The Octopine-Type Ti Plasmid pTiA6 of *Agrobacterium tumefaciens* Contains a Gene Homologous to the Chromosomal Virulence Gene *acvB*

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Although the majority of genes required for the transfer of T-DNA from *Agrobacterium tumefaciens* to plant nuclei are located on the Ti plasmid, some chromosomal genes, including the recently described *acvB* gene, are also required. We show that AcvB shows 50% identity with the product of an open reading frame, designated *virJ*, that is found between the *virA* and *virB* genes in the octopine-type Ti plasmid pTiA6. This reading frame is not found in the nopaline-type Ti plasmid pTiC58. *acvB* is required for tumorigenesis by a strain carrying a nopaline-type Ti plasmid, and *virJ* complements this nontumorigenic phenotype, indicating that the products of these genes have similar functions. A *virJ-phoA* fusion expressed enzymatically active alkaline phosphatase, indicating that VirJ is at least partially exported. *virJ* is induced in a VirA/VirG-dependent fashion by the *vir* gene inducer acetosyringone. Primer extension analysis and subcloning of the *virJ-phoA* fusion indicate that the acetosyringone-inducible promoter lies directly upstream of the *virJ* structural gene. Although the roles of the two homologous genes in tumorigenesis remain to be elucidated, strains lacking *acvB* and *virJ* (i) are proficient for induction of the *vir* regulon, (ii) are able to transfer their Ti plasmids by conjugation, and (iii) are resistant to plant wound extracts. Finally, mutations in these genes cannot be complemented extracellularly.

Gene duplication is a major driving force in molecular evolution. One copy of a duplicated gene often retains the original function, while the other copy is available to evolve new properties. Frequently, the products of duplicated genes may remain functionally similar, while their transcriptional regulation may diverge sharply, so that they are controlled by different regulatory proteins and induced by different stimuli. In cases where gene redundancy proves to be beneficial to the cell, both genes remain under selective pressure even though they code for nearly identical proteins. Examples of this include the *nodPQ* and *cysDNC* operons of *Rhizobium meliloti* (40), the *argF* and *argI* genes of the arginine biosynthetic pathway in *Escherichia coli* (18), the genes encoding the three different isozymes of acetohydroxy acid synthase in *E. coli* (44), and the *pel* genes of the plant pathogen *Erwinia chrysanthemi* (10).

We report here an apparent duplication of genes required for crown gall tumorigenesis by *Agrobacterium tumefaciens*. *A. tumefaciens* is a gram-negative plant pathogen that causes tumor formation on its hosts by transferring a fragment of DNA (T-DNA) from its Ti plasmid to the plant chromosome (53). This T-DNA directs the overproduction of (i) the phytohormones auxin and cytokinin, which cause proliferation of infected plant cells, and (ii) opines, which are used as nutrient sources by the colonizing bacteria (53). The two best-studied types of Ti plasmids are the octopine-type plasmids such as pTiA6 and pTi15955 and the nopaline-type plasmids such as pTiC58 and pTiT37.

Most of the genes required for virulence are encoded within the *vir* region of the Ti plasmid, although several genes required for tumorigenesis are located on the bacterial chromosome. Chromosomal virulence genes have a variety of functions, including attachment to the plant cell (3, 11, 31, 32) or induction of the *vir* regulon (4, 6, 19, 20, 30, 46). Still other chromosomal virulence genes have unknown functions. One

such gene, *acvB*, was the subject of a recent report (51). This gene was required for tumorigenesis on all plants tested but was not required for attachment to plant cells. A weak similarity was noted between *acvB* and an open reading frame, here designated *virJ*, found between *virA* and the *virB* operon of octopine-type Ti plasmids. Importantly, the characterization of *acvB* was carried out by using a strain containing the nopaline-type Ti plasmid pTiT37 (51). *virJ* is absent from a different nopaline-type Ti plasmid (pTiC58 [38]), and we provide evidence that *virJ* is absent from pTiT37 as well.

In this study, we correct two sequencing errors in *acvB* and extend the sequence to the correct 3' end of the gene. VirJ is strongly homologous to AcvB over 215 amino acids, rather than over 35 amino acids as previously reported. *virJ* can complement the tumorigenesis defect of an *acvB* mutant, indicating that the two proteins have at least some functional similarities. VirJ and AcvB have hydropathy profiles that suggest export from the cytoplasm, and we used a *virJ-phoA* fusion to provide evidence for export. This fusion was also used to show that *virJ* is induced by *vir* gene-inducing stimuli. Subcloning and primer extension experiments show that a VirG-regulated promoter lies directly upstream from the *virJ* structural gene.

