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Regulated Expression of a Repressor Protein: FadR Activates *iclR*

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The control of the glyoxylate bypass operon (*aceBAK*) of *Escherichia coli* is mediated by two regulatory proteins, IclR and FadR. IclR is a repressor protein which has previously been shown to bind to a site which overlaps the *aceBAK* promoter. FadR is a repressor/activator protein which participates in control of the genes of fatty acid metabolism. A sequence just upstream of the *iclR* promoter bears a striking resemblance to FadR binding sites found in the fatty acid metabolic genes. The in vitro binding specificity of FadR, determined by oligonucleotide selection, was in good agreement with the sequences of these sites. The ability of FadR to bind to the site associated with *iclR* was demonstrated by gel shift and DNase I footprint analyses. Disruption of *fadR* or inactivation of the FadR binding site of *iclR* decreased the expression of an *iclR*::*lacZ* operon fusion, indicating that FadR activates the expression of *iclR*. It has been reported that disruption of *fadR* increases the expression of *aceBAK*. We observed a similar increase when we inactivated the FadR binding site of an *iclR*$^+$ allele. This result suggests that FadR regulates *aceBAK* indirectly by altering the expression of IclR.

Growth of *Escherichia coli* on acetate or fatty acids results in the induction of the glyoxylate bypass. The enzymes which compose this pathway, isocitrate lyase and malate synthase (Fig. 1), are essential for growth on these carbon sources because they prevent the quantitative loss of the entering carbon as CO_2 in the Krebs cycle (12, 14). Once induced, the flow of isocitrate through this pathway is controlled by the phosphorylation of isocitrate dehydrogenase (IDH), the Krebs cycle enzyme which competes with isocitrate lyase. Phosphorylation of IDH decreases its activity and so forces isocitrate through the bypass (1, 7, 18). This phosphorylation cycle is catalyzed by a bifunctional protein, IDH kinase/phosphatase (15, 16).

The genes which encode the metabolic and regulatory enzymes of the glyoxylate bypass reside in the same operon, *aceBAK* (2, 17, 20). The metabolic enzymes, malate synthase and isocitrate lyase, are encoded by *aceB* and *aceA*, while *aceK* encodes IDH kinase/phosphatase. This operon is expressed from a single promoter during growth on acetate (2).

The *aceBAK* operon is regulated, at least in part, by a repressor protein expressed from *iclR* (13, 20, 24, 33). Mutations in this gene have been shown to result in increased expression of *aceBAK* during growth on repressing media (e.g., glucose). The protein which this gene encodes, IclR, binds to a site which overlaps the −35 region of the *aceBAK* promoter (2, 24, 27). In addition to regulating *aceBAK*, IclR also represses its own expression (8).

The expression of *aceBAK* is induced during growth on either acetate or fatty acids. The glyoxylate bypass is required for growth on fatty acids because, like acetate, fatty acids enter intermediary metabolism as acetyl coenzyme A. The response of *aceBAK* to fatty acids has been suggested to be mediated, in part, by FadR (19, 20). FadR was initially identified because it represses the genes encoding the enzymes of fatty acid degradation (26). It was subsequently shown to activate the transcription of *fabA*, a gene whose product participates in unsaturated fatty acid biosynthesis (9). Mutations in *fadR* result in increased expression of *aceBAK* on repressing media, although the effects of these mutations were smaller than those observed for mutations in *iclR* (19, 20). In this report, we demonstrate that FadR activates the expression of *iclR*.

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MATERIALS AND METHODS

Materials. DNA modification enzymes were obtained from New England Biolabs or Bethesda Research Laboratories. Oligonucleotides were synthesized by either National Biosciences or Bethesda Research Laboratories. All reagents were the best grades available.

Strain construction. The strains used in this study are listed in Table 1. Strains W4680 and RS3032 were obtained from the *Escherichia coli* Genetic Stock Center at Yale University.

