

IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins

Christopher J. Schwartz*, Jennifer L. Giel*[†], Thomas Patschkowski*, Christopher Luther*, Frank J. Ruzicka[‡], Helmut Beinert[‡], and Patricia J. Kiley*^{†§}

*Department of Biomolecular Chemistry, [†]Microbiology Doctoral Training Program, and [‡]Institute for Enzyme Research, University of Wisconsin, Madison, WI 53706

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IscR (iron-sulfur cluster regulator) is encoded by an ORF located immediately upstream of genes coding for the *Escherichia coli* Fe-S cluster assembly proteins, *IscS*, *IscU*, and *IscA*. *IscR* shares amino acid similarity with *MarA*, a member of the *MarA/SoxS/Rob* family of transcription factors. In this study, we found that *IscR* functions as a repressor of the *iscRSUA* operon, because strains deleted for *iscR* have increased expression of this operon. In addition, *in vitro* transcription reactions established a direct role for *IscR* in repression of the *iscR* promoter. Analysis of *IscR* by electron paramagnetic resonance showed that the anaerobically isolated protein contains a [2Fe-2S]¹⁺ cluster. The Fe-S cluster appears to be important for *IscR* function, because repression of *iscR* expression is significantly reduced in strains containing null mutations of the Fe-S cluster assembly genes *iscS* or *hscA*. The finding that *IscR* activity is decreased in strain backgrounds in which Fe-S cluster assembly is impaired suggests that this protein may be part of a novel autoregulatory mechanism that senses the Fe-S cluster assembly status of cells.

cofactor synthesis | metalloproteins | *MarA* | transcriptional regulator | EPR

Fe-S proteins are nearly ubiquitous in nature and are required for many cellular processes. Although Fe-S clusters can be assembled into proteins *in vitro* from Fe²⁺ and S²⁻, it is clear that *in vivo*, this process must be facilitated by protein factors to avoid accumulation of Fe²⁺ and S²⁻ to toxic levels. An important finding in recent years is the discovery of a conserved set of proteins in organisms from bacteria to humans that facilitate assembly of Fe-S clusters into Fe-S proteins (1, 2). In several bacteria, the genes encoding these Fe-S assembly proteins (*IscS*, *IscU*, *IscA*, *Hsc66*, *Hsc20*, and ferredoxin) are organized in a cluster, *iscSUAhscBAfdx*. In this study, we have focused on the regulation of the expression of the genes that encode the Fe-S cluster assembly factors from *Escherichia coli*.

Genetic experiments support the key role of the proteins encoded by *iscSUAhscBAfdx* in Fe-S cluster assembly, because mutations in the *E. coli* genes decrease the activity of many Fe-S proteins (3–5). Furthermore, biochemical studies have begun to provide some insight into the process of Fe-S cluster assembly (1, 6–10). *IscS* is a cysteine desulfurase that procures the sulfur from cysteine for Fe-S cluster assembly (1, 7). *IscU* can form a complex with *IscS* and acquire an unstable [2Fe-2S] cluster that has been proposed to be a source of Fe and sulfur for Fe-S protein assembly (6, 8, 11). *IscA* can also acquire a Fe-S cluster *in vitro* (9). *Hsc66* and *Hsc20*, homologs to molecular chaperones, form a complex *in vitro* with *IscU*, but the exact physiological function of this interaction has yet to be determined (10).

It is also of interest to understand how expression of the Fe-S cluster assembly proteins is controlled. In bacteria, the cellular requirements for Fe-S cluster assembly must vary, because the synthesis of many Fe-S proteins is regulated (12) and environmental conditions arise that lead to the destruction of these

metal centers (13). Thus, it would not be surprising to find that expression of the assembly proteins is regulated to accommodate such changes in Fe-S cluster assembly requirements. A clue that the genes encoding Fe-S cluster assembly proteins might be regulated came from the observation that immediately upstream of *iscSUA* in *E. coli* and several other bacteria, there is a gene (here called *iscR*) whose protein product is similar to the well characterized transcriptional regulator *MarA* (multiple antibiotic resistance) from *E. coli* (Fig. 1). *MarA* is a global regulator of genes induced by antibiotics and is a member of a subfamily of AraC-like transcriptional regulators called the *Mar/Sox/Rob* family (14). Interestingly, *IscR* contains a stretch of amino acids not found in *MarA*, which includes cysteine residues that may coordinate a Fe-S cluster (Fig. 1). Given the proximity of *IscR* to the Fe-S cluster assembly genes *iscSUA*, the similarity of *IscR* to *MarA*, and the possibility that *IscR* contains a Fe-S cluster, we characterized the properties of *IscR* and investigated whether this protein regulates the transcription of the *isc* gene cluster. Our results indicate that *IscR* contains a [2Fe-2S] cluster and acts as a direct negative regulator of the *iscRSUA* operon.

