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Identification of a Complex Operator for *galP1*, the Glucose-Sensitive, Galactose-Dependent Promoter of the *Streptomyces* Galactose Operon

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The *galP1* promoter is responsible for galactose-dependent, glucose-sensitive transcription of the galactose utilization operon of *Streptomyces coelicolor* and *Streptomyces lividans*. We describe the characterization of mutations that were positioned directly upstream of the apparent transcription start site of *galP1* and that resulted in deregulated expression. Certain combinations of base changes within a series of hexamers that lie within two pairs of direct repeat sequences resulted in significant expression from *galP1* in the absence of inducer. These motifs are further implicated in regulation by the observation that DNA fragments containing the hexamers and direct repeat sequences resulted in increased transcription from the chromosomal copy of *galP1* on multicopy plasmids in the absence of galactose. We suggest that these hexamers and direct repeat sequences constitute an operator for the negative regulation of the *Streptomyces gal* operon.

The regulation of carbon utilization is of central importance in gene expression pathways for both morphological development and antibiotic biosynthesis in *Streptomyces* spp. (12). While the analysis of gene regulation in these bacteria is in a very early stage, it is clear from studies to date that the mechanism of catabolite control in *Streptomyces* spp. is different from that in other bacteria. Perhaps the most striking difference between *Streptomyces* spp. and *Escherichia coli* is the observation by Hodgson (21) that, in *Streptomyces coelicolor*, catabolite control does not depend on cyclic AMP (cAMP) levels. In addition, there is evidence that *Streptomyces aureofaciens* may not have a phosphotransferase system for sugar transport (34).

Several catabolite-controlled genes have been cloned from *Streptomyces* spp.; they include those for glycerol (37, 40), agarase (10), amylase (44), galactose (1, 17), and chitin (14, 33, 35). For the glycerol utilization operon of *S. coelicolor* (39), a repressor and an operator similar to their counterparts in *E. coli* have been inferred, but surprisingly, glucose repression of the glycerol utilization operon requires the activity of the glucose kinase gene (23). Transcription of the agarase gene is directed from four promoters that are recognized by four distinct holoenzyme forms (11). Direct repeat sequences have been implicated in the regulation of chitinase genes. Single base changes in the direct repeat sequences of one of the chitinase promoters resulted in glucose-resistant, chitin-independent expression (14, 33a). While the study of catabolite control is an active area of investigation for *Streptomyces* spp., very little is known about the way in which *cis*-acting sequences serve to regulate the expression of given genes or how ancillary proteins are involved in the activation or repression of transcription.

The galactose utilization operon of *S. coelicolor* and *Streptomyces lividans* encodes three structural genes, *galT*, *galE*, and *galK*, that are very similar to their counterparts in *E. coli* at the amino acid level. In fact, the *Streptomyces gal* operon was cloned by complementation of an *E. coli galETK* mutant (1). In *E. coli*, transcription of the *gal* operon is directed by two promoters (4, 32, 38), both subject to repression by the *gal* repressor (3, 9). The two promoters are modulated by the cAMP-cAMP receptor protein complex in opposite ways: transcription from *galP1* is stimulated, and transcription from *galP2* is inhibited (4, 32). Transcription of the *Streptomyces gal* operon is also directed by two promoters but, in contrast to the situation in *E. coli*, these promoters are apparently regulated independently and neither depends on cAMP. Transcription from *galP1* is glucose sensitive and galactose inducible, and transcription from *galP2* is constitutive (17). In addition, the structures of the *E. coli* and *Streptomyces* operons are quite different. In *S. coelicolor* and *S. lividans* spp., the two promoters are separated by the *galT* structural gene and are recognized by two distinct forms of RNA polymerase (45). A novel form of RNA polymerase that directs transcription from *galP1* in vitro has been purified, and a putative RNA polymerase binding site has been identified (7a). The *galE* and *galK* genes are transcribed constitutively, and the *galT* gene is transcribed only under inducing conditions.

Here we report an analysis of the *galP1* promoter. Our efforts to obtain simple, *cis*-acting point mutations within *galP1* by random mutagenesis, which resulted in deregulated expression, were unsuccessful. An inspection of the DNA sequence upstream of the apparent transcription start site of *galP1* revealed the presence of two overlapping potential regulatory motifs: a series of six hexamers that conform to the sequence TNTNAN and two pairs of direct repeat sequences. The hexamers and direct repeat sequences overlap. Such TNTNAN elements have been suggested by Ebright (16) to be the DNA sequences that interact with the helix-turn-helix motif of DNA binding proteins. We introduced multiple base changes within four of the TNTNAN hexamers by using oligonucleotide-directed mutagenesis and analyzed the effects of these changes by primer extension of in vivo RNA and transcriptional fusions to the *xylE* reporter

