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The *Streptomyces galP1* Promoter Has a Novel RNA Polymerase Recognition Sequence and Is Transcribed by a New Form of RNA Polymerase In Vitro

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We report the identification of DNA sequences that determine the activity of the *Streptomyces galP1* promoter and a new form of RNA polymerase holoenzyme that recognizes these sequences in vitro. Base substitutions were introduced throughout the *galP1* promoter region, and bases at positions −34, −36, and −11 with respect to the transcription start site were shown to be required for promoter function. These bases correspond in their positions to regions known to be important for RNA polymerase binding in several classes of eubacterial promoters, but the sequences themselves are not similar to those previously described. The −35 region of the *galP1* promoter consists of six G residues, and base changes in this G hexamer had a dramatic effect on promoter activity. By using *galP1*-containing DNA template, a new RNA polymerase activity was purified from *Streptomyces*. Holoenzyme reconstitution experiments identified a new sigma factor that directs *galP1* transcription in vitro. DNase I protection experiments identified a binding site for this new holoenzyme immediately upstream of the *galP1* transcription start site.

Sequences that signal transcription initiation in bacteria play an important role in gene regulation. Among the best studied of these sequences are those involved in RNA polymerase recognition. The observation that sequences centered around −10 and −35 bp upstream of the transcription start site are highly conserved in many promoters (35), the fact that these sequences are required for promoter activity (46), and the discovery of RNA polymerase heterogeneity in *Bacillus subtilis* (26) supported the model that in most cases the sigma subunit of RNA polymerase makes sequence-specific contacts in these two regions of bacterial promoters. The strongest evidence comes from experiments that show allele specificity of suppression between mutations in the sigma subunit of RNA polymerase and compensating base changes in the DNA sequence of promoters. Amino acid changes in various sigma proteins, σ^H (8, 47), σ^A (22), and σ^E (43) of *B. subtilis* and σ^{70} of *Escherichia coli* (13, 38), show that these proteins contact the −10 and −35 regions of their cognate promoters.

While the RNA polymerase holoenzymes that catalyze transcription in *Streptomyces* spp. are presumed to be functionally similar to those of other bacteria, relatively little is known about sequences that are required for RNA polymerase recognition in this complex bacterium. At least eight RNA polymerase holoenzymes have been identified in *Streptomyces*, and promoters with putative RNA polymerase binding sites similar to known consensus sequences have been described (reviewed in references 3 and 41). A consensus sequence prototypical of eubacteria (TTGACA centered around −35 and TATAAT centered around −10) appears in several *Streptomyces* promoters, and at least four genes whose predicted products resemble *E. coli* σ^{70} have been cloned and characterized (2, 4, 42). One of these genes is apparently essential (4). The existence of an additional sigma factor that may be functionally analogous to σ^{70} was identified by in vitro runoff assays and protein reconstitution experiments (45). As with other bacteria, multiple holoenzymes exist in *Streptomyces* that differ in their recognition specificity and the sigma subunit associated with them. RNA polymerase heterogeneity was discovered in *Streptomyces* by using promoter-containing DNA templates from *B. subtilis* (45), and holoenzymes functionally analogous to σ^A of *Bacillus* and *E. coli* (2, 6, 42, 45), σ^B of *B. subtilis* (6, 34, 45), σ^{28} of *B. subtilis* (7), and σ^E, which occurs in a variety of bacteria (25), have been described in *Streptomyces*. Very little is known, however, about the DNA sequences that signal transcription initiation in *Streptomyces*, and the evidence for the involvement of most of the sequences presumed to be responsible for RNA polymerase recognition is circumstantial. In only a few cases do mutations exist to define bases important for promoter activity. Mutations in the promoters of the aminoglycoside phosphotransferase gene (21) and the beta-lactamase gene (12) of *Streptomyces fradiae* and the tetracenomycin biosynthesis genes (9) of *Streptomyces glaucescens* have been reported, but to date no study has involved both a biochemical and a genetic analysis of RNA polymerase interaction with promoter recognition sequences.

Here we report a biochemical and genetic analysis of the *Streptomyces galP1* promoter. This promoter is responsible for glucose-sensitive, galactose-dependent transcription of the *Streptomyces* galactose utilization operon (11). The transcription start site for *galP1* was determined by S1 nuclease mapping (11), primer extension of in vivo RNA (29), and in vitro runoff assay (44, 45). At high resolution, these techniques identified the same nucleotide as the start site. In a study of *galP1* regulation (29), 21 of the 55 bp upstream of the transcription start site were changed to other bases by site-directed mutagenesis. In that analysis, several potential regulatory motifs were identified, including six hexamers and two pairs of direct repeat sequences that apparently act as an operator for negative regulation of transcription. In this report we describe the analysis of single-base changes within the *galP1* promoter region that affect the activity of the promoter. These mutations are distinct from those previously shown to be involved in regulation (29), in that they affect promoter activity with no

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apparent effect on regulation. These single-base changes were analyzed under inducing conditions by primer extension analysis and transcriptional fusions to the *xylE* reporter gene (20). Mutations at −11, −34, and −36 bp upstream of the transcription start site resulted in a severe reduction in transcription initiation from *galP1*. The locations of these base changes and the phenotypes of the resulting mutations are consistent with the notion that they constitute at least part of the element recognized by RNA polymerase. The DNA sequence centered around −35 consists of six G residues. Most of the single-base changes introduced in this G hexamer resulted in a dramatic increase in expression, suggesting that these changes may relieve negative contacts between the promoter sequence and RNA polymerase. By using *galP1*-containing DNA template, an RNA polymerase activity was identified that transcribes *galP1* in vitro. This enzyme is distinct from those previously described from *Streptomyces* in terms of its recognition specificity and subunit composition. By using in vitro reconstitution experiments, a protein of approximately 45,000 Da was shown to function as a sigma subunit capable of directing *galP1* transcription. This new holoenzyme was also shown to protect the region of the promoter containing the putative RNA polymerase binding site from digestion with DNase I endonuclease. We suggest from the data reported here that the *Streptomyces galP1* promoter has a novel RNA polymerase binding site and is transcribed by a new form of RNA polymerase holoenzyme.

MATERIALS AND METHODS

Strains and growth conditions. *Streptomyces coelicolor* A3(2) wild type (19), M124 (*argA1 proA1 cysD18* SCP1$^-$ SCP2$^-$) (19), J1970 (M124 *hrdD*) (4), or J1980 (*argA1 proA1 cysD18 rpoC*^{HIS} SCP1$^-$ SCP2$^-$) (1) was used for RNA polymerase preparation, and *Streptomyces lividans* 1326 (19) was used for analysis of promoter mutations. *S. coelicolor* J1501 (*hisA1 strA1 uraA1* Pgl$^-$ SCP1 NF SCP2$^-$) (10) and J1970 (*hisA1 strA1 uraA1 hrdD::hyg* Pgl$^-$ SCP1 NF SCP2$^-$) (4) were used to assay *hrdD* activity in vivo. *E. coli* CJ236 (23) was used for propagation of uracil-incorporated M13 phage, JM101 (31) was used for the propagation of M13 replicative-form (RF) DNA, and TB1 (28) was used for propagation of plasmids. Plasmid pUC-sP1 is a pUC18 derivative containing a 200-bp *Sau*3A-*Fun*HI fragment that includes the *galP1* promoter from −117 to +53 with respect to the transcription start site. The fragment was cloned into the *Sma*I site of pUC18. All DNA preparations and manipulations for *Streptomyces* (19) and *E. coli* (38) were carried out as described.

