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DNA-Binding Properties of the BetI Repressor Protein of *Escherichia coli*: the Inducer Choline Stimulates BetI-DNA Complex Formation

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The *betT* and *betIBA* genes govern glycine betaine synthesis from choline in *Escherichia coli*. In an accompanying paper we report that the *betT* and *betI* promoters are divergently organized and partially overlapping and that both are negatively regulated by BetI in response to choline (T. Lamark, T. P. Røkenes, J. McDougall, and A. R. Strøm, J. Bacteriol. 178:1655–1662, 1996). In this paper, we report that the in vivo synthesis rate of the BetI protein constituted only 10% of that of BetA and BetB dehydrogenase proteins, indicating the existence of a posttranscriptional control of the *betIBA* operon. A genetically modified BetI protein called BetI*, which carries 7 extra N-terminal amino acids, was purified as a glutathione *S*-transferase fusion protein. Gel mobility shift assays showed that BetI* formed a complex with a 41-bp DNA fragment containing the −10 and −35 regions of both promoters. Only one stable complex was detected with the 41-bp fragment and all larger promoter-containing fragments tested. In DNase I footprinting, BetI* protected a region of 21 nucleotides covering both the −35 boxes. Choline stimulated complex formation but did not change the binding site of BetI*. We conclude that in vivo BetI is bound to its operator in both repressed and induced cells and that BetI represents a new type of repressor.

For protection against osmotic stress, *Escherichia coli* can synthesize glycine betaine from choline. The *bet* regulon which governs this pathway comprises a choline-sensing repressor encoded by *betI* and three structural genes: *betT* (choline porter), *betA* (choline dehydrogenase), and *betB* (betaine aldehyde dehydrogenase). The *betIBA* genes constitute a transcript, whereas *betT* is on a separate transcript with the opposite orientation. The divergently overlapping *betI* and *betT* promoters initiate transcription 61 bp apart. Both promoters are regulated in the same manner by three external stimuli: choline, oxygen, and osmotic stress (20).

The deduced amino acid sequence of BetI reveals a helix-turn-helix motif near the N-terminal end, and the protein has about the same mass (21.8 kDa) as and displays some homology with the TetR class of repressors (19). In vivo studies show that BetI regulates the *bet* gene expression negatively in response to choline by binding at or near the promoter region. Furthermore, the *bet* promoters remain fully osmotically regulated, but not choline regulated, in a *betI* mutant background. The *bet* genes belong to the ArcA modulon, consisting of genes repressed by anaerobicity, and the binding of ArcA appears to require a sequence of the *betT* side of the overlapping promoters (20).

The action of both repressors and activators of bacteria is commonly antagonized or potentiated by the reversible binding of a specific ligand (inducer or corepressor). For most regulatory proteins (regulators), the ligand binding reduces (e.g., TetR and LacI) or increases (e.g., TrpR and cyclic AMP receptor protein) their binding affinity to their target DNA by several orders of magnitude (14, 24). For these "classical" repressors and activators, transcription is mainly decided by whether the regulator is bound to the operator site.

However, regulators of the MerR family (31), in addition to regulators belonging to the LysR family (reviewed in reference 35), exert their action by more subtle mechanisms. Although most of these regulators are activated by specific ligands, their binding affinity to their operator sites is generally not greatly affected by the ligands. Regulation of the target genes appears to be mediated through modulation of the structure of the DNA-regulator complex in response to ligand binding (2, 33, 35, 41).

Regulator genes are often autoregulated and have divergently overlapping promoters with the gene(s) which they control. A similar overlapping promoter is found in the *bet* regulon, but the organization of the *betIBA* operon is unusual in that *betI* is the first gene on a transcript which also contains two structural genes and that the divergent *betT* and *betI* promoters are regulated in the same way (20). Here we report that the synthesis of BetI is under posttranscriptional control in vivo and that choline enhances the binding of BetI to the operator sequence in vitro. BetI seems to represent a new type of repressor.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used were DH5αF′, GM161 (the genotypes are given in the accompanying paper [20]), W1485 (F^+, wild type; laboratory collection), and BL21 (F^- *hsdS ompT* [38]). The minimal medium used was medium 63 (28) with 20 mM glucose and with or without 0.3 M NaCl added. Rich medium contained (per liter) 20 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 2 g of glycerol, and 50 mmol of sodium phosphate, pH 7.2 (39). The antibiotics used were ampicillin (100 μg ml^{-1}) and kanamycin (60 μg ml^{-1}).

General recombinant DNA procedures. All manipulations with recombinant DNA techniques were carried out by standard procedures as described previously (3, 25). Transformation of ligated DNA was performed by the method described by Inoue et al. (15), using strain DH5αF′ as the recipient.

Plasmid constructs with T7 promoter. A 2.3-kb *Bgl*II-*Hin*dIII fragment of plasmid pFF221 (1), carrying the *bet* region, was isolated and digested with *Mse*I, resulting in a 615-bp *Mse*I fragment carrying the *betI* gene. The fragment was isolated from agarose gel, made blunt with Klenow polymerase, and then ligated into the *Eco*RV site of vector pBluescript II KS(+) (Stratagene). In the resulting plasmid pTP22, the *betI* gene was under control of the T7 promoter of the vector.

Plasmid pTP22 was digested with *Cla*I which cleaves in the *betI* gene and in the

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polylinker of the vector. A 3.8-kb DNA fragment extending from the *Cla*I site of *betI* to a Dam-methylated *Cla*I site in the downstream region of *betA* (20) was isolated from plasmid pFF221, which had been replicated in strain GM161 (*dam*). The fragment was ligated into the corresponding sites of pTP22 in the proper orientation. This created plasmid pTP222.

