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Transcription in vitro promoted by the *Agrobacterium* VirG protein

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Expression of the virulence genes (*vir*) on the hairy-root-inducing plasmid pRiA4 is induced by plant signals in *Agrobacterium* cells through a two-component regulatory system, the VirA–VirG system. We constructed an in vitro transcription system that consisted of the purified VirG protein and the *Agrobacterium* RNA polymerase holoenzyme. Both versions of VirG, the non-phosphorylated form and the VirA-phosphorylated form, were active but showed different patterns of the pH-dependency for transcriptional activation.

Agrobacterium; Positive regulator; Signal transduction; Transcription, in vitro; Two-component regulatory system

1. INTRODUCTION

Transcription of the virulence genes (*vir*) on the hairy-root-inducing plasmids (pRi) and tumor-inducing plasmids (pTi) is induced in *Agrobacterium* cells by plant signals through the VirA–VirG system, which is a two-component regulatory system [1–4]. VirA is anchored at the cell membrane [5,6] and appears to sense either directly or indirectly plant factors concurrent with autophosphorylation [7]. The phosphorylated VirA transfers its phosphoryl group to VirG [8,9]. VirG cooperatively binds to *vir* promoter regions in which the 6-bp vir boxes are located in a helical phase [10–12]. RNA polymerase and VirG molecules could simultaneously interact with the *vir* promoter regions without steric hindrance because the helical phase of the vir boxes is nearly opposite to that of the −35 and −10 regions of the promoters [10,13]. However, the −35 and −10 region sequences, particularly the −35 region sequence, show a low degree of similarity to the respective consensus sequences of *Escherichia coli* promoters; nevertheless *Agrobacterium* constitutive promoters resemble *E. coli* promoters [3,14]. Therefore, RNA polymerase seems to be unable to interact with the *vir* promoters by itself, and the cooperative binding of VirG is likely to guide RNA polymerase to the promoters. Toward understanding of molecular mechanisms of transcriptional activation by the VirA–VirG system, we have now developed an in vitro transcription system depending on VirG.

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Abbreviations: bp, base-pair(s); DTT, dithiothreitol; NTP, ribonucleoside triphosphate (ATP, GTP, CTP and UTP); pRi, hairy-root-inducing plasmid; pTi, tumor-inducing plasmid; *vir*, virulence gene(s); VirA, protein encoded by *virA*; vir box, 6-bp sequence recognized by VirG; VirG, protein encoded by *virG*.

2. MATERIALS AND METHODS

Standard procedures for DNA experiments were previously described [9,15]. *Agrobacterium* RNA polymerase was prepared from GV3100 [16] using the method of Cardarelli et al. [17]. This enzyme preparation was composed of about 35% holoenzyme and 65% core enzymes (as judged from gel electrophoretic patterns), and was used in all transcription experiments unless noted otherwise. The reconstituted holoenzyme and core enzyme that were also used had been prepared by the method of Dayle and Richard [18]. One unit of the polymerase activity was defined as the amount of enzyme required to incorporate 1 nmol of ATP into an acid-insoluble form in 60 min at 37°C, and usually corresponded to about 1 μg of protein. VirG and VirA419 (an *N*-truncated VirA derivative) of pRiA4 were prepared from the respective overproducing *E. coli* strains as described [9,10].

