Control of the *ccd* Operon in Plasmid F

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The F sex factor plasmid of *Escherichia coli* contains a pair of genes, *ccdA* and *ccdB*, whose protein gene products are involved in an unusual feature of plasmid maintenance. The CcdB protein is a cytotoxin that becomes activated when the F plasmid is lost, thereby killing the F⁻ segregant cells. In F⁺ cells, the CcdA protein protects against the lethal effects of CcdB. In the present study we show that *ccdA* and *ccdB* expressions are negatively autoregulated at the level of transcription. Genetic studies showed that repression required at least *ccdB*; *ccdA* alone was without effect, and *ccdB* alone was not examined because it is lethal. Ccd-operator complexes were purified and contained a mixture of both CcdA and CcdB proteins; however, we could not conclude from our results whether CcdA was necessary for DNA binding or autorepression. By using restriction fragments of the promoter-operator region, we obtained results indicating that at least two DNA-binding sites existed for the Ccd protein(s). Subsequent footprinting of the binding sites showed protection over about a 113-base-pair region encompassing the putative promoter-operator and the beginning of the *ccdA* gene.

An F⁺ culture maintains its F plasmids not only by periodic plasmid replication and equipartitioning but also by destruction of F⁻ segregants. The plasmid-encoded *ccdA* and *ccdB* genes are involved in the latter process (7-9, 19). Interestingly enough, at least the *ccdB* gene product, the CcdB protein (M_r 11,700), also participates in F DNA replication initiated at origin V (13) (Fig. 1).

Very little is known about how the *ccd* proteins affect any of their functions. With respect to destruction of F⁻ segregants, nascent F⁻ cells grow and divide normally for two or three generations and then filament and lose their ability to act as colony-forming cells (7, 8). The CcdB protein is necessary for filamentation and is lethal in the absence of CcdA protein (M_r 8,700), which implies that the latter neutralizes CcdB (19). CcdA and CcdB form a complex of about 69,000 M_r in vitro (Tam and Kline, submitted for publication). A comparable interaction in vivo could be relevant to neutralization of the lethal effects of CcdB.

The *ccd* locus has been sequenced (20), and the proteins have been expressed and partially characterized by two-dimensional gel electrophoresis (1). In these earlier publications, the proteins were referred to as H(CcdA) and G(CcdB), but we now refer to them according to the widely employed convention mimicking the name of the genetic locus. Both the sequence and expression data suggested that *ccdA*, *ccdB*, and the *repD* locus might form an operon. The latter locus encodes a site-specific resolvase (12).

Our purpose in the present work has been to define control of *ccd* expression. Our results indicate that expression of *ccdA* and *ccdB* is negatively controlled at the level of transcription. At least the CcdB protein is required for repression, and perhaps CcdA is required as well. The CcdA protein by itself is not regulatory. We have cloned the *ccdA* and *ccdB* genes in expression vectors to overproduce the proteins. Subsequent DNA-binding experiments surprisingly indicated that specific binding of Ccd proteins occurs at several sites spaced over approximately a 113-base-pair (bp) interval which is 5' to and overlaps the beginning of the *ccdA* reading frame. Although not examined extensively, our

genetic experiments indicate that RepD protein has no discernible effect on regulation of the operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study were *Escherichia coli* BK1657 and BK1915. BK1657 is JM109 (26) cured of the F' *lac* plasmid. BK1915 is BL21(DE3), which carries the plasmid plysS, a recombinant between pACYC184 and the T7 lysozyme gene (24; F. W. Studier, personal communication). All other plasmids used in this study are listed in Table 1.

Recombinant DNA methodology. Plasmid DNA was isolated by either the dye-CsCl gradient technique (23) or the modified Birnboim and Doly procedure described by Chen and Seeburg (3). Restriction endonuclease, T4 ligase, and fill-in reactions with Klenow fragment were all performed as suggested by the suppliers of the enzymes or by Maniatis et al. (16).

Overexpression of *ccdA* and *ccdB* genes. Genes to be expressed from the T7 promoter were cloned into the multicloning site of pBSM13- (Stratagene). Recombinant plasmids were transformed into BK1915, which is lysogenic for the lambda derivative DE3 that carries T7 RNA polymerase under the control of *lac* UV5 (24).