Labelling and analysis of Bet proteins. Labelling of Bet proteins expressed from the T7 promoters was based on the method described previously by Tabor and Richardson (39). Cells carrying plasmid pGP1-2 and a T7 expression plasmid were grown at 27°C, heat induced for 20 min at 42°C to produce T7 RNA polymerase from plasmid pGP1-2, and treated with rifampin (0.2 mg ml^{-1}) for another 20 min. The cells were then labelled with [^{35}S]methionine (10 μCi; Amersham) for 5 min at 30°C in medium 63 supplemented with all amino acids except for cysteine and methionine (0.1 mg ml^{-1} each) and with or without 0.3 M NaCl added. The stability of the BetI protein was investigated by labelling cells for 1 min with [^{35}S]methionine, after which the cells were chased with unlabelled methionine (1 mg ml^{-1}), and aliquots for analysis were removed after 0, 5, 15, and 60 min. Samples were heat denatured, and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) adjacent to a molecular size marker (Rainbow marker; Bio-Rad) followed by autoradiography. Quantification of the labelled protein bands was performed for three independent experiments with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Construction of a *GST-betI* fusion plasmid and purification of BetI*. A 1.8-kb *Eco*RI-*Xba*I fragment which contains the coding region of *betI* and starts with an artificial *Eco*RI site 11 bp upstream of its deduced start codon was excised from plasmid pFF440 (20) and ligated into pUC19 (Pharmacia). The *betI* gene was then transferred as an *Eco*RI-*Hin*dIII fragment into the polylinker of vector pGEX-KG (11). A 4.7-kb *Eco*RI-*Apa*I fragment of the resulting plasmid was then ligated into the corresponding sites of the vector pGEX-KT (12). The resulting plasmid pTP210 carries the *betI* gene linked in frame with the gene for glutathione *S*-transferase (GST), under control of the *tac* promoter of the vector. The vector sequence between the coding regions of GST and *betI* contains five codons for glycine (a so-called kinker) followed by codons for a cleavage site for the site-specific protease thrombin. As deduced from the DNA sequence, cleavage of the GST-BetI fusion protein with thrombin created a BetI derivative called BetI* which has 7 extra N-terminal amino acids.

Purification of BetI* was based on the method described previously by Guan and Dixon (11). Strain W1485(pTP210) was grown in rich medium until the A_{600} of the culture reached 1.0. Production of the GST-BetI fusion protein was then induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the growth was continued for another 3 h with vigorous shaking at 37°C. The culture was chilled on ice, and the following steps were performed at 0 to 4°C. The cells were harvested and washed with 20 mM sodium phosphate, pH 7.2, by centrifugation. In a typical experiment, cells from 250 ml of culture were resuspended in 4 ml of phosphate-buffered saline (PBS) (150 mM NaCl, 20 mM sodium phosphate [pH 7.2], 0.1% β-mercaptoethanol) and then disrupted by three passages through a French pressure cell (American Instrument Co., Silver Spring, Md.) operated at 100 MPa. The resulting suspension was added to 1% (vol/vol) Triton X-100, incubated with shaking for 30 min, and centrifuged at 10,000 × *g* for 15 min. The supernatant was mixed with an affinity matrix of glutathione Sepharose (250 μl of 50% slurry in PBS; Pharmacia) and incubated for 1 h with gentle shaking at room temperature (22°C). To remove contaminating compounds, the affinity matrix was washed five times with 0.8 ml of ice-cold PBS and five times with 0.8 ml of thrombin cleavage buffer (TCB; 50 mM Tris [pH 8.0], 150 mM NaCl, 2.5 mM $CaCl_2$, 0.1% [vol/vol] β-mercaptoethanol). Each time, the matrix was collected by centrifugation at 500 × *g* for 5 min (4°C). The matrix was then resuspended in 0.5 ml of TCB, the link between GST and BetI* was cleaved with thrombin (2.7 U; Sigma) at room temperature, and the supernatant containing BetI* was collected by centrifugation. The matrix was washed three additional times with 0.5 ml of TCB, and the supernatants were collected. The protein solution could be stored at −70°C for months without any apparent loss of DNA-binding capacity. Aliquots of purified BetI* were subjected to acidic hydrolysis, and the amounts of amino acids were quantified on an amino acid analyzer (4151 Alpha Plus; LKB Biochrom Ltd., Cambridge, United Kingdom). Maybe because of the unusual alkaline properties of BetI (net positive charge of 12 [19]), the values for protein concentration determined by this method were only 30% of those determined by the colorimetric method of Bradford (5) with a Bio-Rad dye reagent, using ovalbumin as the standard. The former values were therefore used to correct the values obtained by the colorimetric method. The protein samples recovered from each purification step were analyzed by SDS-PAGE (18) and visualized either with Coomassie brilliant blue R (Sigma) or by silver staining (29).

Amplification and labelling of DNA fragments. The PCR mixture for amplification of the *bet* promoter area contained 3 to 5 pmol of each primer, 1 ng of template DNA, 300 nM (each) the four deoxynucleoside triphosphates, and 1 U of DNA polymerase (DynaZyme II; Finnzymes) in standard buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.8], 1.5 mM $MgCl_2$, 0.1% Triton X-100). The following oligonucleotides, purchased from Pharmacia, were used as primers for the synthesis of PCR products (see Fig. 1B): pT2269, GGATCGTTGTCAGGGAAAA CAACAAAATCAG; pT2348, GCTGTGTGAAAGGTCTG; pT2366, CATC CAGTATCCCCACTGTTATTG; pIR2500, CATTTTCGCCACTCCATTCAT CAGC; pIR2521, CGACTGCATCCCCAATTTGG; and pIR2578, CATGCC CACTTCATTTATTGC. The primers were named according to their start coordinates in the *bet* region. One or both primers were routinely 5′ end labelled with [γ-^{32}P]ATP (5,000 mCi $mmol^{-1}$; Amersham) by T4 polynucleotide kinase. PCR was performed in an automated thermal cycler (Perkin-Elmer), with cycles of 94°C for 90 s for denaturing, 58°C for 90 s for annealing, and 72°C for 1 min for extension. The last period of extension was prolonged to 4 min. After 35 cycles, the PCR products were analyzed and purified by electrophoresis on a 6% polyacrylamide gel in half-strength Tris-borate-EDTA (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA [pH 8.0]) and eluted with salt-Tris-EDTA (100 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA). A 155-bp fragment was also prepared with unlabelled primers, digested with *Mse*I, and then labelled as described above.

