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Transcriptional Induction of an *Agrobacterium* Regulatory Gene at Tandem Promoters by Plant-Released Phenolic Compounds, Phosphate Starvation, and Acidic Growth Media

STEPHEN C. WINANS

Department of Microbiology, Cornell University, Ithaca, New York 14853-7201

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Transcription of the *virG* gene of *Agrobacterium tumefaciens* was previously shown to be expressed from two tandem promoters and to be responsive to three stimuli: plant-released phenolic compounds, phosphate starvation, and acidic media. In this report, I describe a set of deletions and other alterations of the 5′ end of *virG* that show that the upstream promoter (P1) is necessary for induction by phenolic compounds and by phosphate starvation, whereas the downstream promoter (P2) is induced by acidic media. Upstream of promoter P1 there are three copies of a family of sequences (*vir* boxes) found near all VirA,VirG-inducible promoters. Site-directed mutagenesis of these sequences showed that *vir* box I and *vir* box III but not *vir* box II are needed for induction of P1 by acetosyringone. Induction of P1 by phosphate starvation requires *vir* box III (or an overlapping site), whereas *vir* box I and *vir* box II are not needed. The relative importance of promoters P1 and P2 in *vir* gene induction was tested by measuring the expression of a *virB*::*lacZ* fusion in strains containing mutations at either promoter P1 or P2. Mutations in either promoter significantly attenuated the expression of *virB*, indicating that both promoters play important roles in *vir* gene induction.

The *vir* regulon of *Agrobacterium tumefaciens* mediates the transfer of bacterial DNA into the nuclei of infected plant cells and the integration of this DNA into the plant genome (reviewed in references 16 and 30). These *vir* genes are transcriptionally activated in response to wound-released phenolic compounds by the VirA and VirG proteins (19, 21). VirA and VirG are members of the family of two-component regulatory systems (13, 17, 25). VirA is a transmembrane protein kinase that phosphorylates itself and VirG (10, 15, 28). VirG binds to *vir* promoters in vitro (11) and is presumed to activate their transcription in vivo.

The intracellular concentration of VirG is rate limiting for *vir* gene induction, since increasing the gene dosage of *virG* increases the efficiency both of *vir* gene induction and of infection (9, 29). Therefore, high intracellular concentrations of VirG may make *Agrobacterium* spp. more responsive to plant-released phenolic compounds, and any treatment that increases the pool size of this protein could dramatically increase pathogenicity. Consequently, an understanding of the transcriptional regulation of *virG* is vital to an understanding of *vir* gene regulation.

In previous experiments, *virG* transcription was found to be enhanced by three types of stimuli. A class of wound-released phenolics such as acetosyringone stimulated transcription of *virG* in a VirA,VirG-dependent fashion, thus allowing positive autoregulation of the gene (21). *virG* was also induced by phosphate starvation (27) and by acidic culture media (23, 27). Responsiveness to these latter two stimuli did not depend on any Ti plasmid-encoded gene. Transcripts of *virG* show two different 5′ ends, suggesting strongly that the gene is transcribed from two promoters spaced about 50 base pairs bp apart (21). Although these promoters were originally designated P_i and P_c (for inducible and constitutive), they will here be referred to as promoters P1 and P2, since each has been found to be transcriptionally regulated. The longer transcript was observed only when bacteria were cocultivated with tobacco suspension cells; the shorter one was detected whether or not bacteria had been cocultivated. The upstream promoter contains three copies of the so-called *vir* box to which VirG protein has been postulated to bind (26). It has been speculated that the downstream promoter might be stimulated by acidic media and by phosphate starvation (27). In this report, I will analyze the rather complex transcriptional regulation of this gene.

MATERIALS AND METHODS

Strains and plasmids. *A. tumefaciens* strain A348 is a derivative of A136 containing pTiA6NC (18). A136 is a derivative of strain C58 lacking the nopaline Ti plasmid pTiC58. pSM243 is a Tn*3*HoHo1 insertion derivative of pVK219 and contains *virA* and a *virB*::*lacZ* fusion (20). pTZ18R was purchased from U.S. Biochemical Corp. The broad-host-range plasmid pUCD2 was obtained from C. Kado (3). RZ1032 was obtained from T. Kunkel. Strain DH5α was purchased from Bethesda Research Laboratories, Inc. pMC1403 (1) was obtained from S. Lory.

