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The Active Form of the KorB Protein Encoded by the *Streptomyces* Plasmid pIJ101 Is a Processed Product That Binds Differentially to the Two Promoters It Regulates

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The *korB* gene of *Streptomyces lividans* plasmid pIJ101 is known to encode an autoregulated protein that also represses transcription of a gene, *kilB*, implicated in pIJ101 transfer and in spreading of the plasmid along mycelia of the recipient. Earlier work has indicated that the primary gene product of *korB* is a 10-kDa protein predicted from the gene sequence (D. S. Stein and S. N. Cohen, Mol. Gen. Genet. 222:337–344, 1990; S. Zamen, H. Richards, and J. Ward, Nucleic Acids Res. 20:3693–3700, 1992). We report here that the 10-kDa KorB protein product is processed in vivo into a 6-kDa peptide that has a 20-fold-greater binding affinity for its operator-promoter target; in addition, the 6-kDa peptide binds differentially to the regulatory regions of the two genes it controls, showing 50-fold-greater affinity for the *kilB* sequence. While both the processed and unprocessed forms of KorB were observed in *Escherichia coli* following *korB* gene expression under control of the bacteriophage T7 promoter, only the 6-kDa peptide was found in *S. lividans* containing pIJ101, implying that this peptide is normally the biologically active form of KorB. The footprint resulting from KorB binding to the *korB* operator sequence overlaps the *sti* locus, which affects pIJ101 copy number and incompatibility as well as the size of zones of inhibited recipient cell growth ("pocks") that form around donor cells during mating. The observed ability of the *korB* gene product to interact with both *sti* sequences and the *kilB* promoter region suggests that it may have a role in coordinating the replication and intramycelial spread of plasmids during and/or following bacterial mating.

Streptomyces spp. are gram-positive sporulating mycelial procaryotes that undergo a process of complex morphological differentiation that leads to the production of spores. The 8.9-kb plasmid pIJ101 found originally in *Streptomyces lividans* ISP5434 (14) is one of the most extensively studied and widely used of streptomycete extrachromosomal elements. The plasmid has been sequenced in its entirety, and regions associated with key plasmid functions such as replication and transfer have been identified (12, 13). Two of the loci involved in plasmid transfer, *tra* (formally *kilA*) and *kilB*, cannot be cloned in *S. lividans* on pIJ101-derived replicons unless corresponding *kil*-override (*kor*) functions are also present either in *cis* or in *trans*. It has been proposed (12) that *tra* is involved in the initial transfer event, while *kilB* and the *spd* genes are needed for spread of the plasmid along the mycelia after initial transfer has occurred. Deletions in *tra* prevent the appearance of "pocks," i.e., zones of growth inhibition of recipient cells that form around donor cells during mating, while mutations in *kilB* reduce pock size (12). Earlier work has shown that each of the *kor* genes encodes a protein that negatively regulates both its own expression and expression of the corresponding *kil* function at the transcriptional level (27). Thus, the autoregulated *kil-kor* system of pIJ101 represents an intricately balanced gene control mechanism that accomplishes regulated plasmid transfer, perhaps in response to developmentally determined signals.

Here we report the partial purification and characterization of the KorB protein and the investigation of certain of its properties. We present evidence that posttranslational processing near the carboxyl terminus of KorB generates a 6-kDa product that binds differentially to its respective target sequences in the operator-promoter regions of the *kilB* and *korB* genes. Additionally, we show that the pIJ101 *sti* locus, which previously has been implicated in plasmid DNA replication and incompatibility (5), interacts with the KorB protein and affects the ability of KorB to bind to its own operator sequence.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. lividans* TK64 *pro-2 str-6* SLP2$^-$ SLP3$^-$ (11) was used as the *Streptomyces* host throughout this study. *Escherichia coli* K38 (24) was the host for protein expression and purification, and *E. coli* DH10 (Life Technologies, Inc., Gaithersburg, Md.) was used for plasmid DNA preparation. The pT7-6 vector (28) was a gift from T. Wang. The pMAL-c plasmid was obtained from New England Biolabs, Beverly, Mass. Plasmids pIJ101 (14) and pDSS299 (27) have been described previously.

Construction of pT7-6-*korB* and pMAL-*korB*. A DNA fragment carrying the *korB* gene was isolated as an *Spe*I-*Bgl*II fragment and introduced into the *Xba*I and *Bam*HI sites of pT7-6 to generate pT7-6-*korB*. To generate pMAL-*korB*, an *Nde*I site was first introduced into the first amino acid codon of the *korB* gene sequence by site-directed mutagenesis. In these experiments, an *Spe*I-*Bgl*II fragment carrying the *korB* gene was inserted into a site generated by *Xba*I and *Bam*HI cleavage of an M13 derivative, M13mp18. Single-stranded DNA was obtained, and site-directed mutagenesis was performed according to the procedures of Kunkel et al. (16, 17). An *Nde*I digest was performed, and the 5′ protruding end was digested with mung bean nuclease and then with *Hin*dIII to isolate the *korB* gene. The restriction fragment carrying the *korB* gene was subsequently inserted into a site generated by *Stu*I and *Hin*dIII

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digestion of the pMAL-c vector (18). The MalE-KorB fusion protein expressed from this construct represented 10% of the total cellular protein. After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel slices containing this protein were used to generate a rabbit antibody against KorB. Amylose affinity chromatography was used to purify the MalE-KorB fusion protein (18).

In vivo protein labeling. In vivo labeling of T7-specific protein products was done according to the method described by Tabor and Richardson (28). Following induction of transcription from the T7 promoter and subsequent rifampin treatment to inhibit synthesis of host mRNA (28), total protein was labeled by a 5-min pulse of [^{35}S]methionine (>1,000Ci/mmol; Amersham Co., Arlington Heights, Ill.). The cells were harvested and resuspended in 60 mM Tris-HCl, pH 8, containing 1% SDS, 1% 2-mercaptoethanol, and 10% glycerol. After the mixture was boiled for 3 min to lyse the cells, cell extracts were analyzed by SDS-PAGE (29). Molecular weights of the protein bands were deduced from size standards (Life Technologies, Inc.) (22, 25, 31).

