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Catabolite Gene Activator Protein and Integration Host Factor Act in Concert To Regulate *tdc* Operon Expression in *Escherichia coli*

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Anaerobic expression of the *tdcABC* operon of *Escherichia coli* requires cyclic AMP and the catabolite gene activator protein (CAP). Purified CAP binds to a 30-bp sequence in the *tdc* promoter between positions −55 and −26, and a mutant CAP site with base substitutions at positions −48, −47, and −45 failed to bind CAP and also drastically reduced the β-galactosidase expression from a *tdcB'-'lacZ* fusion plasmid. Recently, we showed that efficient expression of the *tdc* operon also requires a functional integration host factor (IHF) and an IHF-binding site in the *tdc* promoter between positions −118 and −88. The levels of β-galactosidase activity from the *tdcB'-'lacZ* fusion plasmids were also reduced in an IHF-deficient strain with the wild-type or mutant plasmid CAP sequence. In vitro footprinting experiments revealed that CAP and IHF occupy their specific binding sites on *tdc* DNA when they are present separately or together. These regulatory proteins also induced significant bending of the *tdc* promoter DNA. Our results suggest that CAP and IHF act in concert as positive transcription factors for *tdc* operon expression in vivo.

A 6.3-kb *Escherichia coli* DNA fragment that was cloned and sequenced in our laboratory contains, among other genes, the biodegradative threonine dehydratase operon, *tdcABC* (5, 8, 9). *tdcB* is the structural gene for threonine dehydratase (EC 4.2.1.16) (5), which catalyzes the dehydration of L-serine and L-threonine to ammonia and the corresponding α-keto acids. The *tdcC* gene encodes a novel membrane-associated L-threonine–L-serine permease (29). The product of *tdcA*, which has the helix-turn-helix motif of typical DNA-binding proteins, appears to be involved in positive regulation of the *tdc* operon (22). A separate regulatory gene, *tdcR*, which is located immediately upstream from *tdcABC* in the opposite transcriptional orientation, is also required for maximal expression of the *tdc* genes (27). Recently, we reported (33) that expression of β-galactosidase from a *tdcB'-'lacZ* fusion plasmid was drastically reduced in an integration host factor (IHF)-deficient strain and that purified IHF bound to a region around position −100 on the *tdc* promoter for positive activation of the *tdc* operon. These results clearly indicate that the *tdc* operon is a highly regulated system for efficient transport and metabolism of the hydroxy amino acids threonine and serine in *E. coli*.

In addition to the findings mentioned above, previous work (11, 19, 28) has shown that threonine dehydratase is induced under anaerobic conditions in amino acid-rich media, requires cyclic AMP (cAMP) for its synthesis, and is sensitive to catabolite repression by glucose and other intermediary metabolites. Although the mechanism by which cAMP regulates enzyme synthesis in vivo remains unexplored, it is generally assumed that, like other cAMP-dependent systems, transcription from the *tdc* promoter is subject to stimulation by the cAMP-catabolite gene activator protein (CAP) complex. This notion has been further strengthened by nucleotide sequence data (8, 26) for the 233-bp intercistronic region between *tdcR* and *tdcA* harboring the respective promoters. The *tdc* promoter DNA contains a sequence with a TGTGA motif which exhibits homology to the consensus binding site TGTGA-N_6-TCACA for CAP (6) at around position −40. In this paper we report that the cAMP-CAP complex indeed acts as a positive transcription factor for the *tdc* operon in combination with IHF. Lack of either protein resulted in almost complete loss of *tdc* expression. In vitro gel shift and footprinting experiments showed that CAP and IHF bind simultaneously to the *tdc* DNA and occupy their specific binding sites on the *tdc* promoter. These regulatory proteins also induce significant bending of the *tdc* promoter DNA.

MATERIALS AND METHODS

Materials. Tryptone, yeast extract, and other medium components were purchased from Difco Laboratories, Detroit, Mich. DNA polymerase (Klenow), T4 DNA ligase, and restriction enzymes were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis., or Bethesda Research Laboratories, Gaithersburg, Md. T7 DNA polymerase (Sequenase) and *Taq* DNA polymerase were supplied by United States Biochemicals, Cleveland, Ohio, and Perkin-Elmer Cetus, Norwalk, Conn., respectively. α-^{35}S-dATP (specific activity, >1,000 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. All other chemicals were of reagent grade. Purified IHF (18) was a generous gift from Howard Nash.

Bacterial strains and culture conditions. *E. coli* MC4100 [F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 deoC1 relA1 rbsR ptsF25 flbB5310*] (29) was used for propagation and maintenance of plasmids, and strain DH5αF' [φ80 *d lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*] (29) was used for growing bacterial phages M13mp18 and M13mp19. Strain HP4110 [MC4100 *himA* (P1) (Tet^r)] has been described previously

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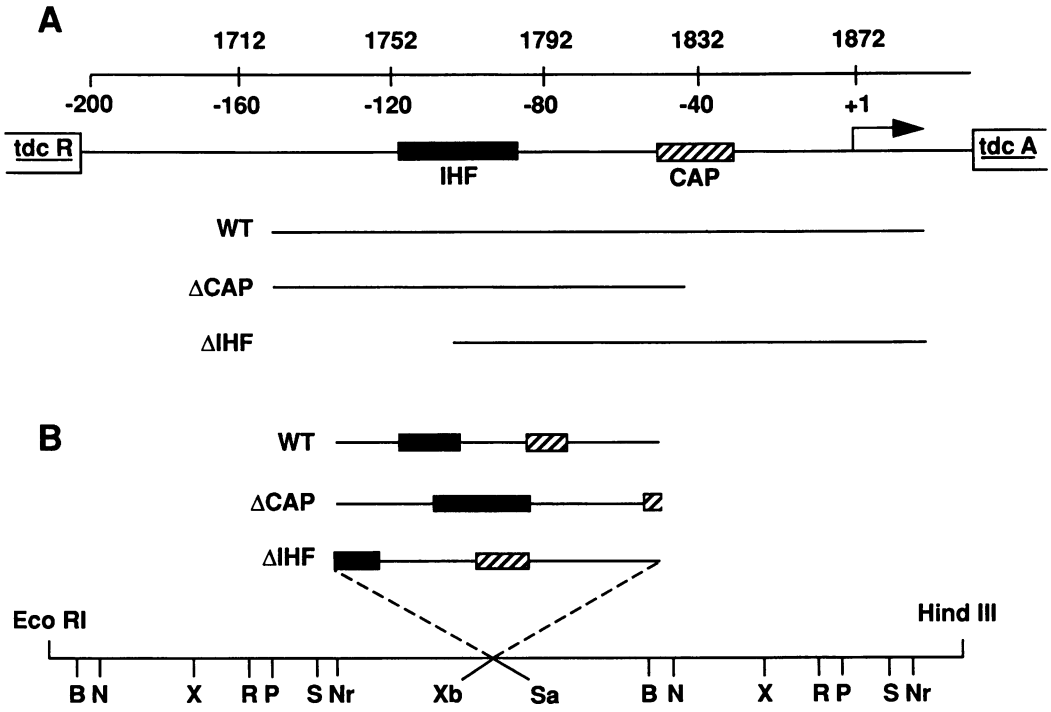

