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Regulation of Expression of the Ethanol Dehydrogenase Gene (*adhE*) in *Escherichia coli* by Catabolite Repressor Activator Protein Cra

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The *adhE* gene encodes ethanol dehydrogenase and is located at min 27.9 of the *Escherichia coli* chromosome. Expression of *adhE* is about 10-fold higher in cells grown anaerobically than in cells grown aerobically and is dependent on both transcriptional and posttranscriptional factors. In this study, a *trans*-regulatory element repressing *adhE* expression was characterized by genetic and biochemical approaches. A mutation down-regulating *adhE* expression was mapped at min 2 of the chromosome. DNA sequence analysis revealed a missense mutation in the *cra* gene, formerly known as *fruR*. The *cra* gene encodes a catabolite repressor-activator protein (Cra) involved in the modulation of carbon flow in *E. coli*. The mutant protein (Cra*) sustained an Arg148→His substitution causing 1.5- and 3-fold stronger repression of *adhE* transcription under anaerobic and aerobic conditions, respectively. By contrast, *cra* null mutants displayed 1.5- and 4-fold increased *adhE* transcription under those conditions. Disruption of the *cra* gene did not abolish the anaerobic activation of the *adhE* gene but diminished it twofold. Cra and Cra* were purified as fusion proteins tagged with an N-terminal 6×His element. In vitro, both fusion proteins showed binding to the *adhE* promoter region and to the control *fruB* promoter region, which is a known Cra target. However, only 6×His-tagged Cra, and not 6×His-Cra*, was displaced from the DNA target by the effector, fructose-1-phosphate (F1P), suggesting that the mutant protein is locked in a promoter-binding conformation and is no longer responsive to F1P. We suggest that Cra helps to tighten the control of *adhE* transcription under aerobic conditions by its repression.

During anaerobic growth in the absence of exogenous electron acceptors, the facultative anaerobe *Escherichia coli* produces fermentation products such as ethanol, glycerol, formate, acetate, D-lactate, succinate, CO_2, and H_2 (5, 31). Two consecutive NADH-dependent reductions, converting acetyl coenzyme A to acetaldehyde and then to ethanol, are catalyzed by the bifunctional ethanol dehydrogenase, AdhE (8, 24, 28). Moreover, this enzyme also functions as a deactivase of pyruvate-formate lyase (13, 14). The AdhE protein can exist as a homopolymer of 96-kDa subunits and is Fe^{2+} dependent for catalysis (10, 13, 19). The *adhE* gene is located at min 27.9 on the chromosome and has been sequenced (10, 14, 16). Expression of *adhE* is transcriptionally regulated and is 10- to 20-fold higher during anaerobic growth than during aerobic growth (2, 16). Recently, we showed that posttranscriptional processing of the *adhE* mRNA by RNase III is required for its translation (1). The level of active ethanol dehydrogenase in a cell is probably also dependent on the rate of oxidative inactivation of the enzyme, as was observed for a corresponding protein of *Klebsiella pneumoniae* (12). It is unlikely that molecular oxygen itself is directly responsible for the transcriptional control because anaerobic respiration of nitrate also strongly curtails *adhE* transcription (2, 16). Anaerobic activation of *adhE* expression, therefore, seems to be related to the degree of accumulation of reducing equivalents. The NADH concentration or the NADH/NAD ratio was suggested as a possible redox signal (16, 20). A crucial test of this hypothesis requires identification of the *trans*-regulatory element(s) for *adhE* and demonstration of an allosteric effect of the nicotinamide nucleotide on this element(s).

Two *trans*-regulatory mutations affecting the level of alcohol dehydrogenase in the cell were reported. The *adhR* mutation at min 72 elevated AdhE activity levels, and the *adhB* mutation at min 28 lowered the AdhE activity levels (5, 6). The genetic nature of these mutations remains to be characterized. In this report, we show that the *cra* (catabolite repressor-activator) gene at min 2 is involved in the transcriptional regulation of *adhE*.

MATERIALS AND METHODS

Bacterial strains, growth media, and reagents. Relevant characteristics and sources of the bacterial strains and plasmids used in this study are given in Table 1. LB and MacConkey media were prepared as described by Miller (21). Minimal medium contained 34 mM NaH_2PO_4 and 64 mM K_2HPO_4 adjusted to pH 7.5 with HCl, as well as 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 300 μM $MgSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, and 0.4% glucose or 0.4% lactose as a carbon source. Antibiotics were purchased from Sigma and were added, when appropriate, at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml. Chloramphenicol was added at 30 μg/ml to glucose-minimal agar and at 10 μg/ml to lactose-minimal agar. Oligonucleotides were custom synthesized by Oligos Etc., Inc. DNA sequence analysis was carried out with Sequenase 2.0 (U.S. Biochemical Co.). All other enzymes were purchased from New England BioLabs. Vent DNA polymerase was used in PCRs. All genetic procedures were conducted as recommended by Miller (21). Enzymatic assays were performed as described or referred to in reference 1.