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed according to the method of Kunkel (23) with the mutagene kit from Bio-Rad (catalog no. 170-3571) according to the manufacturer's instructions. M13mp18, containing a 196-bp *Hin*dIII-*Bam*HI fragment that includes the *galP1* promoter from −69 to +103 with respect to the transcription start site, was used as the template. Fragments containing a single-base change were constructed by annealing a primer containing the desired base change to wild-type *galP1*-containing template DNA. DNA sequences were determined by the dideoxy chain termination sequencing reactions of Sanger et al. (37), carried out with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and the forward (−40) sequencing primer.

***xylE* transcriptional fusions.** To construct transcriptional fusions, the 196-bp *Bam*HI-*Hin*dIII fragments containing base changes were ligated to the 17-kb *Bam*HI-*Hin*dIII fragment of pXE4 containing the *xylE* reporter gene (20). For mutations that resulted in loss of expression from *galP1*, plasmids containing these base changes were constructed several times from independent samples of mutagenized oligonucleotide. Catechol dioxygenase assays were performed as described elsewhere (20).

Primer extension analysis. Cultures for RNA isolation were grown as for catechol dioxygenase assays, collected by centrifugation, and washed once with M56 buffer (10, 28). RNA extraction was carried out as previously described (19). For primer extension experiments, the primer 5′ CGTAAGGCACGCGATGG ATCCCGA 3′ was complementary to the sequence directly downstream from and overlapping the *Bam*HI site of pXE3 (20). RNA (50 μg) was hybridized with 5′-end-labeled primer (5×10^4 cpm), incubated with reverse transcriptase, and treated with RNase as previously described (10). Radioactive DNA was concentrated from the reaction mixture and subjected to electrophoresis in an 8% polyacrylamide gel containing 7 M urea next to Sanger sequencing reactions (37) for the corresponding region of DNA. The fragment shown in the sequencing ladder is part of a 300-bp *Hin*dIII-*Kpn*I fragment containing the *galP1* promoter from −69 to +103 with respect to the transcription start site and part of the *xylE* gene (48) cloned into the *Hin*dIII-*Kpn*I sites of M13mp19.

RNA synthesis. The templates used included a 210-bp *Bam*HI fragment, isolated from pUC-sP1 (44), that includes the DNA sequence of *galP1* from −117 to +96 with respect to the transcription start site; a 340-bp *Bam*HI-*Eco*RI fragment, isolated from pUC-P2:3 (43), that includes the DNA sequence of *galP2* from −200 to +120 with respect to the transcription start site; and a 480-bp *Bam*HI-*Eco*RI fragment, isolated from pCB480 (31, 32), that includes the *veg* promoter from +390 to −95 with respect to the transcription start site. These fragments were separated from digested plasmid DNAs by polyacrylamide gel electrophoresis (PAGE) and were recovered from gel slices by electroelution. Enzyme fractions were assayed for activity by incubation of 1 μl of the column fraction in 40-μl reaction mixtures containing 1 μl of the purified restriction fragment, as previously described (44). Radioactive RNA was concentrated from the reaction mixtures by ethanol precipitation and subjected to electrophoresis in a 6% polyacrylamide gel containing 7 M urea (30).

RNA polymerase preparation. For all experiments except the holoenzyme reconstitution experiment and the DNase I protection assays, RNA polymerase was prepared as follows. A 100-g sample of *S. coelicolor* cells that had been grown in YEME medium (19) containing 34% sucrose was disrupted by passage through a French pressure cell, and RNA polymerase was partially purified by phase partitioning and ammonium sulfate fractionation as previously described (44). RNA polymerase was further purified by application to a 10-ml heparin-agarose column (2 by 5.5 cm), and bound material was eluted with a 0.25 to 0.8 M linear KCl gradient in buffer A (45). Fractions containing the peak of *galP1* transcribing activity as determined by in vitro runoff assay were pooled and applied first to a fast protein liquid chromatography (FPLC) Superose-6 column and then to an FPLC Mono-Q column. Protein from the Mono-Q column was eluted with a 0.10 to 1.00 M KCl gradient, and RNA polymerase activity was again identified by in vitro runoff transcription assays. Proteins contained in the Mono-Q column fractions were subjected to electrophoresis in a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (24) and visualized by staining with the Bio-Rad silver stain kit.

Reconstitution of *galP1*-specific transcribing activity. During the course of this work, *S. coelicolor* J1980, a strain containing an *rpoC*^{HIS} allele, which produces a β′ subunit with six histidine residues at the carboxy terminus, was constructed (1). This modification allows purification of RNA polymerase holoenzymes by a combination of nickel-nitrilotriacetic acid (NTA) agarose affinity chromatography and heparin-Sepharose chromatography (1). RNA polymerase used to reconstitute the *galP1* holoenzyme was isolated from a 70-g sample of *S. coelicolor* J1980 cells that had been grown in SLAB medium (11) and disrupted by passage through a French pressure cell. RNA polymerase was partially purified by affinity chromatography with nickel-NTA agarose (18) and passage through a 5-ml HiTrap heparin-Sepharose column (Pharmacia LKB Biotechnology). Bound material was eluted with a 0.3 to 0.65 M linear KCl gradient. The peak of *galP1* activity was identified by in vitro runoff transcription assays.

Core RNA polymerase was prepared from heparin-Sepharose fractions that eluted near the end of the potassium chloride gradient. These fractions contained significant amounts of core RNA polymerase and showed no specific initiation from any promoters used in this study. Proteins within these fractions were precipitated by using 1/2 volume of 100% ammonium sulfate, resuspended in Superose-6 buffer (6), and applied to an FPLC Superose-6 gel filtration column (6). Proteins contained in fractions from this column were displayed on a 10% polyacrylamide (30:0.8, acrylamide to bis)–SDS gel (24) and visualized by Coomassie blue staining. The fraction containing the highest concentration of core subunits was used as the source of core polymerase.