FIG. 1. Schematic diagrams of the untranslated region between *tdcR* and *tdcA* (A) and plasmid pBend2 with *tdc* DNA insertions (B). (A) The nucleotide numbers above the top line were assigned beginning with the endpoint of 6.3-kb plasmid pEC61 DNA (26). The numbers below the line were assigned with reference to the transcription start site (+1) (arrow) of the *tdc* operon. The solid and cross-hatched boxes represent the IHF-binding site (33) and a sequence exhibiting homology with the consensus CAP-binding sequence, respectively. The DNA fragments WT, ΔCAP, and ΔIHF show the DNA segments harboring the intact binding sites for both IHF and CAP, IHF only, and CAP only, respectively. (B) The same three fragments were cloned into the *Xba*I-*Sal*I cloning sites in plasmid pBend2 (solid line at the bottom). Abbreviations for the restriction enzyme sites in pBend2: B, *Bgl*II; N, *Nhe*I; X, *Xho*I; R, *Eco*RV; P, *Pvu*II; S, *Stu*I; Nr, *Nru*I; Xb, *Xba*I; Sa, *Sal*I.

(33). Strain SA2600 (F⁻ *his relA*) and strain SA2777, a *crp* Cm^r derivative of strain SA2600, were provided by Sankar Adhya, National Institutes of Health. Strain CJ236 [*dut-1 ung-1 thi-1 relA1* (pCJ105)(Cm^r)] (15) was used for generating uracil-substituted DNA.

Bacteria were grown in Luria-Bertani medium (17) supplemented with appropriate antibiotics, as needed, at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; tetracycline, 15 μg/ml; and streptomycin, 25 μg/ml.

For enzyme induction, cells were grown aerobically at 37°C for approximately 12 h in Luria-Bertani medium, washed, and then resuspended in tryptone yeast extract (TYE) medium containing 2% tryptone and 1% yeast extract supplemented with salts and pyridoxine hydrochloride (11) or in synthetic H4 medium of Hobert and Datta (11), which contained four amino acids (threonine, serine, valine, and isoleucine) plus cAMP and fumarate. After anaerobic incubation in still cultures at 37°C for 8 to 12 h, cells were harvested, washed in 0.1 M potassium phosphate buffer (pH 8.0) containing 3 mM AMP and 1 mM dithiothreitol, and then suspended in the same buffer for the enzyme assay.

Plasmids. pSH241 (*tdcR*$^+$ *tdcB'-'lacZ*) and pYW75, a derivative of pSH241 with base substitutions in the plasmid IHF sequence in the *tdc* promoter (*tdcP*), have been described previously (27, 33). pYW74, which had base substitutions in the putative CAP-binding site in *tdcP*, was constructed by site-directed mutagenesis of pSH241 (see below). Plasmids pYW65 and pYW66, the sources of DNA fragments for gel retardation and footprinting experiments, were constructed from pSH241 and pYW74, respectively, as follows: a 169-bp DNA fragment (nucleotides [nt] 1722 to 1890 [26]), which was amplified by using the polymerase chain reaction (PCR) technique (23), primers F40 and R65 (see below), and pSH 241 (or pYW74) DNA as the template, was treated with T4 DNA polymerase and four deoxynucleoside triphosphates and then cloned into the *Sma*I cloning site of pUC19 (16). The orientations of both pYW65 and pYW66 were such that the putative CAP-binding site was closer to the *Eco*RI site on the vector. For DNA bending experiments, the following three DNA fragments were cloned into the *Xba*I (blunt)-*Sal*I cloning sites of plasmid pBend2 (14) (a gift from S. Adhya) (Fig. 1): (i) a 202-bp *Eco*RI (blunt)-*Sal*I fragment from pYW65 (nt 1722 to 1890 plus flanking vector sequences) containing both IHF- and CAP-binding sites, yielding pYW100; (ii) a 123-bp *Afl*II (blunt)-*Sal*I fragment from pYW66 (nt 1722 to 1829 plus flanking vector sequence at the *Sal*I end) containing an IHF-binding site but only one-half of the putative CAP-binding site, yielding pYW88; and (iii) a purified 126-bp PCR-amplified DNA fragment (nt 1765 to 1890) containing the putative CAP-binding site and one-half of the IHF-binding site, yielding pYW99 (the last fragment was generated by using primers F75 and R65 [see below]) and pYW75 template DNA and then a filling-in reaction with DNA polymerase and digestion with *Sal*I). Plasmid pHA7 (a gift from Stephanie Shanblatt, Michigan State University) contained a cloned *crp* gene of *E. coli* in pBR322 (1).

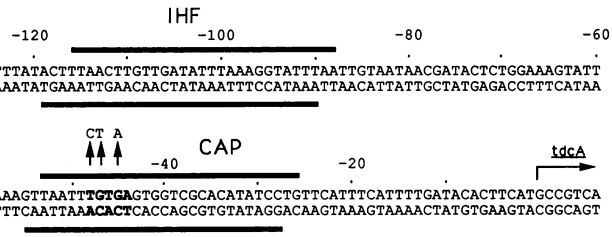

FIG. 2. Nucleotide sequence surrounding the IHF- and CAP-binding sites and base substitutions within the CAP TGTGA motif (boldface letters). The overlined and underlined areas represent sequences of noncoding and coding strands, respectively, protected by IHF and CAP (designated IHF and CAP, respectively) against DNase I digestion (data from the gels shown in Fig. 4 and 5 and from reference 33). The transcription start site of *tdcA* is indicated by the arrow labeled *tdcA*.

PCR. The PCR (23) was performed by using the protocol supplied by Perkin-Elmer Cetus. Double-stranded DNA fragments spanning various regions of the *tdc* promoter were amplified in 100-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, four deoxynucleoside triphosphates (each at a concentration of 200 μM), primers (at concentrations of 300 pM), 200 ng of an appropriate template DNA, and 2 U of *Taq* DNA polymerase. After 30 cycles of amplification (each cycle consisted of a 1-min denaturation step at 94°C, a 2-min annealing step at 55°C, and a 2-min polymerization step at 72°C), the products of the expected sizes were separated by 2% agarose gel electrophoresis and then extracted with a Qiagen gel extraction kit. The fragments were sequenced to verify their nucleotide sequences.

The following oligonucleotides were used as primers for the PCR: (i) F40, 5′ TGACAAAAATCAGGGTT 3′ from nt 1722 to 1738 (26); (ii) F75, 5′ CTTTAGCTTGTCGACATT TAA 3′ from nt 1755 to 1775 (26), with base substitutions A → G at nt 1760 and T → C at nt 1766 and at nt 1769 that yielded a new *Sal*I site (see reference 33); and (iii) R65, 5′ AATTACCTCATTGACGGCAT 3′, which was complementary to the sequence from nt 1871 to 1890 (26). The primers were synthesized by the University of Michigan Biomedical Research Core Facility.

