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Transcription from the P1 Promoters of *Micromonospora echinospora* in the Absence of Native Upstream DNA Sequences

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We demonstrated previously that the 0.4-kilobase DNA fragment from *Micromonospora echinospora* contains multiple tandem promoters, P1a, P1b, P1c, and P2, which are also functional when cloned into *Streptomyces lividans*. We now show by in vitro transcription with *Streptomyces* RNA polymerase that each of these promoters is an authentic initiation site, rather than a processing site for transcripts which initiate further upstream. The DNA sequence requirements for the closely spaced promoters P1a, P1b, and P1c, which are coordinately induced during stationary phase in *M. echinospora*, were examined by deletional analysis in *S. lividans*. The P1a and P1b promoters were functional despite deletion of native sequences 5 and 17 base pairs upstream of each initiation site, respectively. Thus, P1a and P1b had greatly reduced upstream DNA sequence requirements compared with typical procaryotic promoters. In contrast, transcription from promoter P1c was significantly decreased when native sequences 34 base pairs upstream were replaced.

We have been studying promoter structure in the actinomycete *Micromonospora echinospora* (NRRL 15839), which grows as multicellular mycelia and has the capacity to form spores after the growing phase. During the stationary phase, *M. echinospora* produces the calicheamicins (19), a novel family of antitumor antibiotics that causes site-specific double-stranded cleavage of DNA (29).

Previously, we defined a set of multiple tandem promoters within a 0.4-kilobase (kb) DNA fragment that has several novel features (1). The P1 promoter region consists of three transcriptional start sites separated by 12 and 17 nucleotides (nt), which are maximally active during stationary phase, concurrent with calicheamicin production. Promoter P2 is located 80 base pairs (bp) downstream from the P1 region and is maximally active during the exponential phase of the life cycle. The P1 and P2 promoters are potentially useful for the investigation of gene activation in actinomycetes during the transition from exponential to stationary phase, when many secondary metabolites are produced (22).

The P1 and P2 promoters, and an additional site designated Ptk, are recognized by *Streptomyces lividans* transformants harboring the 0.4-kb fragment on a plasmid (1). We have therefore utilized the well-characterized genetic system of *Streptomyces* for more detailed transcriptional analysis. The P1 and P2 promoters belong to the major class of *Streptomyces* promoters that do not function in *Escherichia coli* (12, 15, 16). A consensus sequence has not been identified for this class of promoters. The occurrence of multiple forms of RNA polymerase holoenzyme in *Streptomyces* spp. which direct transcription from different promoters (6, 28) is consistent with the diversity of promoter sequences found in this organism. Recently, four different genes apparently encoding sigma factors were identified in *S. coelicolor* by homology to sequences derived from *E. coli* and *Bacillus subtilis* (26). In addition, the *whiG* locus also encodes a putative sigma factor (7).

In this report, we confirm by in vitro transcription that each transcriptional start site within the 0.4-kb fragment is an actual promoter, rather than a processing site for transcripts which initiate upstream. In addition, we define the upstream boundaries of the P1 promoter cluster by deletional analysis, which suggests that only 5 and 17 bp of native upstream DNA sequence is required for proper initiation at promoters P1a and P1b, respectively. This result contrasts with the majority of well-characterized procaryotic promoters, which contain essential sequences further upstream of the start site of transcription (23).

MATERIALS AND METHODS

Strains and plasmids. *S. lividans* TK54 (13) was the host for all *Streptomyces* plasmids, which are summarized in Fig. 1. The 0.4-kb *Sau*3A1 *Micromonospora* DNA insert containing five tandem promoters (1) was ligated into the *Bam*HI site of the promoter probe plasmid pIJ486 (27), regenerating one *Bam*HI site downstream of the 0.4-kb fragment (Fig. 1, pIJ486-14). Constructs in the *Streptomyces*/*E. coli* shuttle vector pIJ903 (20) were first transformed into *E. coli* HB101 (21) and verified by restriction enzyme digestion or DNA sequence analysis prior to transformation into *S. lividans*. The 0.4-kb insert was excised as a *Hin*dIII-*Bam*HI fragment and subcloned into the corresponding sites of PIJ903, creating pIJ903-14. Derivatives of pIJ486-14 and pIJ903-14 are described in the legend to Fig. 1.

Transcription studies. Runoff transcription from gel-purified DNA fragments, using *Streptomyces* RNA polymerase from previously isolated freezer stocks (5) and dinucleotide priming, have been described (5). Isolation of RNA from *S. lividans* and S1 nuclease protection experiments were described previously (1).

Kanamycin resistance. Overnight cultures of *S. lividans* were prepared by growing each strain tested in GER medium (18) containing 50 μg of thiostrepton per ml. A 10-μl portion of each culture was spotted on plates containing minimal medium (13) supplemented with 2% glucose, 50 μg of histidine per ml, 37 μg of leucine per ml, 50 μg of thiostrepton per ml, and the indicated concentrations of kanamycin (Table 1). A sterile toothpick was used to streak the spot across the plate. Cell growth was scored after 2 days of incubation at 30°C. Growth within the spot or within the entire streak is indicated in Table 1.

