ppGpp regulation of RpoS degradation via anti-adaptor protein IraP

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IraP is a small protein that interferes with the delivery of σ^S (RpoS) to the ClpXP protease by blocking the action of RssB, an adaptor protein for σ^S degradation. IraP was previously shown to mediate stabilization of σ^S during phosphate starvation. Here, we show that *iraP* is transcribed in response to phosphate starvation; this response is mediated by ppGpp. The *iraP* promoter is positively regulated by ppGpp, dependent on the discriminator region of the *iraP* promoter. Sensing of phosphate starvation requires SpoT but not RelA. The results demonstrate a target for positive regulation by ppGpp and suggest that the cell use of ppGpp to mediate a variety of starvation responses operates in part by modulating σ^S levels.

phosphate starvation | σ^{38} | SpoT

Bacteria have evolved sophisticated mechanisms to recognize and respond to a variety of environmental stresses. In *Escherichia coli*, σ^S, the gene product of *rpoS*, is the σ factor of the general stress response and adaptation to stationary phase (1, 2). σ^S controls the expression of ≈100 genes involved in functions that help cells cope with various kinds of stress. σ^S amounts and activity are tightly regulated (3–5).

In exponentially growing cells, the active proteolysis of σ^S is crucial to maintain a low intracellular concentration of this σ factor (6, 7). σ^S becomes stable as cells are starved or are exposed to certain stresses (reviewed in ref. 4). σ^S protein turnover requires the energy-dependent protease, ClpXP, and the adaptor protein, RssB (SprE), which binds directly to σ^S and targets it to ClpXP (8). RssB has an N-terminal domain of the response regulator family, with a conserved site for phosphorylation. Recently, the identification of an anti-adaptor protein, IraP, provided an explanation for how proteolysis of σ^S can be regulated. *In vivo*, σ^S is stabilized after phosphate starvation in a manner that depends on IraP and that is independent of RssB phosphorylation. *In vitro*, IraP inhibits σ^S proteolysis through a direct interaction with RssB (9). Although the inhibitory effect of IraP on σ^S degradation has been observed in response to phosphate starvation, how phosphate starvation is sensed and how this signal affects IraP expression and/or activity has not been described previously. We show in this work that *iraP* mRNA accumulates in phosphate-starved cells, dependent on the alarmone ppGpp, the global regulator of the stringent response. This induction requires the discriminator sequence of the *iraP* promoter. Two proteins in *E. coli*, RelA and SpoT, are capable of synthesizing ppGpp (10, 11); we find that SpoT, but not RelA, is essential for the induction of *iraP* expression in response to phosphate starvation. The positive regulation of *iraP* transcription by ppGpp leads to regulation of σ^S proteolysis.

Results

***iraP* mRNA Accumulates in Response to Phosphate Starvation.** Previous work showed that IraP stabilizes the transcription factor σ^S under phosphate starvation (9). We analyzed by Northern blot the levels of *iraP* mRNA before and after removal of phosphate. Fig. 1*A* shows that *iraP* mRNA was barely detectable in growing cells (0′), and transiently accumulated on phosphate starvation, with a peak of accumulation (≈19-fold increase) 15 min after removal of phosphate from the culture. The signal decreased progressively over the next 45 min (Fig. 1*A*) and became as low as before starvation after 60 min of phosphate starvation (data not shown). Accumulation of *iraP* transcripts was also observed in stationary-phase cells grown in LB (data not shown).

The 5′ end of *iraP* mRNA was mapped by primer extension (Fig. 1*B*). A major product was much more abundant in stationary phase (lane 2) and under phosphate starvation conditions (lanes 3 and 4), suggesting that this transcript was transcribed from a phosphate starvation-induced promoter.

The sequence upstream of this start site includes two possible σ^{70}-dependent promoters (Fig. 1*C*; potential −10 hexamers labeled P1 and P2). We introduced two point mutations within this region, $P_{iraP(-10^{-1})}$ and $P_{iraP(-10^{-2})}$, chosen to bring either P1 or P2 closer to the σ^{70} consensus sequence while disrupting the other possible promoter, and measured the effect of these mutations on *iraP* expression. We reasoned that a mutation that improved the promoter should give increased basal level expression of the transcript. Levels of *iraP* mRNA transcribed from $P_{iraP(-10^{-1})}$ were slightly decreased compared with the wild-type (wt) promoter (Fig. 1*A*). $P_{iraP(-10^{-2})}$ showed a significant increase in *iraP* mRNA amounts before starvation, indicating that this mutation was increasing the basal activity of the *iraP* promoter (Fig. 1*A*). Note that the size of the most prominent band (see 0′ sample for $P_{iraP(-10^{-2})}$) was not detectably different from that of the wt. These data suggest that P1 (taaaaa) (Fig. 1*C*; −10 hexamer in italics) corresponds to the functional −10 hexamer of *iraP*. *iraP* mRNA accumulated after removal of phosphate with both $P_{iraP(-10^{-1})}$ and $P_{iraP(-10^{-2})}$ mutations, indicating that the promoter was still regulated in a phosphate starvation-dependent manner (Fig. 1*A*). The position of the 5′ end corresponds to a 65-nt untranslated leader for *iraP*.

