JOURNAL OF BACTERIOLOGY, July 1996, p. 3982–3984 Vol. 178, No. 13
0021-9193/96/$04.00+0

The Binding Site of the IclR Repressor Protein Overlaps the Promoter of *aceBAK*

BIN PAN, INDIRA UNNIKRISHNAN, AND DAVID C. LAPORTE*

Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

Received 16 January 1996/Accepted 30 April 1996

In *Escherichia coli*, repression of the *aceBAK* operon is mediated by the IclR protein. We used an in vitro oligonucleotide selection technique to determine the consensus recognition sequence for IclR. Mutational analysis confirmed the contribution of this sequence to repression in vivo and identified the −35 element of the promoter.

In *Escherichia coli*, adaptation to growth on acetate or fatty acids requires the induction of the *aceBAK* operon. This operon encodes the metabolic and regulatory enzymes of the glyoxylate bypass. The metabolic enzymes of the bypass are isocitrate lyase (*aceA*) and malate synthase (*aceB*) (2, 3, 12, 15). Once it is induced, the flow of isocitrate through this pathway is regulated by phosphorylation of isocitrate dehydrogenase in a reaction catalyzed by isocitrate dehydrogenase kinase-phosphatase (*aceK*) (1, 5, 11, 13). This pathway is essential for growth on acetate or fatty acids, since it prevents the quantitative loss of the entering carbon as CO_2 in the Krebs cycle.

Transcription of the *aceBAK* operon is initiated from a single site upstream of *aceB*. Although sequences upstream of the start site are similar to the consensus for promoter elements in *E. coli*, the ability of these sequences to promote transcription has not been tested (3).

Expression of *aceBAK* is activated by integration host factor (23) and is repressed by IclR (9, 18, 23, 24). In addition to repressing *aceBAK*, IclR also represses its own expression. In this paper, we determine the sequence requirements for IclR binding and examine the operator-promoter region of *aceBAK*.

The consensus DNA sequence for IclR binding. The DNA sequence required for the binding of IclR was determined by oligonucleotide selection in vitro (21). This technique employs an electrophoretic mobility shift to select sequences which are capable of binding to IclR from a pool of degenerate oligonucleotides (Fig. 1). Following a total of four rounds of selection, the oligonucleotides were cloned into a sequencing vector and the sequences of 44 independent isolates were determined. The binding site consensus was determined by comparing these sequences (Fig. 1). The location of the binding site within the degenerate region of the probe appeared to be distributed randomly among the 44 clones that were sequenced (data not shown).

The consensus IclR binding sequence contains a core that is highly conserved. This core is a palindromic repeat of seven nucleotides separated by one nucleotide which is much less conserved (Fig. 1). Flanking the core consensus are several positions which exhibit a moderate sequence preference.

The IclR binding sites of *aceBAK* and *iclR* contain sequences which exhibit extensive homology with the consensus (Fig. 2). The IclR binding site of *aceBAK* matches the core consensus at 12 of 14 positions. The two mismatched nucleotides occur within the proposed −35 promoter element of *aceBAK*. The IclR binding site of *iclR* matches the core consensus at 13 of 14 positions. The mismatched nucleotide also occurs within a proposed promoter element (6). Several adenines occur upstream of the IclR binding sites of both *aceBAK* and *iclR*. A

A	5	8	15	21	13	20	0	1	0	44	44	40	3	15	30	5	1	2	0	0	34	11	5	6	3	2	5	2
T	10	10	3	7	14	11	39	0	0	0	0	3	33	17	1	38	43	41	2	0	0	19	20	12	11	15	17	10
C	9	2	3	2	3	1	3	0	0	0	0	0	6	7	1	1	0	0	42	44	0	7	6	9	13	12	6	9
G	4	10	10	5	5	4	0	42	44	0	0	1	2	5	12	0	0	1	0	0	10	6	11	14	13	10	9	13