Individual proteins contained within the peak of *galP1* transcribing activity were isolated as follows. Protein from the fraction containing the peak of *galP1* transcribing activity was mixed with loading buffer (24) and subjected to SDS-PAGE (10% polyacrylamide; 30:0.8, acrylamide to bis) (24). The gel was dissected into 18 individual slices, and the proteins were eluted as previously described (15). A small segment of each gel slice was loaded onto a second SDS–10% polyacrylamide gel, and the proteins were visualized by staining with silver (Bio-Rad silver stain kit). The remaining protein from each slice was eluted and then renatured by using GroEL as previously described (2). A 10-μl fraction of renatured protein fraction was added to approximately 0.1 μg of core RNA polymerase and was incubated first at 30°C for 15 min and then at 37°C for 15 min. Reconstituted RNA polymerase holoenzyme activity was identified by in vitro transcription assays with the *galP1*, *galP2*, and *veg* promoter templates described above.

DNase I protection experiments. To map the protected region on the coding strand, a 200-bp fragment which contains the *galP1* promoter sequence from −117 to +53 was prepared by digesting pUC-sP1 with *Hin*dIII, filling in the 5′ overhang with Klenow enzyme and [α-^{32}P]dATP (36), and then digesting with *Bam*HI. This fragment was isolated by PAGE as described above.

RNA polymerase binding to *galP1*-containing fragments was carried out in a solution containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 2 mM dithiothreitol, 10% glycerol, 250 ng of dI-dC/ml, 7 μl of a heparin-Sepharose fraction within the peak of *galP1* transcribing activity, and 0.1 μg of DNA template. After a 30-min incubation at 30°C, various amounts of DNase I (from 200 to 2.5 ng, as indicated in Fig. 8) were added to the reaction mixture, which was then incubated for 3 min at 30°C. DNase I digestion was terminated by phenol-chloroform extraction. DNA was extracted from the reaction by ethanol precipitation, resuspended in formamide loading buffer, and subjected to elec-

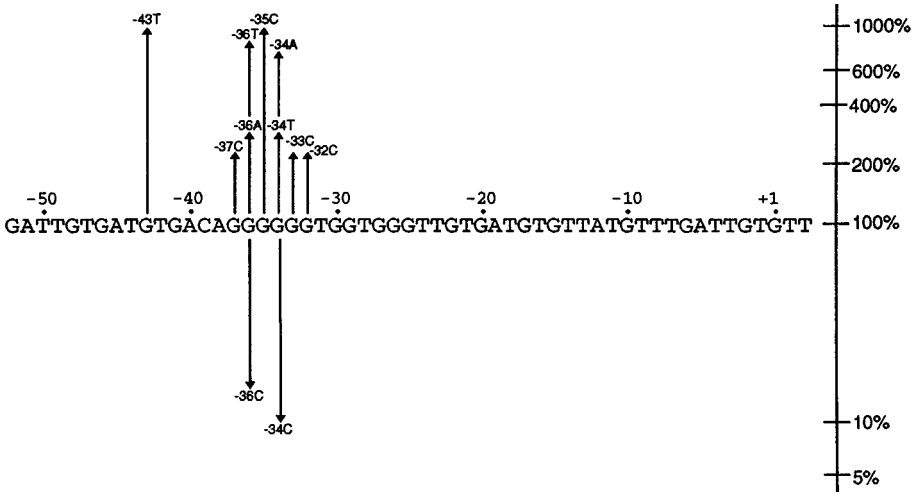

FIG. 1. The effects of single-base-pair substitutions in the *galP1* promoter on expression of *galP1-xylE* transcriptional fusions. The sequence shown is the nontranscribed strand. The transcription start point is indicated as +1. *xylE* levels were assayed after growth of cells on galactose as the sole carbon source. Arrows indicate the activity of each mutated promoter relative to the wild-type promoter, which is given the value of 100%.

trophoresis in a 6% polyacrylamide–7 M urea gel (29, 30). Molecular-size standards were generated by Sanger dideoxy sequencing reactions (37). An oligonucleotide complementary to the 5′ end of the coding stand (5′-GATCCGTCCG CACTCCTCTGCACC-3′) of the *Bam*HI-*Hin*dIII fragment was used to prime the sequencing reactions. The 5′ end of this primer corresponds exactly to the 5′ end of the coding strand fragment.

RESULTS

Analysis of base substitution mutations within the *galP1* promoter region. Centered around −10 and −35 bp upstream of the transcription start site of *galP1* (Fig. 1), positions where RNA polymerase would be expected to bind, are sequences not previously associated with RNA polymerase recognition. In particular, the DNA sequence at −35 consists of six guanidylate residues. In an effort to identify bases important for activity of *galP1*, single-base changes were introduced throughout the *galP1* promoter region, and the effects of these base changes were assayed by transcriptional fusions to the *xylE* reporter gene in cells grown on galactose. In all cases, the DNA sequences of the mutated *galP1*-containing DNA fragments were determined by the method of Sanger et al. (37). In Fig. 1, the levels of expression observed for various mutants are reported as percentages of the level of wild-type *galP1* expression on galactose. Each of the six G residues centered around −35 was changed to other bases. Two of the base changes tested in this analysis, G-to-C transversions at −34 and −36, resulted in severe reduction in expression from *galP1*, suggesting that bases at these positions are required for promoter activity. Interestingly, in most cases, changes in the G hexamer resulted in a dramatic increase in expression from *galP1*, suggesting that the −35 region of this promoter is suboptimal at virtually every position. The most likely interpretation of these data is that the changes in the −35 region that result in increased activity from *galP1* do so because they relieve negative contacts between the DNA sequence and RNA polymerase.

In previous work (29), we reported an analysis of base changes that resulted in significant expression from *galP1* in the absence of inducer or that affected the pattern of expression from *galP1*. Mutations that maintained the requirement for inducer, i.e., that showed low (wild-type) levels of expression on glucose and no significant expression on glycerol, were distinguished from mutations that exhibited a constitutive phenotype, i.e., that showed a significant increase in expression on glucose and glycerol (29). Base changes that affected expression from *galP1* on galactose without affecting the level on glucose (or glycerol [data not shown]) were considered, in this analysis, to be involved in promoter activity and not in glucose or galactose regulation. In Fig. 2, quantitative catechol dioxygenase assays from cells grown on galactose or glucose as the sole carbon source are shown as a histogram. The G-to-T transversion at −36 and the G-to-A transition at −34, which resulted in significantly increased levels of expression from *galP1* on galactose, did not result in constitutive expression. No expression of *galP1* containing either of these base changes was detected in cells grown on glycerol (data not shown). Taken together, the fact that base changes within the G hexamer had a dramatic effect on the activity of *galP1* without affecting the requirement for inducer, the observation that some changes resulted in a severe reduction in activity characteristic of promoter down mutations, and the fact that these bases are in positions upstream of the transcription start site precisely where RNA polymerase would be expected to interact suggest