Antibiotics, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and morpholineethanesulfonic acid (MES) were purchased from Sigma Chemical Co. Sequenase, restriction endonucleases, T4 DNA kinase, T4 DNA ligase, nuclease S1, and exonuclease III were purchased from Bethesda Research Laboratories. Sequenase was purchased from U.S. Biochemical. [^{35}S]dATP was purchased from Dupont, NEN Research Products.

Construction of 5′ deletions. Plasmid pSW104, containing the intact *virG* gene as a *Bgl*II-*Pst*I fragment (25), was digested with *Eco*RI, which cuts the plasmid at a single site 270 bp upstream of the beginning of the structural gene. The DNA was digested with exonuclease III for 1 to 10 min, treated with nuclease S1, phenol extracted, precipitated with ethanol, and then digested with *Bam*HI, which cuts within *virG*. These fragments were then ligated to pMC1403, which had been cut with *Sma*I and *Bam*HI (pMC1403 contains the *lac* operon beginning with the eighth codon of *lacZ* and

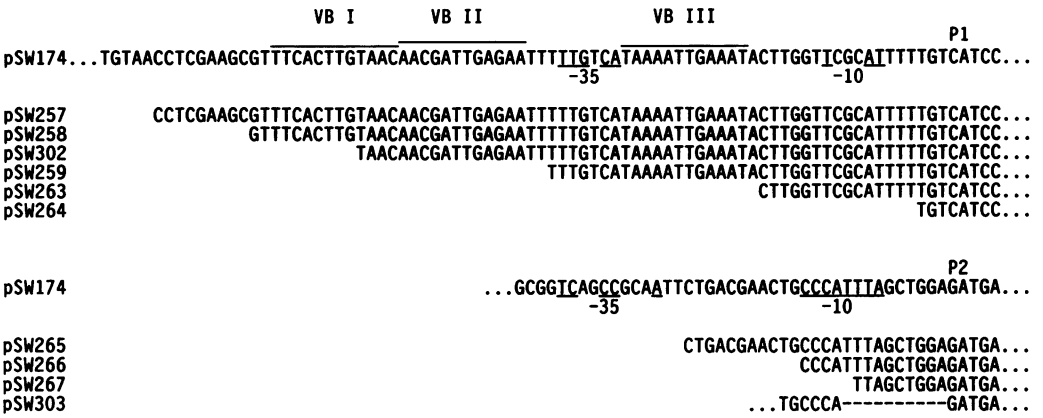

FIG. 1. Deletions of the 5′ regulatory region of *virG*. pSW174 contains *virG* sequences up to −270 bp upstream of the translational start, of which bases −205 to −75 are shown. pSW174 sequences shown on lines 1 and 8 are contiguous and define the wild-type sequence. Overlined sequences represent *vir* boxes I, II, and III. Underlined sequences are similar to *E. coli* consensus vegetative promoters (line 1) or *E. coli* consensus heat shock promoters (line 8). P1 and P2 represent the transcription start sites for promoters P1 and P2, respectively. pSW303 is identical to pSW174 except for the 10-bp deletion indicated. These promoters were used to drive the expression of *virG*::*lacZ* translational fusions as described in Materials and Methods.

preceded by *Eco*RI, *Sma*I, and *Bam*HI sites). The deletions were mapped by restriction endonuclease digestion and ultimately by DNA sequencing. A 10-bp deletion from −2 to −12 bp upstream of P2 was made by digestion pSW167 with *Bst*XI and S1 and ligating. The promoter of the resulting plasmid was cloned into pMC1403 by cutting both plasmids with *Eco*RI and *Bam*HI. These deletions were recloned onto the broad-host-range plasmid pUCD2 by cutting each with *Sal*I and *Pst*I, ligating and transforming strain DH5α to resistance to tetracycline and carbenicillin, and screening for blue colonies. These plasmids were then introduced into *Agrobacterium* strain A348 by transformation (7).