The $P_{iraP(-10^{-2})}$ mutation increases the basal level of *iraP* mRNA by 28-fold, more than the increase seen after phosphate starvation (compare the 15′ lane for wt to the 0′ lane for $P_{iraP(-10^{-2})}$ in Fig. 1*A*). σ^S stability was measured in this strain, without starvation. Consistent with the idea that increasing transcription of *iraP* is sufficient to lead to σ^S stability, σ^S was stabilized in this strain in exponential phase (Fig. 1*D*).

***iraP* mRNA Accumulation Depends on ppGpp.** *iraP* expression increases during phosphate starvation, and IraP is important for σ^S stabilization during phosphate starvation (Fig. 1*A*; ref. 9). Therefore, an obvious candidate for regulating IraP was the two-component regulatory system PhoB/PhoR, which controls the expression of the Pho regulon (12, 13). When extracellular

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Abbreviation: wt, wild type.

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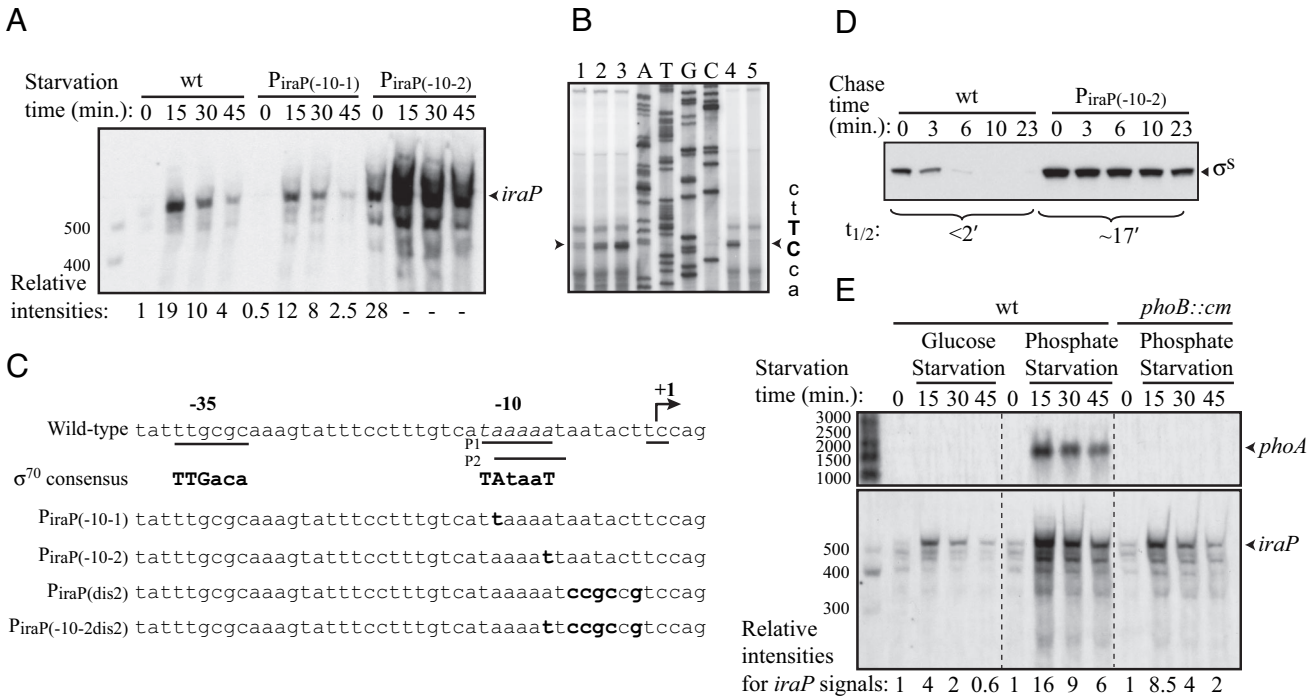