Protein purification and amino acid sequence analysis. For large-scale purification of KorB protein, *E. coli* cells were grown in a HI-Density fermentor (LAB-Line, Melrose Park, Ill.) in 4 liters of SLBH medium (1.1% tryptone, 2.25% yeast extract, and 0.4% glycerol, buffered to pH 7). Frozen cell pellets (20 g) were thawed slowly in an equal volume of 50 mM Tris-HCl, pH 7.4, containing 10% sucrose. The following reagents were added: NaCl to 0.4 M, EDTA to 10 mM, dithiothreitol to 2 mM, phenylmethylsulfonyl fluoride to 0.25 mM, and lysozyme to 400 μg/ml. The mixture was incubated on ice for 1 h and then at 37°C for 15 min. Cell debris was removed by centrifugation at 23,000 × *g* for 1 h at 4°C. The supernatant was dialyzed in 2 liters of TGED buffer (100 mM Tris-HCl [pH 7.9], 1 mM EDTA, 2 mM dithiothreitol, 5% glycerol) containing 25 mM NaCl before being loaded onto a heparin agarose column (20-ml bed volume). The column was washed with the same buffer until no protein was detected in the flowthrough. Bound proteins were eluted with a linear salt gradient (200 ml, 0.025 to 0.8 M NaCl). Active fractions containing *kilB* operator-binding activities were pooled and dialyzed overnight against 10 volumes of TGED buffer containing 0.1 M NaCl and 0.1 M KCl. The dialyzed fractions were loaded onto a *korB* sequence affinity column (5-ml bed volume) equilibrated with the same buffer. The affinity column was prepared according to the procedure of Alberts and Herrick (1); the 600-bp *Sau*3A fragment containing the *korB* operator-promoter sequences described in Fig. 6 was used as the ligand for KorB binding. The column was washed with 200 ml of TGED buffer plus 0.1 M NaCl and 0.1 M KCl before being eluted with a linear salt gradient (50 ml, 0.1 to 1 M NaCl). The *korB*-binding fractions were pooled and stored in small volumes at −80°C. The same purification procedures were followed when *Streptomyces* cultures were used as the source of KorB, except that cells were grown in flasks containing 500 ml of YEME (0.3% yeast extract, 0.5% peptone, 0.3% malt extract, 1% glucose, 34% sucrose) medium and crude extracts were prepared according to the procedure of Thompson et al. (30).

A fraction of the purified KorB protein was transferred onto a Problot membrane (Applied Biosystems, Foster City, Calif.). The region of the membrane that corresponds to the 6-kDa KorB protein was cut out, and Edman degradation was performed in the Protein and Nucleic Acid Laboratory at Stanford University to analyze the sequence of the amino-terminal region of the protein.

Renaturation of proteins from SDS-polyacrylamide gels. The method of Hager and Burgess (9) was employed for renaturation of proteins, except that slab gels instead of cylindrical gels were used and gels usually were not stained before being sliced.

Gel retardation and footprinting assays. DNA fragments carrying *kilB* or *korB* operator-promoter sequences were 5′ end labeled with ^{32}P-deoxynucleotides (>800 Ci/mmol; Amersham Co.) by using the Klenow fragment of DNA polymerase I. *E. coli* cell pellets were resuspended in 25 mM Tris-HCl (pH 8)–5 mM 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride. The cells were sonicated and then centrifuged at 13,000 × *g* for 10 min to remove cell debris.

Binding reactions were done as described by Fried and Crothers (6) with the modification of Miller et al. (20). Protein fractions were added, and the reaction mixtures were incubated at room temperature for 10 min in the binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 0.1 M KCl, 5% glycerol, 10 μg of sonicated salmon sperm DNA [Sigma Chemical, St. Louis, Mo.], and 500 μg of bovine serum albumin [BSA] per ml) and 10,000 cpm of ^{32}P-labeled DNA probe in a volume of 20 μl. The reaction mixtures were loaded onto 5 or 10% nondenaturing polyacrylamide gels (Mini-V8.10 Vertical Gel Electrophoresis System; Life Technologies, Inc.). Electrophoresis was done at 200 V for 45 min to 1 h in 0.5× Tris-borate-EDTA. After electrophoresis, gels were dried and visualized by autoradiography.

The DNase I footprinting method of Galas and Schimitz (8) was modified as follows. The binding reactions were the same as those used for the gel retardation assays except that probes were labeled at only one end. The DNA fragments to be footprinted were digested at one end with appropriate restriction enzymes and end labeled with ^{32}P by using the Klenow fragment of DNA polymerase I; restriction enzyme digestions at the opposing ends were performed to generate DNA fragments in which only one strand was labeled at the 3′ end. After 15 min of incubation, an equal volume of 5 mM $CaCl_2$–10 mM $MgCl_2$ and 5 to 15 ng of DNase I (Worthington Diagnostics, Freehold, N.J.) were added. After 1 min at room temperature, an equal volume of the stop buffer (1% SDS, 20 mM EDTA, 200 mM NaCl, 2 μg of yeast tRNA per ml) was added. The mixtures were phenol extracted, ethanol precipitated, and separated on 8% denaturing polyacrylamide gels. Reactions of DNA sequence ladders containing the target sequences in the regions footprinted were prepared (19) and separated by electrophoresis adjacent to footprinting reactions of the same probe to identify the sites of binding.

Western blot (immunoblot) detection of KorB proteins. Rabbit serum containing KorB-specific polyclonal antibody was obtained from Berkeley Antibody Co. (Richmond, Calif.) and was used without further purification. Protein extracts were separated on 18% high-resolution SDS-polyacrylamide gels (29) before being transferred onto Problot or nitrocellulose membranes. Western blot detection of KorB proteins was performed by using the ProtoBlot AP system (Promega, Madison, Wis.), except that 3% instead of 1% BSA was used in the blocking solution and that a 1:500 dilution of the KorB antibodies was applied in the hybridization.

RESULTS

Synthesis of the *korB* gene product in *E. coli* and partial purification of the KorB protein(s). The *korB* gene product previously has been produced in *E. coli* maxicells (26), in other *E. coli* cells (33), and in vitro by using S30 extracts (33). We employed a bacteriophage T7-based expression system to synthesize sufficient quantities of the KorB protein for purifi-

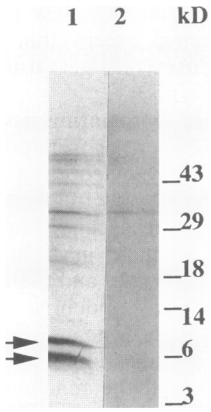

FIG. 1. Identification of the two forms of the KorB protein in *E. coli*. An autoradiogram of an SDS-polyacrylamide gel containing extracts from strains transformed with the pT7-6-*korB* plasmid expressing the *korB* gene products (lane 1) or with the control plasmid, pT7-6, (lane 2) is shown.

cation and study. The plasmid pT7-6-*korB* was constructed, and expression of the KorB protein under the T7 promoter was assessed by SDS-PAGE analysis of [^{35}S]methionine-labeled whole-cell proteins as described in Materials and Methods.