Oligonucleotide-directed mutagenesis of the CAP-binding site. The putative CAP-binding site in the *tdc* promoter was mutagenized by using the method of Kunkel et al. (15), as described previously (33). Briefly, a 1.1-kb *Eco*RI-*Hin*dIII fragment from pSH241 was cloned into M13mp19, and single-stranded uracil-containing DNA was isolated by using strain CJ236. A synthetic 5′-phosphorylated primer, 5′ TAATTCTTAAGTGGTCGCACA 3′, from nt −53 to −33 (Fig. 2), with base substitutions T → C at nt −48, G → T at nt −47, and G → A at nt −45 that created a new *Afl*II site, was annealed to the single-stranded DNA and then extended with T7 DNA polymerase. The mutant clones were screened by digestion with *Afl*II, and the predicted mutation was confirmed (data not shown) by DNA sequencing with a Sequenase kit supplied by United States Biochemicals. Finally, the mutant 1.1-kb *Eco*RI-*Hin*dIII fragment was excised from the replicative form of phage M13mp19 and was cloned into the corresponding *Eco*RI and *Hin*dIII sites of pSH241 to yield pYW74.

Gel mobility shift assay. For the gel mobility shift assay (10), the 208-bp *Eco*RI-*Pst*I fragments from pYW65 (wild-type DNA) and pYW66 (mutant CAP DNA) were uniquely end labeled by filling in the 3′ recessed end with the Klenow fragment of DNA polymerase and α-^{35}S-dATP (24). Portions (approximately 0.05 pmol) of end-labeled fragments were incubated separately for 15 min at 22°C with various concentrations of CAP and/or IHF in a binding buffer (final volume, 20 μl) containing 12 mM Tris-HCl (pH 8.0), 5 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 6% glycerol, 0.1 mM dithiothreitol, 150 μg of nuclease-free bovine serum albumin per ml of reaction mixture, 0.5 μg of denatured, sonicated herring sperm DNA per ml, and, in some cases, 20 μM cAMP. Following incubation, the mixtures were analyzed on 4% polyacrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid, 1 mM EDTA; pH 8.3) supplemented with 20 μM cAMP. During the gel run, 20 μM cAMP was added at 30-min intervals to replenish the buffer. The gels were dried and then subjected to autoradiography (24).

To analyze CAP- and/or IHF-induced DNA bending, three plasmids, pYW88 (Δ CAP, with an IHF-binding site), pYW99 (Δ IHF, with a CAP-binding site), and pYW100 (WT, with both IHF- and CAP-binding sites) (Fig. 1) were digested separately with individual restriction enzymes (*Bgl*II, *Nhe*I, *Xho*I, *Eco*RV, *Pvu*II, *Stu*I, and *Nru*I) to generate DNA fragments which had identical sizes but the protein-binding sites that were present in circularly permuted positions along the length of the DNA (14). The purified fragments were then labeled by the replacement reaction (24), using T4 DNA polymerase and α-^{35}S-dATP, and incubated with CAP and/or IHF prior to gel electrophoresis and autoradiography as described above.

DNase I footprinting. Footprinting experiments were performed essentially as described by Hudson and Fried (13), with minor modifications. The DNA fragments which we used (0.1 pmol) and the binding reaction conditions with CAP and/or IHF were identical to those described above for the gel mobility shift assay. After incubation, the mixtures were treated with 5 ng of freshly diluted DNase I for 90 s, and the reactions were terminated by adding 4 μl of a stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The samples were denatured at 90°C for 5 min and then electrophoresed through 8% polyacrylamide–7 M urea sequencing gels in TBE buffer. The gels were fixed and dried for autoradiography (24). The A > C sequencing reaction for the same DNA fragments was performed by using an alternate method of the Maxam-Gilbert sequencing protocol (2).

Purification of CAP. Multicopy plasmid pHA7 containing the cloned *crp* gene of *E. coli* (1) in strain SA500 (F^- Str^r *his relA*) was used to purify CAP by the method of Boone and Wilcox (3). The purified protein exhibited a molecular weight of 22,000 during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was in agreement with the previously published value, and was judged to be more than 95% pure. The preparation was free of RNase and DNase activities.

Enzyme assay. The threonine dehydratase activity of toluene-treated cells was determined colorimetrically as described previously (11). Specific activity was expressed as nanomoles of α-ketobutyrate formed per minute per milligram of protein. β-Galactosidase activity in sodium dodecyl sulfate-chloroform-permeabilized cells was measured by using the procedure of Miller (17), and specific activity was expressed in Miller units.

Other methods. The general procedures which we used for DNA manipulations, gel electrophoresis, and end labeling were those described by Sambrook et al. (24). Restriction enzyme digestions of DNA were carried out by using the manufacturers' specifications.

TABLE 1. Effect of *crp* mutation on *tdc* expression[a]

Strain (plasmid)	Relevant genotype	Sp act in TYE medium	
		TdcB	LacZ
SA2600	*crp*$^+$	1,238	
SA2777	*crp*	20	
SA2777 (pBR 322)	*crp*/vector	30	
SA2777 (pHA7)	*crp*/*crp*$^+$	1,910	
SA2600 (pMC 1403)	*crp*$^+$/vector		212
SA2600 (pSH 241)	*crp*$^+$/*tdcB'-'lacZ*		3,637
SA2777 (pMC 1403)	*crp*/vector		133
SA2777 (pSH 241)	*crp*/*tdcB'-'lacZ*		157

[a] For information concerning the culture conditions, enzyme assay, and specific activity measurements, see Materials and Methods.

RESULTS

Requirement of CAP for *tdc* expression. Several lines of evidence (11, 19, 28) have indicated that anaerobic expression of the *tdcB* gene requires cAMP. To directly test the proposition that cAMP-mediated gene expression requires the participation of CAP, we measured threonine dehydratase activity from the chromosomally encoded *tdcB* and β-galactosidase activity from a *tdcB'-'lacZ* fusion plasmid (pSH241) in *crp*$^+$ and *crp* strains. Wild-type *crp*$^+$ strain SA2600 produced a high level of dehydratase activity when it was incubated anaerobically in TYE medium (Table 1). Under the same incubation conditions, strain SA2600 transformed with pSH241 had a 17-fold-higher level of β-galactosidase than the level expressed from the chromosomally encoded *lacZ* gene in strain SA2600. In contrast, strain SA2777, which is a *crp* derivative of strain SA2600 and lacks the CAP, had very little dehydratase activity; the β-galactosidase activity from pSH241 was also drastically reduced in the *crp* strain. In a separate experiment (data not shown), strain SA2777(pSH241) produced less than 5% of the LacZ activity observed with strain SA2600(pSH241) in synthetic H4 medium, which contained 4 mM cAMP. The requirement for CAP for enzyme synthesis was further confirmed by transforming strain SA2777 with plasmid pHA7 harboring the cloned *E. coli crp* gene; in the CAP-deficient host, dehydratase activity was completely restored by the plasmid-encoded CAP. Thus, both cAMP and CAP are essential for *tdc* gene expression in vivo.

