The OxyS regulatory RNA represses *rpoS* translation and binds the Hfq (HF-I) protein

Aixia Zhang, Shoshy Altuvia[1], Anita Tiwari, Liron Argaman[1], Regine Hengge-Aronis[2] and Gisela Storz[3]

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA, [1]Department of Molecular Genetics and Biotechnology, The Hebrew University-Hadassah Medical School, 91120 Jerusalem, Israel and [2]Department of Biology, University of Konstanz, 78434 Konstanz, Germany

[3]Corresponding author
e-mail: storz@helix.nih.gov

The OxyS regulatory RNA integrates the adaptive response to hydrogen peroxide with other cellular stress responses and protects against DNA damage. Among the OxyS targets is the *rpoS*-encoded σ^s subunit of RNA polymerase. σ^s is a central regulator of genes induced by osmotic stress, starvation and entry into stationary phase. We examined the mechanism whereby OxyS represses *rpoS* expression and found that the OxyS RNA inhibits translation of the *rpoS* message. This repression is dependent on the *hfq*-encoded RNA-binding protein (also denoted host factor I, HF-I). Co-immunoprecipitation and gel mobility shift experiments revealed that the OxyS RNA binds Hfq, suggesting that OxyS represses *rpoS* translation by altering Hfq activity.
Keywords: Hfq protein/oxidative stress/OxyS RNA/ RNA-binding protein/*rpoS*

Introduction

During exponential growth, the expression of several hydrogen peroxide-inducible proteins is controlled by the transcriptional regulator, OxyR. We recently showed that OxyR also induces the expression of an abundant, 109 nucleotide, untranslated regulatory RNA, denoted OxyS (Altuvia *et al.*, 1997). This small RNA acts as an anti-mutator and a regulator of as many as 40 genes in *Escherichia coli*. One target of OxyS repression is the *rpoS*-encoded σ^s subunit of RNA polymerase. σ^s levels are normally increased during transition into stationary phase or upon starvation, as well as under other stress conditions such as acid shock and osmotic upshift (for reviews, see Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1996). The increased levels of σ^s lead to the induction of genes that have diverse functions including protection against DNA damage, resistance against oxidative, thermal, acid and osmotic stresses, as well as virulence in *Salmonella*. We found that the OxyS RNA represses *rpoS* expression. In strains carrying deletions of *oxyS*, an *rpoS–lacZ* fusion is derepressed after treatment with hydrogen peroxide. Since σ^s and the OxyR transcrip-

tion factor activate some of the same antioxidant genes, we proposed that OxyS RNA repression of *rpoS* evolved to prevent redundant induction of oxidative stress genes (Altuvia *et al.*, 1997).

The expression of *rpoS* is tightly regulated. *rpoS* transcription and translation, and σ^s protein stability are all modulated in response to many different signals, including ppGpp, homoserine lactone, cAMP and UDP-glucose (Loewen and Hengge-Aronis, 1994; Hengge-Aronis, 1996). Multiple regulators of *rpoS* expression have been identified. The RNA-binding protein Hfq, also denoted host factor I (HF-I), was recently found to be essential for *rpoS* translation (Brown and Elliott, 1996; Muffler *et al.*, 1996b). The DsrA RNA is also required for *rpoS* translation at low temperature (Sledjeski *et al.*, 1996). The histone-like protein H-NS has been shown to both repress *rpoS* translation and destabilize the σ^s protein (Barth *et al.*, 1995; Yamashino *et al.*, 1995). In addition, the response regulator RssB and the ClpX/ClpP protease are required for σ^s degradation (Muffler *et al.*, 1996a; Pratt and Silhavy, 1996; Schweder *et al.*, 1996).

We investigated the mechanism of OxyS regulation of *rpoS* and found that the small RNA represses *rpoS* at a post-transcriptional level. We also discovered that OxyS repression of *rpoS* translation requires Hfq and that the OxyS RNA binds the Hfq protein *in vivo* and *in vitro*. These results have led us to propose that OxyS binding alters Hfq activity and thereby prevents *rpoS* mRNA translation.

Results

Several OxyS-regulated genes are also controlled by σ^s

We previously observed that several OxyS-regulated genes are induced in stationary phase (Altuvia *et al.*, 1997). Therefore, we examined whether these genes are regulated by σ^s and whether OxyS repression of these targets is mediated by σ^s. We compared the expression of *fhlA–lacZ*, *yhiV–lacZ*, *yhiM–lacZ*, *gadB–lacZ* and *dps–lacZ* fusions in wild-type and *rpoS*::Tn*10* mutant backgrounds, and examined the effects of constitutively expressing OxyS (poxyS) (Table I). For the strains carrying the pKK177-3 vector control, the activities of all of the fusions were lower in the *rpoS* mutant background compared with the wild-type cells; therefore, σ^s is required for expression of these genes. The extent of OxyS repression of the *fhlA–lacZ* fusions was identical in the wild-type and *rpoS*::Tn*10* mutant backgrounds, indicating that OxyS repression of *fhlA* is not mediated by σ^s. In contrast, OxyS repression of *yhiV*, *yhiM*, *gadB* and *dps* was significantly decreased in the mutant backgrounds, suggesting that OxyS represses these genes, in part, by decreasing *rpoS* expression. We still observed some OxyS repression of *yhiV–lacZ* and

Table I. Effect of *rpoS*::Tn*10* on OxyS-regulated genes

Strain	β-galactosidase activity[a]		
	pKK177-3[b]	poxyS[b]	Fold repression
fhlA–lacZ	56 ± 4	1.0 ± 0.5	56×
fhlA–lacZ rpoS::Tn*10*	27 ± 1	0.5 ± 0.2	54×
yhiV–lacZ	147 ± 19	0.1 ± 0.1	1470×
yhiV–lacZ rpoS::Tn*10*	4 ± 1	0.1 ± 0.1	40×
yhiM–lacZ	667 ± 56	0.5 ± 0.1	1334×
yhiM–lacZ rpoS::Tn*10*	0.7 ± 1.0	14 ± 4	
gadB–lacZ	28 855 ± 2247	135 ± 58	214×
gadB–lacZ rpoS::Tn*10*	211 ± 40	15 ± 4	14×
dps–lacZ	40 967 ± 4580	15 948 ± 9868	3×
dps–lacZ rpoS::Tn*10*	2038 ± 125	1596 ± 89	1×

[a]Average (in Miller units) of three independent experiments. Cells were diluted 1:100 and grown in LB with ampicillin (amp) for 12 h.
[b]amp[r].

gadB–lacZ, and surprisingly, the expression of the *yhiM–lacZ* fusion was induced rather than repressed by OxyS in the *rpoS* mutant background. These results suggest that OxyS is also acting by *rpoS*-independent mechanisms at *yhiV*, *yhiM* and *gadB*.

