JOURNAL OF BACTERIOLOGY, Mar. 1990, p. 1241–1249
0021-9193/90/031241-09$02.00/0

Vol. 172, No. 3

virG, an *Agrobacterium tumefaciens* Transcriptional Activator, Initiates Translation at a UUG Codon and Is a Sequence-Specific DNA-Binding Protein

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The *Agrobacterium tumefaciens* Ti plasmid *virG* locus, in conjunction with *virA* and acetosyringone, activates transcription of the virulence (*vir*) genes. Insertional and deoxyoligonucleotide-directed mutagenesis studies showed that both octopine and nopaline Ti plasmid *virG* genes initiate translation at a UUG codon. VirG protein initiated at this UUG codon was found to be 241 amino acid residues in length and had an apparent molecular mass of 27.1 kilodaltons. A *Salmonella typhimurium trp-virG* transcriptional fusion was constructed to overproduce VirG. *Agrobacterium* cells containing this gene fusion showed a large increase in *virG* activity in the presence of *virA* and acetosyringone. Since the *trp* promoter is not under *virA-virG* control, this result indicates that modification of VirG is necessary for its full activity. VirG overproduced in *Escherichia coli* was purified from inclusion bodies. It was found to be a DNA-binding protein that preferentially bound DNA fragments containing the 5′ nontranscribed regions of the *virA*, *-B*, *-C*, *-D*, and *-G* operons. Significant specific binding to the 5′ nontranscribed region sequences of *virE* was not detected. DNase I footprinting of the upstream regions of *virC-virD* and *virG* showed that VirG binds to sequences around the *vir* box region.

The virulence (*vir*) genes of the *Agrobacterium tumefaciens* Ti plasmid encode proteins required for the pathogenic action of the bacterium (reviewed in references 10, 18, and 44). Of the seven *vir* operons identified thus far, only *virA* and *virG* are expressed in free-living bacteria. Interaction of bacteria with plant cells is required for the transcriptional activation of the other virulence genes (29, 30). These *vir* genes probably mediate the processing and transfer of a segment of the Ti plasmid (T-DNA) from the bacteria to the plant. The T-DNA is found integrated in the plant nuclear genome and encodes, among others, genes responsible for the biosynthesis of the phytohormones auxin and cytokinin. The hormonal imbalance, a result of expression of T-DNA-encoded genes, causes the tumorous growth associated with crown gall tumor disease.

Expression of the *vir* genes is controlled at at least two levels. These genes are regulated positively by *virA* and *virG* in conjunction with plant phenolic compounds such as acetosyringone (AS) and hydroxyacetosyringone (29). These compounds, normally produced in metabolically active plant cells, are present at higher levels in wounded plant cells. In addition, *virC* and *virD* are regulated by a chromosomal locus (*ros*) in a negative manner (1, 2, 33).

Regulation of the regulatory genes, *virA* and *virG*, is more complex. *virA* is constitutively expressed in free-living bacteria and is induced by AS in a *virA-virG*-dependent manner (23, 40). *virG* is transcribed from two promoters producing transcripts that differ in length at the 5′ end by 50 nucleotides. The shorter transcript is produced by free-living bacteria, whereas the longer one is induced in a *virA-virG*-dependent manner (31). Transcription of *virG* is also induced by pH shift or by phosphate starvation (35, 40). The promoter(s) that is activated by these stresses is not known; however, this activation is not *virA-virG* dependent (40).

Gene fusion studies and DNA sequence analysis indicate that one of the regulatory proteins, VirA, spans the bacterial inner membrane (14, 16, 41), whereas the other, VirG, is probably a cytosolic protein (42). VirA is hypothesized to interact with the plant phenolics in its periplasmic domain, leading to the activation of its cytoplasmic domain. Activated VirA is then expected to activate VirG by modification. Modified VirG probably functions as a transcriptional activator (42). Recently, nopaline Ti plasmid VirG was shown to bind DNA nonspecifically when the nontranscribed region of the *virE* operon was used as a probe (21).

Our recent deletion mutagenesis studies indicate that a conserved tetradecameric *vir* box sequence, 5′-dPuT/ATD CAATTGHAAPy-3′ (D = A, G, or T; H = A, C, or T), is required for induction of the *virB* promoter (3). This sequence is presumed to be the VirG-binding site. All inducible *vir* genes of both octopine (pTiA6) and nopaline (pTiC58) Ti plasmids contain this sequence, whereas the noninducible nopaline Ti plasmid *virG* lacks it (3, 32, 39). DNA sequence analysis of pTiA6 *virG* indicates that it can potentially encode a 267-residue polypeptide (42). This study also indicates that there are several potential translation initiation sites for *virG* mRNA. In this study, we demonstrate that a UUG codon, not an AUG codon as presumed earlier (42), is the translation initiation codon for pTiA6 *virG* mRNA. The same codon is also used for translation initiation of the pTiC58 *virG* mRNA. We also report the overproduction of VirG in *Escherichia coli* and its purification and demonstrate that VirG binds specifically to the upstream regions of the *vir* promoters and protects sequences in the vicinity of the *vir* boxes in DNase I protection assays.

MATERIALS AND METHODS

***virG* assay plasmids.** The pTiA6 *virG* was isolated as a 1.25-kilobase-pair (kb) *Sma*I-*Hin*dIII fragment from plasmid pSW104 (42) and was cloned into plasmid vector pUC119 (36) to construct pGP109. Plasmid pGP119 was constructed by introduction of the pTiA6 *virA* and a *virB-lacZ* gene

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fusion into the wide-host-range vector pTJS75 (3, 26). Both plasmids pGP109 and pGP119 were linearized at respective unique *Eco*RI sites and ligated together to construct pGP159. Plasmid pGP159 contains pTiA6 *virA*, *virG*, and a *virB-lacZ* gene fusion. This plasmid, when introduced into avirulent, Ti plasmid-less *A. tumefaciens* A136, supports synthesis of β-galactosidase in the presence of AS (3). All *virG* mutants were constructed in plasmid pGP109 or a derivative, fused to pGP119, and introduced into *A. tumefaciens* A136 for β-galactosidase assays.

pTiA6 *virG* mutant plasmids. Plasmids pGP206 and pGP207 were constructed by linearization of pGP109 with *Eco*47III, followed by ligation with 8-base-pair (bp) *Xba*I linker DNA (dCTCTAGAG) and by linearization of pGP109 with *Tth*III1, followed by mung bean nuclease treatment and ligation in the presence of *Xba*I linker DNA, respectively.

