Identification and Analysis of Transcriptional Regulatory Signals for the *kil* and *kor* Loci of *Streptomyces* Plasmid pIJ101

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Four regulated promoters that direct the transcription of genes (i.e., *korA*, *tra*, *kilB*, and *korB*) involved in the transfer of the *Streptomyces* plasmid pIJ101 were isolated following the in vitro fusion of plasmid DNA fragments to a promoterless gene encoding the *S. lividans* extracellular enzyme β -galactosidase. Introduction of pIJ101 into cells carrying each of these promoter-*lac* fusions resulted in decreased *lac* expression. The sites of initiation of transcription by the promoter sequences were identified by primer extension experiments, and the DNA sequences specifically required for promoter activity and regulation by pIJ101-encoded functions were determined by deletion analysis. The data obtained indicate that the *korB* locus encodes a repressor that regulates its own transcription, as well as transcription of the *kilB* promoter; *korA* and *tra* are transcribed from overlapping divergent promoters that are coregulated by the *korA* gene product. Common DNA sequence domains within coregulated promoters allowed the identification of putative binding sites for each of the *kor* gene products.

Streptomyces spp. are gram-positive, sporulating, mycelial procaryotes that undergo complex morphological differentiation and produce, concomitantly with development, a wide variety of secondary metabolites, including more than 60% of commercially important antibiotics. Notwithstanding the biological and medical importance of these organisms, little is known about the mechanisms that control *Streptomyces* gene expression.

Recently, six plasmid-encoded genetic loci involved in conjugal transfer of the *S. lividans* plasmid pIJ101 (28) and in the associated pocking phenomenon have been identified (26, 28); two of these loci, *kilA* and *kilB*, cannot be cloned on pIJ101-derived replicons unless corresponding *kil*-override (*kor*) functions are also present either in *cis* or in *trans*. Subsequent sequencing of the entire pIJ101 plasmid (27) has identified translational open reading frames (ORFs) that correspond in position to the genetically defined *kil* and *kor* loci (Fig. 1). Mutations in *korA* result in expression of the *KilA* phenotype, which is closely linked to an ORF (i.e., *tra*) required for plasmid transfer (26, 27). Mutations in *korB* affect control of the *kilB* gene, which is required for the pocking phenotype (26–28).

The *kil* and *kor* loci of pIJ101 represent a unique *Streptomyces* regulatory system in which genes encoding regulatory products, as well as the targets for these products, have been identified and sequenced. Moreover, the observation that genetic loci necessary for plasmid transfer are lethal to the plasmid and/or host when uncontrolled by *kor* gene products (26) suggests that finely tuned genetic switches control the transfer process. To elucidate the molecular basis for this regulation, we used a promoterless derivative of the *S. lividans lac* gene (18) to isolate and characterize promoter sequences that initiate transcription for the *tra* (*kilA*), *kilB*, *korA*, and *korB* ORFs; synthesis of the *lac* gene product (i.e., β -galactosidase [β -gal]) served as an indicator of transcriptional activity initiated within DNA segments fused to

lac. Our results identify sequences required for the initiation of transcription from four promoters of the pIJ101 *kil* and *kor* loci, define the sites of action of the *kor* gene products, and establish that the *kor* genes regulate their own transcription, as well as transcription of the *kil* loci.

MATERIALS AND METHODS

Bacterial strains and plasmids. An F' Δ *lac*-150 *proAB* Kan^r derivative (11) of *Escherichia coli* MC1061 *hsdR* *hsdM*⁺ *araD139* Δ (*ara leu*)7697 Δ *lacX74 galU galK strA* (13) was used as the *E. coli* host throughout this study. *E. coli* JM101 *supE thi* Δ (*lac-proAB*) (F' *traD36 proAB lacI*^r Δ M15) (33) and JM109 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ ⁻ Δ (*lac-proAB*) (F' *traD36 proAB lacI*^r Δ M15) (39) were used in sequencing. The *E. coli* cloning and sequencing vectors pBSM13⁺ and pBSM13⁻ were obtained from Vector Cloning Systems. *S. lividans* 1326-9R β gal⁻ SLP2⁺ SLP3⁺ and 1326- Δ β gal SLP2⁺ SLP3⁺ (19) and plasmid p3-20, which is a pBR322-derived replicon containing the *S. lividans lac* gene and encoding extracellular β -gal (18, 19), were gifts of Thomas Eckhardt. *S. lividans* C26::pIJ101, containing the markers *his*-2, *str*-6 and *ath*-8, allowed the selection of prototrophic transconjugants that had received pIJ101 from this strain by mating.

