Spacing of promoter elements regulates the basal expression of the *soxS* gene and converts SoxR from a transcriptional activator into a repressor

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SoxR protein of *Escherichia coli* governs a global response against superoxide-generating agents (such as paraquat) or nitric oxide, and provides broad antibiotic resistance. A redox signal activates SoxR post-translationally to trigger transcription of a second regulatory gene, *soxS*. Activated and non-activated SoxR bind the *soxS* promoter with the same high affinity, but only the activated protein stimulates *soxS* transcription. SoxR acts by an unusual mechanism of positive control: the protein binds the *soxS* promoter between near-consensus –10 and –35 elements that are separated by an unusually long 19 bp (versus the optimal 17 bp). We have constructed and analyzed site-specific deletions that alter the promoter element spacing. Reducing the spacer length to 16–18 bp dramatically elevated basal expression of *soxS* *in vivo* and *in vitro*, and nearly eliminated additional activation by SoxR in response to paraquat. More strikingly, shortening the spacer converted SoxR from an activator into a repressor regardless of paraquat treatment. Gel mobility-shift assays show that repression by SoxR of the promoters with 17 and 16 bp spacers is due to interference with binding by RNA polymerase. Thus, activated SoxR remodels the unusual configuration of the wild-type *soxS* promoter into a highly active form, probably by compensating for the suboptimal distance between the –10 and the –35 elements.

Keywords: DNA binding/*Escherichia coli* promoter/oxidative stress/RNA polymerase/signal transduction

Introduction

In order to initiate transcription, *Escherichia coli* RNA polymerase (RNAP) recognizes and binds specific promoter sequences in DNA occupying a stretch of 50–60 bp determined by both genetic and chemical approaches (Siebenlist *et al.*, 1980; Pivec *et al.*, 1985). Analysis of individual promoters (Jaurin *et al.*, 1982) and comparison of many *E. coli* promoter sequences (Siebenlist *et al.*, 1980; Hawley and McClure, 1983; Pivec *et al.*, 1985; Gralla and Collado-Vides, 1996) revealed the existence of two conserved regions (the –10 and the –35 boxes) to which the sigma 70 (σ^{70}) subunit of RNAP binds, separated by a non-conserved spacer of 17 ± 1 bp (Russell and Bennett, 1982). An A/T-rich region upstream of the –35 box, the UP element, is also present in some strong promoters (Ross *et al.*, 1993) and interacts with the

carboxy-terminal domains of the RNAP α subunits. Activator proteins enhance binding or open complex formation by RNAP at promoters that deviate from the consensus, whereas repressors can impair such a process (Collado-Vides *et al.*, 1991; Gralla and Collado-Vides, 1996). For both positive and negative regulation, the position of regulatory sites in different *E. coli* promoters enables communication with RNAP. With very few exceptions, the majority of repressor binding sites (operators) overlap the promoter elements (Collado-Vides *et al.*, 1991; Gralla and Collado-Vides, 1996). Studies with the *lac* operator revealed that the degree of repression varies with the operator position, and that the highest repression was achieved when the repressor binding site was placed in the –10/–35 spacer region (Lanzer and Bujard, 1988). The MerR/SoxR family of transcriptional activators constitutes a surprising exception to this rule: their binding sites are located between the –10 and the –35 boxes (Summers, 1992; Hidalgo and Dempsey, 1996b), but they exert strong positive regulation.

The *soxRS* locus of *E. coli* regulates an oxidative stress response that includes at least 10 genes involved in counteracting oxidative damage and in providing resistance to multiple antibiotics (Hidalgo and Dempsey, 1996b). The *soxRS* response is triggered when cells are exposed to nitric oxide (NO•) or to superoxide ($O_2^{\bullet-}$)-generating agents such as paraquat (PQ) (Amabile-Cuevas and Dempsey, 1991; Wu and Weiss, 1991; Nunoshiba *et al.*, 1992; Nunoshiba *et al.*, 1993). SoxR protein is evidently the sensor for this response (Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). Activated SoxR protein, a homodimer of 17 kDa subunits containing a pair of [2Fe–2S] clusters (Hidalgo *et al.*, 1995; Wu *et al.*, 1995), triggers expression of the *soxS* gene, whose protein product, SoxS, activates transcription of all the regulon genes (for a review, see Hidalgo and Dempsey, 1996b).