To do single-round transcription in vitro, a template DNA fragment (0.3 pmol) was mixed with VirG (0.25 μg) and RNA polymerase (0.5 units) in 20 μl of either 50 mM Tris-HCl (pH 8.9, 8.5, 8.1, 7.7, 7.3 and 6.9) or 20 mM MES buffer (pH 6.5, 6.0 and 5.5) containing 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.15 mM DTT, and was incubated at 28°C for 13 min. The total volume was then adjusted to 30 μl by adding heparin (200 μg/ml), 100 mM KCl, [α-^{32}P]UTP (10 μCi, 1.2 Ci/mmol, 0.1 mM) and 0.4 mM each of three other NTPs. The indicated concentrations were those in the final solution. After 5 min of reaction at 28°C, the products were separated by 8% polyacrylamide–urea gel electrophoresis. The template DNAs used were the 194-bp *SalI–PstI* fragment and the 365-bp *BanI* fragment (together with their subfragments), which respectively contain the *virE* promoter and the divergent *virCD* promoters of pRiA4 [19,20]. For transcription with the phospho-VirG protein, VirG (0.25 μg) had been phosphorylated at 28°C for 8 min in a 5-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10 μM ATP, and VirA419 (0.25 μg) [9]. Under these conditions approximately 10% of the added VirG molecules were phosphorylated [9]. After pH adjustment by 1 M Tris-HCl or 1 M MES buffer of the noted pH, RNA synthesis was done as above. The 5′-end of transcripts was identified by analyzing cDNA products extended from the synthetic oligodeoxyribonucleotide primers (5′CCTCACCATGAGGGACC3′ and 5′GCACCTCCCTTGAAGGA3′) specific for the *virE* and *virC* mRNAs, respectively [3].

3. RESULTS AND DISCUSSION

3.1. *Transcription promoted by* VirG

Single-round RNA synthesis was done under neutral conditions (pH 7.3) in the presence or absence of VirG with the DNA fragment containing the *virE* promoter, and run-off products were analyzed by gel electrophoresis. One major and a few minor RNA bands appeared only when VirG was added (Fig. 1A). Similar results were obtained with the DNA fragment carrying the *virCD* promoters (Fig. 1B). Their transcriptional directions were examined by trimming the template DNAs and by measuring the resulting size shift of RNA products. It was found that the direction of the major and minor transcripts was the same in each case, coinciding with that of the *virE* and *virC* mRNAs, respectively (data not shown). Therefore, the minor bands presumably correspond to premature or snapback derivatives of the major transcript that did not terminate at the precise end but near the extremity of the template DNA. Gel electropherograms of primer-extension cDNA products (Fig. 2) demonstrated that the start site of each major transcript is exactly identical to that of the *virE* and *virC* mRNAs synthesized in vivo [3]. The reconstituted RNA polymerase holoenzyme was similarly competent for transcription in the presence of VirG, but the purified core enzyme never allowed RNA synthesis (Fig. 1C).

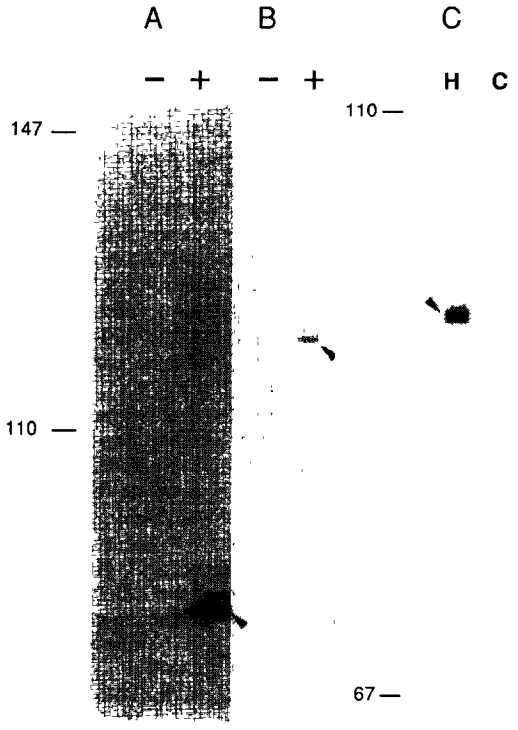

Fig. 1. Gel electrophoresis of RNA synthesized in vitro. (A,B) Transcription was done without (−) or with (+) VirG at pH 7.3 on the template DNA fragment carrying the *virE* promoter (A) or the *virCD* promoter (B). (C) RNA was synthesized with the RNA polymerase holoenzyme [H] or the core enzyme [C]. The size markers shown on the left are the number of nucleotide residues.