OxyS represses stationary-phase and osmotic induction of rpoS

To examine the effect of OxyS on *rpoS* expression, we measured the expression of an *rpoS–lacZ* translational fusion in strains carrying a vector control (pKK177-3) or constitutively-expressing OxyS (poxyS). We first monitored *rpoS–lacZ* expression throughout growth in both rich [Luria-Bertani (LB)] and minimal (M63) media. As shown in Figure 1A, the growth rates of OxyS-expressing cells and control cells were identical. However, the level of β-galactosidase activity in the OxyS-expressing cells was dramatically decreased, showing that OxyS represses the stationary-phase induction of *rpoS* expression in both rich and minimal media. Similarly, we found that *rpoS* induction by high salt was abolished in the poxyS strain (Figure 1B), indicating that the OxyS RNA also modulates the osmotic regulation of *rpoS*. OxyS repression of the stationary phase induction of σ[s] was confirmed by immunoblots (Figure 2; Altuvia *et al.*, 1997). We found that OxyS expression caused a decrease in σ[s] levels in the late-exponential and stationary phase cells in both rich and minimal media.

OxyS inhibits rpoS expression at a post-transcriptional level

Since *rpoS* expression is regulated at multiple levels, we examined the effect of constitutive OxyS expression (poxyS) on transcriptional and translational *rpoS–lacZ* fusions (Table II). The extent of repression observed for the translational fusions was significantly higher than the extent of repression observed for the transcriptional fusion, indicating that OxyS acts primarily at a post-transcriptional level. The translational fusion *rpoS379–lacZ* (RO90) contains the translation initiation region (TIR), while the translational fusion *rpoS742–lacZ* (RO91) carries both the TIR domain and an σ[s] internal turnover element such that the expression of the *rpoS742–lacZ* fusion is regulated at both the level of translation and protein stability (Muffler *et al.*, 1996b; Figure 3). We found that the OxyS RNA
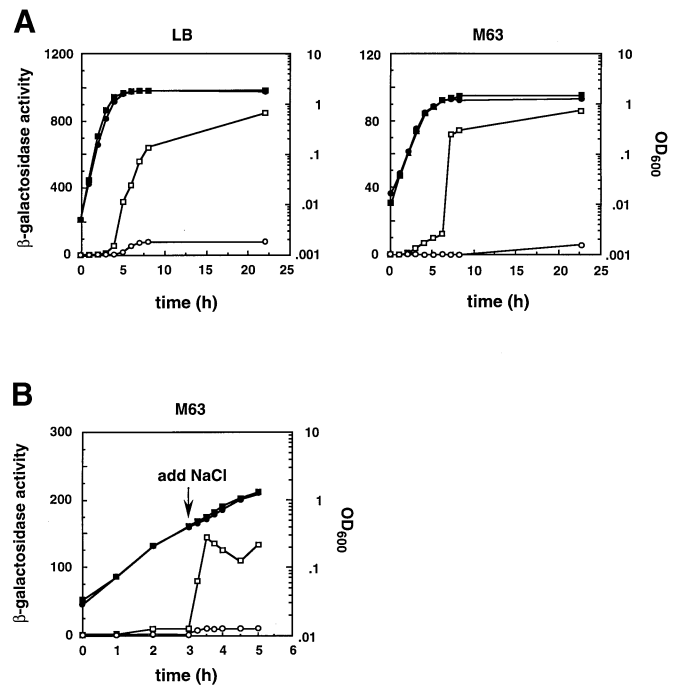
Fig. 1. The OxyS RNA represses stationary phase and osmotic induction of *rpoS*. (**A**) Strains carrying the translational fusion *rpoS742–lacZ* and pKK177-3 (squares) or poxyS (circles) were grown in LB rich medium (left panel) or M63 glucose minimal medium (right panel). (**B**) Strains carrying the translational fusion *rpoS742–lacZ* and pKK177-3 (squares) or poxyS (circles) were grown in M63 glucose minimal medium and treated with 0.3 mM NaCl at the time indicated by the arrow. Optical densities (filled squares and circles) and specific β-galactosidase activities (open squares and circles) were determined along the growth curves.

repressed both of these translational fusions but observed 5-fold stronger repression with the *rpoS742–lacZ* fusion in minimal medium. These findings suggest OxyS inhibits *rpoS* translation in both rich and minimal medium, and may destabilize σ[s] in minimal medium.