For our purposes, BK1915 transformants were grown at 37°C on the M9 medium supplemented with 5% methionine assay medium (Difco Laboratories) described by Levy (14). When cultures reached the mid-log to late-log phase, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.4 mM, and cultures were incubated for 60 min at 37°C. Since T7 RNA polymerase is not sensitive to rifampin, this antibiotic was added to 300 μg/ml to eliminate transcripts from the host cell chromosome. After the addition of rifampin, the cultures were incubated for 120 min at 37°C. In cases where [³⁵S]methionine ([³⁵S]Met) was to be added, 0.6 μCi of [³⁵S]Met per ml of medium was added 30 min after the addition of rifampin.

After the expression of the T7 promoters, the cells were harvested by centrifugation, washed once with fresh M9 medium, and suspended in 0.005 volume of 0.02 M Tris hydrochloride-0.1 M NaCl-0.0005 M dithiothreitol-10% glycerol-0.1% Triton X-100-0.001 M phenylmethylsulfonyl

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FIG. 1. Maps of mini-F, pBK117, and recombinant plasmids. The map units for mini-F are in kilobases (22). The coordinates shown for the *ccd* operon were adjusted to be consistent with the DNA sequencing data of Miki et al. (20). The open reading frames which encode mini-F proteins are shown along with their genetic designation. The *bla* gene encodes for β -lactamase and originates from Tn3 (Table 1). Below pBK117 are the various regions from this plasmid which were cloned, giving the indicated recombinant plasmids. For pBK690, pBK691, pBK701, and pBK703, the junctions between the mini-F sequences and the *trp-lac* ('AZYA) sequences are shown.

fluoride (pH 8.0). Because the *plyS* plasmid produces T7 lysozyme, the cells were easily lysed with three cycles of freezing in dry ice-ethanol and thawing at 30°C.

The viscosity of the lysates was reduced by brief sonication. After sonication the lysates were adjusted to 1.0 M NaCl to reduce protein-DNA interactions, placed on ice, and stirred for 60 min. After this treatment, the lysates were cleared by centrifuging for 30 min at 40,000 $\times$ g. Proteins as opposed to nucleic acids in the cleared lysates were then

precipitated with $(\text{NH}_4)_2\text{SO}_4$ (65% saturation), and the precipitate was suspended and dialyzed against the appropriate buffer.

Gel retardation assays. The binding of cloned proteins to specific DNA sequences was determined as described by Fried and Crothers (5). Protein preparations were diluted in 0.05 M Tris hydrochloride–0.1 M NaCl–0.01 M MgCl_2 –0.01 M 2-mercaptoethanol–1 mg of herring sperm DNA per ml–50 μ g of bovine serum albumin (fraction V) per ml–0.1% Triton