MATERIALS AND METHODS

Enzymes, reagents, and bacterial strains. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories. Acetosyringone was purchased from Aldrich Co. Antibiotics, *o*-nitro-phenyl- β -D-galactoside (ONPG), morpholinoethanesulfonic acid (MES), isopropyl- β -D-thiogalactopyranoside (IPTG), and diethyl pyrocarbonate were purchased from Sigma. Modified T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemical Corp. Avian myeloblastosis virus reverse transcriptase was purchased from IBI. α -³⁵S-dATP, γ -³⁵S-dATP, and [γ -³²P]dATP were purchased from Amersham. A136 is a nalidixic acid-resistant, rifampin-resistant, Ti-plasmidless derivative of C58 (41). A348 and A208 are derivatives of A136 containing pTiA6NC and pTiT37, respectively (41). B119 is a derivative of A208 containing a Tn5 insertion in *acvB* (51). pTi19 and pTi226 are derivatives of pTiA6NC containing Tn3HoH1 insertions in *virG* and *virA*, respectively (42). UIA143 is a derivative of the Ti-plasmidless strain NT-1 containing an erythromycin resistance gene inserted at the *recA* locus (14). Plasmid pNU2 is a derivative of pUC18 containing the *acv* locus cloned as a 3.0-kb *EcoRI* fragment (51). SM10/ λ pir contains the

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pir gene from plasmid R6K and the RP4 *tra* operon on its chromosome (34). pSW191 is a derivative of pTZ18R containing a *P_{lac}-virA* fusion and the 5' half of *virJ* (49). pSW191.60::TnphoA, a derivative of pSW191 containing a TnphoA insertion in *virJ*, was previously isolated during TnphoA mutagenesis of *virA* (49).

DNA sequencing. Plasmids pNU2 and pSW191.60::TnphoA were used as templates for double-stranded DNA sequencing (39) using modified T7 DNA polymerase according to the manufacturer's recommendations, and using both dGTP and dTTP substrates. Oligonucleotide primers were synthesized at the Cornell Biotechnology facility. DNA sequences were analyzed with the computer programs Gene-Pro (Riverside Scientific Co.), Fasta (37), and Blast (1).

Heterologous complementation of an *acvB* mutation. *virJ* was subcloned as a 1.8-kb *SphI-XbaI* fragment into the broad-host-range, tetracycline-resistant plasmid pSW172, creating plasmid pVIK105. pSW172 and pVIK105 were introduced by electroporation into strain SM10/λpir. These plasmids were then introduced into strains A208 (*acvB*⁺) and B119 (*acvB*) by conjugation. The resulting four strains were used to inoculate *Kalanchoe diagraphomontiana* leaves as described previously (30). Photographs were taken 4 weeks after inoculation.

Analysis of *virJ* expression. A *virJ-phoA* fusion from pSW191.60::TnphoA was subcloned as a 4.5-kb *SalI* fragment into the broad-host-range plasmid pSW213 (7), creating plasmid pVIK106. This plasmid was introduced into *A. tumefaciens* A348, A348(pTi226), and A348(pTi19) by triparental mating (12) and assayed for alkaline phosphatase activity (29).

For primer extension analysis, strain A348(pVIK106) was grown to an optical density at 600 nm of 0.6 at pH 5.25 in the presence of 100 μg of acetosyringone per ml and at neutral pH in the absence of acetosyringone. RNA was isolated as described previously (43) from 10 ml of cells. Primer extension analysis (23) was carried out with avian myeloblastosis virus reverse transcriptase and the primer 5'-CGCTCTGCCGCGAGAAACAGT-3'.

Construction of an *acvB* mutant strain. A 540-bp internal fragment of *acvB* was created by PCR (21), using chromosomal DNA from strain A136 and the oligonucleotides 5'-CCCTCTAGAGACGAGACCCGCGCAAGGCG-3' and 5'-CGCGTTCGACCTTGTCGGAAG-3'. This PCR product contained an *XbaI* site at one end and a *SalI* site at the other end. This fragment was cloned into the *XbaI-SalI* site of the suicide vector pKNG101 (22), to create pVIK101. This latter plasmid was used to transform *E. coli* SM10/λpir (34). The vector pKNG101 encodes resistance to streptomycin, has a conditional origin of replication, and has the *sacB* gene, which mediates sucrose sensitivity to *A. tumefaciens* (17). Plasmid pVIK101 was mobilized into *A. tumefaciens* A136 by conjugation. Transconjugants putatively containing pVIK101 integrated into the *acvB* locus by Campbell insertion (34) were checked for sucrose sensitivity and were further characterized by Southern hybridization. The resulting strain is designated A136 *acvB101*.

