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The *sapA* Promoter from *Streptomyces coelicolor* Requires Activation Sites and Initiator-Like Sequences but Not −10 or −35 Sequences

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The *Streptomyces coelicolor sapA* gene encodes a spore coat protein. The *sapA* promoter is regulated developmentally, with maximal expression occurring in aerial hyphae at a late stage of colonial development. The DNA sequences upstream from the transcription start point do not appear to fall into a previously described promoter class. One (or more) putative activation site, required for full activity, is eliminated when 5′ deletions extend to between −178 and −72 bases upstream from the transcription start point. In addition, a downstream activation site is destroyed by removing sequences between positions +40 and +120, relative to the transcription start point, in the absence of an intact upstream region. However, temporal regulation of transcription initiation over the course of the life cycle is maintained faithfully in the absence of these elements, even in the smallest 18-bp *sapAp* fragment containing sequences from positions −8 to +10. Site-specific mutations around the transcriptional start points shift the timing of *sapA* expression to an earlier stage in the developmental cycle. These results suggest that a novel mechanism may be involved in *Streptomyces* late gene expression.

A sophisticated program of gene expression regulates temporal and spatial changes in *Streptomyces* development, in terms of both morphological transformation and secondary metabolic pathways (9, 14). Following spore germination, these gram-positive, filamentous bacteria grow as branching, multinucleoid mycelia. The vegetative network of hyphae in the substrate mycelium secrete a large variety of hydrolytic enzymes and anchor the growing colony to a solid substrate. Nutrient depletion and the onset of the stationary phase then induce formation of a distinct aerial mycelium, production of new secondary metabolites, and, finally, transformation of aerial hyphae into chains of spores.

Very little is known about stationary-phase gene expression in *Streptomyces* spp., largely because most species do not elaborate aerial hyphae or sporulate in submerged cultures, thus complicating biochemical approaches. The plateau in biomass expansion precludes the use of common antibiotic resistance reporter genes for genetic analysis. In addition, colorimetric reporter gene products are difficult to detect against a heavy background of spore and antibiotic pigments.

Most of our current understanding of *Streptomyces* development, at least at the molecular level, has resulted from isolating mutants that fail to sporulate and then cloning the responsible chromosomal loci (7, 8, 10, 13, 23). In an alternative approach, a reverse genetic procedure was employed to yield genes coding for spore-associated proteins (12). The *sapA* gene, which encodes a 13-kDa *Streptomyces coelicolor* spore-associated protein, has been sequenced, and its transcription start point has been located (12, 22). An amino-terminal signal sequence, predicted by the DNA sequence, is presumably cleaved off during secretion. Three other proteins, SapC, SapD, and SapE, are encoded by a large, nonessential, linear plasmid (22). The failure of the reverse genetic procedure to yield amino acid sequence data for SapB led to the suggestion that SapB is assembled nonribosomally in a manner similar to that of peptide antibiotics (20). SapB is known to play a crucial role in spore formation (32).

We are analyzing the developmental control of *sapA* with the intention to exploit it as a gateway to key stationary-phase regulatory genes. In the work reported here, we have defined essential *cis*-acting elements of the *sapA* promoter.

MATERIALS AND METHODS

Bacterial growth. *S. coelicolor* J1501 [*S. coelicolor* A3(2) *hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$] was obtained from K. F. Chater, and *Streptomyces lividans* TK24 (*S. lividans* 66 *str6*) was obtained from J. M. Weber. All expression studies were done with *S. coelicolor* J1501 cells containing luciferase transcription reporter plasmids. After in vitro manipulation, some plasmids were transformed into *S. lividans* TK24 prior to being moved into *S. coelicolor* J1501. The standard *Streptomyces* media and methods used have been described elsewhere (15). A plus appended to the names of media described in this work indicates that 1.5 μg of uracil per ml and 10 μg of histidine per ml were added to the standard recipes.

Luciferase assays. For the experiments shown in Fig. 2, Fig. 5, and Table 1, the strength of light production from colonies was measured by punching out a uniformly sized portion of a lawn of bacteria from a plate with the broad end of a standard yellow 200-μl Pipetman tip. The excess portion of the yellow tip was then removed with a razor blade, and the colony plug (still in the pipette tip) was placed face down in the bottom of a glass scintillation vial. The vial contained 2 μl of *n*-decanal on a thin paper wick to provide a substrate for the luciferase enzyme. The vial was sealed, and photon output was measured with a Turner Designs model 20e luminometer (15-s delay followed by 45-s integration).

For the experiments shown in Fig. 3, measurements were made as described above except that flats were used instead of plates. Previously purified *S. coelicolor* cells carrying various *sapA-luxAB* plasmids were sporulated on R2YE+ plates containing thiostrepton. Spores were collected, titrated, and resuspended in 20% glycerol. They were plated at 5×10^5 CFU on 6 ml of R2YE+-thiostrepton flats in sterile 25-mm glass scintillation vials and incubated with loose caps at 30°C. At each time point, the flats were exposed to 1 μl of *n*-decanal by swapping the vial cap with one that contained the aldehyde on a small paper wick. After 6 min of exposure to *n*-decanal, the flat was placed into the luminometer such that the photon multiplier was facing the top surface of the colony. Photon emission was integrated by the luminometer for 30 s through a 1-cm glass window in the cap (16, 28).

For the experiments shown in Fig. 6, 10 μl of *n*-decanal was added to 1 ml of liquid cultures in scintillation vials. After vigorous mixing, the strength of light production was measured with the luminometer for 60 s.

Construction of a low-copy-number promoter probe plasmid. pHI302, a low-copy-number promoter probe plasmid, was constructed (see Fig. 1) by cloning the promoterless *Vibrio harveyi luxA* and *luxB* genes into the SCP2* derivative pIJ698 (17). A multiple-cloning polylinker was removed from pSL1190 (Pharmacia LKB Biotechnology, Inc.) with *Hin*dIII and *Bam*HI and transferred to pIJ698 in place of 5 kb of nonessential pIJ698 DNA. One of the original two copies of the transcription terminator from phage fd remained in the resulting

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plasmid, pHI301. The *luxA* and *luxB* genes from *V. harveyi* were transferred from pAL1002 on a *Bam*HI-*Bgl*II fragment to the unique *Bam*HI site of pHI301, yielding pHI302.