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gene. We showed by titration experiments that the *galP1* promoter region contains a binding site for a negative regulator and that mutations within the hexamers and direct repeat sequences reduce the ability of this region to titrate the regulator.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmid isolation, and transformation. The *Streptomyces* strains used were *S. lividans* 1326 (28) and *S. coelicolor* A3(2) (22). *Streptomyces* strains were grown in YEME liquid medium containing 34% sucrose (22) or on R2YE (22) for the regeneration of protoplasts and sporulation with 10 μg of thiostrepton (Sigma) per ml for plasmid selection when appropriate. Minimal media used to assay catechol dioxygenase activity have been described elsewhere (24). The *E. coli* strains used were CJ236 (27) for the propagation of uracil-incorporated M13 phage, JM101 (31) for the propagation of M13 replicative-form DNA, and TB1 (Focus **6**:7, 1984) for the propagation of plasmids. *E. coli* strains were grown in LB (5) liquid medium or on LB agar containing 40 μg of ampicillin per ml for plasmid selection. Plasmid DNA was prepared from *E. coli* by alkaline lysis (6) and introduced into $CaCl_2$-treated *E. coli* cells (13). *Streptomyces* protoplasts were transformed as described previously (42).

Construction of promoter mutations. Oligonucleotide-directed mutagenesis was performed with the Mutagene kit from Bio-Rad (catalog no. 170-3571) in accordance with the manufacturer's instructions. M13mp18 containing a 196-bp *Hin*dIII-*Bam*HI fragment, which includes the *galP1* promoter from −69 to +103 with respect to the apparent transcription start site, was used as a template. Fragments containing mutations in a single hexamer were constructed by annealing a primer containing the desired base changes to wild-type *galP1*-containing template DNA. Fragments containing mutations in more than one hexamer were constructed by annealing a primer containing base changes in one hexamer to template DNA containing base changes in the other hexamer. The DNA sequence for each construction was confirmed by subjecting the various promoter-containing fragments to the dideoxy sequencing reactions of Sanger et al. (36) by use of the Sequenase kit and the forward (−40) sequencing primer (primer no. 70736) (both from United States Biochemical Corp., Cleveland, Ohio).

Assay of promoter function with *xylE* fusions. For assay of the effect of various base changes within *galP1*, the 196-bp *Bam*HI-*Hin*dIII fragments containing the promoter mutations were ligated to the larger *Bam*HI-*Hin*dIII fragment of pXE4 (24), generating transcriptional fusions between *galP1* and a promoterless copy of the *xylE* gene contained in pXE4. In all cases, plasmid DNA was isolated after transformation and analyzed to verify the presence of the insert. Catechol dioxygenase activity was detected in cells grown on plates or in liquid medium as described previously (24), except that assays were performed at 30°C. Protein concentrations were determined as described previously (7).

RNA isolation. Cultures were grown as for catechol dioxygenase assays in liquid SLAB medium (17) containing either 1% glycerol, 1% galactose, or 1% glucose. After 2 h of induction, cells were quickly cooled by the addition of an equal volume of ice-cold M56 buffer ([30 mM Na_2HPO_4, 20 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 0.02 mM $Ca(NO_3)_2$, 0.25 mg of $FeSO_4$ per liter]) containing 1 mM NaN_3, collected by centrifugation, and washed once in M56 buffer. RNA was isolated as previously described (22).

Primer extension analysis. The primer used, 5′ CGTAAGG CACGCGATGGATCCCGA 3′, was complementary to the sequence directly downstream from and overlapping the *Bam*HI site of pXE3 (24). Plasmid pXE3 contains the *galP1* promoter cloned upstream of the *xylE* gene, and the *Bam*HI site is located 98 bp downstream of the apparent transcription start site of *galP1* (24). Fifty micrograms of RNA was hybridized with a 5′-^{32}P-end-labelled primer (5×10^4 cpm) in the presence of 0.05 M Tris-HCl (pH 8.3)–0.1 M KCl in a volume of 20 μl by incubation at 90°C for 1 min and then 60°C for 2 min; this step was followed by 15 min of incubation on ice. Six microliters of 5× reverse transcriptase buffer (50 mM Tris [pH 8.3], 6 mM $MgCl_2$, 40 mM KCl, 0.5 mM deoxynucleoside triphosphate), 8 U of RNasin (Promega), and 8 U of reverse transcriptase (Life Sciences, Inc.) were added, and the mixture was incubated for 1 h at 42°C. The reaction was stopped by the addition of 1 μl of EDTA (0.5 M; pH 8). RNase (Sigma) was added to a final concentration of 40 μg/ml, and the mixture was incubated for 15 min at 37°C. Radioactive DNA was concentrated from the reaction mixture by ethanol precipitation, suspended in TE (Tris-EDTA) (pH 8)–1 volume of loading buffer (80% [vol/vol] formamide, 1 mM EDTA [pH 8.0], 0.1% bromophenol blue, 0.1% xylene cyanol), boiled for 5 min, and separated by electrophoresis in an 8% sequencing gel next to the Sanger sequencing reactions (36) 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 sequence from −69 to +103 with respect to the apparent transcription start site and part of the *xylE* gene (48) cloned into the *Hin*dIII-*Kpn*I sites of M13mp19.

