Unusual transcriptional and translational features of the aminoglycoside phosphotransferase gene (*aph*) from *Streptomyces fradiae*

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The aminoglycoside phosphotransferase gene (*aph*) from the neomycin producer *Streptomyces fradiae* encodes an enzyme (APH) that phosphorylates, and thereby inactivates, the antibiotic neomycin. Two promoters were identified upstream of and oriented toward the *aph* coding sequence. One promoter (*aphp1*) initiated transcription at the A of the ATG translational initiation codon, or one to two bases upstream. Mutations made in this promoter region identified functionally important nucleotides and verified that the *aphp1* transcript was translated to yield the APH protein, despite the lack of a conventional ribosome binding site. A second *aph* promoter, *aphp2*, initiated transcription 315 bp upstream of the translational initiation codon but gave transcripts that appeared to terminate before reaching the coding sequence. Multiple transcriptional initiation sites (pA1–pA5) were identified also in the *aph* regulatory region oriented in the opposite direction to *aph* transcription. Promoters for the pA2 and pA4 transcripts overlap with *aphp1* such that down-promoter mutations in *aphp1* also reduce transcription from the overlapping pA promoters.

[Key Words: Antibiotic resistance; divergent transcription; translational initiation; multiple promoters]

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Streptomycetes are Gram-positive mycelial soil bacteria that undergo a complex process of morphological development (Chater 1984). In most *Streptomyces* species this is accompanied by a biochemical differentiation that includes the production of antibiotics and other secondary metabolites, many with important medical and agricultural applications (Berdy 1980). The development of efficient systems for molecular genetic analysis of *Streptomyces* has led to the analysis of RNA polymerase and promoter sequence heterogeneity and its involvement in morphological and biochemical differentiation. These studies have indicated that there are at least eight different RNA polymerase holoenzyme forms, involving at least seven different σ factors, in *Streptomyces coelicolor* A3(2) (Westpheling et al. 1985; Buttner et al. 1988; Takahashi et al. 1988; K.F. Chater, pers. comm.). Attempts to compare promoter sequences also have shown extensive heterogeneity clearly (for review, see Hopwood et al. 1986a). In a striking example of the kind of complexity that can occur, the agarase gene (*dagA*) of *S. coelicolor* A3(2) was shown to be transcribed by at least three, and possibly four, different RNA polymerase ho-

loenzymes (Buttner et al. 1988). It also appears that the *galE* and *galK* genes of *Streptomyces lividans* (Fornwald et al. 1987) are transcribed by two different RNA polymerase holoenzymes from two promoters, *galP1* and *galP2* (J. Westpheling and M.E. Brawner, pers. comm.). These results suggest that multiple promoters recognized by different forms of RNA polymerase holoenzyme might be used widely to regulate the transcription of genes in streptomycetes differentially, perhaps including those for antibiotic production and differentiation.

The level of antibiotic resistance of a producing culture often increases at the onset of antibiotic biosynthesis (Martin and Demain 1980), a phenomenon that may be a requirement for self-protection.

Because biosynthesis generally commences concomitantly with morphological differentiation, studies of the expression of resistance determinants might provide an insight into the mechanisms regulating both antibiotic production and morphological development. The aminoglycoside phosphotransferase gene (*aph*) from *Streptomyces fradiae* was one of the first streptomycete genes to be cloned and sequenced (Thompson et al. 1980; Thompson and Gray 1983). Amino-terminal analysis of the aminoglycoside phosphotransferase protein (APH) (Thompson and Gray 1983) precisely located the transla-

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tional start site. The nucleotide sequence immediately 5' to this site did not contain a conventional ribosome binding site (RBS), even though, when *aph* was expressed in *S. lividans* at a copy number of five to six per chromosome, APH represented 10% of the total soluble protein (Thompson and Gray 1983). Promoter-probing studies (Bibb et al. 1985) identified a fragment that contained the 5' end of the *aph* gene and upstream sequences that functioned as a promoter in *S. lividans*; this promoter-active fragment did not appear to function in *Escherichia coli* and did not contain sequences corresponding to both the -10 and the -35 regions found in the major class of eubacterial promoters (Hawley and McClure 1983) (although potential -10 regions could be identified). These results suggested that *aph* possessed transcriptional and translational regulatory features interestingly different from those commonly found in other bacteria. In this report, we used a variety of techniques to analyze the transcription and translation of the *S. fradiae aph* gene.

Results

Nuclease S1 and exonuclease VII mapping reveals two apparent transcriptional start sites 5' of the *aph* coding sequence

Promoter probing of the *aph* region by Bibb et al. (1985)

identified promoter activity in a 524-bp *Sau3A* fragment that included the amino-terminal end of the APH coding sequence (Thompson and Gray 1983) and 399 bp of 5' upstream sequence. This region (segment 1-7) is shown schematically in Figure 1.

A 575-bp *BglI*-*Bam*HI fragment (segment 2-9, Fig. 1), uniquely labeled at the 5' end of *Bam*HI site 9, was used in nuclease S1 protection experiments to map the 5' end of the *aph* transcripts present in RNA isolated from *S. lividans* TK24(pIJ2951) and from *S. fradiae* 10745/H3 (Fig. 2, lanes c and d, respectively). Taking into account the 1- to 1.5-nucleotide difference in 3' ends that results from nuclease S1 digestion and the chemical sequencing reactions (Hentschel 1985), the 5' end of the RNA was found to correspond to the first nucleotide of the *aph* translational initiation codon (Thompson and Gray 1983) (an A residue indicated as a T on the template sequence shown in Fig. 2). RNA from TK24 (pIJ2951) or TK24(pIJ2955) (containing 400 and 123 bp of 5' upstream sequence, respectively) identified the same potential transcriptional start site (Fig. 3, lanes a and c). This site was identified also by use of the 314-bp *Sst*II-*Taq*I fragment (segment 4-8, Fig. 1) uniquely labeled at the 5' end of *Taq*I site 8, as a probe in nuclease S1 protection experiments with RNA isolated from *S. fradiae* 10745, *S. fradiae* 10745/H3, TK24(pIJ365), TK24(pIJ2951), or TK24(pIJ2955) (data not shown).

Figure 1. Physical map and experimental strategy for analysis of the *aph* promoter region. The region corresponding to the amino-terminal segment of APH is indicated by the broad, stippled arrow. Transcriptional start points are identified by open circles; directions of transcription are indicated with arrows. Hatched lines below the restriction map represent the protected portions of DNA fragments used in the nuclease S1 mapping experiments; the asterisk indicates the labeled 5' end and the two site numbers adjacent to the asterisk identify the fragment originally present in each probe. The dashed arrows represent transcripts generated in vitro from template fragments defined by the two numbers adjacent to the arrows. The *Bam*HI site at position 1 was generated when the 524-bp *Sau3A* fragment (segment 1-7 in this figure) of Bibb et al. (1985) was subcloned into a *Bam*HI site.