An *iclR* disruption was generated by the insertion of a kanamycin resistance gene at the *Mlu*I site in vitro. This site is located at +84 within a coding region of 822 bp. The allele was transferred to the chromosome by recombination (8).

An *iclR*::*lacZ* operon fusion was created by inserting the *Eco*RI-*Mlu*I fragment from *iclR* (33) between the *Eco*RI and *Bam*HI sites of plasmid pCL551 (3). This created a fusion which included 1,232 bp upstream of the transcriptional start of *iclR* and 84 bp from the coding region while deleting 758 bp from the 3′ end of this gene. A translational termination site was inserted between *iclR* and *lacZ*. The allele of *lacZ* which was used includes its own translational start site. This operon fusion was then transferred to the *lac* locus of the chromosome by recombination as previously described (29).

A similar approach was used to insert the intact *iclR*$^+$ gene into the chromosome. The *Eco*RI-*Bam*HI fragment from plasmid pKL5 (33) was recloned into pCL551 (3). This insert includes 1,232 bp upstream of the transcriptional start site as well as the intact *iclR*$^+$ gene. The insert was then transferred to the chromosome at *lac* as described previously (29).

Mutagenesis of the FadR binding site upstream of *iclR*::*lacZ* was carried out by using a Transformer site-directed mutagenesis kit obtained from Clontech Laboratories. Residues at both −56 (T) and −55 (C) relative to the transcriptional start site were changed to G's. The sequence of the oligonucleotide used for mutagenesis was 5′ CAACATTAACTCAGGGGATCAGTTCAG 3′. The fidelity of the reaction was confirmed by sequence analysis (31).

Strain construction was performed by P1-mediated transduction. The genetic background of all the strains used in this study was that found in strain W4680.

Growth conditions and enzyme assays. The minimal medium described by Neidhardt et al. (25), containing 2% glucose, 2% sodium acetate, or 5 mM sodium oleate in 0.5% Brij 58, was used. Cultures were incubated at 37°C in a gyratory shaker, and growth was monitored by light scattering at 600 nm. Assays were performed with cultures in the mid-log phase of growth.

Assays for β-galactosidase were performed on permeabilized cells as described by Miller (23). Enzyme activity was corrected for culture density and calculated in Miller units.

IDH phosphatase was assayed by measuring the release of ^{32}P from phospho-IDH as described previously (15).

Cloning of *fadR*. A clone containing the *fadR* gene was obtained from the

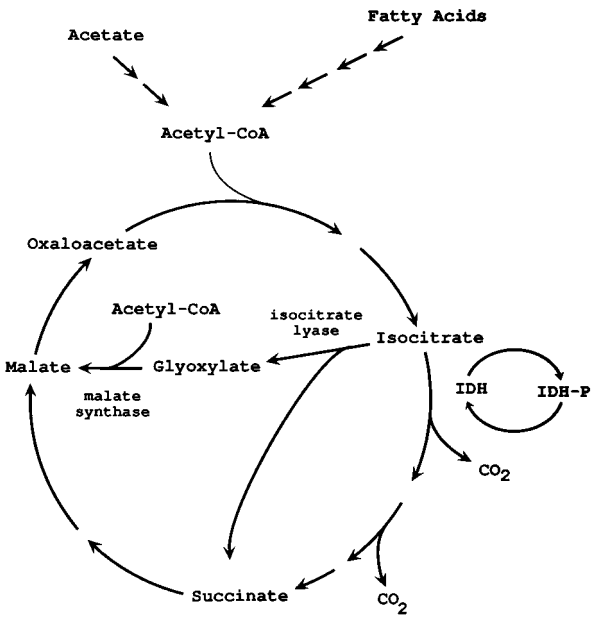

FIG. 1. The Krebs cycle and the glyoxylate bypass. The glyoxylate bypass is composed of isocitrate lyase and malate synthase. This pathway is regulated by phosphorylation of IDH.

