Activities of *virE1* and the VirE1 Secretion Chaperone in Export of the Multifunctional VirE2 Effector via an *Agrobacterium* Type IV Secretion Pathway

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Agrobacterium tumefaciens uses a type IV secretion system to deliver oncogenic nucleoprotein particles and effector proteins, such as the multifunctional VirE2 protein, to plant cells. In this study, we examined the function of *virE1* and its product, the VirE1 secretion chaperone, in mediating VirE2 export. A nonpolar *virE1* null mutant accumulated low levels of VirE2, and *trans* expression of *virE1* in this mutant only partially restored VirE2 abundance. Deletion of *virE1* did not affect transcription but decreased translation of *virE2*, as shown by analysis of *lacZ* transcriptional and translational fusions. VirE2 was stable for a prolonged period, more than 6 h, when it was expressed in *cis* with *virE1*, and it exhibited half-lives of about 2 h when it was expressed in *trans* with *virE1* and less than 10 min when it was expressed in the absence of *virE1*, as shown by pulse-chase experiments. VirE1 stabilized VirE2 via an interaction with a domain near the N terminus of VirE2, as shown by analyses of VirE2 truncation and insertion mutants synthesized in *A. tumefaciens*. VirE1 self-association was demonstrated by using bacteriophage λ cI repressor fusion and pull-down assays, and evidence of VirE1 homomultimerization in vivo was obtained by native polyacrylamide gel electrophoresis and gel filtration chromatography. A putative VirE1-VirE2 complex with a molecular mass of about 70 to 80 kDa was detected by gel filtration chromatography of extracts from wild-type cells, whereas higher-order VirE2 complexes or aggregates were detected in extracts from a *virE1* mutant. Taken together, our findings show that *virE1* contributes in several ways to VirE2 export: (i) *virE1* regulates efficient *virE2* translation in the context of expression from the native P_{virE} promoter; (ii) the VirE1 secretion chaperone stabilizes VirE2, most probably via an interaction with an N-terminal domain; and (iii) VirE1 forms a VirE1-VirE2 complex with a predicted 2:1 stoichiometry that inhibits assembly of higher-order VirE2 complexes or aggregates.

Agrobacterium tumefaciens transfers at least three macromolecular substrates, oncogenic T-DNA, VirE2 single-stranded DNA-binding protein (SSB), and VirF protein, to plant cells during the course of infection (8). Substrate transfer is mediated by a type IV secretion system assembled from the products of the ~9.5-kbp *virB* operon and the *virD4* gene (29). This transfer system is a bona fide conjugation apparatus, as suggested by its ancestral relatedness to transfer systems (Tra) of several broad-host-range plasmids and as demonstrated by its ability to transfer the mobilizable IncQ plasmid RSF1010 to bacterial and plant recipient cells. In recent years, other type IV systems have been shown to contribute to the virulence of several mammalian pathogens. The pathogens utilizing type IV systems during infection include *Helicobacter pylori* (12), *Bordetella pertussis* (3), *Brucella* spp. (31, 42), *Bartonella henselae* (35, 40), and *Legionella pneumophila* (47). A type IV system of *H. pylori* exports CagA to the cytosol of mammalian cells (32, 41, 44), whereas a related system exports pertussis toxin across the outer membrane of *B. pertussis* (3). Other type IV systems are thought to export effector molecules, whose identities are currently unknown, to the mammalian cell cytosol.

Mechanistic studies of the *A. tumefaciens* T-DNA transfer

system and the related conjugal transfer systems of the IncP, IncN, and IncW broad-host-range plasmids have contributed important structure and function information about the type IV systems (8, 29). An area of special interest concerns the mechanism of substrate processing and presentation to the mating channel. Recent work has shown that these systems translocate two general classes of substrates: (i) conjugative plasmids and T-DNA that are translocated as transfer intermediates composed of a single-stranded molecule of DNA covalently bound at the 5' end by a nicking enzyme (4, 7) and (ii) proteins that are translocated independent of DNA (25, 46, 50). Interestingly, export of DNA and protein substrates via type IV machines appears to require common as well as distinct factors at the early stages of substrate processing. In *A. tumefaciens*, T-DNA translocation requires the VirD2 nicking enzyme and the VirD1 auxiliary factor for cleavage at the T-DNA borders, the VirD4 protein, which is thought to physically link the T-DNA processing system and the mating channel, and two membrane-associated proteins, VirC1 and VirC2, whose contributions to T-DNA transfer are largely undetermined (23, 53). By contrast, translocation of protein substrates, such as VirE2 SSB and VirF, does not require VirD1, VirD2, or the VirC proteins but is still dependent on VirD4 (7, 9, 46, 53). In addition, translocation of VirE2 requires a small upstream gene encoding the putative VirE1 secretion chaperone.