Fig. 1. Analysis of *iraP* expression. Cells were grown at 37°C, and total RNA was isolated as described in *Materials and Methods*. (*A*) Northern blot analysis of RNA extracted from exponentially growing cells in Mops glucose in the presence of phosphate (time point 0) and after removal of phosphate at the time points indicated. Strains were as follows: wt (MG1655), AB030 ($P_{iraP(-10-1)}$), and AB031 ($P_{iraP(-10-2)}$). Quantification of the major *iraP* mRNA signal (arrowhead) is shown. No signal was detected in the mock experiment (filtration without phosphate starvation; data not shown). (*B*) Primer extension analysis of *iraP* mRNA. Primer extension assays were carried out as described in supporting information (SI) *Materials and Methods*, with total RNA extracted from MG1655 cells grown in exponential phase (lane 1) and stationary phase (lane 2) in LB or before (lane 5) and after 15 min (lane 4) and 30 min (lane 3) of phosphate starvation in Mops glucose. Signals specific for stationary-phase and phosphate-starvation conditions are indicated by arrowheads. (*C*) Sequence of *iraP* promoter region in *E. coli* K12. The 5′ end of *iraP* mRNA mapped in *B* is indicated with an arrow. Putative −35 and −10 sequences are underlined. Base substitutions introduced within the promoter region of *iraP* in strains AB030 ($P_{iraP(-10-1)}$), AB031 ($P_{iraP(-10-2)}$), AB033 ($P_{iraP(dis2)}$), and AB034 ($P_{iraP(-10-2\ dis2)}$) are indicated in bold. The holoenzyme-σ^{70} consensus sequences are shown. (*D*) Comparison of σ^S half-lives in AB031 ($P_{iraP(-10-2)}$) and wt (MG1655) strains. The half-life of σ^S was measured in cells grown at 37°C in Mops glucose. Protein synthesis was inhibited with chloramphenicol at OD_{600} of ≈0.3 for σ^S half-life in exponential phase. Samples were removed at specific time points and analyzed by immunoblot with an anti-σ^S antibody. Half-lives ($t_{1/2}$) were calculated by regression analysis of the exponential decay of σ^S. (*E*) Effects of *phoB* and glucose starvation on *iraP* expression. Northern blot analysis of RNA extracted from exponentially growing cells in Mops minimal medium (time point 0) and after removal of glucose or phosphate at the time points indicated. Strains used in these experiments are wt (MG1655) and *phoB:cm* (AB026). The same RNA extracts were analyzed by using biotinylated probes to detect *phoA* mRNA and *iraP* mRNA. Representative data are shown.

concentrations of phosphate are low, PhoB is phosphorylated by PhoR and activates the transcription of the Pho regulon. As seen in Fig. 1*E*, the mRNA for *phoA*, a known target of PhoB regulation, is induced under our phosphate starvation conditions and this induction is abolished in a *phoB* mutant, as expected. However, a *phoB* mutation had only a minor effect on *iraP* message accumulation in response to phosphate starvation (≈2-fold less mRNA in the absence of PhoB compared with wt) (Fig. 1*E*). In a *phoB* mutant, σ^S was still stabilized in an IraP-dependent manner during phosphate starvation conditions (data not shown; ref. 9). Therefore, PhoB is not required for *iraP* expression or activity.

We had previously found that σ^S stabilization after glucose starvation was independent of IraP (9). However, an examination of *iraP* expression after glucose starvation showed that *iraP* transcripts were increased ≈4-fold over those in growing cells (Fig. 1*E*, first four lanes), indicating that moderate up-regulation of *iraP* expression also occurs under this stress condition.

The signaling molecule of the stringent response, ppGpp, accumulates in both phosphate- and carbon-starved cells (14–16), and positively regulates σ^S expression under phosphate starvation (17), suggesting that ppGpp might play a role in regulating *iraP* transcription. We monitored the accumulation of *iraP* mRNA in wt and Δ*relA* Δ*spoT* (ppGpp°) strains. Strains carrying these two mutations contain no detectable ppGpp under any starvation condition so far tested (14, 15, 18, 19). Because a ppGpp° strain is unable to grow in minimal medium, phosphate starvation experiments were conducted in morpholinepropanesulfonic acid (Mops) complete medium (see *Materials and Methods*). In this defined rich medium, phosphate starvation led to stabilization of σ^S in an IraP-dependent manner as in minimal medium (data not shown) and *iraP* transcripts accumulated (Fig. 2*A*), although the peak of mRNA may persist longer (compare Figs. 1*A* and 2*A*, 15′ vs. 45′ samples). In the Δ*relA* Δ*spoT* (ppGpp°) strain, *iraP* mRNA levels remained extremely low, even after removal of phosphate (Fig. 2*A*). The requirement for ppGpp was also observed with an *iraP-lacZ* transcriptional fusion (data not shown). The effect of ppGpp on *iraP* induction was analyzed in strains carrying the mutated promoter $P_{iraP(-10-2)}$, which increased *iraP* transcription (Fig. 1*A*). In this strain, a ≈12-fold induction of *iraP* in response to phosphate starvation was observed in the ppGpp$^+$ strain, whereas only ≈3-fold induction was detected in the ppGpp° strain (Fig. 2*B*). Therefore, ppGpp is required for full induction of *iraP* during phosphate starvation.

SpoT Is Required for Phosphate Starvation-Dependent *iraP* Induction. In *E. coli*, accumulation of ppGpp depends on activities of two proteins, RelA and SpoT (10, 11). The ppGpp synthesis activity of the ribosome-associated RelA protein is activated during