Kohara bank (11). This bank is composed of a series of overlapping clones which represent the entire *E. coli* chromosome carried by a λ vector. Clone 11G8(244) carries a fragment which corresponds to 25.5 min on the *E. coli* map, the region which includes *fadR*. This gene was subcloned into plasmid pBR322 on a 1.3-kb *Hin*dIII-*Eco*RV fragment. Comparison of a partial sequence of this insert with the sequence which has been reported for *fadR* (6) confirmed the identity of the clone.

FadR was overexpressed essentially as described by DiRusso et al. (5). The gene was amplified from the original plasmid by PCR. The 3′ primer used for the PCR introduced an *Nco*I site at the translational start site but did not change the sequence of the protein. The PCR fragment was inserted into expression vector pET11d, which employs the T7 promoter. The region that had been amplified by PCR was sequenced following the isolation of the clone to ensure the fidelity of the reaction.

Purification of FadR. The plasmid which expresses *fadR* from the T7 promoter was transformed into strain BL21(DE3). This strain carries the gene encoding T7 RNA polymerase under the control of a *lac* promoter in its chromosome. The culture was grown at 37°C in LB medium containing 100 μg of ampicillin per ml to late log phase. T7 RNA polymerase was then induced by addition of 0.4 mM isopropylthiogalactopyranoside. Following incubation for 2 h, cells were harvested by centrifugation and FadR was purified as described by DiRusso et al. (5).

Assay of FadR binding to DNA. Gel shift analysis was used to demonstrate the binding of FadR to the promoter region of *iclR*. The reaction mixture included 0.8 ng of probe, 10 mM Tris (pH 7.5), 1 mM EDTA, 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 2 μg of poly(dI-dC) in 30 μl. The probe, which included sequences from −128 to −22 relative to the transcriptional start site, was generated by PCR and end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The mixtures were incubated at 20°C for 30 min and then subjected to electrophoresis in a 4.5% polyacrylamide gel at 4°C. The gels were dried and subjected to autoradiography.

DNase I footprint analysis. DNase I footprint analysis of the FadR-DNA complex was carried out essentially as described by Shih and Towle (32). The probe, which included sequences from −128 to +27 relative to the transcriptional start site, was generated by PCR. The 5′ primer (−128 to −110) was end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase prior to PCR. Standards were prepared by digestion of the probe by the Maxam and Gilbert method (21).

Determination of the consensus sequence of the FadR binding site. The method developed by Pollock and Treisman (28) was used to determine the binding site specificity of FadR. This method uses the gel shift technique to select sequences capable of binding to FadR from a pool of random sequences. The probe included a region of 31 random nucleotides flanked by constant regions whose sequences were GCTGCTCGAGTTCTCCTCCCACG (5′ constant region) and GTTCGCGGATCCCGTC (3′ constant region). The probe was initially labeled by including [α-^{32}P]dCTP during the synthesis of the second strand with Klenow fragment. It was subsequently labeled by including [α-^{32}P]dCTP during PCR amplification. Oligonucleotides capable of binding to FadR were selected by the gel shift technique followed by elution from the gel and amplification by PCR. Following six rounds of selection, the amplified products were ligated into plasmid pT7/T3α-18 (22). Forty-four unique isolates were sequenced, and their sequences were compared by using the Pileup program from the Genetics Computer Group package.

RESULTS

A possible binding site for FadR near the *iclR* promoter. Examination of the region upstream of *iclR* revealed a se-