Site-directed mutagenesis. Site-directed mutations using oligonucleotides were made in plasmid pSW167, a derivative of pTZ18R containing *virG*, as described by Kunkel et al. (12). The primer AACCTCGAAGCGTTTTCTAGATAAC AACGATTGAGA was used to make pSW286, the primer TTTCACTTGTAACAATCTAGAAGAATTTTTGTCATA was used to make pSW287, the primer AGAATTTTTGTC ATATCTAGAAAATCATTGGTTCGC was used to make pSW288, and the primer ACTTGTAACTACAATTGAAA ATTTTTGT was used to make pSW289. Where applicable, plasmids were analyzed for a novel *Xba*I restriction site, and all plasmids were ultimately sequenced on both strands. The promoter regions were then introduced into a nonmutagenized version of the same plasmid by cutting with *Bst*XI and *Pst*I and inserting the equivalent *Bst*XI-*Pst*I fragment from pSW164, which contains the *virG* gene cloned into pUC18Cm. Translational fusions were made to *lacZ* by cloning the *Eco*RI-*Bam*HI fragment of each plasmid into pMC1403 that had been digested with *Eco*RI and *Bam*HI. Since these translational fusions contained most of the *virG* gene, they were each cut with *Xho*I and *Bam*HI, treated with nuclease S1, and ligated to remove most of the *virG* gene. These plasmids were then cut with *Pst*I and *Sal*I and introduced into *Pst*I-*Sal*I-digested pUCD2, selecting for resistance to tetracycline and carbenicillin. These plasmids were then introduced into *Agrobacterium* strain A348 by transformation.

For induction assays, 25-ml samples of *Agrobacterium* strains were grown in AB medium (2) supplemented with 40 mM MES (pH 7.0) to an optical density at 600 nm of 0.4, centrifuged, suspended in 1.0 ml of water containing 15% glycerol, and frozen at −70°C. As needed, cells were thawed, diluted to an optical density at 600 nm of 0.05 into 5.0 ml of AB medium supplemented with 40 mM MES (pH 5.25), and cultured at 30°C in culture tubes (20 by 150 mm) in a rotary aerator. After the indicated time intervals, they were assayed for β-galactosidase activity. To assay for induction by phosphate starvation, cells were diluted in AB medium containing 0.1 mM phosphate and 40 mM MES (pH 7.0), cultured for 12 h, and assayed.

Plasmids containing intact *virG* genes containing the wild-type or altered promoters were cloned into pUCD2 by digesting both plasmids with *Eco*RI, ligating, and selecting for resistance to tetracycline and carbenicillin. pSW194 contains both *virG* promoters. pSW305 contains the same 10-bp deletion of P2 as pSW303, thereby attenuating P2. pSW311 contains the same *vir* box I mutation as pSW286, thereby inactivating P1. These plasmids were introduced into strain A136(pSM243) by transformation. Strains were cultured, frozen, used to inoculate broth cultures, and assayed for β-galactosidase activity as described above. Cultures were diluted as required to keep the optical density at 600 nm less than 0.4.

DNA sequencing. Double-strand sequencing of plasmid DNA was done by using Sequenase (U.S. Biochemical) according to procedures recommended by the manufacturer.

RESULTS

In previous work, it was shown that *virG* expression could be stimulated by three different stimuli and that the gene was expressed from two promoters. To confirm the existence of these two promoters and to determine which promoter was stimulated by each of the three stimuli, a series of 5′ deletions of the gene was constructed (Fig. 1). A 10-bp internal deletion of the −12 to −2 region of the downstream promoter was also constructed (Fig. 1). Each altered gene was used to make a *virG*::*lacZ* translational fusion, introduced into *A. tumefaciens*, and tested for its responsiveness to acetosyringone, phosphate starvation, and acidic media.

pSW174, pSW257, and pSW258 were fully responsive to acetosyringone, whereas pSW302 and pSW259 were unable to respond to acetosyringone (Table 1). These data strongly suggest that stimulation of *virG* by acetosyringone occurs at

TABLE 1. Responsiveness of *virG* deletion derivatives to acetosyringone and to acidic media[a]