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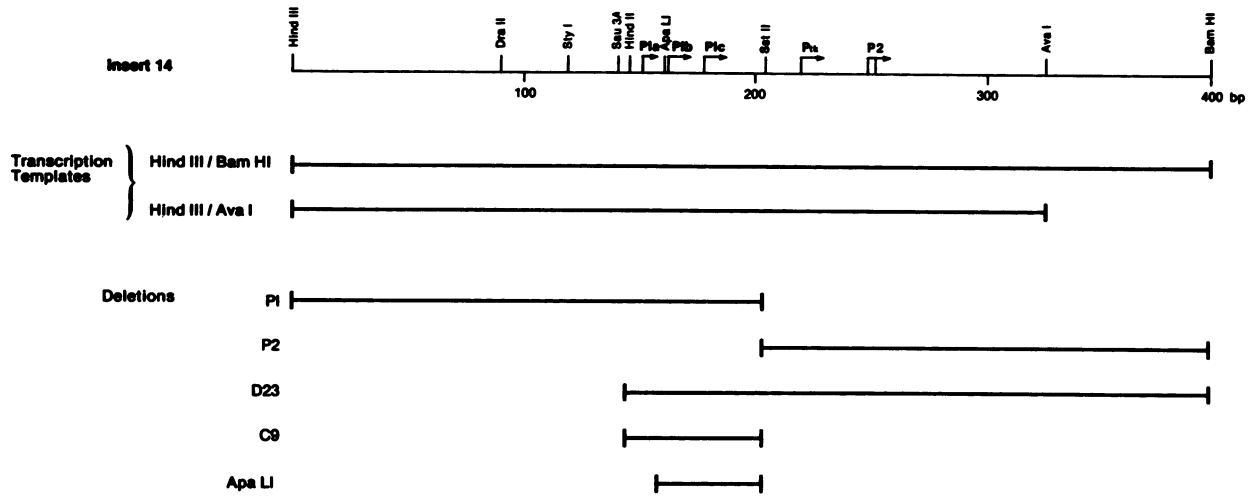

FIG. 1. Restriction map of the 0.4-kb fragment (insert 14) containing multiple tandem promoters. The DNA templates used for in vitro transcription and for dinucleotide priming (Fig. 2 and 3) are as indicated. Restriction fragments of insert 14 were subcloned into plasmid pIJ486 (21; described previously as pPP14 in reference 1) or pIJ903 (20) or both to create the indicated deletion constructs. Subfragment P1 was subcloned into the *Hin*dIII-*Eco*RI sites of pIJ486 and pIJ903. Subfragments P2 and D23 were subcloned into the *Hin*dIII-*Bam*HI sites of pIJ486 and pIJ903. Subfragments C9 and *Apa*LI were subcloned into the *Bam*HI-*Eco*RI sites of pIJ486 and into the *Hin*dIII-*Eco*RI sites of pIJ903. The *Dra*II-*Bam*HI and *Sty*I-*Bam*HI subfragments of insert 14 were subcloned into the *Hin*dIII-*Bam*HI sites of pIJ903. The *Sau*3A-*Bam*HI subfragment of insert 14 was subcloned into the *Bam*HI site of pIJ903 in the orientation shown in Fig. 6.

RESULTS

Previously, we isolated a 0.4-kb fragment from *M. echinospora* containing multiple tandem promoters as defined by S1 nuclease mapping (1). Initiation sites P1a, P1b, and P1c are 102, 90, and 73 bp upstream of promoter P2. These four initiation sites and an additional promoter, Ptk, located 32 bp upstream of P2, are utilized by *S. lividans* transformed with the promoter probe plasmid pIJ486 containing the 0.4-kb fragment (1). These studies showed that P2 can function independently of P1, since a construct deleted for P1 sequences retained P2 promoter activity. Because the three initiation sites of P1 are closely spaced and their recognition sequences may overlap, we were unable to determine whether they can function independently or whether transcripts which appear to initiate from P1b and P1c are actually degradation products of transcripts initiating at P1a.

In vitro transcription from *Micromonospora* tandem promoters. To confirm that P1a, P1b, and P1c represent true initiation sites, the 0.4-kb fragment was used as a template for in vitro transcription with purified RNA polymerase isolated from *Streptomyces* sp. A 399-bp *Hin*dIII-*Bam*HI fragment and a 321-bp *Ava*I-*Bam*HI fragment derived from the 0.4-kb fragment (Fig. 1) were used as transcription templates. Runoff transcripts of 186, 170, 153, 114, and 80 nt, which correspond in size to initiation from promoters P1a, P1b, P1c, Ptk, and P2, respectively, were detected from the *Hin*dIII-*Ava*I template, using RNA polymerase from *S. lividans* or *S. coelicolor* (Fig. 2, lanes 2 and 4). With the *Hin*dIII-*Bam*HI template, these transcripts were lengthened by approximately 80 nt, as expected (lanes 1 and 3), indicating that these RNAs are synthesized from left to right (Fig. 1). Thus, the initiation sites determined from these in vitro transcription studies are in good agreement with previous S1 mapping of RNA produced in vivo (1).

TABLE 1. Kanamycin resistance of *S. lividans* transformed with subfragments of the 0.4-kb *Micromonospora* DNA fragment in pIJ486

Plasmid	Promoter initiation site(s)	Cell growth[a] with given kanamycin concn (μg/ml)					
		0	3	10	30	100	300
pIJ486	None	++	+	−	−	−	−
*Apa*LI	P1b, c	++	++	−	−	−	−
P2	P2	++	++	++	−	−	−
C9[b]	P1a, b, c	++	++	++	+	−	−
P1	P1a, b, c	++	++	++	++	+	−
D23[b]	P1a, b, c, Ptk, P2	++	++	++	++	−	−
14	P1a, b, c, Ptk, P2	++	++	++	++	++	+

[a] +, Growth within the spot; ++, growth within the entire streak; −, no growth. See text.