TABLE 1. Key *E. coli* strains used

Strain	Genotype[a]	Source or reference
W4680	Δ*lacZ39 rpsL melB4*	CGSC[b]
RS3032	*fadR613*::Tn*10 purB58 dadR1 trpA62 trpE61 tna-5* λ$^-$	CGSC
BL21 (λDE3)	pLysS F$^-$ *recA* r_K^- m_K^+ Rif^r	Novagen
ERL5R	*iclR*::*kan* Δ*lacZ39 rpsL melB4*	8
LLG5	*iclR*::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4*	8
LLG6	*iclR*::*kan iclR*::*lacZ* (at *lac*) *rpsL melB4*	8
LLG7	*fadR*::Tn*10 iclR*::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4*	This study
LLG105	*iclR*(FBS$^-$)::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4 fadR*::Tn*10*	This study
LLG106	*iclR*$^+$(FBS$^-$) (at *lac*) *iclR*::*kan rpsL melB4 zah-281*::Tn*10*	This study
LLG107	*iclR*::*kan iclR*(FBS$^-$)::*lacZ* (at *lac*) *rpsL melB4*	This study
LLG108	*iclR*::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4*	This study
LLG109	*iclR*(FBS$^-$)::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4*	This study
LLG1113	*fadR*::Tn*10 iclR*::*lacZ* (at *lac*) *iclR*$^+$ *rpsL melB4*	This study
LLG1115	*iclR*$^+$ (at *lac*) *iclR*::*kan rpsL melB4 zah-281*::Tn*10*	This study
LLG1121	*iclR*::*kan fadR*::Tn*10 iclR*(FBS$^-$)::*lacZ* (at *lac*) *rpsL melB4*	This study
LLG1122	*iclR*::*kan iclR*::*lacZ* (at *lac*) *zah-281*::Tn*10 rpsL melB4*	This study
LLG1123	*fadR*::Tn*10 iclR*::*kan iclR*::*lacZ* (at *lac*) *rpsL melB4*	This study
LLG1124	*iclR*::*lacZ* (at *lac*) *iclR*::*kan rpsL metB4*	This study
SL1070	*fadR613*::Tn*10* Δ*lacZ39 rpsL melB4*	This study

[a] *iclR*::*kan* is a disruption of the *iclR* gene by a gene encoding kanamycin resistance. *iclR*::*lacZ* is an operon fusion between *iclR* and *lacZ* which was inserted in the *lac* locus of the chromosome. *iclR*$^+$ (at *lac*) refers to a wild-type allele of *iclR* inserted at the *lac* locus. FBS$^-$ indicates inactivation of the FadR binding site. Details are given in Materials and Methods.

[b] CGSC, *Escherichia coli* Genetic Stock Center, Yale University.

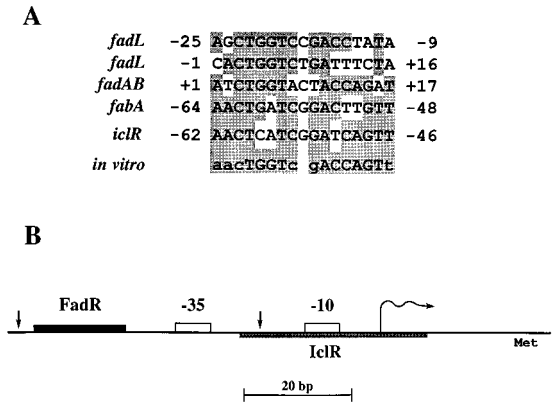

FIG. 2. Possible organization of the region upstream of *iclR*. (A) The sequence of a region upstream of *iclR* (33) is compared with reported binding sites for FadR. The FadR binding site upstream of *fadB* was identified by footprint analysis, while the other binding sites were delimited by deletion analyses (5, 10). Positions are given relative to the start of transcription. The in vitro sequence was determined by oligonucleotide selection (Table 2). Lowercase letters represent nucleotides that occurred in at least 48% of all clones, while capital letters indicate nucleotides that were present in at least 80% of all clones. (B) Organization of the promoter region of *iclR*. The solid box indicates the location of the FadR binding sequence shown in panel A. Open boxes indicate the −10 and −35 promoter elements. The wavy arrow represents the transcriptional start site (8). The shaded box represents the region protected by IclR in footprinting experiments (8). Met indicates the translational start site. The vertical arrows indicate the 3′ endpoints of the fragments used as competitors in the binding assay presented in Fig. 3.

quence which is similar to the binding sites for FadR that are found in a variety of genes (5, 10) (Fig. 2). This site is located just upstream of the *iclR* promoter, a position which is often found for transcriptional activators (4).