Plasmid	β-Galactosidase activity (Miller units)		
	pH 7.0	pH 5.25	pH 5.25, 100 μM AS
pSW174	5.6	105	620
pSW257	5.0	106	653
pSW258	3.5	85	636
pSW302	15.3	99	93
pSW259	6.8	103	87
pSW263	4.8	111	NT[b]
pSW264	5.6	144	NT
pSW265	2.8	4.5	NT
pSW266	3.4	6.8	NT
pSW267	2.5	5.0	NT
pSW303	2.6	20	702

[a] *Agrobacterium* derivatives of strain A348 containing the indicated plasmids were incubated for 12 h in induction broth modified as indicated and assayed for β-galactosidase activity. AS, Acetosyringone.
[b] NT, Not tested.

promoter P1. This hypothesis was confirmed by using a plasmid (pSW303; Fig. 1) containing an internal deletion of part of P2. A strain containing this plasmid was fully responsive to acetosyringone (Table 1), indicating that P2 is not required for this induction. These data also indicate that sequences further than 67 bp upstream of P1 are not required for induction by acetosyringone, whereas some sequence more than 58 bp upstream of P1 is required.

I used the same approach to determine which promoter was required for induction by phosphate starvation. Plasmids pSW174, pSW258, pSW302, and pSW259 were responsive to phosphate starvation, whereas pSW263, pSW264, and pSW265 were nonresponsive (Table 2), suggesting that stimulation by phosphate starvation also occurs at P1. Plasmid pSW303, containing a 10-bp deletion of P2, was also fully responsive (Table 2), confirming that P1 is the phosphate starvation-inducible promoter. Induction by this stimulus does not require any sequence more than 39 bp upstream of P1, whereas some sequence more than 19 bp upstream is required.

Finally, I mapped the sequences required for induction by acidic growth media. Plasmids pSW174, pSW257, pSW258, pSW302, pSW259, pSW263, and pSW264 were responsive to acidic media, whereas pSW265, pSW266, and pSW267 were unable to respond (Table 1). pSW303, which contains a 10-bp deletion in P2, showed a strong deficiency in induction by acidic media (though not a complete loss). Induction by acidic media, therefore, occurs at promoter P2 and does not require sequences more that 51 bases upstream of the P2 start site, whereas it does require sequences more than 29 bases upstream.

TABLE 2. Responsiveness of deletion and alteration derivatives to stimulation by phosphate starvation[a]

Plasmid	β-Galactosidase activity (Miller units) at phosphate concn of:	
	25 mM	0.1 mM
pSW174	6.7	383.7
pSW258	5.2	212.1
pSW302	6.8	315.6
pSW259	10.7	248.1
pSW263	6.0	16.6
pSW264	7.2	15.7
pSW265	3.3	7.7
pSW303	3.5	323.3
pSW286	5.9	261.5
pSW287	6.1	311.1
pSW288	7.5	23.6
pSW289	6.6	287.3

[a] See Table 1, footnote *a*, for experimental procedures.

TABLE 3. Responsiveness of altered promoters to acetosyringone and to acidic media[a]

Plasmid	β-Galactosidase activity (Miller units)			
	pH 7.0	pH 5.25	pH 5.25, 100 μM AS	pH 5.25, 5 μM AS
pSW285[b]	6.6	98	1,595	280
pSW286	6.6	82	78	60
pSW287	5.1	70	1,582	270
pSW289	6.2	91	1,547	280
pSW288	4.9	78	164	73

[a] See Table 1, footnote *a*, for experimental procedures.
[b] pSW285 contains the wild-type promoter.

The region just upstream of P1 contains three copies of a family of sequences found in all VirA,VirG-controlled promoters called *vir* boxes (overlines in Fig. 1 and 2). To assess the roles of these sequences in *virG* induction, mutations altering 6 bp of each *vir* box (and introducing an *Xba*I site) were introduced into the *virG* promoter by site-directed mutagenesis. Since *vir* box II shows a far weaker similarity to the consensus *vir* box than does *vir* box I or *vir* box III, I also introduced three base changes in *vir* box II to make it conform more closely to the consensus sequence. Each of these mutant promoters (Fig. 2) was fused to *lacZ*, introduced into *A. tumefaciens*, and assayed for induction by acetosyringone and by phosphate starvation. A mutation in *vir* box I (pSW286) completely abolished induction by acetosyringone (Table 3). In contrast, neither of the two mutations in *vir* box II (pSW287 and pSW289) had any effect on induction. Finally, a mutation in *vir* box III (pSW288) strongly attenuated induction by acetosyringone, although a residual induction was detected.