Definition of the role of VirE1 for VirE2 export has been the subject of recent investigations. VirE1 interacts with

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VirE2, as shown by the yeast two-hybrid screen (15, 45, 52) and pull-down (15) and immunoprecipitation (52) assays. An interaction domain was localized to the C-terminal half of VirE2, possibly overlapping with regions required for single-stranded DNA binding and for VirE2 self-association (15, 45, 52). An early study suggested that VirE1 stabilizes VirE2 when it is synthesized in *Escherichia coli* (30), although recent work has led to conflicting results regarding this activity in *A. tumefaciens* (15, 16). There is also some evidence that VirE1 prevents VirE2 from aggregating in solution (15). VirE1 is a small, acidic protein with an amphipathic α -helix at its C terminus, the proposed site for interaction with VirE2 (45). Of considerable interest, these physical and functional properties of VirE1 are also features of the Syc chaperone or bodyguard proteins required for export of effector proteins via the type III secretion systems of plant and mammalian pathogens (6, 36).

In this study, we further defined the role of *virE1* for VirE2 translocation. Our findings support a model in which *virE1* acts at the level of translation to ensure efficient *virE2* expression and VirE1 protein acts posttranslationally to stabilize VirE2 and prevent the formation of higher-order nonproductive complexes or aggregates in *A. tumefaciens*.

MATERIALS AND METHODS

Enzymes, chemicals, and reagents. Restriction endonucleases were purchased from Promega (Madison, Wis.), New England Biolabs (Beverly, Mass.), or GIBCO-BRL (Grand Island, N.Y.). The Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase were obtained from Promega. Isopropyl- β -D-thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), carbenicillin, kanamycin, Triton X-100, Coomassie brilliant blue R250, *p*-nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) (AS) was obtained from Aldrich (Milwaukee, Wis.). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G and a Bradford protein assay kit were obtained from Bio-Rad Laboratories (Hercules, Calif.).

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. *E. coli* strains were maintained on Luria-Bertani medium, and *A. tumefaciens* strains were maintained on MG/L medium or on AB minimal salts medium (52). Media were supplemented with antibiotics as follows: for *E. coli*, 50 μ g of chloramphenicol per ml; and for *E. coli* and *A. tumefaciens*, 100 μ g of carbenicillin per ml, 100 μ g of kanamycin per ml, and 5 μ g of tetracycline per ml. For induction of *vir* genes, *A. tumefaciens* cells were grown in MG/L medium to an optical density at 600 nm (OD_{600}) of 0.6, harvested by centrifugation, and inoculated at an initial OD_{600} of 0.25 into induction medium composed of AB minimal salts medium (pH 5.5), 1 mM phosphate, and 200 μ M AS (52). Cultures were incubated with shaking at 22°C for 18 h and then harvested for protein analysis.

Recombinant DNA techniques. DNA manipulations and DNA electrophoresis were performed as described by Sambrook et al. (39). *A. tumefaciens* cells were transformed by electroporation, and plasmids were recovered for physical characterization as previously described (51). DNA sequencing was carried out at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas-Houston Medical School, Houston, Tex., with an ABI 373A DNA sequencer (Perkin-Elmer, Applied Biosystems Division) by using *Taq* polymerase in a thermal cycling reaction. PCR was performed with a Perkin-Elmer Cetus DNA thermocycler by using Pwo DNA polymerase from Boehringer (Mannheim, Germany). Oligonucleotides were purchased from Sigma-Genosys (Woodlands, Tex.).

Construction of *virE1* deletion strain PC3001. To delete *virE1* from pTiA6NC, a 2.5-kb *XbaI-XhoI* fragment (made blunt ended with the Klenow fragment) from pPC732 (carries *P_{virE}::virE2*) was introduced at the *ScaI* site of *sacBR* suicide plasmid pBB50. The new plasmid, pSF1, was recombined onto pTiA6NC by a single crossover event, and sucrose counterselection was used to enrich for double-crossover recombinants lacking the pSF1 plasmid. The *virE1* deletion in strain PC3001 was confirmed by sequencing a PCR product resulting from

amplification across the putative $\Delta virE1$ junction site with primers complementary to the sequences in the *virE* promoter (5'-TCAAGACCCGAGTATGGAT G-3') and the *virE2* gene (5'-TGCCAGAAAGATCCATCGTC-3').