Construction of high-copy-number plasmids containing wild-type and mutant *galP1* promoter fragments. For construction of pMB210 and pMB220, plasmid pIJ351 (26) was linearized with *Pst*I and ligated to *Pst*I-linearized pUC19 derivatives containing either wild-type or mutant *galP1* promoter fragments. The pUC19 derivatives had been constructed by insertion of *galP1*-containing DNA fragment into the *Pst*I-*Bam*HI sites in the pUC19 polylinker (47). pMB210 and pMB220 contained origins of replication and drug resistance genes for replication and selection in either *Streptomyces* spp. or *E. coli*. pMB210 contained the wild-type *galP1* promoter fragment from −69 to +103 with respect to the apparent transcription start site. pMB220 contained the same fragment with transversion mutations in hexamers II and IV (see Fig. 3).

Estimation of the copy number of *galP1* promoter sequences. Total DNA was isolated from *S. lividans* as previously described (22), digested simultaneously with *Sac*II, *Bam*HI, and *Bgl*II, and subjected to electrophoresis through a 1% agarose gel in TBE buffer (pH 7) (29). The DNA was transferred to a nitrocellulose filter and incubated with a labelled probe as previously described (41). The ^{32}P-labelled probe was present in a 10-fold molar excess in relation to genomic DNA. The nitrocellulose filter was prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's reagent (15)–100 μg of denatured salmon sperm DNA per ml for 4 h at 65°C. The filter was hybridized in 5× SSC–1× Denhardt's reagent–100 μg of denatured salmon sperm DNA per ml–^{32}P-labelled probe for 12 h at 65°C and washed three times in 2× SSC–0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature and three times in 0.1% SSC–0.1% SDS for 15 min at 55°C. For detection of *galP1* promoter sequences, a 212-bp fragment containing the sequence from −117 to +96 with respect to the apparent transcription start site of *galP1* was used as a

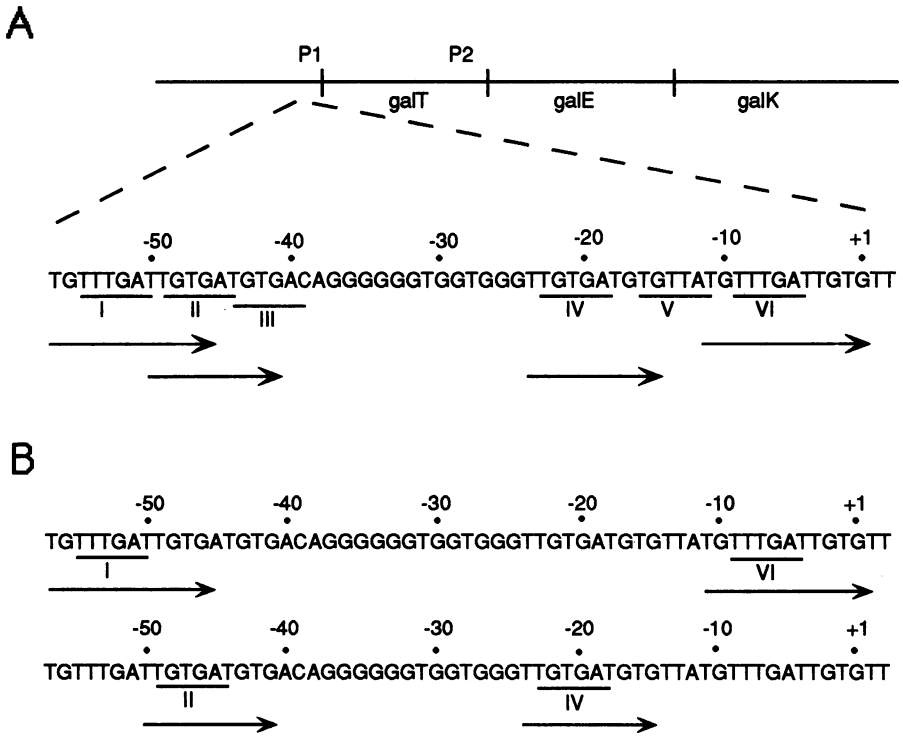

FIG. 1. (A) Structure of the *Streptomyces* galactose utilization operon and DNA sequence of the *galP1* promoter. The apparent transcription start site is indicated as +1; bases 5′ to the start site are labelled as negative numbers. The TNTNAN hexamers are underlined and numbered with roman numerals, and the direct repeat sequences are indicated by arrows. (B) DNA sequence of *galP1* showing the positions of hexamers I and IV relative to the perfect 12-bp direct repeats (top) and the positions of hexamers II and IV relative to the perfect 10-bp direct repeats (bottom).