Assays for *acvB* function. *vir* gene induction assays were carried out with plasmids pCH114 and pCH116 (5). pCH114 contains *virA*, while pCH116 contains a *P_{lac}-virG* fusion and a *virB-lacZ* fusion. These were introduced by electroporation into *A. tumefaciens* A136 and A136 *acvB101*. The resulting strains were assayed for acetosyringone-inducible β-galactosidase activity as described previously (47).

Ti plasmid conjugation assays were carried out with derivatives of strains A208(pSW172), A208(pVIK105), B119(pSW172), and B119(pVIK105) containing plasmid pCF251. pCF251 overexpresses the *traR* gene, a positive activator of Ti plasmid conjugation (16). The four strains were tested for their ability to transfer the Ti plasmid into the recipient *A. tumefaciens* strain U1A143 as described elsewhere (16). Transconjugants were selected on medium containing minimal agar lacking NH₄ with nopaline (167 μg/ml), arginine (2 mg/ml), and erythromycin (200 μg/ml).

The role of *acvB* in sensitivity to leaf extract was tested by using strains A208(pSW172), A208(pVIK105), B119(pSW172), and B119(pVIK105). Strain NM104(pUCD2) was used as a sensitive control (30). These strains were cultured to the exponential phase in minimal medium containing tetracycline (3 μg/ml) and assayed for sensitivity as described previously (30).

Extracellular complementation assays were carried out by inoculating *K. diagraphomontiana* wound sites with equal cell numbers of A136 and either A208(pSW172), A208(pVIK105), B119(pSW172), or B119(pVIK105) and scored for tumorigenesis as described above.

RESULTS

Homology between a Ti plasmid gene and an *A. tumefaciens* chromosomal gene. Octopine-type Ti plasmids such as pTiA6 contain an open reading frame, designated *virJ*, located between *virA* and *virB* (33). To learn more about this region of the plasmid, we used the Blast program (1) to translate its DNA sequence in all possible reading frames and compare the resulting protein sequences with those in the dynamically translated GenBank DNA sequence database. This program identified a similarity to the chromosomal *acvB* gene of *A. tumefaciens*. This was not surprising, as such a similarity was previously reported (51). However, the overall similarity be-

TAGAGTTGCGACGTGTGAGACTGGCAATCAGATTTTCGGTTCGGTGGAGCGGACGGGGG	60
AACTACATGAAAGATCGAAGCGCATAGGTTTCAGTGGATCAAGCCGGCGGACGGCGTTGA	120
TTTGTCCGATCAGGTCGGTCTGTTGCTTGAAGGGTTTACGCCACGATGACACCAC	180
CATCATTGGTGCCAGCAGATTGAAGTAGCCGGAACCGGTGCGGTCAAAAATCGGTGTGG	240
ACGACGGCTGTTGTACAAGAGACGACAGAACCACCGGACCGAGCTTCGCGAGCTTT	300
TGTATCCGTGGCATCCCTGGTTCAGGCGATTGTTTCAGTCCATGAGGCGCTCTCCAAAG	360
GGACCCATATTTTCCGTGCGAGCTTTCTGGTCTTCTTGTGGTGGACTTCTTGAAGTCC	420
CAGCATGGATGTTCTCTTTCGATTTCGAGATCTCAGCAAAAGTTCTCCGCAAACTTCAC	480
<div style="display: flex; justify-content: space-around; margin-top: 10px;"> VB I VB II VB III </div>	
TGCGGGCTTAGGTGTCTTTTGTAACTTGGCAGCAGCTGATATCCGCTTGAACATT	540
AGTCGGAATATCGAGATGTTCCGCTGACAAGGTATCGAATGCGGATAAAATGGTAT	600
M A I K L V L	
TGATACGCTATTTACACTGTTTCTCGCGGACGAGCTGCCTATGCGAATGACCGCGCA	660
I L V F T L F L A A D A A Y A N D R A N	
▽ <i>virJ60</i> ::TnphoA	
ATGGTGTGTCATGTCGTCGAGCGGGCGAAGCTGGAGTGGAGCTTCTCTTCGGGTTTCA	720
G V M W S N G G E A G V R L P L R V F N	
ATGCCAAGCCAGCAAGAACACGGTGGCGATCATTTATTCGGAGACGCTGGATGGCAAA	780
A K P A K A N T V A I I Y S G D A G W Q N	
ATATCGATGAGGTGATTGGTACC...	803
I D E V I G T . . .	