We determined the consensus sequence which is required for binding FadR by oligonucleotide rescue in vitro. This method uses the gel shift electrophoresis technique to isolate binding sequences from a pool of random probes (28) (see Materials and Methods). The sequences of 44 independent isolates were compared to determine the binding specificity of FadR (Table 2).

The consensus binding site for FadR includes 17 bp. This site is composed of a palindrome of 8-bp sequences flanking a single random nucleotide. This consensus is in good agreement with the naturally occurring binding sites of FadR and with the possible site which we identified upstream of *iclR* (Fig. 2).

FadR binding to *iclR*. The ability of FadR to bind to the upstream region of *iclR* was tested by gel shift analysis. Purified FadR produced a single shifted band when it was added to a fragment which contained the putative FadR binding site from *iclR* (−128 to −22 relative to the transcriptional start site) (Fig. 3, lanes 2 and 3). Addition of excess unlabeled probe prevented the formation of the shifted band, suggesting that it was competing for FadR (lanes 4 and 5). In contrast, addition of an overlapping fragment from *iclR* which did not contain the putative FadR binding site (−128 to −66) had no effect on the appearance of the shifted band (lanes 6 and 7). A fragment from the promoter region of *aceBAK* also had no effect on the binding of FadR to the *iclR* upstream sequence (lanes 8 and 9). These results indicated that FadR binds near the promoter of *iclR*.

The precise location of the FadR binding site was determined by DNase I footprint analysis (Fig. 4). A single protected region was detected between −61 and −43 relative to the start of transcription. This region corresponds to the proposed FadR binding site presented in Fig. 2.

FadR activates *iclR* expression. To determine whether FadR regulates *iclR* expression, we constructed an *iclR*::*lacZ* operon fusion and transferred it to the *lac* locus on the chromosome. Disruption of *fadR* resulted in a threefold decrease in *iclR*::*lacZ* expression during growth on glucose or acetate (Table 3), indicating that FadR activates expression of *iclR*.

Although it appeared that FadR activates *iclR*, it was also possible that the effect of the *fadR* mutation on *iclR*::*lacZ* expression had been a secondary consequence of altered cellular metabolism. To address this issue, we inactivated the FadR binding site of *iclR* by mutagenesis. The consensus sequence which we had determined in vitro (Table 2) suggested that conversion of CT at −56 and −55 to GG should greatly reduce the affinity of this site for FadR. This mutation nearly eliminated FadR binding in vitro (data not shown).

Mutation of the FadR binding site decreased expression of *iclR*::*lacZ* by a factor of 5 in an $iclR^+$ $fadR^+$ background (Table 3). This decrease did not result from a change in intrinsic promoter activity, since the mutation had no effect in an *fadR iclR* mutant background (Table 3). These observations confirm our conclusion that FadR activates *iclR* expression.

The effect of inactivation of the FadR binding site was slightly greater than that observed when *fadR* was disrupted in an $iclR^+$ background (Table 3). Furthermore, the mutation in *fadR* actually produced a modest increase in the expression of the *iclR*::*lacZ* fusion gene which carried the inactivated FadR binding site (Table 3). These results were expected since IclR represses its own expression (8). Because these strains were $iclR^+$, mutations in *fadR* would be expected to decrease the level of IclR. This decrease should partially relieve the repression of *iclR*::*lacZ*. Rigorous proof of this explanation would require direct measurements of the level of IclR. This explanation is, however, supported by the effects which we observed when we repeated these measurements in an *iclR* mutant back-

TABLE 2. Determination of the consensus sequence of the FadR binding site[a]

Nucleotide	No. of occurrences in 44 clones																
A	21	32	2	6	1	0	0	9	14	3	41	0	0	41	0	3	6
T	8	3	0	37	1	0	44	3	11	8	1	2	0	3	0	36	22
G	12	6	11	1	41	44	0	0	12	33	2	0	2	0	37	0	6
C	3	3	31	0	1	0	0	32	7	0	0	42	42	0	7	5	10
							*	*									
Consensus	a	a	c	T	G	G	T	c		g	A	C	C	A	G	T	t