Gel mobility shift assay. The gel mobility shift assay described by Fried and Crothers (9) was modified to test the DNA-binding activity of purified BetI*. The standard reaction mixture contained labelled DNA fragments (about 4,000 cpm), purified BetI*, and standard binding solution (50 mM KCl, 0.5 mM dithiothreitol, 0.1 μg of sonicated calf thymus DNA (Pharmacia) μl^{-1}, 0.1% Nonidet P-40 (Sigma), 8% Ficoll-400, 10 mM Tris-HCl [pH 9.0]). In different experiments, the 20-μl reaction mixture was added to various amounts of BetI* (30 ng to 3 μg), and when indicated, it was modified to contain various amounts of choline (100 nM to 100 mM) and increased amounts of KCl (100 to 400 mM). For formation of the BetI*-DNA complex, the reaction mixture was incubated on ice for 20 min. Loading solution (2 μl) (0.2% bromophenol blue, 0.2% xylene cyanol, and 15% Ficoll-400 in water) was then added, and the samples were loaded onto a 6% polyacrylamide gel in half-strength Tris-borate-EDTA. The samples were subjected to electrophoresis at 4°C. Autoradiography of dried gels was carried out at −70°C. For determination of the apparent K_d values, complex formation was determined by densitometric analysis of the dried gels as described for labelling of Bet proteins (see above).

DNase I footprinting analysis. DNase I footprinting on the coding strand of *betT* was carried out as described by Schmitz and Galas (36). A 134-bp operator fragment was labelled in the 5′ end of the *betT* strand (see Fig. 1B). The fragment was incubated for 20 min at 0°C with or without a saturating amount (9 μg) of purified BetI* in 40 μl of standard binding solution (described above) with or without 100 μM choline added. The samples were equilibrated at room temperature for 2 to 3 min, and 4 μl of a solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added. After 1 min, 0.8 or 0.3 U of DNase I (Pharmacia) was added. The digestion was allowed to proceed for 1 min at room temperature and was stopped by the addition of 150 μl of stop solution (50 μl of phenol, 50 μl of chloroform-isoamyl alcohol [24:1], and 50 μl of 5 mM EDTA in water). The samples were immediately vortexed for 15 s. The phenol extraction was followed by one extraction with 100 μl of chloroform-isoamyl alcohol (24:1), and the nucleotides were precipitated by the addition of 1 μl of glycogen (10 mg ml^{-1}), 10 μl of 3 M sodium acetate, and 220 μl of 95% ethanol. The precipitate was washed with 70% ethanol, resuspended in 3 μl of formamide loading buffer (United States Biochemicals), heat denatured, and loaded on a DNA sequencing gel (4.5% polyacrylamide and 6 M urea in Tris-borate-EDTA). As a size marker, a Maxam-Gilbert (27) sequencing ladder (A+G) was generated by using the labelled 134-bp fragment. The dried gels were exposed to autoradiography for 4 to 10 days at room temperature.

RESULTS

Expression of BetI. In order to visualize BetI and investigate the in vivo stability of BetI and the relative amounts of Bet proteins expressed from a *betIBA* transcript, DNA fragments extending from the *Mse*I site in the −10 box of the *betI* promoter (Fig. 1) and encompassing only the *betI* gene (pTP22) or the entire *betIBA* operon (pTP222) were cloned behind the T7 promoter of the vector pBluescript II KS(+). The Bet proteins were expressed, labelled with [^{35}S]methionine, and visualized by SDS-PAGE and autoradiography essentially as described by Tabor and Richardson (39). The experiments were performed with cells grown in medium 63 with or without exposure to osmotic stress generated with 0.3 M NaCl.

Cells of BL21(pGP1-2, pTP22) and BL21(pGP1-2, pTP222) both produced a labelled protein with an apparent mass of 20 kDa, which corresponds to the deduced mass of BetI of 21.8 kDa. This protein band was not seen for the *bet* negative-control cells of BL21[pGP1-2, pBluescript II KS(+)] (Fig. 2A). The BetI protein has not been visualized previously.

Cells containing plasmid pTP222 also produced two strongly labelled proteins with apparent masses of 63 and 54 kDa, which correspond to the deduced masses of BetA (61.9 kDa) and BetB (52.8 kDa), respectively (Fig. 2A). By taking into account the deduced methionine contents of the Bet proteins,