[b] C9 and D23 are deleted of native sequences 5 bp upstream of P1a.

Dinucleotide priming experiments were performed to determine precisely the initiation sites within the 0.4-kb fragment. Because dinucleotides prime initiation at or near the transcriptional start sites to which they are complementary (5), these experiments should rule out the possibility that transcripts result from processing of a larger RNA species by an enzyme present both in vivo and as a contaminant in RNA polymerase preparations in vitro. The 320-nt *Hin*dIII-*Ava*I fragment derived from the 0.4-kb fragment (Fig. 1) was used as a template for dinucleotide priming, using each of the 16 possible pairs of dinucleotides and RNA polymerase isolated from *S. lividans*. Initiation from each promoter P1a, P1b, P1c, and Ptk was primed by one or two dinucleotides (Fig. 3; summarized in Fig. 4), at or within two bases of the start site previously defined by S1 nuclease protection (1).

Initiation from promoter P2 is primed by six different dinucleotides, at or within two bases of the start sites defined by S1 nuclease protection (Fig. 3 and 4). The multiple priming from the P2 region mimics the "ladder" effect observed for this promoter in previous S1 nuclease studies (Fig. 5) (1). Thus, initiation from promoter P2 probably occurs over a small region of DNA, rather than at a single, discrete nucleotide. Illegitimate priming at the ApG start site of P2′ by noncomplementary dinucleotides CpG and UpG

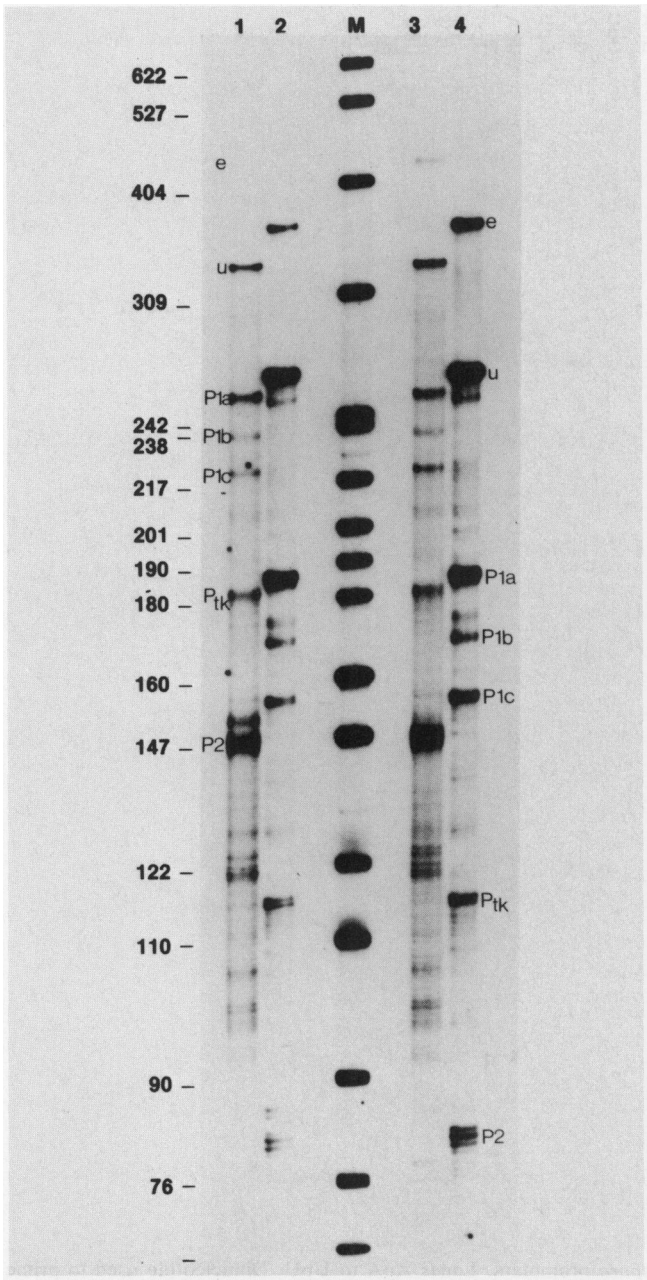

FIG. 2. In vitro transcription from *Micromonospora* promoters. DNA templates derived from the 0.4-kb fragment were incubated with RNA polymerase isolated from *S. coelicolor* (lanes 1 and 2) or *S. lividans* (lanes 3 and 4), in the presence of ribonucleotides as described previously, prior to electrophoresis and autoradiography (5). Lanes 1 and 3, 398-nt *Hin*dIII-*Bam*HI template; lanes 2 and 4, 320-nt *Hin*dIII-*Ava*I template. The sizes, in nucleotides, of molecular weight markers of pBR322 digested with endonuclease *Hpa*II (lane M) are shown on the left. Transcripts corresponding to initiation from promoters P1a, P1b, P1c, Ptk, and P2 are indicated. End-to-end transcripts (e) and artifactual transcripts (u; see text) are also indicated.

and at the ApG start site of P2 by CpG, GpG, and UpG was also detected (Fig. 3 and 4). Illegitimate priming at ApG start sites by *Streptomyces* RNA polymerase, using any dinucleotide having 3'G, has been observed previously at the P1 promoter of the *dagA* gene (5) and is considered artifactual.