probe. For detection of plasmid replicon sequences, a 2.5-kb *Sac*II fragment from plasmid pIJ351 was used as a probe. After autoradiography, bands were cut out and the radioactivity was measured in a liquid scintillation counter. Hybridization to the *galP1* chromosomal fragment served as an internal control to correct for the concentration of DNA present in each lane.

Galactokinase assays. *Streptomyces* cultures were grown in 50 ml of SLAB medium containing 1% glycerol, 10% polyethylene glycol 8000, and 2.5 μg of thiostrepton per ml for 24 h with shaking at 400 rpm in baffled flasks at 28°C. Cells were harvested by centrifugation, washed with SLAB medium, and resuspended in 25 ml of SLAB medium. A 5-ml aliquot was filtered through a prebaked 0.45-μm-pore-size cellulose acetate filter for the determination of dry cell weight. A portion of cell suspension equivalent to 25 mg (dry cell weight) was added to 50 ml of SLAB medium containing 10% polyethylene glycol 8000, 2.5 μg of thiostrepton per ml, and either 1% glycerol, 1% glucose, or 1% galactose. Cultures were incubated at 28°C with shaking for 6 h, harvested by centrifugation, washed with sonication buffer (2), and resuspended in 2 to 4 ml of sonication buffer. Cells were lysed by sonication, and galactokinase activities were determined as described by Wilson and Hogness (46) and as modified by McKenny et al. (30). Galactokinase activities were calculated as nanomoles of [^{14}C]galactose phosphate formed per milligram of protein per minute. Protein concentrations were determined by the method of Bradford (7) with bovine serum albumin as the protein standard.

RESULTS

Construction of base substitution mutations within the *galP1* promoter region. An inspection of the DNA sequence upstream of the apparent transcription start site of *galP1* revealed the presence of two overlapping potential regulatory motifs: a series of six hexamers that conform to the sequence TNTNAN (underlined in Fig. 1A) and two pairs of direct repeat sequences (also shown in Fig. 1A). A closer inspection of the TNTNAN hexamers indicated that hexamers I and VI (Fig. 1B) have the sequence TTTGAT and lie within a pair of perfect 12-bp direct repeat sequences and that hexamers II and IV have the sequence TGTGAT and lie within a pair of perfect 10-bp direct repeat sequences. To test whether these TNTNAN hexamers play a role in *galP1* regulation, we introduced base changes by oligonucleotide-directed mutagenesis. Since single base changes in the "N" positions of these hexamers had essentially no effect on expression from *galP1* (data not shown), either transition or transversion mutations were introduced in all three N positions at once. While the mutations were constructed to change specific bases in the hexamer sequences, it should be noted that base changes within these hexamer sequences also affected the direct repeat sequences overlapping them, and it was not possible in this analysis to separate the two potential regulatory elements. In all cases, the DNA sequences of the mutated *galP1*-containing DNA fragments were determined by the method of Sanger et al. (36).

Analysis of base substitutions by use of *xylE* transcriptional fusions. *xylE* transcriptional fusions were constructed to test

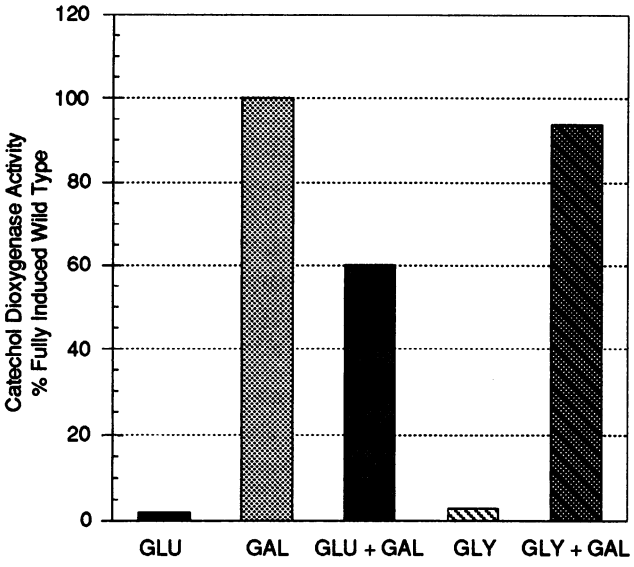

FIG. 2. Histogram of catechol dioxygenase activity assays plotted as a percentage of the fully induced wild-type level. Assays were performed with cells grown on glucose (GLU), galactose (GAL), glycerol (GLY), or combinations as indicated.