FIG. 1. The DNA sequence upstream of *virJ* and the 5' half of the *virJ* gene. The first three bases in the sequence represent the stop codon of *virA*. The boxed T residue represents the apparent transcription start site. Double-underlined residues are the putative -10 and -35 regions, while the single-underlined sequences represent the putative Shine-Dalgarno sequence and ATG start site. Overlined sequences are similar to a family of sequences denoted *vir* boxes that are possible binding sites for phosphorylated VirG protein. Also shown is the locus of the TnphoA insertion used to measure transcription. The region shown in gray is a repeated sequence also found on the T_C-DNA of the octopine-type Ti plasmid and on the pRL7JI plasmid from *R. leguminosarum*.

tween *AcvB* and *VirJ* was far stronger than that previously reported but shifted between the three reading frames in a way that suggested the presence of two reading frame mistakes either in *acvB* or in *virJ* (see below).

This search revealed another apparent sequence homology. A DNA sequence lying between *virA* and *virJ* (shown in gray in Fig. 1) is strongly similar to two sequences, one found between T_L-DNA and T_R-DNA (designated T_C-DNA) on octopine-type Ti plasmids (92% identity over 185 nucleotides) and the other found on plasmid pRL7JI of *Rhizobium leguminosarum* (87% identity over 200 nucleotides [28, 52]). We do not know the origin of these sequences but suspect that they could be mobile genetic elements. We also do not know whether these sequences encode any genes.

Two frameshift corrections in *acvB*. Codons 285 and 359 of *acvB* were suspected of containing reading frame errors. We used pNU2 (51) to determine the DNA sequence of these regions. Both of these regions showed compressions when the labelling and termination mixes contained dGTP (data not shown). In contrast, reaction mixtures containing dITP showed generally inferior sequencing ladders but clearly resolved two bases not reported in the original sequence: a C residue at codon 285 and another C residue at codon 359 (Fig. 2). The corrected *acvB* reading frame did not contain a stop codon within the published sequence. Therefore, the region downstream of the previously suggested end of *acvB* was sequenced. The correct stop codon lies 125 codons downstream from the published stop codon. The two frameshift corrections resulted in a dramatic increase in the similarity between *AcvB* and *VirJ*.

FIG. 2. DNA sequence of the two regions (codons 285 and 359) of the *acvB* gene. Both the top and bottom strands were sequenced as indicated. Arrows indicate the positions of residues not originally reported.

Alignment of AcvB (456 amino acid residues) with VirJ (255 amino acid residues) showed 50% identity over a 215-amino-acid overlap (Fig. 3). Virtually the entire length of VirJ aligns with the carboxyl-terminal half of AcvB.

Heterologous complementation of *acvB* by *virJ*. To determine whether AcvB and VirJ are functionally similar, we tested whether a cloned copy of *virJ* can complement the tumorigenesis defect of an *acvB* mutant. *A. tumefaciens* A208 is a wild-type strain containing the nopaline-type Ti plasmid pTiT37, and it was not known whether this Ti plasmid encoded a functional *virJ* gene. B119 is a derivative of A208 containing a Tn5 insertion in *acvB*. Plasmid pVIK105 is a derivative of pSW172 containing the *virJ* gene from pTiA6. Each plasmid was introduced into both A208 and B119 by conjugation, and the resulting four strains were used to inoculate *K. diagramon-tiana* leaves. As seen in Fig. 4, B119(pSW172) failed to produce tumors (with the exception of one extremely small, spherical tumor on one of the five inoculated leaves), while B119(pVIK105) was fully virulent, indicating that a cloned copy of *virJ* fully restores virulence to the *acvB* mutant. This result also provides strong genetic evidence that pTiT37 does not contain a functional *virJ* gene.

