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NOTES

Resection and Mutagenesis of the Acid pH-Inducible P2 Promoter of the *Agrobacterium tumefaciens virG* Gene

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Transcription of the *virG* gene initiates from two tandem promoters, designated P1 and P2, that are located 50 nucleotides apart. Transcription of the P2 promoter is induced by extracellular acidity. *cis*-acting sites required for P2 activity were identified by constructing and assaying a series of 5′ and 3′ resections and site-directed nucleotide substitutions. Nucleotides between positions −9 and −37 were sufficient for regulated promoter activity. Within this region, nucleotide substitutions at the predicted −10 and −35 regions strongly reduced P2 expression. In addition, alterations in the region between nucleotides −24 and −32 also eliminated or strongly reduced promoter activity. These data suggest that this promoter may be regulated by a positive transcription factor that binds to nucleotide residues in this interval.

Differential gene expression in response to environmental acidity has been well documented and investigated for several *Escherichia coli* operons, including *cadAB*, *adi*, *cycAB*, and *cyoABCDE* (10, 26, 29, 38, 40). This environmental parameter also plays an important role in regulating the expression of the pathogenesis genes of a variety of plant and animal pathogens, including *Salmonella typhimurium*, *Staphylococcus aureus*, *Vibrio cholerae*, *Erwinia amylovora*, and *Agrobacterium tumefaciens* (1, 27, 30, 41, 44). In *A. tumefaciens*, the causative agent of crown gall tumors of dicotyledonous plants, extracellular acidity plays an essential role in at least two stages in pathogenesis. First, acidity is one of three environmental stimuli that activate the expression of the *vir* regulon (36), and it may directly stimulate activity of the transmembrane sensory kinase VirA. Second, acidity acts by a VirA-independent mechanism to stimulate expression of the *virG* gene, whose product is a two-component response regulator that is phosphorylated by VirA (18, 39, 44).

Induction of *virG* by extracellular acidity occurs at a promoter designated P2 and does not require any Ti plasmid-encoded protein (23). The *virG* P1 promoter, in contrast, is activated by phospho-VirG (16, 37, 43, 45) and is also induced by phosphate starvation, probably via a homolog of the *E. coli* PhoB protein (4, 43). Because the induction of *virG* by acidity and by phosphate starvation does not require the VirG protein, these stimuli have been viewed as necessary to establish a pool of VirG sufficiently large to induce the *vir* regulon. This pool of VirG, upon phosphorylation, can then more strongly express *virG* in a positively autoregulated fashion (23).

Induction of the P2 promoter is detectable at pH 6.0 and is stronger at pH 5.25 or 5.0 (39, 43, 44). It is difficult to determine the pH optimum for induction, since pH values lower than 5.0 inhibit cell growth and protein synthesis (23). No other environmental stimulus has been found to induce this promoter as strongly as acidity, although some induction was reported to occur in response to mitomycin C and $CdCl_2$. Induction by these agents does not require RecA, indicating that P2 is not part of an SOS regulon (23).

P2 contains a sequence resembling the −10 sequences of *E. coli* heat shock promoters (43). This led to speculation that P2 might be part of a heat shock regulon. *A. tumefaciens* was subsequently shown to have a heat shock response (22, 33, 34), and a gene encoding a putative heat shock sigma factor similar to σ^{32} was recently cloned (28). However, P2 transcription was not induced by heat shock or by ethanol (23). Perhaps this should not have been surprising. First, the putative heat shock −10 motif of P2 is centered 10 nucleotides upstream of the start site, which is 3 or 4 nucleotides further upstream than its position in heat shock promoters (11). Second, it was recently proposed that members of the α subgroup of proteobacteria have a heat shock consensus motif that differs from P2 at two positions in the −10 region (34). Third, the −35 region of P2 shows no similarity to the −35 regions of heat shock promoters in *E. coli* or of α subgroup bacteria.

Another possibility was that P2 is controlled by some other transcriptional regulator, either an activator or a repressor. Previous efforts in our laboratory to identify *trans*-acting factors that stimulate this promoter have met with little success (25). An alternative approach, used in the present study, is to identify *cis*-acting sites that are required for P2 expression. *cis*-acting sites have been localized in many other promoters (9, 17).