Core T G G A A A T A/g T T T C C A/g

FIG. 1. Determination of the consensus sequence for IclR binding. IclR binding sequences were selected from a pool of degenerate oligonucleotides essentially as described by Pollock and Treisman (21). A single-stranded, 70-base oligonucleotide pool was synthesized by National Bioscience Inc. Each oligonucleotide in the pool was composed of 31 random nucleotides flanked by two constant sequences: 5′ GCTGCTCGAGTTCTCCTCCCACG and GTTCGCGGATCCCGTC 3′. The oligonucleotides were labeled with [α-^{32}P]dCTP during the synthesis of the second strand with a Klenow fragment. Twenty picomoles of the labeled oligonucleotide pool was mixed with purified IclR (4, 6) and subjected to electrophoresis on a 4.5% nondenaturing polyacrylamide gel. The DNA in the IclR-DNA complex was isolated from the gel and amplified by PCR. The amplified DNA was then subjected to a second round of selection. Following a total of four rounds of selection, the DNA product was inserted into pT7/T3α (16) and 44 independent isolates were sequenced. Sequences from the degenerate region of the probe were aligned. Core refers to the consensus sequence from the regions which exhibited the greatest preference for specific nucleotides.

* Corresponding author. Phone: (612) 625-4983. Fax: (612) 625-2163. Electronic mail address: DAVID-L@LENTI.MED.UMN.EDU.

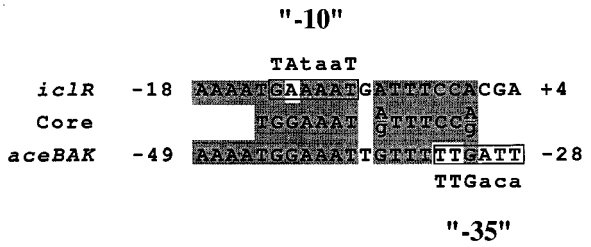

FIG. 2. The IclR binding sites of *iclR* and *aceBAK*. The consensus binding site for IclR (Fig. 1) is compared with the regions of *aceBAK* and *iclR* which are protected by IclR during DNase I footprinting (4, 6). Boxes indicate regions of *aceBAK* and *iclR* which are similar to the −10 or −35 elements of σ^{70}-type promoters in *E. coli* (7). The consensus sequences of these promoter elements are also shown, with bases which exhibit more than 70% conservation presented as capital letters. Prior to this study, these sequences had not been directly shown to stimulate transcription. However, these elements do occur at the expected distances from the transcriptional start sites (3, 6).

preference for adenines in this region was also observed among the oligonucleotides which were selected in vitro (Fig. 1).

Most known transcriptional factors of bacteria or bacterial phage bind to palindromic DNA sequences as dimers (14). Each monomer is thought to recognize one half-site of the dyad (20). The dyad symmetry in the IclR binding sequence suggests that IclR may also bind to DNA in a symmetrical fashion. Consistent with this suggestion, Negre et al. have reported that IclR exists as a dimer in vitro (18).

Mutagenesis of the IclR binding site of *aceBAK*. Mutagenesis was used to assess the contributions of the sequences around the IclR binding site of *aceBAK* to repression and to promoter activity in vivo. Each construct carried a single base pair change. The data presented in Fig. 1 were used to select mutations which would be expected to have a substantial effect on IclR binding. We then determined the effects of these mutations on expression of an *aceB*:*lacZ* operon fusion during growth in minimal glucose medium, a medium which causes IclR-mediated repression of *aceBAK*.

Effects of the mutations on promoter activity were evaluated by measuring *aceB*:*lacZ* expression in an *iclR*::*kan* background (Fig. 3A). Mutations within the sequence which matched the consensus for −35 elements dramatically reduced expression. Mutations at several other positions also decreased the promoter activity, possibly by changing the context of the −35 element.

The extent of IclR repression can be measured for each construct by comparing the expression in *iclR*$^+$ and *iclR*::*kan* strains (Fig. 3A) during growth on glucose (ratios are presented in Fig. 3B). Repression of *aceB*:*lacZ* expression by IclR was reduced after the nucleotides that match the core consensus recognition sequence (indicated by the arrows in Fig. 3) were mutated, demonstrating that these nucleotides mediate IclR repression in vivo. The nucleotide at −39 appears to have dual functions: contributing to both IclR repression and promoter activity. Significant loss of repression was also observed for mutations in the regions which flanked the core consensus. This result is consistent with the observation that IclR exhibits a moderate sequence preference in these regions (Fig. 1).