VirJ export. The hydropathy profiles (25) of VirJ and AcvB suggest that both proteins may have signal sequences and might therefore be exported from the cytoplasm (data not shown). It was previously reported (49) that a *TnphoA* insertion in or near *virJ* showed constitutive alkaline phosphatase activity in *E. coli*. The plasmid carrying that *TnphoA* insertion,

FIG. 3. Amino acid sequence alignment of VirJ with AcvB.

Strain	Genotype		
	<i>acvB</i>	<i>virJ</i>	
A. A208(pSW213)	+	—	
B. A208(pVIK106)	+	+	
C. B119(pSW213)	—	—	
D. B119(pVIK106)	—	+	

FIG. 4. Heterologous restoration of virulence of an *acvB* mutation by a cloned copy of *virJ*.

pSW191.60, contains *virA* and the first 80 codons of *virJ*. We determined the point of insertion of this *TnphoA* by using a DNA primer that hybridizes to the left border of *TnphoA* and found that the *phoA* gene was fused in frame to codon 39 of *virJ*. The fact that a *virJ-phoA* fusion synthesizes enzymatically active alkaline phosphatase supports the hypotheses that *virJ* encodes a protein and that this protein is fully or partly exported.

Transcriptional regulation of *virJ*. The fact that *virJ* is located in the *vir* region of the Ti plasmid raised the possibility that it might be controlled by the VirA/VirG two-component system, as are all other known *vir* promoters (50). To determine whether *virJ* expression is so regulated, we subcloned this *virJ-phoA* fusion (and the *neo* gene of *TnphoA*) as a 4.5-kb *SalI* fragment into the broad-host-range plasmid pSW213 (7), creating plasmid pVIK106. This plasmid contains 297 bp of Ti plasmid DNA, including only 180 bp upstream of the probable *virJ* start codon. pVIK106 was introduced into strains A348 (containing a wild-type octopine-type Ti plasmid), A348(pTi19) (containing a *virG* null mutation), and A348(pTi226) (containing a *virA* null mutation). As seen in Table 1, alkaline phosphatase activity was strongly stimulated by acetosyringone in A348 (pVIK106). Induction occurred only at acidic pH and required functional copies of *virA* and *virG*. pVIK106 fortuitously contains a *P_{lac}* promoter just upstream of the *virJ* promoter, and expression of this promoter is induced by IPTG, since the plasmid also contains *lacI^q*. Addition of IPTG caused alkaline phosphatase expression from all strains containing pVIK106 but not from the strain containing pSW213 (Table 1). From these results, we conclude that *virJ* is expressed from a VirG-inducible promoter located directly upstream of the structural gene.

To localize this promoter more precisely, we carried out primer extension analysis. A primer extension product was visualized by using RNA from acetosyringone-induced cultures but was not seen with RNA from uninoculated cultures (Fig. 5), indicating that the induction described above occurs at the level of transcription rather than at some posttranscriptional step. Although these experiments do not prove that acetosyringone (as opposed to acidic media) is required for transcription, the data described above for a *virJ-phoA* fusion indicate that this is the case. The apparent transcription start site lies 24 nucleotides upstream of the putative translation start of *virJ*. This start site, -10 and -35 motifs, three possible VirG bind-

TABLE 1. Transcriptional induction of a *virJ-phoA* gene fusion

<i>A. tumefaciens</i> strain	<i>virA</i>	<i>virG</i>	<i>phoA</i> fusion	AS ^a	Acidic pH ^b	Alkaline phosphatase activity ^c (U)	IPTG ^d	Alkaline phosphatase activity (U)
A348(pVIK106)	+	+	+	+	+	48	+	62
A348(pVIK106)	+	+	+	—	+	2	+	11
A348(pVIK106)	+	+	+	+	—	2.5	+	ND ^e
pTi226(pVIK106)	—	+	+	+	+	3.5	+	10
pTi19(pVIK106)	+	—	+	+	+	1.5	+	9.5
A348(pSW213)	+	+	—	+	+	1.5	+	1

^a +, addition of 100 µg acetosyringone (AS) per ml; —, no acetosyringone.^b +, pH 5.25; —, pH 7.0.^c Determined as described by Manoil and Beckwith (29).^d +, addition of 1 mM IPTG; —, no IPTG.^e ND, not determined.

ing sites (36, 48), and a possible ribosome binding site are shown in Fig. 1.