Exonuclease VII digestion (Sharp et al. 1980) of a hybridization reaction containing RNA from TK24(pIJ2951) and the *Bgl*II–*Bam*HI fragment (segment 2-9, Fig. 1), uniquely labeled at the 5' end of *Bam*HI site 9, as a probe yielded a protected DNA fragment of similar length to that obtained with nuclease S1 (Fig. 3, lanes b and a, respectively). [As observed previously (Bibb et al. 1986), and as reported by others (Rose and Botstein 1983), exonuclease VII digestion yielded a protected fragment that was ~5–7 nucleotides larger than the corresponding fragments that resulted from nuclease S1 digestion, presumably reflecting the inability of exonuclease VII to digest single-stranded overhangs to

flush ends.] This suggested that the site identified by nuclease S1 mapping corresponded to the 5' end of an *aph* transcript that occurred in vivo rather than to the product of endonucleolytic cleavage in vitro by nuclease S1 at a region of secondary structure. The putative pro-

Figure 2. Nuclease S1 mapping of *aphp1*. The *Bgl*II–*Bam*HI fragment (segment 2-9, Fig. 1) of pIJ2935, uniquely labeled at the 5' end of the *Bam*HI site, was used in hybridization reactions with RNA from strains containing the *aph* gene. Hybridization reactions contained 40 μ g of RNA; the amounts of RNA that gave rise to the observed level of protection are indicated below and were estimated from the proportion of the total sample, after nuclease S1 digestion and precipitation, that was loaded onto the gel: (lane c) 1.6 μ g of RNA from TK24(pIJ2951); (lane d) 8 μ g of RNA from *S. fradiae* 10745/H3; (lane e) 8 μ g of RNA from TK24. Sequence ladders for the *Bgl*II–*Bam*HI fragment are in lanes a (G + A) and b (T + C). (*) Probable start point of transcription.

Figure 3. Exonuclease VII and nuclease S1 mapping of transcripts derived from mutated and nonmutated *aph* promoter regions. The *Bgl*II–*Bam*HI fragment (segment 2-9, Fig. 1) of pIJ2935, uniquely labeled at the 5' end of the *Bam*HI site, was used in hybridization reactions with 40 μ g of RNA from TK24 derivatives containing the *aph* gene with the *aphp1* or *aphp1* plus *aphp2* promoter regions. The amounts of RNA that gave rise to the observed levels of protection are indicated below and were estimated from the proportion of the total sample, after exonuclease VII or nuclease S1 digestion and precipitation, that was loaded on the gel; the level of protection observed in lanes a–c results from 0.53 μ g of RNA, whereas that in lanes f–j and m–p results from 16 μ g of RNA. The protected probe in lane b is the result of digestion with exonuclease VII; all others are the result of digestion with nuclease S1. RNA was used from TK24 derivatives containing the following plasmids: (lanes a and b), pIJ2951 (*aphp1* plus *aphp2*); (lane c), pIJ2955(*aphp1*); (lane f), plasmid-less control; (lane g), pIJ2951 (*aphp1* plus *aphp2*); (lane h), pIJ2952 (–10 mutation in *aphp1*, *aphp2*); (lane i), pIJ2953 (–35 mutation in *aphp1*, *aphp2*); (lane j), pIJ2954 (*Nco*I end fill at *aphp1*, *aphp2*); (lane m), pIJ2955 (*aphp1*); (lane n), pIJ2956 (–10 mutation in *aphp1*); (lane o), pIJ2957 (–35 mutation in *aphp1*); (lane p), pIJ2958 (*Nco*I end fill at *aphp1*). Sequence ladders derived from the *Bgl*II–*Bam*HI fragment are in lanes d and k (G + A) and e and l (T + C). The position of the *aphp1* transcript is indicated by an arrow.

moter that preceded this transcript was designated *aphp1*.

Nuclease S1 mapping experiments using the 396-bp *Bam*HI–*Nco*I fragment (segment 1-5, Fig. 1), uniquely labeled at the 5' end of *Nco*I site 5, as a probe and RNA from *S. fradiae* 10745/H3 or TK24(pIJ2951) failed to reveal a potential transcriptional start site upstream of the *Nco*I site (data not shown). Additional nuclease S1 mapping experiments using as a probe a 210-bp *Bgl*II–*Taq*I fragment (segment 2-3, Fig. 1) uniquely labeled at the 5' end of *Taq*I site 3 and RNA from *S. fradiae* 10745, *S. fradiae* 10745/H3, or TK24(pIJ365) identified another potential transcriptional start site 313 nucleotides upstream of the *aphp1* start site (Fig. 4). This putative promoter was designated *aphp2*. This latter transcript was not detected with the end-labeled probe that contained sequences complementary to mRNA produced from *aphp1* (Fig. 2).

Mutational analysis confirms the *in vivo* promoter activity of *aphp1*

Examination of the nucleotide sequence upstream of the *aphp1* transcriptional initiation site revealed similarity in the –10 region to the consensus sequence for the major class of *E. coli* promoters, but negligible similarity in the –35 region (Hawley and McClure 1983). Site-directed mutagenesis was used to introduce three mutations into the *aphp1* promoter region to demonstrate that the RNA-protected fragment mapping at the *aph* translational start site resulted from initiation at the

aphp1 promoter and that the *aphp1* transcript was translated to yield APH protein.

First, a synthetic oligonucleotide was used to introduce a T → C transition at the highly conserved sixth position of the presumed –10 region (i.e., at the T residue mapping at position –8 relative to the *aphp1* start point; this corresponds to the most highly conserved nucleotide in the –10 region of the major class of eubacterial promoters). Second, a synthetic oligonucleotide was used to generate a 3-bp deletion in the presumed –35 region, removing the 5'-GGC-3' residues mapping at positions –40 to –38 relative to the *aphp1* start point. Third, because the *aphp1* transcriptional start site is included in a *Nco*I recognition sequence (5'-CATGG-3'), digestion with *Nco*I, followed by end-filling with the Klenow fragment of DNA polymerase I and blunt-end ligation, resulted in a 4-bp insertion (which was confirmed by DNA sequencing) at the transcriptional–translational initiation site.

The effects of these mutations on *in vivo* transcription from *aphp1* were determined by nuclease S1 mapping (Fig. 3). RNA from TK24 strains carrying the *aph* gene with the described *aphp1* mutations, in the presence (Fig. 3, lanes g–j) or absence (Fig. 3, lanes m–p) of *aphp2*, was used in hybridization reactions with a *Bgl*II–*Bam*HI probe (segment 2-9, Fig. 1) uniquely labeled at the 5' end of *Bam*HI site 9. The amount of transcript from the unaltered *aphp1* promoter is shown in lane m (pIJ2955, containing *aphp1* only) and lane g (pIJ2951, containing *aphp1* and *aphp2*). The effect of the single nucleotide substitution of T → C in the –10 region is shown in lanes n (pIJ2956, containing *aphp1*) and h (pIJ2952, containing *aphp1* and *aphp2*); the amount of *aphp1* transcript was reduced below the level of detection of this analysis. The –35 region deletion [lane o (pIJ2957, containing *aphp1*) and lane i (pIJ2953, containing *aphp1* and *aphp2*)] severely reduced the amount of *aphp1* transcript and the *Nco*I end-fill [lane p (pIJ2958, containing *aphp1*) and lane j (pIJ2954, containing *aphp1* and *aphp2*)] resulted in a significant reduction in the level of *aphp1* transcript. Transcription from the *aphp1* promoter was not significantly different in the presence (lanes g–j) or absence (lanes m–p) of the upstream *aphp2* promoter in any of these constructs.