Involvement of CAP-binding site in *tdc* promoter. As mentioned above, the nucleotide sequence of the *tdc* promoter region contains a CAP consensus-like sequence between positions −48 and −32 (Fig. 2 with the TGTGA motif (6). To test the possibility that CAP might bind to this site to directly promote *tdc* transcription, we introduced a mutation in this region in *tdcB'-'lacZ* fusion plasmid pSH241 by substituting three bases (T → C, G → T, and G → A at positions −48, −47, and −45, respectively), which yielded pYW74 (Fig. 2). Previous studies indicated that the individual base substitutions G → T and G → A within the TGTGA motif in the *lac* promoter resulted in either low or no binding of CAP (25); the additional base change T → C at position −48 was included to generate a new *Afl*II site to facilitate screening of the mutant DNA. Assays of β-galactosidase activity in Lac$^-$ strain MC4100 transformed with mutant plasmid pYW74 and incubated anaerobically in TYE medium and H4 medium revealed less than 5% of the enzyme activity observed with strain MC4100(pSH241), which harbors the wild-type CAP sequence (Table 2). Thus, the reduced levels of expression of *tdc* in the absence of CAP (as in the *crp* strain [Table 1]) and in the presence of a mutant CAP-binding site in a *crp*$^+$ background [strain MC4100(pYW74)] (Table 2) clearly suggest that the CAP functions as a transcriptional activator when it is bound to the *tdc* promoter at around position −40.

Effect of IHF. We showed previously (33) that efficient expression of the *tdc* operon in vivo requires a functional IHF protein and an IHF-binding site around position −100 in the *tdc* promoter. In view of the absolute requirement for CAP in *tdc* transcription, we thought that it is likely that CAP and IHF act in concert to influence transcription from *tdcP*. Indeed, a comparison of β-galactosidase activities in various *tdcB'-'lacZ* fusions, which harbor the wild-type or the mutant plasmid IHF- and CAP-binding sequences, in strain MC4100 (*himA*$^+$ *crp*$^+$) revealed that a mutation in either the CAP-binding site or the IHF-binding site drastically reduced the fusion-directed LacZ activities (Table 2). Additional experiments showed that in the absence of a functional IHF protein (as in strain HP4110), very little enzyme activity was expressed from either the wild-type or the CAP mutant fusion plasmids. These data, together with the results for *crp* mutations on *tdc* genes shown in Table 1, confirm that both CAP and IHF are essential for *tdc* expression. Presumably, when these regulatory proteins are bound in tandem, they induce optimum promoter conformation in *tdcP* for binding of RNA polymerase.

CAP and IHF binding to the *tdc* promoter. Using gel retardation and DNase I footprinting, Wu and Datta (33) found that purified IHF protected a 31-bp region in *tdcP* between positions −118 and −88. We used the same tech-

TABLE 2. Requirement of CAP-binding site for TdcB-LacZ expression[a]

Strain (plasmid)	Relevant genotype	Plasmid sequence of:		LacZ sp act in:	
		IHF	CAP	TYE medium	H4 medium
MC4100 (pMC1403)	*himA*$^+$/vector			70 (1)[b]	46 (1)
MC4100 (pSH241)	*himA*$^+$/tdcB'-'-lacZ	Wild type	Wild type	5,617 (100)	5,539 (100)
MC4100 (pYW74)	*himA*$^+$/*tdcB'-'lacZ*	Wild type	Substitution	99 (2)	155 (3)
MC4100 (pYW75)	*himA*$^+$/*tdcB'-'lacZ*	Substitution	Wild type	612 (11)	243 (4)
HP4110 (pMC1403)	*himA*/vector			159 (3)	121 (2)
HP4110 (pSH241)	*himA*/*tdcB'-'lacZ*	Wild type	Wild type	411 (7)	212 (4)
HP4110 (pYW74)	*himA*/*tdcB'-'lacZ*	Wild type	Substitution	211 (4)	476 (9)
HP4110 (pYW75)	*himA*/*tdcB'-'lacZ*	Substitution	Wild type	554 (10)	506 (9)

[a] For information concerning the culture conditions, enzyme assay, and specific activity measurements, see Materials and Methods.
[b] The numbers in parentheses are percentages of the wild-type control value.

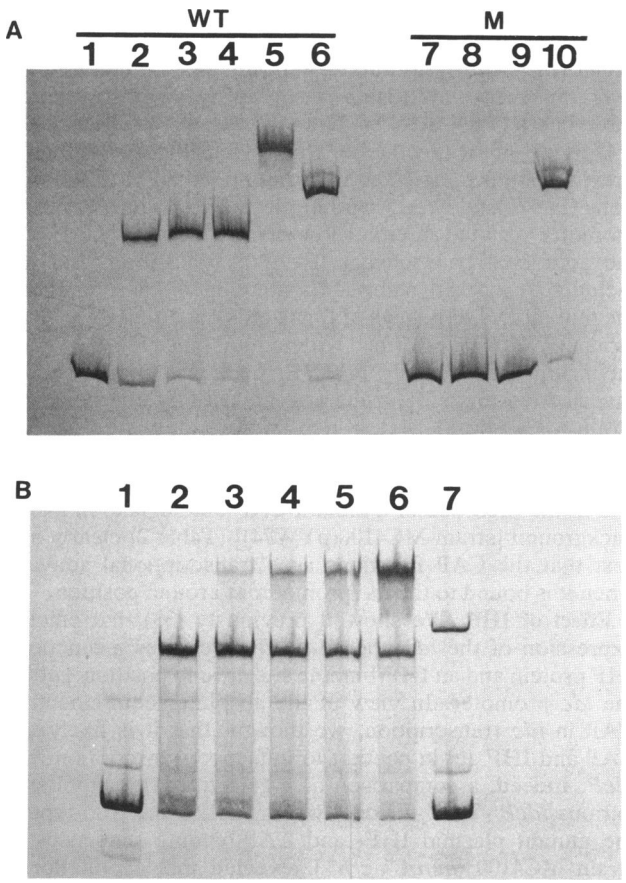

FIG. 3. Mobility shift of *tdc* promoter DNA because of binding of IHF, CAP, and IHF plus CAP. (A) The 208-bp labeled *Eco*RI-*Pst*I fragments from pYW65 (WT) and pYW66 (M) were incubated with various concentrations of purified CAP, IHF, or IHF plus CAP in the presence of 20 μM cAMP and then electrophoresed on a 4% native polyacrylamide gel. The mobilities of free DNA and DNA-protein complexes were visualized by autoradiography. Lanes 1 through 6, wild-type DNA from pYW65 harboring both IHF and CAP sites; lanes 7 through 10, mutant DNA from pYW66 containing the mutant CAP site. Lane 1, free DNA; lanes 2 through 4 and 7 through 9, 50, 100, and 200 ng of CAP, respectively; lane 5, 100 ng of CAP plus 20 ng of IHF; lanes 6 and 10, 20 ng of IHF. (B) The Wild-type DNA from pYW65 was incubated separately with various concentrations of purified IHF in the presence or absence of 50 ng of purified CAP plus 20 μM cAMP prior to gel electrophoresis and autoradiography. Lane 1, free DNA; lane 2, 50 ng of CAP; lanes 3 through 6, 1, 2, 5, and 10 ng of IHF, respectively, with a constant level (50 ng) of CAP; lane 7, 10 ng of IHF. The very faint bands in all of the lanes appear to be minor contaminants.

niques to localize the CAP-binding site on the *tdc* promoter and to answer the question of whether binding of CAP (or IHF) to its own site alters the binding pattern of the second protein. A 208-bp *Eco*RI-*Pst*I fragment from pYW65 containing the wild-type promoter sequence (from nt 1722 to 1890) migrated slowly during gel electrophoresis when the preparations were incubated with increasing concentrations of CAP plus 20 μM cAMP (Fig. 3A, lanes 2 through 4), whereas the same fragment from pYW66, which had a mutation in the CAP consensus site, revealed no shift in mobility under identical incubation conditions (lanes 7 through 9). No mobility shift of the wild-type promoter DNA was detected in the absence of cAMP (Fig. 5; data not shown). Thus, the cAMP-CAP complex appears to bind to the *tdc* promoter at the CAP consensus site.