TABLE 1. Plasmids

Plasmid	Description	Reference or source
pBK50	Derivative of pSC101 carrying an <i>EcoRI</i> <i>kan</i> ⁺ fragment, Km ^r	17
pBK57	pBK50 carrying F sequences 42.84 to 46.13 kb (Fig. 1), Km ^r	17
pBK79	Same as pBK57 except <i>copA48</i> , Km ^r	17
pBK117	<i>Bam</i> HI deletion derivative of mini-F, F sequences 42.84 to 46.13 kb (Fig. 1), Ap ^r	10
pBK542	<i>Bgl</i> III- <i>Bam</i> HI deletion derivative of pBK57 carrying F sequences 45.0 to 46.13 kb, Km ^r	This study
pBK654	<i>Bgl</i> III- <i>Bam</i> HI deletion derivative of pBK57 carrying F sequences 42.84 to 45.0 kb, Km ^r	This study
pBK658	<i>Pst</i> I deletion of derivative of pBK57 carrying F sequences 42.84 to 43.6 kb, Km ^r	This study
pBK660	Same as pBK542 except DNA from pBK79	This study
pBK690	Recombinant plasmid of pBR322 carrying F sequences 42.91 to 43.08 kb as <i>ccdA'</i> - <i>trp lac</i> ('AZYA), Ap ^r Lac ⁺ (Fig. 1)	This study
pBK691	Recombinant plasmid of pBR322 carrying F sequences 42.91 to 43.35 kb as <i>ccdB'</i> - <i>trp lac</i> ('AZYA), Ap ^r Lac ⁺ (Fig. 1)	This study
pBK698	Recombinant plasmid of pGem3 carrying F sequences 42.84 to 43.60 kb	This study
pBK701	Recombinant plasmid pf pTL25 carrying F sequences 42.84 to 43.35 kb as <i>ccdB'</i> - <i>trp lac</i> ('AZYA), Ap ^r Lac ⁺ (Fig. 1)	This study
pBK703	Recombinant plasmid of pTL25 and F sequences 42.84 to 43.60 kb as <i>repD'</i> - <i>trp lac</i> ('AZYA), Ap ^r Lac ⁺ (Fig. 1)	This study
pBK754	Recombinant plasmid of pBSM13- and mini-F sequences 42.84 to 43.60 kb with mini-F sequences 42.91 to 43.01 kb deleted	This study
pBSM13-	Multicopy phagemid carrying T7 and Sp6 promoters, Ap ^r ^{ra}	
pBK800	Recombinant plasmid pBSM13- and mini-F sequences 42.84 to 43.11 kb.	This study
pGem3	Multicopy plasmid carrying Sp6 promoter, Ap ^r ^{tb}	
pTL25	Multicopy promoter fusion vector, <i>trp lac</i> ('AZYA), Ap ^r Lac ⁻	15

^a Purchased from Stratagene, San Diego, Calif.

^b Purchased from Promega Biotec Co., Madison, Wis.

TABLE 2. Regulation of *ccd'*-*trp lac* ('AZYA) fusions by cloned mini-F genes^a

Complementary plasmid	Cloned F genes in heteroplasmid	% Repression of <i>ccd</i> promoter on fusion plasmid ^b :										
		pBK690			pBK691			pBK701			pBK703	
		1	2	3	4	5	6	7	8	9	10	11
pBK50	None	0	0	0	0	0	0	0	0	0	0	0
pBK57	<i>ccdA</i> ⁺ <i>B</i> ⁺ <i>repD</i> ⁺ <i>E</i> ⁺	92	92	93	91	90	91	90	92	97	27	
pBK542	<i>repE</i> ⁺	— ^c	0	36	0	0	0	22	63	63	7	0
pBK654	<i>ccdA</i> ⁺ <i>B</i> ⁺ <i>repD</i> ⁺	64	67		71	62	61	61	67			
pBK658	<i>ccdA</i> ⁺ <i>B</i> ⁺	82	90	71	90	83	94	51	87		7	0
pBK660	<i>copA48</i>	94	90	90	90	88	94	77	78	96	25	25

^a The pSC101 Km^r derivative pBK50 (17) was used as the cloning vector for the variety of genes indicated in the second column. Typical normalized β -galactosidase activities (experiments 1, 4, and 7) when the complementary plasmid was pBK50 were 3,567 U for pBK690, 5,000 U for pBK691, 6,316 U for pBK701, and 365 U for pBK703; comparable values were seen even when pBK50 was absent.

^b Percent repression of the *ccd* promoter was calculated from the corrected specific activity of the β -galactosidase produced by the heteroplasmid cells. The β -galactosidase levels in the heteroplasmid cells containing pBK50 (no cloned F genes) represented 0% repression (first row). The β -lactamase activity of each culture served as an index of fusion plasmid concentration and was used to normalize the actual β -galactosidase activity. For the most part, fusion plasmid concentrations were within a factor of 1.5 of the value of the pBK50 control. However, in the pBK658/690 and pBK658/691 heteroplasmids, the fusion plasmids were elevated from 3.2- to 6.7-fold. Numbered column subheadings indicate experiment numbers.

^c The normalized β -galactosidase activity was 5,235 U, or 1.5 times the pBK50/690 value of 3,567 U.

X-100–5% glycerol (gel retardation buffer). DNA fragments were end labeled with the Klenow form of DNA polymerase I, and 10,000 to 50,000 cpm was added per 40 μ l of reaction mixture. The reaction mixtures were placed on ice for 30 min, loaded on a 5% polyacrylamide gel made with Tris-borate-EDTA buffer (5), and electrophoresed at 1.1 mA/cm of gel length.