Figure 4. Nuclease S1 mapping of *aphp2*. The *Bgl*II–*Taq*I fragment (segment 2-3, Fig. 1) of pIJ2935, uniquely labeled at the 5' end of the *Taq*I site, was used as probe in hybridization reactions with RNA isolated from strains containing the *aph* gene. Hybridization reactions contained 40 µg of RNA, and in each case the entire nuclease S1-resistant reaction product was loaded on the gel. The origin of the RNA used was as follows: (lane c) TK24 (pIJ365) (i.e., +*aph*); (lane d) *S. fradiae* 10745; (lane e) *S. fradiae* 10745/H3; (lane h) no RNA and no nuclease S1 digestion (i.e., full-length DNA probe as size marker); (lane i) *S. lividans* 1326 (pIJ101) (i.e., –*aph*); (lane j) no RNA (i.e., probe DNA after nuclease S1 digestion). Sequence ladders for the *Bgl*II–*Taq*I fragment are in lanes a and f (T + C) and b and g (G + A). (*) Probable start point of transcription.

In vitro transcription studies confirm the nature of *aphp1* and identify oppositely oriented transcripts

RNA polymerase isolated from *S. coelicolor* A3(2) strain M145 was used for *in vitro* transcription studies on the *aph* promoter region. Transcription from *aphp1*, when located on a *Bam*HI–*Bam*HI fragment or on a *Bam*HI–*Sst*I fragment (segments 1-9 and 1-6, respectively, Fig. 1), would be expected to generate transcripts of 214 nucleotides and 115 nucleotides, respectively. Transcription from *aphp1* on an *Eco*RI–*Bam*HI fragment from pIJ2941 (containing segment 4-9, Fig. 1) would be expected to generate a transcript of 214 nucleotides. Figure 5A shows that *in vitro* transcriptions that ran off the *Bam*HI end yielded a major transcript of ~214 nucleo-

tides (lanes b and c); transcriptions that ran off the *Sst*I end yielded the expected transcript of ~115 nucleotides (lane d).

Transcription from *aphp2* would be expected to generate a transcript of ~159 nucleotides when located on a *Bam*HI–*Taq*I fragment (segment 1-3, Fig. 1). In vitro

Figure 5. Transcription in vitro from the *aph* promoter region. Transcription reactions contained 0.1 U of *S. coelicolor* A3(2) strain M145 RNA polymerase and ~0.5 pmole of purified template fragment. Approximately 5% of each of the total in vitro reaction products was loaded in each lane. Labeled fragments of *Hpa*II-digested pBR322 were used as size markers in lanes a of A and B. *aphp1* transcripts running off at *Sst*I site 6 and *Bam*HI site 9 (Fig. 1) would be expected to generate transcripts of 114 and 212 nucleotides, respectively. Variable amounts of end-to-end transcription were observed for each fragment. (A) Transcriptions from the 345-bp *Eco*RI–*Bam*HI fragment of pIJ2941 (lane b), the 602-bp *Bam*HI–*Bam*HI fragment of pIJ2935 (lane c), and the 508-bp *Bam*HI–*Sst*I fragment of pIJ2935 (lane d). (B) Transcriptions from the *Bam*HI–*Bam*HI fragment of pIJ2935 (nonmutated *aphp1*; lane b), pIJ2936 (–10 mutation in *aphp1*; lane c), pIJ2937 (–35 mutation in *aphp1*; lane d), and pIJ2947 (*Nco*I end fill; lane e). Arrows to the right of the figure indicate the position of the *aphp1* transcript (bottom arrow) and of the ~400-nucleotide (top arrow) and 320-nucleotide (middle arrow) transcripts that are oriented in the opposite direction to that of *aphp1*.

transcriptions with this fragment from pIJ2935, however, failed to produce a transcript corresponding to initiation at *aphp2* (data not shown).

Figure 5B shows in vitro transcripts produced from *Bam*HI fragments (segments 1-9, Fig. 1) containing the *aphp1* mutations described above. Lane b shows the expected 214-nucleotide transcript produced from the unaltered *aphp1*. The 3-bp deletion in the –35 region (lane d) significantly reduced the amount of *aphp1* transcript while the T → C transition in the –10 region (lane c) reduced the amount of *aphp1* transcript below the level of detection of this analysis. The 4-bp insertion mutation, which resulted from the end-filled *Nco*I site, also showed a decrease in the amount of *aphp1* transcript (lane e); the *aphp1* transcript was now slightly larger, which indicated that the 4-bp insertion was located within the transcribed region.

The T → C transition in the –10 region of *aphp1* also resulted in the loss of another in vitro synthesized transcript, ~400 nucleotides in size (Fig. 5B, lane c, top arrow). Transcription from an asymmetrically shortened, nonmutated template (i.e., the *Bam*HI–*Sst*I fragment (segment 1-6, Fig. 1) also yielded the 400-nucleotide transcript (Fig. 5A, lane d). Transcription from a second asymmetrically shortened template—the *Sst*II–*Bam*HI fragment (segment 4-9, Fig. 1)—yielded a transcript of ~122 nucleotides (Fig. 5A, lane b). Together, these results predicted the occurrence of a promoter that initiates transcription near to *aphp1* but that is oriented in the opposite direction.

The 3-bp deletion mutation in the –35 region of *aphp1* also reduced the amount of another in vitro-synthesized transcript, ~320-nucleotides in size (Fig. 5B, lane d, middle arrow). Transcription from the asymmetrically shortened *Bam*HI–*Sst*I template (segment 1-6, Fig. 1) did not affect the size of the 320-nucleotide transcript (Fig. 5A, lane d), which suggests that it might also be oriented in the opposite direction to the *aphp1* transcript.

Nuclease S1 mapping indicates the occurrence of multiple transcripts oriented away from the *aph* gene

The in vitro transcription data predicted the presence of two promoters that directed transcription in the opposite orientation to *aphp1*. A 268-bp *Taq*I–*Sst*I fragment (segment 3-6, Fig. 1), uniquely labeled at the 5' end of *Taq*I site 3, was used as a probe in nuclease S1 protection studies to locate the 5' ends of these transcripts; the results are shown in Figure 6. RNA from a strain carrying a plasmid (pIJ2951) with the nonmutated *aphp1* promoter identified four apparent transcriptional start sites, designated pA1–pA4, that were transcribed in the opposite orientation to the *aphp1* transcript (Fig. 6A, lane f; 6B, lane c). On the basis of their size and relative abundance, the in vitro transcripts of ~320 and 400 nucleotides seen in Figure 5 would correspond to initiation in vivo at the pA1 and pA2 promoters, respectively.