The autoradiogram of the gel for the extract from *E. coli* carrying pT7-6-*korB* (Fig. 1) revealed two distinct protein bands whose presence was dependent on T7-initiated transcription of the *korB* gene insert. One of these bands migrated as a 10-kDa protein, characteristic of the previously detected *korB* gene product (26, 33) and corresponding to the molecular mass predicted from the *korB* gene sequence (26). The other migrated at a position expected for a peptide having a molecular mass of 6 kDa. To understand the nature of these *korB*-dependent gene products, we used gel retardation assays to purify protein(s) able to bind to a 200-bp *Bam*HI-*Bgl*II pUC-pIJ101 DNA fragment containing pIJ101 DNA from the *Sal*I (bp 2613) site to the *Bst*EII (bp 2423) site; this segment contains the *kilB* operator-promoter sequence previously shown to be regulated by KorB (26). Figure 2A shows a flow chart of the purification procedures, which were carried out as described in Materials and Methods. Protein profiles after each purification step are shown in Fig. 2B. Active fractions after purification on the DNA affinity column still contained two protein bands migrating at 10 and 6 kDa (Fig. 2B, lane 1), indicating that both of these *korB*-encoded proteins bind to the *korB* operator-promoter sequence.

Amino acyl sequencing of the 6-kDa protein confirmed that it is a product of the *korB* gene and also suggested that the 6-kDa protein was derived by deletion of the carboxyl-terminal end of the previously identified (26, 33) 10-kDa primary gene product of *korB*. Our sequence showed that the first 10 amino acids of the 6-kDa protein (Thr-Gln-Lys-Thr-Pro-Gly-Glu-Ile-Arg-Ala) correspond to those predicted from the nucleotide sequence to be at the amino-terminal end of KorB, except for the terminal *N*-formylmethionine. To further analyze the 6- and 10-kDa proteins encoded by *korB* in *E. coli*, we made an antibody against a fusion peptide that contained the entire *korB* gene product at the C terminus and the *E. coli* protein MalE at the N terminus (see Materials and Methods). With this antibody, *E. coli* extracts containing approximately equal amounts of the two KorB proteins showed a much stronger signal at the 10-kDa position in Western blots (Fig. 3), suggesting that some of the epitopes in the 10-kDa peptide recognized by the polyclonal antibodies are absent in the 6-kDa product.

Treatment of the MalE-KorB fusion protein with the factor Xa protease derived from bovine plasma revealed two separate bands in SDS-PAGE. One band corresponded to the position of the 40-kDa MalE protein (2), while the other showed a molecular mass of 6 kDa instead of the expected 10-kDa size of the full-length product of the *korB* gene, which had been fused to the *malE* gene in the plasmid construct (Fig. 4). The gel retardation patterns produced by binding of the 6-kDa protein to DNA fragments containing the *korB* or *kilB* operator-promoter region were indistinguishable from those caused by binding of the purified 6-kDa KorB protein derived from the primary *korB* gene product. Since the antibody generated against the fusion protein recognizes principally the 10-kDa form of KorB, as indicated above, our failure to liberate a 10-kDa band from the MalE-KorB fusion protein suggested that the Xa protease can cleave within the KorB protein itself, as well as at the MalE-KorB junction.

In contrast to the two forms of the KorB protein detected in *E. coli* extracts by Western blot analysis, only the 6-kDa protein was detected in extracts of *S. lividans* harboring a pIJ101 derivative, pDSS299, grown in liquid cultures, notwithstanding the much higher affinity that the antibodies have for the 10 kD form (Fig. 3, lane 3). Similarly, crude extracts from pDSS299-containing *S. lividans* that were subjected to the same purification procedure used for *E. coli* extracts showed only the 6-kDa protein form of KorB by gel staining (Fig. 2C). To confirm that the absence of the 10-kDa protein in the *Streptomyces* extract was not due to proteolytic degradation in *S. lividans* during or after cell lysis, *E. coli* cells producing the 10-kDa KorB form were mixed with *S. lividans* cells and sonicated together, and the resulting mixture was analyzed by gel electrophoresis. The 10-kDa protein derived from *E. coli* remained intact in the presence of the *S. lividans* extract (data not shown).

Binding activities of the *korB* gene products and characterization of their binding sites. The *kilB*-specific DNA-binding activities of the 6- and 10-kDa forms of KorB were compared. The two proteins isolated from *E. coli* cells were eluted individually from gel slices after electrophoresis, and their respective activities were analyzed by separate gel shift assays with a DNA fragment containing the *kilB* operator-promoter as target. From the relative intensities of the shifted bands containing the radioactively labeled target fragment bound to the 6- or 10-kDa form of KorB (Fig. 2D, lanes 2 and 3), we estimate that the 6-kDa peptide has about 20 times the binding activity of the 10-kDa species. Consistent with the lesser ability of the 10-kDa form of KorB to bind to its own operator-promoter were observations showing that crude extracts containing both forms of KorB produced only a single band shift characteristic of the 6-kDa product (Fig. 5 and 6); extracts from *S. lividans* cells in which only the 6-kDa form was detected showed a band shift pattern similar to the one seen for *E. coli* extracts containing both the 10- and 6-kDa forms (Fig. 5, lanes 3 and 4, and Fig. 6, lanes 2 and 3).

The relative binding affinities of KorB for the *kilB* and *korB* operator-promoter sequences were determined by analyzing the abilities of the binding sites to compete with each other. Binding of KorB to the *kilB* promoter region was monitored in the presence of various amount of the *korB* promoter sequence and vice versa. As seen in Fig. 7, the presence of the *kilB* operator-promoter sequence at a 100-fold excess eliminated KorB binding to the *korB* target. In contrast, the *korB* promoter sequence, even when present at a 200-fold excess, did not affect binding of KorB to its *kilB* target. Reaction constants