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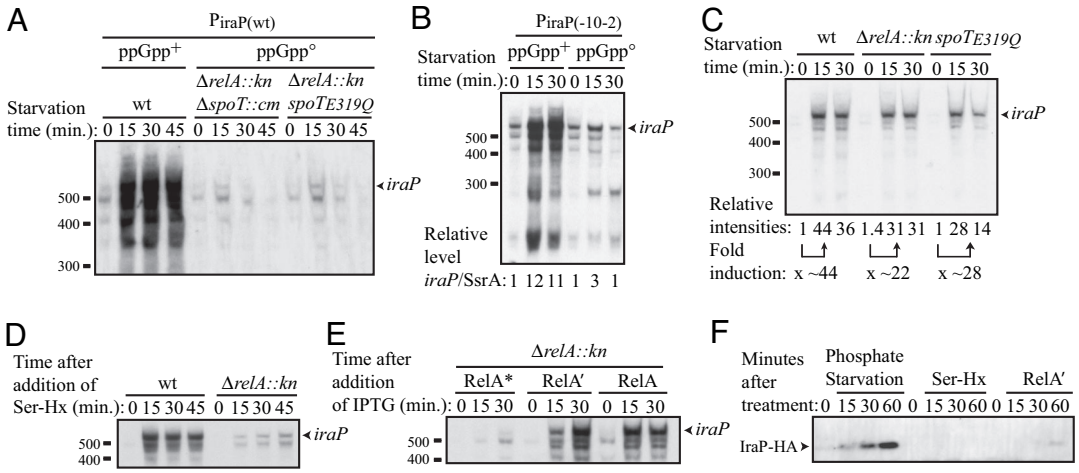

Fig. 2. Effect of ppGpp on *iraP* expression. Cells were grown at 37°C in Mops complete medium (*A*, *B*, and *D*), Mops minimal medium (*C*), or LB (*E*), and starved for phosphate (*A–C*), and total RNA was isolated as described in *Materials and Methods*. Northern blot analyses were conducted as described in Fig. 1. (*A*) Effect of ppGpp on *iraP* promoter activity. The expression of *iraP* was compared in wt (MG1655) and ppGpp° strains (AB029, Δ*relA:kn* Δ*spoT:cm*; AB036, Δ*relA:kn spoT*$_{E319Q}$). (*B*) Effect of ppGpp on the activity of the promoter P$_{iraP(-10-2)}$. Strains used were as follows: P$_{iraP(-10-2)}$ ppGpp$^+$ (AB031); P$_{iraP(-10-2)}$ ppGpp° (AB035). *iraP* transcripts (arrowhead) were normalized to SsrA levels (data not shown); relative amounts are given below the gel. (*C*) SpoT activity is required for *iraP* induction in response to phosphate starvation. mRNA levels in wt (MG1655), Δ*relA:kn* (AB028), and *spoT*$_{E319Q}$ (AB037) were analyzed. (*D*) Effect of the stringent response on *iraP* expression. Cells were cultured in Mops glucose medium supplemented with all amino acids (40 μg/ml each) except serine. Serine hydroxamate (Ser-Hx) (final concentration of 500 μg/ml) was added at $T = 0$ to exponentially growing cells (OD$_{600}$, ≈0.3) to induce starvation, and samples were removed at the indicated time points. *iraP* mRNA levels were compared in wt (MG1655) and Δ*relA:kn* (AB028) strains. (*E*) Effect of ppGpp overproduction on *iraP* expression. Induction of ppGpp was achieved by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to exponentially growing cells (AB028) bearing plasmids pALS13 or pALS10 to overexpress a fragment of the RelA protein, RelA′, which is constitutive for ppGpp synthetic activity or wt RelA protein, respectively. A plasmid pALS14, encoding a truncated and inactive RelA protein, RelA*, was used as a control. (*F*) Immunoblot analysis of IraP expression. Cells (AB040, *iraP-HA*, or AB040 carrying pALS13) were grown as described above for *C* (phosphate starvation), *D* (serine hydroxamate), and *E* (RelA′), and an immunoblot of IraP-HA was conducted as described in *SI Materials and Methods*.

amino acid starvation by the entry of uncharged tRNAs into the A site of the ribosome (20). SpoT is a bifunctional enzyme with both ppGpp synthetase and hydrolase activities (10, 11). Although the regulation of SpoT enzymatic activities is not well understood, SpoT is responsible for ppGpp accumulation in response to carbon, phosphate, fatty acid, and iron starvation conditions (14, 21, 22). We compared *iraP* mRNA levels in wt, *relA*, and *relA spoT* mutant strains. In the absence of *relA*, the kinetics of *iraP* induction was similar to the wt strain, although induction was reduced by 50% in the *relA* mutant (Fig. 2*C*). As mentioned above, in a *relA spoT* mutant strain (ppGpp°), *iraP* transcripts remained extremely low, indicating that *iraP* induction under phosphate starvation can occur in a RelA-independent but SpoT-dependent fashion (Fig. 2, compare *A* and *C*).