Nuclease S1 mapping of RNA from a strain carrying a

plasmid (pIJ2952) with the *aphp1* -10 region T → C transition mutation indicated that the major transcriptional start at pA1 relatively was unaffected by the mutation (Fig. 6A, lane e), whereas the transcript originated at pA2 had been eliminated (Fig. 6A, lane e; 6B, lane d); the minor transcript starts at pA3 and pA4 were no longer detected but another apparent start site, pA5, appeared (Fig. 6B, lane d). The results obtained by use of RNA from a strain carrying a plasmid (pIJ2953) with the *aphp1* -35 region 5'-GGC-3' deletion mutation are shown in Figure 6A, lane d; the amount of transcript that started at pA1 was reduced significantly. The levels

of the pA2, pA3, and pA4 transcripts also appeared lower, presumably because of cleavage of their respective hybrids at the nuclease S1-sensitive heteroduplex that would result from the annealing of the mutant RNAs with the nonmutant probe DNA; the bands that migrated just above the 110-bp marker fragment (Fig. 6A, lane d) locate the position of the 3-nucleotide deletion. The low level of pA2 transcript observed in Figure 6A, lane d, and in Figure 6B, lane e, probably reflects incomplete digestion by nuclease S1 at the position of the 5'-GGC-3' deletion. RNA from a strain carrying a plasmid (pIJ2954) with the end-filled *NcoI* site at the

Figure 6. Nuclease S1 mapping of the pA1–pA5 transcripts. The *TaqI*–*SstI* fragment of pIJ2935 (segment 3–6, Fig. 1), uniquely labeled at the 5' end of the *TaqI* site, was used as probe in hybridization reactions with RNA synthesized in vivo or in vitro. RNA synthesized in vivo was from TK24 or TK24 derivatives with pIJ2951 (nonmutated at *aphp1*), pIJ2952 (–10 mutation at *aphp1*), pIJ2953 (–35 mutation at *aphp1*), or pIJ2954 (*NcoI* end fill at *aphp1*). Electrophoresis was carried out to optimize resolution of the pA1 (part A) and pA2–pA5 (part B) start sites; the end points of the major RNA-protected fragments are indicated by asterisks on the accompanying sequences. (A) Hybridizations contained 20 μ g of in vivo synthesized RNA from TK24 derivatives that contained the following plasmids: (lane c) pIJ2954; (lane d) pIJ2953; (lane e) pIJ2952; (lane f) pIJ2951; (lane g) plasmid-less control. Lane h contains an end-labeled *HpaII* digest of pBR322 to provide size markers. Sequence ladders derived from the *TaqI*–*SstI* fragment are in lanes a (T + C) and b (G + A). The position of the 3-bp deletion in pIJ2953 is indicated by Δ . (B) Hybridizations contained 80 μ g of in vivo synthesized RNA (lanes c–f, j, k) or the products of an in vitro transcription reaction with the purified 314-bp *TaqI* fragment of pIJ2935 (segment 3–8, Fig. 1) as template (lane g). RNA was used from TK24 derivatives that contained the following plasmids: (lane c) pIJ2951; (lane d) pIJ2952; (lane e) pIJ2953; (lane f) pIJ2954; (lane j) plasmidless control. Lane k represents the hybridization products with 80 μ g RNA from *S. fradiae* 10745/H3. Sequence ladders derived from the *TaqI*–*SstI* fragment are in lanes a and h (G + A) and in b and i (T + C).

aphp1 transcriptional start point indicated an increased level of expression at pA1, pA2, and pA3 but no expression at pA4 or pA5 (Fig. 6A, lane c, and Fig. 6B, lane f). RNA from *S. fradiae* demonstrated low levels of transcript from pA2 and pA4 (Fig. 6B, lane k). Figure 6B, lane g, shows the RNA-protected products that resulted from S1 nuclease digestion of a hybridization reaction between unlabeled RNA synthesized in vitro and the same labeled probe DNA that was used in lanes c–f; transcripts corresponding to pA2, pA3, and pA5 were apparent readily. The relative locations of the pA1–pA5 transcripts are indicated in Figures 1 and 8 (below).

Dinucleotide-initiated in vitro transcription from *aphp1*, pA1, and pA2

Specific dinucleotides at a concentration sufficient for in vitro transcript initiation (e.g., 150 μ M), together with a ribonucleotide mixture at a concentration sufficient for transcript elongation but not for initiation (e.g., 2 μ M), can be used to identify transcriptional initiation points (Moran et al. 1981). This technique was used to identify and confirm the *aphp1* and some of the pA transcriptional start points. Figure 7 shows that the *aphp1* transcript was primed specifically only by the CpA and CpC dinucleotides, corresponding to one and two nucleotides, respectively, upstream of the position identified by nuclease S1 mapping in Figure 2.

Figure 7 also shows that the pA1 transcript was primed by GpG, which is consistent with the nuclease S1 mapping results in Figure 6 (although the nuclease S1 mapping suggested a staggered start over 3–4 adjacent nucleotides). The pA2 transcript was primed strongly by the ApG, GpG, and UpG dinucleotides and weakly primed by CpG, GpA, and GpC. The GpA, ApG, and GpG dinucleotides correspond to initiation at the pA2 –1, +1, and +2 positions, respectively, identified by nuclease S1 mapping (see Fig. 6B). The pA2 transcripts initiated by CpG, GpC, or UpG should differ in size relative to the ApG-initiated transcript by –3, –2, and +6 nucleotides, respectively. Because of their size similarity to the ApG transcript it is likely that the CpG-, GpC-, and UpG-primed transcripts represent illegitimate priming at pA2, as observed by Buttner et al. (1987). The origin of the apparent GpA-primed transcript that migrated between the *aphp1* and pA1 transcripts is not known.

Nucleotide sequence of the complex *aph* regulatory region

The transcriptional organization and nucleotide sequence of the *aph* promoter region are shown in Figure 8. The sequence includes corrections (at positions 185, 243, and 342) of typographical errors that occurred in an earlier published version (Bibb et al. 1985). The presumed –10 regions for the *aphp1* and pA2 promoters are underlined to emphasize the physical and functional overlap, on opposite strands, of these two promoters. The deletion in the –35 region of *aphp1* is also underlined; it maps to a position midway between the *aphp1*

Figure 7. Dinucleotide-primed in vitro transcriptions. Dinucleotide-primed transcripts were generated from the 602-bp *Bam*HI fragment (sites 1–9, Fig. 1) of pIJ2935 using *S. coelicolor* A3(2) strain M145 RNA polymerase and [α - 32 P]CTP (>600 Ci/mmole). The positions of the *aphp1*, pA1, and pA2 transcripts are indicated. Lanes R contain the products of a conventional in vitro transcription reaction. Lanes M contain an end-labeled *Hpa*III digest of pBR322 to provide size markers.

and the pA1 transcriptional start points. The position of the mutation in the –10 region of *aphp1*, as well as the complementary strand consequence of this mutation in the –10 region of pA2, is also shown. Figure 9 shows the nucleotide sequences upstream of the seven potential transcriptional start sites that we have identified in the *aph* regulatory region. Comparison with the consensus sequence for the major class of *E. coli* promoters (Hawley and McClure 1983) reveals some similarity in the –10 region but generally little similarity in the –35 region. The locations of the presumed –10 regions for *aphp1* and pA2 are indicated.