The procedure of Kunkel (12) was used for site-specific mutagenesis. Five deoxyoligonucleotides were purchased from Genetics Designs Inc., Houston, Tex. The oligonucleotides and the changes they introduce are as follows: oligonucleotide A (oligo A), dAAGCGCTGTTAACAAGGGT (GTG → GTT change); oligo B, dTTTTAGATTTAAAGG TGAG (first TTG → TTT); oligo C, dTGAGCCGTTTAA ACACGTT (second TTG → TTT); oligo D, dTGAGCCGT TAAAACACGTT (second TTG → TTA); and oligo E, dGGTGAGCCGATGAAACACG (second TTG → ATG). Single-stranded pGP109 DNA (sspGP109 DNA) was mutagenized with oligo A, B, C, or E to yield plasmid pGP156.1, pGP172, pGP173, or pGP194, respectively. sspGP161 (see below) or pGP173 DNA was mutagenized with oligo B or D to construct plasmid pGP174 or pGP185, respectively. Plasmid pGP195 was constructed by mutagenesis of sspGP174 DNA with oligo E. Plasmid pGP161 is a pGP156.1 derivative in which an *Xba*I linker DNA was inserted into the unique *Eco*47III site. All plasmids were later fused to pGP119 to construct the *virG* assay plasmids. In all cases, the orientation that places *virG* coding sequence divergent to the *virB-lacZ* gene fusion was selected. The *virG* mutant plasmids and their wide-host-range derivatives (in parentheses) are as follows: pGP109 (pGP159), pGP156.1 (pGP157.1), pGP161 (pGP165), pGP173 (pGP176), pGP174 (pGP177), pGP172 (pGP179), pGP180 (pGP187), pGP185 (pGP190), pGP194 (pGP196.1), pGP195 (pGP197), pGP206 (pGP209), and pGP207 (pGP210).

All mutants were confirmed by DNA sequence analysis according to the dideoxy-chain termination method on single- or double-stranded DNA templates, using Sequanase (United States Biochemical Corp.) (25).

pTiC58 *virG* mutant plasmids. The pTiC58 *virG* was isolated as a 2.9-kb *Eco*RI restriction fragment from cosmid clone pTHE7 (9) and was cloned into the *Eco*RI site of pUC119 to construct pGP204. sspGP204 DNA was mutagenized with oligo C to construct plasmid pGP214. sspGP214 DNA was mutagenized with oligo D to construct pGP216. The *virG* containing the 2.9-kb *Eco*RI fragment of plasmid pGP204, pGP214, or pGP216 was cloned into the *Eco*RI site of pGP119 to construct plasmid pGP217, pGP218, or pGP219, respectively.

pTiA6 *virG*-overproducing plasmids. The pTiA6 *virG* gene product was overproduced in *E. coli* from a *Salmonella typhimurium trp-virG* fusion plasmid. The *virG* structural gene was isolated as a 0.95-kb *Dra*I-*Hin*dIII restriction fragment from plasmid pGP172. pGP172 has a G → T mutation at position +113 (transcription initiation site, +1) that creates a unique *Dra*I site. The *Dra*I-*Hin*dIII fragment, after filling in with Klenow enzyme in the presence of deoxynucleoside triphosphates, was cloned into the *Sma*I site of pAD9 to construct pGP183. Plasmid pAD9, a derivative of pAD7 (5), contains the *S. typhimurium trp* promoter as a 151-bp *Hin*dIII fragment and the *E. coli rpoC* transcription terminator sequences (28) as a 225-bp *Eco*RI fragment in plasmid pUC8. The *trp* promoter fragment lacks the attenuator sequences (43) and therefore is constitutively expressed in a *trpR* host. Plasmid pGP198 was constructed in an analogous manner except that the *virG* sequences were isolated from pGP195. This plasmid contains an ATG start codon for *virG* instead of the wild-type TTG codon.

Plasmid pGP202 was constructed by cloning a 1.6-kb *Pvu*II fragment containing the *trp-virG* fusion of pGP198 into the *Sma*I site of pUC18 Chl (a chloramphenicol-resistant pUC plasmid). Plasmid pGP208 is a pGP202 derivative in which a 93-bp *Xho*I fragment within the *virG* structural gene was deleted. This deletion removes 31 amino acids from VirG and therefore is expected to yield a 210-residue polypeptide.

Plasmids pGP202 and pGP208 were introduced into *E. coli* GP3 by transformation to construct GP202 and GP208, respectively. *E. coli* GP3 (relevant genotype, *trpR lon-1*) was constructed by P1 *vir* transduction of a *trpR*::Tn*10* (obtained from C. Yanofsky) mutation into *E. coli* AB1899 (relevant genotype, *lon-1*) according to Miller (17).

Other plasmids. The wide-host-range derivatives of pGP183 and pGP198 were constructed by fusing these plasmids with *Eco*RI-cleaved pGP119. The two plasmids were designated pGP188 and pGP199, respectively.

Plasmid pAD1137 was constructed by cloning the 188-bp *Sau*3A fragment (14; A. Das, unpublished data) that spans from −183 to +5 (transcription initiation site, +1 [4]) of the *virA* gene into the *Bam*HI site of pAD1090. Plasmid pAD1138 contains a 330-bp *virB* fragment that spans the region from −108 to +221. An *Ava*II-*Hin*fI restriction fragment (37) containing this region, after filling in, was cloned into the *Hin*cII site of pUC7 to construct pAD1025. The insert was isolated as a *Bam*HI fragment and was cloned into the *Bam*HI site of plasmid pAD1090 to construct plasmid pAD1138. Plasmids pAD1139 and pAD1140 were constructed by cloning the 315-bp *Sau*3A fragment (11) that contains the region from −241 to +74 of the *virC* operon or −203 to +112 of the divergent *virD* operon into the *Bam*HI site of pAD1090. The orientation of this fragment, which places the *virC* promoter oriented toward the *rpoC* terminator, is designated pAD1139; the orientation that places the *virD* promoter oriented toward the *rpoC* terminator is designated pAD1140. Plasmid pAD1090 is a pUC13 Chl derivative in which a *Pvu*II-*Hin*dIII fragment containing the *E. coli lac* promoter sequences were deleted and the *E. coli rpoC* terminator sequences were cloned as an *Eco*RI restriction fragment. This plasmid contains a short duplication of the pUC13 polylinker region.