It was previously determined that all nucleotides required for regulated P2 expression must lie between positions −53 and +707 relative to the transcription start site (23, 43). To test whether any sequences downstream of +1 were required, we synthesized the region between nucleotides −53 and +1 by using the partially overlapping oligonucleotides 5′-CCTG GATCCTCATCTCCAGCTAAATGGGCAGTTCGTCAGA ATT-3′ and 5′-GAGGAATTCTGTCATCCGCGGTCAGC CGCAATTCTGACGAAC-3′ and Klenow DNA polymerase according to a published protocol (6), digested this fragment with *Eco*RI and *Bam*HI, and introduced it between the *Eco*RI and *Bam*HI sites of pRS415, immediately upstream of a promoterless *lacZ* gene (35). The resulting plasmid was digested

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TABLE 1. Responsiveness of 5′ and 3′ deletion derivatives of the P2 promoter to acidic medium[a]

Plasmid	5′ end	3′ end	β-Galactosidase sp act[b] at:		Induction ratio
			pH 7.0	pH 5.1	
pNM102	−195	+707	135	2,036	15
pCH539	−53	+1	121	2,073	17
pCH538	−43	+1	127	1,929	15
pCH537	−33	+1	14	41	3
pCH536 (no insert)			17	33	2

[a] Derivatives of strain A136 containing the indicated plasmids were cultured for 16 h in AB medium supplemented with 50 mM MES (pH 7.0 or pH 5.1) and assayed for β-galactosidase activity. pNM102 was described previously (23).
[b] Miller units.

with *Pst*I and *Sal*I, and the fragment containing the P2-*lacZ* fusion was introduced between the *Pst*I and *Sal*I sites of the broad-host-range plasmid pUCD2 (8), creating pCH539. This plasmid was introduced into *A. tumefaciens* A136 and assayed for β-galactosidase activity in neutral (pH 7.0) and acidic (pH 5.1) media. This plasmid expressed acid pH-inducible β-galactosidase at levels similar to those expressed by a positive control (pNM102 [23]) which contains the region between nucleotides −195 and +707 (Table 1). These results indicate that all *cis*-acting sites lie upstream of the transcription start site.

To learn the approximate location of the furthest upstream site, we used the same approach to construct two additional P2-*lacZ* fusions, creating plasmids pCH538 (nucleotides −43 to +1) and pCH537 (nucleotides −33 to +1). pCH538 showed wild-type expression of β-galactosidase, while pCH537 expressed this enzyme at levels comparable to those expressed by a strain containing pCH536, a plasmid similar to pCH537 but lacking any P2 sequences (Table 1). We conclude that no essential site lies upstream of nucleotide −43 and that some essential site lies between nucleotides −43 and −33. This region contains the putative −35 region, which could well explain the requirement for this interval.

To identify critical residues within this 43-nucleotide region, we created a series of plasmids whose structures are similar to that of pCH538 except for particular site-directed alterations. Most of these plasmids contained mutations of three consecutive nucleotides, although plasmids containing one-, two-, or nine-nucleotide alterations were also constructed (Fig. 1). These plasmids were introduced into strain A136 and assayed as described above. A number of conclusions can be made from these data. First, nucleotides −43 to −39 are dispensable for expression, and similarly, nucleotides −8 to −3 are dispensable. These data indicate that all essential residues are contained between nucleotides −37 and −9 (with the caveat that the region between nucleotides −2 and +1 was not tested). Second, all three-nucleotides alterations in the region between nucleotides −38 and −9 affected β-galactosidase expression. These mutations decreased rather than increased expression, suggesting that P2 regulation is achieved by a positive rather than a negative transcription factor. Third, the severity of these effects varied greatly. As summarized in Fig. 2, most of the three-nucleotide alterations in the left half of this region drastically decreased expression, while most alterations in the right half had more modest effects. Some of these alterations are predicted to overlap the −35 and −10 regions of P2, especially nucleotides −37 to −32 and −14 to −9. Mutations at these positions may therefore have altered contacts between DNA and the σ subunit. However, alterations at other positions are less likely to affect RNA polymerase contact sites. These data provide evidence that there is a site between the −35 and −10 motifs that may be bound by a positive transcription factor.

This region contains an imperfect dyad symmetrical sequence ($GCAATTCN_4GAACTGC$). In many cases, the major grooves of such dyad symmetries provide binding sites for symmetric protein dimers (32). It seemed plausible that if this was true of P2, then such a dimer might not make base-specific contacts at the center of this sequence. To test this, we altered two nucleotides at positions −25 and −26, creating pCH494