Phosphate starvation could be increasing synthesis of ppGpp, decreasing its degradation, or both. A *spoT* mutation alone could not be tested, because it is lethal because of excess accumulation of ppGpp when RelA is present (10). Instead, we compared *iraP* induction in wt cells to cells carrying a nonlethal allele of *spoT*, *spoT*$_{E319Q}$. SpoT can both synthesize and degrade ppGpp; the *spoT*$_{E319Q}$ allele encodes a protein defective for synthesis of ppGpp but retaining the hydrolase activity (R. Harinarayanan and M. Cashel, unpublished work). In the presence of RelA, the *spoT*$_{E319Q}$ mutant showed a pattern of *iraP* expression similar to that in wt cells, although the induction was slightly reduced (<2-fold) in the *spoT*$_{E319Q}$ mutant, suggesting that the ppGpp synthetic activity of SpoT was not required and that the ppGpp synthesized by RelA is sufficient for the response to phosphate starvation (Fig. 2*C*). Consistent with this conclusion, the *relA:kn spoT*$_{E319Q}$ double mutant, which lacks detectable ppGpp (M. Cashel, personal communication), had little or no *iraP* message (Fig. 2*A*). Because ppGpp made from either RelA or from SpoT (Fig. 2*C*) will suffice, we conclude that the basal level of synthesis of ppGpp by either protein is not regulated by phosphate starvation, but that inhibition of the hydrolase activity of SpoT on phosphate starvation allows ppGpp accumulation and *iraP* transcription. Proof of this awaits an understanding of the signaling pathway to SpoT, including identification of mutants in SpoT unable to respond.

Although RelA does not seem to play a critical role during phosphate starvation when SpoT is present, it does play an important role in induction of ppGpp under amino acid starvation (reviewed in ref. 10). Levels of *iraP* transcripts were determined before and after addition of serine-hydroxamate to logarithmically growing cells. A ≈50-fold induction of *iraP* mRNA was observed in wt cells 15 min after amino acid starvation, whereas only a ≈2-fold induction was detected in a *relA* mutant strain (Fig. 2*D*). *iraP* mRNA accumulation was also monitored after artificial induction of ppGpp obtained by overproducing RelA from an inducible promoter on a plasmid. Transcripts for *iraP* rapidly accumulated when ppGpp synthesis was induced with isopropyl-β-D-thiogalactopyranoside in comparison with the uninduced cultures or cells carrying an inactive RelA* protein (Fig. 2*E*). These data indicate that ppGpp accumulation correlates with *iraP* message accumulation and is sufficient to induce *iraP* transcription.

Surprisingly, σ^S degradation was not inhibited after addition of serine hydroxamate, even though *iraP* transcripts were highly induced, and was only partially stabilized after induction of RelA′ (data not shown). This suggests that high ppGpp levels are necessary and sufficient for transcription of *iraP* but not sufficient for IraP translation or activity. To determine whether IraP protein was expressed under these conditions, IraP-HA intracellular amounts were measured by immunoblot. As expected, IraP-HA was induced during phosphate starvation, with levels increasing up to 1 h after starvation began (Fig. 2*F*). However, IraP-HA levels were very low or undetectable after serine-hydroxamate treatment or RelA′ overproduction (Fig. 2*F*). The absence of IraP protein explains why σ^S was not stabilized.

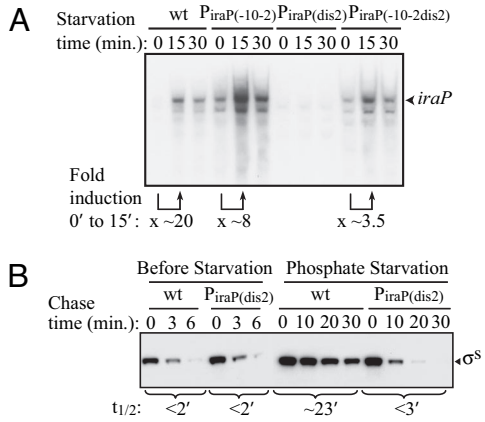

Fig. 3. Effects of promoter mutations on *iraP* expression and activity. Cells were grown at 37°C in Mops glucose medium and starved for phosphate. (*A*) Northern blots for *iraP* mRNA were performed as described in Fig. 1. The expression of *iraP* was compared in wt (MG1655), AB031 ($P_{iraP(-10-2)}$), AB033 ($P_{iraP(dis2)}$), and AB034 ($P_{iraP(-10-2dis2)}$). (*B*) The half-life of σ^S was determined in exponentially growing cells and after 1 h of phosphate starvation as indicated. Protein synthesis was inhibited with chloramphenicol at an OD_{600} of ≈0.3. Samples were removed at specific times and analyzed by immunoblot with an anti-σ^S antiserum. To obtain similar amounts of σ^S at time 0 of each of the chase experiments, the amount of cell extract used for exponential phase cultures was twice the volume used for starved cells. σ^S half-lives ($t_{1/2}$) were determined from plots of intensity vs. time. σ^S degradation was monitored in the wt (MG1655) and AB033 ($P_{iraP(dis2)}$) strains.

ppGpp levels and *iraP* mRNA levels are higher under the conditions of Fig. 2 *D* and *E* than during phosphate starvation (data not shown; refs. 15, 23, 24); possibly high ppGpp levels, while stimulating transcription, may block IraP translation. Because improving the *iraP* promoter is sufficient to give σ^S stabilization (Fig. 1*D*) without starvation, we think it unlikely that any other aspect of phosphate starvation is necessary for IraP translation.