Construction of *virE* expression plasmids. Plasmids expressing *virE* derivatives were constructed as follows. Both *lacZ* transcriptional and translational fusions to *virE2* were made by inserting cassettes at the *StuI* site at bp 36 of *virE2*. For the *lacZ* transcriptional fusions, *StuI-XhoI* fragments of pPC731 and pPC732 were replaced by a 6.3-kbp *SmaI-SalI* fragment containing the *lacZYA* cassette from pRS551 to generate pZZ14 (*P_{virE}::virE1 virE2' lacZYA*) and pZZ15 (*P_{virE}::virE2' lacZYA*). For *lacZ* translational fusions, *StuI-HindIII* fragments of pPC731 and pPC732 were replaced by the *SmaI-HindIII* fragments containing the '*lacZ*' cassette from pMC1871 to generate pZZ16 (*P_{virE}::virE1 virE2'-lacZ*) and pZZ17 (*P_{virE}::virE2'-lacZ*). In these constructs, 12 N-terminal residues of VirE2 were fused to β -galactosidase. For production of His-VirE1, the *virE1* gene carried on a 1.0-kbp *NdeI-XhoI* fragment from pXZ426 was introduced into pET15b to make pZZ3. Plasmid pPC914KS⁺ *NcoI* was constructed as previously described for pPC914KS⁺. *NdeI* (2), except that an *NcoI* site was introduced at the *virB1* start site by oligonucleotide-directed mutagenesis. For expression of *his-virE1* from *P_{virB}*, a ~1.1-kbp *NcoI-XhoI* fragment from pZZ3 was introduced into pPC914KS⁺. *NcoI*, replacing *virB1*. For production of GST-VirE1, the *virE1* gene carried on a ~1.0-kbp *NdeI* (blunt-ended)-*EcoRI* restriction fragment from pXZ426 was introduced into *SmaI-EcoRI*-digested pGEX-2T, resulting in pZZ6. For production of His-VirE2, the *virE2* gene carried on a ~1.6-kbp *NdeI-XhoI* restriction fragment from pPC725 was introduced into pET15b to make pPC730. Plasmids with *ColE1* replication origins that were ligated to IncP broad-host-range plasmid pSW172 or pXZ151 for introduction into *A. tumefaciens* were designated by using the *ColE1* plasmid name plus a B to indicate ligation to the broad-host-range plasmid.

Protein analysis, immunoblotting, and cell fractionation. Proteins were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or with a Tricine-SDS-PAGE system as previously described (37). Vir proteins were visualized by SDS-PAGE, transfer of the proteins to nitrocellulose membranes, and development of immunoblots with goat anti-rabbit antibodies conjugated to alkaline phosphatase and histochemical substrates. For enhanced sensitivity, blots were developed with anti-rabbit antibodies conjugated to horseradish peroxidase, and antibody-antigen interactions were visualized by chemiluminescence (Amersham, Arlington Heights, Ill.). Proteins were loaded on a per-cell-equivalent basis to compare VirE protein abundance in different strains. Molecular size markers were obtained from GIBCO-BRL. Fractionation of *A. tumefaciens* into soluble material (cytoplasm and periplasm) and insoluble material (cytoplasmic and outer membranes) was carried out as previously described (19). Subcellular fractions were applied to SDS-polyacrylamide gels on a per-cell-equivalent basis.

Purification of tagged VirE proteins. Anti-VirE1 and anti-VirE2 antibodies were generated as follows. IPTG-induced BL21(DE3) cells transformed with pPC730 and pZZ6 were used as the starting materials for purification of His-VirE2 and GST-VirE1, respectively; this was done by performing immobilized Ni²⁺ metal affinity chromatography (IMAC) and glutathione affinity chromatography, respectively, as recommended by the manufacturers (Ni²⁺ resin from Novagen and glutathione resin from Pharmacia). Purified His-VirE2 and GST-VirE1 were sent to Cocalico Biologicals, Inc. (Reamstown, Pa.) to produce antibodies in New Zealand White rabbits. His-VirE1 was purified by IMAC with a Co²⁺ resin (Talon resin; Clontech) for analyses of His-VirE1 complexes.