Materials and Methods

Plasmid Construction. *iscR* is annotated as *yfhP* or b2531 in the *E. coli* genome sequence. Plasmid pPK5960 was created by ligating the 985-bp *StuI-EcoRV* fragment containing *iscR* from pPK4071 (5) into the *EcoRV* site of pACYC184 (15). pPK6161 was created by PCR amplification of the *iscR* ORF (+1 to +489 nucleotides) by using primers that contained *NdeI* and *BamHI* restriction enzyme sites for subsequent directional cloning into the expression vector pET-11a (16).

Strain Construction. Chromosomal *lacZ* fusions to the *isc* operon were constructed by using the two-step method of Simons *et al.* (17). DNA fragments to be analyzed for promoter activity were obtained by PCR amplification of chromosomal DNA with *Pfu* DNA Polymerase (Stratagene) following the manufacturer's recommended conditions. PCR fragments (Fig. 2) containing 476 to +74 nucleotides relative to the *iscR* start codon; –396 to +419 nucleotides relative to the *iscS* start codon; –476 to +9 nucleotides relative to the *iscR* start codon, and –226 to +419 nucleotides relative to the *iscS* start codon; and –476 to +9 nucleotides relative to the *iscR* start codon and –226 relative to the *iscS* start codon to the end of the *iscA* ORF were phosphorylated with T4 polynucleotide kinase (Promega) and ligated into

Abbreviation: EPR, electron paramagnetic resonance.

See commentary on page 14751.

[§]To whom reprint requests should be addressed at: 1300 University Avenue, Department of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, WI 53706. E-mail: pjikiley@facstaff.wisc.edu.

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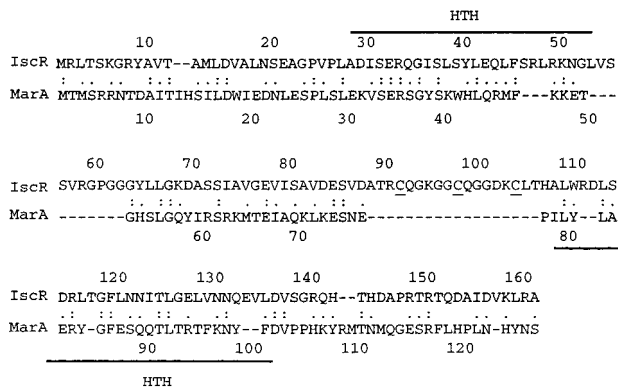


Fig. 1. Similarity of IscR to MarA. Amino acid sequence alignment of IscR and MarA generated with ALIGN showed 20% identical residues (:) and 47% similar residues (.). Solid lines denote the two helix-turn-helix DNA-binding domains of MarA; the three cysteine residues (C92, C98, C104) of IscR that may coordinate the [2Fe-2S] cluster are underlined.

*Sma*I-digested pRS1553 to generate gene fusions to *lacZ* (17). These *lacZ* fusions will be referred to as *iscR'*-*lacZ*, *iscS'*-*lacZ*, *P_{iscRiscS'}*-*lacZ*, and *P_{iscRiscSUA}*-*lacZ*, respectively. *P_{iscRiscS'}*-*lacZ* and *P_{iscRiscSUA}*-*lacZ* have the same promoter region as *iscR'*-*lacZ* but contain an in-frame deletion of *iscR* (from codons 3 to 127) and have a *Bam*HI restriction site in place of codons 3 and 127. The various *isc-lacZ* fusions were then recombined onto λ RS468, and the resulting recombinant λ phages were inserted in single copy at the chromosomal λ attachment site of RZ4500 (18), which was verified by the *ter* excision test (17, 19).

An *iscR* deletion strain, PK5956, was constructed by replacing codons 2–120 of *iscR* with a Kn^R cassette flanked by FRT (*FLP* recognition target) sites from plasmid pKD13 in strain

