Regulation of the integrase and cassette promoters of the class 1 integron by nucleoid-associated proteins

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The integrase IntI1 catalyses recombination of antibiotic-resistance gene cassettes in the integron, a widely found bacterial mobile element active in spreading antibiotic multi-resistance. We have previously shown that resistance cassette recombination rate and specificity depend on the amount of intracellular integrase. Here, we used in vivo and in vitro methods to examine convergent expression of the integrase promoter (P_{int}) and of the cassette promoters (P_{c} and P_{2}) in the prototypical plasmid-borne class 1 integron, In2. Highly conserved P_{int} has near consensus −10 and −35 hexamers for σ^{54} RNA polymerase, but there are 11 naturally occurring arrangements of P_{c} alone or combinations of the P_{c}+P_{2} cassette promoters (note that P_{2} occurs with a 14 or 17 bp spacer). Using a bi-directional reporter vector, we found that P_{int} is a strong promoter in vivo, but its expression is reduced by converging transcription from P_{c} and P_{2}. In addition to cis-acting convergence control of integrase expression, the regulator site prediction program, PRODORIC 8.9, identified sites for global regulators FIS, LexA, IHF and H-NS in and near the integron promoters. In strains mutated in each global regulator, we found that: (1) FIS repressed integrase and cassette expression; (2) LexA repressed P_{int} and P_{2} with the 14 bp spacer version of P_{2} and FIS was necessary for maximum LexA repression; (3) IHF activated P_{int} when it faced the strong 17 bp spacer P_{2} but did not elevate its expression versus LexA-repressed P_{2} with the 14 bp spacer; and (4) H-NS repressed both P_{int} and the 14 bp P_{2} but activated the 17 bp P_{2} cassette promoters. Mobility shift assays showed that FIS and IHF interact directly with the promoter regions and DNase I footprinting confirmed extensive protection by FIS of wild-type In2 integron promoter sequence. Thus, nucleoid-associated proteins, known to act directly in site-specific recombinator, also control integron gene expression directly and possibly indirectly.

INTRODUCTION

The class 1 integron is very important in disseminating multi-drug resistance in bacteria (Hall & Collis, 1995; Stokes & Hall, 1989). It consists of two conserved regions flanking a variable region (Fig. 1a). The central variable region comprises tandemly arrayed gene cassettes which are mobile, non-self-replicating DNA elements encoding an ORF and an integrase-specific recombination site called attC (Hall & Collis, 1995; Hansson et al., 1997). Historically, the two canonical integron components were defined as the conserved region 5' to the resistance gene cassettes, which encodes the integrase gene (intI1), a unique site-specific tyrosine recombinase found in chromosomal islands and on large conjugative plasmids, and an adjacent recombination site, attI, where cassettes are typically inserted (Hall & Stokes, 1993). The promoters we describe here are all contained within this so-called `5' conserved region' of the integron, In2 (Fig. 1b). The 3' conserved region in class 1 integrons lies beyond any mobile resistance gene cassettes and consists of a sulfadiazine resistance gene (sul), a slightly truncated but functional gene for resistance to quaternary ammonium disinfectants (qacEA) and a short ORF of unknown function (ORF5). It is thought that these 3' conserved genes are cassettes which have lost their attC sites and so are no longer mobile. The 3' conserved region is not a subject of this work and is not shown in Fig. 1(b) where it would lie to the far left.

Most gene cassettes lack their own promoters (Stokes & Hall, 1991; Tolmasky & Crosa, 1993) and are transcribed as one transcript by a promoter (P_{c}) located within the divergently transcribed intI1 gene over 200 bp away from
the cassette insertion point attL (Fig. 1, legend) (Hall & Collis, 1995; Recchia & Hall, 1995; Stokes & Hall, 1991). Cassettes closest to attL have higher expression than those situated farther from it (Collis & Hall, 1992). A rare additional cassette promoter (P2) occurs in In2 and a few other integrons due to a 3 bp insertion that generates a 17 bp spacer between the −10 and −35 hexamers of P2. Without this insertion P2 with only a 14 bp spacer is thought to be non-functional; here, we call this promoter P214 and for consistency in this context refer to P2 as P217. The integrase promoter Pint reads convergently towards Pc, and its −10 hexamer overlaps with the −10 hexamer of P217. More research has been done on the transcription of gene cassette arrays than on that of the integrase, but a recent comparison of intI transcription in the prototypical chromosomal Vibrio cholerae integron and the plasmid-borne class 1 integron of Escherichia coli showed that LexA regulates both (Guerin et al., 2009).

Pint is highly conserved in all class 1 integron variants (Zhang et al., 2000), but the cassette promoters are quite varied. There are currently eight Pc variants, three of which sometimes contain P217 resulting in 11 total Pc or Pc + P217 combinations (Jove´ et al., 2010). The initial variants discovered were named according to their strength relative to the Ptac promoter (Le´vesque et al., 1994) and the names of subsequent variants were compared with the same benchmark by quantitative reverse transcription PCR (qRT-PCR) (Papagiannitsis et al., 2009) or lacZ fusions
(Jove et al., 2010). In addition, single base changes 2 bp upstream of the P < 10 hexamer affect promoter strength to varying extents (Burr et al., 2000; Nesvera et al., 1998). Recently, two novel forms of P214 were designated P2m1 and P2m2 but appear to be inactive (Jove et al., 2010). The only current evidence for involvement of trans-acting proteins in cassette promoter(s) regulation comes from transcriptome analysis of the chromosomal superintegron of V. cholerae strain N16961; cassette expression was activated by HapR (a regulator of virulence factor TcpP) and RpoS (stationary phase sigma factor) and repressed by RpoN (nitrogen stress sigma factor) suggesting that cassette expression increases in crowded, stressed or non-replicating bacteria (Yildiz et al., 2004) but whether activation was direct or indirect was not determined.