Purification of SoxR protein revealed important characteristics of its DNA-binding and transcriptional activity. Two forms of the protein were isolated that contained Fe (Fe-SoxR) or lacked detectable metals (apo-SoxR), and both forms bound the *soxS* promoter with equal affinity (Hidalgo and Dempsey, 1994). However, only Fe-SoxR stimulated transcription *in vitro* from the *soxS* promoter, up to 100-fold over the basal transcription seen with RNAP alone (Hidalgo and Dempsey, 1994; Hidalgo *et al.*, 1995). These observations led to our current model for *soxRS* regulation *in vivo* (Hidalgo and Dempsey, 1996b). In this model, the first step in this two-stage transcriptional cascade involves the NO•- or $O_2^{\bullet-}$ -mediated activation of pre-existing SoxR, by a mechanism that could involve both insertion of the iron–sulfur centers and their oxidation (Hidalgo *et al.*, 1995; Hidalgo and Dempsey, 1996a). Activated, Fe-SoxR then induces transcription of the *soxS* gene. The limiting step in this transcription activation

Table I. Strains and plasmids

Series name	Mutation	Plasmid derivatives		Lysogenized strain derivatives	
		pBluescript	pRS551	GC4468 (<i>soxR</i> ⁺)	DJ901 (Δ <i>soxRS901</i>)
Series 40	wild-type	pEH44	pEH40	EH40	EH46
Series 51	–35*, Δ^1 -15		pEH51	EH51	EH57
Series 50	Δ^1 -15	pEH55	pEH50	EH50	EH56
Series 60	Δ^2	pEH64	pEH60	EH60	EH66
Series 70	Δ^1 -24	pEH74	pEH70	EH70	EH76
Series 80	Δ^3	pEH84	pEH80	EH80	EH86
Series 90	Δ^4	pEH94	pEH90	EH90	EH96

event would not be the binding of SoxR to the *soxS* promoter, but an allosteric effect that promotes open complex formation by RNAP (Hidalgo *et al.*, 1995). The proposed binding of non-activated SoxR to the *soxS* promoter *in vivo* requires experimental confirmation.

Two striking features of this SoxR–*soxS* interaction are the unusually long spacing between the –10 and –35 promoter elements and the position of the SoxR binding site. For σ^{70} -dependent *E.coli* promoters, changes in the –35/–10 spacing in different promoters can have important regulatory functions (Pivec *et al.*, 1985). The constitutively weak *soxS* promoter includes –10 and –35 sites that are very close to the *E.coli* consensus, but with an unusual 19 bp spacing (Hidalgo and Demple, 1994). Furthermore, SoxR strongly binds ($K_d \sim 10^{-10}$) and footprints a region that is centered in the spacer region upon a perfect 9 bp inverted repeat (Hidalgo and Demple, 1994). All the members of the MerR/SoxR family of transcriptional activators seem to share this unexpected feature in the interaction with their target promoters. In the case of MerR, it has been proposed that mercury-dependent activation of the target *merT* promoter brings the –10 and –35 promoter elements into better helical alignment through a MerR-mediated DNA untwisting effect (Ansari *et al.*, 1992). Promoter element spacing does play a role in the low-level expression of the *merT* promoter: small deletions in the spacer region conferred an up-promoter phenotype (Lund and Brown, 1989; Parkhill and Brown, 1990).

The intriguing mechanism by which transcriptional activation occurs for the members of this family, as well as an important difference between the SoxR and MerR systems (the repression effect over *merT* expression exerted by uninduced MerR is absent in the *soxS*–SoxR system), prompted us to analyze the effect of promoter element spacing in the constitutive activity of the *soxS* promoter. We conclude from these studies that the 19 bp spacing is required for effective SoxR-mediated activation, and that this unusually long distance prevents SoxR from being a repressor.

Results

Cloning of the wild-type *soxS* promoter into pBluescript and the *lacZ*-based vector, pRS551

Previous studies showed that *soxS* promoter sequences as short as ~180 bp are enough for full activation by RNAP and SoxR *in vitro* (Hidalgo and Demple, 1994; Hidalgo *et al.*, 1995; Wu *et al.*, 1995). However, the shortest version of the *soxS* promoter that we have assayed for

activity *in vivo* by fusion to the *lacZ* gene is a 500 bp long segment that encompasses the whole *soxRS* intergenic region together with ~40 and ~65% of the *soxR* and *soxS* genes, respectively (Nunoshiba *et al.*, 1992). In order to construct *soxS* operator mutants, we decided to use a PCR-mediated mutagenesis method. For this purpose, a 149 bp fragment of the wild-type *soxS* promoter was amplified and subcloned into pBluescript to generate the plasmid pEH44 (see Materials and methods). The same PCR-generated fragment subcloned into pRS551 generated an operon fusion to *lacZ* in plasmid pEH40, and the fusion was inserted into the chromosome of the *soxRS*⁺ strain GC4468, yielding strain EH40 with the fusion present in a single copy. As analyzed by β -galactosidase assay (data not shown), the single-copy fusion in EH40 could be induced by PQ to the same level as the previous reporter strain TN530 with *lacZ* fused to the 500 bp fragment (Nunoshiba and Demple, 1993). Interestingly, the basal (uninduced) level for the fusion for EH40 was significantly lower (2- to 3-fold) than that reported previously for TN530 (data not shown), although the use of slightly different cloning vectors for the construction of the two fusions could explain this difference.

