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The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator

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The SoxR protein of *Escherichia coli* responds to redox signals by activating the transcription of *soxS*, which encodes another transcription activator that directly stimulates oxidative stress genes. We show here that transcription of the *soxR* gene, which is positioned head-to-head with *soxS* in the chromosome, initiates in the intergenic region and is itself repressed by SoxR protein in *in vitro* transcription experiments. Analysis of single-copy operon fusions to *soxR*, combined with the results of Northern blotting experiments, verified this regulation and the transcription start site *in vivo*. The structure of the overlapping promoters is such that the single SoxR-binding site is located in the –10/–35 spacer of the *soxS* promoter, but just downstream of the –10 element of the *soxR* promoter. Activated and non-activated SoxR bind this site equally well, exerting nearly constant repression of *soxR*; activated SoxR simultaneously stimulates the *soxS* promoter ≥30-fold. The functional *soxR* promoter depresses *soxS* transcription when SoxR is not activated and enhances *soxS* transcription when SoxR is activated, as shown by comparing the expression of *soxS′::lacZ* fusions with and without the *soxR* –35 element (induction ratio only ~7-fold). SoxR thus represents a highly polar, redox-regulated transcriptional switch that maximizes the change in expression of *soxS*.
Keywords: activator/repressor/SoxR/*soxS*/transcriptional switch

Introduction

Different cellular responses to environmental challenges are triggered by activation of specific signal transduction pathways. In some cases, there may be only one regulatory step between the stress-activating signal and the downstream response genes. For example, the *Escherichia coli* OxyR protein is the direct link between hydrogen peroxide stress and the activation of defense genes such as catalase, i.e. OxyR is both a sensor and a transcriptional activator (for a review, see Hidalgo and Demple, 1997a). Other circuits involve two or more regulatory proteins, and gene activation frequently is triggered in a series of steps. For example, the two-component systems that respond to anaerobiosis (ArcB/ArcA) or osmolarity (EnvZ/OmpR) constitute signal transduction pathways in which both the membrane sensor protein and the DNA-binding effector are post-translationally activated via sequential phosphorylations (for a review see Wurgler-Murphy and Saito, 1997). The *soxRS* regulon is also switched on in two regulatory steps, but in this case only the SoxR protein pre-exists and is activated during superoxide or nitric oxide stress. Activated SoxR then stimulates transcription of the *soxS* gene, whose protein product in turn induces transcription of all the regulon genes (Hidalgo and Demple, 1997a).

The *soxRS* regulatory locus (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991) up-regulates transcription of at least 12 genes in response to redox-cycling agents such as paraquat (PQ), which produce superoxide intracellularly. Some of the activities induced through the *soxRS* pathway are clearly responsible for diminishing or repairing the damage produced by oxygen radicals (e.g. superoxide dismutase, glucose-6-phosphate-dehydrogenase, DNA repair endonuclease IV). Other *soxRS*-inducible enzymes participate in central metabolism and may replace oxidant-damaged proteins (e.g. fumarase C, aconitase), or stabilize oxidant-sensitive iron–sulfur proteins by maintaining them in the reduced state [e.g. NADPH:ferredoxin (oxidized) oxidoreductase]. Some *soxRS* regulon members are not clearly connected to oxidative stress (e.g. *micF, acrAB*) but instead mediate increased resistance to antibiotics and organic solvents (White *et al.*, 1997).

The level of *soxR* transcript was reported to remain unchanged by superoxide stress (Wu and Weiss, 1991). SoxR protein in solution is a homodimer that contains two binuclear iron–sulfur clusters ([2Fe–2S]; Hidalgo *et al.*, 1995; Wu *et al.*, 1995). During normal growth, the SoxR [2Fe–2S] centers are in the reduced state *in vivo*; upon superoxide stress, the clusters are oxidized and the protein induces transcription of its only known target, the *soxS* gene (Ding *et al.*, 1996; Gaudu and Weiss, 1996; Ding and Demple, 1997; Gaudu *et al.*, 1997; Hidalgo *et al.*, 1997). Both active (oxidized), iron-containing SoxR (Fe-SoxR) and its inactive *in vitro* form lacking iron (apo-SoxR) have the same high binding affinity for the *soxS* promoter (K_D ~10^{-10} M; Hidalgo and Demple, 1994). Reduced Fe-SoxR also seems to bind the *soxS* promoter with high affinity (Gaudu and Weiss, 1996). Thus, SoxR transcriptional activity is not regulated at the level of DNA binding (Hidalgo and Demple, 1997a). As is true for other DNA-binding proteins with high affinity for their target promoters (Ptashne, 1986), the SoxR concentration *in vivo* is kept at a relatively low level (<100 nM) that remains unchanged by oxidative stress (E.Hidalgo, V.Leautaud, and B.Demple, in preparation).

The *soxS* gene encoding the proximal inducer of the individual regulon genes is expressed at only a low level in the absence of the stress (Wu and Weiss, 1992;

Nunoshiba and Demple, 1993; Nunoshiba *et al.*, 1993). Oxidative stress-induced expression of *soxS* occurs upon activation of SoxR protein bound between the –10 and –35 elements of the *soxS* promoter. Activated SoxR powerfully stimulates open complex formation by RNA polymerase (RNAP) by compensating for the abnormally long spacing of the *soxS* promoter elements (19 bp instead of the consensus 17±1 bp in promoters used by σ^{70}-containing RNAP; Hidalgo and Demple, 1997b). The lower affinity of native SoxS protein for its specific DNA targets (K_D values ~10^{-9} M; Li and Demple, 1994, 1996) requires higher SoxS concentrations to be reached in order to achieve transcriptional activation of its target genes. The regulation of *soxS* expression is dynamic: because the half-life of *soxS* mRNA is shortened by oxidative stress, the rapid shut-down of *soxS* transcription after withdrawal of the stress allows the *soxS* mRNA level to fall rapidly (Ding and Demple, 1997).