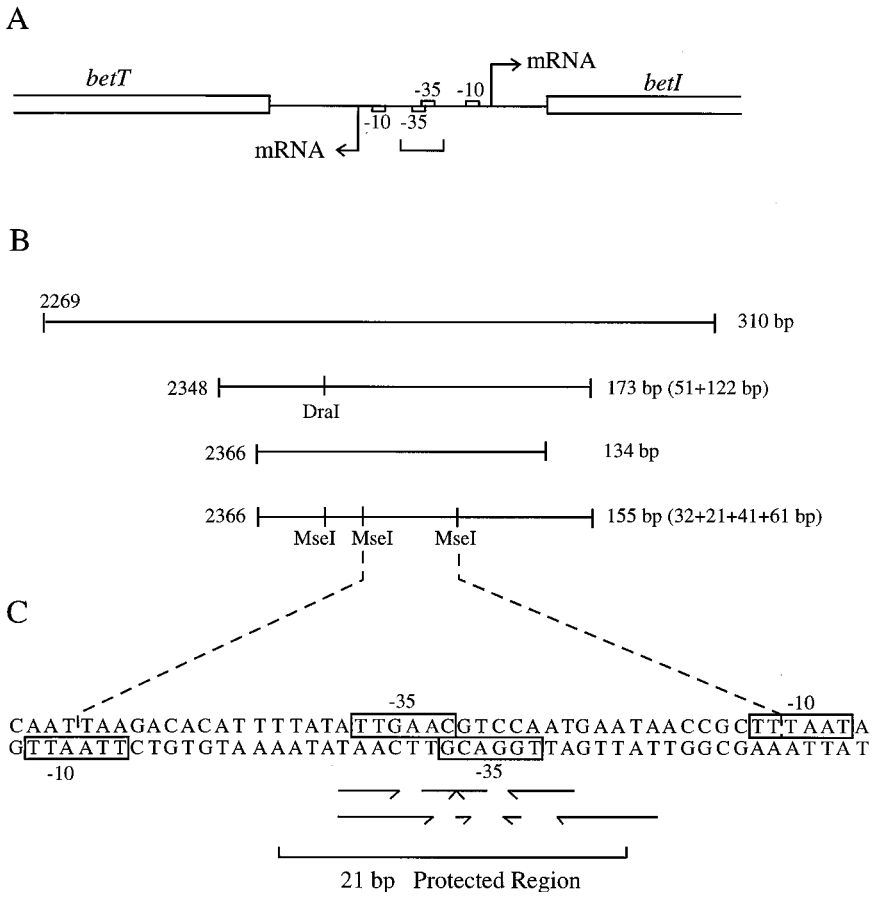

FIG. 1. Schematic presentation of the intercistronic region of *betT* and *betI*. (A) Physical map of the *betI* and *betT* promoters as published in the accompanying paper (20). The bracket shows the binding site of BetI*. (B) Extension and location of PCR-generated DNA fragments used in this investigation. Restriction sites used to cleave the fragments are marked. Lengths of fragments and subfragments (in parentheses) are shown on the right. Coordinates of the left ends of fragments are numbered according to the numbering of the DNA sequence published previously (19). (C) Sequence of the BetI operator and its flanking DNA. Nucleotides protected by BetI* in DNase I footprinting assays are indicated by the bracket, and sequences within this region with dyad symmetry are indicated by half-arrows. In gel mobility shift assays, BetI* formed a complex with the 41-bp *Mse*I fragment (indicated by dashed lines).

densitometric analysis of the protein bands showed that the production of BetI was 10% of the production of BetA or BetB in these cells. Within the range of accuracy there was no difference in the production of BetA and BetB. The same ratios were obtained for cells grown in the presence or in the absence of osmotic stress, showing that the posttranscriptional control is independent of the osmotic conditions of the cell.

When labelled cells of BL21(pGP1-2, pTP22) were chased with an excess amount of unlabelled methionine, the labelled BetI protein remained stable. Densitometric analysis did not show any reduction of the BetI protein band during 1 h of incubation of the cells at either high or low osmolarity. (Data obtained at high osmolarity are shown in Fig. 2B.) Thus, the observed differences in the labelling of the Bet proteins seem to reflect a different rate of synthesis rather than a low level of stability of BetI.

Purification of a genetically modified BetI protein. In plasmid pTP210, *betI* was linked in frame with the gene for GST in the vector pGEX-KT (12). The link was made by using an artificial *Eco*RI site which was previously generated 11 bp upstream of the deduced start codon of *betI* (20). It has previously been reported that the ability to produce a GST fusion protein varies among different laboratory strains of *E. coli* K-12 in an apparently unpredictable manner (10). By screening strains in our laboratory collection, we found that newly transformed cells of W1485(pTP210) displayed a high level of production of the fusion protein after induction of the *tac* promoter with IPTG. The fusion protein was purified from cell extracts by affinity chromatography. After cleavage with the site-specific protease thrombin, a modified BetI protein with 7 extra N-terminal amino acids (Gly-Ser-Pro-Gly-Ile-Pro-Lys) was isolated. This protein was called BetI*. It displayed an apparent molecular mass of 21 kDa, which is in good agreement with the calculated mass of 22.4 kDa. The amino acid composition of BetI* as determined by amino acid analysis corresponded to the composition expected from the DNA sequence. Judging from SDS-PAGE analyzed by silver staining, BetI* was 95% pure (data not shown). Typically, 0.5 mg of purified BetI* was obtained from 250 ml of culture by using the procedure described above. The yield was limited by a weak binding of the GST-BetI* fusion protein to the affinity matrix.