Plasmids and DNA procedures. Both strands of the *cra* and *cra** genes were sequenced with oligonucleotides AO11 (5′-CGTTAGCCCAGCGTTGA), AO12 (5′-ACGTTGTGAACAGCGCC), AO13 (5′-ATATTGATGTCCAGTCCCG), AO14 (5′-CGCAATTTACAGCCCAA), AO15 (5′-CGCACACGTTCTATTG G), AO16 (5′-GTGGTTGGTGCCGATCA), and AO17 (5′-GTCGTTTGCGT TGTTGC).

To construct pAM47-5, a DNA fragment containing the open reading frame (ORF) of *cra* was amplified by PCR with primers AO18 (5′-CCGGATCCATG AAACTGGATGAAATCGCT) and AO19 (5′-CCCCGAAGCTTAGCTACGG

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TABLE 1. Strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics	Source or reference
Strains		
ECL1108	F$^-$ *araD139* Δ*lacU169 rpsL150 relA1 flb-5301 deoC1 ptsF25 adhE*$^+$ Φ(*adhE′-cat-lacZYA*)	2
CSH109	F$^-$ *ara* Δ(*gpt-lac*)*5 rpsL*	21
ECL2166	CSH109 [*adhE*$^+$ Φ(*adhE′-cat-lacZYA*)][a]	This study
CAG12095	Wild type but *zac-3051*::Tn*10*	30
CAG12131	Wild type but *zac-3051*::Tn*10kan*	30
ECL2331	*cra** mutant of ECL1108	This study
ECL2332	ECL2331 (*zac-3501*::Tn*10*)	This study
ECL2171	ECL2166 (*cra* zac-3051*::Tn*10*)	This study
KL723/F′104	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2* λ$^-$ *rac hisG4 rfbD1 mgl-51 recA13 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	17
ECL2175	ECL2171/F′104	This study
ECL2252	ECL2171 (*cra*939*::mini-Tn*10kan*)	This study
ECL2263	ECL2171 (*cra*34*::mini-Tn*10kan*)	This study
Plasmids		
pBluescript II KS(−)	Amp^r cloning vector	Stratagene
pAM24	Amp^r Φ(*adhE′-cat-lacZYA*)	1
pAM34	Amp^r *cra*34*::mini-Tn*10kan*	This study
pAM43	Amp^r *cra*959*::mini-Tn*10kan*	This study
pQE30	Amp^r *T5p*-6×His expression vector	QIAGEN
pAM46-3	Amp^r *T5p*-6×His-*cra** expression plasmid	This study
pAM47-5	Amp^r *T5p*-6×His-*cra* expression plasmid	This study
Phage		
λNK1316	Mini-Tn*10kan c*I857 *P*am80 *nin5 b522 att*	15

[a] *adhE′-cat* encodes the AdhE′-CAT protein fusion (1), except for strain ECL1108 harboring the *adhE′-cat* operon fusion.

CTGAGCAC) with chromosomal DNA of ECL2166 as the template. The PCR product was precut with *Bam*HI and *Hin*dIII and cloned into the corresponding sites of pQE30 (QIAGEN). Construction of plasmid pAM47-5 was verified by sequencing the insert. Plasmid pAM46-3 containing the ORF of *cra**, was constructed in a similar way, except that chromosomal DNA of ECL2171 was used as the template for PCR.

To construct the ECL2166 merodiploid, the Φ(*adhE′-cat-lacZYA*) triple fusion from plasmid pAM24 (1) was integrated into the lambda attachment site of strain CSH109 (21) as described earlier (29). Here, *adhE′* is part of a translational fusion with the chloramphenicol acetyltransferase (*cat*) gene and forms an operon with a promotorless *lacZYA* cassette. The level of LacZ activity in the cell was assayed as a measure of *adhE* transcription. Since the *cat* sequence does not interfere with *lacZ* expression, we will hereafter refer to the triple fusion simply as *adhE′-lacZ*.