Streptomyces plasmids pIJ101, pHYG1, pHYG1:D5 (*KorA*⁺), and pHYG1:D8 (*KorB*⁺) have been described previously (26); pHYG1:D5 carries the entire *korA* ORF, the first 246 codons of the *tra* ORF, and the divergent *korA* and *tra* promoters (27; Fig. 1). pHYG1:D8 contains the *korB* ORF and promoter, the *sti* site, and 400 base pairs (bp) of non-protein-encoding upstream DNA (17, 27; Fig. 1). To construct a shuttle plasmid for use as a negative control, pUC19 (39) was cleaved with *Pst*I and ligated to *Pst*I-cleaved pHYG1, yielding pDSS200 (*KorA*⁻ *KorB*⁻). As a positive control for regulation, a plasmid was constructed (pDSS229 *KorA*⁺ *KorB*⁺) containing a pUC19 derivative carrying a hygromycin resistance determinant (31) inserted into a *Pst*I site (bp 8077) of pIJ101. Cells containing this plasmid were fully competent to carry out mating and pock formation.

Chemicals and enzymes. Chemicals and enzymes were purchased from Boehringer Mannheim Biochemicals, Be-

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FIG. 1. Physical and genetic map of pIJ101 based on the studies of Kendall and Cohen (26, 27) and Kieser et al. (28). ORFs are indicated by open boxes; arrowheads indicate the direction of translation. Those ORFs to which functions can be assigned are labeled appropriately. The location of the incompatibility locus, *sti*, is based on the work of Deng et al. (17). The locations and directions of transcription of promoters discussed in the text are indicated by arrows.

thesda Research Laboratories, Inc., New England BioLabs, Inc., or Sigma Chemical Co.

Cultures, transformations, and nucleic acid manipulations.

E. coli strains were grown at 37°C in LB or on LB agar (1.5%) (34). *Streptomyces* strains were grown at 30°C either in YEME containing 34% sucrose and 5 mM MgCl₂ or in liquid R2YE containing 0.5% yeast extract (22). Growth on plates was on R2YE agar (2%) containing 0.5% yeast extract (22). *Streptomyces* protoplasts for transformations were prepared from cultures grown in S medium containing 1.0% glycine (22). Soft R2YE used for overlays contained 1% agar.

Plasmid DNA was isolated from *E. coli* or *S. lividans* by alkaline lysis (6). *S. lividans* RNA was isolated as described previously (22, 29). Transformations of *E. coli* (15) and *S. lividans* (4, 22), DNA manipulations (32), sequence analysis (14, 36, 39), and Southern blot analysis (37) were performed as described previously. Expression of *lac* was detected by overlaying culture plates with 3.5 ml of soft R2YE containing either 300 µg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Boehringer Mannheim) per ml or 6 ml of soft R2YE containing 5 mg of methyl-umbelliferyl β-galactoside (MUG; Sigma) per ml. Oligodeoxynucleotides were synthesized by the DNA synthesis facility, Department of Cell Biology, Stanford University. Oligonucleotides were purified as described by Carruthers et al. (12).

Primer extension analysis. Transcriptional start sites were determined by primer extension analysis (20). The protocol, which is a modification of one obtained from the laboratory of C. Guthrie, was carried out as follows. RNA (20–40 µg) was first dissolved in a solution containing 25.5 µl of reverse transcription mix (0.1 M Tris [pH 8.0], 0.294 M KCl, 1 µM

EDTA [pH 8.0], 2 µM dithiothreitol, 14 µM MgCl₂, 1 µM dATP, 1 µM dCTP, 1 µM dGTP, 1 µM dTTP), 2 µl of labeled dCTP (800 Ci/mmol), 4 µl of oligonucleotide primer (20-mer) at a concentration of 10 µM, and diethylpyrocarbonate-treated water to a total volume of 50 µl. The mixture was heated to 68°C for 5 min, incubated at 55°C for 30 min, and then incubated at room temperature for 15 min to facilitate annealing of the primer to RNA. One microliter (200 U) of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc.) was added, the mixture was incubated at 42°C for 90 min, 12.5 µl of 0.5 M NaOH was added, and the tube was incubated at 90°C for 3 min. Then, 237 µl of primer extension stop mix (50 mM Tris [pH 7.4], 25 mM HCl) and 300 µl of phenol-chloroform were added, the mixture was vortexed briefly and centrifuged. The aqueous layer was removed, and the primer extension products were precipitated by the addition of 30 µl of 3 M sodium acetate (pH 7.0) and 850 µl of ethanol. The pellet was washed with 70% ethanol, dried under vacuum, suspended in primer extension sample buffer (8 M urea, 20 mM Tris-borate [pH 7.5], 0.1% xylene cyanol, 0.05% bromophenol blue), and electrophoresed on a 6% polyacrylamide sequencing gel.