ppGpp Positively Controls IraP Through a Discriminator Sequence. The data presented above suggested a positive role for ppGpp in the control of *iraP* mRNA levels in response to phosphate starvation (Fig. 2). Promoters positively regulated by ppGpp have A/T-rich discriminators (DNA sequence between the −10 hexamer and transcription start site), whereas promoters negatively regulated by ppGpp have G/C-rich discriminators, suggesting that the discriminator sequence is one important feature for regulation by ppGpp (25–29). Interestingly, the discriminator sequence of the *iraP* promoter is A/T rich (Fig. 1*C*). This sequence was mutagenized to modify the A/T content, and both *iraP* mRNA expression and IraP activity (σ^S stabilization after phosphate starvation) were assayed. In the strain carrying the mutated discriminator $P_{iraP(dis2)}$ (5-nt change; Fig. 1*C*), which contains an extensive conversion of the A/T-rich content to G/C rich, *iraP* mRNA levels failed to accumulate after phosphate starvation and remained extremely low, in a manner that resembles the profile obtained in the ppGpp° strain (compare Figs. 2*A* and 3*A*). In the strain with discriminator $P_{iraP(dis)}$, which partially converts the A/T-rich content to G/C rich (3-nt change; aATAct changed to aCGCct), the induction of *iraP* transcripts was significantly reduced (≈3-fold less than the wt promoter in response to phosphate starvation) (data not shown). These data show a strong correlation between the A/T-rich content of the discriminator and the activity of the promoter. σ^S stability in strains carrying the discriminator mutations, before and after phosphate starvation, correlated with the levels of the transcripts. σ^S was unstable in exponentially growing cells in all of the strains tested and became stable when wt cells were starved for phosphate (Fig. 3*B*). However, in the strain carrying $P_{iraP(dis2)}$, σ^S remained unstable during phosphate starvation, as it is in an Δ*iraP* strain (9). The strain carrying $P_{iraP(dis)}$ showed only a slight decrease in σ^S stability compared with the wt strain, suggesting that the 3-fold decrease in *iraP* transcripts detected in this strain still provides sufficient IraP to protect σ^S (data not shown). These data also strongly suggest that ppGpp acts by increasing initiation of *iraP* mRNA (rather than via an effect on *iraP* message stability, for instance), because mutations in the discriminator that do not change the mRNA sequence eliminate the effects of ppGpp.

To be sure that the discriminator did not simply create an inactive promoter, we combined the $P_{iraP(dis2)}$ mutation with the $P_{iraP(-10^{-2})}$ up-promoter mutation. As noted above, the basal promoter activity of $P_{iraP(-10^{-2})}$ is high, but can be further induced by phosphate starvation (Figs. 1*A* and 3*A*, lanes 4–6). In the strain carrying both the promoter up mutation and the discriminator mutation ($P_{iraP(-10^{-2}dis2)}$), *iraP* transcripts showed only a ≈3.5-fold induction during phosphate starvation compared with an 8-fold induction in a strain carrying the promoter up mutation with the wt discriminator (Fig. 3*A*, lanes 4–6 vs. lanes 10–12). This 3-fold residual induction is very similar to the 3-fold induction seen in the absence of ppGpp (Fig. 2*A*). These data confirm that the discriminator region is important for full induction of *iraP* transcripts during phosphate starvation, and that the change in the discriminator is necessary for the full ppGpp response. In addition, these results demonstrate that converting the A/T-rich discriminator to G/C rich did not convert the promoter to one negatively regulated by ppGpp. This agrees with previous studies on the positive regulation of genes for amino acid biosynthesis by ppGpp; converting the A/T-rich discriminator to G/C rich made these promoters insensitive to stimulation by ppGpp, but did not confer negative regulation (26, 27, 29).

Discussion

ppGpp Activation of *iraP* Transcription Results in σ^S Stabilization. Both the alarmone ppGpp and the stationary-phase σ factor σ^S play roles in the cell's response to a variety of stresses and nutrient depletion (reviewed in ref. 4). ppGpp is necessary for σ^S accumulation; although the mechanism of action has not been clear, no increase in σ^S stability was reported previously (17, 30, 31). Here, we demonstrate that ppGpp accumulation during phosphate starvation promotes transcription of the anti-adaptor IraP, which we have previously shown leads to σ^S stabilization (9). Thus, the cell ties the global monitor of cell health, ppGpp, directly to a global transcription factor that can transcribe a host of stress response genes.

The *iraP* promoter is a novel and clear example of the role of ppGpp in positive regulation of transcription. *iraP* transcripts are barely detectable in exponentially growing cells and accumulate during phosphate starvation. *iraP* transcription is not dependent on PhoB/PhoR, but instead is absolutely dependent on ppGpp. Cells carrying mutations in the AT-rich *cis*-acting discriminator sequence of the *iraP* promoter fail to induce the *iraP* message (Fig. 3*A*), as do cells devoid of ppGpp (Fig. 2*A*). Improving the −10 region increased the basal level of *iraP* transcription, but message levels still increased after phosphate starvation (Figs. 1*A*, 2*B*, and 3*A*), and this increase was still dependent on both the presence of ppGpp (Fig. 2*B*) and the wt discriminator sequence (Fig. 3*A*) for full induction.