Plasmid pAD1073 contains a 201-bp fragment that spans from −146 to +55 of the *virG* gene. It was constructed by cloning an *Eco*RI-*Rsa*I restriction fragment from pSW104 (42) that had been filled in with Klenow enzyme into the *Sma*I site of pAD1072. Plasmid pAD1074 was constructed by cloning the filled-in 618-bp *Sal*I-*Stu*I fragment (38) containing from −193 to +425 of the *virE* operon into the *Sma*I site of pAD1072. Plasmid pAD1072 was constructed by cloning the 225-bp *Eco*RI fragment of pAD9 containing the *E. coli rpoC* terminator sequences into the *Eco*RI site of pGEM1 (Promega Corp.).

Purification of VirG. *E. coli* GP202 was grown overnight at 30°C in TB medium (34) containing 25 μg of chloramphenicol

per ml. The cells were diluted to an optical density at 600 nm of 0.1 in the same medium and grown for 12 h at 37°C. The cells were collected by centrifugation, washed twice with cold 0.8% NaCl, and frozen at −80°C. The cell paste was thawed on ice in protein buffer (25 mM Tris [pH 8.0], 0.1 mM EDTA, 0.02 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10% glycerol) and lysed in a French pressure cell at 10,000 lb/in^2. Inclusion bodies and the membranes were pelleted by centrifugation at 10,000 × *g* for 15 min at 4°C. The pellet was suspended in protein buffer and centrifuged twice to remove contaminating soluble proteins. The pellet was resuspended in protein buffer and was made 1% in Sequanal-grade sodium dodecyl sulfate (SDS) (Pierce Chemical Co.), 5% β-mercaptoethanol, and 10% glycerol. The proteins were solubilized by incubation at 37°C for 30 min, loaded onto a preparative 10% SDS-polyacrylamide gel (13), and electrophoresed overnight at 4°C. After staining of the gel with cold 0.25 M KCl–1 mM DTT the VirG band was excised and electroeluted (8). The electroeluted protein was dialyzed against protein buffer, made 50% in glycerol, and stored at −20°C.

DNA-protein binding assay. DNA-binding ability was measured by a gel retardation assay. The reaction mixture contained, in a total volume of 20 μl, 2.5 ng of radiolabeled DNA (see below), 50 mM KCl, 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 5% glycerol, 2 μg of bovine serum albumin, and VirG, as indicated. Incubation was for 15 min at 30°C. After incubation, the reaction mixture was loaded onto a 6% polyacrylamide gel. The gel was electrophoresed for 2 h at 400 V in 0.5× TBE, dried, and subjected to autoradiography.

Probe for examining binding to the *virA* promoter was prepared by digesting pAD1137 with *Apa*LI, *Eco*RI, and *Pvu*II, producing four fragments, of which the 0.7-, 0.23-, and 0.09-kb fragments were labeled. The 0.7-kb fragment contained the *virA* sequences. Plasmid pAD1138 was digested with *Eco*RI, *Hin*dIII, and *Hae*II to prepare probe for *virB*. This digest produced six fragments, of which 0.38-, 0.23-, 0.16-, 0.05-, and 0.03-kb fragments were labeled, and the 0.38-kb fragment contained the *virB* sequences. To examine binding to DNA from the divergent operons, *virC* and *virD* pAD1139 was digested with *Eco*RI, *Hin*dIII and *Hae*II, producing six fragments, of which the 0.37-, 0.23-, 0.16-, 0.05-, and 0.03-kb fragments were labeled. The 0.37-kb fragment contained the *virC* and *virD* sequences. This fragment migrated slower than expected in a native polyacrylamide gel. Probe for *virE* was prepared by digesting pAD1074 with *Eco*RI, *Hin*dIII, and *Hae*II, producing nine fragments, of which the 0.63-, 0.28-, 0.23-, and 0.1-kb fragments were labeled. The 0.63-kb fragment contained the *virE* sequences. Plasmid pAD1073 was digested with *Eco*RI and *Hae*II to prepare probe for *virG*. This produced eight fragments, of which 0.5-, 0.23-, and 0.1-kb fragments were labeled. Of these, the 0.5-kb fragment contained the *virG* sequences. All probes for gel retardation were end labeled by filling in with Klenow enzyme in the presence of [α-^{32}P]dATP. Because of different labeling efficiencies of the various ends, the specific activities of individual restriction fragments varied considerably.

DNase I footprinting. VirG was incubated with approximately 1 fmol of end-labeled pAD1073 or pAD1140 DNA as described above. The reaction mixtures were then cooled in ice, $MgCl_2$ was added to 5 mM, and 0.4 U of RQ1 DNase (Promega Corp.) was added. After 2 min at 30°C, the reactions were terminated by phenol-chloroform extraction and made 0.4 M in LiCl, and the DNA was precipitated with 2 volumes of ethyl alcohol, using 20 μg of glycogen as a carrier. The precipitate was dried and suspended in 95% formamide–20 mM EDTA with bromophenol blue and xylene cyanol. Samples were denatured by boiling and electrophoresed on a 6% urea–polyacrylamide DNA sequencing gel. After electrophoresis, the gels were dried and subjected to autoradiography.

The probe DNA was prepared by digestion of pAD1073 and pAD1140 DNA with *Xba*I and *Pst*I, followed by filling in with Klenow enzyme in the presence of [α-^{32}P]dCTP. Restriction endonuclease *Xba*I cuts pAD1073 DNA 166 bp upstream of the *virG* transcription initiation site and pAD1140 DNA 76 bp downstream of the *virC* transcription initiation site (which is also 205 bp upstream of the *virD* start site). *Pst*I cuts each of these plasmids just upstream of the *Xba*I site and cannot be radiolabeled by Klenow enzyme. A DNA sequencing reaction of M13mp18 DNA was electrophoresed simultaneously for sizing of DNA fragments.