APH enzyme activities correlate with the effects of the mutations observed at the transcriptional level

APH enzyme assays were carried out on cell-free extracts from TK24 derivatives carrying the *aph* gene with various *aphp1* and *aphp2* promoter combinations (Fig. 10). The construct that contained *aphp1* and *aphp2* (pIJ2951) yielded twice as much APH enzyme activity as the one that contained *aphp1* alone (pIJ2955); the lower activity observed with pIJ2955 may have resulted from loss of *aphp2* or from deletion of sequences important for *aphp1* function. The 5'-GGC-3' deletion in the –35 region of *aphp1* (pIJ2953 and pIJ2957) resulted in ~90% reduction in activity. The T $\rightarrow$ C mutation in the –10 region of *aphp1* (pIJ2952 and pIJ2956) and the *Nco*I end-

fill at the transcriptional–translational start site (pIJ2954 and pIJ2958) both resulted in loss of measurable APH activity. Culturing the mycelia in YEME medium (Hopwood et al. 1985) containing 3 μ g/ml neomycin (a concentration sufficient to inhibit growth of neomycin-sensitive TK24) had no significant effect on APH enzyme levels in either TK24 (pIJ2951) or TK24 (pIJ2955) (data not shown).

An inverse correlation exists between APH levels and neomycin resistance

Neomycin gradient plate assays were carried out on TK24 derivatives containing the *aph* gene with various *aphp1* and *aphp2* promoter constructions (Fig. 10). Loss of resistance was correlated with absence of APH activity (i.e., TK24 with pIJ2952, pIJ2954, pIJ2956, and pIJ2958). Interestingly, an approximate inverse relationship occurred between the observed resistance levels and the APH enzyme levels for cells that contained the plasmids pIJ2951 (*aphp1* and *aphp2*), pIJ2953 (–35 deletion in *aphp1*, *aphp2*), pIJ2955 (*aphp1*), and pIJ2957 (–35 deletion in *aphp1*). While APH levels increased in the order pIJ2957 < pIJ2953 < pIJ2955 < pIJ2951, the resistance levels increased in the reverse order.

Discussion

The aphp1 promoter

The regulatory region of the *aph* gene of *S. fradiae* is unusual and complex. Two promoters, *aphp1* and *aphp2*, occur upstream of and oriented toward the *aph*-coding sequence: *aphp1* initiates at, or 1 or 2 nucleotides upstream of, the *aph* translational start site; *aphp2* initiates 315 nucleotides upstream of the *aph* translational

start site. Site-directed mutagenesis identified nucleotides in the *aphp1* –10 and –35 regions that are important for *aphp1* transcription. The –10 region T $\rightarrow$ C transition resulted in loss of the in vivo- and in vitro-generated *aphp1* transcript, loss of measurable APH activity in cell-free extracts, and loss of neomycin resistance. The dramatic effect of this mutation and its location relative to the *aphp1* start site suggest that the mutagenized T might be analogous to the T commonly found in the sixth position of the *E. coli* –10 consensus sequence (Hawley and McClure 1983); mutation of this highly conserved nucleotide in *E. coli* reduces promoter activity dramatically (Hawley and McClure 1983). The 3-bp deletion introduced in the *aphp1* –35 region (mapping at positions –40 to –38 relative to the *aphp1* start site) significantly reduced, but did not entirely eliminate, *aphp1* transcription.

Translation of the aphp1 transcript occurs in the absence of a conventional ribosome binding site

The T $\rightarrow$ C transition in the –10 region of *aphp1* led to the concomitant loss of *aphp1* transcription, APH activity, and neomycin resistance and indicated that the *aphp1* transcript was translated to yield APH. In the absence of a 5'-nontranslated leader region, the *aphp1* transcript might bind to ribosomes through interaction with a sequence internal to the *aph* coding region; alternatively, the presence of a translational start codon close to the 5' end of the mRNA might be sufficient for ribosome binding. Although this would represent an atypical ribosome–transcript interaction (Stormo et al. 1982) several bacterial transcripts have been identified that apparently lack nontranslated leader regions and conventional ribosome binding sites. Among the eubacteria these include transcripts derived from the bacteriophage

Figure 8. Nucleotide sequence and transcriptional organization of the *S. fradiae aph* regulatory region.

	"-35"	- 17 bp -	"-10"
<i>E. coli</i> :	TTGACA		TATAAT
<i>aphp1</i> :	CCCCGACGAAAGGCGCGGAACGGCGTCTCCGCTCTGCCATGATGCCGCCA		*
<i>aphp2</i> :	TGGCGCGGTGGGGGATTCCGGCCGAACGCGCCGACGCCCATGTGACCGCCT		*
<i>pA1</i> :	GTTCCGCGCCTTTCGTCGGGGCCCGTAGGGGTTTCGGACATCTTGTGCGGGG		***
<i>pA2</i> :	CGGGTACTTCCGGCGCAACGTGCTGTCGTCCATGGGCGGCATCATGGCAGA		*
<i>pA3</i> :	CTGCGTGCCACTCGTGGTGCGGGTACTTCCGGCGCAACGTGCTGTCGTCCA		*
<i>pA4</i> :	TCGTTCACTGCGTGCCACTCGTGGTGCGGGTACTTCCGGCGCAACGTGCTG		*
<i>pA5</i> :	CGCCGGTGAGCTGGTAGACGAAGGCGCCCGAGTCTCCTTCGTTCACTGCGT		*

Figure 9. Transcriptional start points and upstream sequences for the *aph* and *pA* series of promoters. Transcriptional start points identified by nuclease S1 mapping are indicated with an asterisk. The putative -10 regions for *aphp1* and *pA2* are underlined. The nucleotide positions within the *aphp1*, *pA1*, and *pA2* promoter regions at which the T $\rightarrow$ C transition mutation and the 3-bp deletion were carried out are indicated, respectively, by overlying closed circles (●) and (–). A region of sequence similarity preceding the transcriptional start sites of *aphp1* and *aphp2* is indicated by vertical lines. The $-10'$ and $-35'$ consensus sequences for the major class of *E. coli* promoters are also presented for comparison (Hawley and McClure 1983).

λ *cl* gene when transcribed from the pRM promoter (Ptashne et al. 1976), the *tetR* gene from transposon Tn1721 (Klock and Hillen 1986), the *ermE* gene from *Streptomyces erythraeus* (Bibb et al. 1986), and the *sta* gene from *Streptomyces lavendulae* (Horinouchi et al. 1987). Among the archaeobacteria, they include transcripts derived from the bacteriorhodopsin (*bop*; Dunn

et al. 1981), bacteriorhodopsin-related (*brp*; Betlach et al. 1984), and halo-opsin (*hop*; Blanck and Oesterhelt 1987) genes from *Halobacterium halobium*.