Here we used bidirectional transcriptional fusions of the convergent promoters Pint (lacZ reporter) and P1 + P2 or P2 (phoA reporter) of the Tn21 integron (In2) to ask: (i) what is the activity of wild-type Pint after deletion of one or both competing cassette promoters; (ii) what is the activity of P2 with 14 or 17 bp spacers; (iii) are there regulators other than LexA for any of these promoters and, if so, (iv) are their effects direct or indirect?

**METHODS**

**Bacterial strains, plasmids and culture conditions.** Bacterial strains and promoter constructs are described in Table 1. E. coli strains were grown in Luria–Bertani (LB) broth at 37 °C and 250 r.p.m. and supplemented with 100 μg ampicillin (Ap) ml −1 and 50 μg kanamycin (Km) ml −1, the latter only for chromosomal knockout mutants. Plasmid constructs carrying Pint, P1 and P2 (P217), Pint and P2 (P2177) and P1 only (P1) were constructed by PCR amplification of the promoter region of In2 in Tn21 of plasmid NR1 incorporating BamHI and HindIII restriction sites using primer pairs IP212U and IP212L, IPU and IP134L and IPU and IP97L (Table 1). PCR products were run for 5 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 57.4 °C, 30 s at 72 °C and a final elongation step of 5 min at 72 °C. Amplicons were digested with BamHI and HindIII and ligated to BamHI- and HindIII-digested pCBE1 (Schneider & Beck, 1986) which contains translational stop codons in all three reading frames upstream of the lacZ and PhoA initiation codons. The P214 construct carrying Pint and P2 with 14 bp spacer was generated from the P217 construct by PCR amplification with primers IP131U and IP131L using a QuickChange Site-Directed Mutagenesis kit (Stratagene).

**β-Galactosidase assay.** In initial development work we found that the integrase and cassette promoters were all optimally detectable in late exponential to early stationary phase. Overnight cultures in LB broth were diluted 1:20 with fresh LB containing 100 μg Ap ml −1. The cells were grown for an additional 6 h; 1 ml aliquots were taken and cells were pelleted, washed and resuspended (Miller, 1972). Cells were lysed in 100 mM Na2HPO4,7H2O, 20 mM KCl, 2 mM MgSO4, 0.8 mg CTAB ml −1, 0.4 mg sodium deoxycholate ml −1 and 5.4 μl 2-mercaptoethanol ml −1 according to Zhang & Bremer (1995), except lysis was carried out at 25 °C for 30 min in a 96-well microtitre plate. ONPG (100 μl of 5 mg ml −1) was added to each 200 μl reaction, which was incubated at 25 °C for 60 min. During incubation, A492 (ONPG) and OD630 (cell debris) readings of sample wells were carried out 0, 15, 30, 45 and 60 min after addition of ONPG to determine the rate of galactoside hydrolysis in each culture aliquot. Each reported LacZ activity is the average of three independent experiments done in duplicate. Host strain background activity done at the same time was subtracted in each experiment. The t-test was used (P < 0.05) to assess the significance of the difference in expression by a specific promoter in the wild-type host compared with a host strain mutant in a DNA-binding protein.

**Alkaline phosphatase assay.** Cells were prepared as for the β-galactosidase assay except that they were washed and resuspended in 1 mM Tris/HCl, pH 8.0. Cells were lysed with 1 mM Tris/HCl, pH 8.0, 0.8 mg CTAB ml −1 and 0.4 mg sodium deoxycholate ml −1 at 25 °C for 30 min in a 96-well microtitre plate. A 100 μl volume of (SO942)104 phosphate substrate 5 mg ml −1 (Sigma-Aldrich) was added to each 200 μl reaction which was then incubated at 25 °C for 60 min. During incubation, A405 and OD538 readings of sample wells were carried out 0, 15, 30, 45 and 60 min after addition of phosphatase to determine alkaline phosphatase activity. Each reported activity is the average of three independent experiments done in duplicate and host strain background activity measured at the same time was subtracted in each experiment. The t-test was used (P < 0.05) to assess the significance of the difference in expression from a specific promoter in the wild-type host to that same promoter’s expression in a host strain mutant in a DNA-binding protein.