[a] The consensus sequence of the FadR binding site was determined by oligonucleotide selection (see Materials and Methods). The GCG Pileup program was used to align the sequences of 44 independent clones. The consensus derived from this comparison is shown. Lowercase letters represent nucleotides that occurred in at least 48% of all clones; capital letters indicate nucleotides that were present in at least 80% of all clones. Asterisks show positions that were mutated in the FadR binding site of *iclR*::*lacZ*.

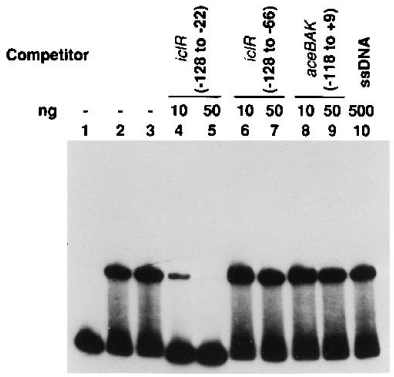

FIG. 3. Binding of FadR near the *iclR* promoter. Binding of FadR to sequences upstream of *iclR* was tested by gel shift analysis. The probe (0.8 ng) included sequences from −128 to −22 relative to the transcriptional start site. Lanes 1 to 3 included 0, 12, and 30 ng of purified FadR. All other lanes included 30 ng of FadR. Lanes 4 and 5, competition with 10 and 50 ng of unlabeled probe; lanes 6 and 7, competition with 10 and 50 ng of a fragment of *iclR* (−128 to −66) which does not contain the predicted FadR binding site; lanes 8 and 9, competition with 10 and 50 ng of sequences upstream of *aceBAK* (−118 to +9 relative to the start of transcription); lane 10, competition with 500 ng of salmon sperm DNA.

ground. In this background, mutations in *fadR* and the FadR binding site of *iclR* had similar effects (Table 3). Furthermore, mutation of *fadR* no longer caused an increase in the expression of the *iclR*::*lacZ* fusion which carried the inactivated FadR binding site (Table 3). These results are consistent with our model in which FadR activates *iclR* expression.

Activation by FadR is dependent on the carbon source. The extent to which FadR activates *iclR* can be estimated from the effect of inactivation of the FadR binding site on the expression of *iclR*::*lacZ* (Table 3). Although FadR activates *iclR* expression about fivefold during growth on glucose, it has only a slight effect during growth on oleate. This finding is consistent with the observation that FadR is inactivated during growth on fatty acids (5, 10).

In a wild-type strain, the levels of expression of the *iclR*::*lacZ* fusion were similar during growth on all three of the carbon sources that we tested (Table 3). This was a surprising result since FadR activates the expression of this construct during growth on glucose and acetate but not during growth on oleate. In contrast, the expression of *iclR*::*lacZ* varied over a range of five- to sixfold in strains carrying mutations in *iclR* and either *fadR* or the FadR binding site of *iclR*::*lacZ* (Table 3). Expression was also sensitive to the carbon source in strains which retained the ability to regulate *iclR*::*lacZ* with either IclR or FadR, although the range was less pronounced (Table 3). We have not determined the reason for this wide variation in the expression of *iclR*::*lacZ* in the mutant strains. However, it appears that FadR regulation and IclR regulation combine to maintain a constant level of expression of *iclR* under the conditions which we have examined.

FadR affects expression of *aceBAK*. Disruption of *fadR* has been reported to increase the expression of *aceBAK* (20). This effect might have resulted from a change in the level of IclR. To test this hypothesis, we constructed full-length clones of *iclR* with and without a functional FadR binding site. These alleles were then transferred to the *lac* locus of an *iclR*::*kan* strain. The strain carrying native *iclR* was functionally *iclR*$^+$, as shown by measuring the expression of IDH phosphatase during growth on glucose and acetate (Table 4). Mutation of either the *fadR* gene or the FadR binding site of *iclR* yielded similar increases in the expression of IDH phosphatase. This increased expression very likely results from decreased expression of IclR.