While the transcriptional initiation point and its distance from the -10 region remained identical to that in the nonmutant *aphp1*, the 4-bp insertion mutation resulted in loss of measurable APH activity and loss of

Figure 10. APH enzyme activities and gradient plate assays of neomycin resistance levels of strains that contain the mutant and nonmutant *aph* promoter regions. The approximate neomycin gradient is indicated below each plate. *NcoI*, -35 and -10 refer to the 4-base insertion, 3-base deletion and T $\rightarrow$ C transition mutations, respectively. APH activity was measured in μ mole of NADH oxidized per minute per milligram of protein. [N.T.] Not tested.

neomycin resistance (Fig. 10). Although the insertion caused somewhat reduced *aphp1* transcript levels, the amount of transcript that remained far exceeded that observed after mutagenesis of the -35 region, which led to reduced but clearly detectable levels of APH activity and neomycin resistance. Thus, it seems likely that the 4-bp insertion affects the translation of the *aphp1* transcript. The apparent inability to translate this transcript into functional APH may reflect the potential frameshift introduced by the 4-bp insertion (e.g., if ribosomes merely attach and initiate translation at the 5' end of the mRNA) or an altered conformation at the 5' end of the *aphp1* transcript that prevents successful interaction with ribosomes.

The precise nature of a RBS and its position within the leader region of a transcript can be expected to influence the level of translation (Stormo et al. 1982). The low level of *cI* protein in λ lysogens is thought to reflect, in part, inefficient translational initiation of the leaderless *cI* transcript derived from pRM (Ptashne et al. 1976). In contrast, APH constitutes as much as 10% of the total soluble protein in *S. lividans* cells that contain the cloned *aph* gene at a copy number of five to six per chromosome (Thompson and Gray 1983); APH levels in such *S. lividans* derivatives have been reported to be 40-fold higher than APH levels in *S. fradiae* (Thompson et al. 1982). We have estimated that *aph* mRNA levels in *S. lividans* derivatives that contain the cloned gene at a copy number of five to six per chromosome are 20- to 30-fold higher than *aph* transcript levels in *S. fradiae* (data not shown). Therefore, the higher levels of APH activity in *S. lividans* may reflect, in part, increased levels of *aph* transcription; nevertheless, the observed levels of enzyme production in *S. lividans* would still appear to require the efficient translation of an apparently leaderless transcript. Recently, Petersen et al. (1988) suggested that complementarity between nucleotides downstream of the translational start codon and those at the 5' end of 16S rRNA might play a role in translational initiation. Although little complementarity could be found between sequences downstream of the *aph* start codon and those at the 5' end of the 16S rRNA of *S. coelicolor* A3(2) (Baylis and Bibb 1987), further analysis of the *aph* transcript might identify structural features that are important for recognition and binding of leaderless transcripts to the 30S ribosomal subunit and might provide further general insight into the process of translational initiation.

The *aphp2* promoter

The *aphp2* transcript was identified with RNA from *S. fradiae* and from *S. lividans* that contained the cloned *aph* gene; however, we did not detect any *aphp2*-initiated transcripts that extended into the *aph* coding region. It is possible that the *aphp2* transcript terminates before reaching the *aph* coding region or that a larger *aphp2* transcript is sufficiently unstable to prevent detection by nuclease S1 mapping. A DNA fragment that contained the *aphp2* promoter region (corresponding to

sites 1–3, Fig. 1) showed promoter activity when assayed in TK24 with the pIJ486 promoter-probe vector of Ward et al. (1986) (data not shown). Thus, *aphp2* probably represents a bona fide promoter that is recognized by the transcriptional apparatus of both *S. fradiae* and *S. lividans*, but the function of its transcript remains to be determined.

Sequence heterogeneity of the *aph* and *pA* promoters

Comparison of the nucleotide sequences immediately 5' to the *aphp1* and *aphp2* transcriptional start points (Fig. 9) reveals a potentially significant region of similarity (10 out of 13 nucleotides are identical). Apart from this similarity there appears to be little in common at the nucleotide sequence level between the different promoters. Given the rather extensive degree of σ factor heterogeneity in streptomycetes (Westpheling et al. 1985; Buttner et al. 1988; Takahashi et al. 1988; J. Westpheling and M.E. Brawner, pers. comm.; K.F. Chater, pers. comm.), it would not be surprising to find that different forms of RNA polymerase holoenzyme transcribe at least some of the different *aph* and *pA* promoters.

APH levels and neomycin resistance

The levels of neomycin resistance of *S. lividans* derivatives that contain the *aphp1* -35 mutation [i.e., TK24 (pIJ2953) and TK24(pIJ2957)] suggest that not all of the intracellular APH protein is active. The -35 deletion results in a dramatic reduction in *aph* transcript levels (Fig. 3) and an 80–90% reduction in cell-free APH activity (Fig. 10). However, the neomycin-resistance levels (Fig. 10) of the derivatives that contain the -35 mutation are apparently higher than those of cells that contain the nonmutated *aphp1*. This suggests that at high intracellular levels [e.g., 10% of total soluble protein as determined by Thompson and Gray (1983)] the APH protein is largely inactive, perhaps as a result of aggregation. The reduced levels of *aphp1* transcript in the -35 deletion mutants, while reducing the total amount of APH synthesized, may actually result in a higher specific activity of intracellular enzyme.

The *pA* promoters and their possible roles

The occurrence and complexity of the oppositely oriented *pA* series of transcripts were unexpected. The physical and functional overlap between *aphp1* and the *pA1* and *pA2* promoters suggests that they might be co-ordinately regulated; a similar possibility exists for the overlapping and divergently transcribed *sph* and *orfIp2* promoters of *Streptomyces glaucescens* (Vogtli and Hutter 1987) and the *ermEp1* and *orfIp3* promoters of *S. erythraeus* (Bibb and Janssen 1987). The T $\rightarrow$ C transition mutation at nucleotide 393 (Fig. 8) resulted in loss of the *aphp1* and *pA2* transcripts. Examination of the *aph* sequence suggests that nucleotide position 393 might identify the highly conserved sixth position T for the *aphp1* -10 region on one DNA strand and also the

highly conserved second position A for the pA2 – 10 region on the opposite strand (Hawley and McClure 1983). This would suggest that the –10 regions of *aphp1* and pA2 overlap, but on opposite strands, in five out of six positions; the binding of RNA polymerase to one promoter presumably would prevent simultaneous binding to the oppositely oriented promoter. Positively or negatively acting ancillary proteins that bind to one promoter region (or to the RNA polymerase species that recognizes that promoter) could act to stimulate or inhibit transcription from the oppositely oriented promoter region.

The pA1–pA5 transcripts presumably are involved in the expression of a gene located upstream of *aph*. Antibiotic-resistance genes are clustered frequently with production genes (e.g., Malpartida et al. 1984; Chater and Bruton 1985; Ohnuki et al. 1985; Murakami et al. 1986; Stanzak et al. 1986; Motamedi and Hutchinson 1987); this raises the interesting possibility that the expression of *aph* is coordinated with the expression of a divergently oriented biosynthetic gene. Distler et al. (1987) have described recently a streptomycin-resistance gene (*aphD*) transcript that also encodes a putative positively acting regulatory gene (*strR*); the authors suggest that coordinate expression of resistance and regulatory functions ensures resistance before the production of a self-inhibitory antibiotic, an idea also suggested by Hopwood et al. (1986b). Additional analysis of the pA transcripts is likely to provide information on their possible involvement in neomycin production and, perhaps, on the general regulatory features of antibiotic resistance and production genes.