Figure 3A also shows that IHF alone caused identical retardation in the mobilities of both the wild-type and CAP mutant DNAs (lanes 6 and 10, respectively). Interestingly, binding of CAP and IHF individually to the wild-type promoter produced different extents of retardation (Fig. 3A, lanes 2 through 4 and 6), and when CAP and IHF were present together, the mobility was reduced more (lane 5) than the mobility shifts seen with either protein alone.

The results of a more detailed experiment on the effects of both IHF and CAP on the DNA mobility shift are shown in Fig. 3B. With increasing concentrations of IHF (1 to 10 ng) at fixed levels of CAP (50 ng) and cAMP (20 μM), there was an increasing amount of the slowest-migrating band (Fig. 3B, lanes 3 through 6) and a concomitant decrease in the amount of material migrating faster through the gel. From these results and the results of control experiments with CAP and IHF alone (lanes 2 and 7, respectively), we concluded that the slowest-migrating DNA had bound CAP and IHF (see below). These data suggest that the two transcription factors can bind to *tdcP* DNA individually as well as together and that binding of one does not appear to interfere with binding of the other. The DNase I footprinting experiments described below showed that IHF and CAP occupy their own unique binding sites whether they are added separately or together.

The uniquely end-labeled noncoding and coding strands of wild-type promoter DNA (from pYW65), which were incubated separately with increasing concentrations of CAP in the presence of cAMP prior to DNase I digestion, protected DNA segments between positions −53 and −26 (Fig. 4A, lanes 3 through 5) and between positions −55 and −28 (Fig. 4B, lanes 3 through 5), respectively. Incubation of the same wild-type promoter DNA with cAMP alone (Fig. 4A, lane 2 and Fig. 4B, lane 2) or with CAP alone (Fig. 4A, lane 6 and Fig. 4B, lane 6) resulted in no protection against DNase I treatment. Under identical incubation conditions CAP plus cAMP or CAP alone or cAMP alone did not protect either strand when the CAP mutant promoter from pYW66 was used for footprinting (Fig. 4A and B, lanes 7 through 11). Thus, the CAP-cAMP complex appears to bind specifically to a 30-bp sequence of the *tdc* promoter DNA, which encompasses the TGTGA motif of CAP. The size of the *tdcP* segment protected by CAP is similar to the sizes observed (between 25 and 30 bp) in several other CAP-dependent promoters of *E. coli* (6).

The footprints of CAP and IHF added in various combinations with and without cAMP to the wild-type *tdc* promoter DNA with end-labeled noncoding and coding strands are shown in Fig. 5A and B, respectively. CAP plus cAMP (Fig. 5A, lane 4, and Fig. 5B, lane 4) and IHF alone (Fig. 5A, lane 6, and Fig. 5B, lane 6) protected two unique segments in both strands (CAP bound between positions −55 and −26 and IHF bound between positions −119 and −88). These same sequences were found to be protected when they were footprinted with individual proteins (33) (Fig. 4). When CAP and IHF were present together (Fig. 5A, lane 5, and Fig. 5B, lane 5), they protected the same unique sequences as they did when they were added separately. Additional control incubation mixtures of *tdc* DNA with the end-labeled noncoding strand (Fig. 5A) and coding strand (Fig. 5B) before DNase I digestion produced the following results: CAP alone did not protect either strand (lanes 3); IHF plus CAP without cAMP and IHF plus cAMP without CAP protected the

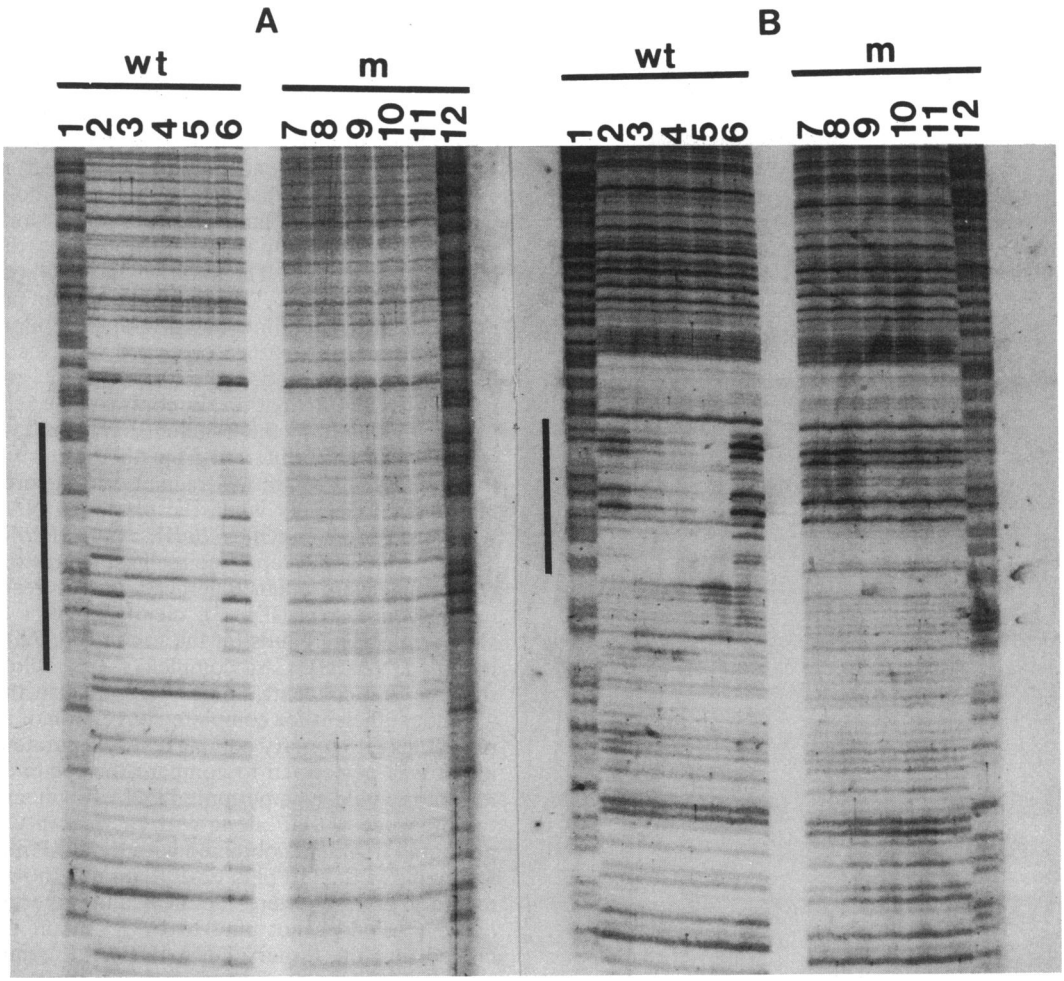

FIG. 4. Footprints of CAP on *tdc* promoter DNA. Samples of uniquely end-labeled wild-type (wt) and CAP mutant (m) DNA fragments were incubated separately with different concentrations of purified CAP with or without cAMP before DNase I digestion. For experimental details, see Materials and Methods. (A) Noncoding strands from wild-type promoter (lanes 1 through 6) and from mutant promoter (lanes 7 through 12). (B) Coding strands from wild-type promoter (lanes 1 through 6) and from mutant promoter (lanes 7 through 12). In both panel A and panel B, lanes 1 and 12 contained A > C sequencing ladders of the corresponding strands of wild-type and mutant DNA fragments. Lanes 2 and 11, free DNA; lanes 3 and 10, 75 ng of CAP; lanes 4 and 9, 150 ng of CAP; lanes 5 and 8, 300 ng of CAP; lanes 6 and 7, 300 ng of CAP. Lanes 2 through 5 and 8 through 11 contained 20 μM cAMP; no cAMP was present in lanes 6 and 7. The regions protected by cAMP-CAP are indicated by vertical bars.