Other procedures. Plasmids were mobilized from *E. coli* to *A. tumefaciens* A136 by triparental mating procedure (6). *A. tumefaciens* A136 is an avirulent strain that lacks a Ti plasmid. Agrobacteria were grown in ABmes medium (AB salts, 0.2% glucose, 25 mM phosphate, 25 mM morpholineethanesulfonic acid [MES] [pH 5.5]) for induction of the *vir* genes. β-Galactosidase enzyme activity was measured according to published procedures (40). Protein concentration was determined by the method of Bradford (Bio-Rad Laboratories).

Restriction and modification enzymes and deoxyoligonucleotide linkers were from New England Bio-Labs, Inc., and Pharmacia, Inc. Radioisotopes were from Amersham Corp.

RESULTS

Identification of the octopine Ti plasmid pTiA6 *virG* translation initiation codon. The DNA sequence of the *virG* locus of the *A. tumefaciens* Ti plasmid pTiA6 and the transcription initiation site for this *virG* mRNA have been determined (4, 31, 42). These analyses indicate that within the first 164 nucleotides of *virG* mRNA there are three commonly used translation initiation codons in the same open reading frame: two AUGs and a GUG at positions 48 to 50, 162 to 164, and 87 to 89, respectively (transcription start site, +1 [4]). None of these potential start sites are preceded by sequences characteristic of a ribosome-binding site (27) normally found to precede bacterial translation initiation sites. There are two UUG codons in this open reading frame as well. UUGs are used rarely as translation initiation codons in procaryotes (7, 15). The two UUGs in *virG* are located at positions 111 to 113 and 126 to 128. The second one is preceded by a putative ribosome-binding site sequence, AAGG, at positions 115 to 118. To overproduce the *virG*-encoded gene product (VirG) in *E. coli*, we considered it essential to identify the *virG* translation initiation codon. If the wrong translation initiation codon is assumed, an in vitro gene fusion could result in an N-terminal extension or deletion that could yield a functionally inactive product. The approach taken to identify the *virG* translation initiation codon was to modify all potential codons by insertional and site-directed mutagenesis and then assay for *virG* activity by measuring its ability to induce a chimeric *virB-lacZ* gene fusion in vivo in the presence of *virA* and AS.

To determine whether either of the two ATGs (for simplicity, the DNA sequence is referred to for discussion of the mutants) is used for *virG* translation initiation, insertion mutants were constructed. An 8-bp *Xba*I linker DNA was

inserted after the first ATG (pGP209) and just before the second ATG (pGP210). The linker insertion would cause a frameshift mutation if translation initiates upstream of the insertion site. The results (Table 1) were as follows. Whereas plasmid pGP209 encoded a functional VirG, that encoded in plasmid pGP210 was functionally inactive. As expected, the wild type VirG produced in plasmid pGP159 was functionally active. This result indicated that neither of the two ATG codons is used for *virG* translation initiation, leaving the GTG codon as a prime candidate. To test this possibility, the GTG was changed to GTT (pGP157.1). This change maintained the valine codon in the polypeptide sequence and did not affect *virG* activity either (Table 1). Therefore, the GTG codon is not the sole translation initiation site for *virG*. We considered the possibility that either the first ATG or the GTG codon could function as the translation initiation codon. To test this, we constructed a double mutant, plasmid pGP165, that contains the GTG → GTT mutation and an *Xba*I linker insertion blocking the first ATG. The VirG encoded by this plasmid was functionally active, indicating that neither the ATG nor the GTG serves as *virG* translation initiation site (Table 1).

Having ruled out the ATGs and GTG as *virG* translation initiation codons, we examined the rare initiation codons (TTGs). When the first TTG was changed to TTT (pGP179), no effect on *virG* activity was observed (Table 1). The same mutation at the second TTG codon (pGP176), however, completely abolished *virG* activity. This indicated that the second TTG at positions 126 to 128 is the *virG* translation initiation codon. The TTG → TTT mutation causes a Leu → Phe codon change. Therefore, the possibility remained that loss of *virG* activity in pGP176 may be due to this point mutation in a protein initiating translation upstream. To test this, we changed the second TTG to a TTA, maintaining this as a leucine codon (pGP190). This change greatly attenuated *virG* activity, confirming our earlier deduction that this codon is the *virG* translation initiation codon (Table 1). We then introduced a TTG-to-ATG mutation to study its effect on *virG* activity and for overproduction of VirG. The TTG → ATG change (pGP196.1) had little effect on *virG* activity (Table 1); however it was not induced as highly as was the wild type.

Analysis of the effect of multiple mutations on *virG* activity provided additional support for our conclusion that the second TTG is the *virG* translation initiation codon. Plasmid pGP177 has all potential translation initiation codons blocked except for the second TTG codon. Agrobacteria containing pGP177 exhibited normal induced levels of *virG* activity (Table 1). When this TTG was modified to TTT, as in pGP187, *virG* activity was almost completely lost. Changing the TTG to an ATG (pGP197) had little effect on *virG* activity.

Nopaline Ti plasmid pTiC58 *virG* initiates translation at a UUG codon. *virG* genes from pTiA6 and pTiC58 are highly homologous both in DNA sequence and in polypeptide sequence; each can complement a mutation in the other gene (21). There are some differences, however. The first potential translation initiation site of pTiC58 *virG*, an ATG codon, is located one codon downstream of the GTG of the pTiA6 *virG*. The GTG codon is not conserved, but both of the TTG codons (see above) are conserved. In a study analogous to that which identified the pTiA6 *virG* translation initiation site, we sought to determine whether the second TTG of pTiC58 *virG* functions as its translation initiation codon. To check this, the same oligonucleotides that were used to mutagenize the pTiA6 *virG* were used to introduce mutations at the second TTG codon of pTiC58 *virG*. These oligonucleotides also introduced an A-to-G change two codons upstream of the second TTG (Table 2). This additional mutation did not change the primary sequence of the encoded polypeptide. Mutations that changed the second TTG either to a TTT (Leu → Phe) (pGP218) or to a TTA (Leu → Leu) (pGP219) led to a complete loss of *virG* activity (Table 2). Since the TTT → TTA change would not change the primary sequence of the encoded polypeptide, these data indicate that the *virG* gene of pTiC58 also initiates translation at the second TTG codon.