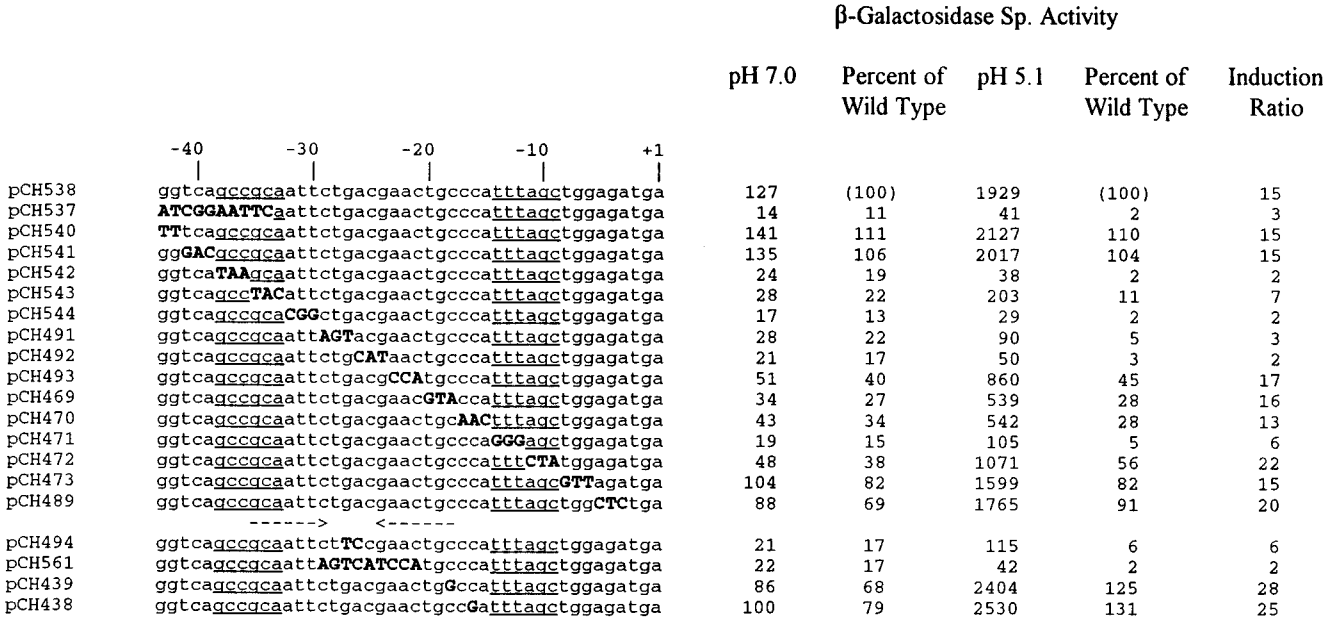

FIG. 1. Responsiveness of mutant P2 promoters to extracellular acidity. Derivatives of strain A136 containing the indicated plasmids were cultured for 16 h in AB medium (6a) supplemented with 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (pH 7.0 or pH 5.1) and assayed for β-galactosidase specific (Sp.) activity. Boldface capital letters indicate nucleotide substitutions. The putative −10 and −35 regions are underlined. The dyad symmetrical sequences located between the −10 and −35 regions are indicated by inverted arrows.

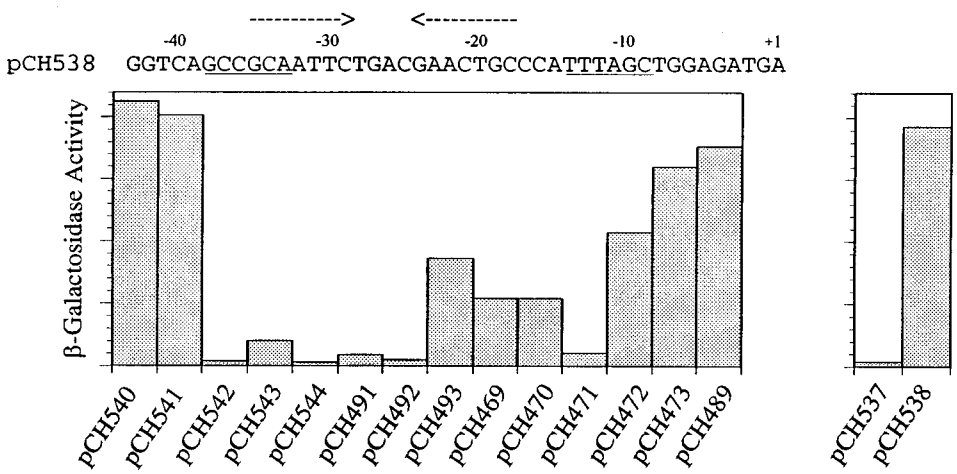

FIG. 2. Graphic representation of induced expression of mutant P2 promoters. Each bar represents the activity with a mutation at the positions directly over that bar. The data are taken from Fig. 1.

(Fig. 1). Contrary to our predictions, this mutant was severely impaired in induction. A nine-nucleotide alteration also severely decreased P2 expression, while two other single-nucleotide mutations, located at nucleotides −16 and −18, showed little if any effect (Fig. 1).