DISCUSSION

FadR was originally identified as a repressor which controls the genes of fatty acid metabolism (26). This protein was subsequently shown to activate transcription of *fabA*, a gene whose product participates in unsaturated fatty acid biosynthesis (9). FadR also activates the expression of *iclR*, the gene which encodes the repressor protein of the glyoxylate bypass operon. FadR accomplishes this task by binding to a site which is just upstream of the *iclR* promoter.

FadR can act as both a transcriptional repressor and an activator. The effect that it has on a promoter appears to depend on the location of its binding site. FadR acts as a repressor when it binds to a site which overlaps the promoter. In contrast, FadR activates transcription when it binds just upstream of the promoters of *fabA* and *iclR*. This finding is consistent with the organization of most *E. coli* promoters: operators often overlap the promoter, while activators usually bind just upstream (4). Presumably, proteins which overlap the promoter interfere with RNA polymerase binding or promoter clearance, while proteins binding to sequences upstream of the promoter have the potential to assist RNA polymerase.

Previous reports have demonstrated that mutations in *fadR* yield increased expression of *aceBAK* (19, 20). However, *aceBAK* does not have a sequence which matches the consensus for FadR binding sites and does not appear to bind FadR in vitro (reference 5 and this study). Our results suggest a mechanism for the effect of *fadR* mutations on *aceBAK* expres-

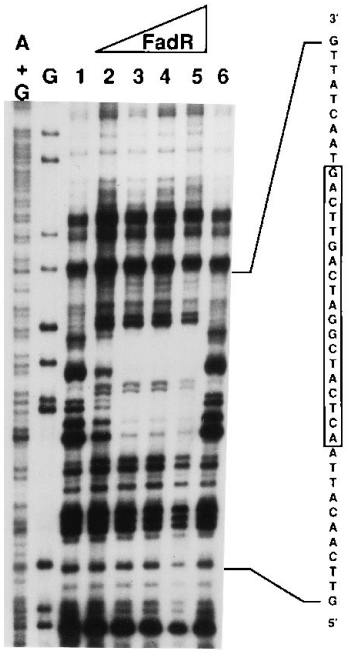

FIG. 4. Identification of the FadR binding site upstream of *iclR*. The FadR binding site was identified by DNase I footprint analysis. The probe, corresponding to sequences from −128 to +27 relative to the start of transcription, was labeled at the 5′ end by T4 polynucleotide kinase. Standards were generated by using the Maxam and Gilbert G and A+G reactions (21). Details are presented in Materials and Methods. Lanes 1 through 6 resulted from reactions containing 0, 0.2, 1.0, 1.2, 6.0, and 0 μg of purified FadR. The sequence protected by FadR is indicated.

TABLE 3. Regulation of *iclR*::*lacZ* expression

Genotype[a]			Relative *iclR*::*lacZ* expression[b] (β-galactosidase activity)		
fadR	FadR site	*iclR*	Glucose	Acetate	Oleate
+	+	+	100	83	105
−	+	+	41	29	86
+	−	+	18	19	82
−	−	+	31	28	76
+	+	−	1,100	300	710
−	+	−	320	120	580
+	−	−	330	100	580
−	−	−	360	110	610

[a] All strains carried an *iclR*::*lacZ* operon fusion at the *lac* locus. The *fadR* mutation resulted from a Tn*10* insertion. FadR site refers to a 2-bp mutation which inactivated the FadR binding site associated with the *iclR*::*lacZ* operon fusion. The *iclR* mutation resulted from insertion of a kanamycin resistance cassette. The strains are otherwise isogenic. Details are given in Materials and Methods. Wild type is indicated as +; mutations are indicated as −. The strains were LLG108, LLG1113, LLG109, LLG105, LLG1124, LLG1123, LLG107, and LLG1121 (from top to bottom).