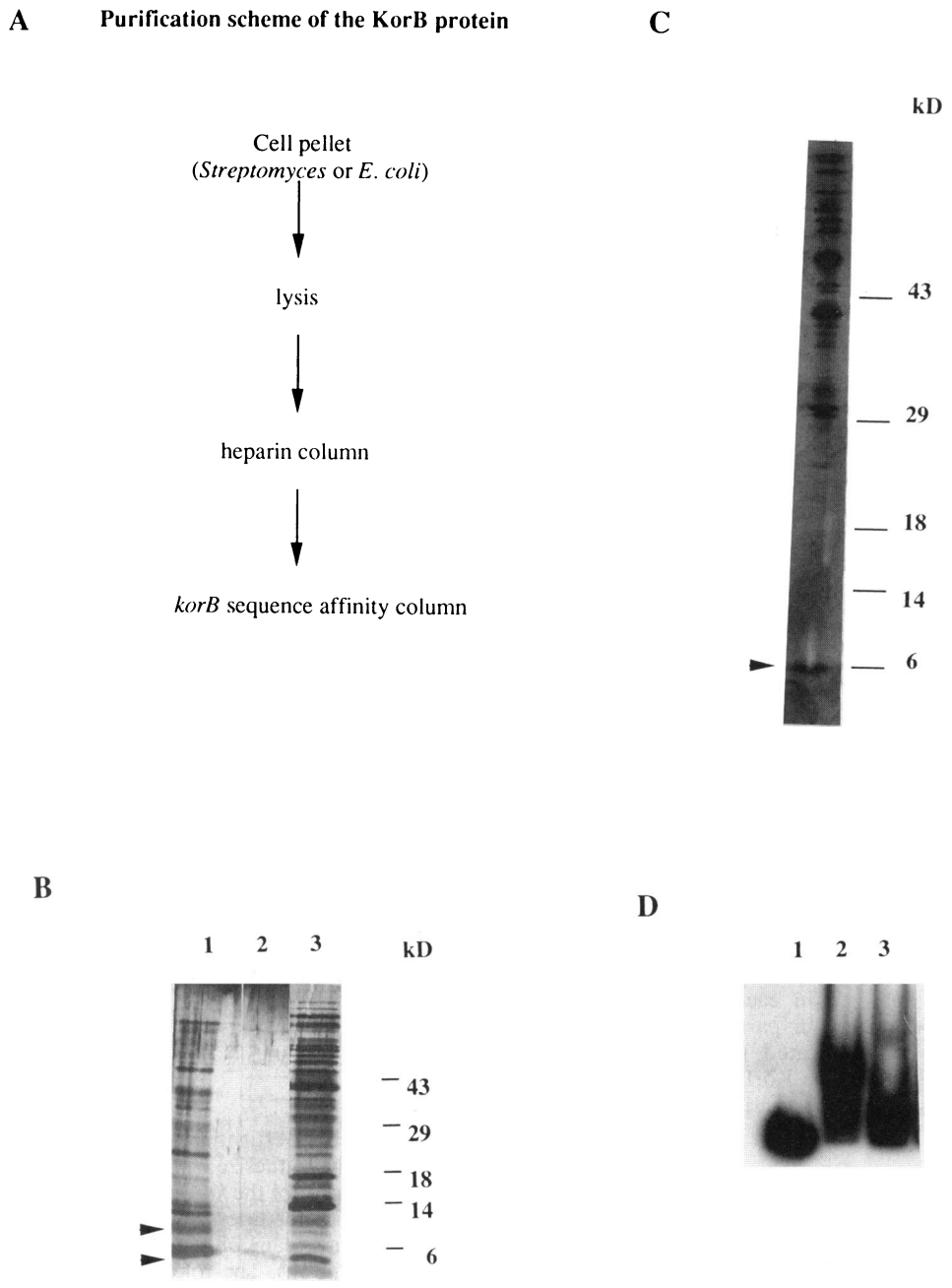

FIG. 2. Partial purification scheme for the KorB proteins and characterization of their DNA-binding activities. (A) Flow chart showing the purification scheme. (B) Active fractions from extracts of *E. coli* cells transformed with pT7-6-*korB*. Following heparin agarose and DNA affinity column chromatography, the active fractions were pooled and separated on an SDS–18% polyacrylamide gel. Lane 3, a fraction from the heparin agarose column that bound to the *kilB* promoter; lane 2, an active fraction collected from the *korB* sequence DNA affinity column; lane 1, the same fraction as in lane 2 but concentrated 50 times by trichloroacetic acid precipitation. The arrowheads show the positions of the two forms of the KorB protein. (C) An extract prepared from *S. lividans* TK64 carrying pDSS299 was subjected to purification by heparin agarose column chromatography. A fraction from the heparin agarose column that possesses activity for binding to both the *kilB* and *korB* operator-promoter regions is shown. The position of the 6-kDa KorB peptide is indicated by the arrowhead. (D) One microgram of each of the two forms of the KorB protein purified from *E. coli* extracts was eluted from an SDS-polyacrylamide gel. The recovered proteins were assayed for their abilities to bind to the *kilB* operator-promoter sequence. Lane 1, migration of the end-labeled *Bam*HI-*Bgl*II fragment (described in Fig. 5) carrying the *kilB* operator-promoter sequences; lanes 2 and 3, migration of the same fragment when mixed with protein recovered from the 6- and 10-kDa bands, respectively.

(*K* values) for KorB binding to the *korB* and *kilB* sequences were calculated from these gels by comparing the percentages of DNA fragment bound to the KorB protein in Fig. 7A, lane 5, and Fig. 7B, lane 4, and assuming that the concentration of the KorB protein in these extracts is constant. The values indicated that the binding affinity of KorB to the *kilB* target sequence was at least 50-fold higher than the binding affinity to the *korB* sequence.

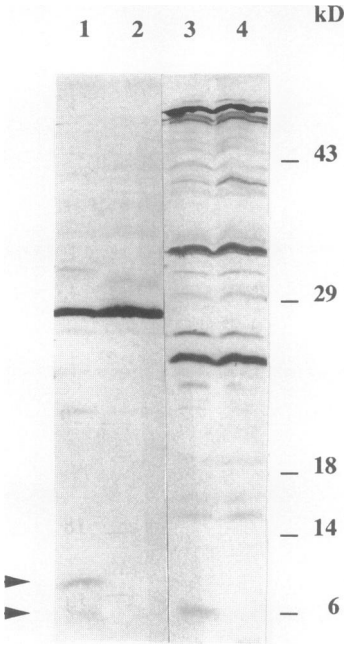

FIG. 3. Western blot analysis showing different forms of the KorB proteins in *E. coli* and *S. lividans*. Twenty micrograms (each) of crude extracts from *E. coli* K38 carrying pT7-6 (lane 2), *E. coli* K38 carrying pT7-6-*korB* (lane 1), *S. lividans* TK64 (lane 4), and *S. lividans* TK64 carrying pDSS299 (lane 3) was separated on an SDS–18% polyacrylamide gel and blotted onto a Problot membrane. The arrowheads show the positions of the 10- and 6-kDa KorB proteins.