OxyS regulation of rpoS is dependent on Hfq

Several factors, including the RNA-binding protein Hfq, the small RNA DsrA, the histone-like protein H-NS, the response regulator RssB and the ClpX/ClpP protease, have been demonstrated to modulate *rpoS* expression at a post-transcriptional level (Barth *et al.*, 1995; Yamashino *et al.*,
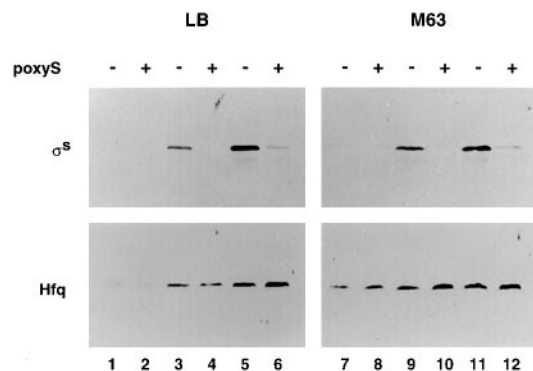
Fig. 2. Effect of OxyS on the cellular levels of σ^s and Hfq. Immunoblot of σ^s (top panels) and Hfq (bottom panels) levels in MC4100 cells carrying pKK177-3 (–) or poxyS (+) grown in LB (lanes 1–6) or M63 glucose (lanes 7–12) medium. The proteins from equal amounts of cells grown to early-exponential phase (lanes 1, 2, 7 and 8), late-exponential phase (lanes 3, 4, 9 and 10), or stationary phase (lanes 5, 6, 11 and 12) were separated by SDS–PAGE and probed with α-σ^s or α-Hfq antiserum.
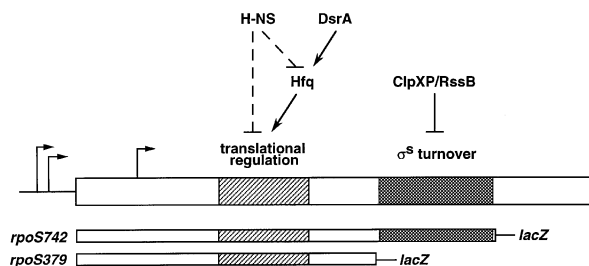

Fig. 3. Structure of the *rpoS* gene and domains proposed to be involved in post-transcriptional regulation (Muffler *et al.*, 1996b). The *rpoS* gene is transcribed from at least three promoters. The translation of the *rpoS* mRNA is repressed by H-NS and activated by Hfq and the DsrA RNA, and σ^s stability is regulated by RssB and the ClpXP protease. The shaded boxes indicated the *rpoS* regions that are required for translational regulation and σ^s turnover. The extent of the *rpoS–lacZ* fusions is indicated at the bottom of the figure.

Table II. Effect of OxyS on *rpoS–lacZ* fusions

Fusion	β-galactosidase activity[a]		
	pKK177-3[b]	poxyS[b]	Fold repression
LB medium			
transcriptional fusion:			
rpoS742–lacZ	17 ± 1	15 ± 2	1×
translational fusions:			
rpoS742–lacZ	423 ± 85	41 ± 16	10×
rpoS379–lacZ	552 ± 59	129 ± 17	4×
M63 glucose medium			
transcriptional fusion:			
rpoS742–lacZ	10 ± 2	5 ± 1	2×
translational fusions:			
rpoS742–lacZ	62 ± 12	1 ± 1	62×
rpoS379–lacZ	132 ± 10	11 ± 2	12×

[a]Average (in Miller units) of three independent experiments. Cells were diluted 1:100 and grown in LB or M63 glucose medium with chloramphenicol (cm) for 12 h.
[b]cm^r.

Table III. Effect of chromosomal mutations on OxyS repression of *rpoS742–lacZ*

Strain	β-galactosidase activity[a]		
	pKK177-3[b]	poxyS[b]	Fold repression
LB medium:			
wild-type	458 ± 49	62 ± 17	7×
hfq1::Ω	75 ± 26	91 ± 22	1×
Δ*dsrA5*...Tn*10*	233 ± 11	49 ± 15	5×
hns8::Tn*10*	747 ± 58	100 ± 14	7×
hns24::Tn*10*	835 ± 78	69 ± 10	12×
rssB::Tn*10*	986 ± 88	172 ± 22	6×
clpX::*kan*	867 ± 71	205 ± 33	4×
clpP::*kan*	1101 ± 70	185 ± 45	6×
M63 glucose medium:			
wild-type	60 ± 17	2 ± 2	30×
hfq1::Ω	6 ± 3	2 ± 1	3×
Δ*dsrA5*...Tn*10*	34 ± 4	2 ± 3	17×
hns8::Tn*10*	358 ± 62	30 ± 8	12×
hns24::Tn*10*	345 ± 48	24 ± 3	14×
rssB::Tn*10*	291 ± 81	45 ± 17	6×
clpX::*kan*	250 ± 81	49 ± 23	5×
clpP::*kan*	410 ± 98	70 ± 32	6×

[a]Average (in Miller units) of three independent experiments. Cells were diluted 1:100 and grown in LB or M63 glucose medium with cm for 12 h.
[b]cm^r.

1995; Brown and Elliott, 1996; Muffler *et al.*, 1996a,b; Pratt and Silhavy, 1996; Schweder *et al.*, 1996; Sledjeski *et al.*, 1996). To determine whether the OxyS RNA acts through any of these regulators, we moved the corresponding mutant alleles into a host strain carrying the *rpoS742–lacZ* fusion and examined the effect of constitutive OxyS expression in these strain backgrounds in both rich and minimal media (Table III). The OxyS RNA repressed *rpoS* expression in all strains except the *hfq1*::*kan* mutant. Thus, OxyS appears to be acting through the Hfq protein. OxyS repression of *rpoS* was 4-fold stronger in minimal medium than in rich medium (30-fold versus 7-fold), consistent with the results in Table II. Interestingly however, OxyS-mediated repression was reduced 5-fold in the *rssB*::Tn*10*, *clpX*::*kan* and *clpP*::*kan* backgrounds in the M63 medium, suggesting that OxyS affects σ^s stability in minimal medium through RssB and the ClpX/ClpP protease.