Activation of VirG is necessary for its transcriptional activator function. The current model of *vir* gene induction is that VirA in response to plant phenolic compounds modifies VirG to activate it. Activated VirG then becomes a transcriptional activator that turns on the expression of all *vir* genes, including itself (39, 42). We wished to examine the role of AS in the modification of VirG without the complication presented by activated VirG inducing its own expression. To maintain a constant level of VirG in the cell in both the presence and absence of AS, we constructed a transcriptional fusion (plasmid pGP188) in which the pTiA6 *virG*

TABLE 1. Identification of the pTiA6 *virG* translation initiation codon

Plasmid[a]	Relevant sequences of 5′ transcribed region of *virG*[b]					β-Galactosidase activity (U)[c]		
	+50 ATG . . 12 codons . .	+89 GTG . . 7 codons . .	+113 TTG . . 4 codons . .	+128 TTG . . 11 codons . .	+164 ATG	−AS	+AS	Δ (fold)
pGP159						5.58	2,330	418
pGP209	X					6.61	2,260	342
pGP210					X	4.76	3.68	0.77
pGP157.1		GTT				10.9	2,610	239
pGP165	X	GTT				7.82	1,850	237
pGP179			TTT			4.09	2,430	594
pGP176				TTT		3.85	14.9	3.9
pGP190				TTA		4.16	69.8	16.8
pGP196.1				ATG		5.86	1,258	214
pGP177	X	GTT	TTT			3.97	1,890	476
pGP187	X	GTT	TTT	TTT		3.72	50.2	13.5
pGP197	X	GTT	TTT	ATG		9.90	1,930	195

[a] All plasmids were introduced into *A. tumefaciens* A136.

[b] Only potential start codon sequences are shown. Numbers at the top indicate residue number relative to the transcription initiation site (+1 according to reference 4). X, Insertion of an 8-bp *Xba*I linker DNA. The changes introduced by mutagenesis are also shown.

[c] Values are averages of three independent assays.

TABLE 2. Identification of the pTiC58 *virG* translation initiation codon

Strain	DNA sequences in region of 2nd TTG[a]	β-Galactosidase activity (U)[b]		Δ (fold)
		−AS	+AS	
A136(pGP217)	GGT GAA CCG TTG AAA CAC GTT Gly Glu Pro Leu Lys His Val	22.6	1,200	53
A136(pGP218)	GGT GAG CCG TTT AAA CAC GTT Gly Glu Pro Phe Lys His Val	2.30	1.98	0.9
A136(pGP219)	GGT GAG CCG TTA AAA CAC GTT Gly Glu Pro Leu Lys His Val	2.70	18.8	7.0

[a] The changes in DNA and protein sequences introduced by mutagenesis are underlined.
[b] Values are averages of three independent assays.

structural gene was fused to the *S. typhimurium* tryptophan (*trp*) operon promoter. This promoter was expressed in agrobacteria at a low level in ABmes minimal medium (Table 3) and was not induced by AS (data not shown). Thus, any AS-induced expression of *virB* in this strain will be due to the modification of VirG. A 13-fold increase in *virB-lacZ* expression was observed in the presence of AS in agrobacteria containing pGP188; cells containing plasmid pGP199, a plasmid containing ATG as the *virG* translation initiation codon instead of the wild-type TTG codon, showed a similar high level of induction (Table 3). However, the TTG → ATG change led to a 3- to 5-fold increase in both basal and induced levels of *virG* activity even though the net induction essentially remained the same. Thus, it appears that VirG can partially induce expression of the *vir* genes in the absence of modification, but for maximal expression, VirG modification is essential.

These constructs also provide additional support for our conclusion that the second TTG is the *virG* translation initiation site. Only 16 bp upstream of the second TTG codon of the pTiA6 *virG* gene are present in these fusion genes and as such lack the first ATG, GTG, and TTG codons. They are fused to the *trp* promoter in a manner such that there is no upstream ATG or GTG codons in frame. There is one TTG trinucleotide in frame in the *trp* sequences, but it is not preceded by a ribosome-binding site sequence and therefore would not be expected to serve as a translation initiation site.

Overexpression and purification of VirG. *E. coli* GP202 containing a *trp-virG* transcriptional fusion was used for VirG overproduction. VirG produced in this strain was sequestered into inclusion bodies and upon cell lysis was pelleted with the membrane fraction (Fig. 1, lane 1). To confirm that this protein was VirG, we constructed an in-frame deletion of 93 bp within the *virG* structural gene. The new plasmid, pGP208, did not synthesize the 27-kilodalton VirG protein but produced a new protein of 23 kilodaltons, as expected (Fig. 1, lane 2). For purification, the cell membrane fraction containing VirG was solubilized with SDS and electrophoresed on a preparative SDS-polyacrylamide gel. The VirG band was excised from the gel, and the protein was electroeluted. The protein obtained was estimated to be greater than 90% pure, with a small amount of contamination by OmpA (Fig. 1, lane 3). A yield of approximately 50% was obtained in these studies.

VirG is a sequence-specific DNA-binding protein. Genetic evidence indicates that VirG is a positive regulator of *vir* gene transcription. This property predicts that VirG may bind to the regulatory region sequences of the genes it controls. To study this activity in vitro, VirG binding to DNA restriction fragments containing promoter regulatory sequences of the inducible genes *virA*, *virB*, *virC*, *virD*, *virE*, and *virG* was examined by a gel retardation assay. Purified VirG was incubated with end-labeled DNA fragments, electrophoresed, and subjected to autoradiography. By comparing the levels of VirG required to retard a particular DNA fragment, the specificity of VirG binding could be assessed in this assay. VirG bound specifically to DNA restriction

TABLE 3. Activation of pTiA6 *virG* upon induction with AS

Strain	*virG* start codon	B-Galactosidase activity (U)[a]		Δ (fold)
		−AS	+AS	
A136(pGP188)	TTG	27.5	362	13.2
A136(pGP199)	ATG	99.1	1,759	17.8

[a] Values are averages of two independent assays.

