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The *chvH* Locus of *Agrobacterium* Encodes a Homologue of an Elongation Factor Involved in Protein Synthesis

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The virulence of *Agrobacterium tumefaciens* depends on both chromosome- and Ti plasmid-encoded gene products. In this study, we characterize a chromosomal locus, *chvH*, previously identified by Tn*phoA* mutagenesis and shown to be required for tumor formation. Through DNA sequencing and comparison of the sequence with identified sequences in the database, we show that this locus encodes a protein similar in sequence to elongation factor P, a protein thought to be involved in peptide bond synthesis in *Escherichia coli*. The analysis of *vir-lacZ* and *vir-phoA* translational fusions as well as Western immunoblotting revealed that the expression of Vir proteins such as VirE2 was significantly reduced in the *chvH* mutant compared with the wild-type strain. The *E. coli efp* gene complemented detergent sensitivity, virulence, and expression of VirE2 in the *chvH* mutant, suggesting that *chvH* and *efp* are functionally homologous. As expected, ChvH exerts its activity at the posttranscriptional level. Southern analysis suggests that the gene encoding this elongation factor is present as a single copy in *A. tumefaciens*. We constructed a *chvH* deletion mutant in which a 445-bp fragment within its coding sequence was deleted and replaced with an omega fragment. On complex medium, this mutant grew more slowly than the wild-type strain, indicating that elongation factor P is important but not essential for the growth of *Agrobacterium*.

Agrobacterium tumefaciens causes crown gall disease in a wide range of dicotyledonous plants. The disease, characterized by neoplastic transformation at the site of infection, results from the transfer and expression of oncogenes from the bacterium to susceptible plant cells (for a review, see reference 27). This transfer process is governed primarily by the products of the *vir* genes located on the Ti plasmid. These genes are tightly regulated and are expressed to a significant level only in the presence of plant signal molecules synthesized by wounded plant cells. The *vir* genes are transcriptionally regulated by the Ti plasmid-encoded VirA/VirG two-component regulatory system (26, 52). The VirA protein senses the plant signal molecules and then transduces the signal by phosphate transfer to the response regulator, the VirG protein. The activated VirG protein is a positive transcriptional activator of itself and all other Ti plasmid-encoded *vir* operons.

Numerous chromosomal virulence genes (*chv*) have also been shown to play important roles in the ability of *Agrobacterium* to transform plants (for a review, see reference 39). In general, the functions of chromosomal virulence genes have not been well elucidated, and mutations in these genes are pleiotropic. Consequently, their precise roles in tumor formation have been difficult to assess. An analysis of a limited number of *chv* mutants suggests that whereas *vir* genes on the Ti plasmid are dedicated solely to specific steps in the interaction of *Agrobacterium* with host plants, the chromosomal virulence genes play important roles in the general physiology of *Agrobacterium* and have been conscripted to play ancillary but significant roles in the interaction of this bacterium with its hosts.

The best-understood chromosomal virulence gene is *chvE*, which codes for a glucose-galactose periplasmic binding protein. It normally functions in the uptake of a number of monosaccharides into the bacterial cell and is also involved in chemotaxis towards these sugars. In the transformation process, this periplasmic protein interacts with these same monosaccharides, all of which are components of the plant cell wall. It then binds to the periplasmic domain of the VirA sensor molecule (26), a requirement for maximum activation of VirG and the subsequent activation of all Ti plasmid-encoded *vir* genes. Depending on the strain, *chvE* mutants are either avirulent or severely attenuated in tumor formation on a wide variety of host plants.

Another *chv* locus, *acvB*, is unusual in that some strains of *A. tumefaciens* have a functional copy (*virJ*) on the Ti plasmid. Only by studying a strain that lacks a copy on the Ti plasmid was this *chv* locus identified as one that is required for T-DNA transfer (29, 41, 54). The relationship between the chromosomal locus and its Ti plasmid counterpart is unknown, as is the precise role that this locus plays in tumor formation. However, the identification of functionally redundant loci raises the possibility that a similar situation may hold for other Ti plasmid or *chv* loci, which complicates their isolation and identification.

Some *chv* genes have homologues in other bacteria that also display close interactions with host cells, either plant or animal. *A. tumefaciens*, *Brucella abortus*, and *Sinorhizobium meliloti* all belong to the same α-2 subdivision of the proteobacteria according to 16S rRNA sequence analysis. These three genera require similar chromosomal loci to establish a relationship

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Agrobacterium strains		
A348	A136(pTiA6)	21
A6007	A348 Pho$^-$ Sm^r	11
A6880	A6007 *chvH*::Tn*phoA*	12
At13000	A348 Δ*chvH*::Ω fragment, contains pWT187kan	This study
At13001	At13000 without pWT187kan	This study
E. coli strains		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*ΔM15	Gibco-BRL
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	U.S. Biochemical Corp.
Plasmids		
pACL2	pRK7813 cosmid clone which complements A6880	12
pSW172	IncP broad-host-range vector with *lac* promoter	14
pSW213	IncP broad-host-range vector with *lac* promoter and *lacI*^{q}	14
pTC110	pUCD2 Δ*Pvu*II-*Eco*RV	13
pBBR1MCS-4	Broad-host-range vector	32
pUC18	Multicopy vector	55
pUC19Ω	*Sma*I Ω fragment in pUC19	10
pPR1068	pMAL-c2 derivative; *Nde*I at the start of MalE	New England Biolabs
pSP329	pTJS75 derivative with α-complementation group and multicloning site from pUC18, IncP, Tc^r	11
pSP329Gm	pSP329 derivative, IncP, Gm^r	D. M. Raineri
pJQ200SK	*sacB* suicide vector	44
pUCD2	IncW broad-host-range vector	16
pBSIIKS+.*Nde*I	pBluescriptIIKS+ derivative; *Nde*I at the start of *lacZ′*	9
pAB2002	Vector for transcriptional fusion with *lacZ*	7
pTC111	*virE2*::*lacZ* translational fusion in pTC110	T. C. Charles, unpublished
pTC112	*virB1*::*lacZ* translational fusion in pTC110	T. C. Charles, unpublished
pUFR047	IncW broad-host-range vector	17
pPR1068-E1+E2	P*tac-virE* operon in pPR1068	18
pUFR047-E1+E2	P*tac-virE* operon in pUFR047	18
pSL59	*virA-phoA* translational fusion	19
pTC234	7.4-kb *Kpn*I-*Sac*I fragment from pACL2 in pSP329	This study
pTC234Ω9A::Tn*5*-B20	Tn*5*-B20 insertion into *chvH* gene of pTC234	This study
pWT131	4.4-kb *Kpn*I-*Bam*HI fragment in pBluescriptIISK+	This study
pWT142	pUC18 carrying a 7.6-kb *Sal*I fragment from A6880, Km^r	This study
pWT151	2.0-kb *Eco*RI fragment of pTC234 in pSW172	This study
pWT154	P*tac-chvH* in pPR1068	This study
pWT155	P*tac-chvH* in pUCD2	This study
pWT158	2-kb *Hpa*I-*Cla*I fragment of pPR1068-E1+E2 in pBBR1MCS-4	This study
pWT159	P*tac-virE1 virE2-lacZ* transcriptional fusion in pBBR1MCS-4	This study
pWT160	*virG-lacZ* translational fusion in pTC110	This study
pWT179	P*tac*-driven *E. coli efp* in pPR1068	This study
pWT181	P*tac*-driven *E. coli efp* in pSP329Gm	This study
pWT183	P*lac-chvH* in pBSIIKS+.*Nde*I	This study
pWT187	1.6-kb *Pvu*II fragment containing P*lac-chvH* in pSW213	This study
pWT187kan	pWT187 plus a kanamycin resistance cassette	This study
pWT188	445-bp *chvH* coding region replaced by Ω in pWT183	This study
pWT191	pWT131 with the 1.29-kb *Nde*I-*Eco*RV fragment replaced by 2.8-kb *Nde*I-*Eco*RV fragment from pWT188	This study
pWT193	6-kb *Kpn*I-*Sst*I fragment from pWT191 in pJQ200SK	This study