β -Galactosidase and β -lactamase assays. The specific activity of β -galactosidase was determined by the colorimetric assays described by Miller (21). The cells to be assayed were grown overnight at 37°C in LB broth (21) containing the appropriate antibiotics. The next day these cells were diluted 1/100 with fresh LB broth (without antibiotics) and incubated at 37°C to an optical density at 500 nm of 0.4 to 0.6. A single assay was done on each culture. Each heteroplasmid genotype was cultured two or three times as indicated in Table 2. β -Lactamase activity was determined on a separate portion of each culture tested for β -galactosidase activity. The β -lactamase assay was described previously (17).

DNase I footprinting. DNase I footprinting experiments were adapted from the procedure of Galas and Schmitz (6). DNA fragments were labeled at the 3' end by fill-in reactions with the Klenow fragment of DNA polymerase I or at the 5' end by using T4 polynucleotide kinase. To obtain a fragment labeled at only one end, the labeled fragment was digested with a second restriction enzyme, and the desired subfragment was purified by electrophoresis. Conditions for binding of proteins to DNA fragments were as described above for gel retardation assays, except the reaction volume was 100 μ l. Under these conditions, 2.5 U of DNase I (Boehringer Mannheim Biochemicals) per ml was added, and the reaction mixture was incubated for 2 min at 20°C. The reaction was halted by the addition of 25 μ l of 3 M ammonium acetate, 0.25 M EDTA, and 0.15 mg of sonicated calf thymus DNA per ml and precipitated with 2.5 volumes of 95% ethanol. The resulting pellet was washed with 70% ethanol and analyzed by gel electrophoresis under conditions that denature DNA as described by Maxam and Gilbert (18).

RESULTS AND DISCUSSION

In vitro construction of *ccd'*-*trp lac* ('AZYA) fusions. To examine regulation of the *ccd* promoter, we made a series of transcriptional fusions between portions of the *ccd* operon and the promoterless *trp-lac* (AZYA) sequences of pTL25

(15). According to the sequencing data of Miki et al. (20), the *ccd* promoter maps at about 42.97 kilobases (kb) and transcribes rightward (Fig. 1). The coordinates for *ccdA* and *ccdB* are 43.045 to 43.260 kb and 43.265 to 43.570 kb, respectively. There are two convenient restriction sites upstream of the promoter: *Bam*HI at 42.84 kb and *Hpa*I at 42.91 kb (Fig. 1). The latter site is 70 bp to the right of the *Bam*HI locus and about 60 bp upstream from the putative –35 sequence of the *ccd* promoter. For our first construction, we used the *Hpa*I-to-*Alu*I restriction fragment (42.91 to 43.08 kb) to produce a transcriptional fusion within *ccdA* proximal to its 5' terminus. This plasmid is pBK690. The fusion juncture in pBK690 and other constructions are depicted in Fig. 1.

trans-acting factors in control of *ccd* transcription. Cells containing a *ccd'*-*lac* fusion plasmid and pBK50 (a pSC101 derivative), the latter with or without cloned F genes, constituted the heteroplasmid system used to study in vivo complementation. Initial analysis of data from heteroplasmid cells indicated that the *ccd* promoter was regulated only when both *ccdA*⁺ and *ccdB*⁺ were present; for example, compare the representative data for the levels of expression given in footnote a of Table 2. These results meant that β -galactosidase output was proportional to fusion plasmid concentration for constructs pBK690, pBK691, and pBK701. The relative concentration of each fusion plasmid was easily monitored by measuring the β -lactamase specific activity it produced, since this activity is proportional to plasmid copy number. Subsequently, the level of β -galactosidase activity from each fusion plasmid was normalized to copy number, i.e., β -lactamase activity. Replica plating studies showed that pBK50 and its derivatives were very stably inherited, in confirmation of earlier studies (17).