The large pHI302 polylinker contains unique restriction enzyme sites for *Hin*dIII, *Stu*I, *Bgl*II, *Bam*HI, and possibly others which have yet to be tested. Upon plasmid preparation by an alkaline lysis method, the yield of pHI302 was comparable to that of pIJ698. There were no detectable plasmid rearrangements after two rounds of sporulation on R5 medium containing thiostrepton. A very low level of light was produced from *S. coelicolor* J1501 carrying pHI302 (see Fig. 2), presumably as a result of some read-through of the transcription terminator.

Deletion mapping. The various deletion derivatives of the *sapA* promoter region are diagrammed in Fig. 3. An 8-kb clone containing *sapA* was a generous gift of J. McCormick (Harvard University). A 330-bp subfragment extending from the *Sph*I site at position −200 to the downstream *Bst*EII site at +120, relative to the *sapA* transcription start point, was transferred into pHI302, creating pHIS10. Additional 5′ deletions were derived from pHIS10, by use of the restriction sites *Sac*II, *Hgi*AI, *Bbv*I, *Fnu*4H, and *Eco*O109I, creating pHIS11, pHIS12, pHIS13, pHIS14, and pHIS15, respectively.

A 0.5-kb *Nar*I fragment, which spans the *sapA* transcription start point, was removed from the plasmid pUJ6 (12) and cloned into pHI302, yielding pHIN1. The 3′ end of the *sapA* DNA in pHIN1 was then trimmed down to the *Bst*EII site, and a *Bam*HI linker was attached to create pHIN2. A 112-bp *Sau*3A fragment that also spans the *sapA* transcription start point was cloned into pBluescript SK(+) (Stratagene, Inc.), leading to pSK19. pSK19 was the substrate for exonuclease III digestion from the 5′ or 3′ ends of the insert. Deletion end points were established by sequencing by the dideoxy method. Representative deletions were recloned into pHI302 as a *Hin*dIII-*Bam*HI fragment to generate pHI317, pHI318, pHI319, pHI320, and pHI331, respectively (Fig. 3).

The smallest *sapA* promoter derivative, carried by pHI341, was generated with a 32-mer oligonucleotide, 5′-GTAAGCTTCAACTGGTGGCTTCAGCGGAT CCG-3′. The oligonucleotide was self-annealed with complementarity at its 3′ end and then converted to double-stranded DNA with the Klenow fragment of *Escherichia coli* DNA polymerase I. After digestion with *Hin*dIII, double-stranded DNA of the correct size was purified from a nondenaturing 12% polyacrylamide gel and cut with *Bam*HI. The resulting 18-bp *Hin*dIII-*Bam*HI fragment was cloned into M13mp18, sequenced, and then transferred into pHI302 to yield pHI341.

Region-specific mutagenesis with degenerate oligonucleotides. Two 28-mer oligonucleotides, 5′-GCAAAGGTCTCAACTGGTGGCTTCAGCA-3′ and 5′-AGGTCTCAACTGGTGGCTTCAGCAACAC-3′, were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, Tex.) in a standard fashion, except that the underlined bases were doped with a small quantity of each of the other three bases at a level designed to yield, on average, one to two base substitutions per molecule synthesized.

Double-stranded replicative form DNA of M13mp18 containing the 320-bp *Sph*I-*Bst*EII *sapA* promoter fragment in the multiple-cloning site was transformed into *E. coli* RZ1032 (*dut ung*), and uracil-containing, single-stranded DNA was prepared from a transformant as described previously (1). Wild-type M13mp18 replicative form DNA (without any insert) was prepared from infected *E. coli* JM103 (*dut*$^+$ *ung*$^+$) cells and digested with *Hin*dIII and *Bam*HI. A gapped duplex DNA, containing only plus-strand DNA for the *sapA* promoter region and double-stranded DNA for the remaining portion of the vector, was formed by annealing (18) the uracil-containing, single-stranded DNA with the denatured linear, thymine-containing mp18 DNA. The 5′-phosphorylated degenerate oligonucleotides were then annealed to the gapped duplex DNA, and the mixture was treated with the Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase. The resulting DNA was transformed into *E. coli* JM103, and the phage DNA in several isolated plaques was sequenced (25). Nine representative *sapA* promoter mutations were recloned onto pHI302 as *Hin*dIII-*Bam*HI fragments, generating pHID4 to pHID28, and then transferred into *S. coelicolor.*

Preparation of mRNA and primer extension reactions. *S. coelicolor* cells containing various plasmids were grown in several parallel 50-ml cultures of YEME+ with 20 μg of thiostrepton per ml and then collected at multiple time points by filtration through disks of Whatman no. 1 paper. Cells were resuspended in 1 ml of Kirby mixture (15) and lysed by vortexing for 2 min with 300 μl of acid-washed 0.45-mm glass beads. After two phenol-chloroform extractions, aqueous material was layered directly onto 1 ml of 5.7 M CsCl in thick-wall polycarbonate tubes. The tubes were spun in a table-top ultracentrifuge (Beckman TL-100; TLA 100.3 rotor) at 100,000 rpm for 3 h. The resulting RNA pellets were dissolved in RNase-free H_2O and stored as ethanol precipitates until needed.