Construction and *in vitro* characterization of *soxS* operator mutants

Using the PCR-based mutagenesis method described in Materials and methods, we constructed a series of mutant *soxS* operators and subcloned them into pBluescript (see Table I and Figure 1). Two series of deletion mutations were engineered that converted the 19 bp promoter element spacing of *soxS* to 18 bp. Series 50 consisted of a set of plasmids and strains with a 1 bp deletion in the 3 bp segment (TTA) between the inverted repeat and the –10 box whereas, in series 70, 1 bp was deleted near the center of the inverted repeat. Series 60 and series 80 contained, respectively, 2 or 3 bp deletions between the –10 and the inverted repeat, yielding spacings of 17 and 16 bp, respectively. Finally, series 90 combined the series 70 and series 80 mutations to remove 4 bp and generate a suboptimal spacing of 15 bp.

We next tested whether SoxR *in vitro* could bind the modified operators, as a prelude to determining the *in vivo* effect of SoxR on expression directed by the mutant promoters. As seen in Figure 2, the *in vitro* affinity of Fe-SoxR for most of the promoter fragments was similar to the affinity for the wild-type *soxS* promoter. However, for the series 90 construct (4 bp deletion), a ≥ 5 -fold higher SoxR concentration was required to yield binding similar to that with the wild-type promoter.

			-35		-10	+1		
						.		
			<u>AAAAGGTATTTAGCGAAATGGAGTTCAATTGAACTCCCTTAATATGAGGGGTTGTCTAC</u>					Spacer size
series 40	(WT):		-----					19-bp
series 51	(-35*):		-----G-----.					18-bp
series 50	(Δ^1 -15):		-----.					18-bp
series 60	(Δ^2):		-----.					17-bp
series 70	(Δ^1 -24)		-----.					18-bp
series 80	(Δ^3):		-----..					16-bp
series 90	(Δ^4):		-----...					15-bp

Fig. 1. Sequence of the *soxS* promoter and positions of the deletion mutations. The -10 and -35 boxes, as well as the origin of transcription, are indicated in the template strand. The 9 bp dyad symmetry for SoxR binding is underlined. The mutation series name and the final spacer size are indicated. Dashed lines indicate conserved nucleotides. A substitution mutation is marked, and nucleotide deletions are represented with dots.

Fig. 2. DNA binding by SoxR to wild-type and mutant *soxS* promoters. Band-shift assays were performed by incubating equivalent amounts of ^{32} P-labeled *soxS* promoter fragments with increasing concentrations (0.1 and 0.5 ng) of purified Fe-containing SoxR. The unbound DNA (D) and the SoxR–DNA complex (C) are indicated.

In previous studies, we demonstrated the ability of Fe-SoxR to activate transcription *in vitro* from a 180 bp wild-type promoter sequence (Hidalgo *et al.*, 1995). We now carried out a similar analysis of the 149 bp promoter fragments using primer extension with a new oligonucleotide to quantify the amount of *soxS* transcript synthesized in the reactions. Plasmids pEH44 (wild-type, 19 bp spacing; series 40) or pEH55 (1 bp deletion, 18 bp spacing; series 50) were incubated with RNAP alone (Figure 3, lanes 1 and 4) or with the active Fe-SoxR (Figure 3, lanes 2 and 5) or the inactive apo-SoxR (Figure 3, lanes 3 and 6). The results demonstrate that, although Fe-SoxR was required for activation of transcription from the wild-type promoter, active SoxR was not required for transcription from the 18 bp spacing mutant promoter. Although apo-SoxR did not affect transcription from the pEH55 promoter detectably, there was slight activation by Fe-SoxR.

Activity in vivo of mutant *soxS* promoters fused to the *lacZ* gene

The *in vitro* transcription assay described above had a limited sensitivity compared with previous experiments (Hidalgo *et al.*, 1995) and did not address the *in vivo* situation. We initially tried to construct *lacZ* fusions to the mutant promoters by using the same strategy described above for the wild-type *soxS* promoter. This effort was unsuccessful, probably due to the high constitutive activity of the deletion mutant promoters and the resulting toxicity of excess β -galactosidase, as has been reported before for other strong promoters (Podkovyrov and Larson, 1995). In fact, all the constructs obtained had modified or deleted part of the *lacZ* gene, or harbored additional mutations that restored the sub-optimal 19 bp spacing or altered

Fig. 3. *In vitro* transcription from the wild-type (19 bp spacing) and mutant Δ^1 -15 (18 bp) *soxS* promoters. pBluescript-derived plasmids containing the wild-type (pEH44; wt) or a 1 bp deletion mutant promoter (pEH55; Δ^1 -15) were incubated with RNA polymerase in the presence or absence of 10 ng of either active (FeSoxR) or inactive (apoSoxR) SoxR in *in vitro* transcription reactions. The primer extension products for the *soxS* and control (*bla*) transcripts are indicated.

some other component of the *soxS* promoter (data not shown). One of these mutant promoters fused to *lacZ* (series 51, containing an A→G mutation in the -35 box, along with the 1 bp spacer deletion; Figure 1) was retained for further study.

