putative PspF- (c) and NtrC- (f) binding sites indicated.

PspF Δ HTH were employed. Each of these activators caused the initiation of transcription (Fig. 6a, b, c). PspF and NtrC-P stimulated transcription at much lower protein levels (about 50 nM) than PspF Δ HTH (1 μM), perhaps because PspF Δ HTH not only does not bind DNA, but also has ATPase activity 30-fold lower than PspF (Jovanovic *et al.*, 1999). We concluded that σ^{54} -RNAP recognized the P6 promoter and formed a closed initiation complex capable of starting transcription *in vitro* provided that any of the ATP-dependent activators was present. Transcription *in vitro* (activated by PspF) was slightly inhibited by IHF (data not shown), which is consistent with the observation that IHF stimulates transcription only with a specific activator (Dworkin *et al.* 1998).

Identification of the transcription start site *in vivo*

In order to identify the 5' end of the *rpoH* transcript from the P6 promoter, primer extension reactions were performed with total RNA isolated from the wild-type (wt) strains: MG1655 or ET8000, and the *rpoN* derivative of ET8000, ET8045. These strains were transformed with two plasmids: pMJ13, overproducing PspF Δ HTH from the *pspF*₈₇₇ gene, inducible by IPTG, and pREP, providing the *lacI* repressor. The specific conditions that would induce transcription from P6 were not known; however, use was made of the observation of Jovanovic *et al.* (1996) that overproduction of PspF Δ HTH activated *pspA* operon expression even in the absence of inducing stimuli. P6

Fig. 6. *In vitro* transcription of the *rpoH* gene from the P6 promoter. Transcription from supercoiled DNA template pTE103-W1-W2 with σ^{54} -RNAP (100 nM). Reaction conditions were as described in Methods. The identified band is the ~500 nt *rpoH* transcript. Activator proteins PspF (a), PspF Δ HTH (b) and NtrC-P (c) were used at the concentrations indicated above each lane.

transcription appeared totally dependent on the overproduction of PspF Δ HTH from pMJ13 induced by IPTG (Fig. 7a, b). The 5' end of the P6 transcript was found at position -30, from the *rpoH* start codon (+1). There was rough proportionality between PspF Δ HTH concentration (dependent on the length of the induction time) and the level of the transcript (Fig. 7a). This transcription was abolished by the *rpoN* mutation (Fig. 7b, lane 9), though PspF Δ HTH was overproduced to the same level in the wt and mutant strains, as confirmed by SDS-PAGE (data not shown). Complementation of the *rpoN* mutation by plasmid pVI688 overproducing σ^{54} restored transcription from P6 (Fig. 7c), if PspF Δ HTH was overproduced simultaneously with the σ factor. These results indicate that the transcription from P6 is σ^{54} -dependent and that the σ^{54} -RNAP is able to direct transcription of the *rpoH* gene from the P6 promoter if an activator is supplied.

Testing factors that might induce transcription from P6

In an attempt to identify conditions that might affect the induction of transcription, heat shock (from 30 °C to 42 or

50 °C), 10% ethanol or CCCP treatments were applied, since these treatments induce transcription of the *pspA-E* operon by the PspF activator (Darwin, 2005). Also, induction of NtrC activity by nitrogen starvation was tested. None of these conditions activated the P6 promoter, as determined by primer extension. The results are consistent with neither PspF nor NtrC being a specific activator of this promoter.

DISCUSSION

We provide evidence that the putative σ^{54} -dependent P6 promoter in the regulatory region of the *rpoH* gene of *E. coli* is able to direct transcription. Binding of the σ^{54} -RNAP holoenzyme to the P6 promoter sequence was demonstrated by EMSA, electron microscopy and DNase I footprinting. The closed complex of the σ^{54} -RNAP and P6 promoter extended from -7 to -38 bp, relative to the transcription start site (-37 to -68 bp relative to the ATG). This is consistent with the size of the DNA area protected by closed complexes observed for other σ^{54} promoters (Buck & Cannon, 1992; Wang *et al.*, 1998). The closed complexes of these promoters require interaction with activators of the AAA+ protein type bound to remote enhancers. IHF is a frequent participant in the process. The presence of an IHF-binding site that corresponded to the consensus sequence (Craig & Nash, 1984; Goodrich *et al.*, 1990) and demonstration of binding to IHF, was consistent with previous findings regarding the structure and functioning of most of the σ^{54} promoter regulatory regions. The dependence of *in vitro* transcription on the AAA+ activators NtrC-P, PspF or PspF Δ HTH (Fig. 6) was shown. Also an analogous situation produced *in vivo* by overproduction of PspF Δ HTH, which does not bind DNA, from pMJ13 in *E. coli* cells resulted in activation of transcription from P6.

The location of the transcriptional start site 30 nucleotides upstream of the *rpoH* coding sequence by primer extension experiments (Fig. 7a, b, c) was in agreement with previous prediction (31 nt upstream according to Pallen, 1999). The start occurred in the statistically determined area for starts from the σ^{54} promoters, i.e. between 8 and 21 nt downstream of the promoter's conserved GC (Barrios *et al.*, 1999). The *rpoN* mutation abolished transcription from P6 as expected (Fig. 7b, lane 9).