and quantitate expression from wild-type and mutant *galP1* promoter fragments. Catechol dioxygenase assays were performed with cells after growth on glucose, galactose, glucose plus galactose, glycerol, or glycerol plus galactose as the sole carbon source(s). The results of these assays for wild-type *galP1* directing *xylE* expression are shown as a histogram in Fig. 2. The level of expression observed on galactose is taken to be the fully induced level, and the results of all other assays are reported as percentages of this level. Essentially no activity was detected in cells grown on glucose or glycerol alone. Approximately 60% of the fully induced expression level was observed after growth on glucose plus galactose, and approximately 95% of the fully induced expression level was observed after growth on glycerol plus galactose. These measurements are in good agreement with those previously reported for galactokinase assays and analysis of *galP1* RNA (17). Taken together, these data indicate that galactose is an inducing carbon source, glucose is a repressing carbon source, albeit a weak one, and glycerol is a neutral carbon source for *galP1* regulation.

Transition mutations in hexamer II, from TGTGAT to TATAAC, resulted in an increase in the induced level of expression from *galP1*, without a significant change in the level of expression on glucose or glycerol (Fig. 3). Transversion mutations in hexamer II, from TGTGAT to TCTCAA, resulted in a dramatic increase in the induced level of expression, more than 1 order of magnitude over the induced wild-type level, without a significant change in the level of expression on glucose or glycerol. Transition mutations in hexamer IV resulted in a modest increase in the induced level of expression, while transversion mutations resulted in an increase in the overall level of expression. The levels of expression on glucose and glycerol were equal to the induced wild-type level, and these mutant promoters retained the ability to be induced by galactose to a level of expression more than four times that of the wild type.

Transversion mutations in hexamers II and IV together resulted in an increase in the level of expression from *galP1* on glucose to a level higher than that of the fully induced wild type. Induction by galactose was equal to seven times that of the wild type, and the level of expression on glycerol was almost four times that of the fully induced wild type. These results are shown as a histogram in Fig. 3, plotted as a percentage of the fully induced wild-type level. When no promoter was present upstream of the *xylE* gene in these constructions, no *galP1*-specific RNA or catechol dioxyge-

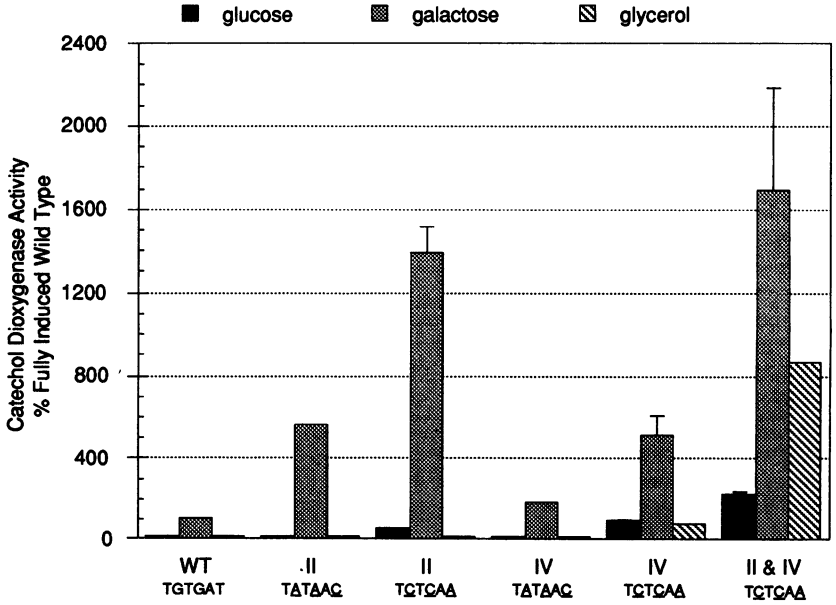

FIG. 3. Histogram of catechol dioxygenase activity assays plotted as a percentage of the fully-induced wild-type (WT) level. Assays were performed with cells grown on glucose, galactose, or glycerol as indicated. Base changes (underlined) indicated in hexamers II, IV, and II plus IV refer to substitutions within the *galP1* promoter sequences.

nase activity was detected (data not shown). In general, we conclude that some changes in hexamer II or IV or hexamers II and IV together result in a dramatic change in the level of expression from *galP1*. Notably, mutations in hexamers II and IV together result in a significant increase in the level of expression in the absence of an inducer.