The spacing mutant promoters fused to *lacZ* were finally constructed by transforming ligation mixtures of pRS551 and the various mutant inserts from the pBluescript derivatives (Table I) into MJC97, a *pcnB* mutant strain. This mutation reduces the copy number of ColE1-based plasmids (Lopilato *et al.*, 1986; Xu and Cohen, 1995).

Fig. 4. β -Galactosidase activities of the wild-type and mutant promoters. WT and mutant promoters were fused to *lacZ* in the multicopy vector pRS551, and the fusions were inserted in the chromosome of strains GC4468 (*soxR*⁺; **A**) or DJ901 (*soxR*⁻; **B**). Activities were measured in the absence (untreated) or presence of 0.25 mM paraquat. The values shown represent the means and standard errors (bars) of two independent experiments.

The resulting fusions were then transferred to the chromosome of GC4468 (a *soxRS*⁺ strain) as described in Materials and methods, yielding the strains indicated in Table I. The *in vivo* activity of the wild-type and mutant promoters in these *SoxR*⁺ strains was assessed by measuring β -galactosidase activity in cells grown in the presence or absence of PQ (Figure 4A). Interestingly, among the altered promoters, the 18 bp spacing mutants (strains EH50 and EH70) showed the highest constitutive and PQ-induced activities, followed closely by the 17 bp mutant (EH60). The 16 bp mutant (EH80) yielded a significantly lower activity than the 18 or 17 bp spacer mutants under both induced and uninduced conditions (Figure 4A). As expected, the 15 bp spacing mutant (EH90) showed a lower basal level of transcription than the other mutants, although this promoter had ~5-fold higher activity with or without PQ treatment than the wild-type promoter under non-inducing conditions. Mutant EH51 (containing a mutation in the -35 box) had very low basal expression and showed only slight induction by PQ. Notably, PQ seemed to induce significant transcription only from the wild-type promoter (up to 40-fold), but not from the mutant promoters (1- to 1.5-fold).

In order to study the effect of SoxR on the intrinsic activity of the different operators, we analyzed expression of the *lacZ* fusion constructs in the chromosome of DJ901, carrying a deletion of the entire *soxRS* locus (Greenberg *et al.*, 1990). The pattern of *soxS* expression in the Δ *soxR* strain (Figure 4B) was clearly different from that seen in the *soxR*⁺ strain (Figure 4A). As seen in Figure 4B, the constitutive β -galactosidase levels effected by the 17 (EH66) and 16 bp (EH86) spacer mutations in the Δ *soxR* strain were increased by 4- to 5-fold over those observed in the *soxR*⁺ strain (EH60 and EH80 in Figure 4A), whereas the rest of the strains retained a comparable level of activity in both the Δ *soxR* and the *soxR*⁺ backgrounds (with the exception of the expected lack of PQ inducibility in the case of the wild-type promoter in the absence of SoxR in strain EH46). When we transferred the fusions to a different Δ *soxRS* background [in strain BW829 (Tsaneva and Weiss, 1990)], the

derepression of the 17 and 16 bp spacing mutants was also observed (data not shown).

Additional evidence for repression exerted by SoxR on the 17 and 16 bp spacer mutants was obtained by varying the expression of the protein. For this purpose, the Δ *soxRS* strains containing the various fusions were transformed with plasmid pSXR, a SoxR expression vector derived from pSE380 (Amáñile-Cuevas and Demple, 1991), or with the vector alone. We confirmed overexpression of SoxR in the strains carrying pSXR by immunoblotting (data not shown), and we measured the transcriptional activity of the fusions by β -galactosidase assay (Figure 5). For the wild-type *soxS* promoter, elevated SoxR expression from the plasmid restored normal constitutive expression and actually enhanced induction by PQ by ~50% (Figure 5A). In the series 50 and 70 promoters (both with 18 bp spacing), increased SoxR levels did not significantly affect the basal or induced levels of fusion expression (Figure 5B and C), although SoxR bound *in vitro* to these promoters with normal affinity (Figure 2). In contrast, for the series 60 and 80 constructs, SoxR exerted strong repression compared with the Δ *soxR* strain (15-fold for series 60 and 34-fold for series 80; Figure 5D and E). Relative to the *soxR*⁺ strain, SoxR overexpression repressed the 17 and 16 bp spacer derivatives ~5-fold (Figure 5D and E). There was no significant effect of SoxR on the expression of the 15 bp spacer derivative (series 90, Figure 5F), consistent with the low affinity of the protein for this operator observed *in vitro* (Figure 2).