We used the spectrum of putative repressor-bearing plasmids described in Fig. 1 and Table 2 to study control of the *ccd* promoter contained in pBK690. The results showed that the *ccd* promoter was strongly repressed whenever *ccdA*⁺ and *ccdB*⁺ were present. With one exception mentioned below, we could see no significant effect on repression by *repE*⁺ or *repD*⁺. By contrast, a mutated form of *repE*, *copA48* (17), which was cloned into pBK660, was always a strong repressor of the *ccd* promoter. The *copA48* mutation removes the autorepressor activity of RepE, allowing higher levels of RepE production (25). We do not know whether the

repression of *ccd* by pBK660 represents the effect of more RepE protein or an alteration in RepE function.

We also observed in the pBK658/pBK690 heteroplasmid that the copy number of pBK690 was increased about threefold over its normal value. Similarly, in the pBK658/pBK691 heteroplasmid pBK691 the copy number was elevated nearly sevenfold above its normal value. Neither the reason nor the significance of these copy number changes is known. In any event, after normalization for the increases, consistently high levels of autorepression of the *ccd* promoter were observed. We also found that pBK542, carrying only *repE*⁺, failed to repress the *ccd* promoter reproducibly, although in the heteroplasmid with pBK701, repression was always seen. The significance of this variability also is not understood.

To determine whether the CcdA protein is the autorepressor, we constructed pBK691, which contains all of the *ccdA* sequences and the first one-third of *ccdB*. This plasmid, like pBK690, lacks the 70-bp *Bam*HI-*Hpa*I fragment that is 5' of the putative *ccd* promoter. Results with pBK691 (Table 2, experiments 4 to 6) were similar to those with pBK690 and indicated that the CcdA protein alone is not an autorepressor. To complete the systematic analysis of the target plasmids, we constructed and tested a plasmid which had the 70-bp *Bam*HI-*Hpa*I fragment and *ccdA*⁺, that is, pBK701 (Fig. 1). The additional DNA in pBK701 had no effect on repression, since the results shown in Table 2 (experiments 7 through 9) were very similar to those found with pBK690 and pBK691. As mentioned above, there was the notable exception that wild-type *repE* (i.e., pBK542) showed modest to moderately strong levels of repression on the *ccd* promoter in the pBK701 heteroplasmid cells.

The *ccd* transcriptional unit is believed to include the *repD* locus, because only 3 bp separate the *ccdB* and *repD* genes (20) and a transcriptional stop signal is not evident in the published sequence (20). To examine control of the *repD* expression through the *ccd* promoter, we constructed a transcriptional fusion in *repD*. The plasmid, pBK703, contains the full *ccd* promoter region as well as *ccdA*⁺ and *ccdB*⁺ (Fig. 1). Repression results are presented in Table 2 (experiments 7 through 11). In heteroplasmid cells containing pBK50 (last line of Table 2), the *ccd* promoter activity in pBK703 was only 5.8% of the activity seen in comparable heteroplasmid cells harboring pBK701. Since pBK703 makes a full complement of CcdA and CcdB proteins, this 94.2% reduction in promoter activity may largely represent the level of autogenous repression. There is, however, a weak promoter for *repD* in the 3' terminus of *ccdB* (12), and this promoter may contribute to the activity seen with pBK703. Hence, autogenous control of *ccd* expression could be greater.

Cloning and expression of *ccdA* and *ccdB*. Our data indicate that one or both Ccd proteins repress *ccd* transcription and therefore should bind to the *ccd* operator. To overproduce the Ccd proteins, the *ccdA* and *ccdB* genes were cloned into the T7 RNA polymerase-dependent expression vector pBSM13-. To be assured that cloned *ccd* genes were expressed only when production of T7 RNA polymerase was induced, we deleted the predicted *ccd* promoter region, that is, the mini-F sequences 42.91 through 43.01 (20). Also, to reduce the number of proteins produced during induction, we deleted all the *repD* sequences 3' of the 43.60-kb coordinate. Thus, only 43 bp of the *repD* gene are contained in the recombinant, which is designated pBK754 (Fig. 1, Table 1).