A primer (*sapA* primer 1) specific for chromosomally encoded *sapA* transcripts was synthesized as a 22-mer oligonucleotide, 5′-TTGCATGGAACGCTTCATG GCA-3′. A *luxA* primer (*luxA* primer 1), used to detect plasmid-encoded *sapA* transcripts, was a 22-mer, 5′-GTTGTTCAAAATCAGGCTCGAT-3′. Purification and labeling of primers were carried out as described by Ausubel et al. (1). For primer extension reactions, 30 μg of RNA was denatured with 10 mM methylmercury(II) hydroxide (Johnson Matthey, Inc.) at room temperature for 10 min and hybridized to 5′ end-labeled primer at room temperature for 5 min in reverse transcriptase buffer (10 mM $MgCl_2$, 100 mM KCl, 50 mM Tris [pH 8.3], 10 mM dithiothreitol, 1.25 mM each deoxynucleoside triphosphate, 28 mM β-mercaptoethanol). Twenty units of avian myeloblastosis virus reverse transcriptase (Seikagaku, Inc.) was added, and the reaction was allowed to proceed for 90 min at 42°C. Products were resolved by electrophoresis on a high-resolution denaturing polyacrylamide gel.

RESULTS

At least one upstream activation site is required for full promoter activity. It was important to define the minimum-sized DNA fragment which contained *sapA* promoter activity, especially because the DNA sequences upstream of the transcription start point do not resemble those of previously sequenced prokaryotic promoters. A low-copy promoter probe plasmid (pHI302) was designed and constructed to facilitate these experiments (Fig. 1). Transcription of *V. harveyi* luciferase genes carried by pHI302 is dependent upon the promoter cloned into the plasmid polylinker.

Cells containing the *sapA-luxAB* fusion plasmid pHIS10, which contains sequences (relative to the transcription start point) from −200 to +120 bp, emit light strongly only at a late stage of colonial development (Fig. 2). These observations are in agreement with earlier studies of *sapA* transcription (12, 16) and suggest that temporal regulation of the *sapA* promoter is maintained faithfully when it is plasmid-borne. A set of deletions of pHIS10 were then tested for light emission during colonial development on solid media (Fig. 2). The activity of the *sapA* promoter decreases as more DNA upstream of the *sapA* transcription start is deleted. Deletion of 5′ sequences up to position −72 reduces promoter activity to about 40%, and the smallest *sapA* promoter fragment containing sequences from positions −8 to +10 shows about 10% of full promoter activity. Surprisingly, all of the deletion mutants retain the late temporal expression typical of the wild-type *sapA* promoter (Fig. 2).

A more complete set of deletions of pHIS10 was tested for light emission during colonial development by use of flats (see Materials and Methods) rather than with separate plugs of colonies removed from a plate. This procedure allows continuous observation of the same cells and avoids potential errors due to a developmental gradient within a colony on plates; however, development of *S. coelicolor* on flats is slower than it is on plates and takes up to 10 to 13 days to complete. Light production profiles, equivalent to those shown in Fig. 2 but extended over a 13-day period, were generated for each plasmid-containing cell type. The activities of each *sapA* promoter construct were then calculated by measuring the area under each curve and are summarized relative to the activity of the promoter on pHIS10 (Fig. 3). The relative activities of the *sapA* promoter measured in plugs (Fig. 2 and data not shown) and flats (Fig. 3) were comparable.

Deletion of upstream sequences of the *sapA* promoter, beginning at the *Sph*I site and moving towards the transcription start point, had no effect until after the *Sac*II site at −178 was reached (Fig. 3). Removal of sequences between positions −178 and −72 caused a twofold drop in promoter activity, with most of the decrease occurring when the region between −137 and −118 is removed. This suggests that a positively acting factor interacts with this upstream region to help RNA polymerase transcribe from the *sapA* promoter. The relative promoter activity of various deletion mutants suggests that more than one positively acting factor might interact with this region, but additional studies would be required for this to be determined rigorously.

Deletions of downstream DNA, from positions +20 to +120, had no effect on a full-length promoter (pHIS9); however, a smaller deletion, from +40 to +120, had a twofold effect on a promoter beginning at −72 (cf. pHI316 and

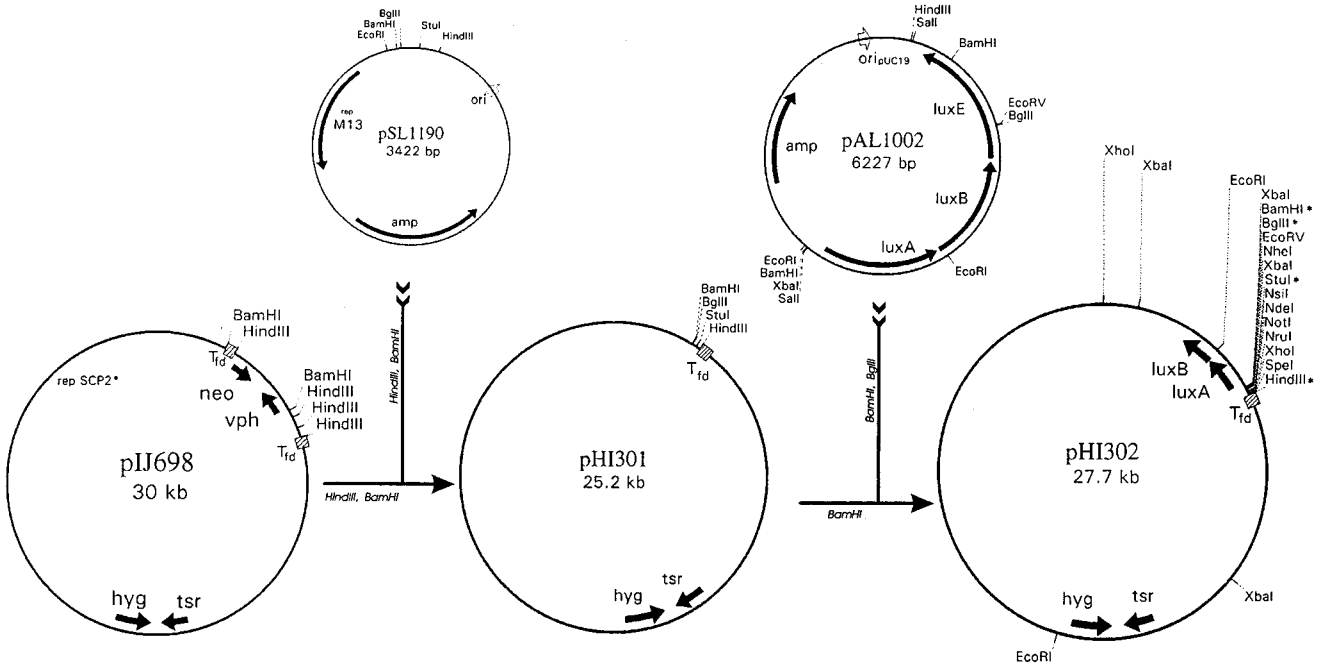


FIG. 1. Construction of pHI302. Known unique sites in pHI302 are designated by asterisks.

pHI317), reducing *sapA* promoter activity of pHI317 to about 25% of that of pHIS10. When the downstream +40 to +120 region was cloned into pHI302, the resulting recombinant exhibited the same level of *lux* expression (data not shown) as that of pHI302 itself, indicating that the region does not appear to contain a promoter. Thus, in the absence of an intact upstream region, downstream activating sequences can be important for *sapA* promoter activity.