Effect of SoxR on binding of RNAP to mutant promoters *in vitro*

The positioning of the binding site for SoxR in the *soxS* promoter between the -10 and the -35 boxes is reminiscent of the sites for repressors that inhibit binding of RNAP (Collado-Vides *et al.*, 1991; Gralla and Collado-Vides, 1996). However, for the wild-type *soxS* promoter, binding of activated SoxR *in vitro* increases the affinity of RNAP modestly (Hidalgo and Demple, 1994). For the mutated *soxS* promoters of series 60 and 80 (17 and 16 bp spacing), the *in vivo* results (Figures 4 and 5) suggested that SoxR

Fig. 5. Effect of SoxR overexpression on the β -galactosidase activities of wild-type and mutant promoter fusions. The activities of WT and mutant promoters were analyzed by comparing their β -galactosidase levels when the fusions were expressed in a *soxR*⁺ background (strains EH40, EH50, EH60, EH70, EH80 and EH90) or in Δ *soxR* strains (EH46, EH56, EH66, EH76, EH86 and EH96) transformed with either a control plasmid (pSE380) or with a SoxR expression construct (pSXR). Activities were measured in the absence (untreated) or presence of 0.25 mM paraquat. The spacer size of the different promoters is indicated in each panel for the different mutant series. For (B) and (C), '-15' and '-24' refer to the deleted base pairs. The values shown represent the means and standard errors (bars) of two independent experiments.

might still bind to the operator sites with high affinity (which was seen *in vitro*, as depicted in Figure 2) but impair the RNAP interaction with the -10 box (see Figure 1). We compared the *in vitro* binding of RNAP to the

different *soxS* promoters in the absence (Figure 6, lanes 1–4 in all panels) or presence (Figure 6, lanes 5–8 in all panels) of saturating amounts of SoxR. As seen previously (Hidalgo and Demple, 1994), binding of Fe-SoxR to the

Fig. 6. Binding of RNA polymerase to wild-type and mutant promoters in the presence and absence of SoxR. Wild-type and the deletion promoter DNA fragments were ^{32} P labeled and incubated without (lanes 1–4) or with (lanes 5–8) 2.5 ng of Fe-containing SoxR. Increasing amounts of RNA polymerase (RNAP; 0, 20, 40 and 80 nM) were then added. Free DNA (e.g. 'ins.44', 'ins.55', 'ins.74', 'ins.64', 'ins.84' and 'ins.94'), SoxR–DNA complexes (CI) and RNAP–(SoxR)–DNA complexes (CII) are indicated.

wild-type *soxS* promoter improved the subsequent binding of RNAP nearly 5-fold (Figure 6A), as determined by densitometric analysis (see Materials and methods). Prior binding of SoxR to the series 50 promoter (18 bp spacer) had no significant effect on subsequent RNAP binding (Figure 6B), and only a 2-fold enhancement of RNAP binding by SoxR was observed for the 18 bp series 70 (Figure 6C). In contrast, SoxR clearly impaired the binding of RNAP to the promoters of series 60 and 80 (17 and 16 bp spacers; Figure 6D and E), by ~3- and 10-fold, respectively. These effects are consistent with the repression exerted by SoxR on these promoters *in vivo*. RNAP binding to the promoter of series 90 (15 bp spacer; Figure 6F) was also reduced 2-fold by SoxR; this blocking effect might be stronger if SoxR could bind this promoter with high affinity relative to RNAP.

Discussion

The first transcriptional event in the response to superoxide or nitric oxide stress in *E. coli* involves the interaction of

activated SoxR with the promoter of the second regulatory gene, *soxS*. We have demonstrated with the construction and analysis of five *soxS* promoter mutants that the 19 bp spacing between the –10 and the –35 boxes of the *soxS* promoter is essential for normal induction by SoxR in the response to PQ, and that deletion of the spacing from 19 bp down to 17 or 16 bp converts SoxR from an activator into a significant repressor. The data presented here suggest that the induction of *soxS* transcription by activated SoxR is achieved by reorienting the –10 and –35 regions to compensate for the 19 bp spacer.