sequence unique for IHF (lanes 7 and 8); and no protection was observed in the absence of CAP and IHF (lanes 2). We believe that these results indicate that CAP and IHF have unique binding sites on *tdcP* DNA and that occupancy of the CAP site by the cAMP-CAP complex does not appear to influence the binding pattern of IHF at its own site and vice versa.

Bending of the *tdcP* region by CAP and IHF. How do the regulatory proteins discussed above influence *tdc* expression when they are bound to two distinct regions of the promoter sequence about 60 nucleotides apart? From several recent studies, it has been firmly established that both CAP and IHF induce DNA bending near their respective target sites (21, 25, 32). Presumably, the protein-induced bends allow flexibility for protein-protein and/or protein-DNA interactions from a distance to optimize the appropriate transcriptional response.

To test the hypothesis that CAP and IHF also might bend the *tdc* promoter DNA, we inserted three different promoter DNA fragments (WT, ΔIHF, and ΔCAP [Fig. 1B]) into the pBend2 plasmid (14) and performed gel mobility shift assays as described above. Figure 6 shows the gel migration patterns of two DNA fragments (ΔCAP harboring the IHF site [Fig. 6A] and ΔIHF containing the CAP site [Fig. 6B]), which were digested individually with various restriction enzymes to generate circularly permuted fragments and then incubated separately with IHF (2 ng) and CAP (30 ng) prior to gel electrophoresis. The relative mobility shift plots of the same data (Fig. 6C and D) clearly reveal that both IHF and CAP induced significant DNA bending. As expected, the permuted fragments containing the protein-binding sites approximately in the middle of the fragment exhibited the least mobility (14, 32), and the centers of the bends corresponded to the binding sites of IHF and CAP. In contrast, the fractions of DNA molecules in these permuted fragments which did not bind proteins had identical mobilities (Fig. 6A

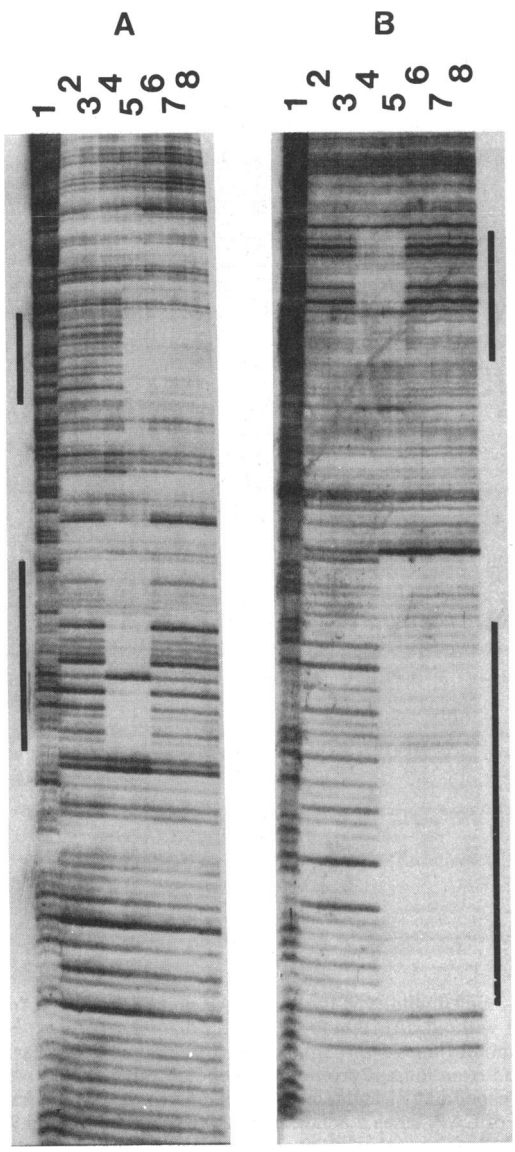

FIG. 5. Simultaneous footprints of CAP and IHF on *tdc* promoter DNA. Samples of uniquely end-labeled wild-type promoter fragments were incubated separately with various combinations of IHF (20 ng), CAP (60 ng), and cAMP (20 μM) before DNase I digestion. For experimental details, see Materials and Methods. (A) Noncoding strands. (B) Coding strands. In both panel A and panel B lane 1 contained an A > C sequencing ladder. Lane 2, free DNA; lane 3, CAP alone; lane 4, CAP plus cAMP; lane 5, CAP plus cAMP plus IHF; lane 6, IHF alone; lane 7, IHF plus CAP; lane 8, IHF plus cAMP. In panel A the short vertical bar at the top and the long vertical bar at the bottom represent DNA sequences protected by IHF and CAP, respectively. In panel B the short vertical bar at the top and the long vertical bar at the bottom represent DNA sequences protected by CAP and IHF, respectively.

and B), indicating that the promoter sequences surrounding the IHF and CAP sites do not have intrinsic bends in the absence of bound protein.

An identical gel mobility shift assay was performed with the wild-type *tdcP* (from nt 1722 to 1890) harboring the binding sites of IHF and CAP (Fig. 1). In the presence of 60 ng of CAP and a subsaturating concentration (5 ng) of IHF, two sets of bands with different mobilities were observed following gel electrophoresis (Fig. 7A); the more slowly migrating bands (at the top) corresponded to the ternary DNA-IHF-CAP complex, and the faster-migrating bands (in the middle) represented the binary complexes of DNA plus CAP (Fig. 3A). The identities of the binary and ternary DNA-protein complexes were established in a separate experiment by comparing the relative mobilities of the wild-type *tdcP* DNA incubated with CAP alone, IHF alone, and CAP plus IHF and then electrophoresed through a single polyacrylamide gel. The relative mobility patterns of the data plotted in Fig. 7B showed that the pattern for the permuted fragments incubated with CAP alone was superimposable on the pattern observed with the less retarded, faster-migrating bands when both CAP and IHF were present during incubation. In contrast, the relative mobility pattern exhibited by the fragments incubated with IHF alone was considerably different from the pattern observed with the more slowly migrating fragments in the presence of CAP plus IHF, especially when the plasmid DNA was digested with restriction enzymes *Bgl*II, *Nhe*I, *Eco*RV, and *Pst*I. These results were reproducible in several experiments and, when they were taken together with the results of the gel retardation assays (Fig. 3), clearly suggest that the more slowly migrating bands (at the top in Fig. 7A) represent the ternary DNA-IHF-CAP complexes. It should be mentioned that the low concentration of IHF used in this experiment was not sufficient for complete conversion of all of the DNA molecules into ternary complexes. A separate control experiment was performed to compare the relative mobility pattern of the wild-type permuted DNA fragments incubated in the presence of IHF alone with the mobility pattern of the permuted fragments obtained with the CAP mutant template incubated with IHF plus CAP (data not shown). These two mobility patterns were identical and superimposable. Because CAP does not bind to CAP mutant DNA, we concluded that the ternary DNA-IHF-CAP complex observed with the wild-type promoter incubated with IHF plus CAP was probably not due to protein-protein interaction between IHF and CAP and then binding of the IHF-CAP complex to the IHF-binding site on *tdcP*.