A-rich linker region is important for OxyS repression of rpoS

Hfq is a 12 kDa, heat-stable, RNA-binding protein. This protein was originally identified as having a role in the replication of the RNA bacteriophage Qβ but was recently found to have more pleiotropic effects including a role in *rpoS* translation (Tsui *et al.*, 1994; Brown and Elliott, 1996; Muffler *et al.*, 1996b, 1997). Studies of Hfq binding to the Qβ and R17 phage RNAs indicate that the protein preferentially binds A-rich sequences (Senear and Steitz, 1976). The OxyS RNA may act on *rpoS* by either affecting the Hfq levels or modulating Hfq action. To test the first possibility, we examined the Hfq levels by immunoblot analysis (Figure 2). No apparent differences were detected in cells carrying poxyS compared with the vector control in both rich and minimal media at all stages of growth, indicating that the OxyS RNA has no effect on the Hfq levels.
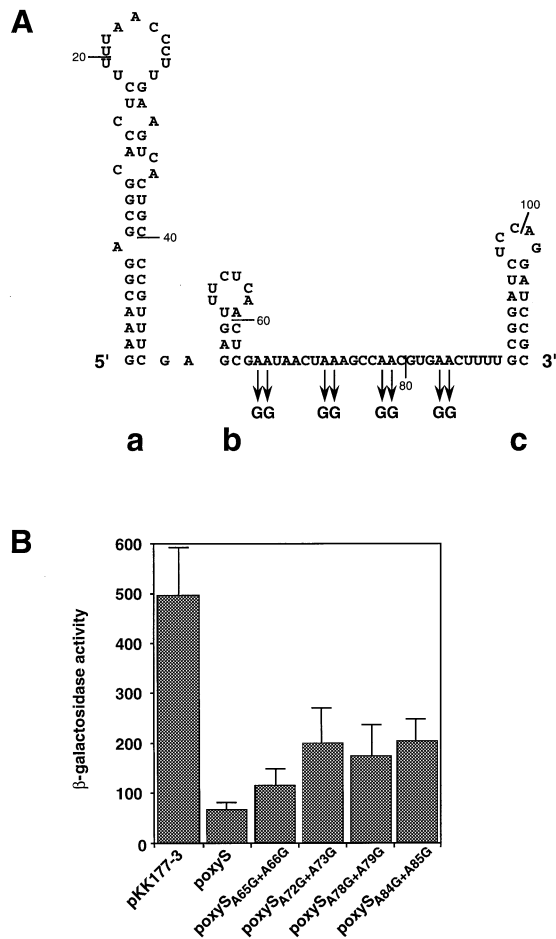
Fig. 4. Mutational analysis of OxyS. (**A**) Secondary structure of the OxyS RNA. The arrows indicate specific nucleotide changes generated by site-directed mutagenesis. Based on the *mfold* (http://www.ibc.wustl.edu/~zuker/rna) program, the secondary structures of stem loops **a**, **b** and **c** of all the mutants are likely to be similar to wild-type OxyS. Northern blots show that the levels of the OxyS$_{A65G+A66G}$, OxyS$_{A78G+A79G}$ and OxyS$_{A84G+A85G}$ RNAs are approximately equivalent to wild-type levels. For unknown reasons, colonies expressing OxyS$_{A72G+A73G}$ showed heterogeneity, and the levels of this RNA were reduced by up to 4-fold in some cultures. (**B**) Effect of mutations on OxyS repression of *rpoS742–lacZ*. The average (in Miller units) of three independent β-galactosidase assays is shown. Single colonies were grown overnight in LB with chloramphenicol (cm).

Several models of the *rpoS* mRNA secondary structure (Brown and Elliott, 1997; S.Bouche, D.Traulsen and R.Hengge-Aronis, unpublished data) predict that the *rpoS* ribosome binding site is occluded, and that Hfq binding to A-rich sequences in this region is required for a conformational change which allows efficient translation of the *rpoS* mRNA. Deletion studies of the OxyS RNA showed that the 27-nucleotide linker region between stem–loops **b** and **c** of the OxyS RNA is required for OxyS repression of *rpoS* (Altuvia *et al.*, 1998). Intriguingly, this linker region contains five AA repeats. To test whether this A-rich region was important for OxyS repression of *rpoS*, we constructed mutants carrying AA to GG substitutions at four positions (Figure 4). All of the strains carrying the mutant plasmids showed less repression than the strain carrying poxyS. Although none of the effects of the individual AA mutations is dramatic, the reduced
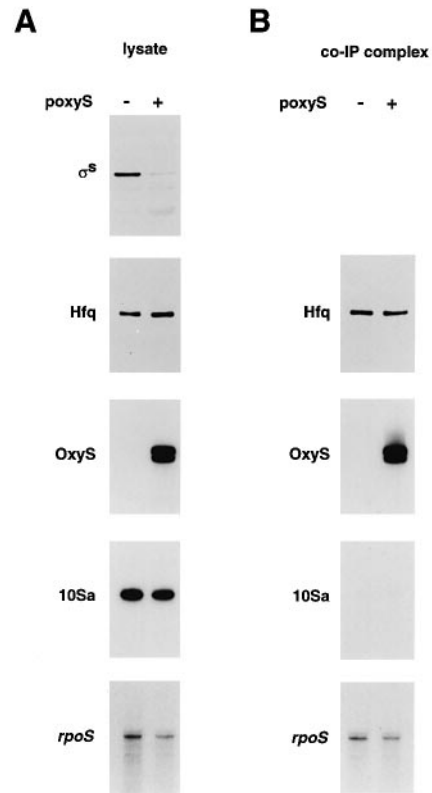

Fig. 5. Co-immunoprecipitation of Hfq and OxyS. (**A**) Levels of the σ^s and the Hfq proteins and the OxyS, 10Sa, and *rpoS* RNAs in the cell lysates of MC4100/pSU-hfq-myc cells carrying pKK177–3 or poxyS. (**B**) Levels of the Hfq protein and the OxyS, 10Sa, and *rpoS* RNAs eluted from a complex (co-IP complex) immunoprecipitated with α-Myc monoclonal antibodies. For the σ^s and Hfq immunoblots, the proteins were separated by SDS–PAGE and probed with α-σ^s or α-Hfq antiserum. For the RNA analysis, RNA was isolated from aliquots of the above samples and subjected to primer extension assays using primers specific to the OxyS or 10Sa RNAs, or to RNase protection assays with an *rpoS* antisense transcript.

rpoS repression is consistent with a role of the linker region. We propose Hfq binds this region, possibly at multiple sites.