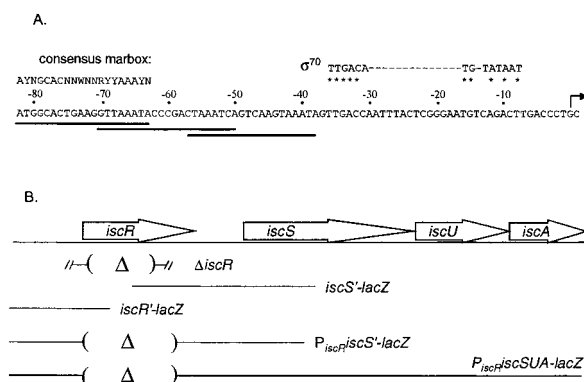


Fig. 2. The *iscR* promoter region. (A) The DNA sequence of the *iscR* promoter region. The transcription start site identified by 5' mapping is indicated by an arrow. Matches to an extended -10 element and a consensus -35 hexamer of the $E\sigma^{70}$ -binding site with a 17-bp spacer region are marked by asterisks. The position of three overlapping marbox-like sequences (sequences that bind MarA) are denoted by the solid lines; -63 to -82 nucleotides contain an exact match to the marbox consensus sequence, whereas the sequences from -38 to -57 nucleotides and -51 to -70 nucleotides match only 9 nucleotides of the 14 conserved marbox nucleotides. (B) The location of the DNA segments used to generate *lacZ* fusions in this study are shown and consist of the following regions: *iscR'* (-476 to $+74$ nucleotides relative to the *iscR* start codon), *P_{iscRiscS'}* (-476 to $+9$ nucleotides relative to the *iscR* start codon and -226 to $+419$ nucleotides relative to the *iscS* start codon), *P_{iscRiscSUA}* (-476 to $+9$ nucleotides relative to the *iscR* start codon and -226 nucleotides relative to the *iscS* start codon to the end of the *iscA* ORF). The *iscR* sequences that were removed to generate the in-frame *iscR* deletions in the *lacZ* fusions or in the chromosome (the line flanked by the hash marks) are indicated by parentheses.

BW25113/pKD46, as previously described (20). This Δ *iscR*:: Kn^R allele was introduced into strains MG1655, PK6361, PK6259, and PK6364 by P1 transduction, creating strains PK4855, PK6367, PK6271, and PK6390, respectively. The Kn^R cassette was subsequently deleted from PK4855 by transforming with pCP20 encoding *FLP* recombinase (20). The resulting Kn^S strain (PK4854) contained the *iscR* start codon, 84 nucleotides of FRT sequence, and the nine terminal codons of *iscR*, leaving an in-frame *iscR* deletion.

To analyze the effect of the loss of IscR, IscS, and HscA on *isc* gene expression, null mutations of the genes encoding these proteins (Table 1) were introduced into the appropriate strains by P1 transduction. To facilitate construction of the Δ *iscS*:: Kn^R derivative, a closely linked (90%) Tet^R marker [*zff*-208::Tn10 from CAG 18481 (21)] was first introduced into PK4331 (5) to obtain PK6311 containing both the Tet^R and Kn^R markers. Because strains containing Δ *iscS*:: Kn^R have a slow growth phenotype and take 2 days to form colonies on plates, selection for the Tet^R marker eliminated obtaining spontaneous Kn^R strains that would also appear in that time period.

β -Galactosidase Assays. To assay promoter activity from the *lacZ* fusion constructs, cells were grown overnight in LB media and subcultured into fresh growth medium at 1:800 dilution. Cells were grown to an OD_{600} of 0.4–0.8, placed on ice, the appropriate antibiotic was added to inhibit further cell growth, and β -galactosidase activity was assayed as previously described (22).

Mapping the 5' End of *isc* mRNA. Total cellular RNA (2–5 μg) was prepared (method from C. Gross, personal communication) from cultures grown in LB media to an OD_{600} of 0.3–0.8 and then hybridized at 65°C to ^{32}P -labeled primers complementary to $+53$ to $+71$ nucleotides of the *iscR* mRNA. Primer extension with Thermoscript Reverse Transcriptase (GIBCO/BRL) was carried out according to the manufacturer's recommendations; the products were separated on a denaturing 6% polyacrylamide gel, and the gel was exposed to either film or a PhosphorImager screen (Molecular Dynamics). To identify the transcription start site, sequencing reactions of pPK4071 (5) by using the same primer were run in parallel.

In Vitro Transcription Reactions. A plasmid template (pPK6511) for *in vitro* transcription reactions was created by ligating the *Sma*I-*Stu*I fragment from pPK4071, containing sequences -161 to $+38$ relative to the *in vivo* mapped transcription start site of *iscR*, into pUC19-*spI'* (23). This plasmid was purified by using the Maxiprep kit (Qiagen, Chatsworth, CA), and *in vitro* transcription reactions containing pPK6511 template DNA (2 nM), IscR (656 nM protein, 200 nM Fe-S cluster), and RNAP $E\sigma^{70}$ holoenzyme (50 nM) (Epicentre Technologies, Madison, WI) were performed as previously described (24) in a Coy chamber.