Studies of many *E. coli* promoters have suggested that spacer length, rather than specific nucleotide sequence, can have important regulatory functions, and that the intrinsic activity of otherwise similar promoters depends only on fluctuations of the spacer distance (Russell and Bennett, 1982; Hawley and McClure, 1983; Pivec *et al.*, 1985). MerR and SoxR constitute the only known examples in which activation of their target genes takes place by compensating for overwinding between the –10

and the -35 elements in σ^{70} -RNAP recognition sites. In the case of the *soxS* promoter, the *in vivo* activities of the different mutant promoters in the strains lacking SoxR systematically revealed the quantitative effects of five different spacing sizes on transcription from an otherwise standard *E.coli* promoter. Although we have not confirmed that the same origin of transcription was used in all the promoter mutants, previous studies demonstrated that the transcriptional initiation site in a synthetic promoter is determined by the -10 region sequence (Rossi *et al.*, 1983), which has been conserved in all our constructs. As suggested for other promoters (Gralla and Collado-Vides, 1996), 17 bp is evidently the optimal spacing for the *soxS* promoter elements; 1 bp deviations from this distance, leading to 18 and 16 bp spacers, reduced the promoter activity by about half. A 15 bp spacer still allows a considerable amount of transcriptional activity, exceeding by ~ 10 -fold the level for the 19 bp spacer.

It is intriguing that the σ^{70} subunit of RNAP is able to bind and trigger promoter activity with such different distances between the two known DNA contacts, the -10 and -35 boxes. Stefano and Gralla (1982) described the 'untwist and melt' model as a mechanism of open complex formation by RNAP. According to this model, RNAP forms unstable closed complexes with promoters due to its inability to make productive contacts with the -10 and the -35 regions. In fact, Werel *et al.* (1991) demonstrated that increasing the flexibility of the spacer DNA by single nucleoside deletions enhances the binding affinity of RNAP. These results are consistent with the hypothesis that favorable orientation of the binding contacts may be achieved by untwisting the DNA between them. The probability of achieving this reoriented state must depend on the length of the spacer, with the consensus spacer length of 17 bp being optimal. Open complex formation would result when the torsional stress exerted by RNAP is relieved by DNA melting around the transcriptional start site (Stefano and Gralla, 1982). In the case of *soxS* activation, the proposed underwinding mechanism exerted by SoxR could both reorient the promoter elements appropriately for RNAP binding, and provide torsional stress that could be relieved by open complex formation by RNAP. Footprinting experiments with DNase I or Cu(I)-orthophenanthroline have already indicated DNA distortion by activated SoxR (Hidalgo and Demple, 1994; Hidalgo *et al.*, 1995), and other experiments are underway. However, a role for protein-protein contacts between SoxR and RNAP has not yet been eliminated and should be addressed.

An important difference between the *merT*-MerR (Summers, 1992) and *soxS*-SoxR systems is that non-activated MerR is a repressor of the *merT* promoter: MerR is required for repression of the *merT* gene (Ross *et al.*, 1989). However, *in vitro* (Frantz and O'Halloran, 1990) and *in vivo* (Heltzel *et al.*, 1990) footprinting analysis indicates that RNAP still binds the promoter in the presence of uninduced MerR. Such a repression effect has never been observed in the *soxS*-SoxR system. Differences between the proteins or the structures of their target promoters could explain this distinction. First, the MerR-binding site consists of a 7 bp inverted repeat interrupted by 4 bp (Summers, 1992), whereas in the case of SoxR we find a perfect 9 bp inverted repeat (Figure 1) which

could differentiate the mode of protein-DNA interaction in these systems. Second, a DNA bend thought to be comprised of two kinks was detected for MerR bound in its inactive state to the *merT* promoter, and this bend is partially released by Hg-dependent activation of the protein (Ansari *et al.*, 1995). The positions of the MerR-induced kinks were identified as two DNase I-hypersensitive sites (Ansari *et al.*, 1995). Although two similar sites are seen for SoxR (Hidalgo and Demple, 1994), which shows no repression activity at the wild-type *soxS* promoter, preliminary experiments suggest that SoxR produces a much less dramatic bend of the *soxS* promoter (J.E.Bradner, E.Hidalgo, A.Ansari and B.Demple, unpublished results). Third, the location of the 18 bp activator site relative to the respective promoter elements is slightly different, the MerR site being shifted 1 bp toward the -10 box compared with the SoxR binding site. It would be of interest to determine the effect of relocating the SoxR operator 1 bp downstream within the spacer region, although a similar move of the MerR operator reduced *merT* activation efficiency (Parkhill and Brown, 1990).