With the *sapA* promoter truncated at +40, removal of upstream sequences from positions −72 to −8 had little effect on promoter activity (i.e., *lux* expression; cf. pHI317 and pHI320 [Fig. 3]), although 5′ deletions extending beyond +1 abolished all transcription to the level of the control plasmid pHI302 (data not shown). Sequences 5′ of position −8 are not essential for *sapA* promoter function, and thus the form of RNA polymerase that recognizes the *sapA* promoter may not utilize the typical prokaryotic −35 and −10 hexamers. However, transcription from the *sapA* promoter is stimulated by a factor(s) that binds between 178 and 82 bases upstream and by a factor(s) that binds downstream of the transcription start site.

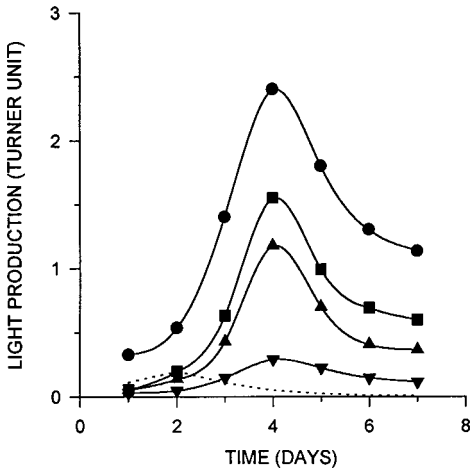


FIG. 2. Time course of light production from *S. coelicolor* J1501 carrying either pHIS10 or other deletion derivatives of *sapA* promoter. See Fig. 3 for a description of the plasmids. A Turner unit is an arbitrary unit of light used with a Turner Design model 20e luminometer. Aerial hyphae formed on day 3, and the gray spore color was evident on day 5. Symbols: circles, pHIS10; squares, pHIN2; triangles, pHI316; inverted triangles, pHI341. Background light production (dotted line) from *S. coelicolor* J1501 containing pHI302 is subtracted from all other values. The relative activities of pHIS10, pHIN2, pHI316, and pHI341 were 1, 0.55, 0.40, and 0.10.

Remarkably, a promoter that contains only 18 bp of *sapA* promoter DNA, from −8 to +10 relative to the start point of transcription, retains 10% maximal activity (cf. pHI341 and pHIS10 [Fig. 3]). Furthermore, this minimal promoter (and all promoter mutants described in Fig. 3) is most active at a late stage of colonial development, at the same time as the intact *sapA* promoter in pHIS10 (Fig. 2).

The 18-bp fragment was cloned into pRS1105 (26) and pRS1108 (28), two other *lux* transcriptional vectors that show only a low level of *lux* expression and then just at an early developmental stage. Both 18-bp-containing recombinants gave the same temporal expression and comparable light production as that of pHI341 (data not shown). Since different sequences provide the 5′ and 3′ borders of the 18-bp *sapA* promoter in the three plasmids (Fig. 4), promoter activity and temporal control of transcription from the *sapA* promoter in pHI341 do not appear to be mediated by upstream and downstream sequences in the vector. *lux* sequences themselves do not regulate temporal expression of promoters, because different timing of expression is demonstrated when the different promoter sequences are cloned on those vectors (reference 28 and results not shown). Thus, the information necessary both for basal activity and temporal regulation of the *sapA* promoter is contained within the 18 bp that span positions −8 to +10.