Binding of BetI* to DNA. We have shown that the promoter activity of a 173-bp fragment (Fig. 1B) is repressed by BetI in vivo (20). The binding specificity of BetI* to this fragment was tested in vitro by performing gel mobility shift assays. The fragment was labelled with ^{32}P on both ends and partially digested with *Dra*I to yield subfragments of 51 and 122 bp (Fig. 1B). Figure 3 shows that BetI* shifted the mobility of the 122-

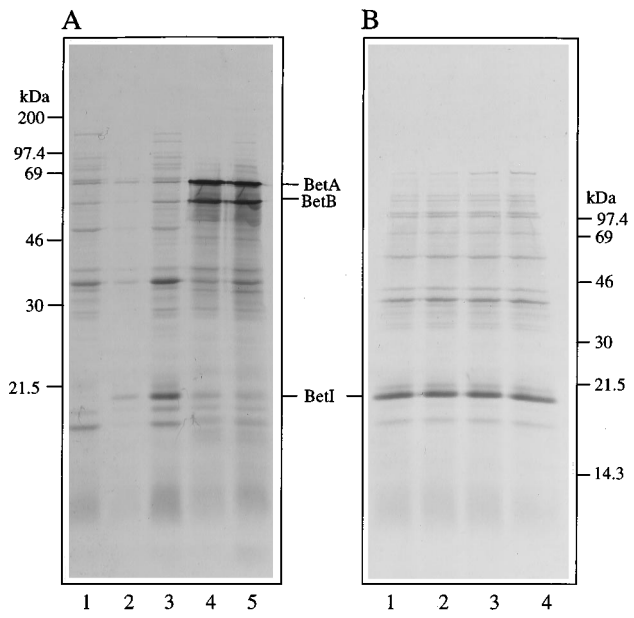

FIG. 2. Expression analysis of the *betIBA* operon. Expression of the *bet* genes from a T7 promoter and labelling of the Bet proteins were performed with cells grown in medium 63 without or with 0.3 M NaCl added. The molecular mass standards and the Bet protein bands are indicated on the autoradiograms generated after SDS–12.5% PAGE. (A) Autoradiogram of cells labelled for 5 min with [^{35}S]methionine. Shown are proteins of unstressed BL21[pGP1-2, pBluescript II KS(+)] (lane 1), osmotically stressed (lane 2) and unstressed (lane 3) BL21(pGP1-2, pTP22 *betI*$^+$), and osmotically stressed (lane 4) and unstressed (lane 5) BL21(pGP1-2, pTP222 *betIBA*$^+$). (B) Autoradiogram of proteins of osmotically stressed BL21(pGP1-2, pTP22 *betI*$^+$) pulse labelled with [^{35}S]methionine for 1 min (lane 1) and chased with an excess amount of cold methionine for 5 min (lane 2), 10 min (lane 3) or 60 min (lane 4).

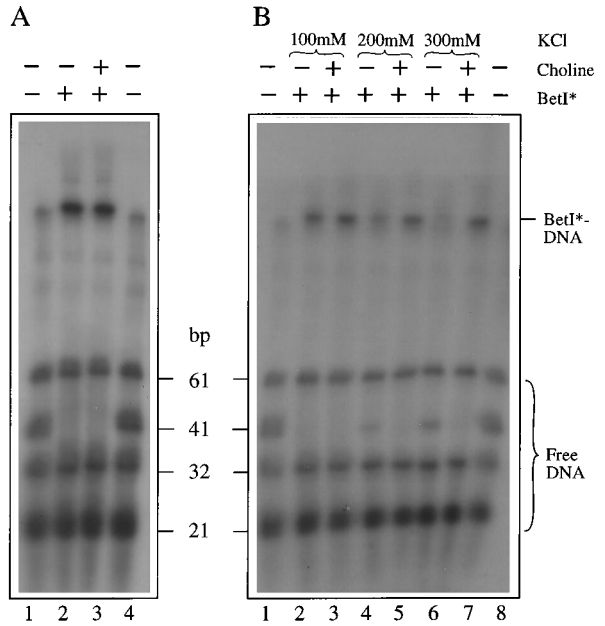

FIG. 4. BetI* DNA-binding assays localizing a binding site of BetI* to a 41-bp *Mse*I intercistronic fragment (Fig. 1C) and demonstrating a positive effect of choline on complex formation. The fragment of 155 bp was generated by PCR and digested with *Mse*I to yield subfragments of 21, 32, 41, and 61 bp (Fig. 1B), which were end labelled. Free DNA and the BetI*-DNA complex are indicated. (A) Results of reactions performed in the presence of 50 mM KCl. Lanes 1 and 4 contained no protein. Lanes 2 and 3 contained 3 μg of BetI* in the presence and in the absence of 100 μM choline, respectively. (B) Results of reactions performed with increasing, inhibitory concentrations of KCl. Lanes 1 and 8 contained no protein. Lanes 2 to 7 contained reaction mixtures with 0.4 μg of BetI* and 100, 200, or 300 mM KCl (as indicated). To the reaction mixtures in lanes 3, 5, and 7, choline (100 μM) was added, as indicated above the lanes.

and 173-bp fragments. The binding of BetI* to these fragments was hindered by the addition of excess amounts of unlabelled corresponding DNA but not by the addition of unrelated plasmid DNA. The mobility of the 51-bp fragment which does not contain the *bet* promoter elements was not shifted. Thus, this fragment served as an internal standard for the specificity of BetI* binding.

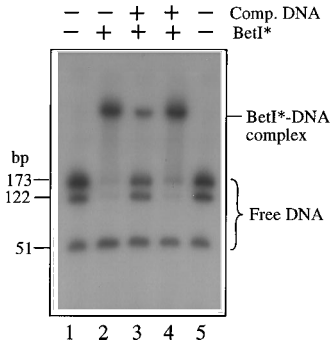

FIG. 3. Gel mobility shift assay demonstrating the specificity of the binding of BetI* to the intercistronic region of the *bet* genes. The PCR-generated fragment of 173 bp which was labelled at both ends was partially digested with *Dra*I to give subfragments of 51 and 122 bp (Fig. 1B). Lanes 1 and 5 contained no protein. Shown are results of reactions with 0.3 μg of purified BetI* (lane 2) and with the addition of excess amounts of unlabelled related DNA (the 173-bp fragment, 10 ng μl^{-1}) (lane 3) or unrelated plasmid DNA (pGEM-3Zf(+), 30 ng μl^{-1}) (lane 4). Free DNA and BetI* complexes formed with the DNA fragments of 122 and 173 bp are indicated.