**Electrophoretic mobility shift assay (EMSA).** PCR amplicons were amplified from plasmids carrying promoters (see above) and cleaned with a QiAquick PCR purification kit (Qiagen). The cleaned P214 PCR product was separated and extracted from the gel with a QiAquick gel extraction kit (Qiagen) to remove plasmid template. FIS, IHF and H-NS at 0, 5, 50, 500, 2500 and 5000 nM were mixed with 5 nM DNA in 10 μl binding buffer 1 [20 mM HEPES (pH 7.5), 100 mM NaCl, 5% (v/v) glycerol, 100 μg BSA ml −1, 2 mM DTT and 1 mM EDTA] for 30 min at 25 °C. Reactions with LexA were carried out at 0, 20, 100, 200, 1000 and 2000 nM with 5 nM DNA in 10 μl binding buffer 2 [10 mM HEPES (pH 7.9), 10 mM Tris (pH 7.9), 5% (v/v) glycerol, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 50 μg BSA ml −1] (Mazon et al., 2004). Mixed-protein EMSAs were carried out in buffer 1 with pre-determined minimum protein binding concentration (5 or 500 nM) for one protein and incubated with 5, 50 or 500 nM concentrations of the other protein (IHF, FIS, LexA or H-NS) present at the start of the reaction. All 10 μl of each binding reaction was immediately loaded onto a 5% nondenaturing polyacrylamide ready gel (Bio-Rad) and run at 25 °C in 1× TBE buffer for 5 min at 120 V, then 40 min at 80 V and stained with SYBR Green (Invitrogen).

**DNase I footprinting.** DNase I footprinting by capillary electrophoresis was performed as described previously (Yindeeyeungyen & Schell, 2000) with slight modifications. The 318 bp PCR product (318amp) was amplified by PCR from NR1 using IPwU and IPwL fluorescent primers (Table 1) and, thus, encodes the wild-type Tn21 In2 integron promoter region including Pcin, P1 and P2 with 17 bp spacer (Figs 1 and 6). This amplicon was produced by PCR in a 100 μl reaction by incubation at 95 °C for 1 min, followed by 30 cycles at 95 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The 318amp was cleaned by using the QiAquick PCR Purification kit (Qiagen). The 15 μl protein–DNA binding reaction was carried out in binding buffer 1 with 2.4 pmol cleaned 318amp and 6.8 μg total protein (0.5–2.2 μg DNA-binding protein plus BSA to total 6.8 μg) and the binding reaction was incubated at 25–26 °C for 30 min. Then, 7.5 μl DNase I (Roche) diluted 1 to 10 000 in D buffer [10 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 5 mM CaCl2, 0.1 mg BSA ml −1] was added to the 15 μl binding reaction and incubated at 25–26 °C for 30 or 35 min. The reaction was stopped with 2.5 μl 0.5 M EDTA (pH 8) and placed at 4 °C. DNA-binding proteins used for footprinting were: FIS at 2,4,
Table 1. Strains, plasmids and primers

<table>
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<tr>
<th>Strain, plasmid or primer</th>
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<th>Source or reference</th>
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<tr>
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<td>lacZ derivative of MG1655</td>
<td>Guerin et al. (2009)</td>
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<td>Plasmids</td>
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<td>pCB267</td>
<td>Carrying promoter region of In2 in Tn21</td>
<td>R. Rownd, University of Wisconsin</td>
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<td>pI2-17</td>
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<tr>
<td>IPwtL</td>
<td>5’-(6-FAM)-TCGCCAGCCAGACAAAAAT-3’</td>
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*CGSC, E. coli Genetic Stock Center; http://cgsc.biology.yale.edu/index.php.

4.8, 12.0 and 24.0 pmol; IHF at 12, 24, 30, 60, 120 and 240 pmol; H-NS at 4.8, 24 and 48 pmol. Footprinting with combinations of DNA-binding proteins was performed with: 24 pmol FIS with 60 pmol IHF; 24 pmol FIS with 24 pmol H-NS; and 24 pmol H-NS with 60 pmol IHF. Footprinting of 318amp was also carried out with LexA and MerR (each at 4.8, 24 and 48 pmol) as negative controls.

A 2 μl sample of the 25 μl footprinting reaction was checked by agarose gel electrophoresis for evidence of successful digestion (results not shown); then 7 μl HPLC-grade water was added, the resulting 30 μl was extracted with phenol: chloroform: isomyl alcohol (25: 24: 1), and the aqueous phase was cleaned by Centri-Sep column (Princeton Separations). Aliquots (2 or 3 μl) of cleaned footprinting reaction (column eluate) were each added to 10 μl deionized formamide and 1 μl 6-carboxy-X-rhodamine (ROX)-labelled DNA ladder (Georgia Genomics Facility) in a 96-well plate for capillary electrophoresis using an ABI3730 sequencer. GeneMapper 4.0 (Applied Biosystems) was used for data analysis. Capillary electrophoresis of a 318amp BbrI partial digest revealed that the fragment sizes estimated by GeneMapper based on the ROX ladder were within 1–4 nt for the top strand and within 0–1 nt for the bottom strand due to the differing mobility of the 2.7, 8.8'-benzo-5'-fluoro-2', 4,7-trichloro-5-carboxyfluorescein (NED) and 6-carboxyfluorescein (6-FAM) fluorescent markers. The positions for the protected nucleotides on the top strand (Fig. 6) were assigned based on a combination of the GeneMapper position estimates, bottom strand protected positions and the predicted FIS binding sites (Figs 1 and 6, Supplementary Table S1, available with the online version of this paper).