The repression effect exerted by SoxR over the 17 and 16 bp mutant promoters is evidently a consequence of steric constraints in the promoter (Figure 7) and is thus mechanistically different from the repression described above in the MerR-*merT* system. SoxR clearly impairs RNAP binding to the shortened promoters, an interference that does not occur for non-activated MerR at the *merT* promoter. In the case of the wild-type *soxS* promoter with 19 bp spacing, and in the 18 bp spacer promoters, the SoxR dimer evidently can bind without significantly diminishing the RNAP-DNA contacts. RNAP binds one face of the promoter DNA across the region from -44 to -13 (Siebenlist *et al.*, 1980); this structure requires SoxR to contact DNA along the DNA face opposite RNAP. Because of this physical proximity, moving the SoxR binding site even closer to the RNAP contact at -10 produces a steric clash that interferes with binding of the polymerase to cause repression.

Materials and methods

Cloning of wild-type and mutant *soxS* promoters in pBluescript

To amplify the wild-type *soxS* promoter sequence from pBD100 (which contains the whole *soxRS* locus; Amabile-Cuevas and Demple, 1991) by PCR, we used primers X (5'-ATGAATTCGCGTTTCGCCACTTCG-3') and Y (5'-GCGCGGATCCTCTTTTCAGTGTGTGT-3'), which correspond to regions -114 to -90 and $+41$ to $+17$ of the *soxS* promoter ($+1$ being the transcriptional start site; Figure 1), and include respectively an *EcoRI* and a *BamHI* site (both underlined). The fragment was purified with a PCR purification kit (Qiagen), digested with *BamHI* and *EcoRI*, and purified again after electrophoresis in a 1% agarose gel, using a gel purification procedure to extract the DNA from the gel slices (Qiagen). The *BamHI*-*EcoRI* fragment was subcloned into the pBluescript KS plasmid (Stratagene), to yield pEH44 (see Table I), with an insert size of 149 bp. The sequences of all the PCR-amplified fragments subcloned into pBluescript and described herein were confirmed by sequencing using pBluescript primers T3 (5'-ATTAACCTCACTAAAG-3') or T7 (5'-AATACGACTCACTATAG-3').

In order to construct most of the mutant operators, pEH44 was used as a template for sequential steps of PCR. Pairs of mutagenic and complementary primers, containing the deletion of interest, were synthesized and used with either the T3 or the T7 primer in a first round of amplification. T3 and a mutagenic primer for the opposite strand were used to amplify the appropriate fragment (182-186 bp) from pEH44; in parallel reactions, T7 primer and a mutagenic primer complementary to

Fig. 7. Model for SoxR-independent and SoxR-dependent transcription of wild-type and mutant *soxS* promoters. The -10 and -35 regions, as well as the origin of transcription of the *soxS* promoter, are indicated for the wild-type (19 bp spacer; top), single deletion mutants (18 bp spacer; middle) and double or triple deletion mutants (17 or 16 bp spacer; bottom). SoxR is represented as a dimer. The large Xs indicate lack of transcription (top and bottom); the small x indicates interference with RNAP binding (bottom).

that in the first reaction were used to amplify a second, overlapping fragment (120–126 bp). Products of the first two reactions (1 μ l of each) were combined in a second round of PCR amplification using the T3 and T7 primers. The resulting PCR products (286–288 bp) containing the complete and mutated *soxS* operator sequences were purified with a PCR purification kit (Qiagen), digested with *EcoRI* and *BamHI*, and purified through agarose gels as described above. The 146–148 bp fragments were then subcloned into pBluescript KS. Following the mutagenesis protocol described above using pEH44 as a template, the plasmids were constructed: pEH55 [using primers del1 (5'-CTTGAGG-ATTATACTCCCC-3') and del2 (5'-GGGGAGTATAATCCTCAAG-3')], pEH64 [from primers del3 (5'-ACTTGAGGTTATACTCCCC-3') and

del4 (5'-GGGGAGTATAACCTCAAGT-3')], pEH74 [from primers del7 (5'-GTTACTTGAGGAATTATACT-3') and del8 (5'-AGTATAATTCCTCAAGTAAC-3')], and pEH84 [from primers del9 (5'-ACTTGAGGTTATACTCCCC-3') and del10 (5'-GGGGAGTATAACCTCAAGT-3')]. In order to generate the insert for plasmid pEH94, pEH74 was used as the template and del9 and del10 as mutagenic primers.