Footprinting analysis localized the specific KorB-binding sequences within both promoter regions (Fig. 8) to 70-bp (*korB*) and 65-bp (*kilB*) DNA segments having extensive homology with each other. To ensure that only one protein-DNA complex was analyzed in each experiment, gel shift experiments were performed in parallel to monitor DNA-binding activities. These indicated that only protein-DNA complexes formed by the 6-kDa KorB protein were observed under the conditions of the footprinting assays.

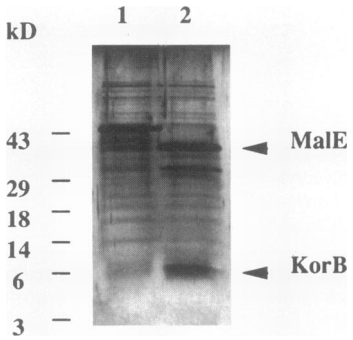

FIG. 4. Factor Xa protease digest of the MalE-KorB fusion protein. One microgram of the MalE-KorB protein partially purified by amylose affinity chromatography (lane 1) was subjected to factor Xa cleavage. The products of the protease digestion, the 40-kDa MalE protein (2) and the 6-kDa KorB peptide, are shown in lane 2. An additional protein band observed in lane 2 shows a molecular mass of 30 kDa, which corresponds to the mass of the large subunit of factor Xa added to the digest (7). The samples were separated on an SDS–18% polyacrylamide gel and silver stained.

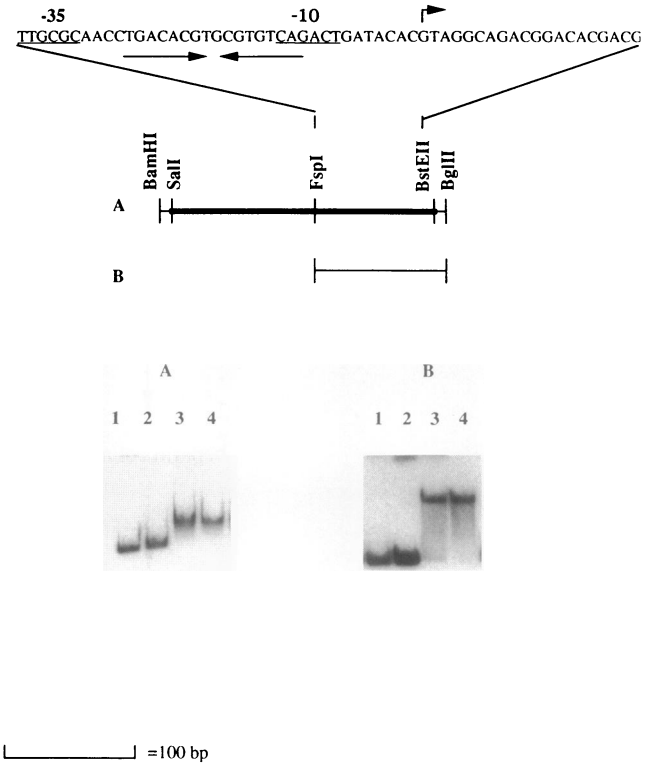

FIG. 5. Gel retardation assay of the KorB protein with *kilB* operator-promoter sequences. Maps of the DNA fragments used are shown at the top. Putative −35 and −10 sequences, the inverted repeat (straight arrows), and the transcription start site (bent arrow) are indicated. The region indicated by a heavy line represents pIJ101 DNA. A ^{32}P-labeled *Bam*HI-*Bgl*II fragment carrying the *Sal*I-*Bst*EII region that carries the two *kilB* promoter sequences (27) (lane 1 in panel A), or the *Fsp*I-*Bgl*II fragment (lane 1 in panel B), which contains only the previously characterized pIJ101A promoter (27), was mixed with *E. coli* extracts containing or lacking KorB (lanes 3 and 2, respectively, in both panels A and B). In both lanes 4, extracts from *S. lividans* TK64 carrying pDSS299 were used. Approximately 10 μg of the crude extract was used in each reaction.

Our initial investigations of KorB-binding sites showed that a chemically synthesized 34-bp DNA fragment containing the inverted repeat of the *korB* promoter (shown as fragment II in Fig. 8B) did not interact with the KorB protein unless the sequence upstream of the *korB* promoter was also present (data not shown). The role of this upstream sequence, which overlaps a genetic locus (*sti*) implicated in pIJ101 replication and incompatibility (5), in the binding of KorB to its own promoter region was investigated by using as target the *korB* inverted repeat attached to different lengths of the adjacent DNA sequence. A chemically synthesized DNA sequence corresponding to the full-length 70-bp segment covered by the KorB footprint (shown as fragment I in Fig. 8B) served as a positive control. As seen in Fig. 9, gel retardation assays showed that only fragments D and E, both of which include sequences upstream of the *korB* promoter, were able to bind to the KorB protein, consistent with our finding that the KorB footprint on the *korB* promoter extends well into the *sti* region.

DISCUSSION

The 243-bp *korB* gene carried by *S. lividans* plasmid pIJ101 is predicted to encode an 80-amino-acid 10-kDa protein. While

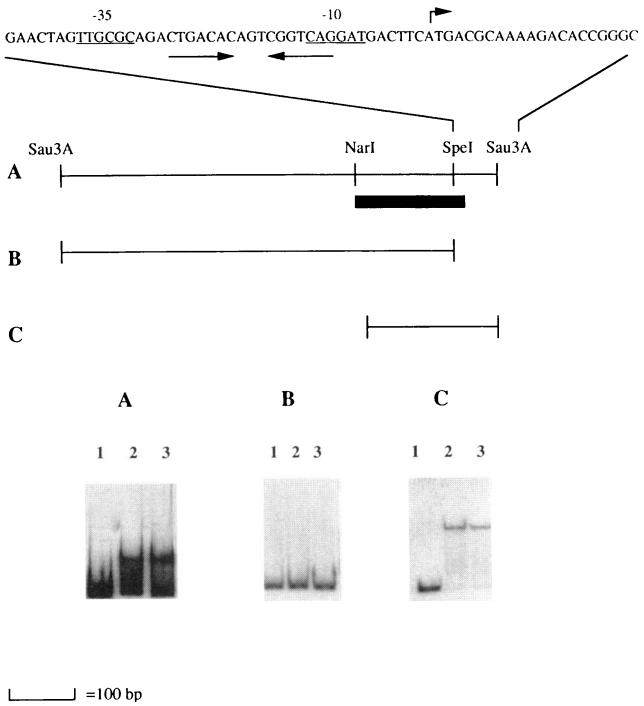

FIG. 6. Gel retardation assay of the KorB protein with the *korB* operator-promoter sequence. Maps of DNA fragments used in the assays are shown at the top. The −35 and −10 regions, the inverted repeat (straight arrows), and the transcription start site (bent arrow) are indicated. Gel shift assays corresponding to each fragment are shown at the bottom. Lanes 1 and 2 on each gel show the migration of the labeled fragment when mixed with *E. coli* extracts lacking or containing KorB, respectively. An extract from *S. lividans* TK64 carrying pDSS299 was used in all lanes 3. The solid bar shows the extent of the *sti* locus as previously described (4).