I also tested these same *vir* box mutations for their effect on induction of P1 by phosphate starvation. A mutation in *vir* box III virtually abolished induction, whereas mutations in *vir* box I or II had no effect on induction (Table 2). As expected, no *vir* box mutation had any effect on induction of P2 by acidic media (Table 3).

Since P1 and P2 control the pool size of the VirG protein, I determined their relative importance in the induction of other *vir* genes. Strains were constructed containing full-length *virG* genes expressed either from both promoters (pSW194), primarily from promoter P1 (pSW305, which contains the same 10-bp internal deletion of P2 as does pSW303), or solely from promoter P2 (pSW311, which contains the same mutation in *vir* box I as pSW286). These strains also contained the cosmid pSM243, which carries *virA* and a *virB*::*lacZ* fusion (20) and did not contain the Ti plasmid. These strains were treated with acetosyringone at two concentrations, and the expression of *virB* was measured over a 36-h period. I tested a low concentration of inducer as well as a high one because at the lower concentration the induction at promoter P1 would be limited, and the relative contribution from promoter P2 would therefore be stronger. Under these conditions, the effects of a P2 mutation might therefore be more pronounced.

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                    VB I      VB II            VB III
...AACCTCGAAGCGTTTCACTTGTAACAACGATTGAGAATTTTIIGTCATAAAATTGAAATACTTGGTICGCAIT...
                                              -35                 -10

pSW286      ...TTTICTAGATAA...
pSW287                ...CAAICTAGAAGA...
pSW289           ...AACIACAATTGAAAAT..
pSW288                                  ...ATAICTAGAAAA...
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FIG. 2. Alterations in the three *vir* boxes of *virG*. In pSW286, pSW287, and pSW288, six contiguous bases (indicated by double underlines) were altered to disrupt each *vir* box. In pSW289, three bases (double underlines) were altered to make *vir* box II conform more closely to the consensus sequence. These promoters were used to drive the expression of *virG*::*lacZ* translational fusions.

Mutations in either promoter significantly affected the induction of *virB* (Fig. 3). As predicted, this effect was somewhat more pronounced at 2 μM than at 50 μM acetosyringone. The mutation in promoter P1 (pSW311) caused a more severe defect in induction than did the mutation in P2 (pSW305). However, the mutation in P2 only partially prevented induction by acidic media (pSW303; Table 1), so these data probably understate the contribution of P2 to *virB* induction. A mutant with a deletion of all of P2 is currently being constructed. The present results nevertheless indicate