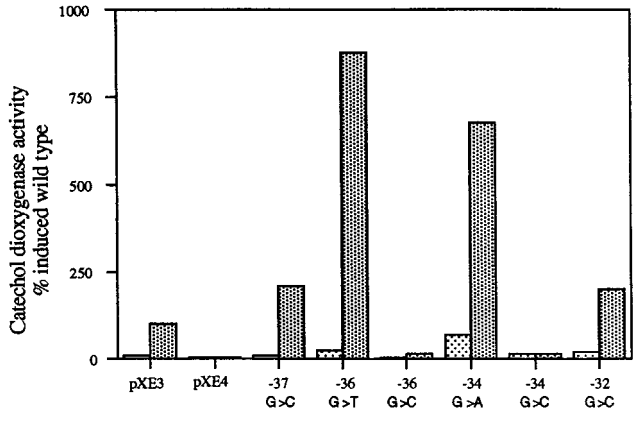

FIG. 2. Histogram showing results of quantitative catechol dioxygenase assays, plotted as percentages of the fully induced wild-type level, with *S. coelicolor* containing pXE3 (wild-type *galP1*), pXE4 (the *xylE* gene with no promoter upstream), or mutant derivatives of *galP1* as indicated, after growth on either glucose (lightly stippled bars) or galactose (heavily stippled bars) as the carbon source.

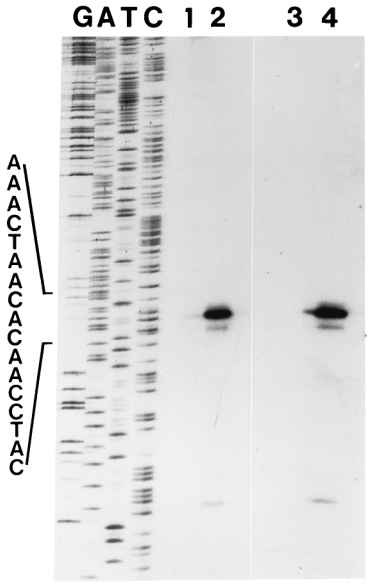

FIG. 3. Primer extension analysis of RNA isolated from cells containing a wild-type copy of *galP1* after growth on glucose (lane 1) or galactose (lane 2), or a copy of *galP1* containing a G-to-T base change at −36 after growth on glucose (lane 3) or galactose (lane 4).

that this sequence constitutes at least part of the element recognized by RNA polymerase.

Assay of *galP1* promoter mutations using primer extension analysis of in vivo RNA. For mutant promoters that showed a change in expression from *galP1*, primer extension analysis was performed to determine whether transcription was initiated at the same start site as for the wild-type promoter. The analysis of one of the mutants, with a G-to-T change at position −36, that showed a dramatic increase in expression is shown in Fig. 3. RNA isolated from cells containing a wild-type copy of *galP1* that had been grown on either glucose (lane 1) or galactose (lane 2) as the sole carbon source was used in primer extension reactions. A *galP1*-specific reverse transcript is clearly visible in lane 2. RNA was also isolated from cells containing a G-to-T change at position −36 in *galP1* that had been grown on glucose (lane 3) or galactose (lane 4). Transcription initiation from this mutant promoter was indistinguishable from that from the wild-type promoter. The fact that RNA isolated from glucose-grown cells gave no signal in these experiments indicates that increased expression from *galP1* in the mutant was dependent on the presence of inducer.

In our analysis of mutants that initially showed a constitutive phenotype (29), one was relevant to this study. By using transcriptional fusions to the *xylE* reporter gene, a base change from a T to a C at −11 resulted in constitutive expression of catechol dioxygenase, the product of the *xylE* gene. Primer extension analysis, however, showed that this change resulted in elimination of transcription from the *galP1* transcription start site. Transcription in this mutant originated instead from upstream of the promoter within the vector sequence, resulting in constitutive catechol dioxygenase activity (data not shown). The fact that a base change at −11 resulted in elimination of activity from the promoter supports the notion that the sequences centered around −10 and −35 bases with respect to the transcription start site of *galP1* are important for RNA polymerase recognition.

Identification of an RNA polymerase activity that transcribes *galP1* in vitro. To examine transcription initiation from

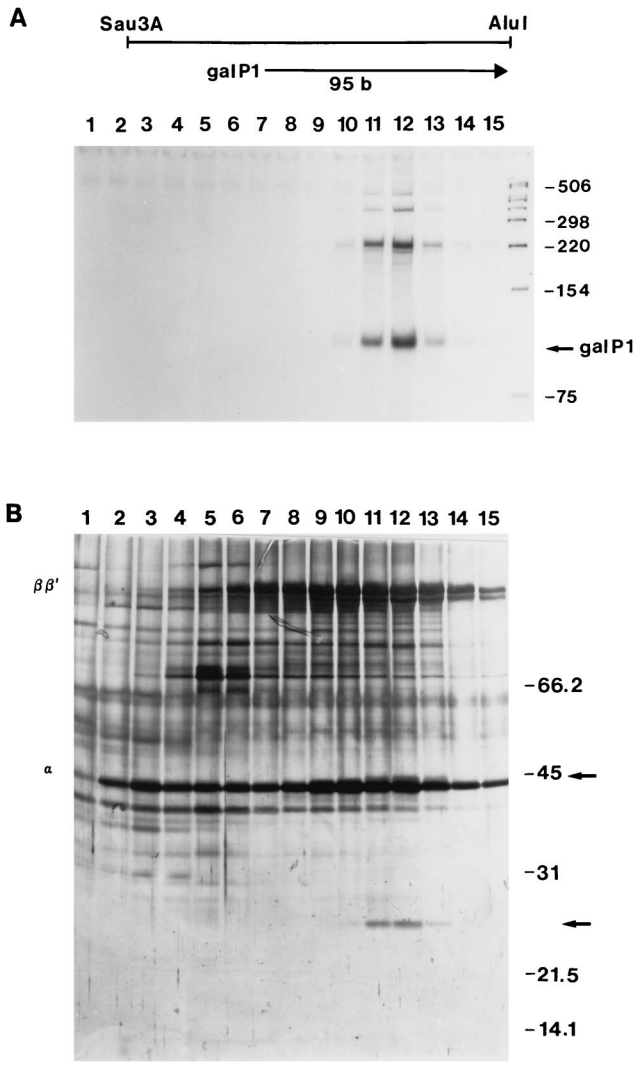

FIG. 4. Elution of *galP1* transcribing activity from an FPLC Superose-6 column with a linear KCl gradient. (A) The *galP1*-containing DNA fragment used in in vitro transcription assays is shown at the top. The size of the predicted transcript (95 bases) is indicated by an arrow. Every column fraction (indicated by the numbers on top) was assayed. After separation on a 7% polyacrylamide gel containing 7 M urea, the runoff transcripts were visualized by autoradiography. The numbers on the right indicate molecular-size markers. (B) The proteins in each fraction were separated by SDS-PAGE and visualized by staining with silver. The number above each lane represents the fraction number. Arrows indicate the positions of the 45,000- and 28,000-Da proteins described. The numbers to the right indicate molecular-size markers.