between the bacteria and their hosts. One set of such genes required for the virulence of *A. tumefaciens* is a chromosomally encoded two-component regulatory system, *chvG* and *chvI* (12, 38). Insertion mutations in either *chvG* (the sensor histidine protein kinase) or *chvI* (the response regulator) render *A. tumefaciens* avirulent. Similar two-component regulatory systems critical for endosymbiosis or virulence were found in *S. meliloti* (15, 40) and *B. abortus* (48). The similarity of these two-component regulatory systems is accentuated further by the contiguous phosphoenol-pyruvate carboxykinase gene in all three species.

Another set of genes required for tumor formation by *A. tumefaciens* is *chvA*/*chvB*. These two genes are concerned with either the synthesis (*chvB*) or the transport (*chvA*) of a cyclic polysaccharide, β-1,2 glucan, into the periplasm. Both *S. meliloti* and *B. abortus* synthesize β-1,2 glucan, and both *ndvA* and *ndvB* are required for effective nodule invasion by *S. meliloti* (20). For *B. abortus*, the *cgs* gene, which complements an *S. meliloti ndvB* mutant and an *A. tumefaciens chvB* mutant, is also required for virulence (28).

In this report we characterize another chromosomal locus, *chvH*, previously shown to be required for tumor formation. We show that this locus encodes a homologue of the *Escherichia coli* elongation factor P (2, 3, 4).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* strains were grown in

MG/L (35) or induction medium (IM) (11) at either 22 or 28°C. *E. coli* strains were grown in Luria-Bertani medium (46) at 37°C. The following antibiotics were used at the indicated concentrations when added to solid medium (in micrograms per millimeter): for *A. tumefaciens*, kanamycin (100), gentamicin (100), and spectinomycin (250); and for *E. coli*, carbenicillin (100), kanamycin (30), gentamicin (5), spectinomycin (50), and tetracycline (15). These concentrations were reduced by one-half for liquid medium.

Construction of plasmids. Several plasmids were constructed to demonstrate that *chvH* is the only gene responsible for the defects of the *chvH* mutant. A 2.0-kb *Eco*RI fragment from pTC234 containing the *chvH* gene was cloned into pSW172 to give pWT151. A 1.4-kb *Nde*I-*Eco*RI fragment starting from the predicted start site of the *chvH* gene was ligated to a 5.3-kb *Nde*I-*Eco*RI fragment of pPR1068 to create pWT154. In this construct, the *chvH* gene is under the control of the P*tac* promoter. A 2-kb *Eco*RV fragment of pWT154 containing the *chvH* gene was cloned into pUCD2 to create pWT155.

To test whether the *E. coli efp* gene can complement the defects of strain A6880, we placed the *E. coli efp* gene under the control of the P*tac* promoter. Two primers, efp-1, GGCCATATGGCAACGTACTATAGCAAC, and efp-2, ACACTGCAGTTACTTCACGCGAGAGAC, were used to amplify the *efp* coding region from DH5α genomic DNA with *Pfu.* *Nde*I and *Pst*I restriction sites were introduced into primers efp-1 and efp-2, respectively. PCR amplification followed the usual methods using *Pfu*: denaturation temperature, 95°C for 45 s; annealing temperature, 55°C for 60 min; and polymerization temperature, 72°C for 2 min. The 0.57-kb *E. coli efp* PCR product (digested with *Nde*I and *Pst*I) was ligated with a 5.3-kb *Nde*I-*Pst*I fragment of pPR1068 to create pWT179. A 1.2-kb *Eco*RV-*Pst*I fragment of pWT179 containing the P*tac*-driven *efp* gene was cloned into pSP329Gm which had been digested with *Sma*I and *Pst*I to create pWT181.

To determine the transcription of the P*tac-virE* operon, pWT159 was constructed as follows. A 2-kb *Hpa*I-*Cla*I fragment of pPR1068-E1+E2 was cloned into *Sma*I- and *Cla*I-digested pBBR1MCS-4, giving pWT158. A 4.5-kb *Eco*RI fragment containing the *lacZ*-Gm^r cassette from pAB2002 was ligated with *Eco*RI-digested pWT158 to create pWT159.

DNA sequencing and analysis. Restriction fragments were subcloned into pBluescript II KS+ or pBluescript II SK+. All double-stranded DNA templates were prepared with a Qiagen kit. Sequencing was completed with universal forward and reverse primers as well as synthetic oligonucleotides deduced from already determined sequences (BRL). DNA sequencing was performed with a BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, Calif.), and the reactions were run on an ABI 377 automated DNA sequencer at the DNA sequence facility of the Biochemistry Department, University of Washington. Both strands were sequenced. DNA sequences were analyzed with GeneJockey. Protein homology searches were performed using the Blastp program at the National Center for Biotechnology Information.

To determine the precise insertion site of Tn*phoA* in the original *chvH* mutant A6880, we cloned the *phoA* and flanking *chvH* region. Total chromosomal DNA of A6880 was digested with *Sal*I, ligated with *Sal*I-digested pUC18, and transformed into strain DH5α, selecting for simultaneous resistance to carbenicillin and kanamycin. The resulting plasmid, pWT142, was used as the template to sequence across the insertion junction by using an oligonucleotide primer, 5′-A CCCGTTAAACGGCGAGCACCGCCGGG-3′ (part of the *phoA* sequence).