A fragment of 155 bp also containing the promoter region was digested with *Mse*I to yield subfragments of 61, 41, 32, and 21 bp (Fig. 1B). Figure 4A shows that BetI* caused a mobility shift of only the 41-bp fragment. This fragment contains the region extending from the −10 box of *betT* to the −10 box of *betI* (Fig. 1C).

Choline stimulates formation of the BetI*-DNA complex. Previous in vivo experiments show that choline causes BetI-dependent induction of the *bet* genes in osmotically stressed cells (20). However, the present gel mobility shift analysis revealed that choline did not hinder but, rather, stimulated BetI*-DNA complex formation. This positive effect could be visualized in binding studies performed with low BetI* concentrations or with high salt concentrations.

At the particular BetI* concentration used in the gel mobility shift analysis depicted in Fig. 5A, hardly any mobility shift occurred in the absence of choline. However, complex formation did occur when choline was added to the reaction mixture. The lowest concentration of choline tested which gave a positive effect was 100 nM, but the effect of choline was more pronounced when the concentration was increased above 1 μM. It should be noted that in this experiment, which was performed in the presence of 50 mM KCl, complex formation still occurred even when the ligand concentration was raised to 100 mM.

It is well known that high salt concentrations counteract specific protein-DNA interactions in vitro (34). To further visualize the positive effect of choline, Fig. 5B shows the result of gel mobility shift assays which were performed with increasing inhibitory concentrations of KCl (100 to 400 mM) in the

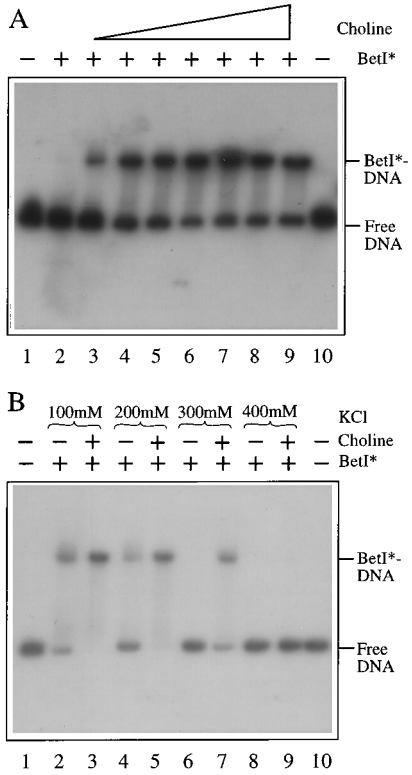

FIG. 5. Gel mobility shift assays demonstrating a positive effect of choline on complex formation with purified BetI*. The labelled fragment was generated by PCR. Free DNA (134-bp fragment, Fig. 1B) and the BetI*-DNA complex are indicated. (A) Results of experiments performed with a limiting amount of BetI* and increasing concentrations of choline. The KCl concentration was 50 mM. Lanes 1 and 10 contained no protein. Lanes 2 to 9 contained reaction mixtures with 0.1 μg of BetI* and 0, 100 nM, 1 μM, 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM choline, respectively. (B) Results of experiments performed with increasing, inhibitory concentrations of KCl with and without 100 μM choline added. Lanes 1 and 10 contained no protein. Lanes 2 to 9 contained reaction mixtures with 0.3 μg of BetI* and 100, 200, 300, or 400 mM KCl (as indicated). To the reaction mixtures in lanes 3, 5, 7, and 9, choline (100 μM) was added, as indicated above the lanes.

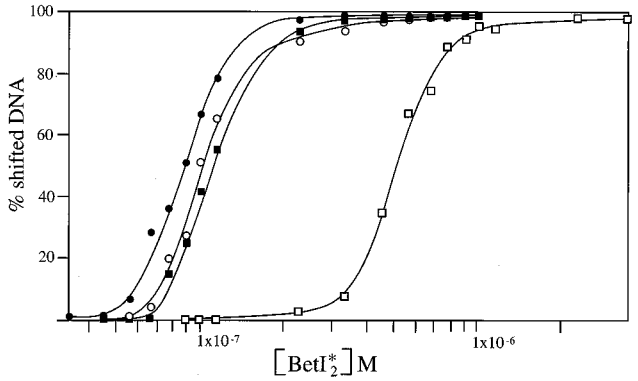

FIG. 6. Influence of KCl and choline on the formation of BetI*-DNA complex. The curves represent typical results obtained by gel mobility shift assays performed with a constant amount of DNA (134-bp fragment, Fig. 1B) and increasing concentrations of purified BetI*. The molarities of BetI* in the reaction mixtures were calculated by assuming that the protein formed a dimer. The percentage of DNA shifted by BetI*, obtained through densitometry, was plotted as a function of log [BetI*$_2$]. Shown are the gel shifts observed in the presence of 50 mM KCl (○), 50 mM KCl and 100 μM choline (●), 300 mM KCl (□), and 300 mM KCl and 100 μM choline (■).

presence or in the absence of choline (100 μM). It appeared that the addition of choline restored the mobility shift, even when the BetI*-DNA interaction was completely prevented by 300 mM KCl. However, in the presence of 400 mM KCl, a mobility shift did not occur either in the presence or in the absence of choline.

The experiments described above were performed with a DNA fragment of 134 bp of the intergenic region (Fig. 1). Gel mobility shift assays with choline and inhibitory concentrations of KCl (100 to 300 mM) were also performed with the mixture of *Mse*I fragments (see above). Figure 4B shows that choline promoted a mobility shift of only the 41-bp fragment. The specificity of BetI* for the 41-bp fragment was also seen when the binding study was performed at a low KCl concentration (50 mM) in the presence of choline (Fig. 4A). Thus, it can be concluded that choline promoted a specific binding of BetI* to the operator and not merely an increased unspecific binding.