To determine the start sites of transcription precisely, nucleotide sequence analysis of a plasmid containing the promoter segment of interest was carried out in parallel using as a sequencing primer the same oligonucleotide that had been used in the primer extension reaction.

S1 analysis. S1 analysis (2) of the 5' ends of RNA transcripts, using end-labeled, double-stranded DNA as a probe, was carried out essentially as described by Belasco et al. (1) with modifications as follows. Twenty micrograms of RNA was annealed to 100 ng of end-labeled double-stranded DNA probe. The mixture of RNA and probe was heated at 90°C for 10 min and transferred to a 55°C water bath for hybridization overnight. After hybridization, 1,000 U of S1 nuclease was added and incubated for 2 to 3 h at 37°C, and the hybrids were precipitated by the addition of 15 µl of 250 mM EDTA and 600 µl of isopropanol. The protected species were suspended in Tris-borate buffer (29) and subjected to electrophoresis on a denaturing urea-polyacrylamide gel (6% acrylamide, 7 M urea, 1× Tris-borate buffer) with appropriate size markers. The gel was fixed, dried down onto Whatman 3MM paper, and exposed to Kodak XAR5 film at –70°C, using an intensifying screen.

RESULTS

Construction of the promoter-probe vector pDSS000. The transcriptional activity of pIJ101-derived promoters was measured by using a plasmid (pDSS000, Fig. 2) that allowed inserted DNA fragments to direct transcription of the *S. lividans lac* gene. This promoter-probe vector, which was constructed from the β-gal-containing plasmid p3-20 (19) and pJAS01, a thiostrepton-resistant (Ts^r) derivative of the *S. lividans* plasmid SLP1.2 (5), has a unique *Bam*HI site immediately upstream of a promoterless derivative of *lac*. The presence of the *t_{oop}* transcription terminator (23) upstream from the *Bam*HI site reduces readthrough transcription into *lac* from pDSS000 vector sequences. SLP1.2 and pBR322 (7) origins of replication allow propagation of pDSS000 in either *S. lividans* or *E. coli*, where selection can be carried out for Ts^r or ampicillin resistance (Ap^r), respectively. *Stu*I sites flank the *Bam*HI cloning site and allow removal of inserts that do not regenerate a sequence susceptible to *Bam*HI cleavage.

FIG. 2. Features of the promoter probe vector pDSS000. See the text for details. kb, Kilobases.

A plate assay was used to visually quantitate β -gal production from promoters introduced into pDSS000. Cells containing pDSS000 itself produced slight but detectable amounts (scored as $\pm$, see below) of β -gal, presumably because of residual readthrough transcription initiated from sequences located 5' of the *lac* gene. However, a significant increase in *lac* activity was observed in some colonies upon ligation of *Sau*3A-generated fragments of *S. lividans* genomic DNA to *Bam*HI-cleaved pDSS000 (data not shown).

We assessed expression by overlaying growing colonies with soft agar containing X-Gal (see Materials and Methods), incubating the plates at 30°C for 1 h, and visually scoring the colonies as follows: colonies that remained white were scored as lacking promoter activity (–); colonies that were white after 1 h but became slightly blue after 4 h were scored as having slight promoter activity ($\pm$); the remainder of the colonies, which showed blue staining at 1 h of incubation, were scored according to their intensity of color at that time, from light blue (+) to dark blue (+++). Parallel results were obtained with the fluorescent substrate MUG, which shows the relative amounts of β -gal production by *S. lividans* transformants in black and white photographs (Fig. 3); the strains shown in the figure had scores ranging from – to +++. Each construct was assayed at least twice and usually three or four times, and the individual assessments of relative promoter activity were found to be highly reproducible. However, attempts at quantitation of β -gal production by cells grown in liquid culture were not similarly reproducible, possibly because of the propensity of *Streptomyces* cells to grow in clusters.