***vir* gene expression assays.** The reporter plasmid pTC111 is derived from pSM358cd (49, 50), which contains a Tn*3*HoHo1 insertion in the *virE2* gene. This resulted in the production of a VirE2-LacZ fusion protein. The reporter plasmid pTC112 is derived from pSM243cd (49, 50), which contains a Tn*3*HoHo1 insertion in the *virB1* gene, resulting in the production of a VirB1-LacZ fusion protein. pWT160 is derived from pSM321cd (49, 50) and contains a Tn*3*HoHo1 insertion in the *virG* gene, resulting in a chimeric VirG-LacZ fusion protein that has approximately 130 amino acids of the VirG protein located at the amino terminus. pSL59 is a *virA-phoA* translational fusion plasmid (19). The reporter plasmids were introduced into A348 and A6880 by electroporation. *vir* gene expression assays were performed basically according to published methods (43). Alkaline phosphatase assays were performed as described previously (19).

Protein gels and Western immunoblotting. Protein analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to standard protocols (1). Gels were either stained with Coomassie blue or processed for Western blot analysis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) using the Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad Laboratories) and detected with the ECL Western blotting analysis system (Amersham Life Science). To detect VirA and VirB proteins, proteins were transferred to nitrocellulose in a Tris-glycine-methanol transfer buffer using a Transblot apparatus (Hofer, San Francisco, Calif.). *vir* genes in *A. tumefaciens* were induced as described previously (11). A348 and A6880 cells were harvested from overnight cultures grown in MG/L medium and washed once with induction medium containing 200 μM AS. The cells were then diluted into fresh induction medium containing 200 μM acetosyringone (AS) to an optical density at 600 nm (OD_{600}) of 0.1 and then induced for 20 h at 28°C (or 16 h at 22°C for VirA and VirB proteins). Total crude extracts were prepared and subjected to electrophoresis (the polyacrylamide concentrations were 7% for VirA; 10% for VirB, VirE2, and VirD2; and 12% for VirG, VirJ, ChvE, and Ros) and immunoblotting analysis as described previously (6, 43). Polyclonal antibodies against proteins of VirA (a gift from S. Winans) or VirB (6), VirE2 and VirD2 (18), and VirJ (41) were used to detect Vir proteins. A monoclonal VirG antibody (a gift from S. Jin) was used to detect VirG. Antiserum specific to ChvE (43) or Ros (34) was used to detect ChvE and Ros, respectively. Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

Southern blot analysis. *A. tumefaciens* genomic DNA for hybridization analysis was prepared by a published method (12). Genomic DNA was digested with restriction enzymes, subjected to gel electrophoresis, and transferred to a Hybond-N+ membrane under alkaline conditions. The membranes were hybridized in Church buffer (0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA) at 65°C. Two 30-min washes were performed in 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for low-stringency conditions. For high-stringency washes, two 30-min washes in 0.5× SSC–0.1% SDS and one 30-min wash in 0.1× SSC–0.1% SDS were performed. Radiolabeled probes were prepared by a random oligonucleotide labeling procedure using the Ready To Go DNA labeling beads (without dCTP) from Amersham Pharmacia Biotech Inc.

Construction of strain At13000. To determine whether *chvH* is essential for viability of *Agrobacterium*, strains were constructed that contained a single functional copy of *chvH* under the control of the inducible *lac* promoter. The 1.4-kb *Nde*I-*Eco*RI fragment starting from the predicted start site of *chvH* was ligated with pBSIIKS+.NdeI digested with *Nde*I and *Eco*RI to create pWT183. In this construct the expression of *chvH* was under the control of the *lac* promoter. A 1.6-kb *Pvu*II fragment containing the P*lac*-driven *chvH* was cloned into blunt-ended *Eco*RI-digested pSW213, which can replicate in *Agrobacterium* and contains *lacI*^{q}, resulting in pWT187. A 1.6-kb *Bam*HI fragment containing a kanamycin resistance cassette was inserted at the *Bam*HI site of pWT187 to give pWT187kan.

To construct a deleted version of the *chvH* mutation, pWT183 was digested with *Hin*dIII and *Cla*I, deleting a 445-bp fragment within the *chvH* coding region. A 2-kb *Sma*I fragment containing the spectinomycin-resistant Ω cassette was inserted between these sites to give pWT188. The 2.8-kb *Nde*I-*Eco*RV fragment containing Ω was subcloned into the large *Nde*I-*Eco*RV fragment of pWT131 to create pWT191. The 6-kb *Kpn*I-*Sst*I fragment of pWT191 was treated with T4 DNA polymerase and cloned into *Sma*I-digested pJQ200SK, which resulted in construct pWT193. pJQ200SK contains two selection markers for the subsequent homologous recombination step: *aacC1*, conferring gentamicin (Gm) resistance, and *sacB*, conferring sucrose sensitivity. In construct pWT193, a 445-bp *Hin*dIII-*Cla*I fragment within the coding sequence of *chvH* was replaced by the Ω fragment. Both pWT187kan and pWT193 were transformed into A348, and Kan^r Sp^r colonies were selected (single crossover). The resulting strains had a total of three *chvH* copies: a functional *chvH* copy controlled by the *lac* promoter, a *chvH* wild-type gene, and a deleted version of the *chvH* gene replaced by the Ω fragment. To isolate strains that carried a single functional *chvH* gene controlled by the *lac* promoter, sucrose selection was carried out on these Kan^r Sp^r colonies. The Kan^r Sp^r colonies were grown overnight in MG/L medium containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and then spread onto AB plates (11) containing 5% sucrose, 1 mM IPTG, spectinomycin, and kanamycin. The Suc^r Kan^r Sp^r colonies were then tested for the loss of the Gm^r marker on plates containing IPTG. The Suc^r Kan^r Sp^r Gm^s colonies were further characterized by Southern blotting to identify the strain (At13000) which had a single functional *chvH* gene controlled by the *lac* promoter in a plasmid and a deleted version of *chvH* in the chromosome.

Assays for virulence. Virulence assays were performed on *Kalanchoe daigremontiana* leaves. *A. tumefaciens* cells were grown in liquid MG/L to mid-log phase, then pelleted by centrifugation, concentrated, and deposited onto leaves wounded with a toothpick. Inoculated plants were grown for 2 to 5 weeks before tumor formation was scored.

Nucleotide sequence accession number. The nucleotide sequence of the 4.4-kb *Kpn*I-*Bam*HI fragment carried on pTC234 has been submitted to the GenBank database and assigned accession number AF177860.