Purification of 6×His-Cra and 6×His-Cra* proteins. The 6×His-tagged Cra and Cra* proteins were purified from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *E. coli* XL-1 Blue cells (Stratagene) transformed with pAM47-5 and pAM46-3, respectively, by using Ni-NTA resin (QIAGEN). Protein purification was performed at 4°C under native conditions as recommended by QIAGEN. Protein elution occurred between concentrations of 0.1 and 0.2 M imidazole. Dialysis of proteins was performed at 4°C against 100 mM sodium phosphate (pH 8.0), 1 mM Na_2EDTA, 150 mM NaCl, 10 mM β-mercaptoethanol, and 25% glycerol. The resulting proteins were quantified by the Coomassie Plus protein assay reagent (Pierce). Protein samples were stored at −20°C.

Preparation of radioactively labeled DNA fragments and a band shift assay. All DNA fragments for radioactive labeling were amplified by PCR using chromosomal DNA of ECL2166 as the template. Primers AO1 (5′-GCGACATTA GTAACAGCC) and AO20 (5′-TATCTAGTTGTGCAAAACATGC) were used to amplify the 350-bp DNA fragment containing the *adhE* promoter. Primers AO21 (5′-TTTACGGCTTTCCTTGCGTGC) and AO22 (5′-CAGCATGC CATTGACGTAGCC) were used to amplify the 320-bp DNA fragment containing the *fruB* promoter (23). Primers AO11 and AO15 (see above) were used to amplify the 300-bp internal fragment of *cra*. Purification of PCR products, gel extraction of DNA fragments, and removal of enzymes and nonincorporated nucleotides were done by using corresponding kits from QIAGEN. End labeling of the purified DNA fragments was performed with T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; NEN Life Science Products, Inc.). Band shift assay was carried out essentially as described by Lynch and Lin (18), except that the DNA concentration used was 0.2 nM and the protein concentrations used ranged from 0 to 350 nM.

RESULTS

Isolation of *adhE* regulatory mutants. The ECL1108 merodiploid strain, bearing an intact *adhE* gene at min 27.9 and the *adhE′-lacZ* fusion at min 17 of the *E. coli* chromosome, was used to isolate *adhE* regulatory mutant (Table 1). The cells were randomly mutagenized with ethyl methanesulfonate and then screened for mutants that displayed either increased expression of both *adhE* and *adhE′-lacZ* during aerobic growth or decreased expression during anaerobic growth. Initial screening of mutants was carried out on MacConkey-lactose agar plates (aerobic incubation of plates is assumed, unless indicated otherwise). Colonies with an overall red color stronger than normal were considered potential candidates for increased *adhE′-lacZ* expression under aerobic conditions. Since the center of a colony is under semianaerobic conditions, colonies with a red color lighter than normal in the center of a colony were considered potential candidates for decreased expression of *adhE′-lacZ* under anaerobic conditions. The subsequent screening for *trans*-acting *adhE* regulatory mutations was carried out by checking for a parallel increase or decrease in both AdhE and LacZ activity levels in cells grown aerobically or anaerobically. No mutants with increased aerobic expression or lacking anaerobic expression of both *adhE* and *lacZ* were found by this procedure. On the other hand, three independent mutants of ECL1108 that displayed decreased *adhE* and *lacZ* expression under both aerobic and anaerobic conditions were found. These mutant strains showed AdhE and LacZ activities that were 3-fold lower than those of ECL1108 under anaerobic conditions and 1.5-fold lower under aerobic conditions (data not shown). We will refer to these strains as AdhE-down mutants. All three AdhE-down mutations mapped in the min 2 region by P1 transduction and showed >75% linkage to the *zac-3051*::Tn*10* marker (30). One

TABLE 2. Expression of *adhE* and *adhE′-lacZ* in wild-type ECL2166 and AdhE-down mutant ECL2171

Strain	AdhE activity[a]			LacZ activity[b]		
	O_2^+	O_2^-	Ratio[c]	O_2^+	O_2^-	Ratio[c]
ECL2166	7 ± 2	117 ± 20	18.4 ± 4.8	150 ± 12	734 ± 37	5.1 ± 0.9
ECL2171	5 ± 1	43 ± 12	7.7 ± 1.2	103 ± 14	230 ± 22	2.5 ± 0.5

[a] AdhE activity is expressed as the rate of NAD reduction in nanomoles per minute per milligram of total cellular protein. Standard deviations were calculated from data from 10 independent experiments.
[b] LacZ activity is expressed in Miller units.
[c] Average of individual O_2^-/O_2^+ ratios of enzymatic activities.