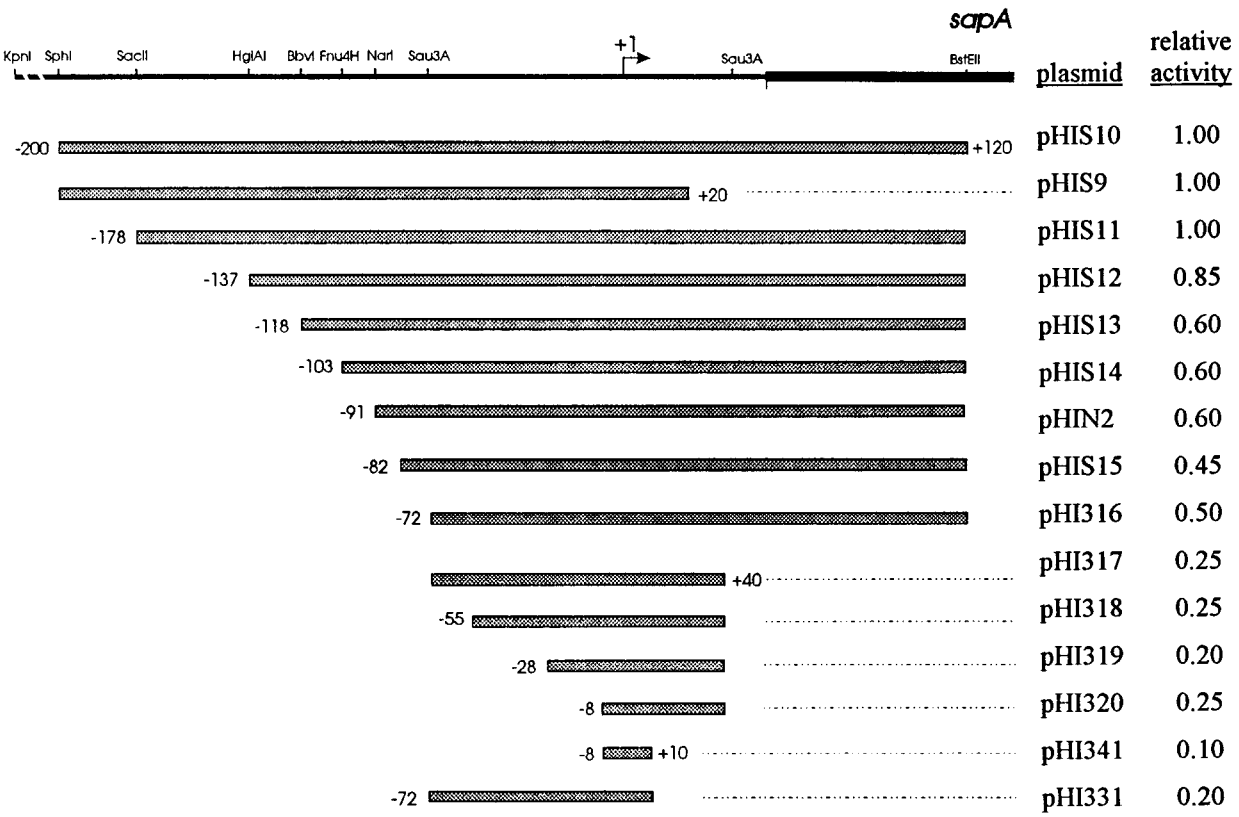


FIG. 3. Deletion analysis. The shaded bars represent DNA from the *S. coelicolor sapA* region cloned into pHI302. There are two *sapA* transcription start points at adjacent base pairs (13); numbering of the region is based on the first of these start points and denoted by +1. Relative transcription activity was computed as follows. Light from four parallel samples was measured every 12 h, the values were averaged, and background light emission from cells containing pHI302 (no *sapA* DNA) was subtracted. After graphing the results, relative transcription activities were calculated by measuring the area under each curve and dividing it by the area measured for pHIS10. The relative activities of the truncated *sapA* promoters are the means of two independent experiments.

Sequences around the transcription start site are important for the temporal expression from the *sapA* promoter. The fact that a small DNA segment from −8 to +10 has the *sapA* promoter activity with normal developmental timing, combined with similar results from the *Micromonospora echinospora* P1a promoter (see Discussion), inspired us to mutagenize this region and perform luciferase time course experiments with several mutants. About 5×10^4 spores of each mutant were spotted as separated circles with 0.5-cm diameters, arranged vertically as a column on R5 plates. Every day for the next 7 days, a new column of mutant spores was spotted on the same plate. On day 8, each horizontal row of colonies showed a morphological gradient, from substrate mycelia to sporulating cells, on the plates (Fig. 5A). Light production from the plate was photographed in the dark with the aid of a light intensifier (Fig. 5B), and light intensity from constant surface areas of *Streptomyces* cells was quantitated with a luminometer. Data obtained with six mutants are shown in Fig. 5C, and a summary of the data obtained with the nine mutants tested is presented in Table 1.

Single base-pair changes at position +2 (pHID24 and pHID28) shift light production from the *sapAp-lux* fusion to an earlier stage in the developmental cycle. Double base-pair changes at positions +1 and +2 (pHID4) or at positions +2 and +3 (pHID15) have an even greater effect, maximum light production occurring after only 2 days of incubation. Thus, at least in part, temporal regulation of *sapA* is determined by the first 3 bp transcribed, a result consistent with the maintenance of temporal regulation of *lux* activity with the minimal 18-bp *sapA* promoter present in pHI341 (Fig. 2 and 3). The same 3

	-50	-40	-30	-20	-10	+1
sapA	AACATCTGCC	AACGACGTAC	AACCCCCGAA	GGTGCAAAGG	TCTCAACTGGTGGCTTCAGC	
pHI341	TGTCTGGAAA	GACGACAAAA	CTTTAGATCC	GGCCAAGCTT	GGTCAACTGGTGGCTTCAGC	
pS966	CCCTCGAGGT	CGACGGTATC	GATAAGCTTG	ATATCGAATT	CCTCAACTGGTGGCTTCAGC	
pS959	CTGCTCTAGA	GCACGTTGCA	TGCCTGCAGG	TCGACTGTAG	AGTCAACTGGTGGCTTCAGC	

FIG. 4. The sequences, in different plasmids, 5′ of the *sapA* promoter truncated at position −8. The *sapA* promoter fragment is underlined and is the same in all constructs. The sequence at the top is the natural *sapA* sequence. The sequences labeled pHI341, pS966, and pS959 are derived from pHI302 (this work), pRS1108 (28), and pRS1105 (26), respectively.