RESULTS

Promoter strength of Pint, Pc and/or P2 when alone or in competition

Variation in cassette promoters (Lévesque et al., 1994; Papagiannitsis et al., 2009) represents only part of the integron transcription mechanics because Pc and P2 converge on, and the latter partially overlaps, the integrase promoter (Pint) by 3 bp. Owing to the strong predictions (Supplementary Table S1 and see below) of multiple overlapping binding sites for four distinct nucleoid-associated proteins we chose traditional deletion analysis,
rather than point mutations, as a first step in dissecting regulation of these three promoters, since single mutations in any of them could also have affected one or more of the others (Fig. 1b). We found that \( P_{int} \) expressed best alone (in the \( Pi \) construct), increasing 6.5-fold in the wild-type host when both competing cassette promoters \( P_c \) and \( P_2 \) were removed (Fig. 2a, b, black bars). Surprisingly, deletion of \( P_c \) (see the \( Pi217 \) construct) dramatically weakened \( P_{int} \) expression (Fig. 2a, black bar) making it almost 12-fold less active than when alone (Fig. 2b, black bar); the possible bases for this result will be discussed below. Moreover, without \( P_c \), additional removal of three CCCs to make the construct with a 14 bp spacer, \( Pi214 \), further diminished \( P_{int} \) expression (Fig. 2a, black bars) likely due to formation of a LexA site by the three-base deletion (Fig. 1, green line; and see below) (Guerin et al., 2009).

Previous tests of cassette expression in Tn2603 established that \( P_{217} \) accounted for approximately 90 % of total cassette expression (Papagiannitsis et al., 2009). Our \( phoA \) transcriptional fusions to \( P_{217} \) alone (construct \( Pi217 \)) and to \( P_{217} + P_c (Pi217c \) construct) confirmed that \( P_c \) contributed little to total cassette expression (Fig. 2c, black bars). It has been assumed that \( P_2 \) with a 14 bp spacer is non-functional. Indeed, although not completely inactive, construct \( Pi214 \) had less than 1 % of \( P_{217} \) function (Fig. 2d, black bars).

Predicted FIS, IHF, H-NS and LexA transcriptional regulator binding sites

To identify possible regulators of the integron promoters we used the virtual footprint tool PRODORIC (http://www.prodoric.de) (Münch et al., 2003) to search the entire In2
integron promoter region for all known regulatory protein binding sites. PRODORIC predicted several potential binding sites for FIS, H-NS and IHF (Fig. 1b), multifunctional proteins that can serve as recombination accessory elements, nucleoid compaction proteins and global transcriptional regulators of many distinct metabolic pathways (Dame et al., 2001; Dorman, 2009; Gottesman, 1984; Luijsterburg et al., 2006; Navarre et al., 2006). All such proteins have highly degenerate 10–18 bp sites, so their identification is based on a probability score (Supplementary Table S1; Fig. 1b) (Münch et al., 2003). Recent research has shown LexA repression of $P_{\text{int}}$ in the $V.\ cholerae$ super-integron (Guerin et al., 2009) and PRODORIC predicted strong LexA sites on the top and bottom strands of $P_{214}$ (construct $P_{214}$; Supplementary Table S1; Fig. 1b) occurring by virtue of a CCC-deletion in four of the 11 currently known natural promoter variants.

**FIS and LexA repress integron promoters**

To assess in vivo a possible role for each of these proteins we obtained *E.\ coli* strains with a single mutation in each, as well as their respective parental strains. Compared with expression in the wild-type background, when FIS was absent, expression of the integrate promoter $P_{\text{int}}$ increased (Fig. 2a, dark grey bars) by 20% when competing against cassette promoters with or without $P_c$ (constructs $P_{217c}$ and $P_{217}$). When both cassette promoters were absent (construct $P_c$; Fig. 2b, dark grey bar) the already higher $P_{\text{int}}$ expression increased another 2.5-fold without FIS. Interestingly, lack of FIS strongly derepressed $P_c$ facing the $P_{214}$ promoter (construct $P_{214}$; Fig. 2a, dark grey bar) although in the *fis* host LexA should be bound there. For the cassette promoters, the increase in the already high expression of $P_{217}$ (constructs $P_{217c}$ and $P_{217}$) averaged 30% without FIS (Fig. 2c, dark grey bars). Surprisingly, even the weak expression of $P_{214}$ increased 3.5-fold in the absence of FIS (construct $P_{214}$; Fig. 2d, dark grey bar) despite its non-ideal shorter spacer region and the expected strong repression by LexA (Guerin et al., 2009). All of these observations are consistent with stabilization by FIS of LexA repression at $P_{214}$ see also below. The alternate possibility, that lack of FIS simply increased the copy number of all plasmids, is less likely because the opposing promoters on the same vector do not increase to the same magnitude. This is particularly striking in construct $P_{214}$ where expression from integrate promoter $P_c$ increased over 10-fold but that from the cassette $P_{214}$ increased only 3.5-fold. Moreover, the size differences in these plasmids are minuscule and would not lead to copy number changes simply on the basis of replication time in this CoEl1-type replicon; the entire promoter insert is only 212 bp of a total 7858 bp for the full-length reporter vector and in its shortest form, the construct $P_c$ is only 115 bp shorter.