Isolation of DNA segments containing regulated promoters from *S. lividans* plasmid pIJ101. A pBR322-derived plasmid containing a pIJ101 *Pst*I fragment (bp 2776 to 8077) (27) (Fig. 1) that encompasses most of the transfer region of the plasmid was partially digested with *Sau*3A, ligated to *Bam*HI-cleaved pDSS000, and introduced by transformation into *S. lividans* 1326- $\Delta\beta$ gal. Fifty *lac*-expressing transformants were chosen for further study.

pIJ101 was transferred from *S. lividans* C26::pIJ101 into each of the 50 strains, and six clones that showed a conse-

FIG. 3. β -gal activity detected by plate assay after overlay with MUG (see the text). Spores were spread ("patched") onto an R2 medium plate containing 0.2% yeast extract and 2% agar. Cells were incubated at 30°C for 2 days. The plate was then overlaid with 6 ml of soft R2YE agar containing 5 mg of MUG per ml (total concentration in plates, 1 mg/ml). Plates were photographed 15 min after overlay, using Polaroid type 55 film and UV illumination; exposure was for 7 s at f4.5 with a yellow filter. Expression was scored as follows: – (A), $\pm$ (B), + (C), ++ (D), and +++ (E).

quent reduction of β -gal activity (from +++ or ++ to + or $\pm$) were identified. Plasmid DNA prepared from each of these isolates was reintroduced into strain 1326- $\Delta\beta$ gal, and selection for pDSS000-derived plasmids was carried out by growth on media containing thiostrepton. Each of the six DNA preparations yielded transformants that once again expressed β -gal at a high (+++ or ++) level, indicating that the decreased β -gal activity observed after the introduction of pIJ101 had resulted from the presence of pIJ101 and not from a structural alteration of the *lac* promoter or gene.

Localization on plasmid pIJ101 of cloned promoter-active DNA fragments. Initial Southern blot analysis of six pIJ101-regulated cloned promoter-active DNA fragments showed that they corresponded to multiple clonings of two discrete regions of pIJ101. The inserts were excised from two representative plasmids, pDSS013, and pDSS014, by *Stu*I digestion and further examined by hybridization to Southern blots of restriction endonuclease-digested pIJ101 DNA. Additionally, the *Stu*I fragments containing the cloned DNA inserts were introduced into *Hinc*II-digested pBSM13⁺ and pBSM13[–] to facilitate sequencing and comparison of the insert sequence with the previously determined sequence of pIJ101 (27).

The insert present in pDSS013 included two separate *Sau*3A fragments, a 110-bp fragment derived from pBR322 and a 218-bp fragment that extended in pIJ101 from bp 5870 to 6088 (27). In pIJ101, this 218-bp *Sau*3A fragment overlaps the region between the *tra* and *korA* ORFs (27; Fig. 1); in pDSS013, the fragment is oriented so that the *lac* gene is downstream from a DNA sequence near pIJ101 coordinate 5870, which normally is 5' of the *tra* ORF.

The 218-bp *Sau*3A fragment was isolated and ligated to *Bam*HI-digested pDSS000, yielding plasmids pDSS031 and

pDSS034, each of which contained the fragment in a different orientation with respect to the *lac* gene. Transformants containing either of these plasmids produced β -gal; moreover, introduction of pIJ101 into cells containing either pDSS031 or pDSS034 resulted in a decrease of promoter activity from strong (+++) to weak (+) (e.g., see Fig. 6). Thus, the cloned fragment was transcriptionally active in both orientations and this activity was regulated by pIJ101. The location of the 218-bp fragment on pIJ101 indicated that it includes a region containing divergent promoters that direct expression of the *tra* and *korA* ORFs (27; Fig. 1).

The transcriptional DNA insert in pDSS014 also had resulted from the ligation of two separate *Sau*3A fragments. The 508-bp fragment adjacent to the *lac* gene extends in pIJ101 from bp 7183 to 7689; the first coordinate is proximal to *lac* in pDSS014 and is adjacent in pIJ101 to the ORF identified as *korB* (27; Fig. 1); its orientation in pDSS014 suggests that the putative promoter present on the cloned fragment corresponds to the *korB* promoter. A second 130-bp *Sau*3A insert in pDSS014 was not characterized further.

Isolation of the *kilB* promoter. Earlier work in our laboratory (26, 27) has shown that the *kilB* locus is controlled in *trans* by the product of the *korB* gene and also that the *kilB* ORF is just downstream from a pIJ101 locus found previously by S1 mapping to initiate transcripts (9). Various DNA manipulations were performed so that a 187-bp *Bst*EII (bp 2425)-to-*Sal*I (bp 2612) fragment carrying the promoter 5' to the *kilB* ORF could be excised from subclones as a *Bgl*II-*Bam*HI fragment. Ligation of this fragment to *Bam*HI-cleaved pDSS000 DNA yielded transformants expressing β -gal and containing a plasmid, designated pDSS017, that carried the putative *kilB* promoter in the appropriate orientation relative to *lac*. Introduction of pIJ101 into strains carrying pDSS017 resulted in a decrease in β -gal expression.