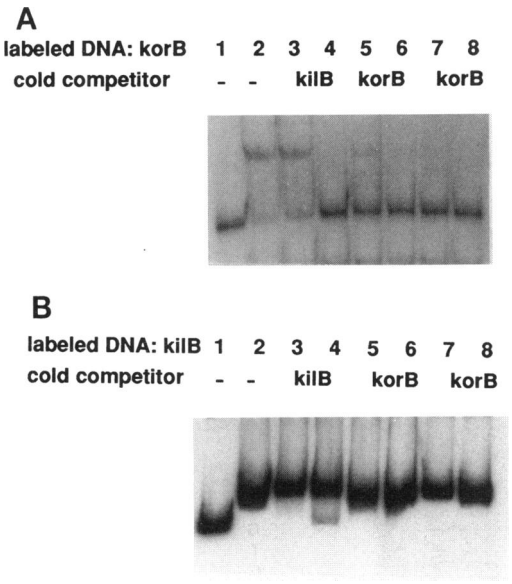

FIG. 7. Competition gel retardation assay. (A) A chemically synthesized DNA fragment carrying the entire sequence included in the KorB footprint (described as fragment I in Fig. 8B) was end labeled. (B) The 200-bp *Bam*HI-*Bgl*II fragment (described in Fig. 5) carrying the *kilB* operator-promoter was labeled. In each case, approximately 10 fmol of the labeled fragment (lanes 1) was mixed with 10 μg of *E. coli* extracts containing KorB (lanes 2), and a nonradioactive DNA fragment carrying the *kilB*-specific sequence was added at 50- and 100-fold molar excesses as the competitor DNA (lanes 3 and 4, respectively). Similarly, a nonradioactive fragment containing the *korB* operator-promoter sequence was added at 50-, 100-, 150-, and 200-fold molar excesses to compete with binding to the labeled fragments (lanes 5, 6, 7, and 8, respectively, in both panels).

a primary gene product of this size does in fact result from *korB* gene expression, our results indicate that the 10-kDa KorB protein is processed posttranslationally to a truncated 6-kDa protein during normal cell growth. Both forms of KorB were found in *E. coli* following expression of the *korB* gene under control of the bacteriophage T7 promoter; however, only the 6-kDa peptide, which was estimated to have a 20-fold-greater binding affinity for target DNA sequences, was detected in *S. lividans* cells. Binding occurred preferentially to the *kilB* promoter, consistent with the biological role of the *korB* gene product as a regulator of the lethal *kilB* gene at intracellular concentrations that still allow its own synthesis.

Protease Xa from bovine plasma is known to cleave preferentially at the tetra-amino-acid sequence Ile-Glu-Gly-Arg but to also be able to cleave at a number of related amino acid sequences (21). Our data show that this protease recognizes an internal site in the 10-kDa KorB protein while leaving the 6-kDa form intact. While we identified a 6-kDa band after digestion of the MalE-KorB fusion protein with protease Xa, the band shift characteristic of the 6-kDa protein was not increased when we treated *E. coli* extracts containing both the 10- and 6-kDa forms with the factor Xa protease (28a), suggesting that internal cleavage of the 10-kDa protein by protease Xa does not occur at precisely the same location as in vivo processing of the protein. These data also imply that the actively binding 6-kDa protein observed after protease Xa digestion of the MalE-KorB fusion protein was generated in vivo by cleavage of the 10-kDa form near its C-terminal end, leaving a 6-kDa fragment bound to MalE.

Results from amino acyl sequencing of the 6-kDa protein support the view that processing of the KorB protein in vivo occurs near the carboxyl-terminal end: we found that the amino-terminal end of the protein is congruent with the amino acid sequence predicted from the sequence of nucleotides in the *korB* gene. The peptide present in the 6-kDa band starts with threonine, the second amino acid in the sequence of the predicted KorB protein.

In previous studies of *korB*-dependent protein synthesis carried out in *E. coli* maxicells (26), only the 10-kDa protein was detected, indicating that significant processing did not occur. Is the production of the 6-kDa form of KorB in the strains reported here an artifact of nonspecific protease activities present in preparations we have used? We believe that this is unlikely because (i) the 10-kDa protein was highly stable during the lysis procedures used, and we did not observe further processing of the 10-kDa protein in vitro in cell extracts; (ii) a significant amount of the 6-kDa KorB protein was detected when extracts were made by adding cells directly to SDS buffers at boiling temperature to avoid protease attacks during cell lysis; and (iii) the 10-kDa product has very weak DNA-binding activity relative to the activity shown by the 6-kDa processed product, implying that most of the biological activity of KorB resides in the processed form. Our finding that only the 6-kDa form was found in *S. lividans* grown under conditions in which plasmid transfer characteristically does not occur (10) is consistent with this notion.