Figure 2A shows the proteins stained by Coomassie blue

FIG. 2. Expression and electrophoretic analysis of Ccd proteins. Sodium dodecyl sulfate-polyacrylamide (20%) gels of lysates from pBSM13- and pBK754 carrying strains BK1919 and BK1920, respectively, are shown in panels A and B. Gels were prepared as described by Laemmli (11) and loaded with 20 μ g of protein from induced cultures labeled with [³⁵S]Met (see Materials and Methods) per lane. The gel was first stained with Coomassie blue (A) and then dried and exposed to X-ray film for 16 h (B). STD lanes are ¹⁴C-labeled size standards of 43, 25.7, 18.4, 14.3, 6.2, and 3.0 kDa (Bethesda Research Laboratories, Inc.).

in lysates made from induced cultures carrying either pBSM13- (BK1919) or pBK754 (BK1920). [³⁵S]Met was present during the induction period to label nascent proteins (Fig. 2B). It was evident the BK1920 has an altered protein profile from that of BK1919 (Fig. 2A). Detection of the ³⁵S label by autoradiography (Fig. 2B) showed that most of the [³⁵S]Met was incorporated into four proteins in the BK1920. These proteins had molecular masses of 25.6, 18.1, 11.1, and 6.1 kilodaltons (kDa). By comparison, much less ³⁵S label was induced in BK1919, and most of the label was in two proteins having molecular masses of 25.6 and 18.1 kDa. Because these lysates were prepared from cultures treated with rifampin, we assume that the four labeled proteins produced by BK1920 were from mRNA transcribed from T7 promoters. We believed the 18.1-kDa species to be T7 lysozyme (4; F. W. Studier, personal communication) and the 11.1- and 6.1-kDa species to be from the cloned mini-F DNA in pBK754 (i.e., *ccdA* and *ccdB*; Table 1). The identity of the 25.6-kDa species is unknown.

We tentatively concluded that the 11.1- and 6.1-kDa species in BK1920 lysates were CcdB and CcdA proteins, respectively, based on their uniqueness and size. However, Miki et al. (20) calculated from the gene sequence that CcdA is 8.3 kDa and CcdB is 11.7 kDa. Bex et al. (1) reported higher molecular masses for these proteins, that is, 10.5 and 12.0 kDa, respectively. Our value for CcdB is in reasonable agreement with the above, but our value of 6.1 kDa for CcdA is not. This created the concern that we were not actually expressing Ccd proteins. Alternatively, our sodium dodecyl sulfate-polyacrylamide gel electrophoresis measurement may have been responsible for the inconsistency, or perhaps CcdA is easily proteolyzed. We examined this issue by sequencing the amino terminus of the 11.1- and 6.1-kDa

FIG. 3. Assay of DNA binding activity toward the *ccd* operator in lysates from induced BK1919 and BK1920 cultures. The protein levels in lysates from BK1920 (lanes 1 through 4) and BK1919 (lanes 5 through 8) were diluted to final concentrations of 75 $\mu\text{g/ml}$ (lanes 1 and 5), 7.5 $\mu\text{g/ml}$ (lanes 2 and 6), 3.8 $\mu\text{g/ml}$ (lanes 3 and 7), and 1.9 $\mu\text{g/ml}$ (lanes 4 and 8) with assay buffer and tested for specific DNA binding. The labeled DNA for this assay was the mini-F fragment of 42.84 to 43.35 kb digested with *HpaII* (see Fig. 1 and text). The lane marked STD shows normal mobility of free, labeled DNA. The arrow shows the fragment carrying the predicted *ccd* promoter (20).

proteins. Expression of CcdA and CcdB proteins was induced in the standard way, and the proteins were partially purified by ion-exchange chromatography. Then the CcdA protein was separated from CcdB protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and each protein was electroblotted onto an Immobilon membrane (Millipore Corp.). The proteins were then eluted from the membrane, and the first 15 amino acid residues were determined. In both cases, the amino acid sequence was exactly as indicated by Miki et al. from the DNA sequence (20; data not shown). Hence, we conclude that our expression system is inducing overproduction of the Ccd proteins.