AdhE-down mutant, ECL2331, was used for further characterization of the unknown gene.

The AdhE-down mutation in ECL2331 was linked to the *zac-3051*::Tn*10* marker and backcrossed into the parental ECL1108 merodiploid by P1 transduction. Desired transductants were identified by screening for decreased anaerobic levels of both AdhE and LacZ. One transductant, ECL2332, with lowered transcription from the *adhE* promoter was chosen for further analysis.

To test whether or not the Tn*10* insertion at min 2 affects *adhE* transcription, P1 transduction was used to replace the *zac-3051*::Tn*10* marker in ECL2332 by *zac-3051*::Tn*10kan* from CAG12131 (30). Some Kan^r transductants displayed wild-type levels of AdhE, and some retained the AdhE-down phenotype. It is unlikely that replacement of *zac-3051*::Tn*10* with *zac-3051*::Tn*10kan* changed the effect of Tn*10* on the surrounding sequences, because both insertions are identical except for the difference between the antibiotic resistance genes borne by the transposon. As expected, more than 75% of the Kan^r transductants displayed AdhE and LacZ activity levels identical to those of wild-type strain ECL1108 (data not shown). This observation indicated that the Tn*10* insertions themselves, placed at min 2, did not interfere with regulation of *adhE*.

Indications for the dominance of the AdhE-down mutation. To facilitate further characterization of the AdhE-down mutation, we first optimized the conditions under which the expression of *adhE* and *adhE′-lacZ* in wild-type and mutant strains could be distinguished by growth on agar plates. We found that strains expressing *adhE′-lacZ* at lower levels showed a clear anaerobic growth disadvantage on lactose-minimal agar. On the other hand, strains expressing *adhE* at lower levels showed a clear anaerobic growth disadvantage on glucose-minimal agar, which is in agreement with the failure of *adhE* null mutants to grow anaerobically on glucose (8).

By using the above criteria, we checked if the AdhE-down mutation in ECL2332 can be complemented by a pBR322-based library of *E. coli* (3). However, no Amp^r transformants of ECL2332 were able to grow normally on lactose-minimal agar under anaerobic conditions. We also tried to complement the AdhE-down mutation with the F′104 episome, which harbors the *E. coli* chromosomal region spanning at least *thr* (min 0) and *argF* (min 6.3) (17). Since ECL2332 was not suitable as a recipient for F′104 because of lack of selective markers, we constructed another merodiploid strain, ECL2166 [F$^-$ Δ(*gpt-pro*) *adhE*$^+$ *adhE′-lacZ*], which required the *pro* genes of F′104 to grow on medium without proline. The *tet* marker at min 2 in ECL2332 was used to transfer the AdhE-down mutation into the ECL2166 background by P1 transduction. Tet^r transductants were screened for the mutant phenotype by assaying AdhE and LacZ activity levels in anaerobically grown cells. One such AdhE-down transductant, ECL2171, displayed decreased anaerobic expression of both the *adhE* and *adhE′-lacZ* genes (Table 2). F′104 was introduced into ECL2171 (Tet^r) by conjugation with KL723/F′104 (17) and selection of exconjugants on glucose-minimal agar plates containing tetracycline at 10 μg/ml but no proline. No complementation of the AdhE-down mutation by F′104 was observed. Since both the F′104 episome and the pBR322-based chromosomal library failed to complement the AdhE-down mutation, it seemed likely that this mutation was dominant.

Suppression of the AdhE-down mutation by transposon mutagenesis. Assuming that the AdhE-down mutation is dominant, we subjected ECL2171/F′104 to mini-Tn*10kan* mutagenesis to disrupt the dominant mutant gene on the ECL2171 chromosome. F′104 was maintained to complement the disrupted gene, in case the gene proved to be essential. The λNK1316 phage (15) was used to deliver the mini-Tn*10kan* transposon into ECL2171 (*zac-3051*::Tn*10*)/F′104. Selection of the Kan^r insertion mutants and screening for elevated *adhE′-lacZ* expression were done under anaerobic conditions on lactose-minimal agar supplemented with kanamycin at 50 μg/ml and tetracycline at 10 μg/ml. The mini-Tn*10kan* insertions from 54 purified clones were backcrossed into a clean background of AdhE-down mutant ECL2171 by P1 transduction, and AdhE and LacZ activity levels were assayed in the resulting Kan^r transductants grown anaerobically in LB containing 0.1 M morpholinepropanesulfonic acid (MOPS; pH 7.4) and 0.4% glucose. Only three backcrossed mini-Tn*10kan* insertions suppressed the AdhE-down phenotype, and these insertions were linked to *zac-3051*::Tn*10* (Fig. 1). We also backcrossed