The *soxR* and *soxS* genes are arranged head-to-head in the *E.coli* chromosome, with their translation start sites lying only 85 bp apart (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991). Transcription of *soxS* begins in the intergenic region both *in vivo* (Wu and Weiss, 1991) and *in vitro* (Hidalgo *et al.*, 1995). Primer extension experiments suggested that *soxR* transcription initiates within the *soxS* structural gene, 200 bp upstream of the *soxR* coding region (Wu and Weiss, 1991). Although the possible transcriptional regulation of *soxR* has not been explored, the opposing polarities and overlapping mRNAs of *soxR* and *soxS* might be expected to affect their expression. We have, therefore, systematically examined *soxR* transcription and its influence on *soxS* expression. Here we demonstrate that the *soxR* and *soxS* promoters are actually overlapping rather than convergent, with their –10 elements positioned on opposite strands at the same site. Furthermore, SoxR binding to the single site that mediates *soxS* activation also represses *soxR* transcription under all conditions. Transcription from the *soxR* promoter may also diminish *soxS* transcription further when SoxR is not activated.

Results

Determination of the in vitro start site for soxR transcription

One potentially interesting feature of *soxRS* regulation arose when the transcriptional start sites of the *soxR* and *soxS* genes were determined (Wu and Weiss, 1991): a predicted 167 nucleotide mRNA overlap led to speculation that the overlapping transcripts and opposing transcriptional polarities could have a regulatory role (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991). Nevertheless, no effect based on such an overlap has yet been reported. We decided to determine the initiation site for *soxR* transcription by primer extension analysis of *soxR* mRNA synthesized *in vitro*, which would circumvent the otherwise low sensitivity of primer extension with total cellular mRNA. The template used in the *in vitro* transcription reactions, pBD100, should allow the synthesis by σ^{70}-containing RNAP of *soxR*, *soxS* and *bla* transcripts (Figure 1A). Primer extension products of *in vitro* transcripts are shown in Figure1B.

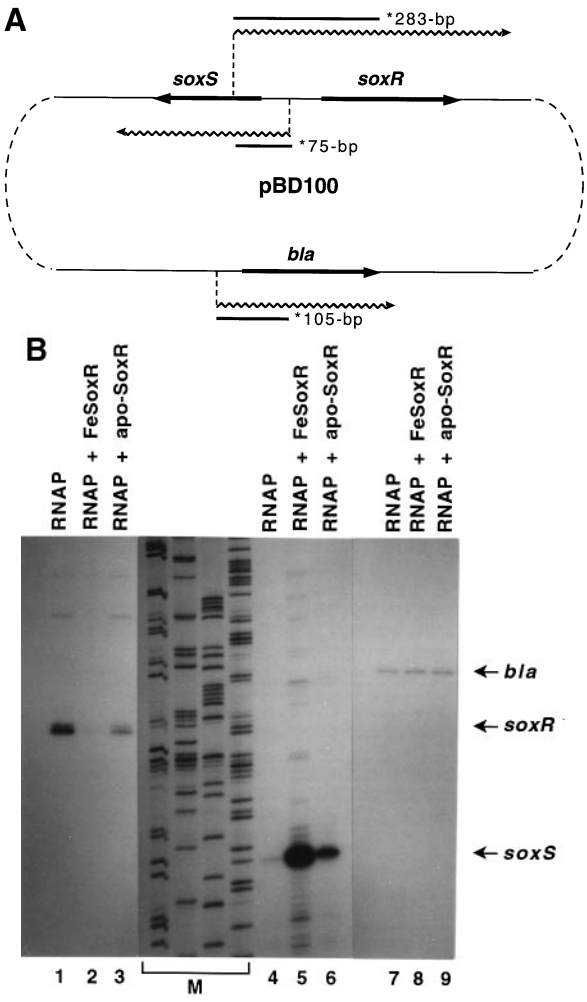

Fig. 1. Transcription of *soxR* and *soxS in vitro*. (**A**) Transcript map of the template plasmid pBD100. The wavy lines indicate *in vitro* transcripts previously identified for the *soxS* and *bla* genes (Hidalgo and Demple, 1997b), and the *in vivo soxR* transcript proposed by Wu and Weiss (1991). The adjacent solid lines indicate the primer extension products and their sizes expected for these transcripts using the primers described in Materials and methods. (**B**) *In vitro* transcription modulated by SoxR protein. Reactions were performed using pBD100 as the template, and σ^{70}-containing RNAP (1 U; ~1 μg) alone or in the presence of transcriptionally active Fe-SoxR (10 ng) or inactive apo-SoxR (10 ng). Three equal aliquots were removed and incubated individually with oligonucleotides that anneal downstream of the *soxR* (lanes 1–3), *soxS* (lanes 4–6) or *bla* (lanes 7–9) transcription start sites to mediate the primer extension reactions (see Materials and methods). Plasmid pEH44 (Hidalgo and Demple, 1997b) was sequenced using the T7 primer (New England Biolabs), and the products were used as size markers (M). The positions of the primer extension products of the *soxS*, *soxR* and *bla* transcripts are indicated.

Transcription of the *bla* gene was constant whether SoxR protein was present or not (Figure 1B, lanes 7–9). As previously shown, the amount of *soxS* mRNA was strongly dependent on the presence of active Fe-SoxR (Hidalgo and Demple, 1994; Figure 1B, lanes 4–6); the smaller *soxS* activation by apo-SoxR depends on the topological state of the DNA template and has been explored in work to be reported elsewhere (E.Hidalgo and B.Demple, in preparation). The previously reported initiation site for *soxR* at position –212 (Wu and Weiss, 1991) would generate a 283 nucleotide

product from primer 2 (Figure 1A). Instead, a major 93 nucleotide product was generated by *in vitro soxR* transcription (Figure 1B, lane 1). This observation located the *soxR* transcription start site at position –22. The same *soxR* initiation site was determined using two other oligonucleotides for primer extension (data not shown). Consistent with this assignment, close to consensus –10 and –35 boxes are present at suitable distances from this initiation site (see Figure 5A below). Furthermore, the effect of SoxR protein on *soxR* transcription *in vitro* was the inverse of its effect on *soxS*: addition of Fe-SoxR completely inhibited the transcription of *soxR* (Figure 1B, lane 2), and apo-SoxR seemed to have a partial inhibitory effect (Figure 1B, lane 3).