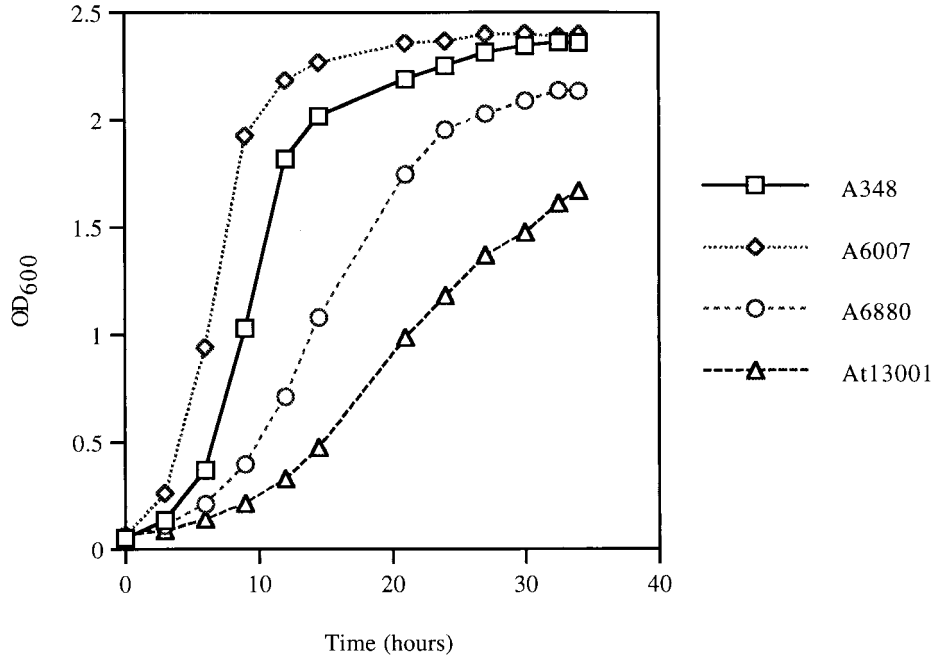

FIG. 1. Growth curves. At time zero, overnight cultures were diluted in MG/L at a starting OD_{600} of 0.06 and incubated at 28°C with shaking. At the indicated times, the OD_{600} was measured and plotted against time. The growth curves shown represent a typical experiment.

RESULTS

General features of *chvH* mutant A6880. The *chvH* mutant A6880, obtained by Tn*phoA* mutagenesis of A6007 (11, 12), is an avirulent, pleiotropic mutant. It is far more sensitive to detergents such as SDS, sodium deoxycholate, and Sarkosyl than the parent strain (12) and also to carbenicillin compared to the parental strain (data not shown). This suggests that the integrity of the outer membrane is impaired. The growth rate of the mutant on an enriched medium is somewhat slower than that of the wild-type strain (Fig. 1).

***chvH* locus encodes a protein similar to elongation factor P.** To gain insight into the possible function of *chvH*, we first isolated a cosmid clone, pACL2, which complemented the detergent sensitivity and restored virulence to *chvH* mutant A6880 (12). We subcloned a 7.4-kb *Kpn*I-*Sac*I fragment from pACL2 into pSP329 to make plasmid pTC234. This plasmid also complemented the *chvH* mutant. A partial restriction map of pTC234 was then constructed (Fig. 2). Tn*phoA* (36, 37) and Tn*5*-B20 (47) were used to mutagenize the 7.4-kb fragment in order to localize the region required for complementation of strain A6880. Several insertions in a 2.0-kb *Eco*RI fragment (the insert in pWT151) (Fig. 2) abolished complementation (data not shown).

The 4.4-kb *Kpn*I-*Bam*HI fragment of pTC234 was sequenced (GenBank accession number AF177860), and three open reading frames (ORFs) were identified (ORF1, ORF2, and ORF3) (Fig. 2). The first one, extending from nucleotides 771 to 2225, is preceded by a ribosome-binding site (GGGA AA) and encodes a putative protein of 484 amino acids with a predicted molecular mass of 51,000 Da. The second ORF (nucleotides 2346 to 2915, complementary strand) with its po-

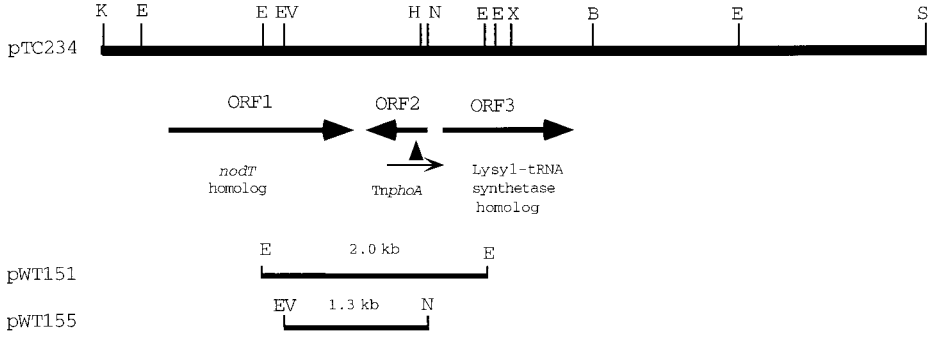

FIG. 2. Restriction map of *A. tumefaciens* chromosomal DNA inserts in pTC234, pWT151, and pWT155. Restriction sites are labeled as follows: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; S, *Sac*I; X, *Xho*I. The arrowhead under ORF2 indicates the site of the Tn*phoA* insertion, while the arrow underneath indicates its orientation.

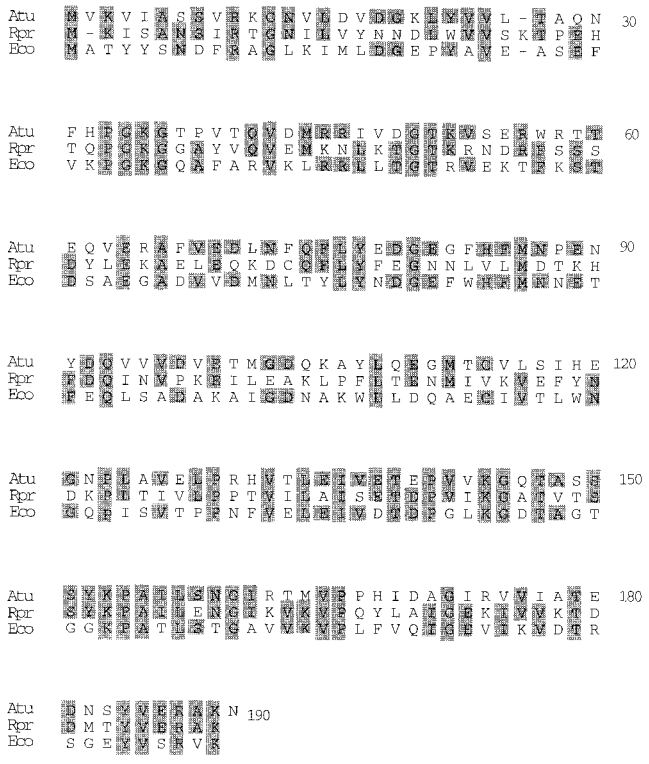

FIG. 3. Amino acid alignment of the *A. tumefaciens* (Atu) elongation factor P protein with the elongation factor P proteins from *R. prowazekii* (Rpr) (AJ235271) and *E. coli* (Eco) (X61676). Amino acids are represented by the single-letter code. The Malign program was used for the comparison. Amino acid positions are indicated on the right. Amino acid residues that are identical in two of the sequences are shaded.

tential ribosome-binding site (AGGAAG) encodes a putative protein of 189 amino acids with a predicted molecular mass of 21,000 Da. A third ORF was identified between nucleotides 3167 and 4231, which codes for a predicted protein of 354 amino acids with a molecular mass of 39,000 Da.