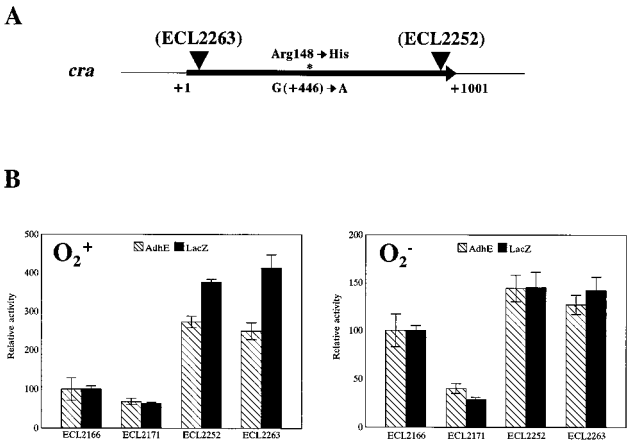

FIG. 1. (A) Schematic representation of the *cra* gene, the *cra∗* mutation, and two mini-Tn*10kan* insertions in strains ECL2263 and ECL2252. The symbols ∗ and ▼ indicate, respectively, the locations of the *cra∗* mutation and the mini-Tn*10kan* insertions. (B) Relative AdhE and LacZ activity levels in ECL2166 (*cra*$^+$), ECL2171 (*cra∗*), ECL2263 (*cra*), and ECL2252 (*cra*) under aerobic (O_2^+) and anaerobic (O_2^-) conditions. Relative activity was calculated separately for O_2^+ and O_2^- as a percentage of the AdhE or LacZ activity level in ECL2166. For absolute values corresponding to the 100% activity of strain ECL2166, see Table 2.

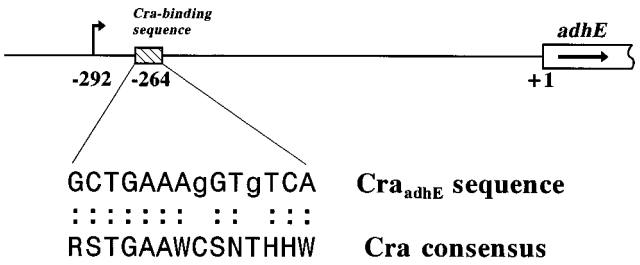

FIG. 2. Schematic representation of the putative Cra-binding sequence in relation to the *adhE* coding sequence and the −292 transcription start. The −264 position corresponds to the G nucleotide at the 5′ end of the Cra-binding motif of *adhE*. R is G or A; S is C or G; W is A or T; H is A, T, or C; and N is A, C, G, or T.

the mini-Tn*10kan* insertions into ECL2171/F′104, and the results were comparable to those obtained with ECL2171 (F^-) (data not shown). This indicated that the gene disrupted by the mini-Tn*10kan* transposon is not essential, and therefore, F′104 was omitted from later experiments. Two independent chromosomal insertion mutants, ECL2263 and ECL2252, with mini-Tn*10kan* linked to *zac-3051*::Tn*10* were further analyzed to reveal the nature of the regulatory element.

Sequencing of the mutated gene. *Pst*I fragments of ECL2263 and ECL2252 chromosomes containing the mini-Tn*10kan* insertions were cloned into the *Pst*I site of pBlueScript II KS(−), resulting in plasmids pAM34 and pAM43, respectively. Sequence analysis of the DNA fragments flanking the mini-Tn*10kan* insertion showed that both plasmids contained the same target gene, *cra*, involved in cyclic AMP (cAMP)-cAMP receptor protein (CRP)-independent global modulation of carbon flow (22, 25). The mini-Tn*10kan* insertion sites were located at positions +34 for pAM34 and +959 for pAM43 (the +1 nucleotide is the first guanidine in the starting GTG codon of *cra*) (Fig. 1A). Both plasmids contained the same single missense mutation, G(+446)→A, in the *cra* gene resulting in the amino acid substitution Arg148→His. This mutated gene was designated *cra**.