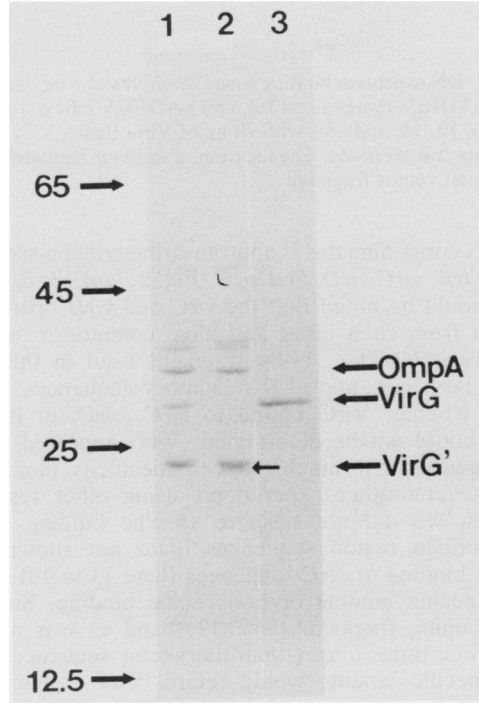

FIG. 1. Analysis of the overproduction and purification of VirG. Proteins were separated on a 12.5% SDS-polyacrylamide gel as described by Laemmli (13). Numbers at the left indicate positions of molecular size marker proteins in kilodaltons. Locations of VirG, the deletion derivative VirG', and OmpA are indicated. Lanes: 1, GP202 insoluble fraction, 20 μg; 2, GP208 insoluble fraction, 20 μg; 3, purified VirG, 2 μg.

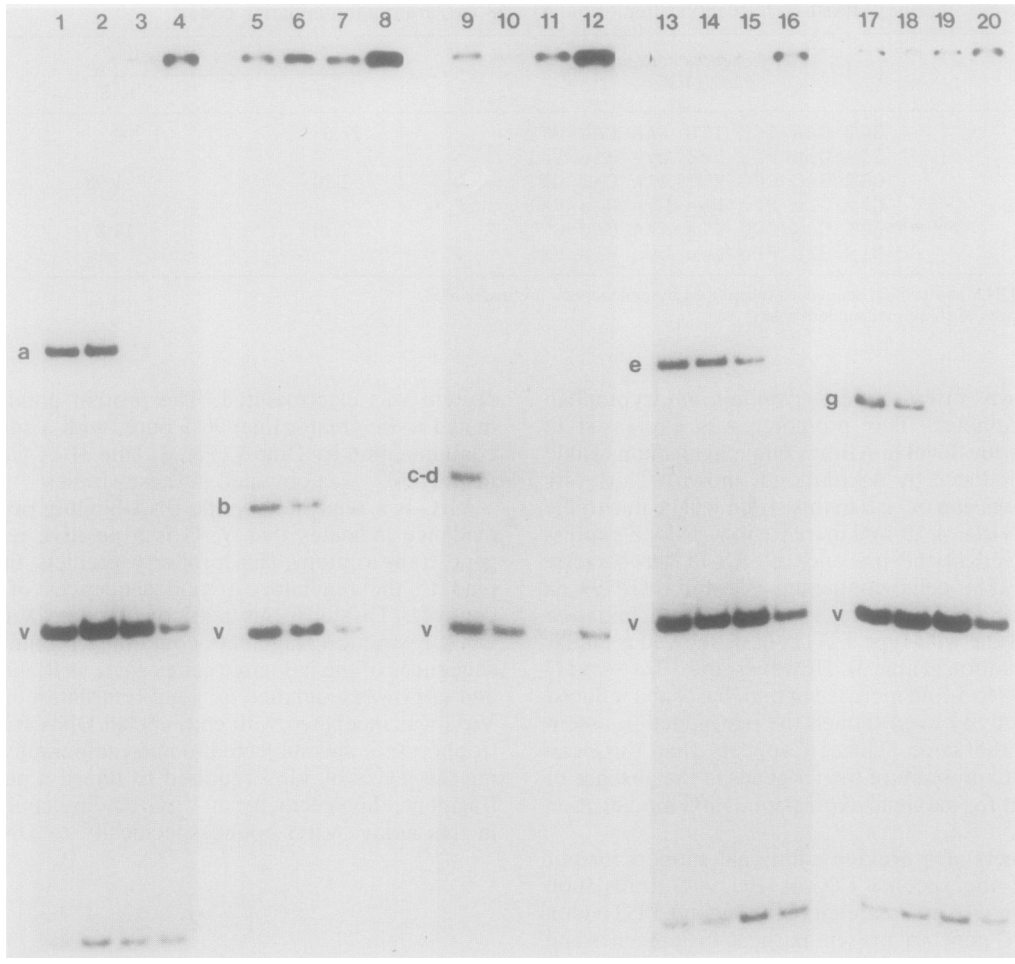

FIG. 2. DNA-protein binding assay. Samples (2.5 ng each) of end-labeled pAD1137 (lanes 1 to 4), pAD1138 (lanes 5 to 8), pAD1139 (lanes 9 to 12), pAD1074 (lanes 13 to 16), and pAD1073 (lanes 17 to 20) were incubated without VirG (lanes 1, 5, 9, 13, and 17), with 20 ng of VirG (lanes 2, 6, 10, 14, and 18), with 70 ng of VirG (lanes 3, 7, 11, 15, and 19), and with 140 ng of VirG (lanes 4, 8, 12, 16, and 20) as described in Materials and Methods. The location of each *vir* regulatory fragment is indicated on the left by the letter of the locus it is from. v, Location of a common vector fragment.