Lack of LexA very modestly increased (~5–10%) expression of $P_{\text{int}}$ facing $P_{217}$ with or without $P_c$ (constructs $P_{217c}$ and $P_{217}$; Fig. 2a, white bars) compared with wild-type since there is no LexA site in these promoters. But, as expected, with $P_{214}$ absence of LexA resulted in a 23-fold derepression of $P_{\text{int}}$ (construct $P_{214}$; Fig. 2a, white bar), much greater than in the absence of FIS alone (note that FIS is still present in the *lex* host). Interestingly, $P_{\text{int}}$ alone (construct $P_i$) was derepressed twofold without LexA, probably because this construct retained half each of its two LexA sites (top and bottom strands), including the more conserved 5’-CTG of the 16 bp LexA (top strand) binding site (Münch et al., 2003). For the cassette promoters, absence of LexA consistently derepressed the integrase promoters $P_{217}+P_c$ (construct $P_{217c}$), $P_{217}$ alone (construct $P_{217}$) (Fig. 2c, white bars) and even the weak expression of $P_{214}$ (Fig. 2d, white bar) but none of these differed significantly from the values for the wild-type host. Thus, within the limits assayable by transcriptional fusions, the unexpected observations that lack of FIS compromised LexA repression of both promoters $P_{214}$ and $P_c$ suggests either that FIS stabilizes LexA occupancy via their closely adjacent sites in the integron promoters region (Fig. 1b) or that absence of FIS lowers cellular abundance of LexA. Again, altered copy number of the reporter vector itself is an unlikely explanation of these results because in the construct $P_{214}$, the integrase promoter increased 23-fold but the cassette promoter $P_{214}$ increased insignificantly compared with the wild-type host.

**IHF may activate integron promoters**

Two sites for IHF binding were predicted in the integron promoters region (Fig. 1). In the absence of IHF, $P_{\text{int}}$ expression decreased 30% compared with the wild-type when it was facing $P_c$ with or without $P_c$ (constructs $P_{217c}$ and $P_{217}$; Fig. 2a, medium grey bars). The low LexA-repressed expression of $P_{\text{int}}$ facing $P_{214}$ decreased to practically nothing without IHF (construct $P_{214}$; Fig. 2a, medium grey bar). When unencumbered by convergent promoters or repressors, $P_{\text{int}}$ expression still declined by approximately 20% when IHF was absent (construct $P_c$; Fig. 2b, medium grey bar). The cassette promoters also decreased 15–25% without IHF (constructs $P_{217c}$ and $P_{217}$; Fig. 2c, medium grey bars), but there was no change in LexA-repressed $P_{214}$ (construct $P_{214}$; Fig. 2d, medium grey bars). Thus, within the limits assayable by transcriptional fusions, IHF may be a weak activator of $P_{\text{int}}$ and of the cassette promoters not subject to LexA repression. Alternatively, loss of IHF might cause a slight decrease in copy number of all plasmids relative to the wild-type parental hosts.

**H-NS represses the integrase promoter and activates cassette promoters not repressed by LexA**

Between one and three H-NS binding sites were predicted for both forms of $P_c$ (Fig. 1), so we examined the effect of eliminating H-NS, which binds A/T-rich curved DNA regions (Navarre et al., 2006; Shao et al., 2008). Without H-NS, $P_{\text{int}}$ expression increased compared with the
wild-type host in all promoter constructs, except in P_{214} where it was, surprisingly, undetectable (Fig. 2a, b, light grey bars; this strain grew very slowly). The extent of \( hns \) derepression of \( P_{int} \) varied widely in the other promoter constructs; without H-NS \( P_{int} \) increased fivefold in P_{214}, but only 40% in P_{1} and <10% in the wild-type construct P_{217c} (Fig. 2a, b, light grey bars). The H-NS effect on P_{1} was unexpected as this construct lacked any predicted H-NS binding site; its 3' junction with the vector adds a single adenine, not enough to be predicted as an H-NS site. In contrast, expression from the cassette promoters P_{212} + P_{c} and P_{217} alone (constructs P_{217c} and P_{217f}; Fig. 2c, light grey bars) decreased 35–40% in the absence of H-NS, consistent with its having a modest activating effect on these promoters not subject to LexA repression. However, P_{214} expression increased 60% without H-NS (construct P_{214f}; Fig. 2d, light grey bars). This is surprising as the LexA sites of P_{214} are superimposed on one of its two putative H-NS sites, suggesting that they might compete for occupancy. However, derepression of P_{214} in the absence of H-NS suggests that, as with FIS above, LexA repression may be stabilized here by H-NS. Here, again plasmid copy number changes cannot explain expression differences in these promoters. With construct P_{217c}, expression of P_{int} increases in the \( hns \) host while cassette expression decreases. For construct P_{214}, expression of P_{int} declines to almost nothing but cassette expression drops by only 40% and in construct P_{214f}, expression of P_{int} increases fivefold but that of P_{214} is only 60% higher than in the parental host.