OxyS co-immunoprecipitates with Hfq

To test for an interaction between the OxyS RNA and the Hfq protein *in vivo*, we carried out immunoprecipitation experiments. We first constructed an Hfq derivative carrying a C-terminal Myc tag (pSU-hfq-myc). The Myc-tagged Hfq protein appeared to have wild type activity since the protein could restore normal levels of *rpoS–lacZ* expression in an *hfq1::kan* mutant strain (data not shown). MC4100/pSU-hfq-myc cells carrying pKK177-3 or poxyS were grown to early stationary phase in LB medium. Immunoblots of lysates from these cells showed that, as expected, σ^s levels were reduced in the poxyS strains compared with the pKK177-3 strain (Figure 5A). In contrast, both strains had equally high levels of the Myc-tagged Hfq protein. α-Myc monoclonal antibodies were then used to immunoprecipitate the Myc-tagged Hfq protein. Equal quantities of Hfq protein were precipitated from the pKK177-3 and the poxyS strains (Figure 5B). The RNA in the immunoprecipitated complexes was extracted and assayed for the presence of OxyS. The OxyS RNA was clearly co-immunoprecipitated from the
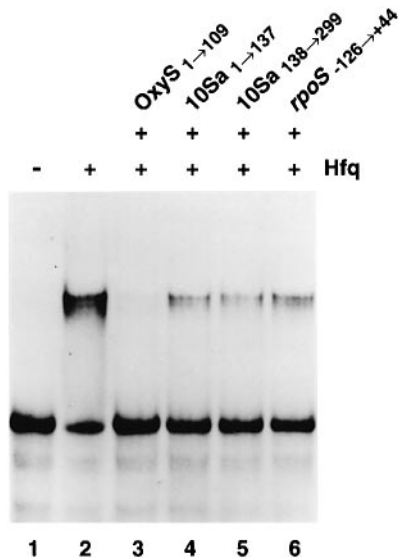
Fig. 6. Gel mobility shift analysis of OxyS binding to Hfq. [α-^{32}P]OxyS run-off transcript (2 fmol) was incubated without (lane 1) or with 2 pmol purified Myc-tagged Hfq protein (lanes 2–6). Unlabeled run-off transcripts (1 pmol) of OxyS$_{1\to109}$ (lane 3), 10Sa$_{1\to137}$ (lanes 4), 10Sa$_{138\to299}$ (lanes 5), or *rpoS*$_{-126\to+44}$ (lanes 6) were added as competitors.

poxyS extracts (Figure 5B). The co-immunoprecipitation was specific since the OxyS RNA was not precipitated by the cells expressing Hfq without a Myc tag (data not shown). As another specificity control, we assayed for the presence of the 362-nucleotide 10Sa RNA which also carries several AA repeats and whose levels are 3-fold higher than the OxyS RNA (Altuvia *et al.*, 1997). While the 10Sa RNA was clearly present in the cell lysate, we could not detect this control RNA in the precipitated complex (Figure 5B). Together, these results indicate that the OxyS RNA binds Hfq *in vivo*.

OxyS binding to the Hfq protein may prevent *rpoS* translation by competing with the *rpoS* mRNA. Alternatively, Hfq may simultaneously bind OxyS and the *rpoS* mRNA, leading to the formation of a ternary complex which cannot be translated. We tested for the presence of the *rpoS* mRNA in the complex which immunoprecipitated with Hfq. Interestingly, equal amounts of *rpoS* mRNA were precipitated from cells carrying both pKK177-3 and poxyS (Figure 5B). Thus, *rpoS* binding to Hfq is not affected by high levels of OxyS, suggesting that the OxyS and *rpoS* RNAs do not compete for the same site on Hfq.

OxyS binds Hfq in vitro

To further verify OxyS binding to Hfq, *in vitro*-synthesized OxyS RNA was incubated with purified Myc-tagged Hfq protein and examined by a gel mobility shift assay (Figure 6). Incubation with Hfq clearly led to gel retardation of the *oxyS* transcript showing that Hfq interacts with the *oxyS* RNA. To test for specificity we added a 500-fold excess of unlabeled RNAs. These experiments showed that the OxyS–Hfq interaction is specific since the binding was strongly competed by the OxyS$_{1\to109}$ transcript, but much less efficiently by the similarly-sized 10Sa$_{1\to137}$, 10Sa$_{138\to299}$ or *rpoS*$_{-126\to44}$ transcripts.

Discussion

The *E.coli* OxyS is a small untranslated RNA, induced by oxidative stress (Altuvia *et al.*, 1997). This novel RNA acts as a pleiotropic regulator of the expression of as many as 40 genes. We have examined OxyS regulation of its target genes and found that the regulatory RNA represses a subset of the target genes, *yhiV*, *yhiM*, *gadB* and *dps*, through the RNA polymerase σ^s subunit encoded by *rpoS* (Table I). OxyS repression of *dps* is completely dependent on σ^s, while OxyS repression of *yhiV* and *gadB* is partially σ^s-dependent. The effect of OxyS on *yhiM* expression is more complex. In an *rpoS*$^+$ background OxyS represses *yhiM*, while the RNA activates *yhiM* in an *rpoS*::Tn*10* mutant strain. This observation suggests that OxyS regulates *yhiM* expression by two different mechanisms, one being σ^s-dependent and the second being σ^s-independent. Further characterization of the two mechanisms should be interesting, especially since another OxyS-regulated gene recently identified also shows opposing effects of OxyS in the presence and absence of *rpoS* (T.Schar and G.Storz, unpublished data).

Previous studies have shown that *rpoS* expression is controlled on multiple levels, and several regulators have been characterized. Our results suggest that OxyS represses *rpoS* translation in both rich and minimal medium. OxyS also appears to affect σ^s stability in minimal medium. The effect on σ^s stability may be mediated through the response regulator RssB and/or the ClpX/ClpP protease, but not through the histone-like protein H-NS. It is interesting to note that a mutation in *rssB* was isolated in a screen for mutations that affected the ability of OxyS to repress the expression of a *yhiV–lacZ* fusion (Muffler *et al.*, 1996a). Why the effect on proteolysis is more predominant in minimal medium and how OxyS affects σ^s stability is not clear. Possibly, OxyS destabilizes σ^s by affecting the levels of RssB or ClpX/ClpP, or by modulating the activities of these proteins.