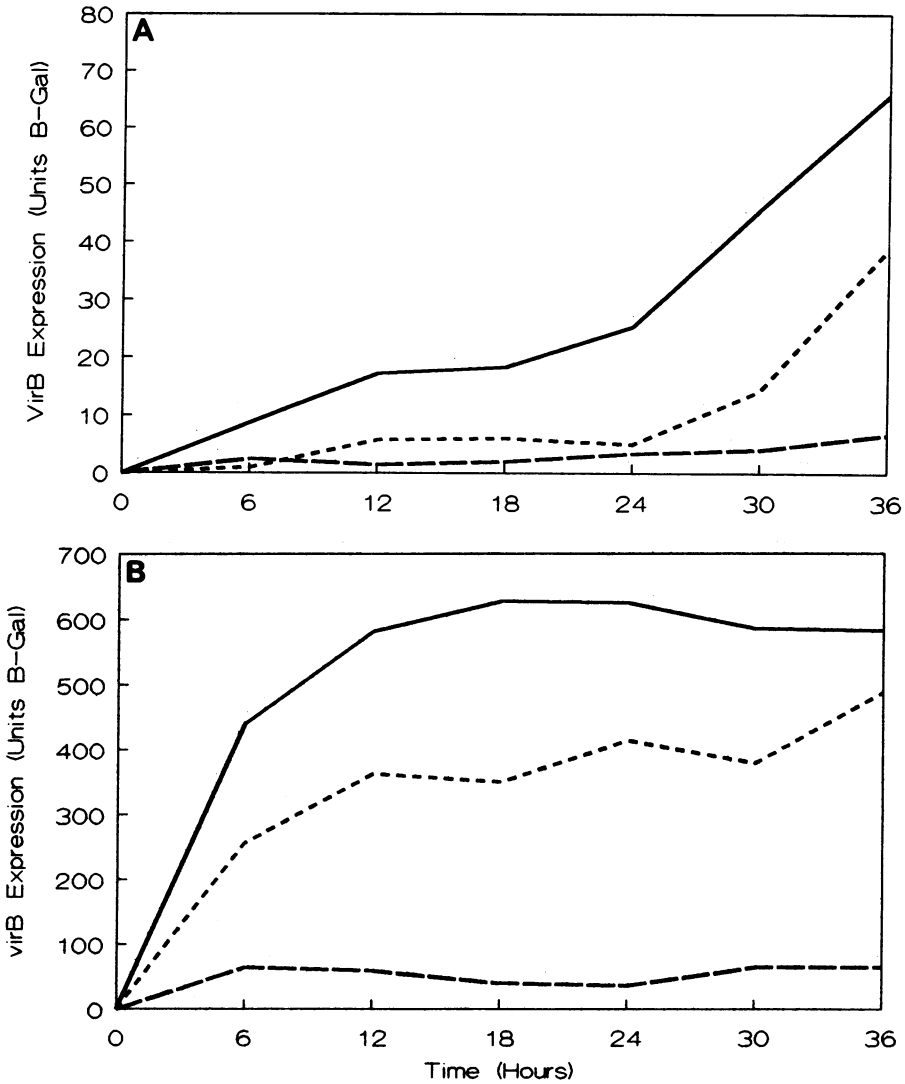

FIG. 3. Induction of *virB* by acetosyringone in A136(pSM243)(pSW194) (——), A136(pSM243)(pSW305) (-----), and A136(pSM243)(pSW311) (– – –). pSW243 is a cosmid containing *virA* and a *virB*::*lacZ* fusion. pSW194 contains the wild-type *virG* gene and expresses *virG* from both P1 and P2. pSW305 contains a leaky mutation in P2 and therefore expresses *virG* primarily from P1. pSW311 contains a mutation in *vir* box I and therefore expresses *virG* solely from P2. Cultures were incubated in induction broth containing 2 (A) or 50 (B) μM acetosyringone and were diluted into fresh medium as needed to prevent the optical density at 600 nm from rising above 0.4. Data shown are averages of duplicate cultures. B-Gal, β-Galactosidase.

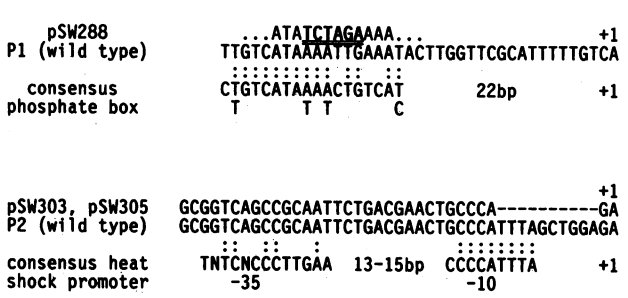

FIG. 4. Similarities between *virG* promoters and *E. coli* promoters induced by phosphate starvation (A) or by heat shock stresses (B). Line 1 represents sequence alterations in *vir* box III of pSW288 that abolish induction by phosphate starvation. Line 4 represents a 10-bp deletion in pSW303 and pSW305 that causes an attenuation in induction by acidic media.

that both promoters play an important role in efficient induction of *virB* and presumably of other *vir* genes.