Materials and methods

Bacterial strains, transformation, and culture conditions

Standard media and methods of culture and transformation of *S. lividans* were as described by Hopwood et al. (1985). *S. lividans* derivative TK24 (SLP2[–], SLP3[–], *str*; Hopwood et al. 1983) was used as recipient in all *Streptomyces* transformation experiments. *S. fradiae* ATCC 10745 and 10745/H3 were grown in tryptone soya broth (Hopwood et al. 1985). Strain 10745/H3 (a spontaneous mutant of ATCC 10745 with an increased level of neomycin resistance; Komatsu et al. 1981) was the strain from which the *aph* gene was cloned initially (Thompson et al. 1980). In addition to the constructs described in this report, *S. lividans* 1326 (pIJ101) and *S. lividans* TK24 (pIJ365) were used as sources of RNA lacking and containing *aph* transcripts, respectively; pIJ365 is a derivative of pIJ101 that contains the *aph* coding and regulatory sequences (Kieser et al. 1982). Levels of neomycin (Nm) resistance were assayed on MMT agar Nm gradient plates (Ward et al. 1986) that were incubated at 30°C for two days.

Culture conditions for *E. coli* were as in Schottel et al. (1981). *E. coli* K12 F[–] *lacZ* ΔM15 *recA* (Ruther et al. 1981), the recipient in all *E. coli* transformation experiments, was transformed as in Cohen et al. (1972, 1973); transformants were selected on L agar (Lennox 1955) that contained 200 μg/ml of carbenicillin (Cb) with or without 40 μg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 11.9 μg/ml of isopropyl β-D-thiogalactopyranoside (IPTG).

DNA isolation, manipulation, and characterization

Streptomyces plasmid preparations were as in Hopwood et al. (1985). Small-scale *E. coli* plasmid preparations were carried out by a modification (S.Y. Chang, pers. comm.; Hopwood et al. 1987) of the method of Ish-Horowitz and Burke (1981); large-scale preparations were by the procedure of Timmis et al. (1978). Plasmids were maintained during liquid cultivation by selection with 10 μg/ml of thiostrepton for *Streptomyces* cultures or 200 μg/ml of carbenicillin for *E. coli*. DNA fragments were isolated and purified from electrophoresis-grade low-melting-point agarose (BRL) according to the manufacturer's recommendations or by electroelution.

Restriction endonucleases and T4 DNA ligase (Anglian Biotechnology or BRL), calf intestine alkaline phosphatase (CIAP; Boehringer–Mannheim), DNA polymerase I (Klenow fragment; Anglian Biotechnology), nuclease S1 (Sigma), exonuclease VII (BRL), and polynucleotide kinase (BRL) were used according to the manufacturers' recommendations.

RNA isolation

RNA was isolated from stationary phase cells by a modification (C.P. Smith, pers. comm.; Hopwood et al. 1985) of the methods of Kirby et al. (1967) and Covey and Hull (1981).

Plasmid constructions

A series of plasmid manipulations were carried out to place the mutagenized *aph* promoter region back into its normal position relative to the coding sequence. For ease of manipulation, a 605-bp *Bam*HI fragment (segment 1–9, Fig. 1; Bibb et al. 1985) that contained the *aphp1* and *aphp2* promoters was cloned into various pUC18 and pUC19 (Yanisch-Perron et al. 1985) derivatives. The remainder of the *aph* gene, truncated at the amino-terminal end, was cloned as a 703-bp *Bam*HI–*Sst*II fragment (Thompson and Gray 1983) into the polylinker of pUC18 to yield pIJ2934. In one set of constructions, the *aphp2* promoter region was removed by deletion of the 271-bp *Bam*HI–*Sst*II fragment (segment 1–4, Fig. 1). When necessary for ligation, incompatible 5'– or 3'–protruding ends were made blunt by the 5' to 3' polymerase activity (Maniatis et al. 1982) or the 3' to 5' exonuclease activity (Henikoff 1984) of the Klenow fragment of *E. coli* DNA polymerase I. Many of the manipulations in *E. coli* were carried out with pUC18 derivatives constructed in our laboratory that contained a *Bgl*II site flanking one or both ends of the pUC18 polylinker (G.R. Janssen and M.J. Bibb, unpubl.).

Mutations were introduced into the promoter-containing *Bam*HI fragment (described below) and the fragment was then ligated to *Bam*HI-cut pIJ2934 to reconstitute the *S. fradiae aph* gene. The reconstructed *aph* gene, with or without mutations, was then cloned into the *Bgl*II site of a derivative of the *Streptomyces* plasmid pIJ425 (Ward et al. 1986) from which the Tn5-derived *neo* gene had been deleted. Cloning into the *Bgl*II site was carried out so that the orientation of *aph* was always the same and vector-initiated transcriptional readthrough into *aph* was prevented by the transcriptional terminator from phage fd located immediately upstream of the *Bgl*II site (Ward et al. 1986).

A listing and partial description of relevant plasmids is given in Table 1.

Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis of the *aphp1* promoter

region was carried out essentially as in Zoller and Smith (1982), but without alkaline sucrose gradient enrichment of covalently closed circular molecules. An M13mp19 (Norrand et al. 1983) derivative that contained the *Bam*HI–*Acc*I fragment and that extended from nucleotide 1 to nucleotide 481 (Fig. 8) was used as template. The synthetic oligonucleotides 5'-TGCCAT-GACGCCGCC-3' and 5'-CCCGACGAAAGCGGAACGGC-3' were used to mutagenize the –10 and –35 regions of *aph*p1, respectively. Mutant phages were detected by plaque hybridization (Benton and Davis 1977) using the mutagenic primers labeled at the 5' end with [γ - 32 P]ATP (3000 Ci/mmol) and polynucleotide kinase (Maxam and Gilbert 1980) as probes. After plaque purification and reprobing, 396-bp *Bam*HI–*Nco*I fragments (segment 1-5, Fig. 1) that contained each of the mutagenized promoter regions were recombined with the 212-bp *Nco*I–*Bam*HI fragment (segment 5-9, Fig. 1) to yield pUC18 and pUC19 derivatives (see above) that contained the 605-bp *Bam*HI fragment (segment 1-9, Fig. 1). The occurrence of the expected mutations was confirmed by sequencing *Bam*HI–*Acc*I segments derived from representative plasmids by the method of Maxam and Gilbert (1980).