Measurement of VirE2 turnover in AS-induced *A. tumefaciens* cells. *A. tumefaciens* strains were grown to an OD_{600} of 0.5 in AB minimal medium (pH 7.0), washed, pelleted, and resuspended at a 1:5 dilution in induction medium. Cells were induced for 6 h (required because there was a long lag in induction of *vir* gene expression [5]), pelleted, and resuspended in 5 ml of induction medium supplemented with Pro-Mix ([³⁵S]methionine-[³⁵S]cysteine; Pharmacia) at a final concentration of 5 μ Ci/ml. Cells were pulse-labeled for 30 min at 22°C with shaking, and then excess cold methionine and cysteine were added at final concentrations of 25 mM. A 1-ml culture aliquot was removed (zero time), and aliquots were removed at various times during continued incubation of the cell cultures at 22°C with shaking. Washed cell pellets were resuspended in 600 μ l of a solution containing 50 mM Tris (pH 8.0), 50 mM MgCl₂, 20% sucrose, 0.1 mg of DNase per ml, 0.1 mg of RNase per ml, and 1 mM PMSF. Cells were broken by sonication and centrifuged at 14,000 $\times g$ for 15 min to remove the cell debris. Anti-VirE2 antisera and protein A-Sepharose were used to precipitate VirE2 protein from the clarified culture supernatants (24). Briefly, 5 μ l of VirE2 antisera was incubated with culture supernatants at 4°C for 3 h, and then 50 μ l of 10% (wt/vol) protein A-Sepharose in Tris buffer (50 mM Tris [pH 8.0], 1 mM PMSF) was added and the slurry was incubated at 4°C for 2 h. Precipitable