Mutations that caused a deficiency in induced expression also caused a decrease in basal-level expression (Fig. 1). This suggests that any regulatory factor or factors required for induced expression are also required for basal levels of expression. Deficiencies in induced expression appeared to be more severe than deficiencies in basal expression. However, a portion of *lacZ* expression probably does not originate from P2, since pCH536, which lacks this promoter, expresses this gene at detectable levels (Table 1). When this background level is deducted, the deficiencies in basal and induced levels of expression are similar (data not shown).

To determine whether P2 is expressed in *E. coli*, plasmid pCH539 was introduced into *E. coli* MC4100 and assayed for β-galactosidase activity under acidic conditions. MC4100 (pCH539) expressed β-galactosidase at the same low levels as a promoterless control (pCH536) (data not shown). To test whether the P2 promoter is induced by low pH in *Rhizobium meliloti*, an IncP group plasmid containing a P2-*lacZ* fusion (pSW165) was introduced into *R. meliloti* RM1021. The resulting strain did not shown appreciably elevated levels of β-galactosidase activity in response to acidic pH (data not shown). Evidence was previously obtained that the *A. tumefaciens chvI* and *chvG* genes are required for P2 expression (24). The plasmid pNM157, which contains the *chvGI* operon, was therefore introduced into MC4100(pSW264), which contains a P2-*lacZ* fusion (43). pNM157 did not stimulate expression of P2 in *E. coli* (data not shown). This plasmid was previously shown to activate transcription of the *phoA* gene of this *E. coli* strain (24), indicating that these genes are expressed and that their products are stable and active.

This study has necessitated a reevaluation of our previous model for regulation of the P2 promoter. As described above, we had previously suggested that P2 expression required an alternative σ factor. With the exception of σ^{54} promoters, promoters in gram-negative organisms that use alternative σ factors rarely require additional transcription factors (13). This model would therefore predict that no *cis*-acting sites other than the −10 and −35 motifs should exist. Our discovery of an essential site between these regions suggests that a positive regulatory factor may be required for P2 expression. If so, then P2 may well utilize the vegetative σ factor of *A. tumefaciens*, despite the poor similarity between P2 and vegetative promoters.

In *E. coli*, many vegetative promoters with defined transcriptional start sites have been analyzed for conserved sequences, and these studies revealed that only the −10 and −35 hexamers are conserved (14, 15). These two hexamers are the regions directly contacted by the σ subunit of RNA polymerase, while alternative sigma factors recognize different sequences at these regions (13). In addition, the nucleotides in the TG motif immediately upstream of the −10 hexamer sometimes play an important role in the expression of some promoters (19, 20), while the UP element located upstream of the −35 region also plays an important role in some promoters (31). The length of the spacer region between the −10 and −35 sites is also important, since deletion or insertion of even one nucleotide often dramatically changes promoter strength (12). However, many reports have indicated that single-base substitutions in the region between the −10 and −35 sites usually do not cause significant changes in transcriptional activity, and promoters with multiple substitutions in this region often retain at least 50% of wild-type activity (5, 12, 15).

Very few positive transcriptional regulators have been described as contacting the region between the −10 and −35 motifs. Perhaps the best characterized protein that binds in this region is MerR protein of Tn*501*, which binds to a dyad symmetrical sequence centered at position −23.5 (2, 3). In the absence of Hg^{2+}, MerR represses *merT*, while the addition of Hg^{2+} causes a repressor-to-activator conversion. This is caused by an Hg^{2+}-induced unwinding and bending of the helix, which aligns the −10 and −35 sites. While it is possible that P2 is regulated by a similar mechanism, mutations between the −35 and −10 sites do not increase expression at neutral pH, suggesting that this hypothetical P2 regulator acts only as an activator rather than as both an activator and a repressor. Other proteins that activate transcription from sites between the −35 and −10 motifs include the LysR-type protein IlvY of *E. coli* (42), the PhoB protein of *E. coli* (21), and, curiously, the *A. tumefaciens* VirG protein acting at the P1 promoter (43).

In future work, it will be important to identify the putative P2 transcriptional activator. One interesting candidate that has already been described is the chromosomally encoded ChvI protein, since *chvI* null mutants are completely deficient in P2

induction (7, 24). ChvI is strongly similar to PhoB (24), and as described above, PhoB acts at sites whose positions are similar to that of the binding site of the putative P2 activator. Although the *chvGI* operon did not regulate P2 in *E. coli* (see above), it is possible that ChvG and ChvI cannot function at this promoter in this heterologous host. If ChvI directly regulates P2, it may be possible to activate P2 in *A. tumefaciens* by overexpression of ChvG and/or ChvI, and it may also be possible to isolate point mutations in the *chvGI* operon that express P2 constitutively.

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