A database search of the predicted amino acid sequence of the three polypeptides revealed that ORF1 has a high level of identity with NodT from *Rhizobium leguminosarum* (52% identity and 68% similarity) (51). NodT is a member of a growing family of outer membrane proteins found in a wide variety of gram-negative bacteria, including several pathogens (42). ORF3 shows a high degree of similarity to lysyl-tRNA synthetase from a large number of bacteria.

ORF2 contains the site of the Tn*phoA* insertion of the avirulent mutant A6880, which has been designated *chvH*. Comparing the predicted amino acid sequence of ChvH with the protein database revealed that ChvH is similar at the amino acid sequence level to a range of elongation factor P proteins. Elongation factor P has been implicated in peptide bond synthesis (2). The predicted amino acid sequences for the *A. tumefaciens* ChvH and the elongation factor P proteins for *Rickettsia prowazekii* and *E. coli* are aligned in Fig. 3 using the Malign program. The identity and similarity at the amino acid level are 39 and 62% for *R. prowazekii* and 36 and 57% for *E. coli*, respectively.

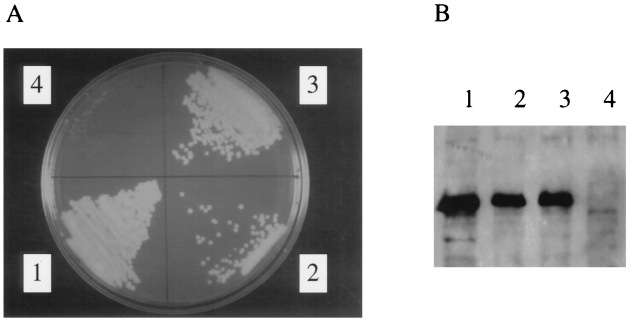

FIG. 4. (A) Functional complementation of *chvH* mutant A6880. Cells were grown on MG/L plates containing 0.2 g of SDS per liter at 28°C and observed after 3 days. (B) Measurement of VirE2 expression. *A. tumefaciens* strains were grown in IM containing 200 μM AS at 28°C for 20 h. Cells were pelleted, and total crude extracts were subjected to immunoblotting analysis as described in Materials and Methods. Strains: 1, A6007; 2, A6880(pWT155); 3, A6880(pWT181); 4, A6880(pSP329Gm).

Tn*phoA* is a transposon that can fuse alkaline phosphatase lacking a signal peptide to the amino-terminal sequences of the proteins into whose genes it inserts. Active fusions expressing alkaline phosphatase can arise only when this transposon inserts in genes encoding secreted or membrane-spanning proteins. Sequence analysis suggests that ChvH is a soluble protein. The Tn*phoA* insertion in A6880 is located between bp 123 and 124 of the *chvH* ORF, and *chvH* is in the opposite orientation relative to the *phoA* gene of Tn*phoA* (Fig. 2), although the insertion was initially identified after screening a collection of mutants that expressed alkaline phosphatase activity. However, when we checked colonies of A6880 growing on MG/L plates containing XP (5-bromo-4-chloro-3-indolylphosphate), they were white, as would be predicted. Two potential rho-independent transcriptional terminators (nucleotides 2244 to 2279 and 2299 to 2326) were identified, one downstream of the *chvH* gene and the other downstream of the *nodT* homologue. The isoelectric point of ChvH, calculated from the sequence, is 5.13.

Complementation of the *chvH* mutation. To prove that the Tn*phoA* insertion is actually responsible for the several defects observed in strain A6880, we complemented the mutation with *chvH*-containing subclones of the complementing cosmid. Two plasmids, pWT151 and pWT155, were constructed (see Fig. 2 and Materials and Methods). pWT151 contains the *chvH* gene under the control of its native promoter and flanking DNA sequences, while pWT155 contains the *chvH* gene under the control of the *tac* promoter. Both pWT151 and pWT155 complemented the detergent sensitivity (Fig. 4A) and restored virulence of A6880 on *K. daigremontiana* (for complementation by pWT155, see Fig. 5). These data strongly suggest that disruption of *chvH* is responsible for both the avirulence and detergent sensitivity of A6880.

We also tested whether the *E. coli efp* gene could complement the *chvH* mutation. The *efp* gene from *E. coli* was cloned under the control of the *tac* promoter to form plasmid pWT181. This gene (pWT181) complemented the detergent sensitivity of the *chvH* mutant strain (Fig. 4A) and increased the growth rate (data not shown). Most importantly, the *E. coli*

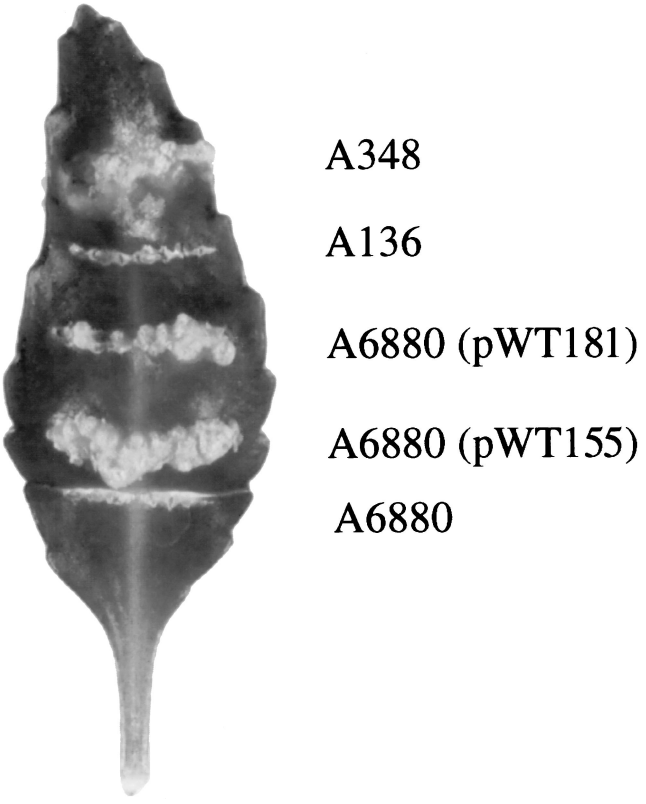

FIG. 5. Tumorigenesis assay on *Kalanchoe* leaves. The assay was performed as described in Materials and Methods.

efp gene (pWT181) restored virulence to A6880 (Fig. 5), although it took longer for tumors to appear and the tumors were smaller than those formed by the construct with the *tac* promoter-driven *chvH* itself (pWT155). Thus, the *tac* promoter-driven *E. coli efp* can phenotypically complement the *chvH* mutant, although the *E. coli* gene does not function optimally in *A. tumefaciens*. *chvH* and *efp* are functionally homologous.