Induced *ccd* gene products bind to specific *ccd* DNA sequences. The experiment shown in Fig. 3 compares the DNA-binding activity of lysates from induced BK1920 and BK1919 toward various segments of the *BamHI-SmaI* mini-F fragment (42.84 to 43.35 kb). In this experiment, the 42.84- to 43.35-kb DNA was digested with the *HpaII* restriction endonuclease to produce three fragments. The coordinates of their termini are 42.84 to 43.11 kb, 43.11 to 43.22 kb, and 43.22 to 43.35 kb (Fig. 1). The largest fragment (Fig. 3, arrow) carried the putative *ccd* promoter-operator (20). The BK1919 lysate elicited strong binding to the largest fragment at a dilution which had 75 μg of protein per ml (lane 5), but further dilution to 7.5 μg of protein per ml (lane 6) produced trivial binding to DNA. In contrast, the BK1920 lysate elicited strong binding to the largest fragment at 3.8 μg of protein per ml (lane 3) and showed detectable binding at 1.9 μg of protein per ml (lane 4). Therefore, the activity of BK1920 for specific DNA binding was much higher in BK1920 by at least 30- to 40-fold. This high-titer specific activity was absent in BK1920 cultures not subjected to induction (data not shown).

Recently it has been reported that the extent of retardation of the DNA in gel electrophoresis assays is dependent more upon the charge of the DNA-binding protein than on the mass of the DNA-protein complex (2). Hence, different species of DNA-binding proteins can be expected to dem-

onstrate different effects on the mobility of a given DNA fragment. Such is the case with the BK1919 and BK1920 lysates. In Fig. 3 there is clearly a difference between the mobility of the DNA-protein complex formed by the two lysates. This result suggests that induction of BK1920 produces a different DNA-binding protein than that found in BK1919. In general, the results are consistent with the notion that BK1920, when induced, overproduces Ccd proteins and that at least one protein specifically binds to *ccd* DNA. If this interpretation is correct, then purified *ccd* DNA-protein complexes should contain one or both of the Ccd proteins.

Characterization of proteins complexed to *ccd* DNA. Before we were aware of how much protein influenced the position of DNA retardation, we ran a gel retardation assay with ^{35}S -labeled proteins from induced BK1920 and unlabeled *ccd* DNA. We had hoped to find that DNA-protein complexes migrated at a different rate than free proteins; they did not (data not shown). To overcome this problem, we took advantage of the fact that gel chromatography conditions could be defined wherein free proteins were well separated from DNA-protein complexes (Fig. 4A). Preliminary tests showed that ^{35}S -labeled proteins at the concentration used in Fig. 4A nonspecifically bound to the herring sperm DNA in the standard DNA binding buffer, i.e., these proteins co-chromatographed with herring sperm DNA in the void volume of the column (data not shown). However, the addition of KCl (150 mM final concentration) to the chromatography buffer prevented nonspecific binding to herring sperm DNA (Fig. 4A). The addition of KCl also prevented binding to pBSM13-, the vector used to make pBK800 (data not shown). In the presence of 150 mM KCl and pBK800, a recombinant plasmid containing the *ccd* operator (Table 1 and below), we found that a fraction of the labeled proteins eluted in the void volume of the gel column (Fig. 4A). Subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of crude lysate (Fig. 4B, lane 1) and a pool of fractions 9 and 10 representing *ccd*-bound proteins (Fig. 4A) showed that the latter contained just the 11.1- and 6.1-kDa labeled proteins characterized by size and amino-terminal sequencing as CcdA and CcdB (Fig. 4B, lane 2).

From genetic results alone, we know that at least CcdB is required for repression and that CcdA alone cannot repress. Construction of a $\Delta\text{ccdA } \text{ccdB}^+$ genotype to see whether *ccdB* alone is responsible for repression is not possible because CcdB alone is toxic (19). Use of an in vitro transcription system and purified CcdB should allow us to determine whether CcdB alone represses. However, at present such an approach also is not possible, because CcdB always copurifies with CcdA under nondenaturing conditions (Tam, unpublished observations). Thus, further work is needed to determine whether one or both of Ccd proteins constitute the autorepressor.

Locating the *ccd* operator. (i) Gel retardation analysis of restriction fragments. The above experiments clearly showed that the mini-F fragment 42.84 to 43.11 kb (Fig. 3) was bound by the Ccd proteins. To localize the binding site(s) more accurately, we digested this fragment with the restriction endonuclease *HpaI*, *AccI*, or *AluI* (Fig. 1). The fragments produced by each of these enzymes are shown in lanes 1, 6, and 11, respectively, of Fig. 5. Each set of fragments was then used as a substrate for gel retardation assays.