Transition mutations in hexamer VI had no effect on transcription from *galP1* (data not shown), while transversion mutations resulted in a level of expression from *galP1* on glucose, galactose, or glycerol equal to the fully induced wild-type level, as determined by catechol dioxygenase assays (data not shown). Transcription in these mutants, however, did not originate from the same start site as that observed for wild-type *galP1* (see below). Nonconservative changes in hexamer I also resulted in constitutive expression from *galP1*, as determined by catechol dioxygenase assays, but transcription in these mutants was complex, as determined by primer extension analysis (see below).

Assay of *galP1* mutations by primer extension analysis of in vivo RNA. For each case of deregulated expression from mutated *galP1* promoters, primer extension analysis was performed to determine whether transcription from the mutated promoters initiated at the same base as from the wild-type promoter. Analysis of mutations in hexamer VI revealed that transcription from the start site observed for wild-type *galP1* was severely reduced. In these mutants, an additional transcript originating from within the *galP1* promoter-containing fragment but upstream of the wild-type start site was detected. Base substitution mutations in hexamer I resulted in reduced transcription originating from the wild-type start site and an additional transcript originating upstream of the *galP1* promoter-containing fragment (data not shown). The absence of wild-type transcription initiation and the presence of additional transcripts in these mutants compromised their analysis with respect to *galP1* regulation. These mutants are, however, potentially important for analysis of the DNA sequences recognized by RNA polymerase (see Discussion).

Analysis of mutations in hexamer II or IV or hexamers II and IV together indicated a single transcription start site indistinguishable from that of the wild type. As shown in Fig. 4, for wild-type *galP1*, very little *galP1*-specific RNA was detected in cells grown on glucose or glycerol, while RNA in galactose-grown cells was readily detected. For the mutant promoter with transversion mutations in hexamers II and IV, a significant amount of *galP1*-specific RNA was detected in cells grown on glucose or glycerol. While the relative amount of RNA in cells grown on glucose or glycerol was increased in the mutant strain, the absolute amount of RNA detected under these conditions did not correlate well with the catechol dioxygenase activities shown in Fig. 3. The amount of *galP1*-specific RNA detected in mutant cells grown on glucose or glycerol was less than that detected in wild-type cells grown on galactose. Equal amounts of RNA were examined in each primer extension experiment, but the conditions used may not have allowed a direct comparison of the mutant and wild-type strains. Taken together, the results of the *xylE* fusion and primer extension analyses of mutations within hexamers II and IV indicated that these DNA sequences might be a binding site for a negative regulator of transcription.

Repressor titration by *galP1* in *trans*. To investigate the possibility that the regulation of *galP1* transcription involves negative control, we constructed plasmids to increase the number of copies of the *galP1* promoter region in the cell. If this DNA region contained a binding site for a negative

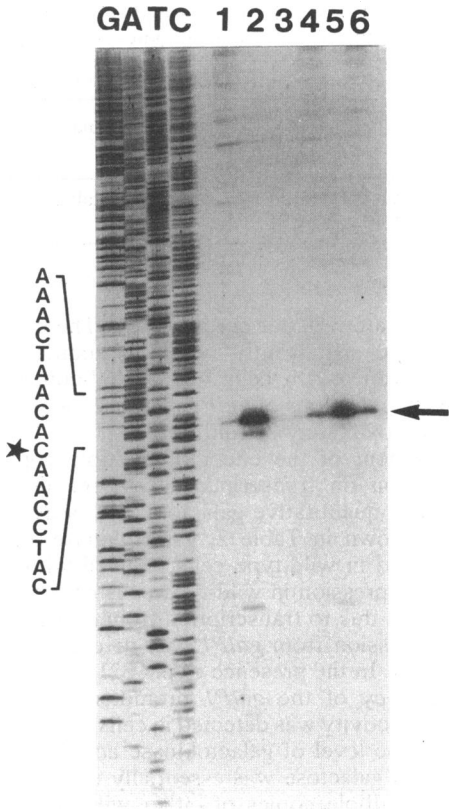

FIG. 4. Primer extension analysis of RNA isolated from cells containing a wild-type copy of *galP1* after growth on glucose (lane 1), galactose (lane 2), or glycerol (lane 3) or *galP1* with transversion mutations in hexamers II and IV as indicated in Fig. 3 after growth on glucose (lane 4), galactose (lane 5), or glycerol (lane 6). The arrow indicates a primer extension product that corresponds to initiation from *galP1*. The sequence on the left is the DNA region around the apparent transcription start site for *galP1*, shown by a star.