In vivo activity of the soxR promoter fused to the lacZ gene

According to the *in vitro* experiments described above, the SoxR protein seems to play an inhibitory role on the expression of the *soxR* gene. In a first attempt to confirm whether SoxR represses transcription of its own gene *in vivo*, we fused a 0.35 kb DNA fragment of the *soxRS* locus to the *lacZ* reporter gene (see Materials and methods; Figure 2A). This fragment contained both the previously reported initiation site and the newly determined site (designated, respectively, '1' and '2' in Figure 2A). The fusion was inserted in the chromosome of strains GC4468 (Δ*lac soxRS*$^+$) and DJ901 (Δ*lac* Δ*soxRS*) to yield strains VL20 and EH200, respectively. The first indication that the level of expression of the *soxR′::lacZ* fusion depended on SoxR was the different behavior of the two strains on MacConkey–lactose media. In principle, only strains producing significant levels of β-galactosidase from the operon fusions should utilize sufficient lactose to modify the pH of the medium and convert the indicator dyes to their red color. In this assay, VL20 (*soxRS*$^+$, *soxR′::lacZ*) was scored as Lac$^-$ due to a very low level of expression of the *soxR′::lacZ* reporter, in contrast to EH200 (Δ*soxRS*, *soxR′::lacZ*) (Figure 2B, left panel). However, in X-Gal plates, the level of β-galactosidase expressed in VL20 cells was actually sufficient to register them as Lac$^+$ (compared with the Δ*lac* strain DJ901; Figure 2B, right panel). Thus, the ~22 units of β-galactosidase expressed in VL20 are below the limit of detection on MacConkey–lactose. A more detailed analysis of the ability of these media to distinguish Lac$^+$ from Lac$^-$ bacteria has shown that the sensitivity threshold for X-Gal corresponded to expression of ~9 Miller units of β-galactosidase, determined by direct activity assay of culture samples from liquid media (Miller, 1992), whereas the MacConkey indicators required at least ~36 Miller units to score as Lac$^+$ (data not shown).

We then transformed EH200 with an expression vector, pSE380, or its derivatives pSXR and pSXS, which contain, respectively, the *soxR* and the *soxS* genes (Amábile-Cuevas and Demple, 1991). Strain EH200 carrying the vector pSE380 (Figure 2B, left panel) or pSXS (data not shown) was still Lac$^+$ on MacConkey–lactose plates. This result implies that the previously reported SoxS autorepression (Nunoshiba *et al.*, 1993) does not affect *soxR* transcription. However, EH200 carrying pSXR was Lac$^-$ in MacConkey–lactose (Figure 2B, left panel). This result confirms that SoxR represses transcription of its own gene *in vivo*.

The degree of *soxR* autorepression was quantified by measuring the β-galactosidase activity of VL20 and EH200 cells grown in liquid cultures. As seen in Figure 2C, wild-type levels of SoxR repressed expression of the *soxR* promoter by ~20-fold. Treatment with PQ to convert SoxR to the activated state had little effect on repression of *soxR* in strain VL20 (Figure 2C).

Comparing the expression of the *soxR′::lacZ* reporter inserted in strains VL20 and EH200 demonstrated autoregulation by SoxR, but did not address the location of the *soxR* transcriptional start site because that fusion contained the entire upstream region well into the *soxS* gene (Figure 2A). We therefore constructed a new series of *lacZ* fusions with a promoter fragment containing only 79 bp of DNA upstream from the *soxR* ATG start codon, yielding strains EH40R (*soxR*$^+$) and EH46R (Δ*soxR*) (Figure 3A). The same fragment in the opposite orientation places the *lacZ* reporter gene under the control of the *soxS* promoter (Hidalgo and Demple, 1997b; Figure 3A). As found for VL20, EH40R was Lac$^-$ on MacConkey–lactose plates, whereas EH46R, like EH200, was clearly Lac$^+$; plasmid complementation demonstrated that SoxR was responsible for the Lac$^-$ phenotype on MacConkey–lactose plates (data not shown). We determined β-galactosidase activities in liquid cultures of strains EH40 (*soxR*$^+$, *soxS′::lacZ*), EH40R (*soxR*$^+$, *soxR′::lacZ*) and EH46R (Δ*soxR*, *soxR′::lacZ*) transformed with either vector alone (pSE380) or the SoxR expression vector pSXR. As reported previously (Hidalgo and Demple, 1997b), there was an ~30-fold, SoxR-dependent induction of the *soxS′::lacZ* fusion in strain EH40 following PQ treatment (Figure 3B). The basal and induced β-galactosidase levels directed by the *soxR′::lacZ* fusion in strain EH40R were both 9 Miller units. The lack of SoxR in strain EH46R-pSE380 derepressed the *soxR′::lacZ* fusion ~20-fold and, once again, providing plasmid-encoded SoxR (in EH46R-pSXR) repressed the fusion (Figure 3B).

In vivo quantitation of the soxR transcript

In order to confirm the newly determined transcriptional start site of *soxR* and the autorepression exerted by its gene product, we initially attempted primer extension analysis using total mRNA, but without success (data not shown). The low level expression of *soxR* reported by the operon fusions described above is consistent with these negative results. We instead quantified the *soxR* transcript in total cellular mRNA by Northern blot analysis. As shown in Figure 4A, TN531 has a truncated *soxR* gene: any residual gene product from this construct is unable to activate transcription of *soxS in vivo* (Nunoshiba *et al.*, 1992; Nunoshiba and Demple, 1993). We used as a probe a DNA fragment containing the 5′ half of *soxR*, which would detect both wild-type and truncated *soxR* transcripts. For the start site reported by Wu and Weiss (1991), these would be molecules of 0.7 and 0.4 kb from the intact and truncated *soxR* genes, respectively (Figure 4A). The intergenic initiation site was expected to yield products of 0.5 kb (intact *soxR*) and 0.2 kb (truncated *soxR*) (Figure 4A). After visualization of marker RNAs (1.77, 1.52, 1.28, 0.78, 0.53, 0.40, 0.28 and 0.15 kb) with methylene blue, the filters were hybridized with a *soxS*-specific probe as