galP1 in vitro, promoter-containing DNA fragments were tested with partially purified RNA polymerase in runoff transcription assays as previously described (44). RNA polymerase that had been purified by phase partitioning was applied to a heparin-agarose column, and fractions containing *galP1* transcribing activity, as indicated by in vitro runoff assays, were pooled and applied first to an FPLC Superose-6 gel filtration column and then to an FPLC Mono-Q column. RNA polymerase was eluted with a linear KCl gradient and used in in vitro runoff assays. RNAs generated in these assays were displayed on a polyacrylamide gel and visualized by autoradiography. The results of these assays are shown in Fig. 4A. RNA corresponding in size to the runoff transcript expected for *galP1* was generated in reactions using fractions 10 through 14, indicating

that these fractions contained a form of RNA polymerase that could recognize and initiate transcription from the *galP1* promoter. No transcript was detected in reactions using fractions 10 through 14 with the *galP2* (11) or *ctc* (16) promoter-containing template (reference 44 and data not shown). When similar preparations of *galP1* transcribing activity were used, no transcript originating from the correct start site was detected in reactions using mutant *galP1*-containing DNA fragments in which either of the G residues at positions −34 and −36 was changed to C (21a). Proteins contained in fractions from this FPLC Mono-Q column were displayed on an SDS-polyacrylamide gel and stained with silver (Fig. 4B). The β, β′, and α subunits of this RNA polymerase are clearly visible and indicated. Proteins of approximately 28,000 and 45,000 Da were observed to coelute precisely with the *galP1* transcribing activity. The concentrations of these proteins in each fraction correlated closely with the amount of RNA generated from the *galP1* template.

To test whether either or both of these proteins could function as sigma for *galP1* transcription, we attempted to reconstitute these proteins with core RNA polymerase in vitro. Initial attempts were unsuccessful even when *groEL* protein was added to the renaturation reaction (2). With this method of RNA polymerase purification, the putative sigma proteins were present in very low concentrations (the protein gel shown in Fig. 4B is stained with silver), and even though sigma is catalytic in the transcription reaction, and in vitro runoff assays are very sensitive, the amount of renatured protein may not have been sufficient. During the course of this work, a strain of *S. coelicolor* was constructed that contains an *rpoC*^{HIS} allele which produces a β subunit of RNA polymerase with six histidine residues at the carboxy terminus (1). This modification allows purification of RNA polymerase holoenzymes by nickel-NTA agarose affinity chromatography and was used in the reconstitution experiments described below.

Reconstitution of *galP1* holoenzyme. To identify the sigma factor that directs in vitro transcription of *galP1*, we performed holoenzyme reconstitution experiments. RNA polymerase was purified from a strain containing an *rpoC*^{HIS} allele by nickel-NTA agarose affinity chromatography followed by binding to a heparin-Sepharose column. A single heparin-Sepharose fraction located within the peak of *galP1* transcribing activity was used. Proteins contained in this fraction were separated on SDS-PAGE, and the gel was dissected into 18 slices. The proteins within each slice were eluted, separated on SDS-PAGE, and stained with silver. A picture of this gel is shown in Fig. 5. Lane 1 contains proteins from gel slice 1 taken from the top of the original gel, and slice 18 is from the bottom of the original gel. Slice 10 contained the α subunit of RNA polymerase. In all cases the gel slices contained more than one protein. The 28,000- and 45,000-Da proteins identified as potential sigma proteins were located in slices 16 and 9, respectively. Proteins from the gel slices were renatured by using *groEL* protein (2) and were reconstituted with *Streptomyces* core RNA polymerase. Runoff transcription assays using reconstituted RNA polymerase are shown in Fig. 6. Fig. 6A shows results of runoff transcription with *galP1*-containing template. Lane H22 contains the peak fraction of *galP1* transcribing activity from the heparin-agarose column fraction used to generate the gel slices. A runoff transcript the size (96 bases) predicted for initiation from *galP1* is abundant in this lane. Lane C contains core RNA polymerase alone, and no runoff transcript was detected. Lanes 1 through 18 contain proteins contained in gel slices 1 through 18 and core RNA polymerase. Specific *galP1* transcription was reconstituted only by slice 9 (Fig. 6A, lane 9). We conclude that a protein contained in gel slice 9, which corresponds to an apparent molecular size of approximately 45,000 Da, is capable of acting as sigma for *galP1* transcription in vitro. No transcript was observed with reconstitution of protein from fraction 16 (lane 16), which corresponds to the size range of 28,000 Da. The 28,000-Da protein shown in Fig. 4 that coeluted with *galP1* transcribing activity may be a degradation product of the 45,000-Da sigma, may have independent sigma activity not detected in this analysis, or may have no role in *galP1* transcription.

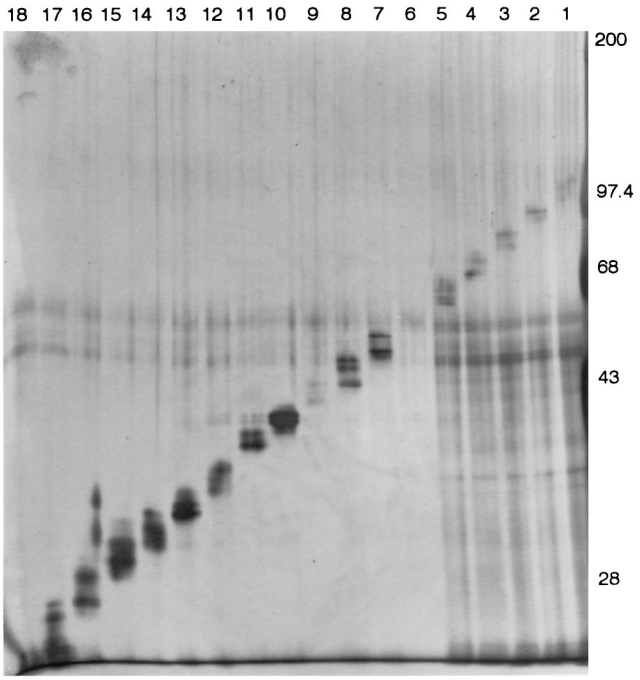

FIG. 5. Silver-stained SDS-polyacrylamide gel of proteins used in RNA polymerase reconstitution assays. The lane numbers shown at the top of the gel correspond to gel slices from the top (lane 1) to the bottom (lane 18) of the original gel. Molecular-size standards, given in kilodaltons, are indicated on the right of the gel.