regulator, an increase in the number of binding sites might titrate this regulator and allow expression from the intact, chromosomal operon in the absence of an inducer. A DNA fragment containing the sequence of *galP1* from −69 to +103 with respect to the apparent transcription start site was cloned into pIJ351 (a derivative of the pIJ101 replicon). The reported copy number of pIJ101 is 40 to 300 per chromosome (26). For determination of the total copy number of the *galP1* promoter sequences and the approximate plasmid copy number in cells containing these plasmid constructions, total DNA was isolated and digested simultaneously with *Sac*II, *Bam*HI, and *Bgl*II. This digestion resulted in the generation of a 1.5-kb *Bgl*II fragment containing the chromosomal copy of *galP1*, a 0.25-kb *Bam*HI fragment containing the plasmid copy of *galP1*, and a 3.3-kb *Bam*HI-*Sac*I fragment containing the part of the pIJ351 replicon that did not include *galP1* sequences. These digests were probed in Southern hybridization experiments first with a fragment that contained the DNA sequence of *galP1* from −117 to +96 with respect to the apparent transcription start site for *galP1* and then with a 2.5-kb *Sac*II fragment that was part of the pIJ351 replicon. Bands identified by hybridization were removed, and the radioactivity was measured in a liquid scintillation counter. The results of these experiments are shown in Table 1. In these experiments, the copy number of pIJ351 was estimated

TABLE 1. Determination of plasmid copy number and concentrations of *galP1* promoter sequences

S. lividans strain	Plasmid	cpm in the presence of the:		
		galP1 chromosomal fragment	*galP1* plasmid fragment	pIJ351 replicon fragment
JW287	pIJ351	120	None detectable	30,400
JW288	pMB210	154	2,908	35,406

to be approximately 20 per chromosome. The copy number did not change significantly with the addition of *galP1* promoter sequences. In cells with *galP1*-containing plasmids, the concentrations of *galP1* promoter sequences were increased approximately 20-fold.

For assessment of the effect of multiple copies of the promoter region on transcription from the chromosomal copy of *galP1*, quantitative galactokinase assays were performed. As shown in Table 2, galactokinase activity was readily detected in wild-type cells induced with galactose. The level of expression in wild-type cells grown on glucose or glycerol was due to transcription from *galP2*, not *galP1*, since no expression from *galP1* was detected under these conditions (17). In the presence of pMB210, which contains a wild-type copy of the *galP1* promoter, an increase in galactokinase activity was detected in cells grown on glucose or glycerol. The level of galactokinase activity detected in cells grown on galactose was essentially unchanged by the presence of multiple copies of either wild-type or mutant *galP1* in these experiments. As shown in Table 2, the quantities (nanomoles) of [^{14}C]galactose phosphate were similar in strains containing various *galP1*-containing plasmids. The values shown represent *galP1*-specific galactokinase activities. These results indicate that the presence of multiple copies of the *galP1* sequence (contained on pMB210) resulted in transcription from *galP1* (the chromosomal copy) in the absence of an inducer. We conclude from these experiments that the DNA sequence of *galP1* between −69 and +103 with respect to the apparent transcription start site contains a binding site for a negative regulator (repressor) of transcription and that the presence of multiple copies of *galP1* partially titrates this repressor, allowing some expression in the absence of an inducer.

Predictions of the model in which the TNTNAN hexamers act as part of a repressor binding site are that mutations within these hexamers that affect regulation in vivo might cripple the ability of this site to bind the repressor and that a mutated promoter fragment, present in multiple copies, might not titrate the repressor as well. To test these predictions, we used a promoter fragment with transversion mutations in hexamers II and IV in titration experiments. In the presence of pMB220, which contains a mutant *galP1* promoter, the chromosomal copy of *galP1* was induced to some extent but not as much as with the wild-type *galP1* promoter (Table 2). The differences observed between induction by the wild-type and mutant promoters were reproducible. The data shown in Table 2 represent the results of six experiments done in duplicate.

DISCUSSION

We have used oligonucleotide-directed mutagenesis to introduce specific multiple base changes within four of six hexamers that overlap two pairs of direct repeat sequences within the *galP1* promoter region. Base substitution mutations in hexamers II and IV (Fig. 1), that overlap a pair of perfect 10-bp direct repeat sequences, resulted in a dramatic increase in the level of expression from *galP1*. Mutations in hexamers II and IV together resulted in a significant increase in the level of expression from this galactose-dependent promoter in the absence of an inducer. These results suggest that these hexamers and/or direct repeat sequences act as part of an operator element for the regulation of *galP1*. This conclusion is supported by the observation that the presence of multiple copies of the wild-type *galP1* promoter "induces" expression of the chromosomal operon and that mutant promoters containing base changes within hexamers II and IV show a reduced ability to induce expression of the chromosomal operon.