Regulation of the *chvH* gene. Plasmid pTC234Ω9A::Tn*5*-B20 is a derivative of pTC234 in which Tn*5*-B20 has inserted into the 2.0-kb *Eco*RI fragment containing the *chvH* gene. The Tn*5*-B20 insertion also abolished the complementation ability of pTC234. Tn*5*-B20 forms an operon fusion with the gene into which it inserts. We transformed A348 with pTC234Ω9A::Tn*5*-B20. The cells were grown in (i) AB (pH 7.5) or AB plus 50 mM MES (morpholineethanesulfonic acid, pH 5.5) or IM with and without AS. The β-galactosidase activities were determined. The results showed that *chvH* gene is constitutively expressed, independent of acid conditions and phenolic compounds (data not shown).

Reduced expression of virulence genes in *chvH* mutant. Since the *chvH* mutation rendered cells avirulent, we investigated how well the Ti plasmid-encoded *vir* genes were expressed in the mutant. An appropriate level of expression of the *vir* genes is critical for tumor formation. Plasmid pTC112 containing the *virB1*::*lacZ* translational fusion was introduced into the *chvH* mutant and the wild-type strain. As shown in Table 2, expression of the *virB-lacZ* fusion was reduced 80% in the *chvH* mutant compared with the wild-type strain under the same conditions. We also introduced a *virE2-lacZ* translational fusion on a plasmid (pTC111) into the same strains. The VirE2-LacZ fusion protein in the *chvH* mutant was assayed under inducing and noninducing conditions. Under noninducing conditions, we observed that the basal level of VirE2-LacZ expression was reduced approximately 70% in the *chvH* mutant compared with the wild-type strain. Under inducing conditions, the expression of VirE2-LacZ was reduced approximately 85%.

The above results clearly show that the expression of VirB and VirE2 is reduced in the *chvH* mutant. Because the *vir* operons are under the control of the VirA/VirG two-component system, *chvH* might reduce the level of VirB and VirE2 proteins indirectly by affecting the expression of the two-component system. Therefore, the level of VirA was determined by assaying the *phoA* activity of a *virA-phoA* translational fusion (pSL59) in the two backgrounds. As shown in Table 2, the induction of VirA expression by AS in the wild-type strain agrees with previous observations (45, 53), while the expression of VirA was only induced slightly less in the *chvH* mutant. To measure VirG expression, we used *virG-lacZ* translational fusion plasmid pWT160. Under noninducing conditions, VirG-LacZ expression was reduced 40% in the *chvH* mutant (Table 2). In the presence of AS, the reduction was substantially greater (approximately 75%), probably in part because of the positive autoregulation of VirG (52) (Table 2).

We also analyzed the expression of *vir* genes by measuring the accumulation of several Vir proteins by Western immunoblotting. As shown in Fig. 6, the levels of VirB8, -9, -10, and -11 were reduced dramatically in the mutant cells compared with those of the wild-type strain. VirE2 and VirJ were undetectable in the *chvH* mutant. The level of VirD2 was markedly reduced but still detectable. The levels of both VirA and VirG were significantly reduced. The expression of VirE2 was also measured in the *chvH* mutant transformed with the P*tac*-driven

TABLE 2. Effect of *chvH* mutation on expression of *lacZ* and *phoA* translational fusions to *vir* genes[a]

Fusion (plasmid)	β-Galactosidase expression (Miller units)			
	chvH strain (A348)		*chvH*::Tn*phoA* strain (A6880)	
	IM	IM + 100 μM AS	IM	IM + 100 μM AS
virB-lacZ (pTC112)	ND	1,357 ± 5	ND	285 ± 6 (80%)
virE-lacZ (pTC111)	46.6 ± 1.0	3,208 ± 46	15.1 ± 1.0 (70%)	519 ± 15 (85%)
virG-lacZ (pWT160)	77.5 ± 1.5	1,536 ± 39	48.0 ± 2.4 (40%)	360 ± 10 (75%)
virA-phoA (pSL59)	6.9 ± 0.7	59 ± 1.5 (8.6)	6.9 ± 0.2	42 ± 1.0 (6)

[a] Data are means ± standard errors of the mean for three samples. The percentages in parentheses represent the reduction in expression in the *chvH* mutant relative to the wild-type strain. The numbers in parentheses are the fold increases in induction upon adding AS. The units are Miller units. ND, not determined.

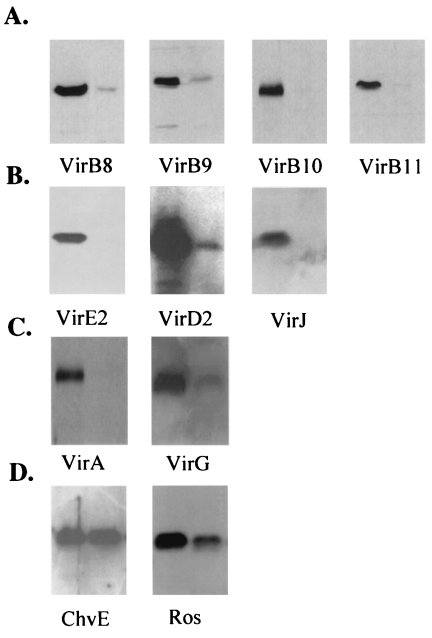

FIG. 6. Effect of the *chvH* mutation on the production of virulence-related proteins. *A. tumefaciens* strains A348 and A6880 were grown in IM containing 200 μM AS at 28°C for 20 h (or 22°C for 16 h for VirA and VirB proteins). Cells were pelleted, and total crude extracts were subjected to immunoblotting analysis as described in Materials and Methods. To detect VirB proteins, loading was standardized so that the samples in each lane represent equivalent numbers of cells. To detect other proteins, equal amounts of total protein from A348 and A6880 were loaded. The wild-type strain A348 is on the left, while the *chvH* mutant A6880 is on the right.