[b] Cultures were grown to mid-log phase on minimal medium containing the indicated carbon source. Assays for β-galactosidase were performed as described in Materials and Methods. β-Galactosidase activity is expressed relative to that observed for the wild-type strain (*fadR*$^+$ *iclR*$^+$ with a wild-type FadR binding site for *iclR*::*lacZ*) during growth on morpholinepropanesulfonic acid (MOPS) glucose (1,200 Miller units). The values shown are the averages of seven or more independent experiments. The standard errors were all less than 12%.

sion: loss of FadR should result in decreased expression of IclR. This decrease in IclR would yield the observed increase in *aceBAK* expression. Consistent with this expectation, mutation of the FadR binding site of *iclR* produced an increase in *aceBAK* expression which was very similar to that produced by disruption of *fadR*.

Although FadR and IclR mediate the responses of a variety of genes to the carbon source, they also appear to play the paradoxical roles of ensuring that the transcription of *iclR* does not change under these same conditions. We have made similar observations in assays using an *iclR*::*lacZ* gene fusion, suggesting that the level of the IclR protein may be constant as well (unpublished observation). In the absence of FadR and IclR, the expression of an *iclR*::*lacZ* operon fusion varied over a fivefold range on the carbon sources that we tested.

IclR probably acts as a general feedback inhibitor of *iclR*, maintaining constant expression under a variety of conditions. Unlike its behavior in regulating *aceBAK*, IclR repression of its own expression is only modestly sensitive to the carbon source (8). Integration host factor (IHF) may be responsible for this differential control by IclR. IHF, which binds just upstream of the *aceBAK* promoter, enhances the response of IclR to the carbon source. In contrast, *iclR* does not have an IHF site and so is much less sensitive to the carbon source (30).

FadR may act specifically to prevent an increase in IclR during growth on fatty acids. This increase was observed during growth on the fatty acid oleate in strains that lacked FadR or the FadR binding site of *iclR*::*lacZ* (Table 3). FadR prevented this increase by activating *iclR* transcription on carbon sources such as glucose and acetate but not on oleate. This is an unusual mechanism, since *E. coli* would generally prevent increased expression of a gene by repression. However, repression by FadR would not solve this problem for *iclR* since FadR is inactivated during growth on fatty acids (references 5 and 10 and this study). Instead, FadR appears to prevent an increase in the expression of *iclR* during growth on fatty acids by activating the transcription of this gene on all other carbon sources.

TABLE 4. Effect of the FadR site of *iclR* on *aceBAK* expression

Genotype[a]			IDH phosphatase[b] (mU/mg)	
fadR	FadR site	*iclR*[c]	Glucose	Acetate
+	+	wt	0.5	12
+	NA[d]	−	10	26
−	+	wt	4.0	18
+	+	wt (*lac*)	0.8	12
+	−	wt (*lac*)	9	19

[a] The *fadR* mutation resulted from a Tn*10* insertion. FadR site refers to a 2-bp mutation which inactivated the FadR binding site associated with *iclR*$^+$. The *iclR* mutation resulted from insertion of a kanamycin resistance cassette. The strains are otherwise isogenic. Details are given in Materials and Methods. The wild type is indicated as +, while mutations are indicated as −. The strains were LLG5, LLG6, LLG7, LLG1115, and LLG106 (from top to bottom).

[b] Cultures were grown to mid-log phase on minimal medium containing the indicated carbon source. Assays for IDH phosphatase were performed as described in Materials and Methods. The values shown are the averages of three or more independent experiments. The standard errors were all less than 20%.

[c] Intact *iclR*$^+$ was expressed either from its normal locus (wt [wild type]) or from a construct inserted at the *lac* locus [wt (*lac*)]. Strains which expressed *iclR*$^+$ from the *lac* locus had *iclR*::*kan* at the *iclR* locus.

[d] NA, not applicable.

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