Transversion mutations in hexamer VI resulted in a severe reduction in initiation from the apparent wild-type transcription start site of *galP1*. This result is not surprising, since this hexamer overlaps the putative −10 RNA polymerase binding site. In fact, mutations centered around −10 and −35 bp upstream of the apparent wild-type transcription start site resulted in a severe reduction in the level of expression from *galP1* (29a), indicating that these bases may be part of the element involved in RNA polymerase recognition. Transversion mutations in hexamer I resulted in a reduced level of expression from the wild-type transcription start site and the appearance of a new transcript apparently originating upstream of the promoter-containing fragment from vector DNA. While the results obtained with mutations in hexamers I and VI compromised interpretation of these mutations with respect to *galP1* regulation, they may be informative for identifying the sequence elements involved in RNA polymerase recognition.

We suggest from our analysis of hexamers II and IV that the hexamers and/or direct repeat motifs within the promoter region of *galP1* constitute an operator for negative regulation

TABLE 2. Induction of the chromosomal wild-type *gal* operon by multiple copies of the *gal* operator[a]

S. lividans strain	Plasmid	Sp act of galactokinase			
		% of fully induced level in cells grown in:			nmol of galactose phosphate in cells grown in galactose
		Glycerol	Galactose	Glucose	
1326		15 ± 5	100	20 ± 3	185 ± 20
JW287	pIJ351	18 ±5	100	21 ± 1	160 ± 18
JW288	pMB210	38 ± 10	100	51 ± 2	164 ± 5
JW289	pMB220	31 ± 5	100	38 ± 5	194 ± 10

[a] Cells were assayed after growth on glucose, galactose, or glycerol as indicated. *S. lividans* 1326 contains no plasmid. pIJ351 is a high-copy-number plasmid with no *galP1* sequences. pMB210 is a composite of pIJ351 and pUC19 containing a wild-type copy of *galP1*. pMB220 is a composite of pIJ351 and pUC19 containing *galP1* with transversion mutations in hexamers II and IV as indicated in Fig. 3.

of the galactose utilization operon. While we have no explanation for the fact that base changes in hexamer I, which is outside the putative RNA polymerase binding site, affect transcription initiation, it is clear that further analysis of the promoter region and a biochemical analysis of *trans*-acting factors are required to explain the regulation of transcription from *galP1*.

Our results indicate that the mechanism of *galP1* regulation in *S. coelicolor* and *S. lividans* is different from that of its counterpart in *E. coli*. The *E. coli gal* repressor binds to two sites of dyad symmetry (25) and affects the transcription of both of the *gal* promoters. In *S. coelicolor* and *S. lividans*, the *galP2* promoter is separated from the *galP1* promoter by more than 1 kb of DNA, which includes the *galT* structural gene, and the regulation of these promoters is essentially independent. The evidence presented here indicates that negative regulation of the *galP1* promoter may involve direct repeat sequences. While repressor binding to direct repeat sequences is not unknown in prokaryotes, it is very unusual. In *E. coli*, the best examples of catabolite-controlled genes are *araC* (8, 18) and *malT* (43). The AraC protein binds direct repeat sequences to effect both positive and negative regulation of the arabinose operon, and direct repeat sequences apparently serve to facilitate positive activation of the maltose operon by MalT. In lambda and in phage P22, the CII protein (20) and the C1 protein (19), respectively, bind direct repeat sequences to activate transcription from each of three distinct promoters. The only other example in *Streptomyces* spp. of a catabolite-controlled promoter in which *cis*-acting elements have been identified is the *chi-63* promoter (14). Single base changes in a pair of perfect direct repeats that overlap the putative RNA polymerase binding site of this promoter result in glucose-resistant, chitin-independent transcription. While the DNA sequences are different, it is interesting to note that the position of the direct repeat sequences in *galP1* with respect to the putative RNA polymerase binding site is very similar to that of the direct repeat sequences in the *chi-63* promoter. We emphasize that our analysis does not distinguish between the hexamers and direct repeat motifs. It is formally possible that either the hexamers or the direct repeat sequences per se are irrelevant to regulation. If they are involved in regulation, the structure and organization of the hexamers and direct repeats of the *Streptomyces galP1* promoter may suggest that more than one repressor binds to these motifs or that one repressor binds with different affinities to different sites.

In *E. coli*, cAMP is required for the activation of *galP1*. In *S. coelicolor*, cAMP is apparently not involved in catabolite control, and we have no evidence for positive activation of the transcription of *galP1*, although this possibility is not excluded by our analysis. The observation that a 20-fold increase in the concentration of *galP1* promoter sequences results in only a 2-fold increase in expression from *galP1* leaves open the possibility that a positive activator is required.

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