**In vitro binding of LexA, FIS, IHF and H-NS to the integron promoter region**

The behaviour of integrase and cassette promoters in \( lex, fis, ihf \) and \( hns \) mutants might arise directly from the lack of physical interaction of the promoters with the corresponding proteins or indirectly via the regulation of some other necessary transcription factor by these proteins. We first used EMSAs of each promoter amplicon with purified LexA, FIS, IHF and H-NS to examine this point. As expected, at a 1 : 1 molar ratio, LexA readily formed a single complex with amplicon P_{214f}; however, it did not retard all of the DNA encoding P_{int} + P_{217} at 100-fold molar excess (amplicon P_{217f}; Fig. 3a, b, lanes 2–4). Even at 1000-fold excess (Fig. 3b, lane 5) some free DNA remained. In contrast, equimolar FIS gave five conformers with the wild-type promoter amplicon, P_{217c} (Fig. 3c, lane 2), leaving very little free DNA. This electrophoresis pattern suggests that a single FIS protein binding to any of the several binding sites can give rise to at least five electrophoretically distinct conformers of the amplicon. The conformer distribution became more homogeneous at higher protein excess (Fig. 3c, lanes 3–5) and no free DNA was visible even at just a 10-fold molar excess of FIS (lane 3). FIS had similarly high affinities for the other three promoter amplicons (Fig. 3d), forming fewer conformers consistent with the removal of predicted FIS sites in the smaller amplicons. However, for even the smallest promoter amplicon (P_{1}, lane 2), >90% of the amplicon is retarded at a 1 : 1 protein : DNA ratio, consistent with a high affinity for FIS. Thus, LexA and FIS act directly in this promoter region and, in this assay, FIS has a higher affinity for any one of its sites than LexA does for its two overlapping sites.

To further examine the *in vitro* interactions of FIS, IHF and H-NS with the promoter region of P_{217c} DNase I footprinting was performed using a 318 bp amplicon (318amp) including the wild-type In2 integron promoter region, in which the first position shown for P_{217c} (Fig. 1) corresponds to position 50 of the 318amp (Fig. 4). FIS

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**Fig. 3.** Binding of LexA or FIS to amplicons of the integron promoter constructs. (a, b) LexA (0, 5, 50, 500 or 5000 nM, lanes 1–5, respectively) bound to (a) amplicon P_{214} (5 nM; lanes 2–5) or (b) amplicon P_{217} (5 nM; lanes 2–5). (c) FIS (0, 5, 50, 500 or 5000 nM, lanes 1–5, respectively) bound to amplicon P_{217c} (5 nM; lanes 2–5). (d) FIS (0 nM, odd-numbered lanes; 5 nM, even-numbered lanes) bound separately to each amplicon P_{1} (lanes 1, 2), P_{214} (3, 4), P_{217} (5, 6) and P_{217c} (7, 8). Free (F) DNA is indicated by a line. LexA conformers 1 and 2 are indicated by lines (a, b); FIS conformers 1, 2, 3, 4, 5 and 6 are indicated by a bracket and FIS conformers 6, 7 and 8 are indicated by lines (c). Details of each construct are shown in Fig. 1 and in the summary in Fig. 7.
showed clear regions of DNase I protection for both the top and bottom strands at 12 pmol (results not shown) and 24 pmol (see Supplementary Figs. S1 and S2 available with the online version of this paper), 5:1 and 10:1 molar excess over the DNA, respectively. The large cassette-proximal (left-side of Figs 1 and 4) protected region covers from the P135 hexamer to well beyond the Ps, 35 hexamer (positions 54–126, Fig. 4). The integrase-proximal (right side of Figs 1 and 4) FIS-protected region covers the −10 hexamer and spacer of Ps just up to the Ps, 35 hexamer (positions 193–219, Fig. 4). The seven predicted FIS binding sites on the top strand match well to the FIS-protected regions (Supplementary Fig. S1). Only five FIS binding sites were predicted for the bottom strand, but the protected nucleotides (positions 59–89, 99–126, 181–218, Fig. 4) match well to those protected on the top strand and indicate that FIS binding to the top strand also protects the bottom strand from DNase I cleavage (Supplementary Fig. S2). Hypersensitive sites were widely distributed with four (one purine and three pyrimidines) on the top strand (Fig. 4, black arrows) and ten (three purines and seven pyrimidines) on the bottom strand (Fig. 4, red arrows).

IHF formed one to three discrete complexes in EMSAs with all promoter amplicons but only at 100- and 1000-fold molar excess (Fig. 5a–d), typical of other IHF binding interactions which are of relatively low affinity (Ali et al., 2001; Arfin et al., 2000; Yang & Nash, 1995). The largest amplicon P1217c contained two predicted IHF sites (11 and 14) and was the most effective in forming electrophoretically stable conformers, as evidenced by its leaving the smallest amount of free DNA (Fig. 5a, lane 4). The P1217 amplicon with one predicted IHF site showed diffuse retardation by IHF at 10:1 and 100:1 protein molar excesses (Fig. 5b, lanes 3 and 4). Although only amplicons P1217c, P1217 and P214 have IHF sites detectable by PRODORIC, these EMSA observations suggested that the in vivo activator role for IHF seen with all promoter configurations might involve direct interaction. However, DNase I footprinting with IHF showed no protection until 60 pmol, a 25:1 ratio of IHF to amplicon, also consistent with low-affinity binding (data not shown). IHF can affect gene expression indirectly by interacting with other transcriptional regulators (Freundlich et al., 1992); thus, IHF may affect 

H-NS bound to the amplicons P1217c, P1217 and P214, (Fig. 5e–g) but not to P1 (Fig. 5h). The only promoter lacking a predicted H-NS site (Fig. 1). H-NS bound in a range of 1- to 100-fold protein molar excess producing one to three formers, but the patterns shown occurred only half of the time for undetermined reasons. DNase I footprinting with 48 pmol H-NS, a 20:1 H-NS to amplicon ratio, gave a digest fragment pattern nearly identical to the DNase I protection pattern observed with the negative control proteins LexA and MerR at the same molarity (data not shown). Since two standard in vitro methods gave equivocal results we did not resolve whether the in vivo affects of H-NS on this system are direct or indirect. H-NS binding requires curved DNA (Schro¨der & Wagner, 2002) whose formation in vitro may be affected by the length and overall composition of the target DNA.