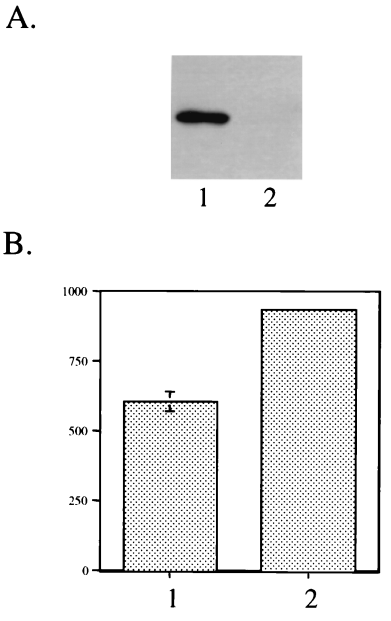

FIG. 7. (A) Measurement of VirE2 protein levels with a P*tac*-driven *virE* operon in A348 and A6880. Cells were grown to mid-log phase in MG/L at 28°C, and total crude extracts were prepared as described in Materials and Methods. Equal amounts of total crude extracts (20 μg) from each strain were loaded. Lanes: 1, A348(pUFR047-E1+E2); 2, A6880(pUFR047-E1+E2). (B) Determination of transcription level of the *tac* promoter in A348 and A6880. A348 and A6880 were transformed with the reporter plasmid pWT159. The cells were grown in AB medium at 28°C and harvested at an OD_{600} of about 1.0. β-Galactosidase activity (Miller units) was monitored as described in Materials and Methods.

chvH gene (pWT155) and the P*tac*-driven *E. coli efp* gene (pWT181). As shown in Fig. 4B, both the *A. tumefaciens chvH* gene (pWT155) and the *E. coli efp* gene (pWT181) restored the expression of VirE2.

Both kinds of analysis indicate that the expression of the two-component system, especially VirG, the transcriptional activator for all of the *vir* genes including itself, was reduced. Therefore, the reduced levels of Vir proteins could in part be explained by the reduced levels of the VirA and VirG proteins. We conclude that the wild-type *chvH* locus is essential for full expression of *vir* genes encoded by the Ti plasmid.

The levels of two chromosomally encoded proteins were also analyzed in the *chvH* mutant by Western blotting. As shown in Fig. 6, the level of the ChvE protein was about the same in the *chvH* mutant as in the wild-type strain, whereas the level of the Ros protein was significantly reduced. It appears that the level of different proteins is affected differently by the *chvH* mutation.

ChvH functions at the posttranscriptional level. It has been reported that elongation factor P is involved in peptide bond synthesis in *E. coli* (2, 3, 4). To confirm the involvement of ChvH at the posttranscriptional level, we measured the expression of the VirE2 protein in A348 and A6880. To bypass the VirA/VirG two-component system, which adds another level of complexity to the expression level, we used the P*tac-virE* operon construct pUFR047-E1+E2. Figure 7A shows that the synthesis of VirE2 was significantly reduced in the *chvH* mutant compared with the wild-type strain. When the transcription of the P*tac-virE* operon was determined by using *lacZ* transcriptional fusion plasmid pWT159, the transcription of the P*tac-virE* operon was not affected by the *chvH* mutation (Fig. 7B). Therefore, we conclude that ChvH functions at the posttranscriptional level. According to the data shown in Fig. 7B, translation of native β-galactosidase was not affected by the *chvH* mutation.

***A. tumefaciens* gene encoding elongation factor P is present as a single copy in the genome.** Aoki et al. (4) reported that in *E. coli*, the *efp* locus is essential for viability. The Tn*phoA* insertion in the *chvH* mutant is within the coding region of the gene encoding elongation factor P. These results raised the possibility that *Agrobacterium* might have two copies of the gene encoding elongation factor P.

A genomic Southern blot analysis was performed to determine if the gene encoding elongation factor P is a single copy. The 0.5-kb *Nde*I-*Cla*I internal fragment of the *chvH* gene was used to probe a Southern blot of A348 genomic DNA individually digested with a panel of restriction enzymes. Employing low-stringency wash conditions (see Materials and Methods), only those fragments expected from the restriction map of the *chvH* locus itself were observed (data not shown). This strongly suggests that only one copy of the gene encoding elongation factor P is present in the *A. tumefaciens* genome.

Is *chvH* essential for viability of *Agrobacterium*? The above data suggest that only one copy of the gene encoding elonga-

tion factor P is present in *Agrobacterium*. Therefore, two possibilities exist: (i) the gene encoding elongation factor P is essential for the viability of *Agrobacterium* but the Tn*phoA* insertion is leaky, and (ii) the gene is not essential for the viability of *Agrobacterium*. To distinguish between these two possibilities, we constructed a deletion mutant of *chvH* and characterized the resulting strain.

We first placed the *chvH* gene under the control of the IPTG-inducible *lac* promoter (in plasmid pWT187kan; see Materials and Methods). This construct was then transferred into *A. tumefaciens* A348 by electroporation. We then exchanged the wild-type copy of *chvH* with the deleted version of the *chvH* gene (a 445-bp *Hin*dIII-*Cla*I fragment was deleted) in the presence of IPTG, giving strain At13000 (see Materials and Methods).

To determine whether *chvH* is an essential gene, we first streaked out the At13000 cells on MG/L plates lacking IPTG to see whether the depletion of the ChvH protein stopped cell growth. The depletion did not stop cell growth. The cells formed colonies on solid medium and also increased in optical density in liquid medium, suggesting that *chvH* is not an essential gene. If this is indeed the case, it should be possible to cure strain At13000 of plasmid pWT187kan without affecting cell viability. To this end, we grew strain At13000 under nonselective conditions overnight in liquid medium and plated out the culture for single colonies on MG/L plates containing spectinomycin. Among 300 colonies, 20 colonies were kanamycin sensitive. These were candidates for cells that had lost the plasmid. Southern blot analysis of 10 of these colonies confirmed that they indeed did not contain pWT187kan (data not shown). The cured strain was named At13001. Since a strain which lacked the *chvH* gene could be isolated, the gene product is not essential for cell viability.

Elongation factor P is necessary for optimum growth of *A. tumefaciens*. We investigated the effect of elimination of the *chvH* locus on cell growth in rich medium, MG/L. As shown in Fig. 1, the deletion mutant At13001 grew significantly more poorly than the Tn*phoA* insertion mutant A6880, suggesting that the original mutant was leaky for elongation factor P activity. The deletion mutant also exhibited a longer lag time than the Tn*phoA* insertion mutant. The effect of an introduced wild-type *chvH* gene and the *E. coli efp* gene on the growth of strain At13001 was also studied. As expected, the *chvH* gene expressed from the P*tac* promoter (pWT155) increased the growth rate to nearly the level of the wild-type strain A348 (data not shown). Interestingly, the *E. coli efp* gene expressed from the P*tac* promoter (pWT181) increased the growth rate to about the same extent as the introduced *chvH* gene (data not shown). As expected, strain At13001 is avirulent. Both pWT155 and pWT181 restored virulence to strain At13001 (data not shown).