The size of the largest *bet* fragment tested in gel mobility shift assays was 310 bp (Fig. 1B). Similar to all smaller fragments tested, this fragment gave only one mobility shift both in the presence and in the absence of choline (data not shown), indicating that BetI* formed only one stable complex with DNA of the *bet* promoter region. The positive effect of choline was also seen when complex formation was inhibited with NaCl or potassium glutamate. With increasing BetI* concentrations, the BetI*-DNA complex displayed a gradually increased mobility shift, but in all cases only one complex was seen (data not shown).

Apparent equilibrium dissociation constants (K_d) for the BetI*-DNA complex. In order to quantify the affinity of BetI* for the target DNA and the influence of KCl and choline on the complex formation, the apparent equilibrium dissociation constants (K_d) for the BetI*-DNA complex were determined by performing gel mobility shift analysis under various conditions and subjecting the dried gels to densitometric analysis. For each set of conditions, the amounts of DNA, KCl (50 or 300 mM), and choline (0 or 100 μM) were kept constant and the amount of BetI* was varied. Three independent measurements were made for each set of conditions. Data were analyzed by plotting the percentage of DNA shifted versus log [BetI*$_2$]. Typical examples of the plots obtained are shown in Fig. 6. In the semilogarithmic plots, the apparent K_d is the concentration of BetI* at which 50% of the DNA forms a complex.

It appeared that in the presence of 50 mM KCl, choline did not significantly reduce the measured average values for K_d. However, in each individual experimental series, complex formation occurred at a somewhat lower BetI* concentration in the presence than in the absence of choline. The average K_d values determined with 50 mM KCl added were 95 ± 5 and 110 ± 10 nM, respectively, in the presence and in the absence of choline. With 300 mM KCl added to the reaction mixtures, the positive effect of choline on complex formation was more pronounced (Fig. 6). The average K_d values determined were 120 ± 25 and 420 ± 150 nM with and without choline, respectively. The K_d values were calculated by assuming that BetI* formed a dimer (see below).

DNase I footprinting analysis. To further localize the BetI binding site, DNase I footprinting of the *betT* strand was conducted both in the presence and in the absence of choline. It appeared that the −20 to −40 region of the *betT* promoter was strongly protected by BetI*. This region corresponds to the −22 to −42 region of the divergent overlapping *betI* promoter. No differences in the band pattern of the sequencing gel were

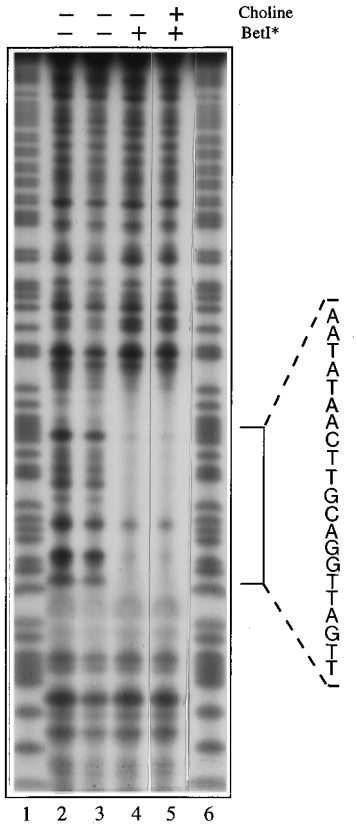

FIG. 7. BetI* DNase I footprinting on the *betT* coding strand. Shown are the results of a Maxam-Gilbert (27) sequencing reaction (A+G) of the 134-bp fragment (lanes 1 and 6). In lanes 2 to 5, the labelled 134-bp fragment was present at a constant amount. Lanes 2 and 3 show DNase I cleavage of the target DNA without protein, using 0.8 and 0.3 U of DNase I, respectively. In lanes 4 and 5, the target DNA was treated with DNase I (0.3 U) when complexed with a saturating amount of BetI* (9 μg) without and with 100 μM choline added, respectively, as indicated above the lanes. The protected region is indicated by the bracket, and the DNA sequence of this area is shown.

observed whether or not 100 μM choline was present in the reaction mixture (Fig. 7).

Inspection of the nucleotide sequence of the protected area revealed the presence of two sequences of dyad symmetry which are candidates for the recognition site of BetI: (−36) ATTGGAC*GTTCAAT and (−41) TATTGATTGG*ACGT TCAATA. The asterisks show the centers of symmetry. The underlined bases are indicated with half-arrows in Fig. 1C. The numbers in parentheses refer to the start position relative to the *betT* transcript. The existence of sequences of dyad symmetry indicates that BetI binds to the protected region as a dimer (or tetramer).

DISCUSSION

In the accompanying paper we report that the divergently overlapping *betI* and *betT* promoters display sequence similarity and that in vivo both promoters are negatively regulated in the same manner by the choline-sensing repressor BetI. The activities of the *bet* promoters were always found to be higher in a *betI* than in a *betI*$^+$ background, but the repression mediated by BetI was reduced in response to an external supply of choline (20). In the present investigation, we report that purified BetI*, which is a genetically modified BetI protein with 7 extra N-terminal amino acids, can specifically bind to the *bet* promoter region at its axis of symmetry. We also report that the inducer choline caused a somewhat enhanced binding of BetI* to the operator in vitro. Within the resolution of our DNase I footprinting and gel mobility shift analyses, the observed binding site of BetI* was the same in the absence and in the presence of choline. The possibility that the prolonged N-terminal end of BetI* affected the binding properties cannot be excluded, but we believe that our in vitro data indicate the in vivo mechanism of choline regulation. Our interpretation of the present data is therefore that BetI is unusual among negative regulatory proteins in that it is bound to its operator site even during induction.