Phenotypic characterization of *cra* null and cra* mutations. The *trans*-regulatory effects of the *cra* mutations on the transcription of *adhE* were studied by assaying the activity levels of AdhE and LacZ in *cra*$^+$, *cra**, and *cra* null backgrounds (Fig. 1). As expected, the *cra** mutation repressed *adhE* and *adhE′-lacZ* expression under both anaerobic and aerobic growth conditions. Inactivation of *cra*, on the other hand, resulted in elevated levels of *adhE* and *adhE′-lacZ* expression under those growth conditions, suggesting that Cra acts as a repressor of *adhE*.

A recent compilation of data on Cra-mediated gene regulation indicates that binding of Cra upstream from a promoter activates gene transcription, whereas downstream binding causes gene repression (22). We found a DNA sequence similar to the consensus for Cra binding downstream from the *adhE* promoter (Fig. 2), consistent with *adhE* repression by Cra. This putative Cra-binding motif, however, differed from the Cra consensus by two conserved nucleotides. Therefore, DNA-binding studies in vitro were essential to confirm Cra interaction with the *adhE* promoter region.

In vitro studies of Cra and Cra* function. Protein binding to the *adhE* promoter was studied by a band shift assay. The 6×His-tagged Cra and Cra* proteins were overproduced and purified by one-step affinity chromotography. It is unlikely that the histidine tag at the N terminus of the purified proteins interferes with their biochemical function, since the ECL2166 (Cra$^+$)/pAM46-3 merodiploid producing 6×His-Cra* displayed the Cra* (or AdhE-down) phenotype, as judged by the impaired ability of this strain to grow anaerobically on glucose- or lactose-minimal agar (data not shown). For convenience, we will refer to the purified proteins simply as Cra and Cra*. A protein-binding assay was carried out on a 350-bp DNA fragment containing the *adhE* promoter. A 320-bp DNA fragment with two Cra-binding sites downstream from the *fruB* promoter was used as a positive control, and a 300-bp internal fragment of *cra* lacking the Cra-binding consensus was used as a negative control. As expected, both Cra and Cra* bound to the *adhE* promoter fragment, resulting in a single band shift (Fig. 3). A double band shift was observed on the *fruB* promoter fragment, consistent with the presence of two Cra-binding sites. No binding was observed, even at the highest concentrations of proteins, on the internal *cra* DNA fragment. These results suggested that Cra does bind directly to the *adhE* promoter region. Moreover, both Cra and Cra* bound to DNA with similar affinities and both proteins bound more strongly to the *fruB* promoter fragment than to the *adhE* promoter fragment. This difference in binding could be due to the difference between the Cra-binding motifs of the *adhE* and *fruB* genes.

We also addressed the question of the mechanism by which the *cra** mutation increases the repression of *adhE* transcription. Stabilization of a repressor-DNA complex could result in stronger repression. However, comparable affinities of Cra and Cra* binding to DNA ruled out the possibility of a more stable binding of Cra* to the *adhE* promoter (Fig. 3). Another possibility is that Cra* is locked in a conformation insensitive to fructose-1-phosphate (F1P) or fructose-1,6-bisphosphate ($F16P_2$). F1P and $F16P_2$ can displace Cra from DNA in vitro at micromolar and millimolar concentrations, respectively, and are thought to be the effectors in vivo (22). To test the hypothesis of locked conformation, we examined Cra and Cra* binding to the *adhE* and *fruB* promoter fragments with or without F1P. Fructose-6-phosphate (F6P) was used as a control of F1P specificity for Cra. Indeed, F1P was able to displace Cra but not Cra* from the *adhE* and *fruB* promoters (Fig. 4). Both proteins remained bound to DNA in the presence of F6P. Glucose-6-phosphate, NAD, NADH, NADP, NADPH, and ATP also failed to interfere with the binding of Cra or Cra* to DNA even at a concentration of 5 mM (data not shown). These results suggested that the Arg148→His mutation in Cra* locks the protein in a conformation insensitive to F1P or $F16P_2$. This conclusion is supported by the slower growth of *cra** colonies than *cra*$^+$ colonies on fructose-minimal agar, in contrast to their equal growth rates on glucose-minimal agar.