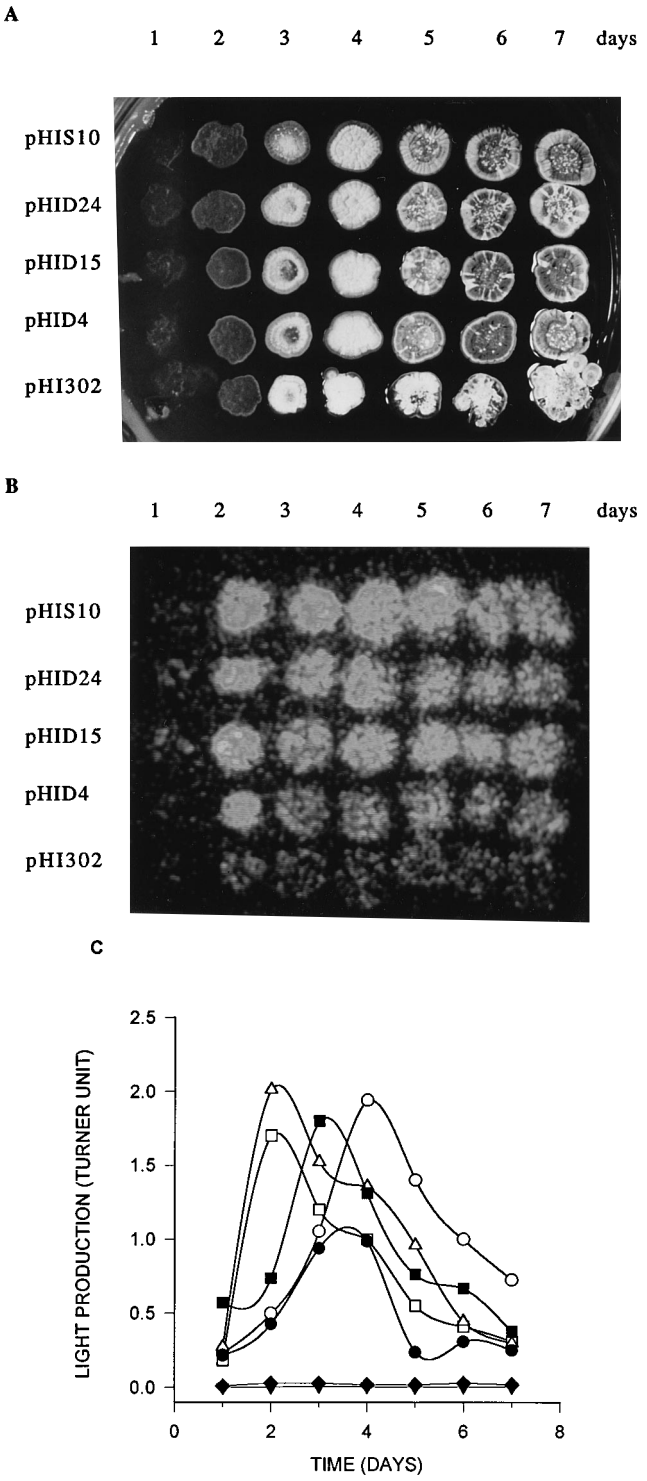


FIG. 5. Time course of light produced by *S. coelicolor* J1501 containing various mutant *sapA* promoters fused to *lux*. See Table 1 for the mutant promoter sequence for each plasmid. (A) After 7 days of incubation, the plate was photographed under light illumination. Aerial hyphae formed on day 3, and the gray spore color was evident on day 5. (B) The same plate as that described for panel A was also photographed in the dark with the aid of a light intensifier. (C) Light production was measured quantitatively as in Fig. 2. Symbols: open circles, pHIS10; open squares, pHID4; open triangles, pHID15; closed squares, pHID24; closed circles, pHID28; closed diamonds, pHID17; closed inverted triangles, pHID22.

bp are also important in promoter activity since a G-to-T change at position +3 (pHID26) inhibits *lux* expression almost 2-fold, deleting position +1 (pHID17) inhibits *lux* expression more than 20-fold, and deleting positions +2 and +3 (pHID22) abolishes *lux* expression. Sequence changes in the promoter just upstream of the transcription start site (pHID6 and pHID13) also reduce promoter strength but do not change the timing of *lux* expression. Thus, sequences on both sides of the transcription start site are important for *sapA* promoter activity. Transcription from the wild-type *sapA* promoter begins dominantly at +1 T and less frequently at +2 G (12) (see Fig. 7). The result that a deletion of the +1 T-A base pair has such a severe effect on the level of transcription suggests either that the nucleotide at position +2 cannot function efficiently as a transcription start site or that +1 is essential for the recognition of *sapA* promoter by a transcriptional machinery.

Primer extension of *sapA* mRNA transcripts. The dispensability of −35 and −10 regions for the proper temporal control of the *sapA* promoter was unexpected, and it seemed possible that positional effects of the promoter on a plasmid were causing some distortion of transcription. Primer extensions of *sapA* mRNA transcripts were done to make sure that *sapA* transcription from the plasmid starts at the same site with the same temporal control as the wild-type, chromosomal promoter.

Most time course experiments of luciferase activity were performed with solid media, since morphological differentiation of *S. coelicolor* could be monitored at the same time. However, the *sapA* promoter is activated in early-stationary-phase liquid cultures and the SapA protein is secreted, even though cells do not undergo morphological changes (12). To show that the *sapAp-lux* fusion had comparable activities in liquid cultures and solid media, light production from *S. coelicolor* containing the *lux* fusion plasmids was monitored during growth in rich media. As judged by cell density, cells reached the early stationary phase 44 h after inoculation, and light production from the *sapAp-lux* fusion peaked after 48 h (Fig. 6). Without any promoter cloned, pHI302 produced a low level of light only during exponential growth in liquid cultures. The relative activities of the deletion derivatives of the *sapA* promoter in this experiment are similar to those measured on solid media (cf. Fig. 3 and 6).

Typical primer extension results with RNA from liquid cultures are shown in Fig. 7. *lux* primers detect *sapA* transcripts only from the plasmid, and *sapA* primers detect *sapA* transcripts only from the chromosome. Primer extension experiments with RNA from *S. coelicolor* cells (containing pHIS9) demonstrate that the same transcription start is utilized for both the plasmid-borne promoter and the chromosomal promoter. *sapA* expression peaks in an early stationary phase, regardless of the location of the promoter. These results are also consistent with S1 analyses of the transcription start sites of the chromosomal *sapA* promoter (12). When RNA from *Streptomyces* cells containing pHI302 (no *sapA* promoter inserted) was used, only transcripts from the chromosomal *sapA* promoter were detected (compare lanes 1 and 5). These results suggest that the expression of the plasmid-borne *sapA* promoter is comparable to that of the chromosome-borne *sapA* promoter because they show the same late temporal control and use the same transcription start sites.