Increased ppGpp during phosphate starvation has been documented previously (15, 16) and is necessary for the proper induction of certain genes of the Pho regulon (15, 16). The strong tie of phosphate starvation to ppGpp-dependent transcription of IraP confirms and extends these findings and provides a

new physiological role for ppGpp accumulation under these conditions.

ppGpp has been shown to be required for σ^S synthesis as well (17, 30, 31). The combination of an effect on synthesis and stabilization of the protein ensures that under stress conditions that lead to elevated ppGpp, σ^S will be rapidly available.

Alternative Stresses and IraP. Amino acid starvation is the best known inducer of ppGpp, and, in fact, *iraP* mRNA levels are high when the amino acid starvation response is invoked by treatment of cells with serine hydroxamate; this increase is fully RelA dependent, as expected (Fig. 2*D*). However, increased ppGpp is not always sufficient to lead to σ^S stabilization (data not shown; ref. 30), most likely because IraP protein is not produced (Fig. 2*F*). Thus, in cells subjected to serine hydroxamate, significantly less IraP protein was detected than in cells subject to phosphate starvation. Whether this reflects a specific defect in IraP translation or increased IraP instability under these conditions (high ppGpp via RelA), or reflects the general inhibition of translation previously described for conditions of high ppGpp (23, 32) has not been addressed. However, these results suggest that moderate levels of ppGpp (high enough to stimulate transcription but not high enough to inhibit translation) are the signal that is needed for invoking IraP stabilization of σ^S.

At least two other conditions that lead to moderate ppGpp induction also lead to σ^S stabilization. Nitrogen starvation induces ppGpp (33), and IraP-dependent stabilization of σ^S is seen under nitrogen starvation (9). Glucose starvation, which also causes increases in ppGpp (14, 34), has a modest effect in increasing *iraP* transcription (Fig. 1*E*). Whereas σ^S is stabilized by glucose starvation, IraP is not necessary for the stabilization (9, 35). It is possible that redundant anti-adaptors and/or other mechanisms contribute to stabilization under this condition (36). Overall, we predict that any stress condition that leads to moderate accumulation of ppGpp is likely to lead to IraP synthesis, contributing to stabilization of σ^S.

Secondary Levels of IraP Control. In the discriminator mutant or the ppGpp° strain, mRNA levels increased only ≈3-fold after phosphate starvation, instead of the 8- to 12-fold induction of isogenic cells in which ppGpp can act at *iraP* (Figs. 2*B* and 3*A*). The residual induction in the absence of ppGpp may reflect a modest effect of PhoB (Fig. 1*E*) and/or direct or indirect effects of phosphate starvation on mRNA stability.

Not yet addressed is how cells recover from phosphate starvation or other stresses. Whereas the ppGpp-dependent increase in *iraP* transcription is relatively transient (see Fig. 1*A*), the IraP protein, which is stable (9), continues to accumulate (Fig. 2*F*). If the stress is reversed, σ^S degradation rapidly resumes within 15 min (data not shown). Increased synthesis of RssB, for instance, or synthesis of an activator of RssB or inactivator of IraP, might allow σ^S degradation to recommence in the presence of IraP. Reactivation of RssB cannot rely solely on the phosphorylation of RssB because a RssB point mutant blocking phosphorylation is still able to degrade σ^S during the recovery phase (35) and sensitivity to IraP is not perturbed by such a mutation (9).

Promoter Characteristics for Positive Regulation by ppGpp. Positive regulation of transcription by ppGpp has primarily been described for amino acid synthesis operons. Our findings for the *iraP* promoter are consistent with those observations but extend them. As found for the *his* operon and *thr* operons (27, 29), changing the "discriminator" sequence to decrease the AT content destroyed expression of the mRNA in the context of an otherwise wt promoter, and a GC-rich discriminator does not lead to negative regulation by ppGpp (Fig. 3*A*). However, unlike the *his* promoter (27), improving the −10 region of the *iraP* promoter still allowed significant induction by ppGpp (via phosphate starvation) (Figs. 1*A*, 2*B*, and 3*A*).

The direct activity of ppGpp on transcriptional regulation requires the coregulator DksA (37). The activity of a P_{iraP}-*lacZ* transcriptional fusion in stationary phase was ≈2- and ≈5-fold reduced in *dksA* and ppGpp° mutant strains, respectively (data not shown). Thus, the *iraP* promoter fulfills all of the expectations for a target of direct activation by ppGpp, although this has not yet been demonstrated *in vitro*, and extends the positive action of ppGpp beyond amino acid synthesis operons to regulation of an anti-adaptor, and through that, to the accumulation of the RpoS σ factor. Our results, as well as recent reports of the direct positive action of ppGpp and DksA on promoters important for virulence in *E. coli* and *Shigella* (38, 39), strongly suggest that the global effects of ppGpp are even broader than previously thought.