DISCUSSION

Cra was originally discovered to be the repressor of the fructose operon, FruR (9, 11). The protein was subsequently shown also to regulate, in a cAMP-independent manner, other operons involved in the dissimilation of carbon compounds (22, 25). This broad role of Cra in the regulation of carbon flow has recently become more fully articulated (26). In the absence of the effectors, F1P and $F16P_2$, the Cra protein can act either as a repressor or as an activator, depending on the target promoter. Negative control is exerted by Cra on a number of operons involved in glycolysis permitting derepression of the glycolytic pathways by F1P and/or $F16P_2$ in the presence of exogenous six-carbon compounds. Positive control is exerted by Cra over a number of operons involved in gluconeogenesis, permitting induction of the gluconeogenic pathways by F1P and/or $F16P_2$ in the presence of nonfermentable compounds. Derepression of the glycolytic network and deactivation of the gluconeogenic network by the effectors of Cra also help to

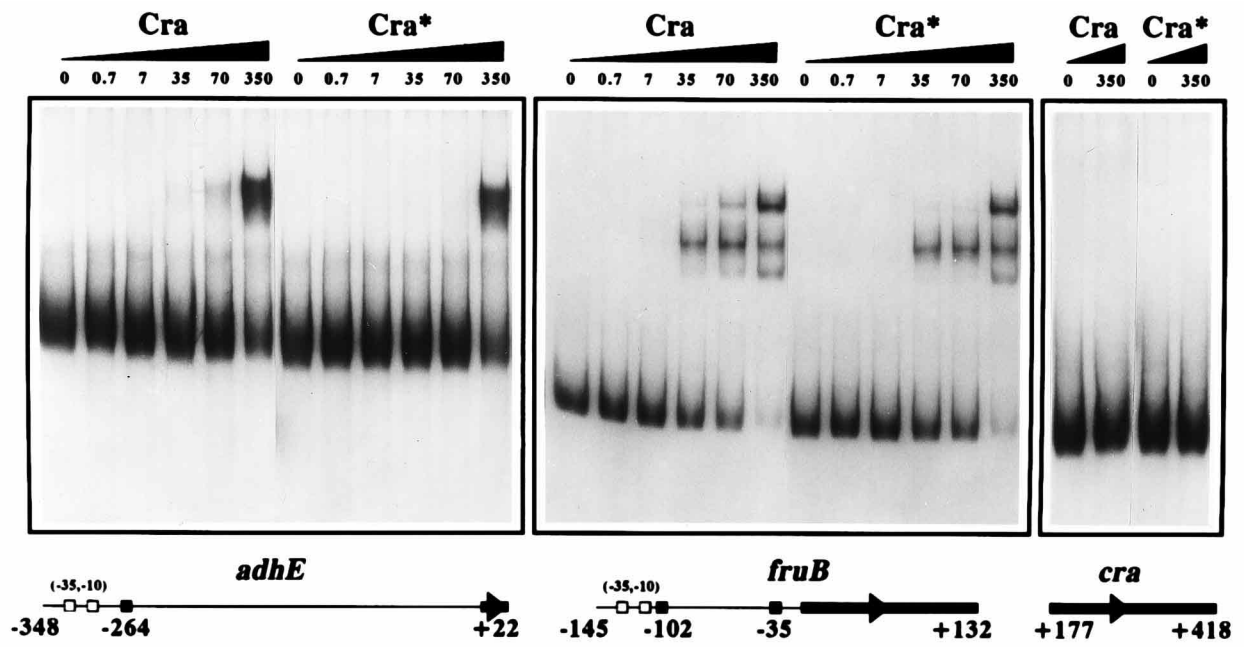

FIG. 3. Results of DNA band shift assays demonstrating Cra and Cra* binding to the *adhE* and *fruB* promoter regions but no binding to the *cra* gene region lacking the Cra-binding sequence. Nanomolar concentrations of proteins are given above the lanes. Diagrams of the PCR fragments used in this assay are shown below the panels. The empty boxes and the (−35, −10) symbols represent the promoters of the studied genes. The sections of coding sequences are drawn in bold bars with arrows. The solid boxes represent Cra-binding sequences. Coordinates of the 5′ ends of Cra-binding sequences and of 5′ and 3′ ends of PCR fragments relative to a corresponding gene are presented below each scheme. The +1 nucleotide always corresponds to the first nucleotide of the starting codon of a gene.

ensure the preferential utilization of carbon sources such as glucose and fructose.

Heretofore, it has not been suspected that regulation by Cra extended also to operons involved in terminal fermentation pathways, e.g., reduction of acetyl coenzyme A into acetaldehyde and then into ethanol by AdhE. Our results showed that Cra repressed *adhE* expression 1.5-fold during anaerobic growth on LB-glucose and 4-fold during aerobic growth. This regulatory range is consistent with the typical two- to fourfold magnitude of a Cra-mediated repression-activation (4, 27). It would not be surprising if additional Cra-regulated fermentation pathways were identified.