When a different pair of primers (*luxA* primer 2 and *sapA* primer 2 [Fig. 7]) was used in a primer extension experiment to map *sapA* transcripts with the same RNA as that described above, comparable results were obtained (data not shown). Despite repeated attempts, *sapA* transcripts from a promoter deleted for sequences upstream of position −8 could not be

TABLE 1. Region-specific mutations in *sapA* promoter

Plasmid[a]	Mutated position[b]	Relative promoter activity[c]	Temporal control
pHIS10	None	1	Normal
pHID4	+1 and +2 (TG to GT)	1.05	Premature expression
pHID6	−2 (addition of G)	0.8	Normal
pHID13	−5 to −2 (ACTG to CGGA)	0.5	Normal
pHID15	+2 and +3 (GG to CA)	0.85	Premature expression
pHID17	+1 (deletion of T)	<0.05	Constitutive expression
pHID22	+2 and +3 (deletion of GG)	0	Background only
pHID24	+2 (G to A)	0.95	Premature expression
pHID26	+3 (G to T)	0.6	Normal
pHID28	+2 (G to T)	0.5	Slightly premature expression

[a] All plasmids are derivatives of pHIS10 (*sapA* promoter of positions −200 to +120), mutated with degenerate oligonucleotides. See Materials and Methods for details.
[b] The positions given are in reference to the sequence TCAACTGGTGGCTTCAGC, from position −8 to position +10. The underlined nucleotide is position +1.
[c] Promoter activity is calculated as in Fig. 2.

detected, probably because the signal falls below the sensitivity of primer extension experiments.

DISCUSSION

One approach to understanding *S. coelicolor* development is to probe the transcriptional control machinery of aerial hyphae at late stages in the cell cycle. Specifically, we selected the promoter region of the *sapA* spore coat gene because it is one of the few stationary-phase genes that has been characterized in *Streptomyces* spp. The availability of luciferase promoter probe vectors, which are ideally suited for analysis of stationary-phase gene expression, allowed us to analyze both *cis*- and *trans*-acting components (see Results and below, respectively) that are necessary for *sapA* promoter activity.

The time-dependent expression of luciferase fused to the *sapA* promoter on a multicopy plasmid and its temporal coincidence with *sapA* mRNA accumulation have already been established (12). In this study, we used a low-copy, SCP2-derived replicon (pHI302) to obtain better stability and to minimize potential titration of any *trans*-acting factors. The temporal profiles of light production from the low-copy and multicopy vectors were the same. In addition, the same time courses of luciferase production, both from solid and liquid cultures, are observed when the *sapAp-lux* construct was integrated into the chromosome at the bacteriophage ϕC31 *attB* site (16).

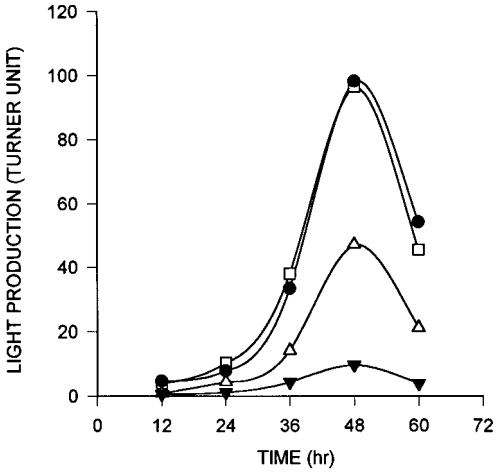
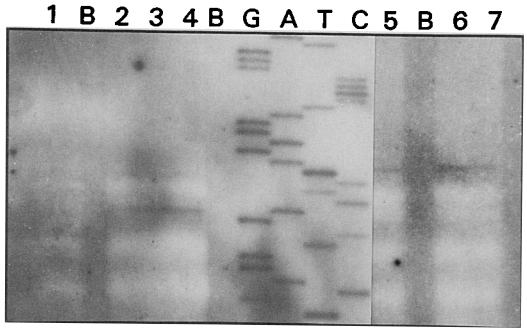


FIG. 6. Time course of light production from J1501 liquid cultures of *S. coelicolor* containing various 5′ deleted *sapA* promoters fused to *lux*. Spores (5×10^4) of each strain were inoculated into 50 ml of YEME+ liquid medium and incubated at 30°C with vigorous shaking. For each time point, 1 ml was removed and light production was measured as described in Materials and Methods. Cultures reached an early stationary stage at about 44 h. Symbols: circles, pHIS10; squares, pHIS9; triangles, pHI316; inverted triangles, pHI341. The relative activities of pHIS10, pHIS9, pHI316, and pHI341 were 1.00, 0.95, 0.45, and 0.10.

Inspection of the sequences 5′ to the transcription start point of *sapA* does not reveal an *E. coli*-like consensus around positions −35 and −10. This is not surprising because *S. coelicolor* is known to code for at least seven alternative RNA polymerase σ factors (4–6, 31). It also seems quite reasonable to expect a specialized σ factor to be involved in *Streptomyces* spore compartment-specific expression, particularly in light of the *Bacillus subtilis* paradigm (21). A large number of *Streptomyces* sp. promoters have been sequenced, but they do not appear homologous to the *sapA* promoter around −35 and −10 (29). However, this apparent uniqueness of the *sapA* promoter may be due to the paucity of studies on stationary-phase gene expression.

Starting with a 330-bp fragment that extended from −200 to +120 bp relative to the transcription start site of the *sapA* promoter, 5′ deletions identified a putative sequence element(s) essential for full transcriptional activity. There was no observable change in *sapA* promoter-driven luciferase production until deletions reached 178 bp upstream of the transcription start point. A drop in transcription efficiency occurred by removal of sequence from −178 to −72, with most of the decrease occurring when the region between positions −137 and −118 is removed. Remarkably, even an 18-bp promoter that extends from −8 to +10 relative to the transcription start point retained 10% of the activity of the undeleted promoter.