**Competition and cooperation among the regulatory proteins**

The maximum copy numbers of FIS and IHF occur at very different periods of the growth cycle: in very early exponential phase there are 30,000 FIS dimers per cell and only 6000 dimers of IHF (Ali Azam et al., 1999). However, in late exponential phase, IHF peaks at 27,500 dimers per cell, when FIS has decreased to less than 1000 (Ali Azam et al., 1999). H-NS has a pattern similar to IHF and LexA’s cellular concentration does not vary so dramatically with growth phase but increases when DNA is damaged (Ali Azam et al., 1999; Kelley, 2006). So, we used competitive EMSA to determine whether these proteins affect each other’s binding to the integron promoter region (Fig. 6) and we used combinations of FIS, IHF and H-NS in DNase I footprinting to determine the effect of two proteins on wild-type promoter region binding. FIS+IHF, LexA+IHF and FIS+LexA were incubated with the wild-type promoter amplicon P1217c.
(Fig. 6a) or with short spacer amplicon P_{214} (Fig. 6b, c) where an IHF site is predicted to overlap with the LexA site (Fig. 1b). IHF only competed with FIS or LexA binding at very high excesses such as could occur in late exponential phase (Fig. 6a, b, lanes 7 and 8). FIS and LexA were incubated with the amplicon of short spacer P_{214} containing adjacent FIS and LexA sites. LexA clearly altered the distribution of FIS conformers at lower molar ratios of FIS (Fig. 6c, compare lanes 3 and 4) but not at higher FIS ratios. In footprinting with FIS + IHF or FIS + H-NS, the protection patterns were indistinguishable from the pattern seen with FIS alone (results not shown). The DNase I protection observed with IHF + H-NS was identical to that observed with 60 pmol IHF alone. Thus, H-NS did not increase the non-specific binding of IHF to the 318amp wild-type integron promoter region.

**DISCUSSION**

The class 1 integron family has many well-studied cassette promoter variants, but the integrase promoter (P_{int}) is highly conserved and until recently there was no information on P_{int} expression, or any on regulators affecting P_{int}
and/or the cassette promoters. Here we established that alone \( P_{\text{int}} \) is a relatively strong promoter and its 6.5-fold weaker expression in its natural configuration in In2 can be ascribed to direct competition with convergent \( P_c \) and \( P_2 \). Note that the leader mRNA for the \( lacZ \) gene is 109 bp longer in the \( P_{i217c} \) construct than it is in the two constructs lacking \( P_c \) (\( P_{i214} \) and \( P_{i217} \)). Counterintuitively, bringing the \( LacZ \) reading frame 145 bp closer to \( P_{\text{int}} \) decreases the absolute \( LacZ \) activity of the two \( P_{\text{int}} \) reporters, \( P_{i217} \) and \( P_{i214} \) (Figs 1 and 2a, black bars). In \( P_{i217} \) the 50\% decrease in \( LacZ \) activity suggests that the secondary structure in the integrase mRNA might delay turnover of the \( LacZ \) mRNA. The further drop in \( LacZ \) activity with construct \( P_{i214} \) more likely results simply from the formation of a LexA site immediately downstream of the \( P_{\text{int}} \) –10 hexamer (Fig. 1b). Finally, in construct \( P_1 \), the highest absolute activity of \( P_1 \) (Fig. 2b) is achieved by removing most of that LexA site. In contrast, the removal of cassette promoter \( P_1 \) modestly strengthens PhoA activity driven by the 17 bp \( P_{217} \) promoter (Fig. 2c, black bars); this might also suggest a protective effect of the secondary structure in the long cassette mRNA leader which traverses the same region as the integrase mRNA. Of course, the weak 14 bp spacer form of \( P_{214} \) is most profoundly affected by its spacer defect (Fig. 2d) as also noted in other recent work (Guerin et al., 2009). The remaining scant expression of cassette promoter \( P_{214} \) may only be read through from an unidentified vector promoter, although its 3.5-fold derepression in the absence of FIS (which strongly binds the \( P_{i214} \) amplicon) suggests that this might be residual intrinsic activity of this promoter. Note that the derepressed MerR promoter with a 15 bp spacer can produce >1500 PhoA Miller units with this same vector (Ross et al., 1989).

Here we show for the first time, to our knowledge, in late-exponential-phase cells, that FIS directly represses the integrase and the cassette promoters, apparently assisting LexA, and that IHF may indirectly activate \( P_{\text{int}} \) and the cassette promoters. Since both EMSA and footprinting show weak or no interaction of IHF with amplicons containing these promoters, its direct involvement seems unlikely, and neither LexA nor FIS affects IHF binding. The small expression decrease from \( P_{\text{int}} \) and the cassette promoters in \( ihf \) mutants might arise from a decrease in plasmid copy number. Our work also shows that in vivo HN activates \( P_{\text{int}} \) in settings where it converges with only \( P_{217} \) (Fig. 2a) or is repressed by LexA (in \( P_{214} \), Fig. 2a).
Although EMSA data tentatively suggest direct H-NS interaction, this conclusion is tempered by the reproducibility of EMSA and the absence of DNase I protection by H-NS on the In2 integron promoter region.