IscR Purification. IscR was purified from *E. coli* B strain PK22 (BL21 Δ *crp*-*bs990* Δ *fur*) harboring *iscR* under the control of the inducible T7 RNA polymerase promoter on plasmid pPK6161. Cell growth, protein overproduction, harvesting of cells, and preparation of anaerobic cell extracts were carried out as described previously (25). In an anaerobic chamber (Coy) containing 5% CO_2 , 5% H_2 , and 90% N_2 , the supernatant was loaded at a flow rate of 1 ml/min onto a 5-ml HiTrap Heparin column (Amersham Pharmacia) by using an Amersham Pharmacia LCC-501 Plus-based FPLC system. The column was washed with 10 ml of anaerobic buffer A (50 mM Tris-HCl, pH 7.4/10% glycerol/0.1 M KCl) containing 1 mM DTT, 1.7 mM dithionite, and 0.1 mM phenylmethylsulfonyl fluoride. IscR was eluted from the column in a linear gradient of 0.1–1.0 M KCl in anaerobic buffer A at a flow rate of 1 ml/min. Fractions containing IscR were pooled and loaded at a flow rate of 0.2 ml/min onto a 5-ml

Table 1. Strains and plasmids used in this study

	Genotype	Source
<i>E. coli</i>		
Strain		
RZ4500	MG1655 <i>lacZ</i> Δ145	Ref. 18
W3110 <i>hscA2-cat</i>		L. E. Vickery, personal communication
PK6307	RZ4500 <i>λiscS'</i> - <i>lacZ</i>	Transduction of RZ4500 with <i>λiscS'</i> - <i>lacZ</i>
PK6311	RZ4500 <i>ΔiscS</i> ::Kn ^R , <i>zff-208</i> ::Tn10	P1 transduction of Tn10 from CAG18481 (21) into PK4331 (5)
PK6361	RZ4500 <i>λP_{iscR}iscS'</i> - <i>lacZ</i>	Transduction of RZ4500 with <i>λP_{iscR}iscS'</i> - <i>lacZ</i>
PK5956	BW25113/pKD46 <i>ΔiscR</i> ::Kn ^R	Transformation of BW25113/pKD46 (20)
PK6367	PK6361 <i>ΔiscR</i> ::Kn ^R	P1 transduction from PK5956
PK6259	RZ4500 <i>λP_{iscR}iscSUA</i> - <i>lacZ</i>	Transduction of RZ4500 with <i>λP_{iscR}iscSUA</i> - <i>lacZ</i>
PK6271	PK6259 <i>ΔiscR</i> ::Kn ^R	P1 transduction from PK5956
PK6364	RZ4500 <i>λiscR'</i> - <i>lacZ</i>	Transduction of RZ4500 with <i>λiscR'</i> - <i>lacZ</i>
PK6390	PK6364 <i>ΔiscR</i> ::Kn ^R	P1 transduction from PK5956
PK6391	PK6364 <i>ΔiscS</i> ::Kn ^R , <i>zff-208</i> ::Tn10	P1 transduction from PK6311
PK6389	PK6364 <i>hscA2-cat</i>	P1 transduction from W3110 <i>hscA2-cat</i>
PK4855	MG1655 <i>ΔiscR</i> ::Kn ^R	P1 transduction from PK5956
PK4854	MG1655 <i>ΔiscR</i>	Resolution of Kn cartridge with pCP20
Plasmids		
pPK5960	<i>Stul</i> -EcoRV 985-bp <i>iscR</i> fragment cloned into pACYC184, Cm ^R	This study
pPK6161	+1 to +489 <i>iscR</i> fragment cloned into pET-11a, Ap ^R	This study
pPK6511	-161 to +38 <i>iscR</i> promoter fragment cloned into pUC19- <i>spf'</i> , Ap ^R	This study

BioRex70 (Bio-Rad) column on the same FPLC system. IscR was eluted from the column in a linear gradient of 0.1–1.0 M KCl in anaerobic buffer A at a flow rate of 0.2 ml/min and concentrated by passing the eluent over a 1-ml BioRex70 column and eluting with 0.8 M KCl-containing buffer A.

Iron, Sulfide, and Protein Determinations. The iron (26) and sulfide (27) content of IscR were analyzed in duplicate. Protein concentrations (reported here as monomers) were determined by a biuret procedure as described previously (28) and standardized against the FNR protein (28).

Electron Paramagnetic Resonance (EPR) Spectroscopy. After recording the spectrum of the protein as obtained on anaerobic purification, [2Fe-2S] IscR was oxidized by exposing the purified protein to air for 10 min and then frozen. For reduction of the air-oxidized sample, the sample (200 μl) was thawed and transferred to a 5-ml vessel with two sidearms, suitable for gassing and evacuation (29). The sample was degassed, and 5 μl of a dithionite solution (containing a 5-fold molar excess of dithionite) was added through a sidearm during argon flow. The vessel was closed and placed in an anaerobic chamber, where the sample was transferred to an EPR tube and frozen.