Other known prokaryotic regulators which remain bound to the operator independently of the presence or the absence of ligand are the members of the MerR and the LysR families. MerR binds to a single operator site at the overlapping *merR* and *merT* promoters both in the presence and in the absence of the inducer Hg^{2+}. In the absence of the ligand, MerR negatively autoregulates its own expression and functions as a repressor of the target genes. The presence of ligand switches MerR to become an activator of the target genes, whereas it remains a repressor of its own gene (32). The mechanism of activation appears to be a stereospecific modulation of the DNA-protein complex (reduced DNA bending and underwinding) caused by the inducer, promoting the formation of an activated transcription complex (2).

The LysR family of regulators is diverse, but they all have certain common structural characteristics. Almost all negatively autoregulate their own expression, and many are transcribed divergently to target genes. Typically, they have two binding sites located in the overlapping promoter region for independent regulation of each transcript, and they are activators of the target genes in the presence of ligand, which does not greatly affect their affinity for DNA (references 21, 35, and 42 and references therein). It is notable that LysR regulators which regulate several target genes may act as repressors for a subset of these genes (35, 37). A few have been identified as repressors only of their target genes (NolR [6], CitR [16], and CatM [30]). Furthermore, some are multifunctional regulatory proteins with a dual, repressor-activator function for the same target genes (GbpR [7], NocR [26], and GcvA [43]). DNA modulation has been reported for many of the regulators belonging to the LysR family (AmpR [4], CynR [21], CatR [33], and OccR [41]). For OxyR, the ability to activate transcription seems to correlate with the ability to bind cooperatively with RNA polymerase (17).

On the basis of its amino acid sequence and mode of action, BetI seems to represent a new type of prokaryotic repressor. BetI-mediated regulation is distinguished from that of MerR and LysR in that both target promoters are regulated in the same manner from a single operator site. This mode of action correlates with the findings that the relative position of the BetI operator is similar in both orientations (i.e., it covers the −35 boxes of both the *betT* and *betI* promoters) and that the position of the binding site apparently is unaffected by the presence of choline. The present in vitro data revealed only one DNase I protected region and the formation of only one BetI*-DNA complex for the promoter fragments tested in gel mobility shift assays. However, an increased retardation of this complex was seen when elevated BetI* concentrations were used in gel mobility shift assays (data not presented). It should be noted that the in vivo experiments presented in the accompanying paper showed that overproduction of BetI caused a more pronounced repression of longer (e.g., 173-bp) promoter fragments which were linked to a *cat* reporter gene on a multicopy plasmid than of shorter (89-bp) ones. Furthermore,

overproduction of BetI resulted in a strong repression of the *bet* expression, also in the presence of choline (20). These data suggest that BetI, at least when present in unphysiologically large amounts, may bind to an extended region of *bet* or aggregate with the BetI protein already bound to the major operator site.

The biological advantages, if any, of BetI residing on the operator in both repressed and induced states are not obvious. This mode of action implies, however, that BetI is needed both in the presence and in the absence of choline, which may be a reason why *betI* is the first gene in the *betIBA* operon. But BetI is obviously produced in lesser amounts than the BetA and BetB dehydrogenase proteins, as would be expected for a regulatory protein. The latter finding is in agreement with the previous data from experiments with minicells, in which [^{35}S]methionine-labelled BetA and BetB, but not BetI, were detected (19).

Compared with those observed for most regulators, the apparent dissociation constant for BetI* binding to its operator is rather high (100 nM under optimal in vitro conditions). We cannot exclude the possibility that the seemingly high apparent K_d was caused by the extra N-terminal amino acid residues of BetI* or partial inactivation during the purification procedure, but the low affinity may, rather, reflect that there is always a high BetI level in the cells. From previous reports, the cytoplasmic water content of *E. coli* cells is shown to be 2.6 μl per mg of cell protein (23); the BetB activities of induced and repressed cells are 0.1 and 0.01 U per mg of cell protein, respectively, at 37°C (22); the specific activity of BetB is about 100 U per mg at 37°C (calculated from measurements made at 25°C [8]); and the mass of BetB is 52.8 kDa (19). It can thus be calculated that the cytoplasmic content of the BetB monomer is about 1 to 10 μM. Since the content of BetI is 10% of this amount (see above), the estimated concentration of the BetI dimer varies from 50 to 500 nM in repressed and induced cells, respectively. These values for the BetI concentration are close to the observed K_d, although the local concentration of BetI near the promoter may be higher than the calculated average for the cytoplasm. Thus, within the range of accuracy of these calculations, the proposed mode of action for BetI seems plausible.

It has been suggested previously that potassium glutamate, which is accumulated in osmotically stressed cells, may be a central signal for induction of osmotically regulated genes (13). A proposed mechanism of osmotic induction of *proU* is that potassium glutamate reduces the binding of H-NS to the *proU* promoter regulatory region (40). Along this line of thinking, we have considered the possibility that a hindrance of BetI binding to the operator by elevated cytoplasmic concentrations of potassium glutamate could contribute to osmotic induction of the *bet* promoters in vivo. However, on the basis of our finding that the *bet* promoters remain fully osmotically regulated in a *betI* mutant (20), together with the observation that the increase in K_d of BetI* caused by increasing [K^+] from 50 to 300 mM was rather small (fourfold) and nearly abolished by choline, we have discarded this explanation. Thus, although this salt effect was useful in our attempt to elucidate the effect of choline on BetI-DNA interaction, we consider the decreased in vitro BetI*-binding affinity caused by elevated salt concentrations to be unimportant for the in vivo osmoregulation of the *bet* promoters.

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