We summarize our observations of these interactions in late exponential phase cells in Fig. 7. Our model system, In2, expresses cassettes with the strong P217 (Fig. 7, left panel) which we examined with (upper) and without (lower) the ‘weak’ version of the distant Pc promoter. Removal of Pc results in only a slight increase in cassette expression but even a small increase in the strong P2 promoter might explain the 50% decrease in converging Pint expression in this construct. Some or all of the remaining FIS and IHF sites function in vivo and in vitro in both constructs depicted in the left panel. Curiously, loss of H-NS affected Pint expression differently in these constructs and varied with cassette promoters as indicated by the slow growth rate (not shown) and low LacZ production in the hns mutant strain carrying cassette promoter P217 in construct P1217. The decline in cassette expression is not severe in construct P1217 when compared with wild-type P1217c, so an H-NS effect on integrase expression cannot simply be accounted for by possible plasmid instability.

In promoter constructs with P214 (Fig. 7, middle panel), the extent to which IHF or H-NS might compete with LexA binding if a direct interaction occurs would depend on the affinity of LexA for its integron binding sites relative to its chromosomal binding sites. Of course, when the SOS response is enabled by DNA damage, LexA repression of integrase and cassette promoters would be relieved, but through most of the period of active growth, promoters could still be modulated by H-NS and IHF. The apparent cooperation between LexA and FIS at this promoter warrants further examination; FIS may alter H-NS or IHF binding in or near the LexA site, facilitating LexA binding, or it might simply interact with LexA. Lastly, without converging cassette promoters, the integrase promoter alone (Fig. 7, right panel) is still controlled to a great degree by FIS which will be largely absent during most of exponential growth and into stationary phase.

This work brings to light a previously unknown dimension in the control of integron behaviour, i.e. the involvement of three global regulators, FIS, IHF and H-NS with implications for growth cycle effects in addition to the recently noted LexA-mediated stress response (Guerin et al., 2009). In other recent work we have shown an increase in integron recombination products during the transition from exponential to stationary phase and also in cells artificially overexpressing the integrase (Shearer & Summers, 2009); these phenomena likely involve modulation of cellular integrase concentrations by the proteins we have examined here.

The most widely found variant of P2 is P214 (Jove et al., 2010) in which cassette expression depends entirely on various constitutive P2 promoters and is strongly repressed, as is P2 by LexA, aided by FIS. LexA repression is released by SOS-inducing stresses including sublethal doses of antibiotics like trimethoprim, ciprofloxacin and β-lactams (Butala et al., 2009; Drlica & Zhao, 1997; Guerin et al., 2009; Lewin & Amyes, 1991; Miller et al., 2004). Integrons with the rare very strong P217 cassette promoter have traded LexA repression for promoter competition to control integrase expression from P2 but have retained some measure of repression by FIS, which is abundant in very early exponential phase. Overexpression of recombinases can be dangerous to the cell, and expression of antibiotic resistance genes when they are not needed is metabolically costly. Thus, it is clear why the P217 variant promoter occurs in only 8.7% of the currently known class 1 integron population (Jove et al., 2010). All of the promoter variants have the same predicted number of each type of global regulator sites as the P1217c construct and its derivatives examined here, although there are slight variations in the positions (data not shown). Thus, involvement of nucleoid-binding proteins is an even more conserved feature of gene expression in class 1 integrons than any single variant of the highly variable P2 promoter itself. The mechanistic details of their contributions to the evolutionary success of these formidable agents of antibiotic multi-resistance will be the subject of future work.

**Fig. 7.** Summary of integron gene expression influenced by global regulators. Potential binding by LexA and global regulators to promoter constructs in late exponential to early stationary phase cells. Although FIS and IHF bend DNA 40–90° (Kostrewa et al., 1991) and 140° (Rice et al., 1996), respectively, we do not represent this here since the bend directions are not known.
ACKNOWLEDGEMENTS

We thank Anna Karls (University of Georgia) for purified FIS and IHF, helpful advice regarding this work and comments on the manuscript, Didier Mazel (Institut Pasteur) for the lex strain and its parent and Sylvie Rimsky and Colin Corcoran (École Normale Supérieure de Cachan, France) for purified H-NS and an H-NS overexpression plasmid. We also thank Natalie Strynadka (University of British Columbia) for providing the LexA overexpression plasmid and John Little (University of Arizona) and Ishita Mukerji (Wesleyan University) for purified wild-type LexA and IHF, respectively. We thank Ken Jones (Georgia Genomics Facility, University of Georgia) for his aid in footprinting and we also thank Georgia colleagues Sidney Kushner and John Maurer for valuable comments on the manuscript. This work was supported in part by United States Department of Agriculture (USDA) grant 99-35212-8680 and National Science Foundation (NSF) grant 06-26940 to A. O. S. and manuscript. This work was supported in part by United States Department of Agriculture (USDA) grant 99-35212-8680 and National Science Foundation (NSF) grant 06-26940 to A. O. S. and was submitted in partial fulfillment of the requirements for the Ph.D degree by C. A. C.

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Edited by: U. Dobrindt.