Transcription Directs Function: IraP in *Salmonella*. In *E. coli*, the major promoter for *iraP* is the ppGpp-dependent promoter described here. However, in *Salmonella*, *iraP* is regulated both by phosphate starvation and by limiting Mg^{2+} (40). Consistent with the role of ppGpp in increasing *iraP* transcription after phosphate starvation, transcription of *iraP* increases in response to limiting Mg^{2+}, regulated by the PhoQ/PhoP two-component system; IraP is required for stabilization of σ^S in response to limiting Mg^{2+} (40). Therefore, in *Salmonella*, as in *E. coli*, transcriptional regulation of *iraP* is the primary signal for when IraP will act, but an additional set of regulatory signals are present, so that IraP helps the cell respond to both the general (ppGpp-dependent) stress and the specific (PhoQ/PhoP-dependent) stress.

Regulation and Physiological Function of ppGpp During Phosphate Starvation. The two-component regulatory system PhoR/PhoB regulates the expression of phosphate starvation stress response genes (12, 13), but our findings add to evidence that ppGpp plays an important role in this stress condition as well. ppGpp had previously been shown to be required for induction of certain genes of the Pho regulon (*phoA* and *pstS*) (15, 16).

ppGpp is synthesized in *E. coli* by RelA and SpoT and degraded by the hydrolase activity of SpoT. Our results and others (15) suggest that SpoT rather than RelA is responding to phosphate starvation, and that the hydrolase activity is specifically responsible for transmitting signals during phosphate starvation. Regulation of SpoT hydrolase activity has been proposed (41), but how SpoT is sensing the phosphate starvation stress signal is still unknown. Acyl carrier protein, a cofactor necessary for the biosynthesis of fatty acids, had recently been reported to regulate SpoT activity through direct interaction in response to perturbation of fatty acid synthesis (42). Whether acyl carrier protein regulates SpoT activity under additional stress conditions such as phosphate starvation or there are different proteins or metabolites that interact with SpoT under each stress condition remains to be determined.

From our work, we propose that the use of ppGpp to stimulate σ^S accumulation, rather than PhoB/PhoR, reflects the cell's need for a general stress response, with cross-resistance to a variety of stresses, rather than a limited targeted response. The link between ppGpp and *iraP* transcription and function reinforces how complex the network of signals affecting σ^S is.

Materials and Methods

Bacterial Strains and Plasmids. The genotypes of *E. coli* K12 strains and plasmids used in this work, all derivatives of MG1655, are described in SI Table 1; oligonucleotides used as probes and for strain construction are described in SI Table 2. The construction of strains is described in *SI Materials and Methods*.

Media and Growth Conditions. Cells were grown in LB or Mops minimal medium (Teknova, Hollister, CA) supplemented with 0.4% glucose, 1.32 mM K_2HPO_4, and 1 μg/ml thiamine (Mops glucose media). For growth of ppGpp° strains, Mops medium was supplemented with 1× EZ solution (Teknova) containing amino acids and vitamins (Mops complete media). All liquid cultures were grown under aerobic conditions at 37°C; growth was monitored as OD_{600}. Exponential phase and stationary phase correspond to an OD_{600} of ≈0.3 and ≈3, respectively. Stationary-phase cells were obtained 2 h after the break in the exponential growth curve. For starvation experiments, cells were first grown overnight in Mops medium containing K_2HPO_4 and glucose, and then subcultured into fresh Mops glucose medium at an OD_{600} of 0.01 (≈1:400 dilution) and grown to mid-logarithmic phase (OD_{600} of ≈0.3). Cells were washed twice by filtration with prewarmed (37°C) Mops without K_2HPO_4 (phosphate starvation medium) or without glucose (carbon starvation medium) and resuspended in the same volume of starvation medium, and incubation at 37°C was continued.

For eliciting the stringent response, Mops glucose medium was supplemented as before but containing 40 μg each of 19 aa per ml, omitting serine. Serine hydroxamate (0.5 mg/ml) was added at an OD_{600} of ≈0.3, and samples were taken at the indicated times.

RNA Isolation and Northern Blot Analysis. Cells were grown in LB or in Mops media as described above and at the time indicated cells from 10 to 15 ml of culture were harvested and resuspended in 1 ml of TRIzol reagent (Invitrogen, San Diego, CA). All subsequent purification steps were carried out according to the manufacturer's instructions. Concentrations of RNA samples were determined by measuring the OD_{260}. Analysis of *iraP* and SsrA by Northern blot was performed with ≈2 μg of total RNA per sample separated on an 6% urea-acrylamide sequencing gel (SequaGel; National Diagnostics, Atlanta, GA) in 1× TBE buffer at 65 V for 2 h and transferred onto a positively charged nylon membrane (0.2 μm) at 200 mA for 1 h in 0.5× TBE buffer at 4°C. For detection of the *phoA* transcript, ≈3 μg of total RNA per sample was separated on a 1% denaturing agarose gel as described previously (43). Detection was as described in ref. 44 by using the specific biotinylated probes Bio-yaiB, Bio-SsrA, Bio-phoA (SI Table 2). Signal intensities were quantified by using with ImageJ software (National Institutes of Health).

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