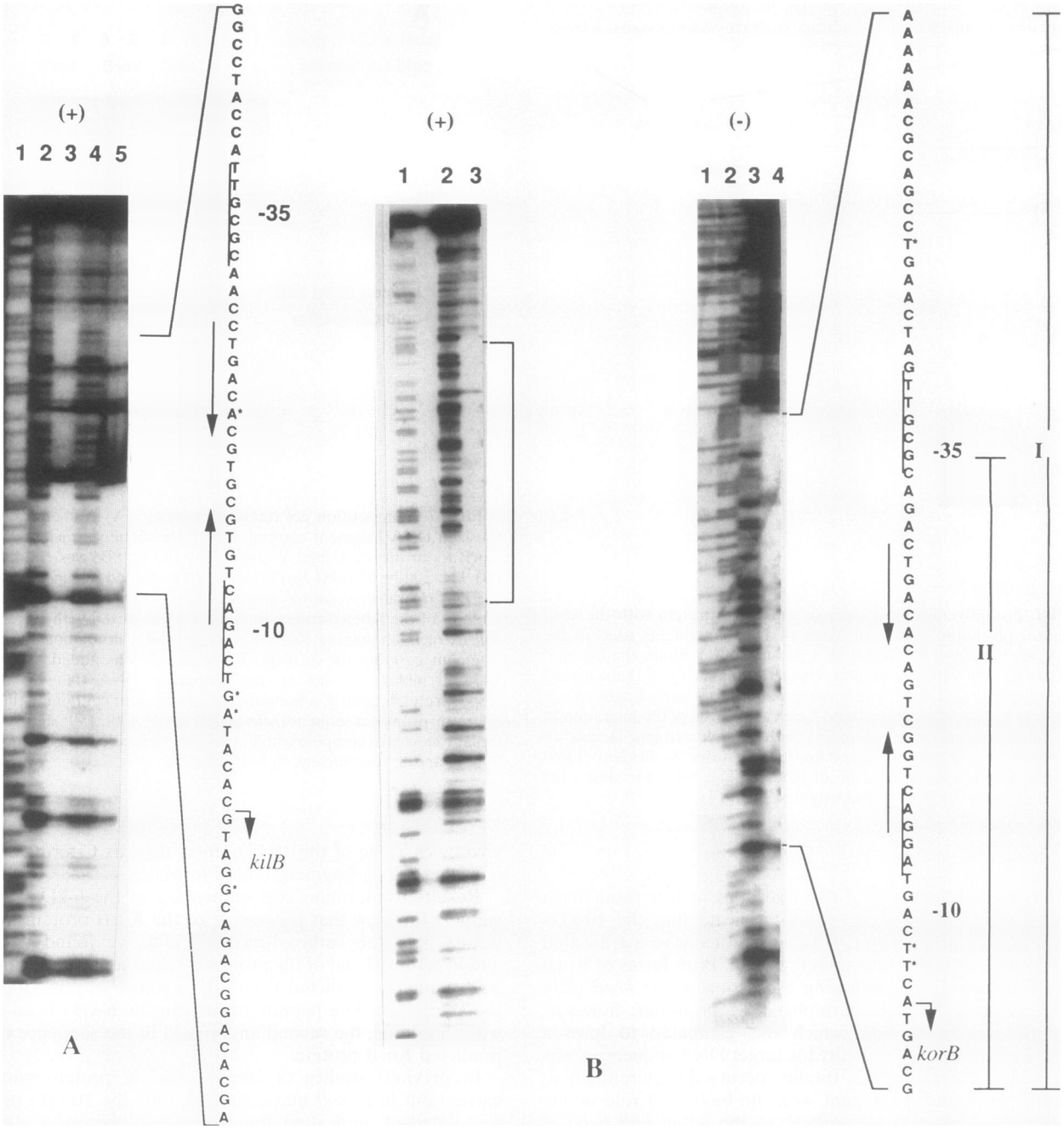

FIG. 8. DNase I footprinting analysis of the *kilB* (A) and *korB* (B) promoter regions. (A) The 200-bp *Bam*HI-*Bgl*II fragment (described in Fig. 5) carrying the *kilB* promoter region was 5′ end labeled on the strand containing the coding sequence, as described in Materials and Methods, and mixed with extracts containing (lanes 3 and 5) or not containing (lanes 2 and 4) KorB. Ten nanograms (lanes 2 and 3) or 20 ng (lanes 4 and 5) of DNase I was added to the reaction mixtures. The sequence in the region covered by the 65-bp KorB footprint is shown in brackets; bases with asterisks represent DNase I-hypersensitive sites. A Maxam-Gilbert sequencing ladder (A+G reaction) of the same fragment is shown in lane 1. (B) The 250-bp *Nar*I-*Sau*3A fragment (described in Fig. 6) carrying the *korB* promoter was end labeled on either the plus (coding) (+) or the minus (−) strand. Extracts containing KorB [lane 3 in (+) and lane 4 in (−)] or not containing KorB [lane 2 in (+) and lane 3 in (−)] were mixed with the fragment before being treated with 20 ng of DNase I. The DNA sequence in the 70-bp KorB footprint is shown in brackets. Bases with asterisks represent DNase I-hypersensitive sites. Maxam-Gilbert sequencing ladders of the fragments are shown in lane 1 (A+G reaction) in (+) and lanes 1 (A reaction) and 2 (A+G reaction) in (−). I and II indicate the sequences of the two DNA fragments chemically synthesized for binding analysis.

Our current findings, together with earlier evidence that uncontrolled expression of the *kilB* gene prevents growth of *S. lividans* (12), suggest that the synthesis and processing of the product of the incompletely repressed *korB* gene during normal cell growth yields an intracellular concentration of the 6-kDa peptide sufficient to repress expression of the lethal *kilB* gene, whose operator binds the repressor more efficiently. Upon mating, the cell fusion thought to be associated with

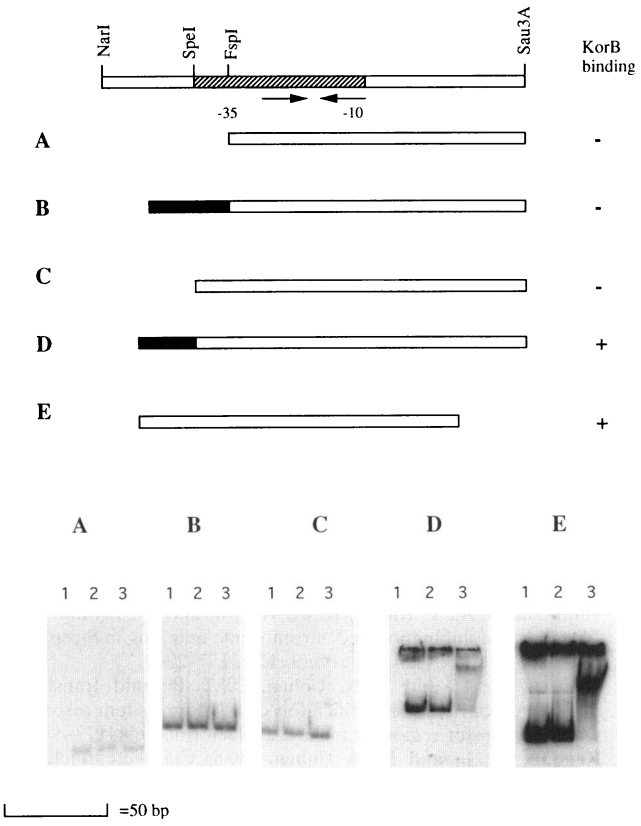

FIG. 9. Analysis of KorB binding specificity encoded by the sequence upstream of the inverted repeat in the *korB* operator-promoter region. The inverted repeat was isolated as *Fsp*I-*Sau*3A and *Spe*I-*Sau*3A fragments (A and C). These fragments were also cloned onto *Sma*I-*Bam*HI- and *Xba*I-*Bam*HI-digested pUC19, respectively. They were then isolated as *Eco*RI-*Hin*dIII fragments so that extra sequences from the pUC19 multiple cloning sites are at both ends of these fragments (B and D). A DNA sequence corresponding to the 70-bp KorB footprint described as fragment I in Fig. 8B was synthesized chemically and served as the positive control (E). Gel retardation assays of each of these constructs are shown at the bottom. In each case, the end-labeled fragment was incubated either with (lanes 3) or without (lanes 2) KorB and then loaded onto polyacrylamide gels. Lanes 1 are controls showing the migration of the extract-free fragments. The striped and solid regions represent segments containing *korB* promoter and nonspecific sequence from the pUC19 plasmid, respectively.