High-resolution mapping of the transcription start sites for *korB*, *kilB*, *tra*, and *korA* mRNA. Transcription start sites for mRNA encoded by the four regulated promoters were mapped by primer extension on transcript templates (20), using as primer an oligonucleotide complementary to the 5' end of the *lac* gene (Fig. 4). For pDSS031 (p-*tra*) and pDSS034 (p-*korA*), a single band representing DNA molecules initiated at a unique site was seen. For pDSS014 (p-*korB*) and pDSS017 (p-*kilB*), two separate extension product bands were seen. No reproducible bands were seen in the lanes used for primer extension analysis of RNA obtained from plasmid-free *S. lividans* 1326- $\Delta\beta$ gal cells.

The transcription start sites in relation to the *kil* and *kor* promoter regions are indicated in Fig. 5. For *kilB*, two mRNA 5' ends were mapped, the downstream terminus at a locus previously termed the pIJ101A promoter by Buttner and Brown (9). The major start site is shown for the *korB* transcript, and the single start sites for the divergent transcripts from the *tra* and *korA* promoters are shown. Upstream from the mapped transcription start sites, each of the four promoter regions contains sequences similar to the -10 and -35 regions of promoters transcribed by *E. coli* RNA polymerase carrying the σ^{70} subunit (21). These sequences are underlined and labeled.

The more prominent of the 5' termini identified for *korB* mRNA occurred precisely at the A of the first codon (AUG) of the predicted ORF (bp 7208). A band representing a possible second 5' terminus, located at a site 2 bp downstream from the first (at bp 7206) and within the *korB* ORF, was also seen. The *korB* and *kilB* promoters have considerable sequence similarities (indicated in Fig. 5 by shaded

FIG. 4. High-resolution mapping of the transcription start sites of pIJ101 promoters by primer extension analysis. Lane 1, RNA was prepared from *S. lividans* 1326- $\Delta\beta$ gal cells containing pDSS014 (*korB* promoter) (A), pDSS017 (*kilB* promoter) (B), pDSS031 (*tra* promoter) (C), and pDSS034 (*korA* promoter) (D). The primer used was the 20-bp deoxynucleotide 5'-CGGCGACTACTGAGTCACGG-3', which is complementary to a sequence within the *lac* gene 5' noncoding region immediately downstream of the *Bam*HI cloning site in pDSS000 (16). Lane 2, For each primer extension a negative control reaction was included, using *S. lividans* 1326- $\Delta\beta$ gal cells that do not carry a plasmid. At the same time, sequence analysis of the promoter region giving rise to each RNA product was done. Each primer extension product was subjected to polyacrylamide gel electrophoresis alongside the negative control lane and alongside the corresponding sequencing reactions for that promoter.

boxes), including an inverted repeat structure located between the -35 and -10 regions of both promoters.

The start site determined for the *tra* transcript was at bp 5939, suggesting that the *tra* promoter corresponds to the pIJ101B promoter identified by Buttner and Brown (10). Transcription towards *korA* starts at bp 5992, 53 bp from the start site for *tra*, and proceeds in the opposite direction. The -35 region of the *tra* promoter is located between the -10 and -35 regions of the *korA* promoter, while the *korA* -35 region is located between the -10 and -35 regions of the *tra* promoter (Fig. 5). Two inverted repeat sequences are within the region containing these divergent promoters: one is a

FIG. 5. Sequences of DNA segments upstream of the transcription initiation sites. The sequences in the vicinity of the mapped transcription start sites are shown. The bent arrows show the start sites of transcription. Putative -10 and -35 regions of the *kilB* and *korB* promoters are underlined. Shaded boxes show the similar regions in the *korB* and *kilB* promoters. Straight arrows show inverted repeats and half inverted repeats in the region. Certain relevant restriction sites within the sequences are shown. The box around the *tra* promoter indicates the extent of the synthetic oligonucleotide discussed in the text.

12-bp inverted repeat with a 4-bp spacer (bp 5949 to 5964), lying between the *tra* promoter -10 and -35 regions and overlapping the *korA* promoter -35 region. The second is an imperfect 10-bp inverted repeat with a 3-bp spacer (bp 5959 to 5971), lying between the *korA* promoter -35 and -10 regions and partially overlapping the *tra* promoter -35 region. Within this segment are additional short stretches of DNA that have homology to the inverted repeats.

Regulation of promoter activity by *korA* and *korB*. To study the effects of the *korA* and *korB* gene products on the *kil* and *kor* promoters, plasmids containing one or both of the *kor* genes were introduced together with pDSS000 derivatives containing one of the four promoters into *S. lividans* 1326- $\Delta\beta$ gal, and β -gal expression directed by the four promoters was determined (Fig. 6 and Table 1).