Results

IscR Negatively Regulates *iscR* Expression *in Vivo*. Inspection of the DNA sequence upstream of *iscR* revealed the presence of DNA sequences similar to those that MarA binds -151 to -106 nucleotides upstream of the *iscR* initiation codon (Fig. 2). We considered it a reasonable possibility that IscR might bind these sequences, because IscR contains regions similar to the helix-turn-helix DNA-binding domains of MarA (Fig. 1). Furthermore, the location of the putative binding sites upstream of *iscR* led us to hypothesize that IscR might regulate its own synthesis. Two lines of evidence suggest that *in vivo* IscR is indeed a regulator of *iscR* transcription. First, we compared β-galactosidase levels of both *iscR*⁺ and *ΔiscR*::Kn^R strains containing the

iscR upstream region fused to the reporter gene, *lacZ* (*iscR'*-*lacZ*), on a λ phage integrated at the λ attachment site (Fig. 2). These results showed that expression of the *iscR'*-*lacZ* fusion increased 17-fold in the absence of IscR (Fig. 3). When *iscR* was provided *in trans* on plasmid pPK5960, β-galactosidase activity returned to wild-type levels, demonstrating that the effect is attributable to *iscR* (Fig. 3; *piscR*). Second, mapping of the 5' end of the *iscR* mRNA by primer extension assays showed that one major 5' end was identified 67 nucleotides upstream of the *iscR* start codon in both an *iscR*⁺ strain (MG1655) and a strain containing an in-frame deletion of *iscR* (PK4854) (Fig. 4). In addition, this appears to be an IscR-responsive transcript, because levels were increased in a strain lacking IscR (Fig. 4). Taken together, these data indicate that IscR negatively regulates transcription from a promoter located upstream of *iscR*.

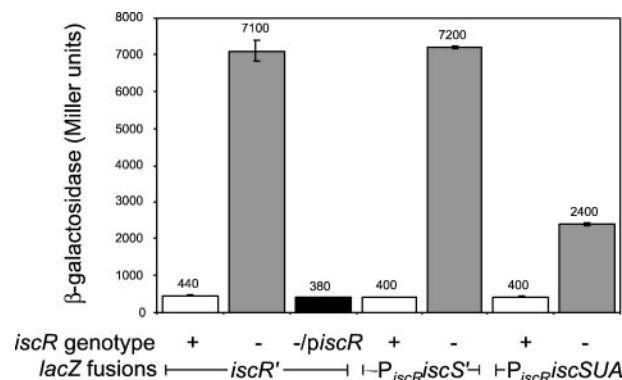


Fig. 3. IscR negatively regulates expression of *iscR**SUA*. β-Galactosidase activity was measured from strains containing *lacZ* fusions to the DNA regions shown in Fig. 2. IscR⁺ strains (open bars) contain wild-type *iscR*, and IscR⁻ strains (filled bars) contain the *ΔiscR*::Kn^R allele; *piscR* is plasmid pPK5960. The data represent the average activity of three independently isolated strains.

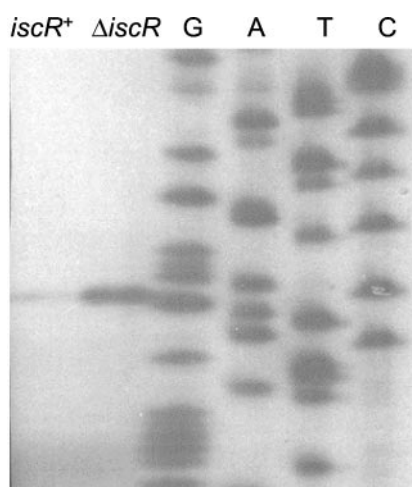


Fig. 4. Primer extension assays of *iscR* mRNA. An autoradiograph of primer-extended RNA products from either MG1655 (*iscR*⁺) or PK4854 (*ΔiscR*), by using an *iscR*-specific primer. Sequencing ladders carried out with the same primer are included as a reference.

IscR Contains a [2Fe-2S] Cluster. To further characterize IscR and determine whether it directly represses *iscR* transcription, we purified the protein from cells that overexpress IscR. Because IscR contained a series of Cys residues, we reasoned that it might contain a Fe-S cluster; therefore, the protein was purified under anaerobic conditions by using the overexpression scheme previously shown to allow isolation of the FNR protein, which contains an oxygen-sensitive Fe-S cluster (25). By SDS/PAGE, the protein as obtained on purification had a molecular weight of approximately that of the predicted size of 17,336 (data not shown). In addition, IscR had a reddish color, and the broad absorption features between 500 and 600 nm in the absorption spectrum were typical of a protein containing a [2Fe-2S] cluster (data not shown) (30). Anaerobically purified IscR showed an EPR signal with *g* values at 1.99, 1.94, and 1.88, typical for the reduced (1+) state (Fig. 5), which accounted for one spin per 2 S²⁻. The behavior of the signal toward changes of temperature