Results with the *HpaI* fragments showed that the binding site was to the right of the *HpaI* site at 42.91 kb (lanes 1 through 5). Results with the *AluI*-digested fragments indicated that the binding sites was to the left of 43.08 kb (lanes

FIG. 4. Isolation of a ³⁵S-labeled protein-DNA complex by gel filtration chromatography on Ultragel ACA34 (LKB Instruments, Inc.). (A) Lysates from ³⁵S-labeled BK1920 were mixed with an equal volume of gel retardation assay buffer with 16 pmol of pBK800 DNA (●) or without pBK800 (▲). The mixture was chromatographed on a 0.9- by 11.0-cm column equilibrated with gel retardation assay buffer minus carrier DNA and bovine serum albumin but containing 150 mM KCl (see the text). The void volume of the column was 1.5 ml, the flow rate was 10 ml/h, and 0.15 ml fractions were collected. Each fraction was checked for ³⁵S-labeled protein by liquid scintillation counting. (B) Fractions 9 and 10 from the mixture containing pBK800 were pooled, lyophilized, and analyzed in sodium dodecyl sulfate-20% polyacrylamide. The autoradiograph of this gel is shown. Lane 1 is BK1920 crude lysate used in this experiment, and lane 2 shows the pooled fractions 9 and 10. Each lane contains 40,000 cpm. The molecular mass of each band is shown.

12 through 15). Thus, 42.910 to 43.080 kb defines a 170-bp region that contains the binding site. This region contains an *AccI* site at 43.01 kb. Binding results obtained with *AccI*-digested DNA showed that both fragments interacted with the Ccd proteins. It should be noted that with each set of restriction fragments, identical dilutions of BK1920 lysate were used. With the *HpaI*- and *AluI*-digested DNAs, sharp, clean bands of retardation were produced; but with *AccI*-digested DNA, two smeared, largely nondistinct bands were produced at all but the highest protein concentration (lane 7). This suggested to us that at least two binding sites exist and that the disconnected sites are not as effective in associating Ccd protein as the intact *ccd* locus. Confirmation and identification of multiple binding sites was determined by "footprinting" with DNase I.

(ii) **Mapping Ccd protein-DNA interaction by footprinting.** Induced lysates of BK1920 were prepared, serially diluted, and examined by the gel retardation assay to determine the concentration of cell proteins that lacked nonspecific binding. Dilution at 1/100 (100 µg of protein per ml) or greater showed specific binding to the *ccd* operator fragment (data not shown). To footprint, lysates were diluted serially at 1/100 to 1/800, mixed with ³²P-end-labeled sequences 42.91 to 43.07 kb, and treated with pancreatic DNase I. Only sequences on one strand were protected (Fig. 6); results with the unprotected strand are not shown. Each lane in Fig. 6 was loaded with an equal amount of DNA to facilitate direct comparison of band intensities. Briefly, the greatest protection was seen at dilutions of 1/100 (lane 2) to 1/400 (lane 4) and extended from 42.957 to 43.070 kb, a distance of over 113 bp. One should note that this protection overlaps the beginning of the *ccdA* open reading frame at 43.045 kb. At a dilution of 1/800 (lane 5), protection was seen over the narrower range of about 42.957 kb to somewhere between

FIG. 5. General localization of Ccd protein-DNA-binding sites. The retardation assay was done with a BK1920 lysate. Mini-F fragments with coordinates 42.84 to 43.11 were digested with either *HpaI* (lanes 1 through 5), *AccI* (lanes 6 through 10), or *AluI* (lanes 11 through 15). The digested mini-F DNA was incubated with protein concentrations of 0 µg/ml (lanes 1, 6, and 11), 50 µg/ml (lanes 2, 7, and 12), 25 µg/ml (lanes 3, 8, and 13), 12.5 µg/ml (lanes 4, 9, and 14), and 10 µg/ml (lanes 5, 10, and 15). In each digest, the largest DNA fragment carries the predicted promoter.

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