In addition to the transcripts which initiate at promoters P1a, P1b, P1c, Ptk, and P2, full-length transcripts (399 nt for the *Bam*HI template and 321 nt for the *Ava*I template) were detected in vitro (Fig. 2, band e). These apparently correspond to end-to-end transcription (4). More striking is the prominent transcript of 265 nt from the *Ava*I template (Fig. 2, lanes 1 and 3, band u) which corresponds to the band of 345 nt from the *Bam*HI template (lanes 3 and 4, band u). This transcript is not observed in vivo in *M. echinospora* or in *S. lividans* (1) and could result from either initiation upstream of P1 (at region U, Fig. 4) or internal termination (at region U) of transcripts initiating at the *Bam*HI or *Ava*I end of the template. In the dinucleotide priming experiment (Fig. 3), each of the 16 dinucleotides can prime transcription from region U. This lack of specificity indicates that this promoter activity is an artifact of in vitro transcription, resulting from either starts at region U or starts from the right end of the template which terminate at region U.

Deletion analysis of the P1 promoter cluster. The three promoters of the P1 cluster are very closely spaced; P1a is 12 bp upstream of P1b and 29 bp upstream of P1c. These promoters are known to be coordinately activated during stationary phase in *M. echinospora* (1). However, they exhibit considerable activity during the growing phase in *S. lividans* (data not shown). We examined the DNA sequence requirements for the activity of promoters P1a, P1b, and P1c by constructing a series of deletions in *S. lividans*. These deletions were analyzed for promoter activity by their ability to drive the kanamycin resistance gene of the promoter-probe plasmid pIJ486 and also by S1 nuclease mapping of transcripts.

Kanamycin resistance of P1 deletion constructs. Plasmid pIJ486 (27) contains a promoterless *aphII* gene encoding kanamycin resistance, with an upstream polylinker for promoter insertion. A transcription termination site (from bacteriophage fd) directly upstream of the polylinker terminates transcripts initiating within the vector and ensures that transcription of *aphII* is from the inserted promoter (27). The amount of kanamycin resistance conferred is an indication of promoter strength (27).

Various subfragments of the 0.4-kb insert were subcloned into pIJ486 (Fig. 1). The copy number of the various deletion plasmids in *S. lividans* transformants is similar, as determined by ethidium bromide staining of plasmid DNA. Table 1 summarizes the kanamycin resistance of *S. lividans* bearing these constructs. The parent construct, pIJ486-14, confers resistance to 300 μg of kanamycin per ml. The P1 promoter cluster (pIJ486-P1) confers resistance to 100 μg of kanamycin per ml and the P2 promoter (pIJ486-P2) confers resistance to 10 μg of kanamycin per ml, indicating that the P1 and P2 regions can act independently and that the P1 region, in *S. lividans*, contains stronger promoter activity than the P2 region. We demonstrated previously by S1 nuclease protection that P2 is active in pIJ486-P2, but that Ptk activity is lost, apparently because the Ptk promoter recognition sequence is disrupted by cleavage at the *Sst*II restriction site (1).

Native DNA sequence 5 nt upstream of P1a were deleted from the 0.4-kb fragment by subcloning of the *Hin*dII/*Bam*HI subfragment, creating pIJ486-D23 (Fig. 1). This plasmid conferred resistance to 30 μg of kanamycin per ml, which is higher than the 10-μg/ml kanamycin resistance observed for P2 alone. These data suggest that either one or more of the P1 promoters are functional in this construct or P2 activity is enhanced by the presence of new upstream sequences. To distinguish between these possibilities, a