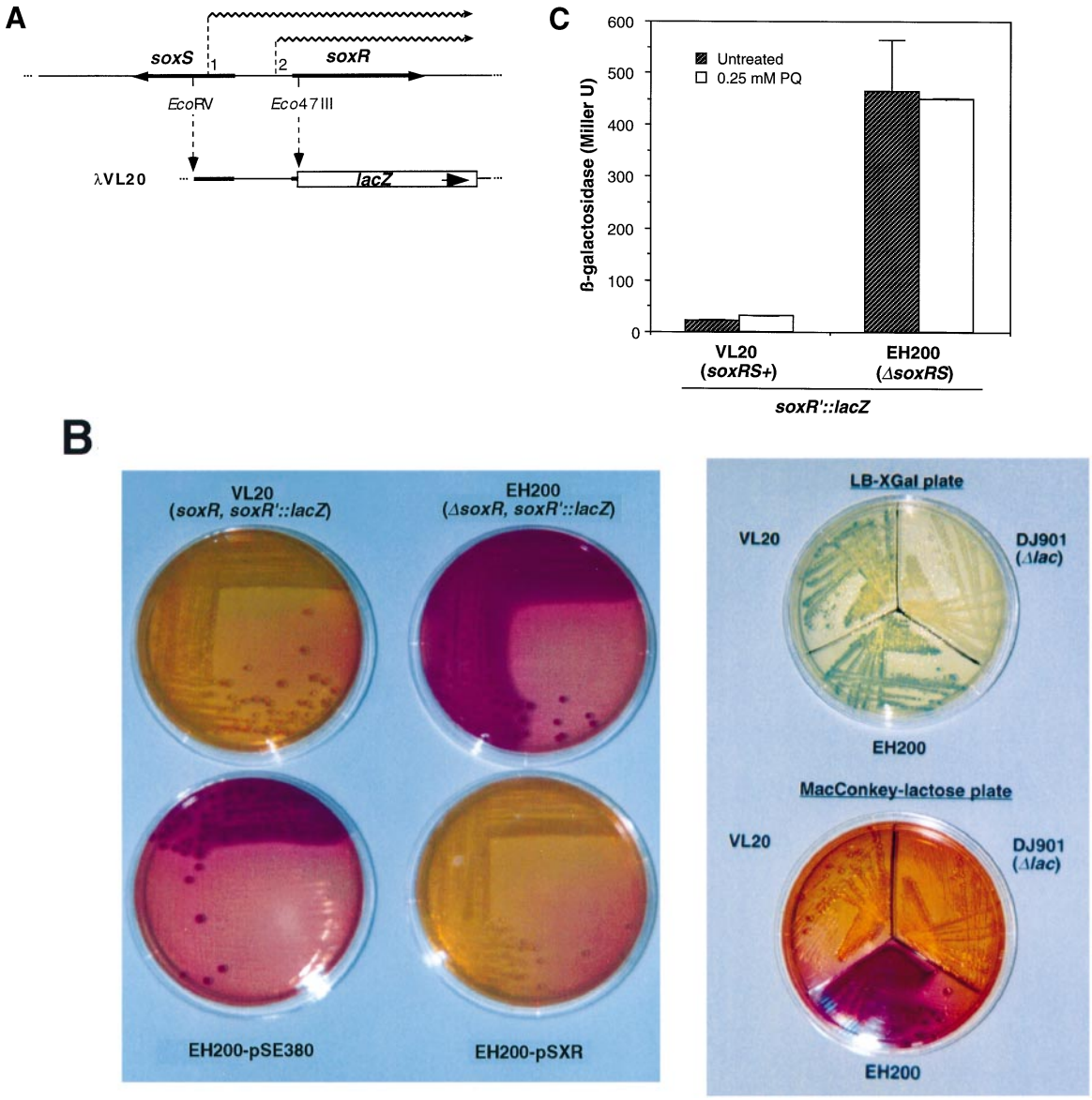

Fig. 2. SoxR represses transcription of its own gene *in vivo*. (**A**) Construction of *soxR′::lacZ* operon fusions. A 0.35 kb fragment containing the *soxR* promoter with the two putative transcription initiation sites (labeled 1 and 2) was fused to *lacZ*, and a phage λ derivative (λVL20) inserted in the chromosome of GC4468 (*soxRS*$^+$; to yield strain VL20) or DJ901 (Δ*soxRS*; yielding strain EH200). (**B**) Lac phenotypes of VL20 and EH200 on solid media. Strains VL20, EH200 and EH200 transformed with the SoxR expression vector pSXR or with the parent vector (pSE380) were spread on MacConkey–lactose plates and incubated for 15 h (left panel). Strains VL20, EH200 and DJ901 (control for no β-galactosidase activity) were also spread on LB-X-Gal plates (upper right) or MacConkey–lactose plates (lower right). (**C**) The β-galactosidase level of VL20 and EH200 grown in liquid cultures. The activity of the *soxR′::lacZ* reporter fusion (λVL20) was measured in the absence (untreated) or presence of PQ, and is indicated in Miller units (Miller, 1992). The *soxRS* genotype of the strains is shown; both contain the *soxR′::lacZ* fusion.

a positive control for the *in vivo* activation of SoxR protein by PQ. As expected, only strains containing full-length *soxR* showed PQ-inducible expression of the *soxS* or *soxS′::lacZ* transcripts (Figure 4B, lanes 2 and 6). The blots were then stripped and re-hybridized with the *soxR*-specific probe. For both strain GC4468 (Figure 4B, lanes 1 and 2) and strain TN521 (Figure 4B, lanes 5 and 6), this analysis revealed a *soxR* transcript of 0.5 kb, as estimated against co-electrophoresed size markers. This result is consistent with an *in vivo* transcription start site for *soxR* in the intergenic region between *soxR* and *soxS*, rather than within *soxS* gene (see Figure 4A). A 2-fold induction of *soxR* mRNA by PQ (lanes 2 and 6) compared with untreated cells (lanes 1 and 5) detected in both strains was consistent with a similar small effect on expression of the two *soxR′::lacZ* fusions described above (strain VL20, Figure 2C; strain EH40R, Figure 3B).