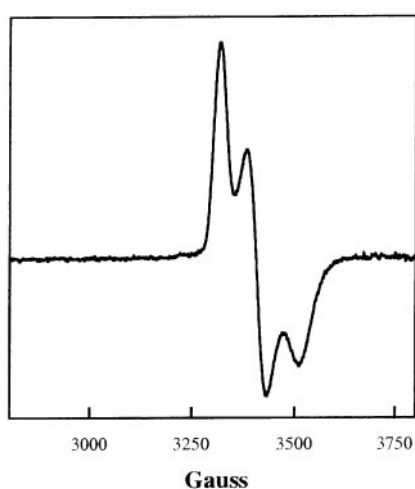


Fig. 5. EPR spectrum of IscR. EPR spectrum (first derivative) of IscR as obtained on purification under anaerobic conditions. The protein concentration was 1.75 mM, S²⁻ 1.11 mM and Fe 1.16 mM. The spectra were collected at a frequency of 9.2254 GHz, microwave power of 10 μW, modulation amplitude of 0.4 mT (4 G), modulation frequency of 100 kHz, scan time of 4 min, and temperature of 20 K. The *g* values are 1.99, 1.93, and 1.88.

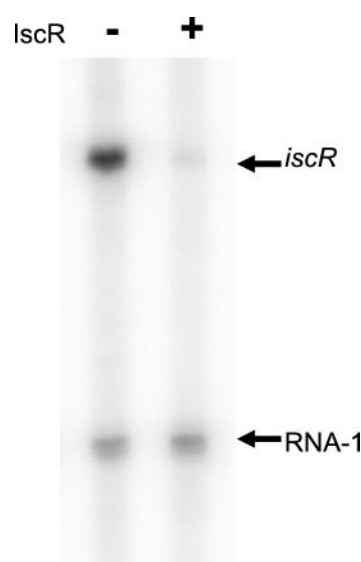


Fig. 6. IscR represses transcription from the *iscR* promoter. A phosphorimage of the products from *in vitro* transcription reactions containing the *iscR* promoter plasmid, pPK6511 (2 nM) and Eσ⁷⁰ (50 nM) (lanes 1 and 2). The reaction in lane 2 also contained IscR (656 nM protein; 200 nM Fe-S cluster). The arrows indicate the transcripts corresponding to *iscR* and RNA-1.

and microwave power indicated that the Fe-S cluster in IscR has a particularly low spin relaxation rate. These features together, the optical spectra, the spin vs. sulfide stoichiometry, and the low spin relaxation rate, leave no doubt that IscR contains a [2Fe-2S] cluster. When the sample was thawed, oxidized by shaking in air for 10 min, and refrozen, the only signal observed was at *g* = 4.3, typical for a high-spin ferric iron accounting for only 4% of the iron in the sample, and the color of the protein changed from red to brown, indicative of [2Fe-2S]¹⁺ cluster conversion to the 2+ oxidation state. After reduction (see *Materials and Methods*) the signal at *g* = 1.99, 1.94, 1.88 was again observed with no significant loss in intensity, demonstrating that the cluster undergoes reversible oxidation reduction. Sulfide and iron analysis of four IscR preparations showed that the stoichiometry of S²⁻ per mol of IscR ranged from 0.36 to 0.63 with an average Fe to S²⁻ ratio of 1.2. In summary, these results demonstrate that IscR contains a [2Fe-2S] cluster, but that these preparations are not completely occupied with Fe-S clusters, a finding not unusual for overexpressed Fe-S proteins (25).

IscR Acts as a Repressor *in Vitro*. To establish that IscR was directly affecting transcription from its own promoter, we carried out *in vitro* transcription reactions by using an *iscR* promoter template. In the presence of only Eσ⁷⁰ RNAP and supercoiled template DNA, a transcript of 155 nucleotides was observed in addition to the control RNA-1 transcript (Fig. 6). The 155-nt transcript corresponds to the *iscR* transcript, because its production depended on the *iscR* insert, and this product is of the size expected according to the *in vivo* 5' end-mapping data in Fig. 4. Addition of IscR protein to the transcription reactions reduced the abundance of the *iscR* transcript 38-fold (±2), whereas no change in the level of RNA-1 transcript was observed. This result indicates that IscR directly represses transcription from its own promoter.

***iscRSUA* Expression Is Under the Control of the IscR-Responsive Promoter.** To test whether IscR is also a regulator of the downstream *iscSUA* genes, we measured β-galactosidase activity from strains containing a *lacZ* fusion starting from the same region 5' to *iscR* but ending within *iscS* or at the end of *iscA* and

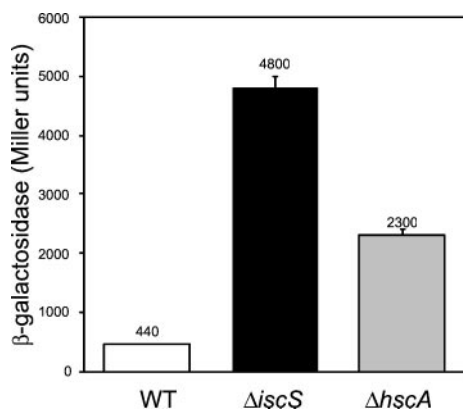


Fig. 7. IscR activity is decreased in strains lacking Fe-S cluster assembly proteins. β-Galactosidase activity was measured from strains containing a *lacZ* fusion to the *iscR* promoter (Fig. 2) and derivatives containing *hscA2-cat* or Δ*iscS*::Kn^R. The data represent the average activity of three independently isolated strains.