To assess the selectivity of this reconstructed holoenzyme, in vitro transcription reactions were also performed with DNA fragments containing the *S. lividans galP2* promoter or the *B. subtilis veg* promoter. The *galP2* template was chosen because previous work (44) had shown that the *galP1* and *galP2* promoters are recognized in vitro by two distinct RNA polymerase activities. As shown in Fig. 6B, no *galP2* transcript (120 bases) was observed in reactions containing either H22 holoenzyme or holoenzyme reconstituted with protein from gel slice 9. The *veg* promoter was chosen because it contains the sequences TTGACA and TACAAT centered around −35 and −10, respectively, with a spacing of 17 bp. Promoters with this recognition sequence are known to be transcribed by at least five holoenzymes in *Streptomyces*, including the *hrd* gene products. The *hrdB* gene product, σ^{hrdB}, is 66,000 Da, and the *hrdD* gene product, σ^{hrdD}, is 45,000 Da. Specific transcription from the *veg* promoter (Fig. 6C) was reconstituted by proteins contained in gel slices 6 and 7 (lanes 6 and 7) and gel slice 9 (lane 9). Slices 6 and 7 contained a 66-kDa protein, and this activity most likely corresponds to the *hrdB* gene product. Slice 9 contained two proteins of approximately 45,000 Da, one of which likely corresponds to the *hrdD* gene product.

We conclude from these reconstitution experiments that a protein of approximately 45,000 Da acts as sigma for *galP1*.

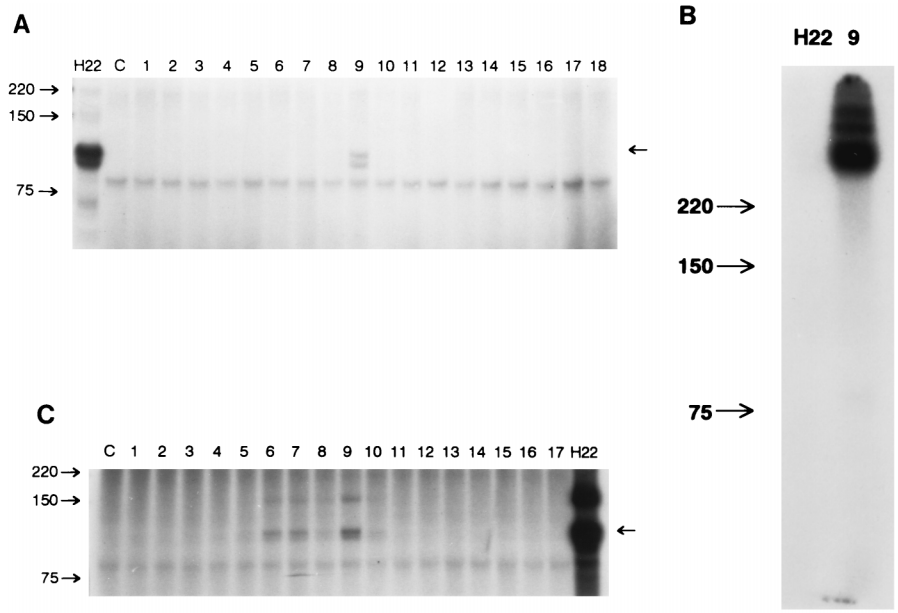

FIG. 6. In vitro reconstitution assays. Proteins extracted from gel slices 1 through 18 were renatured, added to *S. coelicolor* core RNA polymerase, and used in in vitro transcription assays. Shown to the left of each autoradiogram are the positions of the molecular-size standards, which consisted of end-labeled *Hin*f-digested pBR322 DNA. In each case the position of the runoff transcript is indicated by an arrow. (A) RNA generated from the *galP1*-containing template. Lane H22, the peak of *galP1* transcribing activity after elution from heparin-agarose; lane C, core RNA polymerase alone; lanes 1 through 18, samples of proteins eluted from gel slices 1 through 18 as shown in Fig. 5. (B and C) RNA generated from the *veg*-containing and the *galP2*-containing templates, respectively.

This protein is similar in size and perhaps identical to the protein identified by coelution with the *galP1* transcribing activity.

The *gal* operon is expressed in a *hrdD* mutant, and the *galP1* promoter is transcribed in vitro by RNA polymerase prepared from a *hrdD* mutant. Since a sigma protein of approximately 45,000 Da is known to be the product of the *hrdD* gene (4, 42), and since the gel slice used to reconstitute *galP1* activity clearly contained two proteins, one of which is likely to be σ^{hrdD}, we wanted to explore the possibility that σ^{hrdD} was actually responsible for *galP1* transcription. It was not possible to cleanly separate the two proteins contained in gel slice 9, so two approaches were taken to address this issue. First, expression of the *gal* operon was examined in a *hrdD* mutant. *S. coelicolor* J1970 (4) contains a *hrdD* mutation that was generated by insertional inactivation of the *hrdD* open reading frame. Strain J1508 is an isogenic strain that contains a wild-type *hrdD* allele. Equal numbers of spores of each strain were plated on minimal medium containing galactose as the sole carbon source, and no differences were detected either in survival of the spores to colonies or in growth rate (data not shown). We conclude from this experiment that σ^{hrdD} is not required for *galP1* expression in vivo.

Second, RNA polymerase prepared from the same *hrdD* mutant, J1970, was tested in in vitro transcription assays using *galP1*-containing DNA template. As shown in Fig. 7, RNA polymerase purified from the *hrdD* mutant (M124 *hrdD*) or from M124, which contains a wild-type *hrdD* allele, transcribed the *galP1* promoter with equal efficiency in vitro. As controls in this experiment, *galP2*- and *veg*-containing templates were also tested with *hrdD* RNA polymerase. Transcription from these promoters in vitro is apparently not affected by the absence of σ^{hrdD}. We conclude from these experiments that transcription of the *galP1* promoter does not depend on *hrdD* either in vitro or in vivo. We suggest that one of the 45,000-Da proteins detected in the holoenzyme reconstitution experiments is distinct from *hrdD* and is, in fact, responsible for transcription of *galP1*.

Localization of an RNA polymerase binding site within the *galP1* promoter. To test the ability of RNA polymerase contained in fraction H22 to bind to the *galP1* promoter and to identify a binding site if one existed, DNase I nuclease protection experiments were performed. The coding strand was labeled with ^{32}P and incubated with heparin-Sepharose fraction H22. This fraction contained the peak of *galP1* transcribing activity and was used for the RNA polymerase holoenzyme reconstitution experiments. In preliminary experiments, gel retardation assays indicated that the *galP1*-containing DNA frag-

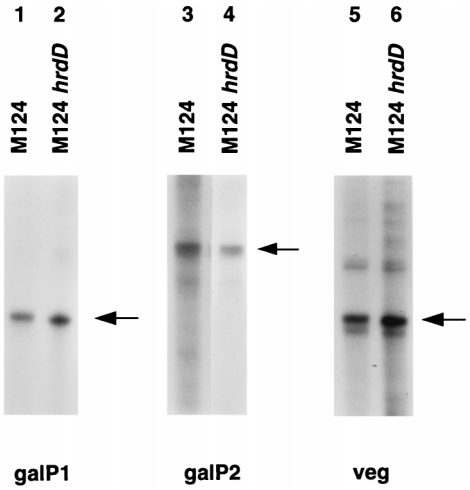

FIG. 7. Runoff transcripts generated from *galP1*, *galP2*, and *veg* promoter-containing templates by using RNA polymerase purified from a strain containing a wild-type (M124) or mutant (M124 *hrdD*) *hrdD* allele.