fragments containing the 5′ nontranscribed region sequences of *virA*, *virB*, *virC-virD*, and *virG* (Fig. 2, lanes 3, 6, 10, and 19). It should be noted that the *virC* and *virD* operons are divergent from each other and have common 5′ nontranscribed regions. The 315-bp fragment used in this study contains both *virC* and *virD* regulatory sequences. Consequently, whether VirG bound to *virC*, *virD*, or both sequences could not be determined. VirG appeared to bind *virE* sequences with much reduced specificity (lanes 13 to 16). In several other experiments using other restriction fragments, we did not observe specific binding to *virE* nontranscribed region sequences (data not shown). The low-level binding to *virE* sequences (lane 13 to 16) may be due to specific binding or nonspecific binding. Since the *virE*-containing fragment (lane 13, band e) was approximately three times longer than the vector sequences (band v), nonspecific binding would retard band e significantly more than it would retard band v. In comparison, the restriction fragment containing *virA* sequences (lane 1, band a) was slightly larger than the *virE* fragment (lane 13, band e). The *virA* fragment was completely retarded by 70 ng of VirG (lane 3, band a), whereas under similar conditions the *virE* fragment was only partially retarded (lane 15, band e). In all cases, we observed partial or complete retardation of other radiolabeled fragments at higher protein concentrations (lanes 4, 8, 12, 16, and 20). These results are in agreement with results previously obtained with other DNA-binding proteins. At a low VirG concentration (20 ng), almost complete retardation of the *virC-virD*-containing fragment was observed (lane 10, band c-d). Under similar conditions, only partial or no retardation of *virA*-, *virB*-, *virE*-, and *virG*-containing fragments was noted (lanes 2, 6, 14, and 18). This higher affinity of VirG for the *virC-virD* sequences may be due to the presence of the regulatory sequences of the two operons in the same restriction fragment or due to stronger binding to either or both sequences. The VirG used in these studies had a low level of OmpA contamination (Fig. 1, lane 3). To determine whether OmpA was contributing to DNA binding, we isolated VirG from an *E. coli ompA* strain and performed DNA-binding studies. No difference in DNA binding was observed in these studies (data not shown).

Gel retardation assays clearly showed that VirG bound specifically to DNA fragments containing the regulatory regions of *virA*, *virB*, *virC*, *virD*, and *virG*. To localize the VirG-binding sites on these fragments, a DNase I protection assay was used. End-labeled DNA fragments containing the regulatory regions of the *virC-D* and *virG* genes were incu-

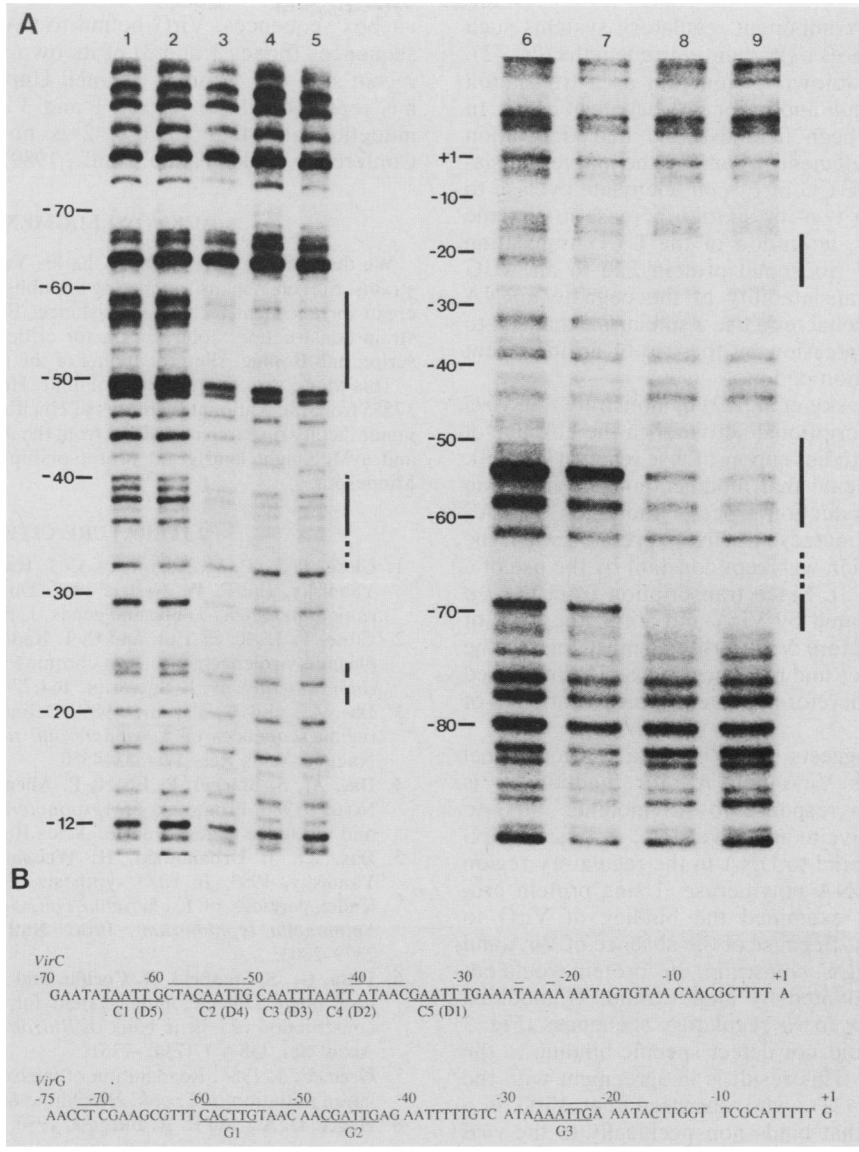

FIG. 3. DNase I footprinting analysis. DNase I footprinting of the *virC-D* regulatory region (A, lanes 1 to 5) and the *virG* regulatory region (A, lanes 6 to 9) and the relevant DNA sequences of the regulatory regions (B) are shown. (A) Numbers to the left indicate positions with respect to transcription initiation sites of *virC* and *virG* (+1 of reference 4). Solid bars to the right indicate the protected regions; broken bars indicate regions adjacent to protected areas that are deficient in DNase I cleavage products, as a result of which their protected or unprotected status could not be determined. DNA-binding reactions were performed in the absence of VirG (lanes 1 and 6), and in the presence of 20 ng (lanes 2 and 7), 200 ng (lanes 3 and 8), 2 μg (lanes 4 and 9), and 6 μg (lane 5) of VirG as described in Materials and Methods. A sequencing ladder was used to define the positions of the protected regions. (B) Sequence of the *virC* and *virG* 5′ nontranscribed region, numbered with respect to start of transcription. Protected regions are overlined, and the hexameric core regions of the *vir* boxes are underlined. Each *vir* box is named according to the locus and its position.

bated with VirG, treated with DNase I, denatured, and electrophoresed in a urea-polyacrylamide sequencing gel (Fig. 3A).