Consistent with the autorepression model, strain TN531, lacking a functional SoxR, showed an ~20-fold derepression (Figure 4B, lanes 7 and 8) relative to TN521 (Figure 4B, lanes 5 and 6). These strains produced a shorter *soxR* transcript (Figure 4B), as expected if transcription of the truncated gene begins in the intergenic region and utilizes the bi-directional terminator present in the fusion vector (see Figure 4A).

Effect of divergent soxR transcription on soxS transcription

The newly determined *soxR* transcription initiation site indicates that the *soxR* and *soxS* promoters are interdigit-

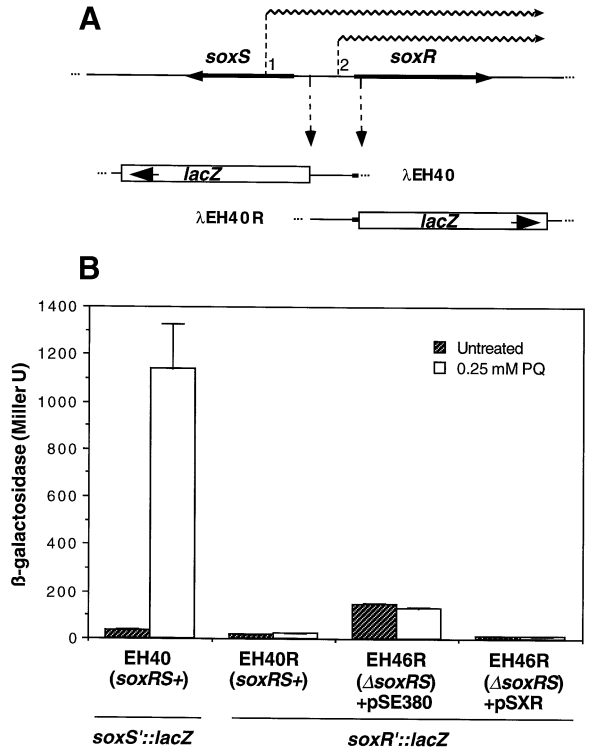

Fig. 3. Regulated expression of *soxR* from a proximal promoter. (**A**) Construction of a smaller *soxR′::lacZ* fusion. A 0.14 kb insert containing only the newly determined *soxR* transcriptional start site (indicated as 2), but lacking the previously reported site (1), was fused to *lacZ*, and the λ derivative (λEH40R) inserted into a *soxRS*$^+$ background (yielding strain EH40R) or into a Δ*soxRS* strain (to yield EH46R). A similar construct, with the same fragment fused in the opposite orientation to *lacZ*, has been characterized as a reporter of *soxS* expression (Hidalgo and Demple, 1997b); insertion of its λ derivative (λEH40) in a *soxRS*$^+$ background yielded strain EH40. (**B**) β-Galactosidase assays in liquid cultures. The activity of *soxS′::lacZ* (EH40) and *soxR′::lacZ* (EH40R, and EH46R transformed with pSE380 or the SoxR expression plasmid pSXR) was measured with or without treatment with PQ. The *soxRS* genotypes, plasmids and fusions present in the strains are shown.

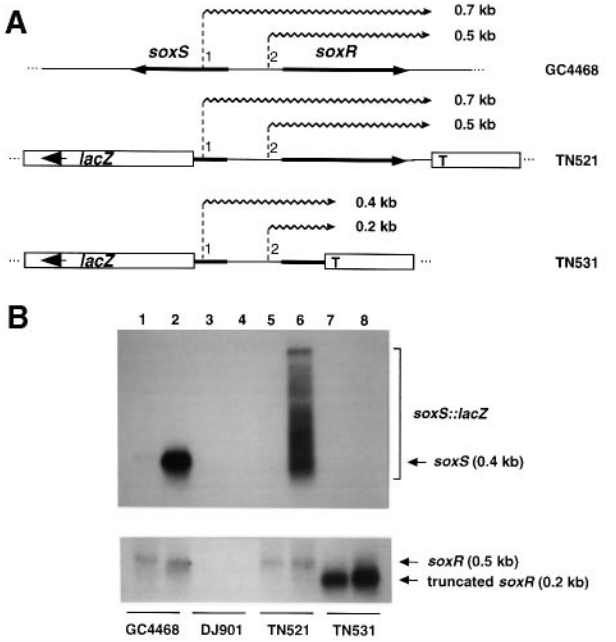

Fig. 4. Northern blot analysis of *soxR* expression. (**A**) Scheme of the putative *soxR* transcripts from different genetic backgrounds. Depending on whether *soxR* transcription starts at position 1 [as reported by Wu and Weiss (1991)] or at position 2 (this work), the two possible sizes for the *soxR* transcript are indicated for strains GC4468, TN521 or TN531. For strain TN531, the expected transcripts are ~0.3 kb shorter because the truncated *soxR* gene in this construct is fused to a terminator sequence (T) in the vector. (**B**) Total RNA from strains GC4468 (lanes 1 and 2), DJ901 (Δ*soxRS*; lanes 3 and 4), TN521 (lanes 5 and 6) and TN531 (lanes 7 and 8) was obtained. The bacteria had been grown for 60 min in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of PQ. After blotting, the filters were hybridized with *soxS*-specific (upper panel) or *soxR*-specific (lower panel) probes. The approximate sizes of the transcripts are indicated in parentheses, determined using molecular weight markers run in parallel (see Materials and methods). These markers electrophoresed as follows (distance from origin): 1.77 kb (5.0 cm), 1.52 kb (5.3 cm), 1.28 kb (5.6 cm), 0.78 kb (6.4 cm), 0.53 kb (7.0 cm), 0.40 kb (7.4 cm), 0.28 kb (7.8 cm) and 0.15 kb (8.4 cm). The migration of the *soxR* transcript (7.0 cm) corresponds to a size of 0.53 kb, shown as 0.5 kb on the figure; the migration of the *soxS* transcript (7.3 cm) corresponds to 0.41 kb, shown as 0.4 kb on the figure.

ated (Figure 5A). Evidently, binding of SoxR to potentiate activation of the *soxS* gene simultaneously inhibits transcription of *soxR*, because the SoxR-binding site (Hidalgo and Demple, 1994) covers the *soxR* transcription initiation site (see Figure 5A). We tested whether the presence of the divergent *soxR* promoter affects the expression of *soxS*.