The Cra protein binds to a specific DNA sequence (23), the consensus of which was successively refined with studies of additional Cra-controlled promoters (7, 22). The position of this sequence relative to a promoter determines Cra function: binding sites upstream from the promoter for activation and binding sites downstream from the promoter for repression (26). A single Cra-binding site was found downstream from the *adhE* promoter, consistent with the derepression observed in

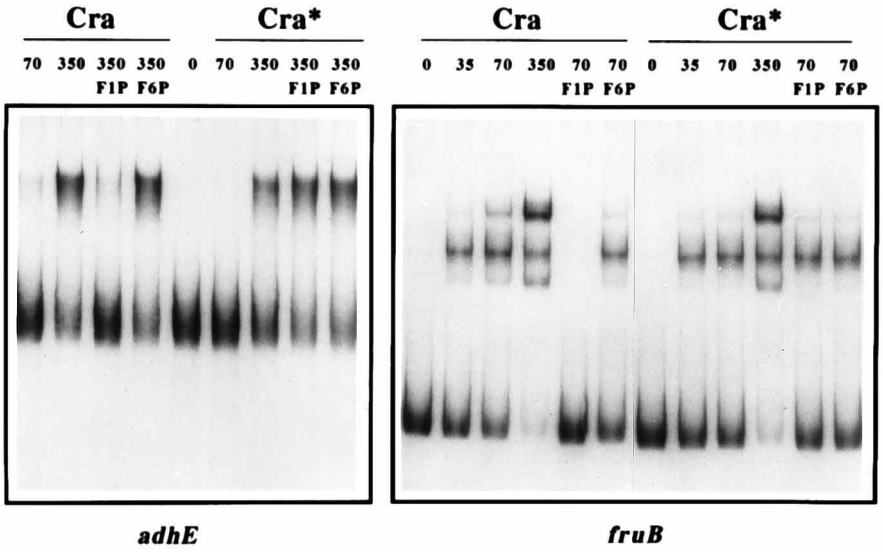

FIG. 4. Results of a DNA band shift assay representing Cra and Cra* binding to the *adhE* and *fruB* promoter regions. DNA fragments are the same as those described in the legend to Fig. 3. F1P and F6P were added at final concentrations of 100 μM.

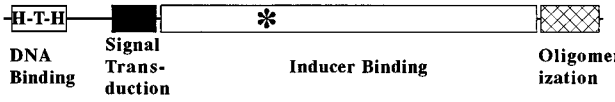

FIG. 5. Schematic representation of putative functional domains of the Cra protein. The domains were derived by alignment of the Cra and LacI protein sequences (11). The asterisk marks the location of the Cra* mutation. H-T-H, helix-turn-helix domain.

the *cra* null mutant. The in vitro DNA retardation assay showed that Cra binds to a region on the 5′ side of the ORF, as predicted by the consensus sequence. Affinity of Cra binding to the *adhE* promoter region was 5- to 10-fold lower than that for the *fruB* promoter region. This may explain the relatively modest 1.5- to 4-fold effect of *adhE* repression by Cra observed in our study, in contrast to the 20-fold repression of the *fru* operon (9).

The mutation G(+446)→A in the coding sequence of *cra** resulted in the Arg148→His replacement. No difference, however, was observed in the affinity of Cra and Cra* binding to the *adhE* or *fruB* promoter region. The wild-type Cra protein binds to DNA as a homotetramer and is readily displaced from DNA in vitro by micromolar concentrations of F1P and millimolar concentrations of $F16P_2$ (23), leading to derepression of negatively controlled promoters and deactivation of positively controlled promoters. Our results indicated that the enhanced *adhE* repression in the mutant strain was due to the inability of the effectors to displace Cra* from DNA because the protein was locked in the binding conformation. It is noteworthy that the amino acid substitution occurred in the putative inducer binding domain (Fig. 5). The locked conformation of Cra* could also explain the dominance of the *cra** mutation over the cra^+ allele. How this mutation affects formation of homo- and heterotetramers and their biological activities remains to be elucidated.

The stronger repression of *adhE* by Cra under aerobic than anaerobic growth conditions gives the appearance of a redox control, but in fact, this difference in gene expression may reflect F1P and $F16P_2$ levels. What should be emphasized is that in a *cra* null mutant, *adhE* expression remained several-fold higher anaerobically than aerobically. Although the true respiratory transcriptional regulator remains to be identified, the work described in this study should constitute a further step toward a holistic understanding of *adhE* regulation.

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