When the *virC-D* regulatory region sequences were used as a substrate, regions from −35 to −40 and from −50 to −59 were protected against DNase I digestion (Fig. 3A, lanes 3 to 5). Two additional bands at positions −22 and −25 were also protected. Similar studies using a fragment containing the *virG* regulatory sequences showed that regions from −16 to −27, from −48 to −60, and from −68 to −72 were protected (lanes 8 and 9). The DNA sequences of the 5′ nontranscribed regions, the sequences protected by VirG against DNase I degradation, and the locations of the central core regions of the *vir* box sequences are shown in Fig. 3B.

DISCUSSION

DNA sequence analysis by Winans et al. (42) predicted that *virG* of pTiA6 could encode a polypeptide of 267 amino acids. This prediction was based on the assumption that the first AUG of the largest open reading frame is used for *virG* translation initiation. We have shown that a UUG codon, located 26 codons downstream of the first AUG codon, is used to initiate *virG* translation (Table 1). A UUG codon located in an analogous position is also used to initiate translation of the *virG* mRNA of pTiC58 (Table 2). Thus, both *virG*s encode polypeptides of 241 amino acid residues and have predicted molecular masses of 27.1 kilodaltons. These proteins are highly homologous to each other and to

other members of two-component regulatory systems such as OmpR, SfrA, and PhoB over their entire lengths (24, 42).

The UUG codon is known to function as a translation initiation codon in *E. coli* and other procaryotes (7, 15). In several cases, it has been demonstrated that translation initiation at UUG is inefficient. Changing the native translational initiation codon UUG of *E. coli* adenylate cyclase to GUG or AUG led to a two- or sixfold increase in enzyme activity (22). Similarly, alteration of the UUG translation initiator of the *E. coli* ribosomal protein S20 to an AUG appears to increase translatability of the cognate mRNA (19). It is likely that agrobacteria use a similar mechanism to down-regulate *virG* expression by the use of an inefficient UUG translation initiation codon.

The studies of Rogowsky et al. (23) demonstrate that *virG* can function as a transcriptional activator in the absence of induction by AS. Our studies support these results (Table 3). These results also indicate that modification of VirG is an important part of the induction process. Induction with AS caused a 12- to 15-fold increase in *virB* expression when the level of *virG* transcription was kept constant by the use of a *trp-virG* fusion (Table 3). Since transcription from the *trp* promoter is not modulated by VirA and VirG, the level of *virG* mRNA, and therefore VirG protein, must remain the same in both the absence and presence of AS. The increased activity of VirG must therefore be due to a modification(s) of the protein.

Genetic evidence suggests that VirG is a transcriptional activator that requires VirA and AS for function. It is proposed that VirA in response to AS modifies VirG to change it from an inactive to an active state. Activated VirG would be expected to bind to DNA in the regulatory region and to interact with RNA polymerase. Using protein produced in *E. coli*, we examined the binding of VirG to inducible *vir* promoters. Because of the absence of VirA and AS in the overproducing *E. coli* strain, this protein would not be expected to be activated by modification. Unmodified VirG bound specifically to *vir* regulatory sequences (Fig. 3 and 4). However, we did not detect specific binding to the *virE* regulatory region. This result is in agreement with the data of Powell et al. (21), who observed that VirG is a DNA-binding protein that binds nonspecifically to the *virE* regulatory region. The reason for the lack (or reduced level) of VirG binding to *virE* sequences is not apparent. *vir* gene induction studies indicate that *virE* is induced to a high level (30), yet VirG did not show significant binding to a restriction fragment containing the *virE* regulatory region sequences. Several plausible explanations can account for this. First, the VirG used in this study is probably in the unmodified form, although some modification by an *E. coli* homolog of VirA, such as EnvZ, cannot be ruled out. Modified VirG may have a higher affinity, greater specificity, or both. Second, the position of the *vir* box relative to the promoter (−10, −35 region) sequence is important for induction of at least one *vir* gene, *virB* (3). A combination of the location of *vir* box sequence and binding affinity for a *vir* box sequence(s) together probably control the induction of individual *vir* genes.

DNase I protection studies showed that the binding sites of VirG lie around the *vir* box sequences of *virC-D* and *virG*. Both *virC* and *virG* contain multiple *vir* box sequences (Fig. 3B). DNase I footprinting analysis showed that VirG does not bind to all of these sequences. One binding site of VirG mapped to the second of the five *vir* box sequences of *virC*. The second site (−36 to −41) mapped to sequences between the highly conserved hexameric region (dCAATTG) of two *vir* box sequences. VirG bound to two of the three *vir* box sequences (boxes 1 and 3) of its own upstream region. In a recent study, S. Winans (Cornell University, Ithaca, N.Y.) has reported that *vir* boxes 1 and 3 are required for *virG* induction and that *vir* box 2 is not (Fallen Leaf Lake Conference, Lake Tahoe, Calif., 1989).

ACKNOWLEDGMENTS

We thank Steve Farrand and Charles Yanofsky for cosmids and strains, Steven Winans for sharing unpublished results, Mark Mersereau for excellent technical assistance, Betsy Kren for help with strain construction, John Larkin for critical reading of the manuscript, and Bonnie Allen for typing of the manuscript.

This work was supported by Public Health Service grant GM 37555 from the National Institutes of Health. A.D. is the recipient of junior faculty research award 170 from the American Cancer Society and a McKnight land grant professorship from the University of Minnesota.

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