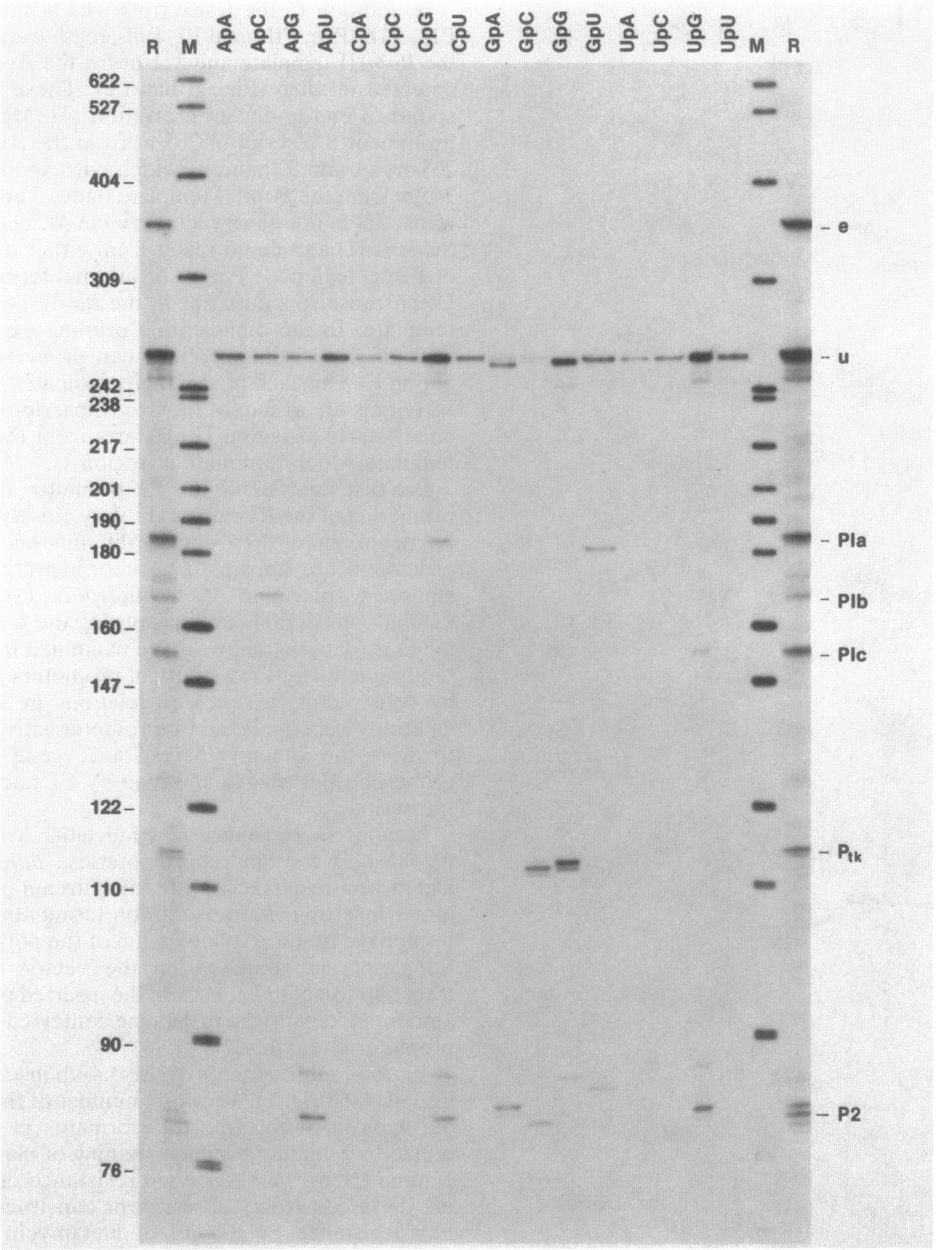

FIG. 3. Dinucleotide-primed in vitro transcription from *Micromonospora* promoters. Lanes ApA to UpU, Dinucleotide used to prime transcription from the 0.4-kb *Hin*dIII-*Ava*I DNA fragment, using *S. lividans* RNA polymerase; lane R, conventional runoff transcripts (Fig. 2). The sizes, in nucleotides, of molecular-weight markers of pBR322 digested with endonuclease *Hpa*II (lane M) are shown on the left.

plasmid containing only the P1 promoters (beginning at −5 relative to P1a), but lacking P2, was constructed by subcloning the 60-bp *Hin*dII-*Sst*II fragment into pIJ486 (Fig. 1, C9). This fragment conferred resistance to 30 μg of kanamycin per ml (Table 1), which is comparable to the D23 construct and significantly higher than that of the control plasmid, pIJ486. Thus, at least one of the P1 promoters remains active, despite the removal of native DNA sequences up to 5 bp upstream of the P1a initiation site.

S1 nuclease protection of P1 transcripts. To determine the P1 promoter(s) responsible for conferring kanamycin resistance in the deletion constructs, S1 nuclease transcriptional mapping experiments were carried out. A single-stranded antisense hybridization probe was prepared by labeling the 0.4-kb fragment at its unique *Ava*I site (Fig. 1). The probe was annealed to RNA isolated from *S. lividans* harboring pIJ486-D23, digested with S1 nuclease, and analyzed by polyacrylamide gel electrophoresis and autoradiography. All five promoters, P1a, P1b, P1c, Ptk, and P2, were functional in this construct (Fig. 5, lane 7). This result was especially striking since only 5, 17, and 34 nt of native upstream sequence remain intact for promoters P1a, P1b, and P1c, respectively. The 254-nt *Hin*dII-*Ava*I fragment of the 0.4-kb insert was included as a molecular-weight marker (lane 8) because it is 4 nt longer than the P1a transcript size determined previously (1). This marker migrates more slowly than, and can be distinguished from, the band which corresponds to transcripts initiating at P1a. Thus, the P1a band is