The presence in *trans* of a plasmid carrying either the *korB* gene or the entire pIJ101 plasmid reduced *lac* expression from both the *kilB* and *korB* promoters (Fig. 6 and Table 1). *korB* was more effective in repressing β -gal expression directed by the *korB* promoter than by the *kilB* promoter. While *korA* did not affect β -gal activity directed by either the *kilB* or *korB* promoter, both *korA* and the entire pIJ101 plasmid in *trans* decreased β -gal activity directed by the *tra* and *korA* promoters. A plasmid carrying the *korB* gene did not affect β -gal expression from either the *tra* or *korA* promoter.

***korA* and *korB* act by decreasing *lac* mRNA synthesis directed by the *tra* and *korB* promoters, respectively.** The effects of the *korB* and *korA* genes on *lac*-containing transcripts initiated at the *korB* and *tra* promoters, respectively, were examined by S1 analysis (2), which provided a sensitive means of assessing the regulation of transcripts initiated at known sites (Fig. 7). A doublet consisting of two DNA fragments, each about 350 nucleotides long, was seen in the lane containing RNA isolated from cells carrying both pDSS014 (*korB* promoter) and pDSS200 (*KorA*⁺ *KorB*⁻) (Fig. 7A, lane 4); these bands were the expected size for RNA molecules initiated at the previously mapped start sites for *korB* transcripts. However, no protected bands were observed in the lanes that contained RNA from cells lacking

FIG. 6. Regulation of promoter activity by *korA* and *korB*. Plasmids pDSS014, pDSS017, pDSS031, and pDSS034 were individually introduced by transformation into *S. lividans* 1326- $\Delta\beta$ gal protoplasts along with plasmid pDSS200, pDSS299, pHYG1:D5, or pHYG1:D8. Transformants showing resistant to both thiostrepton (the kind gift of S. J. Lucania, E. R. Squibb & Sons) and hygromycin (Calbiochem-Behring) were isolated and screened for β -gal expression. Spores from each of the transformation mixtures were spread on plates containing thiostrepton and hygromycin. After 48 h of incubation they were overlaid with soft R2YE containing MUG and were photographed 15 min later. Plate 1, pDSS014 (*korB* promoter) plus *kor* constructs; plate 2, pDSS017 (*kilB* promoter) plus *kor* constructs; plate 3, pDSS031 (*tra* promoter) plus *kor* constructs; plate 4, pDSS034 (*korA* promoter) plus *kor* constructs. The *kor* constructs were pDSS200 (*KorA*⁺ *KorB*⁻) (A, E, I, and M), pHYG1:D5 (*KorA*⁺ *KorB*⁻) (B, F, J, and N), pHYG1:D8 (*KorA*⁻ *KorB*⁺) (C, G, K, and O), and pDSS299 (*KorA*⁺ *KorB*⁺) (D, H, L, and P).

TABLE 1. Regulation of pIJ101 promoters by the *korA* and *korB* genes^a

lac-expressing plasmid (promoter)	β-gal activity			
	pDSS200 (no <i>kor</i> gene)	pDSS205 (<i>korA</i>)	pDSS208 (<i>korB</i>)	pDSS299 (<i>korA korB</i>)
pDSS014 (<i>korB</i>)	+++	+++	+/-	+/-
pDSS017 (<i>kilB</i>)	+++	+++	++	++
pDSS031 (<i>tra</i>)	+++	+	+++	+
pDSS034 (<i>korA</i>)	+++	+	+++	+

^a Plasmids containing the four cloned promoters were introduced by cotransformation with constructs containing different *kor*-carrying plasmids (Fig. 6). After selection, β-gal activity was determined as described in the text.

pDSS014 or from cells containing both pDSS014 and a plasmid carrying *korB* (Fig. 7A, lanes 3 and 5, respectively).

A strongly visible protected DNA fragment about 400 nucleotides long was protected by RNA prepared from cells containing pDSS031 (*tra* promoter) and pDSS200 (Fig. 7B, lane 4). This fragment corresponded in size to a transcript initiating at the previously mapped *tra* promoter start site. The amount of protected DNA was sharply reduced in the presence of *korA*, as only a faint band was visible with RNA prepared from cells carrying both pDSS031 and pHYG1:D5 (Fig. 7B, lane 5). No band was seen for RNA preparations from 1326-Δβgal cells that lacked the pDSS031 plasmid (Fig. 7B, lane 3).