Our genetic studies showed that OxyS repression of *rpoS* translation is mediated through the RNA-binding protein, Hfq. Since OxyS had no effect on the Hfq levels, the RNA must repress *rpoS* by affecting Hfq activity. The Hfq protein preferentially binds A-rich sequences (Senear and Steitz, 1976), and we observed that the linker region between stem–loops **b** and **c** of OxyS contains five AA repeats. We propose that one or more Hfq molecules binds this linker region since mutations of the AA sequences decrease OxyS repression of *rpoS*. The essential role of the linker region is supported by deletion studies of Altuvia *et al.* (1998). While a mutant carrying a 5′-deletion of stem–loops **a** and **b** (poxyS$_{\Delta1-63}$) still repressed *rpoS*, a mutant carrying a 5′-deletion of stem–loops **a** and **b**, and the linker region (poxyS$_{\Delta1-90}$) did not repress *rpoS* even though this deletion mutant was still able to regulate *fhlA*, an OxyS target found to be repressed by an antisense mechanism.

Co-immunoprecipitation and gel mobility shift experiments revealed that the OxyS RNA binds the Hfq protein. We previously calculated there to be ~4500 molecules of *oxyS* per cell after treatment with hydrogen peroxide (Altuvia *et al.*, 1997). Hfq has also been reported to be abundant although the exact levels of the protein are controversial (Carmichael *et al.*, 1975; Kajitani *et al.*,

1994). While the nature of the OxyS–Hfq interaction needs to be analyzed, it is reasonable to expect that the observed levels of OxyS are adequate to modulate Hfq activity.

Based on our current results and other *rpoS* structure-and-function studies (Brown and Elliott, 1997; S.Bouche, D.Traulsen and R.Hengge-Aronis, unpublished data), we propose the following model to explain OxyS RNA repression of *rpoS* translation. In the absence of other regulators, a secondary structure encompassing the *rpoS* mRNA Shine–Dalgarno sequence interferes with ribosome binding. Hfq recognizes and binds A-rich sequences in the vicinity of the ribosome binding site. This binding releases the *rpoS* Shine–Dalgarno and allows translation. When the OxyS RNA is induced by oxidative stress, OxyS binds Hfq and prevents Hfq from acting. Interestingly, the DsrA RNA contains a sequence complementary to the *rpoS* message. Thus, DsrA may act to induce *rpoS* expression by pairing with the *rpoS* message and melting the secondary structure (Majdalani *et al.*, 1998).

OxyS binding to Hfq may compete with *rpoS* mRNA binding. Alternatively, Hfq may simultaneously bind OxyS and the *rpoS* mRNA leading to the formation of a ternary complex which cannot be translated. The findings that high levels of OxyS do not affect *rpoS* co-immunoprecipitation with Hfq, and that a *rpoS* transcript encompassing the putative Hfq binding site did not compete with OxyS binding in a gel mobility shift assay, suggest that Hfq may simultaneously bind the OxyS and *rpoS* transcripts, but additional binding studies are warranted. Regardless, it is clear that the OxyS RNA specifically binds the Hfq protein.

It is intriguing how many regulators act to modulate σ^s levels in response to starvation, osmotic shock, acid shock, low temperature, and in the case of OxyS, oxidative stress. The finding that the hydrogen peroxide-induced OxyS RNA represses *rpoS* expression appeared counterintuitive since σ^s controls the expression of several genes that protect against oxidative stress. We proposed that OxyS regulation of *rpoS* may provide a mechanism to fine tune the expression of antioxidant activities and prevent the redundant induction of *katG*, *gorA* and *dps*, by both the hydrogen peroxide-specific OxyR transcription factor and the general stress factor σ^s (Altuvia *et al.*, 1997). In addition, OxyS repression of *rpoS* would prevent the induction of many broadly protective σ^s-dependent proteins whose synthesis would be costly and unnecessary as long as the OxyR-regulated response can alleviate the stress condition. An important direction for future work will be to clearly elucidate the interactions among all of the regulators of *rpoS* under different stress conditions.

Materials and methods

Bacterial strains and growth conditions
The bacterial strains used in this study are listed in Table IV. Cultures were grown under aeration at 37°C in LB rich medium or in M63 minimal medium supplemented with 2 mg/ml glucose, 20 μg/ml vitamin B_1 and 1 mg/ml casamino acids (Miller 1972). Growth was monitored by measuring the optical density at 600 nm (OD_{600}). Ampicillin (amp, 50 μg/ml) or chloramphenicol (cm, 25 μg/ml) was added where appropriate. The *rpoS*::Tn*10*, *hfq1*::Ω, Δ*dsrA5*...Tn*10*, *hns8*::Tn*10*, *hns24*::Tn*10*, *rssB*::Tn*10*, *clpX*::*kan*, and *clpP*::*kan* mutant alleles (Loewen and Triggs, 1984; Shi *et al.*, 1993; Tsui *et al.*, 1994; Muffler

et al., 1996a; Sledjeski *et al.*, 1996) were moved into the desired strain backgrounds by P1 transductions (Silhavy *et al.*, 1984).

Site-directed mutagenesis
All DNA manipulations were carried out using standard procedures. The OxyS mutants carrying the specific nucleotide changes were generated by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). poxyS (pGSO85, Altuvia *et al.*, 1997) DNA was subjected to PCR using primers to create the desired mutations; poxyS$_{A65G+A66G}$ [#663 (5′-GAG TTT CTC AAC TCG GGT AAC TAA AGC CAA CG) + complementary #664], poxyS$_{A72G+A73G}$ [#665 (5′-TCA ACT CGA ATA ACT GGA GCC AAC GTG AAC TT) + complementary #666], poxyS$_{A78G+A79G}$ [#667 (5′-CGA ATA ACT AAA GCC GGC GTG AAC TTT TGC GG) + complementary #668], and poxyS$_{A84G+A85G}$ [#669 (5′-ACT AAA GCC AAC GTG GGC TTT TGC GGA TCT CC) + complementary #670]. The presence of the mutations was confirmed by DNA sequencing. The levels of the mutant RNAs were examined by Northern blot analysis: total RNA isolated from cells grown for 12 h was separated on a 6% polyacrylamide–urea gel, transfered to a nylon membrane by electroblotting, and probed with a γ-^{32}P-end-labeled primer (5′-CCT GTG TGA AAT TCT TAT CC, corresponding to pKK177-3 sequence upstream of the *oxyS* coding sequence).