We interpret these data, taken together, as evidence that σ^{54} -RNAP binds the P6 promoter and forms a closed complex able to initiate *rpoH* gene transcription *in vitro* and *in vivo*, provided that an ATP-dependent activator is present. However, the specific activator and conditions inducing transcription from the P6 promoter remain unknown. This makes assessment of the physiological importance of P6 difficult, but evolutionary conservation of the P6 sequence (Pallen, 1999) speaks for its usefulness in as yet unrecognized conditions.

The P6 promoter influenced transcription starting from the upstream promoters (P1-P5) of the *rpoH* gene. Formation

Fig. 7. Transcriptional start site from the P6 promoter of the *rpoH* gene. The arrows indicate positions of the transcripts from the P3, P4, P5 and P6 promoters [locations of transcriptional start sites relative to the ATG start codon (+1) of the *rpoH* gene are shown in parentheses]. (a) Primer extension reactions were carried out with total RNA isolated from *E. coli* MG1655(pMJ13, pREP) before (lane 1) and 30 (lanes 2 and 3) or 60 (lanes 4 and 5) min after induction of PspFΔHTH overproduction from pMJ13 by addition of IPTG. (b) Primer extension reactions were carried out with total RNA isolated from ET8000(pMJ13, pREP) and its *rpoN* derivative ET8045(pMJ13, pREP) before (lanes 6 and 8) and 30 min after (lanes 7 and 9) induction of PspFΔHTH overproduction by addition of IPTG. (c) Primer extension reactions were carried out with total RNA isolated from ET8045(pMJ13, pREP, pVI688) before (lane 10) and 30 min after (lane 11) induction of PspFΔHTH and σ^{54} overproduction from pMJ13 and pVI688, respectively.

of the stable, closed complex of σ^{54} -RNAP at the P6 promoter can be a spatial hindrance for the RNAP holoenzyme. Such a complex cannot be formed in the *rpoN* strain. Accordingly, an increased level of the transcription from the upstream promoters in the *rpoN* cells was observed (Fig. 7b, lanes 8 and 9) but not further explored. The sigma competition for the core RNAP, mediated by the alarmone ppGpp (Sze & Shingler, 1999; Maeda *et al.*, 2000; Jishage *et al.*, 2002; Laurie *et al.*, 2003; Nyström, 2004; Magnusson *et al.*, 2005) could also play an important role in the complicated regulation of *rpoH* transcription. Since core RNAP is limiting for transcription, the amount of σ^{70} bound to RNAP is increased in the absence of σ^{54} .

In a paper by Reitzer & Schneider (2001), the authors mentioned unpublished results of experiments in which it was not possible to demonstrate the existence of a transcript from the P6 promoter in nitrogen-limited cells, which meant NtrC was not the activator of this transcription. We confirm this opinion. These results are also consistent with the negative result of our *in vitro* studies on PspF and NtrC-P binding to the *rpoH* regulatory region (Fig. 5).

There is a possibility that the unidentified enhancer for the activator may be found further upstream or downstream of the 707 bp (W1-W2) region tested. The enhancers binding

ATP-dependent activators for the σ^{54} promoters are usually situated about 100–160 bp upstream of the coding region; they may rarely be found 700 bp apart, but a larger distance is not excluded (Gralla & Collado-Vides, 1996). Belitsky & Sonenshein (1999) described an enhancer located 1.5 kb downstream of the σ^{54} promoter of the *rocG* gene of the *Bacillus subtilis*. We are currently working on the construction of transcriptional fusions that should be helpful in a search for P6 activating conditions.

Since IHF binds in the *rpoH* regulatory region, it might be expected that it plays a role in *rpoH* transcription regulation. However, under the conditions of our experiment on *in vitro* transcription from P6, with PspF as an activator, IHF slightly inhibited the reaction (results not shown). Considering the results of Dworkin *et al.* (1998), who found that IHF facilitated transcription initiation only when a specific activator was used, it has to be accepted that, as long as the specific activator is unidentified, the significance of the IHF binding cannot be evaluated. Nevertheless, the IHF binding seems to be a strong argument for its regulatory role in transcription from P6. Nyström (1995) reported that IHF overproduction caused the induction of the *rpoH*-dependent heat shock response and proposed that this effect could reflect an influence of IHF on the activity of one or several *rpoH* promoters. Our results lend support to this notion.

One can speculate on the role of transcription from P6. Recently attention was paid to biofilm formation. This is an interesting process from the point of view of the development of unicellular organism cooperation leading to a kind of bacterial community. Beloin *et al.* (2004) found that biofilm formation involves, among other processes, the stress response. The *pspA*–*E* operon was strongly induced. The expression of *ibpB* and *ibpA*, genes for small heat shock proteins, increased 40- and 20-fold, respectively, compared with expression during the exponential growth phase of planktonic cells, in conditions inducing biofilm formation. The *clpB* and *dnaK* genes were also induced (Schembri *et al.*, 2003). Elevated expression of these genes is dependent on σ^{32} , the *rpoH* gene product. The molecular mechanism of this induction remains obscure.

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