Identification of DNA sequences required for promoter activity and regulation. The sequence determinants required for transcription directed by the *korB*, *kilB*, *tra*, and *korA* promoters were predicted from the primer extension analyses described above. To directly identify these promoter sequences, deletion derivatives of transcriptionally active DNA segments were tested for their ability to direct *lac* expression in pDSS000 and to be regulated in *trans* by *korA* and/or *korB*.

Deletion analysis of the *korB* promoter region showed that a fragment extending from bp 7245 (*SpeI*) to bp 7184 (*Sau3A*) (Fig. 5) and containing the *E. coli*-like promoter identified upstream of the *korB* ORF conferred fully regulated promoter activity. However, deletion of the *SpeI*-to-*Sau3A* segment of DNA or insertion of a *Clal* linker into the *FspI* site in the putative -35 region of the *korB* promoter (Fig. 5) resulted in a loss of promoter activity, as did introduction of the *korB* promoter region into pDSS000 in the reverse orientation. Insertion of a *BglII* linker into the *SpeI* site immediately upstream of the putative -35 region did not interfere with either promoter activity or regulation.

Deletion analysis of the *kilB* promoter region contained in pDSS017 showed that a segment of DNA extending from bp 2425 (*BstEII*) to immediately upstream of bp 2501 (the first T of the putative -35 region; Fig. 5) was sufficient to confer promoter activity. This fragment contains the *E. coli*-like promoter sequence identified previously within the *kilB* promoter region (9; Fig. 5). Surprisingly, transcriptional activity from this fragment was regulated more strongly by pIJ101 than activity encoded by the larger *BstEII*-*SalI* fragment present in pDSS017. Insertion of either *Clal* or *HpaII* linkers into the *FspI* site of this construct (within the putative *kilB* -35 region; Fig. 5) resulted in a complete loss of promoter activity. However, insertion of a *Clal* linker at the corresponding location in pDSS017, which contains additional DNA extending to the *SalI* site at bp 2612, 21 bp further upstream from the sequence shown in Fig. 5, resulted in unregulated weak transcriptional activity. Thus, it appears

FIG. 7. Regulation of *korB* and *tra* promoter activity by *korB* and *korA*, respectively. The *S. lividans lac* gene contains an *XmnI* site approximately 300 nucleotides downstream from the *Bam*HI promoter-cloning site of pDSS000. *EcoRI*-*XmnI* DNA fragments from pDSS014 and pDSS031, containing cloned *korB* and *tra* promoter inserts, respectively, were isolated and labeled radioactively at the *XmnI* ends for use as S1 hybridization probes. For the analysis of regulation by *korB*, RNA was prepared from 1326-Δβgal cells containing either no plasmid, pDSS014 and pDSS200, or pDSS014 and pHYG1:D8. S1 protection analysis was carried out using the labeled DNA fragment from pDSS014. For analysis of regulation by *korA*, RNA was prepared from 1326-Δβgal cells containing either no plasmid, pDSS031 and pDSS200, or pDSS031 and pHYG1:D5. The S1 hybridization probe was the labeled DNA fragment from pDSS031. (A) Regulation of the *korB* promoter. Lane 1, Size markers; lane 2, reannealed probe alone; lane 3, probe plus RNA from *S. lividans* 1326-Δβgal; lane 4, probe plus RNA from *S. lividans* 1326-Δβgal containing pDSS014 and pDSS200; lane 5, probe plus RNA from *S. lividans* 1326-Δβgal containing pDSS014 and pHYG1:D8 (*korB*). (B) Regulation of the *tra* promoter. Lane 1, Size markers; lane 2, reannealed probe alone; lane 3, probe plus RNA from *S. lividans* 1326-Δβgal; lane 4, probe plus RNA from *S. lividans* 1326-Δβgal containing pDSS031 and pDSS200; lane 5, probe plus RNA from *S. lividans* 1326-Δβgal containing pDSS031 and pHYG1:D5 (*korA*).

that a second unregulated promoter is present between bp 2612 and 2501, upstream of the putative *kilB* promoter; it seems likely that this promoter is responsible for both the second transcriptional start site identified by primer extension analysis and the weak *lac* expression shown by pDSS017 in the presence of pIJ101. No *lac* expression was seen when the fragment containing the *kilB* promoter was introduced into pDSS000 in the opposite orientation.

The sequences responsible for expression and regulation of both the *tra* and *korA* promoters were found to be completely contained within the 83-bp *TaqI* fragment (bp 5935 to 6018; Fig. 5). To distinguish the sequences responsible for *tra* promoter activity from those responsible for

korA promoter activity, a 41-bp synthetic oligonucleotide was designed (represented by the boxed sequences in Fig. 5) and inserted into pDSS000. However, this synthetic *tra* promoter showed barely detectable transcriptional activity in either orientation, indicating that determinants in addition to the transcriptional start site and the -10 and -35 regions are necessary for the normal functioning of the *tra* promoter.