DISCUSSION

A series of deletions and other alterations of the *virG* regulatory region was constructed to localize *cis*-acting sites required for induction by three classes of treatments known to stimulate expression of this gene. Induction by acetosyringone required promoter P1 and *vir* boxes I and III, whereas induction by phosphate starvation required P1 and *vir* box III (or an overlapping sequence). Finally, induction by acidic media required P2. Although the data presented here do not show that these stimuli necessarily acted at the level of transcription, we have recently obtained evidence by using a transcriptional *virG*::*lacZ* fusion (rather than the translational fusions described here) that each stimulus does indeed act at the level of transcription (N. Mantis, M. Sessler, and S. C. Winans, unpublished data). Both promoters played a significant role in the induction of *virB* and presumably other promoters, although a mutation in P1 had a greater effect than did a mutation in P2.

I propose that VirG protein may bind to *vir* boxes I and III to allow induction by acetosyringone. Confirmation of this hypothesis was found by A. Das and co-workers (personal communication), who showed by footprinting techniques that VirG protein binds to *vir* boxes I and III but not to *vir* box II. It is unusual for a transcriptional activator to bind DNA downstream of the −35 sequence, although the MerR protein, which regulates a mercury resistance operon of transposon Tn*21*, and the PhoB gene, which regulates *Escherichia coli* genes inducible by phosphate starvation (see below), both have binding sites in this region (6, 14). These proteins have been proposed to bind to the face of the DNA opposite that bound by RNA polymerase, and VirG may have similar properties. Since *vir* boxes I and III are 33 bp apart, molecules of VirG protein binding each site would lie on the same face of the helix.

The DNA sequence of promoter P1 provides an important clue as to how it may respond to phosphate starvation. P1 contains a sequence strongly similar to a so-called *pho* box found in the −42 to −22 region of a group of *E. coli* promoters that are inducible by phosphate starvation (14; Fig. 4A). *E. coli* genes that are inducible by this treatment are regulated by the PhoB protein, which is homologous to VirG. The consensus *pho* box differs from the *virG* sequence at only 4 of 18 positions, and the two are found at almost identical positions with respect to their promoters. Since this site overlaps *vir* box III, it was disrupted in pSW288, which was indeed deficient in induction by phosphate starvation. The most probable explanation is that *A. tumefaciens* has a *pho* regulon similar to that of *E. coli* and that *virG* is part of this regulon.

Similarly, the DNA sequence of P2 contains a clue as to how it responds to acidic growth media. Unlike other *vir* promoters (5), P2 has little if any similarity to *E. coli* vegetative promoters. However, it has a strong similarity to *E. coli* heat shock-inducible promoters (4; Fig. 4B), whose expression depends on the heat shock σ^{32}. Genes under control of this regulon are inducible by a wide variety of environmental stresses, including high temperature, ethanol, alkaline pH, and heavy metals (22). We have recently obtained data that P2 is induced by all these stimuli in addition to acidic media and that all of these treatments do induce the heat shock response in *A. tumefaciens* (Mantis et al., unpublished data). These data strongly suggest that P2 is a member of the heat shock regulon.

If both of these hypotheses are correct, then *virG* would be inducible by three different systems, each of them a global regulatory system. Since the intracellular concentration of VirG protein is rate limiting for *vir* gene induction, increased expression of *virG* by phosphate starvation or by heat shock inducers could greatly stimulate the expression of the *vir* regulon. What advantage would *Agrobacterium* strains gain from inducing *virG* in response to phosphate limitation or heat shock stimuli?

Since *Agrobacterium* strains are essentially soil bacteria that are facultatively pathogenic, they may, when confronted with a wounded plant, make a decision as to whether to try to infect that plant (27). Perhaps these two stimuli, both of which can be thought of as environmental stresses, could, by increasing the pool size of VirG, play some role in that decision. Since phosphate is often scarce in nature, and since crown gall tumors could be a source of phosphate, a limitation in this nutrient could provide a signal that plant infection would be an appropriate survival strategy. Similarly, soils and plant wounds contain a variety of toxic compounds, including phytoalexins, which may induce the heat shock response in *Agrobacterium* strains. Such compounds may provide a second category of chemical signals in addition to the phenolic compounds recognized by VirA. We have recently obtained evidence (Mantis et al., unpublished data) that plant wounds do indeed contain one or more compounds capable of inducing P2.

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