Because the IHF- and CAP-binding sites on the wild-type promoter DNA fragment used for the bending assay were situated asymmetrically with respect to the ends of the permuted fragments generated by the restriction enzymes (Fig. 1B), it was not feasible experimentally to locate the center of the bend due to binding of IHF plus CAP. Nevertheless, the relative mobility pattern of the ternary DNA-IHF-CAP complex shown in Fig. 7 clearly reveals the presence of bent DNA, and the extent of bending appears to be different from the pattern exhibited by the binary DNA-IHF complex. Additional experiments will be needed to analyze more precisely the location and extent of DNA bending and to understand the role of bent promoter DNA in *tdc* operon expression.

DISCUSSION

A number of previous studies (11, 19, 28) have indicated that there is an absolute requirement for cAMP for anaerobic induction of the threonine dehydratase encoded by the *tdcB* gene in *E. coli*. In this study two lines of evidence (experiments with the *crp* mutant and gel mobility shift and footprinting analyses with purified CAP and the cloned *tdc* promoter DNA) provided direct evidence for the first time that cAMP and CAP are required for expression of the

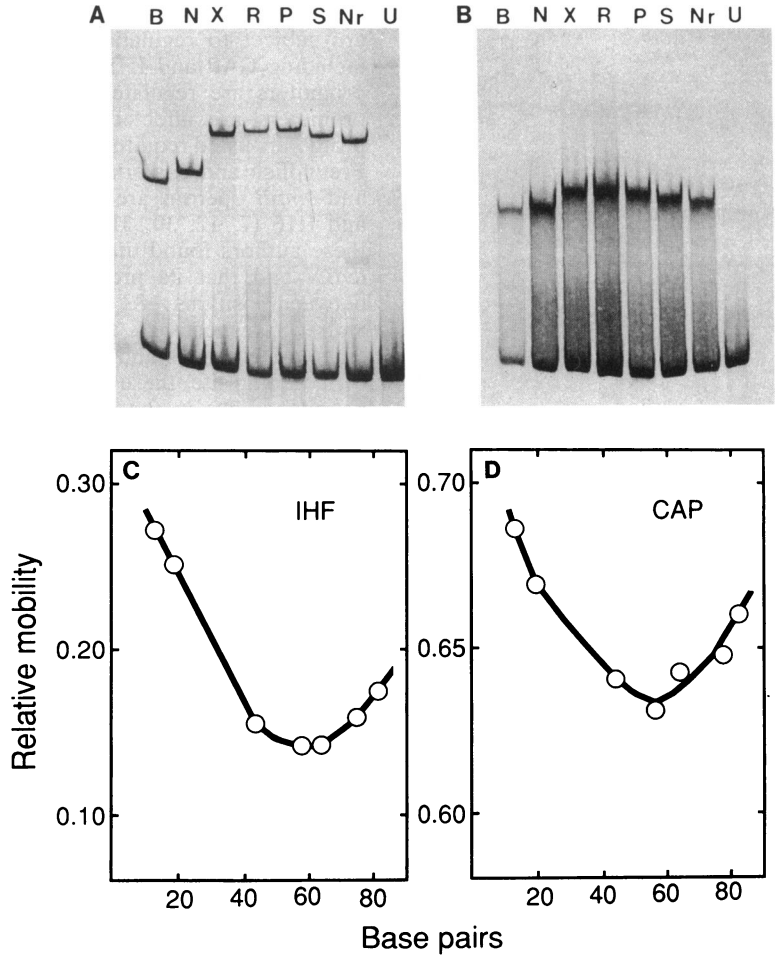

FIG. 6. (A and B) Mobility shifts of permuted DNA fragments from plasmid pBend2 containing *tdc* DNA insertions. (C and D) Relative mobility plots of the same fragments. (A) Plasmid pBend2 harboring ΔCAP DNA template (see Fig. 1B) was digested separately with various restriction enzymes (lane B, *Bgl*II; lane N, *Nhe*I; lane X, *Xho*I; lane R, *Eco*RV; lane P, *Pvu*II; lane S, *Stu*I; lane Nr, *Nru*I), and then the 238-bp fragments were purified by gel electrophoresis. The fragments were labeled by using α-^{35}S-dATP, incubated with 2 ng of IHF, and then subjected to polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. (B) The 237-bp ΔIHF template DNA from plasmid pYW99 was digested with various restriction enzymes (see above), and the labeled fragments were incubated with 30 ng of CAP and then electrophoresed prior to autoradiography. In both panel A and panel B lane U contained the *Bgl*II fragment from either pYW88 or pYW99 not incubated with CAP or IHF. (C and D) Relative mobility plots of permuted DNA fragments from the ΔCAP template and from the ΔIHF template, respectively. The numbers of base pairs indicated on the *y* axes represent the distances of the individual restriction enzyme sites from the *Eco*RI ends of the pBend2 plasmids (see Fig. 1B).

tdcABC operon. In the presence of cAMP, purified CAP binds to a 30-bp sequence in the *tdcP* region between positions −55 and −26 encompassing a 17-bp consensus-like sequence with a TGTGA motif (6). The center of the CAP-binding site is located at position −41, which is identical to the position found in several CAP-dependent promoters in *E. coli* (4). A mutant CAP site with three base substitutions (T → C at position −48, G → T at position −47, and G → A at position −45) within the TGTGA sequence failed to bind CAP and drastically reduced β-galactosidase expression from a *tdcB'-'lacZ* fusion plasmid. The exact binding site for RNA polymerase on *tdcP* has not been determined yet; however, the region downstream from the CAP site, which contains weak −35 and −10 sequences, may be assumed to recognize RNA polymerase to initiate transcription. The proximity of the CAP- and polymerase-binding sites and the CAP-induced DNA bending observed in this study and at other CAP sites (25) might suggest that there is a direct CAP-RNA polymerase interaction to regulate *tdc* transcription (20). It should be mentioned in this context that the locations of the CAP sites in various promoters vary considerably from positions around nt −40 to −60 and −70 and that in some cases multiple CAP-binding sites have been found (4, 20). Furthermore, the cAMP-CAP complex is capable of both positive (activation) and negative (repression) regulation or both in terms of gene expression (12). In general, the most activatable promoters are known to harbor CAP sites at around position −40 (4).