Temporal control of *sapA* promoter is of special interest. Although the various deletion derivatives altered promoter strength, the developmentally late temporal induction that is characteristic of wild-type *sapA* expression was preserved, even when native DNA upstream of position −8 and downstream of position +10 was removed (pHI341). The upstream vector DNAs, consisting of a portion of a polylinker and the fd transcription terminator, were identical in all of the constructs shown in Fig. 3. The possibility that the adjoining vector DNA was providing necessary *cis*-acting sites was tested by providing other, unrelated DNA segments in place of the polylinker at the 5′ end of the *sapA* segments (Fig. 4). However, there was no change in luminescence profiles for the deletion mutants carrying the different adjoining vector sequences. Consistent with the deletion mapping data, which implicated sequences



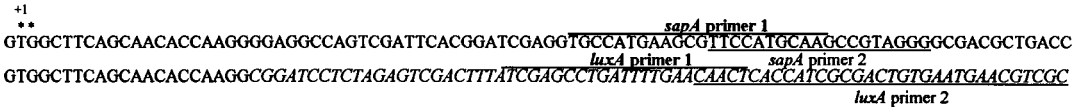


FIG. 7. Primer extension analysis. Plasmid and chromosomal *sapA* transcription start sites were mapped at various times after inoculation. The transcription start points and the primers used are shown at the bottom of the figure. The wild-type *sapA* sequence (upper sequence) is shown along with the *sapA-luxA* junction from pHIS9 (lower sequence). Italicized DNA represents the luciferase sequences from the vector pHI302. The *sapA* transcription start sites are highlighted with asterisks. The major transcription start site is numbered +1. Black lines represent the sequences that the various primers are made against. The plasmid-specific 22-mer (*luxA* primer 1) was used for the reactions shown to the left of the mp18 sequence ladder (with −40 primer; lanes G, A, T, and C) in the upper portion of the figure (lanes 1 to 4). The 22-mer (*sapA* primer 1) specific for the chromosomal transcript was employed in the reactions of the right-hand lanes (lanes 5 to 7). Left-hand lanes (*luxA* primer 1): 1, pHI302 (vector), 48 h; 2, pHIS9, 36 h; 3, pHIS9, 48 h; 4, pHIS9, 60 h; B, blank. Right-hand lanes (*sapA* primer 1); 5, pHI302, 48 h; 6, pHIS9, 48 h; 7, pHIS9, 60 h; B, blank.

around the transcription start point in the temporal control of the *sapA* promoter, single or double base-pair changes at +1 to +3 changed the timing of *sapA* expression to an earlier stage in development (Fig. 5 and Table 1).

There may be precedents for the *sapA* type of promoter. −35 region sequences are not required for the late temporal control and minimal promoter activity of bacteriophage T4 late promoters. Gp55, a novel sigma factor coded by T4, recognizes a −10, but not a −35, region of T4 late promoters, and RNA polymerase is enhanced by interactions with other accessory proteins (33). In *Streptomyces* spp., it has been suggested that −35 region sequences may not important for a few promoters (for a review, see reference 29). Other late promoters in *Streptomyces* spp., such as promoter A1 of *Streptomyces aureofaciens*, Pr4 in the *redD* region of *S. coelicolor*, and *orf2,3P* and *orf1p* in the *actII* region of *S. coelicolor*, show sequence homologies to the *sapA* promoter around the transcription start point(s). Deletion and/or substitution studies need to be done to determine whether control of these promoters is similar to that of the *sapA* promoter. Perhaps the closest analogous promoters are P1a and P1b from *M. echinospora*. Baum et al. (2, 3) have shown that P1a and P1b do not require native DNA further than 5 and 20 bp upstream from the transcription start point, respectively. There are some intriguing parallels between these *M. echinospora* promoters and the *sapA* promoter. First, both organisms are members of the order *Actinomycetales*. Second, both the *sapA* and P1 promoters drive transcription of a stationary-phase gene. Third, there is a small block of sequence identity, from −2 to +4, between the *sapA* and P1a promoters. Fourth, both a 2-bp deletion at +2 and +3 of the *sapA* promoter (Table 1, pHID17) and a spontaneous 1-bp deletion at either +2 or +3 (from GG to G) on the P1a promoter (24) abolish transcriptional activity of the promoter to the basal level. Fifth, removal of downstream sequences of the *sapA* promoter from +40 to +120 lowers promoter activity (Fig. 3), and mutations downstream from P1b reduce its activity. However, this may be an indirect effect resulting from increasing the strength of the nearby P1c promoter (19).

Many eukaryotic promoters transcribed by RNA polymerase II have been shown recently to lack a TATA element which might correspond to a prokaryotic −10 region. In such TATA-less promoters, the initiator element, which encompasses the transcription start site, is critical in positioning RNA polymerase II. Various initiator elements have been described and classified by sequence similarities. These initiator elements are recognized specifically by initiator-binding proteins that interact with components of the basal transcription machinery, thereby providing a means of forming a transcription-competent complex (30).

Temporally regulated transcription from the 18-bp *sapA* promoter does not fit well with current models of promoter recognition by most prokaryotic sigma factors that invoke key contacts to sequences around −35 and −10 bp upstream (11, 27, 34). Therefore, as in eukaryotic TATA-less promoters, it is possible that a protein binding at or near the transcription start site mediates formation of a transcription complex. This accessory protein(s) may be a novel factor or a new class of sigma factor, which minimally requires only positions −8 to +10 of the promoter for binding and which possibly contacts RNA polymerase at a different site from that of typical sigma factors. Another possibility is that σ recognizes sequences at, or just downstream from, the transcription start site and then slides upstream to form an open complex. Third, an entirely novel RNA polymerase, lacking the normal $\alpha_2\beta\beta'\sigma$ motif, might be responsible for *sapA* transcription.

In an effort to identify the *trans*-acting regulator(s) of *sapA*, two second-site mutations that specifically alter the developmental pattern of light production from colonies carrying the *sapAp-lux* gene fusion were isolated independently (16). These two mutations (*esa1* and *esa2*) showed the same phenotype,

namely, premature light production from *sapA-lux* fusions. Thus, *esa* might encode a factor(s) that determines the timing of *sapA* expression. Expression of *esa* may be itself temporally regulated in a way similar to the temporally regulated σ factors. A synthetic *sapA* promoter DNA column may be used to affinity-purify the binding protein(s) of this promoter. Rifamycin-resistant *sapA* transcription experiments may be used to test the possibility of a novel RNA polymerase involved in late gene expression in *Streptomyces* spp.

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