Assays for AcvB or VirJ function. In an effort to learn more about the functions of AcvB and VirJ, we undertook a variety of genetic experiments. An *acvB* mutation was constructed in strain A136 by Campbell insertion mutagenesis (see Materials and Methods), creating strain A136 *acvB101* (this strain does not have a Ti plasmid and therefore does not contain *virJ*). To determine whether *acvB* is required for *vir* gene induction, we introduced plasmids pCH114 (which contains *virA*) and pCH116 (which contains *P_{lac}-virG* and a *virB-lacZ* fusion) into strains A136 *acvB101* and A136. β -Galactosidase activity was assayed from cultures grown at pH 7.0 and 5.25 with either 100 or 2 µM acetosyringone (conditions that induce the *virA/virG* regulatory system). No significant difference in β -galactosidase activity was observed between the two strains (data not shown). We conclude that *acvB* mutants block transfer at some step other than *vir* gene induction.

Since Ti plasmids contains two independent but homologous DNA transfer systems (T-DNA transfer to plant nuclei and conjugal transfer to bacterial recipients [13, 53]), we decided to check whether *acvB* mutants are deficient as conjugation donors of the Ti plasmid. Triparental mating (12) was used to introduce plasmid pCF251 into strains A208(pSW172), A208(pVIK105), B119(pSW172), and B119(pVIK105). pCF251 overexpresses the Ti plasmid *traR* gene, resulting in high levels of conjugation (16). These strains all contain a nopaline-type Ti plasmid and do not contain *virJ*. The four resulting strains were tested for their ability to transfer the Ti plasmid into an *A. tumefaciens* conjugal recipient. All four strains donated their Ti plasmids with

equal efficiencies, indicating that AcvB is not required for conjugal DNA transfer.

Another possible explanation for the avirulence of *acvB* mutants is that they could be hypersensitive to phytoalexins or other toxic compounds found at wound sites. Another chromosomal gene (*chvI*) was recently identified as having this property (6, 30). To test this hypothesis, A208(pSW172), A208(pVIK105), B119(pSW172), B119(pVIK105), and the *chvI* mutant NM104(pUCD2) were used to test for sensitivity to leaf extract (see Materials and Methods). Leaf extract did not inhibit growth of any strain except the sensitive control, indicating that AcvB is not required for viability in the wound environment.

Several *vir* mutations have the interesting property of being complemented extracellularly, either by providing a second *A. tumefaciens* strain expressing the protein (8, 35) or by expressing the protein in the plant host (9). We determined whether an *acvB*⁺, Ti-plasmidless strain (A136) could rescue the ability of an *acvB* strain to cause tumors. Each of the strains A208(pSW172), A208(pVIK105), B119(pSW172), and B119(pVIK105) was coinoculated with strain A136 into *K. diogeniana* wound sites. *acvB* mutants were not complemented by coinoculation.

DISCUSSION

The product of the recently identified virulence gene *acvB* was reported to be 38% identical over 35 amino acids with the putative product of the *virJ* gene found in the *vir* region of octopine-type Ti plasmids but not found in nopaline-type Ti plasmids (51). By aligning VirJ with all three reading frames of AcvB, we observed that two reading frame changes in either gene would extend this homology up to the end of the published *acvB* sequence. Sequencing the two regions of *acvB* where similarity with *virJ* shifted between reading frames confirmed that two errors had indeed been made in *acvB*. In addition, we sequenced the region downstream of the reported sequence and found that *acvB* is 125 codons longer than previously reported. AcvB and VirJ share 50% identity over a 215-amino-acid overlap. This means that virtually the entire VirJ protein (255 amino acids) is homologous with the carboxyl-terminal half of AcvB (456 amino acids). Heterologous complementation of an *acvB* mutation by *virJ* indicates that the two proteins share at least some degree of functional similarity. It is not known whether the amino-terminal half of AcvB has additional properties that are not conserved in VirJ.

We have shown that tumorigenesis by a nopaline plasmid requires either *acvB* or *virJ*. We do not know whether octopine

FIG. 5. Primer extension analysis of the *virJ* transcript, using a wild-type *A. tumefaciens* strain containing *virJ* on a low-copy-number plasmid. RNA was collected from cells cultured at pH 5.25 with 100 µM acetosyringone (lane 2) and from cells cultured at neutral pH without acetosyringone (lane 1).