The DNA sequence upstream from the CAP site in the *tdc* promoter also contains an IHF-binding site between positions −118 and −88. We have shown recently (33) that this IHF-binding site and a functional IHF protein are essential for *tdc* operon expression; lack of either one reduces *tdc* expression by about 90%. The results summarized in this paper clearly show that in an IHF-deficient strain with a wild-type *tdcP* CAP sequence or in an IHF-positive host

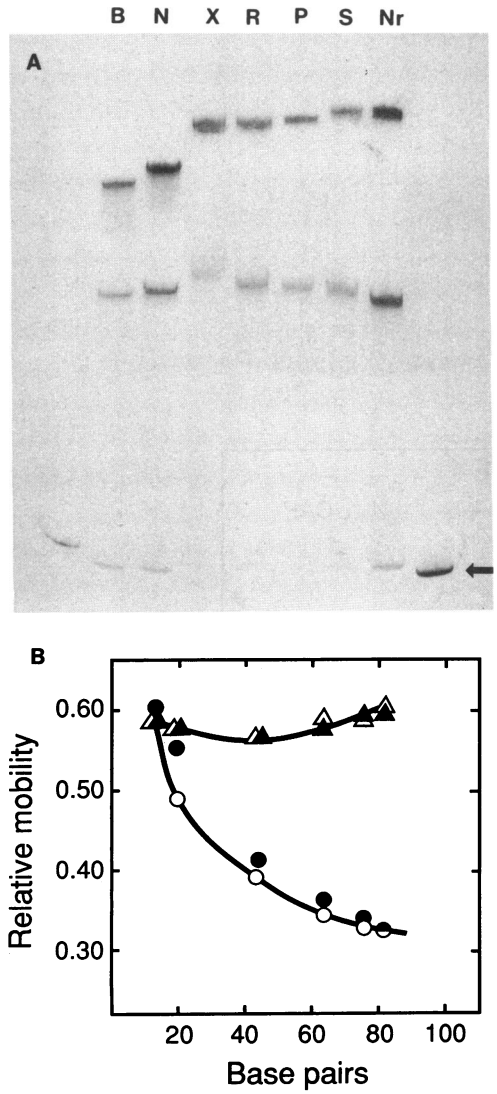

FIG. 7. Mobility shifts (A) and relative mobility plots (B) of permuted DNA fragments of the 317-bp wild-type *tdc* promoter isolated from plasmid pYW100 after restriction enzyme digestions. For experimental details, see the legend to Fig. 6 and Materials and Methods. (A) Individual DNA fragments were incubated separately with 60 ng of CAP plus 5 ng of IHF prior to electrophoresis. The arrow indicates the position of free DNA that was not complexed with proteins. Lane B, *Bgl*II fragment; lane N, *Nhe*I fragment; lane X, *Xho*I fragment; lane R, *Eco*RV fragment; lane P, *Pvu*II fragment; lane S, *Stu*I fragment; lane Nr, *Nru*I fragment. (B) Symbols: ○ and △, relative mobility patterns of the more slowly migrating and the faster-migrating sets of DNA-protein complexes, respectively (see panel A); ●, relative mobility pattern of the slowly migrating DNA-protein complexes observed when the permuted DNA fragments were incubated only with 5 ng of IHF; ▲, relative mobility pattern of the fast-migrating DNA-protein complexes observed when the permuted fragments were incubated only with 60 ng of CAP.

with a mutant CAP sequence or vice versa *tdc* expression is drastically reduced (Table 2). Thus, the two proteins act in concert as positive transcription factors when they are bound to their respective target sites on *tdcP* DNA.

Collado-Vides et al. (4) recently searched the Medline data base to obtain information on 119 procaryotic promoters that are subject to regulation by various regulatory proteins, including CAP and IHF. These authors found that 28 σ^{70} promoters are regulated by CAP and at least seven σ^{54} promoters are affected by IHF. However, none of the promoters listed requires both CAP and IHF. More recently, Freundlich and coworkers reported that the *E. coli ilvBN* and *ompB* operons are subject to regulation by both CAP and IHF (7, 12, 30, 31). In separate experiments (7, 30), these authors found that CAP and IHF positively regulate *ilvBN* and that its promoter DNA binds CAP and IHF between positions −82 and −44 and between positions −99 and −73, respectively. Because CAP and IHF occupy overlapping DNA sites and IHF enhances a natural bend at its own binding site, these authors proposed that the antitermination activity in the *ilvBN* operon is dependent on the IHF-mediated conformation change in the promoter leader DNA (30). However, the combined effects of IHF and CAP in altering promoter conformation remain to be established by binding experiments with IHF plus CAP. On the other hand, the *ompB* promoter is negatively regulated by IHF, and CAP exhibits both a positive effect and a negative effect in controlling multiple *ompB* transcriptional start sites (12, 31). IHF binds to three sites on the *ompB* promoter between positions −117 and −82, −55 and −18, and −10 and +35, whereas CAP occupies a single site between positions −69 and −36. Deletion experiments revealed that the two distal IHF sites (with reference to transcription start sites) have no effect on *ompB* expression. Thus, the functional IHF site between nt −10 and +35 resides about 65 nucleotides downstream from the CAP site and overlaps the two major transcription start sites (at nt +1 and +23), which are inhibited and activated, respectively, by the cAMP-CAP complex. An understanding of this complex transcription pattern involving these regulatory proteins would require a detailed analysis of *ompB* promoter conformation upon simultaneous binding of CAP and IHF.

The situation with the *tdc* promoter appears to be distinct from the situations observed with *ilvBN* and *ompB*. In *tdcP*, the IHF-binding site is located approximately 60 bp upstream from the CAP-binding site. IHF and CAP can simultaneously bind to their unique sites on *tdcP* DNA, and both proteins are capable of bending DNA at their target sites, at least in vitro. Thus, it is possible to envisage that while bending of promoter DNA by IHF or CAP, separately, may not create the optimum promoter conformation to activate transcription, binding of both proteins might bend DNA into a new conformation to allow transcription to proceed. This notion is consistent with the observation (Fig. 7B) that the DNA-IHF-CAP complex exhibited a different extent of DNA bending than that exhibited by either the DNA-IHF or the DNA-CAP complex. However, because IHF and CAP are not the sole activators for promoting *tdc* transcription, an alternative mechanism involving DNA looping is a more likely explanation for the concerted action of IHF and CAP. It has been found that both TdcR and TdcA, which have helix-turn-helix motifs, appear to be involved in efficient expression of the *tdc* operon (22, 27). Although the DNA-binding sites of these proteins have not been identified yet, a preliminary deletion analysis indicated that a *cis*-acting sequence in the *tdcP* region is required for *tdcR* action on the *tdcABC* operon (27). It is tempting to speculate that the products of *tdcR* and/or *tdcA* may occupy a DNA-binding site(s) upstream from IHF and that the CAP- and -IHF-induced DNA bending may allow the promoter DNA to loop around to juxtapose these proteins with CAP or with the

RNA polymerase. If correct, this hypothesis is compatible with the general observation (4) that IHF plays an important accessory role in augmenting the function of other transcriptional factors to regulate transcription. Obviously, a detailed analysis of the mechanism of activation of *tdc* transcription must await a careful study of TdcR and TdcA with respect to their DNA-binding sites and their interaction, if any, with the cAMP-CAP complex bound to *tdcP* DNA.

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