DISCUSSION

The *korA* and *korB* genes of the *Streptomyces* plasmid pIJ101 regulate four distinct promoters that normally direct transcription of the *tra*, *kilB*, *korA*, and *korB* open translational reading frames (27; Fig. 1). *korA* and *korB* regulate both their own transcription and transcription of the *tra* and *kilB* genes, respectively. Primer extension analysis showed that transcripts from the *kilB* promoter were initiated at the same site reported for a promoter previously termed pIJ101A (9); a second promoter that showed weak, unregulated activity appears to be present upstream of the *kilB* promoter. Initiation of transcripts at the *tra* promoter occurs at a site previously identified for the pIJ101B promoter (10). Each of the four promoters contains, upstream of the transcriptional start sites, sequences similar to those found in *E. coli* promoters transcribed by RNA polymerase holoenzyme carrying the σ^{70} subunit (21). Thus, they may be transcribed by the *Streptomyces* RNA polymerase holoenzyme that carries the 35-kilodalton sigma factor, σ^{35} , which can direct transcription from *E. coli* consensus-type promoters (24, 38).

Surprisingly, transcription initiation from the *korB* promoter occurs at the A residue of the predicted AUG translational start codon for the *korB* ORF. This raises the possibility that the *korB* mRNA is translated in the absence of a conventional ribosome-binding site. Although rare, such occurrences have been noted before with the *aph* and *ermE* genes of *Streptomyces* spp. (3) and the λ cI and Tn1721 *tetR* genes from *E. coli*. (30, 35).

Deletions and/or insertions indicate that the *E. coli*-like sequences in both the *korB* and *kilB* promoters are necessary and sufficient for both transcriptional activation of *lac* and regulation by *korB*. While an 83-bp segment of DNA that contains the transcription start sites for both the *tra* and *korA* promoters initiated *korA*-regulated transcriptional activity in both directions, a synthesized segment of DNA that contains a subset of the sequences of this fragment, namely the transcription start site and the putative -10 and -35 regions for the *tra* promoter, showed only weak promoter activity. This finding suggests that determinants required for full activity of the *tra* promoter are located outside of the -10 and -35 regions, as has been seen previously for certain other promoters (8, 25). Alternatively, reduction in activity of the synthesized *tra* promoter sequence may be related to the overlapping arrangement of the *tra* and *korA* promoters; in studies of the overlapping, divergently transcribed *tetA* and *tetR* promoters of Tn10, Daniels and Bertrand (16) found that certain mutations that reduced the promoter activity of *tetA* resulted in a concomitant reduction in *tetR* promoter activity, even though the mutations did not affect the -10 or -35 region of the *tetR* promoter. Possibly, deletion of the *korA* promoter leads to an analogous concomitant decrease in *tra* promoter activity.

S1 analyses showed that *kor*-mediated repression of the *korB* and *tra* promoters occurs at the level of transcription. Inverted repeats within the highly similar *kilB* and *korB* promoter regions (Fig. 5) may be operator sites for the binding of the *korB* product. The overlapping arrangement of

the sequences comprising the *tra* and *korA* promoters suggests that the *korA* gene product may simultaneously bind to and repress the initiation of transcription from both promoters. We speculate that one or both of the inverted repeat sequences present between the promoters (Fig. 5) may be required for such binding.

Kendall and Cohen (26) have shown that the *KilB* phenotype is overridden in *trans* by the *korA* or *korB* genes. While the work reported here indicates that the *kilB* promoter is regulated by *korB*, significant transcriptional repression of the *kilB* promoter by *korA* was not detected. It therefore appears that the override of the *KilB* phenotype by *korA* may not occur at the level of transcription. The basis for this effect is currently under study.

What is the biological role of these complex transcriptional regulatory systems on the pIJ101 plasmid? The involvement of the *kilB* and *tra* ORFs in the conjugal transfer of plasmids has been established (26–28), suggesting that regulation of these loci is involved in modulating the transfer process. Supporting this notion are results of preliminary experiments suggesting that the *tra* and *korA* promoters are induced transiently during mating. The DNA region containing these divergent promoters thus appears to serve as a genetic switch that determines whether specific transfer functions are expressed.

The identification in *Streptomyces* spp. of regulated transcription signals, as well as their putative regulators, should be useful for investigating the control of gene expression in these developmentally complex organisms. Experiments to be reported elsewhere elucidate further, by mutational analysis of the *korA* and *korB* ORFs, the role of the *kor* genes as transcriptional regulators.

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