The mutational analysis indicates that a region upstream of *aceBAK* has two opposing functions: binding RNA polymerase and the IclR repressor protein. A similar situation appears to exist for *iclR*. The sequence requirements for the binding of IclR and RNA polymerase are distinct (Fig. 2), necessitating a balance between these functions. For both operators, maximal repression appears to have been sacrificed in the interest of

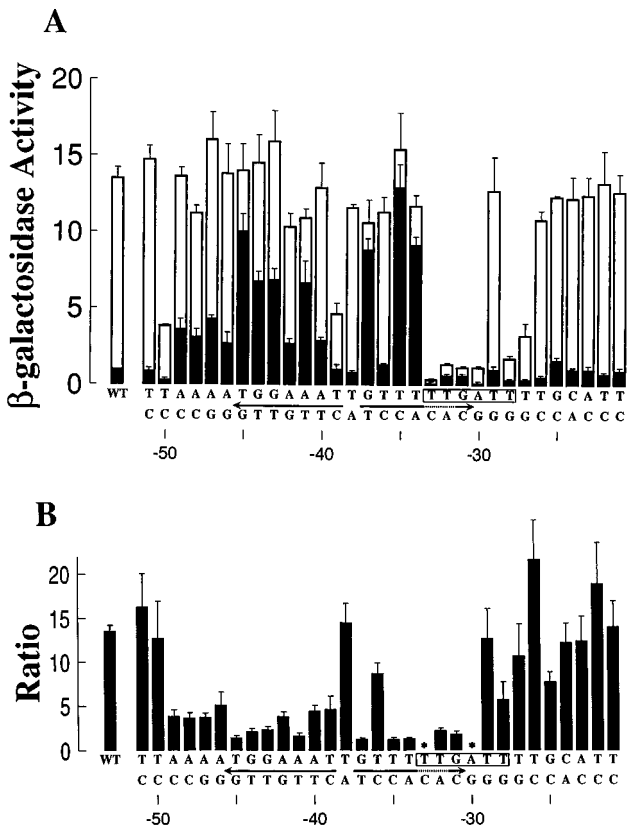

FIG. 3. Mutational analysis of the IclR binding site of *aceBAK*. Single base mutations were introduced into the operator region of an *aceB*:*lacZ* operon fusion either by PCR with a contaminated oligonucleotide (8) or by oligonucleotide-directed mutagenesis with the Transformer site-directed mutagenesis kit from Clontech Laboratories Inc. These operon fusions included 195 bp upstream of the transcription start site and the first 223 bp of the coding region of *aceB* (23). The presence of a single mutation in each construct was confirmed by sequence analysis (10). The *aceB*:*lacZ* fusions were then introduced into the *lac* locus of the chromosomes of congenic strains W4680 (*iclR*$^+$) and ERL5R (*iclR*::*kan*) (6, 22). Cultures were grown on minimal glucose medium (19) to mid-log phase at 37°C and then assayed for β-galactosidase activity (17). (A) The β-galactosidase activities are expressed relative to that observed with the wild-type IclR binding site in an *iclR*$^+$ strain. Solid columns indicate expression in an *iclR*$^+$ strain, while the open columns represent expression in an *iclR*::*kan* strain. The wild-type sequence is shown immediately below the *x* axis, while the single base mutations are shown at the bottom. Arrows indicate the IclR recognition sequence, with the broken line indicating mismatches. A sequence matching the consensus for the −35 elements of the promoters is boxed. (B) The ratio of the activities determined in the *iclR*::*kan* and *iclR*$^+$ strains. In two cases (each marked with an asterisk), the very low activity from the *iclR*$^+$ strain resulted in a calculated ratio that was statistically unreliable.

producing a stronger promoter. However, reducing the affinity of IclR for the operator associated with the *aceBAK* promoter might be beneficial, since it would enhance induction of this operon during growth on acetate. Similarly, reducing the affinity of IclR for the *iclR* operator might be necessary to provide an appropriate cellular level of IclR. This need to balance competing functions is a problem which must be resolved whenever a DNA sequence binds more than one protein factor.

This research was supported by grant DK40486 from the National Institutes of Health.

We thank Dennis Livingston for a critical reading of the manuscript.