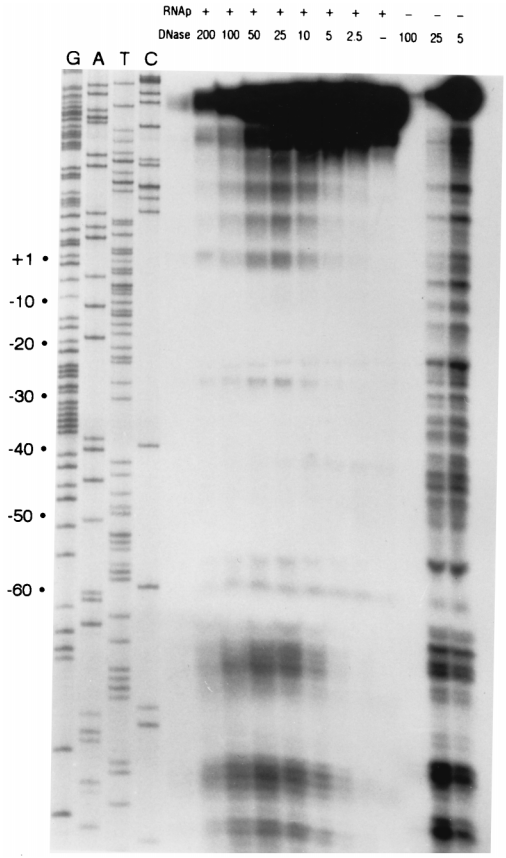

FIG. 8. DNase I nuclease protection assays. The presence (+) or absence (−) of RNA polymerase (RNAp) in the DNA binding reactions is indicated at the top. The amount of DNase I used in each reaction is also shown above each lane as nanograms of enzyme. To identify the DNase I-protected region, molecular-size standards were generated by Sanger dideoxy sequencing. The individual reactions are indicated as G, A, T, and C. The position of the *galP1* promoter sequence (−60 to +1 of the coding strand) within the sequencing reactions is shown to the left. The 5′ end of the primer used to generate the sequencing reactions corresponds exactly to the 5′ end of the fragment used for the protection assays.

ment was retarded only by fractions within the peak of *galP1* transcribing activity (data not shown). RNA polymerase contained in fraction H22 (shown in Fig. 6) protected a region of the *galP1* promoter from −56 to +1 with respect to the transcription start site on the coding strand (Fig. 8). In particular, regions centered around −10 and −35 were protected, indicating possible contacts between RNA polymerase and the DNA in these regions. We point out that formation of the *galP1* RNA polymerase complex did not require ribonucleoside triphosphates. We conclude that RNA polymerase contained in fraction H22 makes stable contacts in the −10 and −35 regions of the *galP1* promoter DNA.

DISCUSSION

We present evidence for DNA sequences upstream of the transcription start site of *galP1*, not previously associated with RNA polymerase recognition, that are required for promoter activity and a new form of RNA polymerase holoenzyme that recognizes these sequences in vitro. Base substitutions within the *galP1* promoter region were introduced by oligonucleotide-directed mutagenesis, and mutated promoter-containing fragments were assayed by transcriptional fusions to the *xylE* reporter gene and primer extension analysis. Mutations at −11, −34, and −36 with respect to the transcription start site resulted in severe reduction in expression from *galP1*, indicating that bases in these positions are required for promoter function. The DNA sequence of the −35 region consists of six G residues, a sequence not previously observed to be involved in RNA polymerase recognition. With *galP1*-containing DNA template, a transcribing activity which is apparently new in terms of its recognition specificity and subunit composition was identified. Holoenzyme reconstitution experiments identified a protein of approximately 45,000 Da (apparent molecular size) that acts as sigma for *galP1* transcription. DNase I nuclease protection experiments identified a binding site for this polymerase just upstream of the transcription start site of *galP1.*

We had established previously that the DNA sequence from −69 (29) to +98 (20) bp with respect to the transcription start site of *galP1* was sufficient for promoter activity and regulation. While there are important exceptions (reviewed in reference 14), it is well established that for many promoters, bases centered around −10 and −35 bp upstream of the transcription start site are critical for RNA polymerase binding (13, 38, 43, 47). Mutations identified in this study that affected promoter activity under inducing conditions (the level of expression on galactose), without affecting regulation (still requiring galactose for expression) indicate that bases in the −10 and −35 region of the *galP1* promoter region in positions where RNA polymerase would be expected to bind are required for promoter function. We suggest that these sequences constitute at least part of the promoter element recognized by RNA polymerase.

The putative −35 RNA polymerase recognition element of *galP1* exhibits two unusual features. First, it consists of six G residues. Bacterial promoters are characteristically AT rich, and polypurine tracks are not usually observed in −10 or −35 recognition elements. Second, the *galP1* −35 element is apparently suboptimal at every position. The −10 and −35 regions of bacterial promoters normally conform in most positions to the consensus for their particular class, and changes away from consensus generally weaken the activity of the promoter, while changes toward consensus strengthen it. The fact that substitutions at every position in the *galP1* −35 region can strengthen the promoter raises the possibility that it may not function in the conventional sense as a polymerase recognition element, making specific major-groove contacts with the sigma protein. It may instead be a structural element of the promoter. Another explanation is that the G hexamer is involved in interaction with an activator or repressor. This interpretation is less likely, since in no case did mutations in this hexamer affect regulation of *galP1*. Taken together, these data support the conclusion that the G hexamer is involved in some capacity in facilitating RNA polymerase recognition. A T-to-C change at position −11 resulted in a severe reduction in expression from *galP1*, indicating that at least one base in the −10 region is required for promoter function.

It is formally possible that the mutations at −11, −34, and −36 result in increased binding of a repressor and that this increased binding renders the promoter uninducible. In fact, another interesting feature of the −35 region of *galP1* is its similarity to the sequence GCGGGGC, which is thought to be a binding site for CreA, a glucose-responsive repressor protein in the *Aspergillus nidulans alcA* gene (17). While we have no evidence that a CreA-like protein exists in *Streptomyces*, the similarity is perhaps noteworthy. While we have no data to eliminate the possibility that this region of *galP1* is a repressor binding site, for the reasons stated previously, we feel that this interpretation is unlikely.

In support of the interpretation that the −10 and −35 regions of this promoter are, in fact, RNA polymerase binding sites is the observation that in all cases, expression from *galP1* in these mutants was dependent on the presence of inducer. If the increased expression from base changes at −10 and −35 were the result of inactivating a repressor binding site, expression in the absence of inducer would be expected. Mutations that affected regulation of *galP1* were isolated in previous work (29) and were shown to be located in a series of hexamers and direct repeat sequences that overlap the putative RNA polymerase binding site.