containing an in-frame deletion of *iscR* ($P_{iscR}iscS'-lacZ$ and $P_{iscR}iscSUA-lacZ$, respectively; Fig. 2). Regardless of the 3' endpoint fused to *lacZ*, expression was increased in strains lacking IscR, demonstrating that IscR regulates transcription of the entire *iscRSUA* gene cluster (Fig. 3). To determine whether IscR regulates *iscSUA* expression from the promoter identified upstream of *iscR* or from an unknown promoter located within the intragenic region (115 nucleotides) between *iscR* and *iscS*, we measured β-galactosidase activity from a strain containing a *lacZ* fusion that was under control of the region that extends from -396 to +419 nucleotides relative to the *iscS* start codon (*iscS'-lacZ*). Overall, very little β-galactosidase activity was detected, and the *iscR* deletion had no effect on expression of the *iscS'-lacZ* fusion, suggesting that this region, which includes the 281 terminal nucleotides of *iscR*, has no promoter activity (data not shown). Therefore, we conclude that *iscSUA* must be cotranscribed with *iscR* from the major IscR-responsive promoter.

IscR Activity Is Affected by Fe-S Cluster Assembly Mutants. IscS has been previously characterized as a cysteine desulfurase, and strains deleted for *iscS* display deficiencies in Fe-S cluster assembly (3–5). Therefore, we wanted to test whether the defects in Fe-S cluster assembly that occur in an *iscS* deletion strain would affect IscR activity. Indeed, we observed that *iscR'-lacZ* expression was increased in strains in which *iscS* was replaced with a Kn^R cartridge (Fig. 7). Strains containing null mutations in *hscA* lack Hsc66 and are defective in Fe-S cluster assembly, similar to strains containing an *iscS* deletion (3, 5), so we also assayed IscR activity in a strain containing an insertion in *hscA* (*hscA2-cat*). As shown in Fig. 7, *iscR'-lacZ* expression was also increased in cells lacking Hsc66, indicating that IscR activity was decreased. Therefore, the loss of two different gene products implicated in Fe-S cluster assembly, IscS or Hsc66, leads to increased expression from the *iscR* promoter, suggesting that the Fe-S cluster of IscR is important for IscR-dependent regulation. IscR activity was not completely eliminated in the Δ*iscS*::Kn^R or the *hscA2-cat* strains, because the β-galactosidase levels were not as high as that in an Δ*iscR* strain. This finding is not surprising, because previous results showed that strains deleted for *iscS* or *hscA* still possess some residual Fe-S cluster assembly activity (5).

Discussion

IscS, IscU, and IscA have been implicated in facilitating Fe-S cluster assembly both *in vitro* and *in vivo*. In this study, we found that a previously uncharacterized regulator, IscR, regulates

expression of the genes encoding these proteins. We have shown that IscR contains a [2Fe-2S] cluster and is a transcriptional repressor of the *iscRSUA* operon. Because our data also suggest that the Fe-S cluster of IscR may be important for its function as a transcriptional regulator, we propose that IscR participates in an autoregulatory loop to maintain the appropriate levels of cellular Fe-S cluster synthesis.

***iscR* Is Cotranscribed with *iscSUA*.** Our data indicate that IscR represses transcription initiation from a single promoter upstream of *iscR*. Both an extended -10 element and a nearly consensus -35 hexamer are present upstream of this start site (Fig. 2), consistent with this promoter being transcribed by Eσ⁷⁰ RNA polymerase. In addition, we found that deletion of *iscR* led to derepression not only of *iscR* but also of *iscSUA*. Because no additional promoter element was identified between *iscR* and *iscS* by using *lacZ* fusions, the simplest interpretation of these data is that *iscR* is cotranscribed with *iscSUA* and that transcription of *iscRSUA* originates from a single promoter upstream of *iscR* that is repressed by IscR. Furthermore, the genomic organization of *iscRSUA* is conserved in many β- and γ-proteobacteria, or, as in the case of some Gram-positive bacteria, *iscRS* is conserved. This suggests that the autoregulation of Fe-S cluster assembly genes by IscR is not unique to *E. coli*.

IscR, a Member of the Mar/Sox/Rob Family, Functions as a Repressor.

IscR is the first MarA/Rob/SoxS family member, to our knowledge, that has been shown to directly function as a repressor. Although the exact location of the IscR-binding site(s) within the *iscR* promoter region is unknown, it must reside within the region from -161 to +38 nucleotides relative to the start site of transcription, because this region was sufficient for IscR to decrease transcription *in vitro*. Within this region (-82 to -38 nucleotides relative to the *iscR* transcription start site; Fig. 2), three sequences with similarity to MarA-binding sites were found (31, 32). If these represent sequences to which IscR binds, then these sites are in a position atypical for binding of a repressor. Additional studies are necessary to determine what sequences in the *iscR* promoter are critical for IscR DNA binding and repression and the mechanism of IscR repression.