REFERENCES

1. **Borthwick, A. C., W. H. Holms, and H. G. Nimmo.** 1984. The phosphorylation of *Escherichia coli* isocitrate dehydrogenase in intact cells. Biochem. J. **222:**797–804.
2. **Brice, C. B., and H. L. Kornberg.** 1968. Genetic control of isocitrate lyase activity in *Escherichia coli*. J. Bacteriol. **96:**2185–2186.
3. **Chung, T., D. J. Klumpp, and D. C. LaPorte.** 1988. Glyoxylate bypass operon of *Escherichia coli*: cloning and determination of the functional map. J. Bacteriol. **170:**386–392.
4. **Cortay, J. C., D. Negre, A. Galinier, B. Duclos, G. Perriere, and A. J. Cozzone.** 1991. Regulation of the acetate operon in *Escherichia coli*: purification and functional characterization of the IclR repressor. EMBO J. **10:** 675–679.
5. **Garnak, M., and H. C. Reeves.** 1979. Phosphorylation of isocitrate dehydrogenase of *Escherichia coli*. Science **203:**1111–1112.
6. **Gui, L., A. Sunnarborg, B. Pan, and D. C. LaPorte.** 1996. Autoregulation of *iclR*, the gene encoding the repressor of the glyoxylate bypass operon. J. Bacteriol. **178:**321–324.
7. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. **11:**2237–2255.
8. **Higuchi, R.** 1990. PCR protocols. A guide to methods and applications. Academic Press, Inc., San Diego, Calif.
9. **Kornberg, H. L.** 1967. The regulation of anaplerotic enzymes in *E. coli*. Bull. Soc. Chim. Biol. **49:**1479–1490.
10. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using miniprep plasmid DNA for sequencing double stranded templates with Sequenase™. BioTechniques **6:**544–546.
11. **LaPorte, D. C., and T. Chung.** 1985. A single gene codes for the kinase and phosphatase which regulate isocitrate dehydrogenase. J. Biol. Chem. **260:** 15291–15297.
12. **LaPorte, D. C., P. E. Thorsness, and D. E. Koshland, Jr.** 1985. Compensatory phosphorylation of isocitrate dehydrogenase: a mechanism for adaptation to the intracellular environment. J. Biol. Chem. **260:**10563–10568.
13. **LaPorte, D. C., K. Walsh, and D. E. Koshland, Jr.** 1984. The branch point effect: ultrasensitivity and subsensitivity to metabolic control. J. Biol. Chem. **259:**14068–14075.
14. **Lewin, B.** 1990. Genes IV. Cell Press, Cambridge, Mass.
15. **Maloy, S. R., and W. D. Nunn.** 1982. Genetic regulation of the glyoxylate shunt in *Escherichia coli* K-12. J. Bacteriol. **149:**173–180.
16. **Mead, D. A., E. Szczesna-Skorupa, and B. Kemper.** 1986. Single-strand DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1:**67–74.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Negre, D., J.-C. Cortay, A. Galinier, P. Sauve, and A. J. Cozzone.** 1992. Specific interactions between the IclR repressor of the acetate operon of *Escherichia coli* and its operator. J. Mol. Biol. **228:**23–29.
19. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for enterobacteria. J. Bacteriol. **119:**736–747.
20. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53:**293–321.
21. **Pollock, R., and R. Treisman.** 1990. A sensitive method for the determination of protein-DNA binding specificities. Nucleic Acids Res. **18:**6197–6204.
22. **Resnik, E., and D. C. LaPorte.** 1991. Introduction of single copy sequences into the chromosome of *Escherichia coli*: application to gene and operon fusions. Gene **107:**19–25.
23. **Resnik, E., B. Pan, N. Ramani, M. Freundlich, and D. C. LaPorte.** 1996. Integration host factor amplifies the induction of the *aceBAK* operon of *Escherichia coli* by relieving IclR repression. J. Bacteriol. **178:**2715–2717.
24. **Sunnarborg, A., D. Klumpp, T. Chung, and D. C. LaPorte.** 1990. Regulation of the glyoxylate bypass operon: cloning and characterization of *iclR*. J. Bacteriol. **172:**2642–2649.