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
DH5 α	λ - ϕ 80 <i>dlacZ</i> Δ <i>M15</i> Δ (<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (rK ⁻ mK ⁺) <i>supE44 thi-1 gyrA relA1</i>	GIBCO-BRL
BL21(DE3)	F ⁻ <i>ompT hsdS_B</i> (rB ⁻ mB ⁻) <i>dcm gal</i> λ (DE3)	Novagen
AG1688	MC1061 F'128 <i>lacI</i> ^q <i>lacZ</i> ::Tn5	J. Hu
<i>A. tumefaciens</i> strains		
A136	Strain C58 cured of pTiC58	49
A348	A136 containing octopine Ti plasmid pTiA6NC	21
PC1000	A348 derivative with the <i>virB</i> operon deleted from pTiA6NC	19
KE1	A348 with a Kan ^r cassette in place of the <i>virE</i> operon	30
At12516	A348 with a Spc ^r cassette in place of the <i>virE2</i> gene	20
PC3001	A348 with a <i>virE1</i> gene deletion	This study
LBA4404	pTiA6NC lacking T-DNA	33
Bacteriophage λ KH54	<i>cI</i> ⁻ lambda phage	27
Plasmid vectors		
pUC118	Crb ^r , cloning vector	39
pUC119	Crb ^r , cloning vector	39
pBSIISK ⁺	Crb ^r , cloning vector	Stratagene
pBSIISK ⁺ . <i>NcoI</i>	Crb ^r , pBSIISK ⁺ with an <i>NcoI</i> site at the translational start site of <i>lacZ</i> α	2
pBSIISK ⁺ . <i>NdeI</i>	Crb ^r , pBSIISK ⁺ containing an <i>NdeI</i> site at the translational start site of <i>lacZ</i> α	2
pET15b	Crb ^r , cloning vector for construction of N-terminal His-tagged proteins	Novagen
pGEX-2T	Crb ^r , cloning vector for construction of N-terminal GST ^r -tagged proteins	Pharmacia
pSW172	Tet ^r , broad-host-range IncP cloning vector	5
pXZ151	Kan ^r , pSW172 with an <i>nptII</i> gene	52
pPC914KS ⁺ . <i>NdeI</i>	Crb ^r , pBSIISK ⁺ with <i>P_{virB}::virB1</i> , <i>NdeI</i> site at <i>virB1</i> start site	2
pPC914KS ⁺ . <i>NcoI</i>	Crb ^r , pBSIISK ⁺ with <i>P_{virB}::virB1</i> , <i>NcoI</i> site at <i>virB1</i> start site	This study
pMC1871	Tet ^r , <i>lacZ</i> translational fusion vector, promoterless <i>lacZ</i> gene lacking a ribosome-binding site and first eight nonessential codons	Pharmacia Biotech
pRS551	Crb ^r , <i>lacZYA</i> transcriptional fusion vector	N. Kleckner
pBB50	Kan ^r Chl ^r , pBCSK ⁺ with <i>sacBR</i> and <i>nptII</i> genes in polylinker	2
pZ150	Crb ^r , pBR322-based vector	26
pTAD180	Crb ^r , pBR322-based vector expressing <i>P_{lacUV5}::cI'-virB11</i>	13
pZ157	Crb ^r , pBR322-based vector expressing <i>P_{lacUV5}::cI</i>	26
pKH101	Crb ^r , pBR322-based vector expressing <i>P_{lacUV5}::cI[1-102]</i>	26
<i>virE</i> expression plasmids		
pXZaE1	Crb ^r , pACT2.2 yeast vector with <i>virE1</i> gene	52
pXZ27	Crb ^r , pBSIISK ⁺ expressing <i>P_{virB}::virE2</i>	52
pXZ46	Crb ^r , pBSIISK ⁺ expressing <i>P_{lac}::virE2</i>	52
pXZ761-769 series	Crb ^r , pBSIISK ⁺ . <i>NdeI</i> with <i>P_{virB}::virE2.i31</i> insertions	52
pXZ201-212 series	Crb ^r , pBSIISK ⁺ with <i>virE2</i> truncations expressed from <i>P_{virB}</i>	52
pXZ420	Tet ^r , pSW172 with <i>P_{virE}::virE2</i>	X.-R. Zhou
pXZ425	Tet ^r , pSW172 with <i>P_{virB}::virE2</i>	52
pXZ426	Tet ^r , pSW172 with <i>P_{virE}::virE1</i>	X.-R. Zhou
pPC725	Chl ^r , pBCSK ⁺ . <i>NdeI</i> with <i>P_{lac}::virE2</i> , <i>NdeI</i> site at 5' end of <i>virE2</i>	52
pPC730	Crb ^r , pET-15b expressing <i>P_{T7}::virE2</i> by insertion of a 1.6-kb <i>NdeI-XhoI</i> fragment containing <i>virE2</i> gene from pPC725	This study
pPC731	Crb ^r , pBSIISK ⁺ . <i>NdeI</i> with <i>virE1</i> and <i>virE2</i> coexpressed from <i>P_{virE}</i>	52
pPC732	Crb ^r , pBSIISK ⁺ . <i>NdeI</i> with <i>virE2</i> expressed from <i>P_{virE}</i>	52
pSM358	Kan ^r Crb ^r , <i>virE2'-lacZ</i> translational fusion as a result of Tn3HoHo1 insertion, IncP replicon	43
pSF1	Kan ^r , pBB50 with <i>P_{virE}::virE2</i> on a 2.5-kb <i>XbaI-XhoI</i> fragment (blunt ended) in <i>SacI</i> site	This study
pZD1	Crb ^r , pBSIISK ⁺ with <i>virE2</i> Δ 39-84 expressed from <i>P_{virB}</i>	This study
pZZ1	Crb ^r , pBR322 plasmid expressing <i>P_{lacUV5}::cI'-virE2</i> , 1.6-kb <i>NdeI-XhoI</i> fragment containing <i>virE2</i> from pPC725 substituting for <i>virB11</i> fragment of pTAD180	This study
pZZ2	Crb ^r , pBR322 plasmid expressing <i>P_{lacUV5}::cI'-virE1</i> , 0.75-kb <i>NdeI-XhoI</i> fragment containing <i>virE1</i> from pXZaE1 substituting for <i>virB11</i> fragment of pTAD180	This study
pZZ3	Crb ^r , pET-15b expressing <i>P_{T7}::virE1</i> by insertion of a 1-kb <i>NdeI-XhoI</i> fragment containing <i>virE1</i> gene from pXZ426	This study
pZZ4	Crb ^r , pBSIISK ⁺ . <i>NcoI</i> expressing <i>P_{lac}::his-virE1</i> by insertion of a 1.1-kb <i>NcoI-XhoI</i> fragment from pZZ3	This study
pZZ6	Crb ^r , pGEX-2T expressing <i>P_{lac}::gst-virE1</i> by insertion of 1.0-kb <i>NdeI</i> (blunt-ended)- <i>EcoRI</i> fragment carrying <i>virE1</i> gene from pXZ426 into <i>SmaI-EcoRI</i> sites of pGEX-2T	This study
pZZ9	Crb ^r , pBSIISK ⁺ expressing <i>P_{virB}::virE1</i> by substitution of 1.0-kb <i>NdeI-XhoI</i> fragment from pZZ3 for <i>virB1</i> fragment of pPC914KS ⁺	This study