plasmid transfer (4) would be expected to cause dilution of the repressor and consequently derepression of the *kilB* gene, as well as full derepression of *korB*. We speculate that transient expression of *kilB* leads to the inhibition of cell growth of recipients (i.e., "pocking" [3]), as proposed initially by Kendall and Cohen (12), and, by a mechanism that is still quite unclear, to spread of the plasmid along mycelia (14). This notion is consistent with evidence that mutations in *kilB* affect pock size (12). The differential binding of KorB to the *korB* and *kilB* operators observed in the work reported here would be expected to reestablish full repression of the *kilB* promoter, ending the inhibition of cell growth while still allowing basal activity of the *korB* promoter. Our inability to detect the 10-kDa form of KorB in *S. lividans* is consistent with the notion that this form normally is present only transiently. Detection of the 10-kDa form of the *korB* gene product in *E. coli* may be a consequence either of the highly efficient production of the protein that occurs under the control of the bacteriophage T7 promoter or, alternatively, of less-efficient processing of KorB in this bacterial host.

The processing of other repressor proteins to modify their function has been observed previously. For example, the RepE protein involved in the replication of the F plasmid of *E. coli* exists in at least two forms; removal of the first 17 amino acids eliminates the replication initiation function of the protein but increases its specific DNA-binding affinity (15). It is also known that specific proteolysis is required for the DNA-binding activity of the p65 subunit of the NF-κB complex; homodimers of the full-length p65 do not bind to DNA unless the carboxyl terminus is deleted (23).

The DNA sequences specifically required for regulation of the *kilB* and *korB* genes were originally identified by deletion

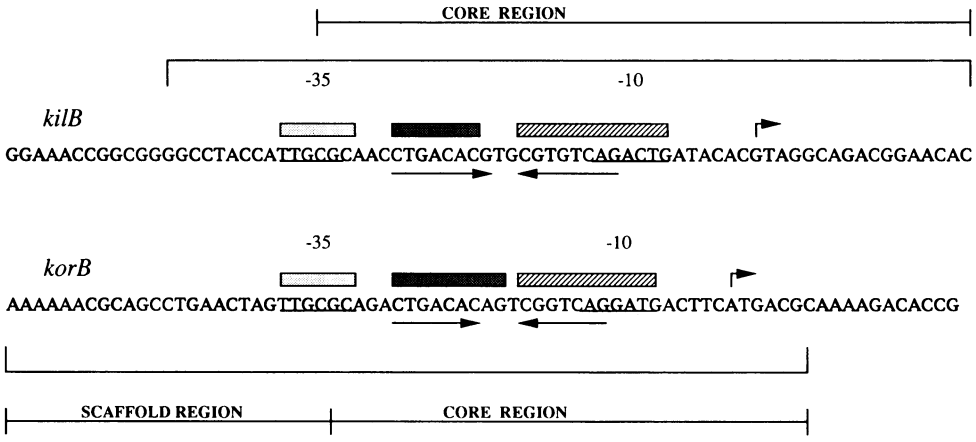

FIG. 10. Summary of KorB binding to the *kilB* and *korB* promoter regions. The sequences of the *kilB* and *korB* operator-promoter regions are shown. The bent arrows show the start sites of transcription, while straight arrows indicate the inverted repeat. Putative −10 and −35 regions are underlined, and boxes show the regions of homology between the two promoters. Sequences within the brackets represent regions covered by the KorB footprints. The sequences of the core and scaffold regions identified by gel shift analysis are indicated.

analysis (27) and subsequently were shown to bind to KorB protein (33). We now have found that a DNA segment (the scaffold region [Fig. 10]) outside the known *korB* operator is included within the KorB footprint and that interaction of KorB with this segment enhances the stability of the complex that KorB forms with its operator sequence. While replacing even part of the wild-type DNA sequence between the *Spe*I site and the *korB* operator (the core region [Fig. 10]) abolishes KorB binding, as indicated by gel shift assay (Fig. 9B), replacement of the entire region distal to the *Spe*I site (i.e., the scaffold region [Fig. 10]) by nonspecific sequences still allowed binding to occur (Fig. 9D), although with an affinity of less than 6% of the normal level (28a). In contrast, binding of KorB to the *kilB* operator-promoter region required only sequences corresponding to the *korB* core region downstream of the *Fsp*I site; these encode the pIJ101A promoter for the *kilB* genes (27) and the associated inverted repeat (Fig. 10). The sequence encoding a second previously identified *kilB* promoter (27) is not required for KorB binding.

Zaman et al. (33) have reported that *E. coli* extracts containing a *korB*-encoded protein that they estimated to be 10 kDa in mass yield a footprint on the *korB* operator-promoter region that is similar to the core region *korB* footprint we observed with the 6-kDa processed product. However, these workers reported no interaction of KorB with the scaffold region that we find is included in the footprint. Whether the extracts studied by Zaman et al. contained the 6-kDa form of the protein is unclear, as is the basis for the different footprints.

The KorB footprint on the regulatory region for the *korB* gene covers part of a locus previously shown to encode a function termed strong incompatibility (*sti*) (5) and known also to contain a site where second (lagging)-strand DNA synthesis begins (5). It has been suggested (26) that the *korB* gene may partially or completely overlap a locus encoding Cop, a *trans*-acting function identified on the basis of its ability to influence the copy number of pIJ101 derivatives by acting on the *sti* locus (5). Our finding that the KorB protein interacts physically with the *sti* locus supports the notion that the Cop and KorB functions are synonymous. As lagging-strand synthesis is known to occur in the recipient following conjugative transfer of *E. coli* plasmids such as F (32), we speculate that the KorB-*sti* interaction may regulate a similar process during or following transfer of pIJ101 in *Streptomyces* spp. This speculation is consistent with previous findings showing that insertions into the *sti* site affect pock size (12). Thus, the KorB protein may function biologically to coordinate plasmid transfer with conjugative DNA replication.

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