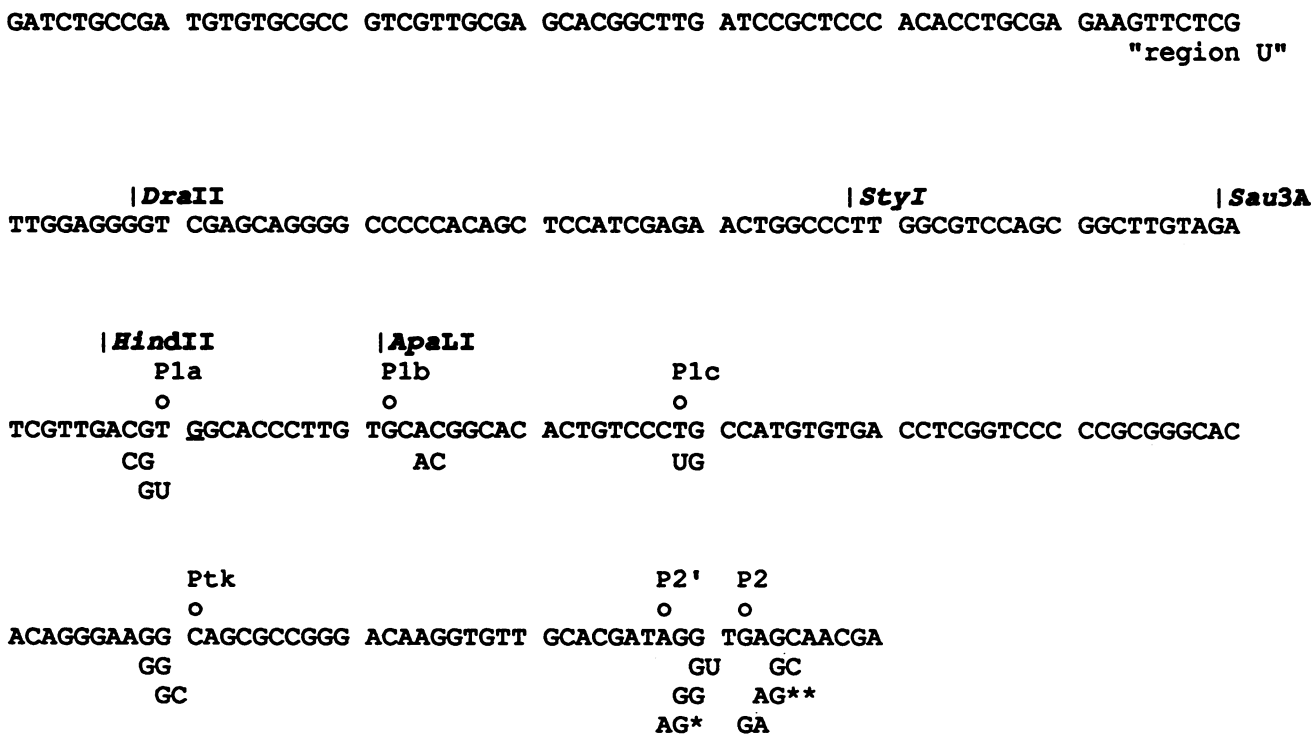

FIG. 4. Transcriptional start sites within the 0.4-kb *Micromonospora* DNA fragment. The DNA sequence of nucleotides 1 to 260 (1) is shown. Sites mapped by dinucleotide priming (Fig. 3) are indicated by the complementary dinucleotides below the DNA sequence. *, Artifactual priming by non-complementary dinucleotides CpG and UpG was also observed at this site. **, Artifactual priming by noncomplementary dinucleotides CpG, GpG, and UpG was also observed at this site. o, Promoters determined by S1 mapping of RNA synthesized in vivo (1). The underlined nucleotide was inadvertently omitted from the previously published sequence (1).

complementary to a transcript initiating from promoter P1a, and not to a transcript initiating from an upstream promoter, which would protect the probe to the *Hin*dII site.

The amount of transcription from the D23 deletion construct compared with the parent 0.4-kb fragment was similar for promoters P1a, P1b, Ptk, and P2 (cf. lanes 6 and 7). In contrast, initiation from P1c was somewhat diminished in the D23 deletion construct, suggesting that sequences upstream of −34 of P1c are required for wild-type levels of transcription from this promoter. The partial loss of P1c function is consistent with the reduced level of kanamycin resistance for *S. lividans* carrying plasmid pIJ486-D23, compared with pIJ486-14 (Table 1).

It is possible that fusion of the vector to the *Hin*dII site of the 0.4-kb insert created sequences which can substitute for the native upstream promoter elements. To address this possibility, the D23 subfragment of the 0.4-kb insert was subcloned into plasmid pIJ903, which provides another upstream environment for the evaluation of transcripts from this deletion construct. S1 nuclease protection of RNA isolated from *S. lividans* bearing pIJ903-D23 demonstrated that all five promoters, P1a, P1b, P1c, Ptk, and P2, were active (Fig. 5, lane 4). Since the DNA sequences of pIJ903-D23 and pIJ486-D23 differ significantly from that of the intact 0.4-kb fragment 5 bp upstream of P1a (Fig. 6), it is unlikely that these vectors created upstream promoter elements. Instead, it appears that utilization of the P1 promoter cluster was independent of sequences upstream of the *Hin*dII site.

The level of P1c transcripts in *S. lividans* containing plasmid pIJ903-D23 is somewhat reduced compared with *S. lividans* harboring pIJ903-14 (lanes 3 and 4). These results are consistent with the findings for deletion construct pIJ486-D23 (lanes 6 and 7) and support the notion that sequences upstream of −34 of P1c may be required for wild-type levels of transcription from this promoter. We also observed that despite the high copy number of pIJ486 relative to pIJ903 (100 to 200 versus 1 to 2 copies per cell [20, 27]), there was less than a fivefold difference in the steady-state level of transcripts detected from each promoter.

Additional 5′ deletions of the 0.4-kb fragment in pIJ903 were constructed by removing native DNA sequences 73, 32, and 11 nt upstream of the P1a start site at the *Dra*II, *Sty*I, and *Sau*3A restriction sites, respectively (Fig. 1). The 3′ end of each construct is the *Bam*HI site (Fig. 1). In each case, S1 nuclease protection of RNA isolated from *S. lividans* harboring these constructs demonstrated that P1a, P1b, P1c, Ptk, and P2 were active (data not shown). Thus, the pIJ903-*Sau*3A construct provides another example that P1a and P1b can function in the absence of extensive upstream native sequence (Fig. 6). Since P1c appears fully functional in the *Sau*3A construct, these data suggest that the recognition sequence for P1c may reside within 40 bp upstream of the P1c initiation site (Fig. 6).