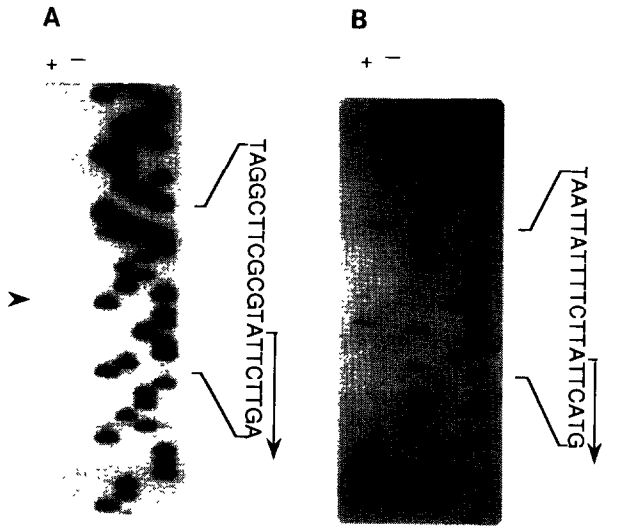

Fig. 2. Gel electrophoresis of primer-extension cDNA products with the DNA fragment carrying the *virE* promoter (A) and the *virCD* promoter (B). Two lanes marked by (+) and (−) contain cDNA synthesized in the reaction mixture with and without VirG, respectively. The remaining four lanes are the sequence ladder for identification of the RNA start site (A, G, C and T from the left). DNA sequences of the relevant regions are shown on the right side at which the deduced RNA start site is indicated by an arrow.

Naturally VirG alone had no RNA synthesizing activity (data not shown). These results clearly show that VirG and the RNA polymerase holoenzyme are needful and sufficient proteins for VirG-dependent transcription. In addition, the holoenzyme requirement verified that VirG does not work as an alternative σ factor but as a positive regulator of transcription, as supposed from the characteristic helical phase of the vir boxes [10] as well as on the analogy of other two-component regulatory systems (e.g. [21,22]).

As described in section 1, VirG exists in two alternative forms, non-phosphorylated and phosphorylated [8,9]. The VirG protein sample used above was likely to contain non-phosphorylated molecules for the following reasons. Although, as reported by Lukat et al. [23], low molecular weight phospho-donor compounds may have phosphorylated VirG during preparation, its extent appears to be fairy low because no induction of *vir* expression occurs in *Agrobacterium* cells without plant factors and/or VirA [3]. We hereafter call VirG prepared from *E. coli* the non-phosphorylated VirG. It was thus apparent that even the non-phosphorylated version of VirG is able to promote faithful transcription from *virE* and *virC*. Nevertheless the *virCD* template DNA used contains both of the divergent *virC* and *virD* promoters [3], we detected only the *virC* mRNA. A failure to synthesize the *virD* mRNA under the conditions used might be related to probable complex partnership of the two regulatory systems for *virD* in *Agrobacterium* cells, the plasmid-derived VirA–VirG system and the host-chromosomal Ros regulatory system [24].

3.2. *Dependency on pH of the in vitro transcription system*

To examine the pH-dependency of this in vitro transcription system, RNA synthesis was done with the *virE* template DNA at various pHs (9.1–5.5) as described in section 2. After separation of RNA products by gel electrophoresis, the major RNA band was measured. The results indicated that significant RNA synthesis occurs at pHs between 6.5 and 8.1 with the optimum of pH 8.1 (Fig. 3, the back bars). Since binding of VirG to DNA, formation of the initiation complex, and RNA synthesis were all done at each specified pH, the observed pH-dependency should have occurred during these processes.