While *galP1* may represent a new class of promoter in *Streptomyces*, we point out that there are sequences upstream of the transcription start site of *galP1* similar to a class of promoter that is prototypical in eubacteria. The sequence GTGACA occurs centered around −40, and the sequence TATGTT occurs centered around −10. In fact, a G-to-T change in the hexamer at −40 that makes it TTGACA increases expression from *galP1*. There are several observations, however, that argue against the involvement of these sequences in RNA polymerase recognition. The spacing between these hexamers is 24 bp, a distance that would not normally allow interaction with RNA polymerase (27, 33, 40). Base changes centered around −40 were shown to affect the regulation and not the level of expression of *galP1* (29). Interaction between these sequences and RNA polymerase might require facilitated interaction. Transcription of *galP1* with partially purified RNA polymerase in vitro is relatively strong, and no factors have yet been identified that are required in addition to RNA polymerase for transcription in vitro. We emphasize, however, that the existence of such factors, and the importance of these sequences and their potential interaction with RNA polymerase, is not excluded by our analysis.

If the −10 and −35 sequences identified in this mutational analysis are involved in RNA polymerase recognition, they represent a new class of promoter and would require a new form of RNA polymerase holoenzyme for transcription initiation. In fact, the notion that *galP1* contains a novel RNA polymerase binding site is supported by the observation that when *galP1*-containing DNA fragments were used in in vitro transcription assays, an apparently new RNA polymerase activity was identified. Partial purification of this activity revealed the presence of a 45,000-Da protein whose concentration closely correlated with *galP1* transcribing activity. Reconstitution of proteins in this size range with core RNA polymerase resulted in holoenzyme activity that directed transcription initiation from the *galP1* promoter. Furthermore, fractionated RNA polymerase containing this activity was shown to protect the −10 and −35 regions of the *galP1* promoter from DNase I nuclease digestion.

The gel slice used to reconstitute *galP1* activity clearly contained two proteins, and a sigma protein of approximately 45,000 Da is known to be the product of the *hrdD* gene (4, 42). In fact, it is likely that one of the proteins contained in the gel slice used to reconstitute *galP1* transcribing activity was, in fact, σ^{hrdD}. In support of this is the fact that transcription from the *veg* promoter, which has the sequences TTGACA at −35 and TACAAT at −10 with 17-bp spacing, was detected by reconstituted enzyme from proteins contained in this gel slice. We argue that the reconstituted activity we detect for *galP1* is not *hrdD*. This conclusion is based on two lines of evidence. First, cells containing an insertion into the *hrdD* gene are able to grow on galactose as the sole carbon source, indicating that the *gal* operon is expressed in absence of σ^{hrdD}. Second, RNA polymerase isolated from a *hrdD* mutant transcribed the *galP1* promoter efficiently in vitro. In addition, σ^{hrdD} is known to be one of a family of proteins in *Streptomyces* that are very similar to σ^{70} of *E. coli*. The *hrd* genes were identified by homology to regions of σ^{70} that have been shown by allele-specific suppression analysis to contact the sequences TTGACA and TAT AAT with a spacing of 17 bp. It is extremely unlikely that σ^{hrdD} would recognize these sequences (which are present in *galP1*) at a spacing of 24 bp (as they are in *galP1*) or that σ^{hrdD} would recognize six G residues as a −35 sequence. We suggest that the proteins from the gel slice containing two proteins of approximately 45,000 Da reconstituted two distinct holoenzymes, $E\sigma^{hrdD}$ and a new holoenzyme that transcribes *galP1* specifically.

```
          -40        -30        -20        -10        +1
           .          .          .          .         .
galP1   ACAGGGGGGTGGTGGGTTGTGATGTGTTATGTTTGATTGTG
gylR    ATTGGCGGGAGGTCGGCATGGACCGGTAGTGTTCGGCATTG
```

FIG. 9. DNA sequences of the regions upstream of the transcription start sites (underlined) of the *galP1* and *gylR* promoters. Identical bases are indicated by bold letters. The G hexamer of the −35 region of the *galP1* promoter is underlined.

While the presence of this 45,000-Da protein correlates closely with *galP1* transcribing activity in vitro, and fractions containing this activity are not able to initiate transcription from mutant *galP1* promoters in vitro, and we can demonstrate that protein within the active fraction was able to bind the promoter region of *galP1*, we emphasize that we have no direct evidence at this time that the 45,000-Da protein is involved in *galP1* transcription in vivo. The most convincing evidence would be to show that a mutation in this 45,000-Da protein eliminated *galP1* expression in vivo. While efforts to clone the gene that encodes this protein by "reverse genetics" and to generate a mutation in this gene in order to test its effect on *galP1* transcription in vivo are in progress, there are several technical problems that make this experiment difficult. Attempts to determine the amino acid sequence of the 45,000-Da protein have been unsuccessful for two reasons. First, this protein exists in very low concentrations and has never been detected with Coomassie blue staining by conventional methods of RNA polymerase purification. Only the more sensitive silver staining of gels has allowed visualization of the 45,000-Da protein, and isolating enough protein to obtain a sequence has been difficult. It is possible that the use of an $rpoC^{HIS}$ strain will allow more efficient purification of RNA polymerase and will result in increased yields of the *galP1* sigma. This possibility is being tested. Second, in part because of the low concentration of this protein with respect to other holoenzyme forms and because of its migration in polyacrylamide gels, it has not been possible to cleanly separate it from contamination with other proteins of similar size or from the α subunit of RNA polymerase, which is abundant in these fractions.

While the sequences in the −10 and −35 regions of *galP1* are unusual, *galP1* does show striking similarity to the promoter for the *gylR* gene (Fig. 9), and this similarity may support the notion that *galP1* is an example of a new class of promoter. Twenty-two of the 40 bp upstream of the transcription start sites for *gylR* and *galP1* are identical, and especially conserved are bases centered around −10 and −35. The *gylR* promoter directs transcription of a repressor protein involved in regulation of the glycerol utilization operon of *S. coelicolor* (39). The transcription start site of this promoter was determined by high-resolution S1 nuclease mapping and in vitro runoff transcription assays (39). Like that of *galP1*, transcription of *gylR* is

carbon source dependent and is somewhat repressed by glucose. Interestingly, *gylR* also has G residues centered around −35, except that instead of six G residues, there is a C in the third position. This base change in *galP1* results in a dramatic increase in expression. So far there are no reported mutations within *gylR* that affect activity, and nothing is known about which form of RNA polymerase transcribes this promoter. While *galP1* and *gylR* are very similar, we note that the putative RNA polymerase binding sites for these promoters are different from those inferred in other catabolite-controlled promoters (5, 39, 44). It is not likely, therefore, that there is a specialized sigma factor involved in the regulation of catabolite control in *Streptomyces.*

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