A plasmid containing a more extensive deletion, which was missing native sequences 1 nt upstream of P1b, was constructed by subcloning the 43-bp *Apa*LI-*Sst*II fragment of the 0.4-kb insert into pIJ486 (Fig. 1). This construct was sensitive to 10 μg of kanamycin per ml (Table 1), indicating that promoter activity was virtually abolished. Transcripts could not be detected by S1 analysis (data not shown).

DISCUSSION

We demonstrated previously that a 0.4-kb DNA fragment from *M. echinospora* contains a complex transcriptional

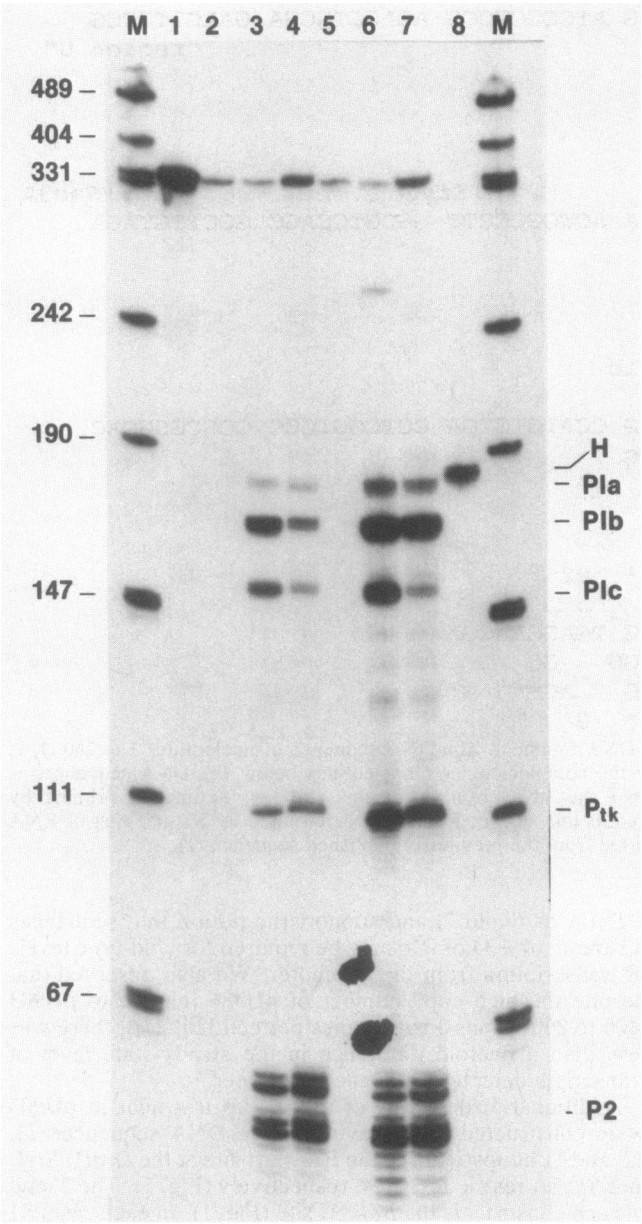

FIG. 5. Mapping of transcriptional start sites in the *Hin*dII deletion constructs. A single-stranded, 325-nt *Hin*dIII-*Ava*I probe labeled with T4 polynucleotide kinase at the *Ava*I site of the 0.4-kb fragment was hybridized to 10 μg of RNA isolated from *S. lividans* transformed with the indicated plasmids. After S1 nuclease digestion, samples were subjected to electrophoresis through a sequencing gel and autoradiographed. Lane 1, Undigested probe (5% of the amount used in each hybridization); lane 2, pIJ903; lane 3, pIJ903-14; lane 4, PIJ903-D23; lane 5, pIJ486; lane 6, pIJ486-14; lane 7, pIJ486-D23; lane 8, double-stranded *Hin*dIII-*Ava*I probe digested with *Hin*dII prior to denaturation and electrophoresis. The positions of protected probe corresponding to promoters P1a, P1b, P1c, Ptk, and P2 and of probe digested with *Hin*dII (H) are indicated. The sizes, in nucleotides, of pUC19 digested with *Hpa*II (lane M) are shown on the left.

regulatory region of multiple tandem promoters, which are differentially regulated during the *Micromonospora* life cycle (1). As discussed by Hopwood et al., tandem promoters may provide a mechanism for graded gene expression under different growth conditions (12). Analysis of the essential elements of the P1 promoter region, in particular, may lead to a better understanding of gene regulation during the stationary phase, when antibiotics such as calicheamicin are produced.

Each of the *Micromonospora* promoters is recognized in vivo by *S. lividans*. Tandem promoters have been documented in *Streptomyces* spp. for the genes encoding glycerol utilization, streptomycin, α-amylase, agarase, tyrosinase, and the resistance genes for erythromycin, thiostrepton, and neomycin (5, 8, 11, 12, 14, 15). The distances between the tandem promoters is generally 50 to several hundred base pairs. In contrast, *Micromonospora* promoter P1a is 12 bp upstream of P1b, which is 17 bp upstream of P1c. We now show, by runoff transcription and dinucleotide priming, that each promoter, P1a, P1b, P1c, Ptk, and P2, is active in vitro. Each of these promoters is therefore a bona fide initiation site for RNA synthesis and not a processing site for transcripts which initiate further upstream. For each promoter, the initiation site determined in vitro is within 2 bp of the site mapped previously in vivo (1).