Our finding that IscR is a repressor of *iscRSUA* does not exclude the possibility that IscR may also function as a transcription activator like other members of the AraC family (14). In this regard, it is important to note that we did not observe any large growth defects with the *iscR* deletion strain. Furthermore, Tokumoto and Takahashi (3) found that normal levels of several Fe-S proteins were present in strains deleted for *iscR*. Together, these data indicate that if IscR does have other targets, changes in their expression apparently cause no large growth defects or large changes in levels of Fe-S proteins.

Expression of *hscBAfdx*. Downstream of the *isc* operon are the genes encoding a ferredoxin and the chaperone homologs, Hsc66 and Hsc20, which have also been implicated in promoting Fe-S cluster assembly (1). Although it is logical to hypothesize that expression of these genes may also be under the control of the major IscR-responsive promoter, two independent transcription start sites have been previously mapped upstream of each of the *hsc* genes, suggesting that they may be transcribed separately from the *isc* genes (33, 34). However, in some bacteria, such as *Azotobacter vinelandii* (1), the intergenic space between the *iscA* and *hscB* is smaller than in *E. coli*, leaving open the possibility that, in such cases, the Fe-S cluster assembly genes may be cotranscribed. Experiments that address whether *hscBAfdx* expression is regulated by IscR in *E. coli* should provide additional insight as to how expression of these genes is controlled.

The Role of the [2Fe-2S] Cluster in Controlling IscR Function. Our finding that IscR contains a [2Fe-2S] cluster raises the question of how this cluster might function in regulation of IscR repressor activity. The observation that IscR activity is decreased in mutants lacking the Fe-S cluster assembly proteins, IscS and Hsc66, has led us to consider that the ability of IscR to function as a repressor depends on whether it contains a [2Fe-2S] cluster. This is a particularly attractive hypothesis, because the three IscR cysteine residues, which may coordinate the Fe-S cluster, are located in a region that in MarA separates the two helix-turn-helix DNA-binding motifs (14). Thus, if IscR has DNA-binding domains organized in a manner similar to other members of this family, the presence of the Fe-S cluster might affect IscR repressor function by influencing its DNA binding activity. In this case, we would expect that IscR is a monomer like other members of this family (14) and contains one [2Fe-2S] cluster, although this hypothesis has not been tested experimentally. This model would also imply that the [2Fe-2S] cluster would have one noncysteine ligand, because only three Cys are present in IscR. Nevertheless, it is also possible that the oxidation state of the cluster further modulates IscR function as has been found with the [2Fe-2S]-containing transcription factor, SoxR (35). However, preliminary experiments suggest that oxidation of IscR does not cause any large changes in the ability of IscR to repress the *iscR* promoter *in vitro* (data not shown).

Nonetheless, the finding that IscR activity is decreased in mutants lacking the Fe-S cluster assembly proteins, IscS and Hsc66, has led us to consider that there may be a link between the levels of [2Fe-2S] IscR and the rate of Fe-S cluster formation catalyzed by the Fe-S cluster assembly proteins. This type of homeostatic model would predict that when Fe-S cluster assembly becomes rate limiting (i.e., all Isc proteins are engaged in Fe-S cluster assembly), levels of [2Fe-2S] IscR would decrease as a result of a diminution in its rate of synthesis, and repression of *iscRSUA* would be relieved. The resulting increase in the Isc

assembly proteins would subsequently lead to an increase in the rate of Fe-S cluster formation, causing levels of [2Fe-2S] IscR to rise, thus reestablishing repression of the *isc* operon. If this model were correct, then we would expect that Fe-S clusters would be assembled only when there is a need. For example, it predicts there would be an increase in Fe-S cluster synthesis in response to conditions known to damage Fe-S clusters, such as oxidative stress. In support of this notion, it has been recently reported that *iscR* expression is increased by high external concentrations of hydrogen peroxide (1 mM) (36).

In summary, we have presented data showing that IscR is a transcriptional repressor of *iscRSUA*. This repression appears to depend on the Fe-S cluster assembly proteins, providing a mechanism by which Fe-S cluster assembly can be coupled to cellular requirements for synthesis or repair of Fe-S proteins. Because the synthesis or repair of individual Fe-S cluster proteins is subject to input from a variety of physiological and environmental signals, this type of mechanism would provide an additional level of global regulation to ensure that Fe-S clusters are synthesized when there is an increased demand. Future work will aim to elucidate the mechanism of IscR repression, determine whether other targets exist for IscR, and study how the activity of this protein is modulated.

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