β-galactosidase assay
β-galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galacto-pyranoside (ONPG) as a substrate (Miller, 1972).

Immunoblot analysis
To determine the cellular levels of σ^s and Hfq, MC4100 cells carrying pKK177-3 or poxyS (pGSO5) were grown for 2, 6 and 12 h in LB, or 4, 8 and 12 h in M63 medium. Aliquots (1 ml) were centrifuged, and equal absorbance units were suspended in Laemmli buffer (1 OD_{600}/100 μl). The proteins (5 μl) were separated on SDS–12% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. The blots were probed with a 1:4000 dilution of α-σ^s antibody (Lange and Hengge-Aronis, 1994) or a 1:4000 dilution of α-Hfq antibody (Kajitani *et al.*, 1994).

Primer extension assay
RNA samples were subjected to primer extension assays using primers specific to OxyS [#188 (5′-GCA AAA GTT CAC GTT GG)] and 10Sa [#620 (5′-TTG CGA CTA TTT TTT GCG GC)]. The RNA samples were incubated with 0.5 pmol of a γ-^{32}P-endlabeled primer for 5 min at 65°C, and then quick-chilled on ice. After the addition of dNTPs (1 mM each) and AMV reverse transcriptase (10 U, Life Sciences) the reactions were incubated for 1 h at 42°C. The cDNA products were then analyzed on an 8% polyacrylamide–urea gel.

RNase protection assay
Fragments carrying the *rpoS* coding sequence (from +24 to +304, relative to A of the initiating AUG) were generated by PCR [#683 (5′-GGC AAG CTT CCA GAC GCA AGT TAC TCT CGA) and #684 (5′-GGC GAA TTC TCA TGA TTT AAA TGA AGA TGC)]. The DNA fragments were digested with *Eco*RI and *Hin*dIII, and cloned into the corresponding sites of pGEM2 (Promega). The [α-^{32}P]*rpoS* antisense transcript was made from the *Eco*RI-linearized plasmid by *in vitro* transcription with T7 RNA polymerase. The RNase protection assays were carried out using the RPAII kit (Ambion). The RNA samples were hybridized with 1.5×10^6 c.p.m. of [α-^{32}P]*rpoS* antisense transcripts overnight at 45°C and then digested with RNase A (0.5 U) and RNase T1 (20 U) for 30 min at 37°C. Subsequently, the samples were treated with proteinase K, extracted with phenol, precipitated with ethanol in the presence of 5 μg yeast tRNA, and analyzed on a 6% polyacrylamide–urea gel.

Immunoprecipitation assay
To construct pSU-hfq-myc, fragments carrying the *E.coli hfq* gene linked to the *myc* tag at the C-terminus were generated by PCR [#654 (5′-ACG AAT TCG ATG GCT AAG GGG CAA TCT) and #655 (5′-CCA AGC TTT CAA TTC AAG TCC TCC TCG CTG ATC AGC TTC TGC TCC ATT GAT TCG GTT TCT TCG CTG TCC T)]. To construct the control plasmid pSU-hfq, *hfq* was amplified by PCR [#654 and #680 (5′-CCA AGC TTA TTC GGT TTC TTC GCT GTC C)]. The DNA fragments were then digested with *Eco*RI and *Hin*dIII and cloned into the corresponding sites of pSU18 (Bartolomé *et al.*, 1991). Cultures of MC4100/pSU-hfq-myc carrying pKK177-3 or poxyS (pGSO5) were grown to early stationary phase (OD_{600} = 1.5) in LB medium with amp

Table IV. Bacterial strains and plasmids

Strain	Relevant genotype	Source or reference
MC4100	Δ(arg-lac)U169	N.Trun
GSO39	MC4100 φ*yhiV–lacZ* (kan^r)	Altuvia *et al.* (1997)
GSO40	MC4100 φ*yhiM–lacZ* (kan^r)	Altuvia *et al.* (1997)
GSO43	MC4100 (λRS45 *dps–lacZ*) (kan^r)	Altuvia *et al.* (1997)
GSO44	MC4100 (λRS45 *gadB–lacZ*) (kan^r)	Altuvia *et al.* (1997)
GSO69	GSO39 *rpoS*::Tn*10* (kan^r, tet^r)	this study
GSO70	GSO40 *rpoS*::Tn*10* (kan^r, tet^r)	this study
GSO73	GSO43 *rpoS*::Tn*10* (kan^r, tet^r)	this study
GSO74	GSO44 *rpoS*::Tn*10* (kan^r, tet^r)	this study
RO200	MC4100 [λRZ5 *rpoS742–lacZ*] (amp^r)	Lange and Hengge-Aronis (1994)
RO91	MC4100 [λRZ5 *rpoS742–lacZ* (hybr)] (amp^r)	Lange and Hengge-Aronis (1994)
RO90	MC4100 [λRZ5 *rpoS379–lacZ* (hybr)] (amp^r)	Lange and Hengge-Aronis (1994)
GSO59	RO91 *hfq1*::Ω (amp^r, kan^r)	this study
GSO60	RO91 Δ*dsrA5...zed*3069::Tn*10* (amp^r, tet^r)	this study
GSO61	RO91 *hns8*::Tn*10* (amp^r, tet^r)	this study
GSO62	RO91 *hns24*::Tn*10* (amp^r, tet^r)	this study
GSO63	RO91 *rssB*::Tn*10* (amp^r, tet^r)	this study
GSO64	RO91 *clpX*::*kan* (amp^r, kan^r)	this study
GSO65	RO91 *clpP*::*kan* (amp^r, kan^r)	this study