The P1a and P1b promoters are unusual because they do not appear to require specific DNA sequences at small distances upstream of initiation. These deletion experiments were carried out in *S. lividans* and are therefore only suggestive of what might occur in the native *Micromonospora* host. The deletion analysis suggests that as little as 5 bp of sequence upstream of P1a, and 17 bp upstream of P1b, is sufficient for transcription from each of these promoters. Although it is possible that sequences contributed by the vector recreate upstream promoter sequences, we consider this unlikely due to the differences between the vector sequences joined to the deletions (Fig. 6). The *ermE* promoter of *S. erythraeus* is likewise functional in the absence of its −35 region, albeit with significantly reduced efficiency (2). The P_{re} promoter of phage λ (17) and the *E. coli gal* promoters (24) are other examples of promoters recognized by bacterial RNA polymerase which do not require −35 regions. The importance of DNA sequences at least 40 bp upstream from the start site of RNA transcription, however, is a general feature of procaryotic promoters (23). In contrast, the class III promoters of phage T7 require less upstream DNA sequence, from −17 to +6 bp, actually overlapping the start site of RNA transcription (9). These promoters are recognized by an RNA polymerase encoded entirely by the phage genome. Similar results have been obtained for phage T4 late promoter (10). In eucaryotes, a more extreme example is the recognition sequence of RNA polymerase III, which is internal to the 5S RNA gene (3).

The P1a and P1b promoters may be transcribed by one of the several forms of RNA polymerase that have been reported for *Streptomyces* spp., which use the common core subunits and differ in the sigma subunit (6). In *B. subtilis*, new forms of RNA polymerase appear during sporulation (25); a similar mechanism in *Micromonospora* spp. could account for the temporal regulation observed in that organism.

The promoters of the P1 region are very closely spaced; P1a is 12 bp upstream of P1b and 29 bp upstream of P1c. Thus, their recognition sequences may overlap. In addition, transcription from these promoters is coordinately induced during stationary phase in *M. echinospora*. It is possible that utilization of one promoter would enhance, or even be essential for, the utilization of other promoters. Our analysis suggests that promoter P1c may be different from P1a and P1b. The deletion of native sequences 34 bp upstream diminishes P1c activity. Thus, in terms of upstream se-

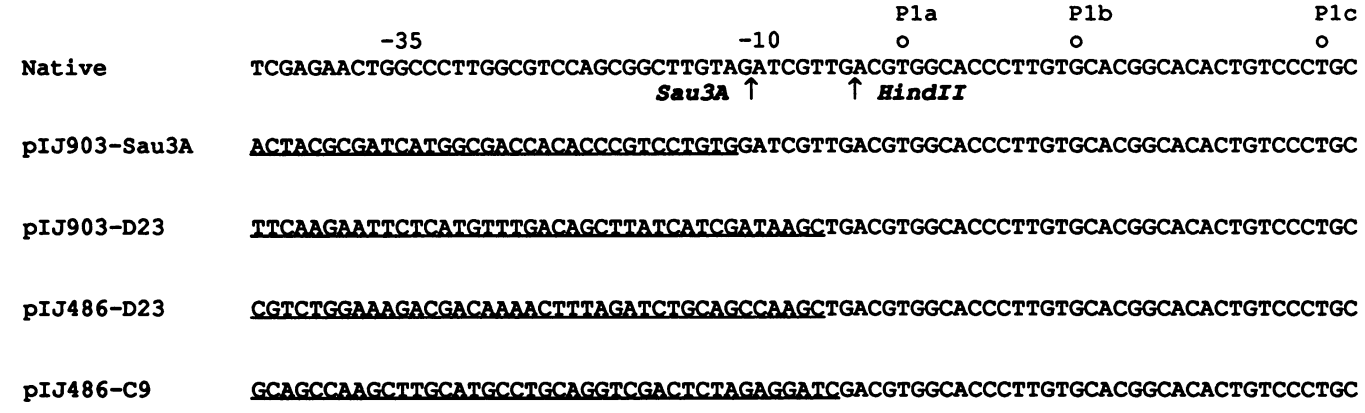

FIG. 6. DNA sequences upstream of the P1 promoter region for various deletion constructs. The positions of P1a, P1b, and P1c (o) are indicated. Sequences differing from the native construct are derived from the vector and are denoted by underlining.

quence requirements, P1c appears to be more similar than P1a and P1b to typical procaryotic promoters. These results may indicate that promoter P1c is transcribed by a different form of RNA polymerase than P1a and P1b. The dinucleotide priming experiments suggest that the P1 promoters can act independently, since particular dinucleotides prime transcription only from P1a, P1b, or P1c. It is possible, however, that initial binding of RNA polymerase(s) at one of the promoters is essential for activity at other promoters.

It will be useful to determine the effects of single nucleotide mutations in the P1 region on the utilization of P1a, P1b, and P1c. We would also like to determine whether more than one form of RNA polymerase is responsible for transcription from promoters P1a, P1b, P1c, and P2 and, if so, whether these forms are differentially expressed in *Micromonospora* spp. Such studies should contribute to an understanding of gene regulation during stationary phase, when antibiotics are produced.

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