Nuclease S1 and exonuclease VII transcript mapping

DNA probes for nuclease S1 or exonuclease VII mapping of the *aph*p1 start site were prepared by digesting pIJ2935 with *Bam*HI or *Taq*I, followed by dephosphorylation with CIAP and labeling of 5' ends with [γ - 32 P]ATP (3000 Ci/mmol) and polynucleotide kinase as described by Maxam and Gilbert (1980). Subsequent digestion of the labeled *Bam*HI fragment with *Bgl*I (site 2, Fig. 1) produced in a 575-bp *Bgl*I–*Bam*HI fragment (segment 2-9, Fig. 1) uniquely labeled at the 5' end of *Bam*HI site 9. Subsequent digestion of the labeled *Taq*I fragment with *Sst*II (site 4, Fig. 1) produced a 314-bp *Sst*II–*Taq*I fragment (segment 4-8, Fig. 1) uniquely labeled at the 5' end of *Taq*I site 8. A similar procedure was used to map the *aph*p2 start site. In brief, pIJ2935 was digested with *Taq*I, dephosphorylated, labeled as above, and digested with *Bgl*I to generate a 210-bp *Bgl*I–*Taq*I fragment (segment 2-3, Fig. 1) uniquely labeled at the 5' end of *Taq*I site 3.

DNA probes for nuclease S1 mapping of the pA series of transcripts were prepared by digesting pIJ2935 with *Taq*I, followed by dephosphorylation and labeling as described above. Subsequent digestion with *Sst*I (site 6, Fig. 1) produced a 268-bp *Taq*I–*Sst*I fragment (segment 3-6, Fig. 1) uniquely labeled at the 5' end of *Taq*I site 3.

RNA (40 or 80 μ g) was mixed with 0.02–0.03 pmole of 32 P-labeled DNA ($\sim 1\text{--}2 \times 10^6$ Cerenkov cpm/pmol) and incubated at 85°C for 15 min in 20 μ l (for 40 μ g RNA) or 40 μ l (for 80 μ g RNA) hybridization solution (Favaloro et al. 1980); the temperature was then lowered to 65°C and hybridization continued for an additional 6–8 hr. Hybridization reactions were then treated as in Bibb et al. (1986). Nuclease S1 and exonuclease VII-resistant hybrids were analyzed by electrophoresis in 6 or 8% polyacrylamide gels containing 7 M urea (Sanger and Coulson 1978) followed by autoradiography at –70°C with intensifying screens.

In vitro transcriptions

RNA polymerase that had been purified from *S. coelicolor* A3(2) strain M145 but not fractionated into different holoenzyme forms (Buttner et al. 1988) was kindly provided by Dr. M.J. Buttner. Transcription reactions were carried out as described by Buttner et al. (1987), except that mixtures were allowed to equilibrate for 5 min at 30°C in the presence of 0.33–0.42 μ M [α - 32 P]CTP (10 μ Ci per reaction of >600 Ci/mmol [α - 32 P]CTP)

and 0.4 mM each of ATP, GTP, and UTP prior to the addition of RNA polymerase [0.1 units, as defined by Buttner and Brown

Table 1. Description of plasmids

Plasmid	<i>aph</i> promoter status	Comments
pUC18	—	plasmid vector for DNA manipulations in <i>E. coli</i> (Yanisch-Perron et al. 1985)
pIJ2934	—	703-bp <i>Bam</i> HI– <i>Sst</i> II carboxy-terminal <i>aph</i> fragment (Thompson and Gray 1983) in pUC18
pIJ2935	p2 + p1	605-bp <i>aph</i> <i>Bam</i> HI fragment (segment 1-9, Fig. 1) in pUC18
pIJ2941	p1	331-bp <i>aph</i> <i>Sst</i> II– <i>Bam</i> HI fragment (segment 4-9, Fig. 1) in pUC18
pIJ101	—	<i>Streptomyces</i> plasmid (Kieser et al. 1982) used in various <i>aph</i> constructs
pIJ365	p2 + p1	<i>aph</i> gene in pIJ101-derivative (Kieser et al. 1982)
pIJ425	—	pIJ101-derivative (Ward et al. 1986) used for construction of <i>aph</i> plasmids
pIJ2951 ^a	p2 + p1	pIJ2951–pIJ2958 <i>aph</i> gene with nonmutant promoter region
pIJ2952 ^a	p2 + p1(–10)	<i>aph</i> gene with T → C mutation in the –10 region of <i>aph</i> p1
pIJ2953 ^a	p2 + p1(–35)	<i>aph</i> gene with 3-bp deletion in the –35 region of <i>aph</i> p1
pIJ2954 ^a	p2 + p1(<i>Nco</i> I)	<i>aph</i> gene with <i>Nco</i> I end-fill at the <i>aph</i> transcriptional–translational start site
pIJ2955 ^b	p1	<i>aph</i> gene with nonmutant <i>aph</i> p1; p2 deleted
pIJ2956 ^b	p1(–10)	<i>aph</i> gene with T → C mutation in –10 region of <i>aph</i> p1; p2 deleted
pIJ2957 ^b	p1(–35)	<i>aph</i> gene with 3-bp deletion in the –35 region of <i>aph</i> p1; p2 deleted
pIJ2958 ^b	p1(<i>Nco</i> I)	<i>aph</i> gene with <i>Nco</i> I end-fill at the <i>aph</i> transcriptional–translational start site; p2 deleted

^a pIJ2951–pIJ2954 are pIJ425 derivatives that contain the *aph* structural gene with 400 bp (i.e., segment 1-5, Fig. 1) of 5'-flanking sequence.

^b pIJ2955–pIJ2958 are pIJ425 derivatives that contain the *aph* structural gene with 123 bp (i.e., segment 4-5, Fig. 1) of 5'-flanking sequence.

(1985)]. Reactions were stopped on ice and were precipitated at -20°C with an equal volume of isopropanol and 0.1 volume of precipitation mix [5 $\mu\text{g}/\text{ml}$ of tRNA, 2 M sodium acetate (pH 5)]. Precipitates were dissolved in 250 μl of 0.3 M sodium acetate and were reprecipitated on ice with an equal volume of isopropanol to remove unincorporated label. Pellets were washed with ethanol, dried, and dissolved in 10 μl of 10 M urea, $0.5 \times \text{TBE}$ (45 mM Tris-base, 45 mM boric acid, 4 mM EDTA) and 2 μl of loading dye (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, $0.5 \times \text{TBE}$). Heat-denatured transcripts were analyzed on 6% polyacrylamide–7 M urea gels (Sanger and Coulson 1978). A heat-denatured, ^{32}P -labeled *HpaII* digest of pBR322 (Sutcliffe 1979) was used to provide size standards.

Dinucleotide-initiated in vitro transcriptions were performed by the method of Moran et al. (1981), except that the incubations were performed at 30°C with 0.1 units of purified but unfractionated *S. coelicolor* A3(2) strain M145 RNA polymerase using the transcription buffer of Buttner et al. (1987).

Aminoglycoside phosphotransferase (APH) assays

APH assays were performed as in Thompson et al. (1982). In brief, ADP resulting from the ATP-linked phosphorylation of neomycin was coupled through pyruvate kinase and lactate dehydrogenase to the oxidation of NADH (quantified spectrophotometrically at 340 nm). APH activity was expressed as micromoles of NADH oxidized per minute per milligram of protein. Enzymes and reagents were obtained from Boehringer–Mannheim. Protein estimates were as in Bradford (1976).

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Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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