A new *soxS′::lacZ* fusion deleted for the –35 site of the *soxR* promoter (Figure 5A) was constructed and inserted in a *soxRS*$^+$ strain (yielding EH120; see Table I) and in a Δ*soxRS* strain (EH126; see Table I). Expression of this truncated fusion was compared with that in strains EH40 and EH46, in which the *soxS′::lacZ* fusion contained the whole *soxR* promoter (Figures 3 and 5A). In the presence of SoxR (in EH120), the modified fusion was still inducible by PQ, although to a 2-fold lower level than seen for the unmodified promoter (in EH40; Figure 5B). Most notably, however, there was a 2-fold increase in the basal expression ('Untreated'; Figure 5B) of the truncated compared with the intact *soxS* promoter. Essentially the same elevated basal expression was measured for the truncated *soxS* promoter in the *soxR*$^+$ strain (EH120) and the Δ*soxR* strain (EH126; Figure 5B), which is not expected if the interference is due to the binding of SoxR protein to its target site in the *soxS* promoter (Figure 5A). Therefore, divergent transcription of the *soxR* gene seems to modulate the activity of the *soxS* promoter.

Discussion

The *soxRS* genes govern the expression of an oxidative stress response, with the expression of the SoxS activator depending on the transcription-stimulating activity of SoxR. Here we show that the expression of the *soxR* gene is itself regulated by SoxR, which is a new function for this protein. Revised mapping of the *soxR* transcription initiation site now establishes that the *soxR* promoter overlaps that of *soxS*. Binding of SoxR to its DNA site in the *soxS* promoter, which is essential for activating *soxS* transcription, inhibits RNAP from initiating transcription of *soxR*.

We have shown here that the *soxR* and *soxS* promoters are not only divergently transcribed, but also have overlapping elements. Both activated and non-activated SoxR have high affinity for the DNA-binding site in the *soxS*

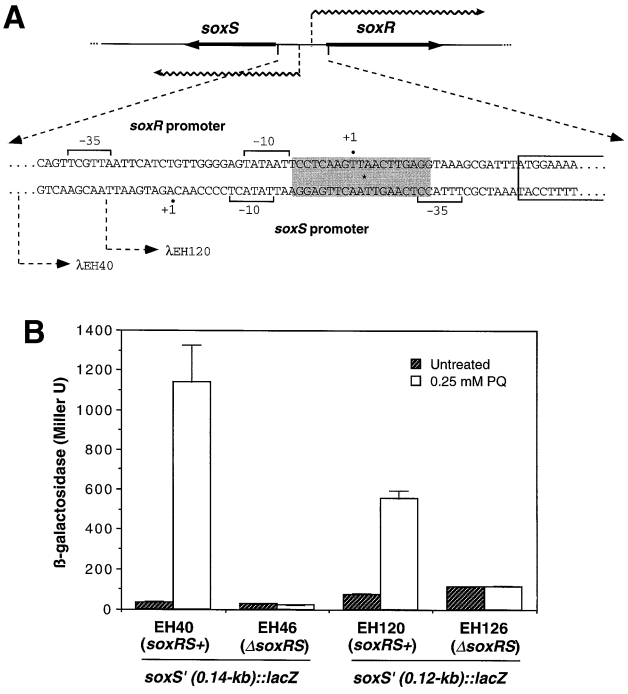

Fig. 5. Influence of the *soxR* promoter on *soxS* expression. (**A**) Construction of a Δ*soxR′*, *soxS′::lacZ* fusion. The positions of the *soxR* (upper strand) and *soxS* (lower strand) transcription start sites (+1), and the respective –10 and –35 boxes are indicated on the sequence shown. The shaded box corresponds to the SoxR-binding site (Hidalgo and Demple, 1994); the asterisk indicates the center of dyad symmetry in this site. The beginning of the *soxR* coding sequence is indicated by an open box. A fragment containing the *soxS* promoter, as well as the whole *soxR* promoter in the opposite strand, previously had been fused to *lacZ* (λEH40; Hidalgo and Demple, 1997b). The new construct (λEH120) excludes the –35 box of the *soxR* promoter. The fusions were inserted in a wild-type background (yielding strains EH40 and EH120, respectively) or in a Δ*soxRS* background (yielding strains EH46 and EH126, respectively). (**B**) β-Galactosidase assays of bacteria grown in liquid cultures. The activities of the *soxS′::lacZ* fusions were measured after 60 min growth in the absence (untreated) or the presence of PQ. The *soxRS* genotypes, and the type of *soxS′::lacZ* reporter fusion present, are shown.

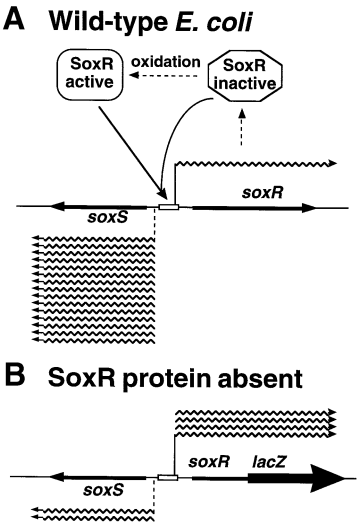

Fig. 6. A model for SoxR-mediated activation and repression from a single site. (**A**) Under inducing conditions, only activated SoxR triggers transcription of *soxS*; both active and inactive SoxR bind to its DNA site (white box) and strongly repress transcription of the *soxR* gene. (**B**) In the absence of a functional SoxR, *soxS* transcription remains low even under inducing conditions, whereas *soxR* transcription is constitutively de-repressed.

promoter (Hidalgo and Demple, 1994; Gaudu and Weiss, 1996). Footprinting studies (Hidalgo and Demple, 1994; Hidalgo *et al.*, 1995) provide no indication of a second SoxR-binding site in the *soxR–soxS* region investigated here. Thus, without changing its occupancy of this single operator site, SoxR simultaneously exerts constant repression of one gene and conditional activation of the neighboring gene. The polarity of the dual repressor/regulated activator functions of SoxR and the homologous MerR protein (Summers, 1992) seems to constitute a unique example of gene regulation through a single binding site.