A 32-kDa protein accumulates in the *chvH* mutant. The effect of the *chvH* mutation in A6880 on the protein profile was examined on an SDS-PAGE gel. Figure 8 shows representative data for the patterns of soluble proteins in the *chvH* mutant A6880 and its parental strain A6007 when equal amounts of protein were loaded on the gel. The most striking difference is that a 32-kDa protein accumulated in the *chvH* mutant. Several other less striking differences were observed between the mutant and parental strains. The 32-kDa protein also accumulated in the *chvH* deletion mutant At13001 (data not shown). We are in the process of identifying this protein.

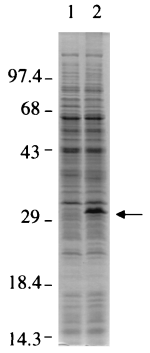

FIG. 8. Protein profiles of *chvH* mutant A6880 and its parental strain A6007. *A. tumefaciens* A6007 and A6880 were grown in IM containing 200 μM AS at 28°C for 20 h. Cells were pelleted, and total crude extracts were subjected to SDS-PAGE analysis. Equal amounts of total crude extracts (20 μg) from each strain were loaded. Lanes: 1, A6007; 2, A6880. The arrow indicates the 32-kDa protein.

DISCUSSION

In this study, we have characterized the *chvH* chromosomal virulence locus, which was originally identified by Tn*phoA* mutagenesis as being required for virulence. Sequence analysis indicates that *chvH* encodes a protein homologous to the elongation factor P protein of *E. coli* (2, 3, 4). This sequence homology is strongly supported by the observation that expression of the *efp* gene of *E. coli* can complement the *chvH* mutant and restore all of the phenotypic alterations resulting from the mutation in *Agrobacterium*. This is the first demonstration that an *E. coli* gene can complement the avirulent phenotype of an *A. tumefaciens* mutant and speaks to the highly conserved nature of this protein.

The exact role of elongation factor P in protein synthesis is not clear. It was originally isolated from a complex of 70S ribosome-AUG-formyl[^{35}S]Met-tRNA and added puromycin (22, 23). This factor stimulated the synthesis of *N*-formyl-methionyl-puromycin and was thought to be involved in peptide bond synthesis. Further studies indicated that elongation factor P was more effective in increasing the efficiency of peptide bond formation between formyl[^{35}S]Met-tRNA and amino acids with small *R* groups, such as glycine (24). A recent study indicates that bacterial elongation factor P is homologous to the eukaryal/archaeal eIF-5A (33), with the highest homology at the N-terminal 90 residues. Factor eIF-5A was originally isolated from a high-salt wash of rabbit reticulocyte ribosomes and was thought to be involved in translation initiation (31). eIF-5A enhanced the synthesis of methionyl-puromycin in vitro, suggesting that eIF-5A is involved in the formation of the first peptide bond in translation (8). In *Saccharomyces cerevisiae*, two genes (*TIF51A* and *TIF51B*) code for eIF-5A. Although deletion of both eIF-5A genes was lethal, the complete depletion of eIF-5A in the cell led to only a 30% drop in the first round of protein synthesis (30). These authors suggested that eIF-5A is not absolutely necessary for general protein synthesis in eukaryotic cells but is essential for the translation of a subset of specific mRNAs.

A recent study concluded that *efp* was essential for viability and was required for protein synthesis in *E. coli* (4). We have not found this to be the case in *A. tumefaciens*. We have clearly shown that *A. tumefaciens* has a single copy of *chvH*, which can be disrupted without loss of viability, although the cells grow more slowly and are no longer virulent. Our observations are consistent with the supposition that elongation factor P increases the efficiency of formation of peptide bonds involving aminoacyl acceptors that bind poorly to the ribosome in its absence (25) and argue against the notion that elongation factor P is required for general protein synthesis.

The virulence genes of *A. tumefaciens* for which *chvH*-dependent expression was examined in the present study appear to belong to a class of genes whose optimal translation depends on elongation factor P. Expression of other genes that were tested was not affected to the same extent. The elongation factor P-dependent genes might code for particular sequences of amino acids, perhaps near the start of translation, that are exceptionally dependent on elongation factor P for translation. An example of this type of specificity is the observation that when the *Bacteroides fragilis efp* gene was introduced into *E. coli*, the *B. fragilis* glutamine synthetase activity increased in *E. coli* but the activity of *B. fragilis* sucrase was unaffected (1). The significance of the control of *A. tumefaciens* virulence gene expression at the posttranscriptional level is not immediately apparent. Further experimental work is necessary to characterize the role of specific amino acid sequences on the elongation factor P dependence of translation.

We have demonstrated that at least some of the *vir* genes are regulated posttranscriptionally in a *chvH*-dependent manner. When *virE2* transcription was driven by the *tac* promoter and thus uncoupled from VirG control, the level of VirE2 protein was drastically reduced in the *chvH* mutant strain, although the levels of *virE2* transcription in the *chvH* mutant and the wild-type strain were comparable (Fig. 7). The reduction in the levels of Vir proteins in the *chvH* mutant thus appears to be determined at two stages. In addition to the direct effects on translation of *vir* mRNA, the reduction in the levels of all Vir proteins could be partly due to the reduced levels of VirA and especially VirG, which is rate limiting for *vir* gene induction (14). The effect of the *chvH* mutation on the expression of VirG is likely due to a direct effect on the translation of VirG. However, it is possible that this reduction results from overproduction of the 32-kDa protein acting on the translation of the *vir* genes. In this case, the role of *chvH* would be indirect. The reduction in Vir protein levels at the posttranscriptional level raises the possibility that elongation factor P serves as a posttranscriptional regulator of *vir* gene expression, perhaps in concert with a second regulatory factor. Identification of the 32-kDa protein that accumulated in the *chvH* mutant might provide an insight into this possibility.

The simplest explanation for the avirulence of the *chvH* mutant is that the levels of key proteins required for T-DNA transfer are reduced profoundly. These include VirB8, VirB9, VirB10, and VirB11 as well as the single-stranded-DNA-binding protein VirE2. We would predict that the levels of other Vir proteins would also be depressed. However, the possibility that the *chvH* gene product contributes in other ways to tumorigenesis cannot be ruled out. In this regard, it may be significant that a number of chromosomal avirulent mutants have in common a lack of integrity in their outer membrane (12).

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