Plasmid	Relevant genotype	Source or reference
pGSO5	p*oxyS* (amp^r)	Altuvia *et al.* (1997)
pGSO85	p*oxyS* (cm^r)	Altuvia *et al.* (1997)
pGSO78	p*oxyS*$_{A65G+A66G}$ (cm^r)	this study
pGSO79	p*oxyS*$_{A72G+A73G}$ (cm^r)	this study
pGSO80	p*oxyS*$_{A78G+A79G}$ (cm^r)	this study
pGSO81	p*oxyS*$_{A84G+A85G}$ (cm^r)	this study
pGSO82	pSU18-*hfq* (cm^r)	this study
pGSO83	pSU18-*hfq-myc* (cm^r)	this study
pGSO99	pGEM2-*rpoS*$_{+24\rightarrow+304}$ (amp^r)	this study
pGSO100	pSP64-*oxyS*$_{1\rightarrow109}$ (amp^r)	this study
pGSO101	pSP64-*10Sa*$_{1\rightarrow137}$ (amp^r)	this study
pGSO102	pSP64-*10Sa*$_{138\rightarrow299}$ (amp^r)	this study
pGSO103	pSP64-*rpoS*$_{-126\rightarrow+44}$ (amp^r)	this study

and cm. The cells from 30 ml of culture were collected and lysed by three freeze-thaw cycles in 1.5 ml of lysis buffer (10 mM Tris–HCl pH 8.0, 250 mM NaCl, 10 mM MgCl$_2$ and 1 mM dithiothreitol) with 150 µg lysozyme. The samples were then treated with DNase I (740 U) and RNasin (200 U) for 20 min on ice. After centrifugation, the supernatant was treated as follows: one aliquot (0.25 µl) was analyzed by immunoblotting as described above. Total cellular RNA from a second aliquot (490 µl) was isolated by phenol extraction, and then 2 µg of RNA was subjected to primer extension assays using primers specific to OxyS and the 10Sa RNA, or to RNase protection assays with *rpoS* antisense transcript. A third aliquot (500 µl) was subjected to immunoprecipitation as follows: protein A–Sepharose beads (250 µl of a 1:1 slurry; Pharmacia) were incubated with α-Myc monoclonal antibodies (25 µl of 9E10; Santa Cruz Biotechnology) and lysis buffer (1 ml) for 2 h at room temperature, and then washed three times with lysis buffer. The washed beads (50 µl of a 1:1 slurry) was mixed with the supernatant (500 µl) and incubated for 2 h at 4°C. The beads were subsequently collected and washed three times with lysis buffer. Finally, the pellet was resuspended in 500 µl of lysis buffer and 2.5 µl was analyzed by immunoblotting. RNA was isolated from 490 µl (with 100 µg yeast tRNA as carrier) and 10 µg of RNA subjected to primer extension or RNase protection assays.

Purification of Myc-tagged Hfq
Cell pellets from 25 ml of cultures (grown to OD$_{600}$ = 1.5 in LB) of MC4100/pSU-hfq-myc or MC4100/pSU-hfq (as a control) were resuspended in 1.5 ml lysis buffer (10 mM Tris–HCl pH 8.0, 250 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA) containing 150 µg lysozyme, and lysed by three freeze-thaw cycles and subsequent sonication. The lysates were then treated with DNase I (740 U) and RNase A (15 µg) for 15 min on ice. After centrifugation, the cleared lysates were incubated with protein A–Sepharose cross-linked to α-Myc monoclonal antibodies (150 µl of a 1:1 slurry, prepared as described in Harlow and Lane, 1988) for 2 h at 4°C. The immunoprecipitated complexes were washed three

times with lysis buffer and the proteins were eluted in 150 µl of lysis buffer by heating for 30 min at 95°C. An aliquot was examined on an SDS–polyacrylamide gel stained with Coomassie Blue as well as by immunoblotting to verify that the Myc-tagged Hfq protein isolated from the MC4100/pSU-hfq-myc cells was purified to near homogeneity.

Gel mobility shift assay
The RNAs used for the mobility shift were obtained as follows. PCR was used to amplify OxyS$_{1\rightarrow109}$ [#689 (5′-CTT GAA TTC TAA TAC GAC TCA CTA TAG GGA AAC GGA GCG GCA CC) and #690 (5′-TAC AAG CTT GCG GAT CCT GGA GAT CCG CAA AAG TT)], 10Sa$_{1\rightarrow137}$ [#694 (5′-CTT GAA TTC TAA TAC GAC TCA CTA TAG GGG CTG ATT CTG GAT CG) and #695 (5′-TAC AAG CTT GCT CTA AGC AGG TTA TTA AGC TGC TA)], 10Sa$_{138\rightarrow299}$ [#696 (5′-CTT GAA TTC TAA TAC GAC TCA CTA TAG GGC AGG CTC TCT CCC TAG CCT) and #697 (5′-TAC AAG CTT GTC AGT CTT TAC ATT CGC TTG CCA GC)], and *rpoS*$_{-126\rightarrow+44}$ (from –126 to +44 relative to A of the initiating AUG) [#698 (5′-CTT GAA TTC TAA TAC GAC TCA CTA TAG GGC ATT TTG AAA TTC GTT AC) and #699 (5′-TAC AAG CTT GCA TCT TCA TTT AAA TCA TGA ACT TT)]. The DNA fragments were then digested with *Eco*RI and *Hin*dIII, and cloned into the corresponding sites of pSP64 Poly(A) (Promega). The [α-^{32}P]OxyS transcript and the unlabeled competitor RNAs were prepared from the *Hin*dIII-linearized plasmids by *in vitro* transcription with T7 RNA polymerase (Gibco-BRL).

The gel mobility shift assays were carried out as follows. Purified Myc-tagged Hfq (2 pmol) and [α-^{32}P]OxyS$_{1\rightarrow109}$ transcript (2 fmol, 3×10^4 c.p.m.), without or with unlabeled OxyS$_{1\rightarrow109}$, 10Sa$_{1\rightarrow137}$, 10Sa$_{138\rightarrow299}$, or *rpoS*$_{-126\rightarrow+44}$ transcript (1 pmol), were incubated in a 20 µl reaction in RNA binding buffer (10 mM Tris–HCl pH 8.0, 50 mM NaCl, 50 mM KCl, 1 mM MgCl$_2$) for 10 min at 37°C, and mixed with 2 µl loading dye (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol). The mixtures were analyzed on 5% polyacrylamide gels in 0.5× TBE buffer at 150 V for 2 h, and subjected to autoradiography.

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