Transcriptional activation or repression is often based on enhancing or impairing, respectively, RNAP interactions with target promoters (Collado-Vides *et al.*, 1991; Dove *et al.*, 1997). Interference with the productive interactions, for example by steric hindrance by a bound protein, may be achieved more simply than enhancing productive interactions of RNAP with a promoter. A detailed analysis (Gralla and Collado-Vides, 1996) of *E.coli* transcriptional activators indicated that most can repress transcription of their own or other genes under physiological conditions when the protein-binding sites are located within an 'exclusive zone of repression' downstream of –30. The SoxR protein fits this pattern: it occludes *soxR* transcription by covering the transcriptional start site. However, it now appears that the proposed 'activators-forbidden' zone (Gralla and Collado-Vides, 1996) should be restricted to the region downstream of the –10 element: both SoxR and the homologous MerR protein (Summers, 1992) exert powerful activation from binding sites located between the –10 and –35 elements of their target promoters.

The transcriptional activation mechanism of the SoxR/MerR family of regulators may result from structural distortion of DNA (Ansari *et al.*, 1995; Hidalgo *et al.*, 1995), although a role for protein–RNAP interactions has not been eliminated. It is also important to determine why non-activated SoxR does not repress transcription of *soxS*, even though the protein occupies a site more typical of repressors. In fact, the simple deletion of 2 bp from the –10/–35 spacer in the *soxS* promoter converts SoxR into a repressor that interferes with RNAP binding (Hidalgo and Demple, 1997b). Therefore, natural selection solved two opposing problems in evolving SoxR as an activator: enhancing initiation by RNAP when SoxR is activated, without impairing RNAP binding when the bound SoxR is in the non-activated form.

SoxR autorepression is a feature shared with many prokaryotic transcriptional activators that repress transcription of their own genes (Gralla and Collado-Vides, 1996). Autorepression allows the amount of a regulator to be held within fixed limits: an excess will immediately reduce its own synthesis, a deficiency will allow increased synthesis. Such a mechanism is probably more effective at maintaining a relatively constant, low-level expression than just having a poor promoter, which cannot compensate when the level of the gene product falls too low.

The autorepression exerted by SoxR is almost constant, whether or not SoxR is activated by superoxide stress (see Figures 2C and 3B). Overexpression of SoxR reduces this

Table I. Strains used in this work

Strain	Genotype	Source/reference
DJ901	Δ*soxRS* derivative of GC4468	Greenberg *et al.* (1990)
EH40	GC4468 lysogenized with λEH40 (see Figure 5A)	Hidalgo and Demple (1997b)
EH40R	GC4468 lysogenized with λ(*soxR′::lacZ*) fusion (0.15 kb promoter)	this work
EH46	DJ901 lysogenized with λEH40 (see Figure 5A)	Hidalgo and Demple (1997b)
EH46R	DJ901 lysogenized with λ(*soxR′::lacZ*) fusion (0.15 kb promoter)	this work
EH120	GC4468 lysogenized with λEH120 (see Figure 5A)	this work
EH126	DJ901 lysogenized with λEH120 (see Figure 5A)	this work
EH200	DJ901 lysogenized with λ(*soxR′::lacZ*) fusion (0.35 kb promoter)	this work
GC4468	K12 *rpsL thi soxR*$^+$ *soxS*$^+$	Greenberg *et al.* (1990)
MC4100	F′Δ*lacU169 araD139 thiA rpsL relA*)	Casadaban (1976)
TN521	DJ901 lysogenized with λ(*soxR*$^+$, *soxS′*^{b}*::lacZ*) fusion	Nunoshiba and Demple (1993)
TN531	DJ901 lysogenized with λ(Δ*soxR, soxS′::lacZ*) fusion	Nunoshiba and Demple (1993)
VL20	GC4468 lysogenized with λ(*soxR′::lacZ*) fusion (0.35 kb promoter)	this work

repressed level only a further <2-fold (from 16 to 9 Miller units; see Figure 3B). These observations indicate that the SoxR DNA-binding site is nearly fully occupied *in vivo*, in accord with previous conclusions (Hidalgo and Demple, 1997b). Nevertheless, we observed a small (<2-fold) derepression of *soxR* in PQ-treated cells (Figure 4B; see text). This effect could be due to subtle changes in the SoxR–DNA contacts upon activation, for which there is some evidence from footprinting assays (Hidalgo and Demple, 1994; Hidalgo *et al*., 1995).

The *soxS* and *soxR* promoters constitute clear examples of how densely packed genetic information can be in *E.coli*, with overlapping promoter elements and a unique binding site for both repression and inducible activation. This dense packing seems ultimately to maximize the responsiveness of *soxS* to induction: elimination of the *soxR* promoter increased the basal level of *soxS* transcription and decreased its induced level (Figure 5B), changing the induction ratio from a typical ~30-fold to ~7-fold. Changes in *soxS* promoter DNA structure resulting from *soxR* transcription may underlie these effects. Possible mechanisms include the supercoiling produced by actively transcribing RNAP (Liu and Wang, 1987) and contacts between SoxR and RNAP bound to the *soxR* promoter on the same face of the DNA (Figure 5A).