plasmids have a similar requirement. In fact, *virJ* was previously reported to be dispensable for tumorigenesis (33). However, several lines of reasoning suggest that octopine plasmids do require this function. First, it is important to bear in mind that Ti plasmids are conjugal and therefore can in principle be found in diverse chromosomal backgrounds. The very fact that nopaline plasmids do not encode *virJ* suggests that *acvB* must be ubiquitous among agrobacteria. Otherwise, nopaline plasmids would be virulent only in some bacterial strains. To our knowledge, this has never been reported, although admittedly, few combinations have been tested. If *acvB* is ubiquitous among *A. tumefaciens* strains, then the observation that *virJ* is not required for tumorigenesis could be explained by heterologous complementation. Second, it seems highly unlikely that octopine plasmids would encode a function that only nopaline plasmids require. The very fact that octopine plasmids encode *virJ* suggests that it, like its homolog *acvB*, might play some important role in tumorigenesis.

The documentation of *vir* gene duplication forces us to reassess reports that certain other *vir* genes are dispensable for tumorigenesis (2, 24, 26, 45), as it may be that other *vir* genes are present in two copies. This would be reminiscent of the extensive gene duplication found in *Rhizobium* spp., both of *nod* genes required for nodulation of plant hosts and of a variety of other genes (27).

The hydropathy plot of VirJ suggests that its amino terminus might initiate Sec-dependent secretion from the cytoplasm. Analysis of an enzymatically active *virJ-phoA* fusion supported this hypothesis. The apparent lack of a second hydrophobic domain suggests that VirJ might be fully released from the cytoplasmic membrane rather than having a transmembrane topology. We do not know whether VirJ is periplasmic or exported across the outer membrane, but the lack of intercellular complementation provides suggestive evidence that the protein may not be released from bacterial cells.

This *virJ-phoA* fusion also permitted analysis of the transcriptional regulation of *virJ*. Expression of other *vir* genes is stimulated by phenolic compounds and requires the VirA/VirG two-component system (50). The expression of *virJ* requires exactly the same factors (Table 1). The transcriptional regulation of this gene seems highly appropriate given its location within the *vir* region and its function in tumorigenesis. Primer extension analysis (Fig. 5) showed that a transcript is made only under inducing conditions and that the apparent start site lies 24 nucleotides upstream from the translational start. As shown in Fig. 1, upstream of the probable initiation codon there are a ribosome binding site motif, -10 and -35 regions, as well as three possible VirG binding sites. One of these possible sites is located between the -35 and -10 regions. Such a position is unusual for a VirG binding site, although a site at this position is found in the P1 promoter of the *virG* gene (47). It is not known whether *acvB* is also regulated by VirG, although the lack of upstream *cis* elements similar to VirG binding sites suggests that it may not be.

The role of VirJ (and AcvB) in tumorigenesis is not known. The formation of a single tumor in one of five leaves inoculated with an *acvB* mutant could be interpreted to suggest that *acvB* mutants attenuate rather than abolish tumorigenesis, although this tumor may also have been caused by a reversion of the mutation. It was shown that VirJ and AcvB are not involved in the induction of the *vir* operon. Moreover, strains lacking *virJ* and containing a mutation in *acvB* are not sensitive to plant wound exudates, unlike *chvI* mutations (30). Mutations in *acvB* cannot be complemented extracellularly, unlike *virE* and *virF* mutations (8, 35). The fact that this protein is exported from the cytoplasm might suggest that it is mecha-

nistically involved in T-DNA transfer, even though it is not required for Ti plasmid conjugation.

It is far from clear how this presumptive gene duplication occurred, especially considering that it is not found in all Ti plasmids. Duplication might have occurred by a nonhomologous chromosomal breakage and reunion event or as the product of an aberrant transposition. The repeated sequence found upstream of *virJ* could even have played some role in such an event. It is also unclear why this duplication occurred. In other examples of gene duplication, the isofunctional genes are frequently subject to differential regulation. Examples of this include the *nodPQ* and *cysDNC* genes in *R. meliloti* (40), the *groEL* genes of *Bradyrhizobium japonicum* (15), the *argF* and *argI* genes of *E. coli*, and the three acetohydroxy acid synthases of *E. coli* (44). It would therefore not be surprising if transcription of these two genes responded to different environmental stimuli. In fact, there are no well-documented examples of a chromosomal gene that is activated by VirG, and the *acv* operon does not contain sequences similar to the family of VirG binding sites. It is therefore plausible that *acvB* is not regulated by VirG and that the induction of *virJ* by VirG during infection helps to ensure sufficient quantities of this protein for efficient crown gall tumorigenesis.

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