3.3. *Transcription with the phosphorylated VirG protein*

To find the functional difference between the non-phosphorylated and phosphorylated forms of VirG, both versions of VirG were separately subjected to in vitro transcription at pH 6.9 with the *virE* template DNA, and RNA products were analyzed by gel electrophoresis. The results (Fig. 4A) indicated that phospho-VirG has a notably higher activity than non-phospho-VirG. Similar results were obtained with the *virCD* template DNA (Fig. 4B). The pH-dependency of transcriptional activation by phospho-VirG was tested with the *virE* template DNA as in the preceding section (Fig. 3, the front bars). Much RNA synthesis occurred in rather acidic conditions (pH 6.5–6.9), but less did in neutral conditions (pH 7.3–8.1). Phospho-VirG was satisfactorily stable in the reaction periods under the various pH conditions used (data not shown). Therefore, these results clearly show that the pH-dependency for transcriptional activation of the phosphorylated VirG differs from that of the non-phosphorylated VirG. No accurate quantitative comparison was available for RNA synthesized with the two versions of VirG, but roughly comparable amounts of RNA were produced at each optimum pH. Since the polymerizing reaction is likely to entirely depend on RNA polymerase but not VirG, the different pH-dependency remarked with the two forms of VirG is presumably derived from the difference in: (1) the affinity of VirG toward the vir boxes, as in the cases of PhoB and OmpR regulators [25,26], (2) the ability to guide RNA polymerase to the *vir* promoter for the initiation complex, or (3) a combination of these two characteristics. Although the exact biological meaning of the activity exerted by the non-phosphorylated VirG is unknown, it is probable that non-phospho-VirG is also functional in vivo, at least in a later period upon induction, for the following reasons. The concentration of VirG in *Agrobacterium* is low just after induction by plant signals, but gradually increases with time because of inducible expression of the *virG* gene itself [3], implying that non-phospho-VirG gradually accumulates in the cell owing to a limited capacity of phosphotransfer from VirA to VirG. Therefore, it appears that phospho-VirG predominantly operates at an initial stage after induction, while both forms of VirG contribute to transcriptional activation at later stages.

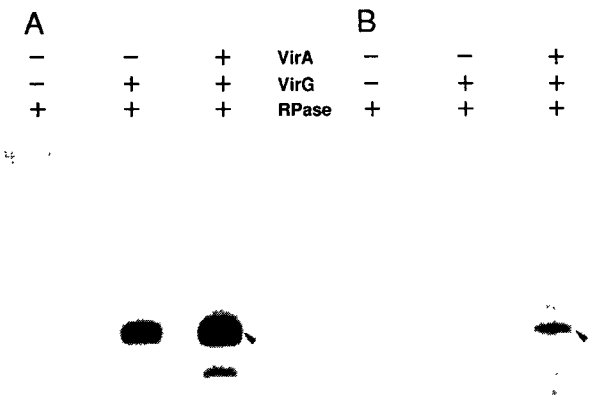

Fig. 4. Gel electrophoresis of RNA synthesized with non-phospho-VirG and phospho-VirG. Transcription was done at pH 6.9 with the *virE* template DNA (A) or with the *virCD* template DNA (B). The presence (+) or absence (−) of VirA, VirG, and RNA polymerase in reaction mixtures is indicated at the top of each lane.

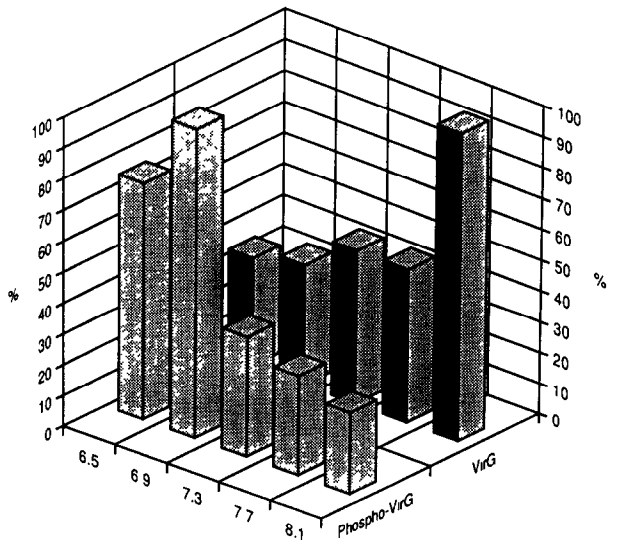

Fig. 3. Transcription promoted by non-phospho-VirG (back bars) and phospho-VirG (front bars) under various pH conditions. The abscissa shows pH, and the ordinate indicates the amount of the major transcript relative to that at the optimum pH (pH 8.1 for non-phospho-VirG and pH 6.9 for phospho-VirG).

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