Materials and methods

In vitro transcription and primer extension

In vitro transcription reactions were performed following a procedure previously described (Hidalgo and Demple, 1996). The DNA template used, plasmid pBD100 (Amábile-Cuevas and Demple, 1991), contains both the *soxR* and *soxS* genes, and the *bla* gene encoding β-lactamase, which acted as an internal control for RNAP (Epicentre Technologies) activity *in vitro*. The *soxS* transcript, the *soxR* transcript and the control *bla* transcript were quantified by primer extension with, respectively, primer soxS-1 (Hidalgo and Demple, 1996; the extended product on *soxS* mRNA has an expected size of 75 bp), primer 2 (located 283 bp downstream from the *soxR* initiation site reported by Wu and Weiss, 1991) and primer pBR-1 (Hidalgo *et al*., 1995; extension from this primer on *bla* mRNA yields a product of 105 bp).

Construction of soxR′::lacZ operon fusions

A blunt-ended *Eco*47III–*Eco*RV fragment from plasmid pCA2710 (Amábile-Cuevas and Demple, 1991) containing the *soxR* promoter (from –314 to +29 bp, +1 being the first base pair of the initiator methionine codon of *soxR*) was ligated into *Eco*RV-digested pRS550, a pBR322-derived vector designed to prepare operon fusions directing the expression of *lacZ* and *lacY* (Simons *et al.*, 1987). The ligation mixture was transformed into DH5α cells, and pVL20 was isolated. All the pRS550 constructs described herein were confirmed by sequencing the inserts with primer M13-40 (New England Biolabs), which hybridizes within the vector sequence and allows sequencing of the upstream cloned promoter. A *recA*$^+$ strain, MC4100 (Table I), was transformed with pVL20. Bacteriophage λRS45 was used to transfer the *soxR′::lacZ* fusions from MC4100 with pVL20, into the chromosome of GC4468 (*soxRS*$^+$; Greenberg *et al*., 1990), as described by Simons *et al*. (1987). The resulting GC4468-derived lysogen, strain VL20 (Table I), was confirmed as described previously (Hidalgo and Demple, 1997b). We used UV induction (Silhavy *et al*., 1984; Hidalgo and Demple, 1997b) to generate phage lysates of VL20. Lysogens were then obtained by infecting DJ901 (Table I) with the fusion phage lysate (Simons *et al.*, 1987) and identification of Lac$^+$ colonies on MacConkey–lactose agar (Difco), to yield strain EH200 (Table I). The DJ901-derived strains were transformed with the SoxR expression vector pSXR, the SoxS expression vector pSXS and the control vector pSE380 (Amábile-Cuevas and Demple, 1991).

A smaller version of the *soxR* promoter (from –79 to + 63, +1 being the translation initiation base of *soxR*) was fused to *lacZ* by releasing the *soxS* promoter insert of pEH40 (Hidalgo and Demple, 1997b) with *Eco*RI and *Bam*HI and further ligation with *Eco*RI–*Bam*HI-digested pRS550 to yield plasmid pEH40R (this construction simply reverses the orientation of the promoter fragment; see Figure 3A). The procedure described above was then used to generate lysogens of this *soxR′::lacZ* fusion inserted in the chromosomes of GC4468 and DJ901, yielding strains EH40R and EH46R, respectively (Table 1). Again, the DJ901-derived strain, EH46R, was transformed with pSE380 and its derivatives pSXR and pSXS (Amábile-Cuevas and Demple, 1991).

A small fragment containing the wild-type *soxS* promoter (from base pair –108 to base pair +9, with +1 being the *soxS* transcriptional start site; Figure 5A) was amplified by the polymerase chain reaction (PCR) from pBD100 (Amábile-Cuevas and Demple, 1991), using primers E (5′-GTTCAGTTCGTGAATTCATC-3′) and X (5′-ATGAATTCTGCGTTTCGCCACTTCG-3′), each including an *Eco*RI site (underlined). The fragment was purified, digested and subcloned into *Eco*RI-digested pRS550 as described previously (Hidalgo and Demple, 1997b), to yield pEH120. Using the procedure described above, this *soxS′::lacZ* fusion was inserted into the chromosomes of GC4468 and DJ901, resulting in strains EH120 and EH126, respectively (Table I).

Isolation and analysis of total mRNA

Total mRNA was obtained from 2 ml of exponentially growing cells using a commercial kit (RNeasy; Qiagen), following the manufacturer's instructions. The amounts of *soxR* and *soxS* transcripts were quantified by primer extension as described above, or by Northern blot analysis. Total RNA from different strains, as well as 5 μg of a 0.15–1.77 kb RNA ladder as a molecular weight marker (Life Technologies) were loaded on formaldehyde-containing, 1.5% agarose gels (Ausubel *et al.*, 1987), electrophoresed, and transferred to nylon membranes (GeneScreen Plus; New England Biolabs) using a Turboblotter (Schleicher and Schuell). After staining the nylon membranes with methylene blue as indicated by the manufacturer to visualize the molecular weight markers and rRNA (a loading control), the blots were hybridized with *soxS*- or *soxR*-specific probes previously labeled with a random primer system (Life Technologies). A *soxS*-specific probe was prepared by PCR amplifying the whole *soxS* gene from the pBluescript derivative pSOXS

(Amábile-Cuevas and Demple, 1991) with primers T3 and T7 (Stratagene). A *soxR* probe was amplified similarly from pSOXR (Amábile-Cuevas and Demple, 1991), then column purified (Qiagen), digested with *Bsm*I and *Kpn*I, electrophoresed on a 2% agarose gel, and the ~0.25 kb fragment containing the 5′ half of *soxR* isolated with QIAquick (Qiagen).

Determining β-galactosidase expression from operon fusions

The ability of the different lysogens to metabolize lactose was determined by spreading the cells either on MacConkey–lactose agar plates (Difco) or on LB agar plates (Miller, 1992) containing 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). To quantify the β-galactosidase levels in liquid media, lysogens containing the different operon fusions were inoculated into LB broth (Miller, 1992) containing 30 μg/ml kanamycin and 30 μg/ml streptomycin, and incubated at 37°C for ~16 h with vigorous shaking. Inocula from these overnight cultures were diluted 100-fold into 1 ml of fresh medium in duplicate tubes and incubated at 37°C for exactly 60 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 with shaking. The samples were then placed on ice. β-Galactosidase activity in SDS–$CHCl_3$-treated cells was determined as described by Miller (1992).

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