Construction of *soxS*::*lacZ* operon fusions

The *BamHI*–*EcoRI*-digested, 149 bp wild-type *soxS* promoter fragment described above was ligated into *BamHI*–*EcoRI*-digested pRS551, a pBR322-derived vector for *lac* operon fusions (Simons *et al.*, 1987). The ligation mixture was transformed into DH5 α cells, and pEH40

(Table 1) was isolated. The same procedure was used in attempts to subclone the mutant operators, but the resulting plasmids contained not only the designated deletions but also additional mutations. One of these, pEH51, contained an A→G mutation in the -35 box and was retained for further study. In order to construct fusions of the deletion mutant promoters to *lacZ*, the *Bam*HI-*Eco*RI-digested mutant fragments from the pBluescript derivatives (pEH55, pEH64, pEH74, pEH84 and pEH94) were purified after gel electrophoresis and ligated into *Bam*HI-*Eco*RI-digested pRS551. The ligation mixtures were transformed into MJC97 [*F*⁻ Δ (*lacX74*) *galE galK thi rpsL Δ phoA(PvuII) pcnB24-1 zad1::Tn10*]; the *pcnB* mutation reduces the copy number of pBR322 derivative plasmids, therefore decreasing the possible toxicity of β -galactosidase overexpression (Podkovyrov and Larson, 1995). The pRS551-derived plasmids were named pEH50, pEH60, pEH70, pEH80 and pEH90 (Table I). Bacteriophage λ RS45 was used to transfer the *soxS'*::*lacZ* fusions from MC4100 [*F*⁻ *lacU169 araD139 thiA rpsL relA*] carrying pEH40 or pEH51, or MJC97 carrying pEH50, pEH60, pEH70, pEH80 or pEH90, into the chromosome of GC4468 (*soxRS*⁺; Greenberg *et al.*, 1990) as described by Simons *et al.* (1987). The resulting GC4468-derived lysogens, EH40, EH51, EH50, EH60, EH70, EH80 and EH90, were selected by their resistance to kanamycin, and confirmed by their Lac⁺ Amp^S phenotype (Simons *et al.*, 1987). The resistance to the kanamycin selection procedure could not be used to insert the fusions into the chromosome of DJ901 [as GC4468 but Δ (*soxR-zjc2205*)*zjc2204::Tn10*]. Instead, we used UV induction (Silhavy *et al.*, 1984) to generate phage lysates of the different fusion derivatives already inserted into GC4468. Lysogens were then obtained by infecting DJ901 with the appropriate fusion phage lysate (Simons *et al.*, 1987) and identification on McConkey lactose plates, to yield strains EH46, EH57, EH56, EH66, EH76, EH86 and EH96 (Table I). The DJ901-derived strains were transformed with the SoxR expression vector pSXR and the control vector pSE380 (Amábile-Cuevas and Demple, 1991).

In vitro transcription

The ability of RNAP to initiate transcription from two different *soxS* promoters in the presence and absence of SoxR was analyzed *in vitro* following a procedure previously described (Hidalgo and Demple, 1996a), except that the template plasmids used were pEH44 (containing the wild-type *soxS* promoter) or pEH55 (containing the *soxS* promoter with a 1 bp deletion between the -10 and the -35 boxes; see Table I). The *soxS* transcript and a control *bla* transcript were quantified by primer extension with, respectively, primer T7 (located in pBluescript, 99 bp downstream from the initiation of transcription in the *soxS* promoter insert) and primer pBR-1 (Hidalgo and Demple, 1996a).

DNA-protein binding

Plasmids containing either wild-type or mutated *soxS* promoters (pEH44, pEH51, pEH55, pEH64, pEH74, pEH84 and pEH94) were digested with *Bam*HI and *Eco*RI and their inserts isolated by extraction from agarose gel slices after electrophoresis, as described above. The 146–148 bp fragments were labeled at the 5' end with [γ -³²P]ATP and T4 polynucleotide kinase (Promega) (Sambrook *et al.*, 1989), and purified from unincorporated nucleotides on a small Sephadex G50 column. The DNA fragments (each at a final concentration of ~0.1 nM) were incubated with the indicated amounts of SoxR and analyzed by gel electrophoresis (Hidalgo and Demple, 1994). For the RNAP binding experiments, 2.5 ng of Fe-SoxR were incubated where indicated with the DNA for 10 min at 25°C, followed by the addition of the indicated amounts of σ ⁷⁰-containing RNAP (Pharmacia) and further incubation at 37°C for 5 min in 20 μ l reaction mixtures (Hidalgo and Demple, 1994). Apparent dissociation constants (*K*_d) were estimated from analysis of the gels by scanning densitometry with a BioImage system (Millipore) as previously described (Hidalgo and Demple, 1994).

soxS induction experiments

Lysogens containing the different operon fusions were inoculated into LB broth (Miller, 1992) with 30 μ g/ml of kanamycin and 30 μ g/ml of streptomycin and incubated at 37°C for ~16 h with vigorous shaking. The overnight cultures were diluted 100-fold into 1 ml of fresh medium in duplicate tubes and incubated at 37°C for exactly 90 min. PQ was then added at a final concentration of 0.25 mM to one of each pair of tubes, and incubation continued for 60 min. The samples were then placed on ice. β -Galactosidase activity in SDS-CHCl₃-treated cells was determined as described by Miller (1992).

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