Continued on following page

TABLE 1—Continued

Bacterial strain or plasmid	Description	Source or reference
pZZ12	Crb ⁺ , pBSIIKS ⁺ expressing <i>P_{virB}::his-virE1</i> by substitution of 1.2-kb <i>NcoI-EcoRI</i> fragment from pZZ3 for <i>virB1</i> fragment of pPC914KS ⁺ <i>NcoI</i>	This study
pZZB13	Crb ⁺ Tet ^r , pZZ12/pXZ426 cointegrate plasmid expressing <i>P_{virB}::his-virE1</i> and <i>P_{virE}::virE1</i>	This study
pZZ14	Crb ⁺ , pBSIIKS ⁺ <i>NdeI</i> expressing <i>P_{virE}::virE1 virE2' lacZYA</i> transcriptional fusion	This study
pZZ15	Crb ⁺ , pBSIIKS ⁺ <i>NdeI</i> expressing <i>P_{virE}::virE2' lacZYA</i> transcriptional fusion	This study
pZZ16	Crb ⁺ , pBSIIKS ⁺ <i>NdeI</i> expressing <i>P_{virE}::virE1 virE2'-lacZ</i> translational fusion	This study
pZZ17	Crb ⁺ , pBSIIKS ⁺ <i>NdeI</i> expressing <i>P_{virE}::virE2'-lacZ</i> translational fusion	This study
pZZB45	Crb ⁺ Tet ^r , pZZ4/pXZ425 cointegrate plasmid expressing <i>P_{lac}::his-virE1</i> and <i>P_{virB}::virE2</i>	This study
pZZB46	Crb ⁺ Tet ^r , pZZ12/pXZ425 cointegrate plasmid expressing <i>P_{virB}::his-virE1</i> and <i>P_{virB}::virE2</i>	This study
pZZB65	Crb ⁺ Tet ^r , pZZ12/pXZ420 cointegrate plasmid expressing <i>P_{virB}::his-virE1</i> and <i>P_{virE}::virE2</i>	This study
pZZB66	Crb ⁺ Tet ^r , pZZ9/pXZ420 cointegrate plasmid expressing <i>P_{virB}::virE1</i> and <i>P_{virE}::virE2</i>	This study
pZZB67	Crb ⁺ Tet ^r , pXZ426/pXZ27 cointegrate plasmid expressing <i>P_{virE}::virE1</i> and <i>P_{virB}::virE2</i>	This study
pZZB68	Crb ⁺ Tet ^r , pXZ426/pPC732 cointegrate plasmid expressing <i>P_{virE}::virE1</i> and <i>P_{virE}::virE2</i>	This study

^a GST, glutathione-S-transferase.

complexes were recovered by centrifugation at 10,000 × *g* for 1 min. The pellet was washed with 1 ml of Tris-EDTA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF) and resuspended in 25 µl of 50 mM Tris (pH 8.0) and 25 µl of SDS loading buffer. The resuspended material was boiled and analyzed for the presence of VirE2 by SDS-PAGE and immunoblotting. VirE2 abundance was estimated by scanning densitometry of autoradiographs.

Gel filtration chromatography and native PAGE. Total soluble proteins from *A. tumefaciens* extracts or soluble His-VirE1 purified by Co²⁺ affinity chromatography was applied at a concentration of 5 mg of protein/ml of 50 mM Tris (pH 8)–50 mM MgCl₂ to a gel filtration column (30 by 1.5 cm) prepared with Toyopearl MW55 resin (TosoHaas, Montgomeryville, Pa.) according to the manufacturer's instructions. Material was fractionated with a Pharmacia Gradi-fraction chromatography system at a flow rate of 0.6 ml/min, and 1.2-ml fractions were collected. The fractions were analyzed for the presence of VirE1 or VirE2 by SDS-PAGE and immunostaining as described above. The protein molecular size markers used included apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (BSA) (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12 kDa). Size markers were electrophoresed both together with and independent of the test sample.

For native gel electrophoresis, purified His-VirE1 was electrophoresed through a nondenaturing 15% polyacrylamide gel, and the gel lane containing His-VirE1 was excised and placed horizontally on a Tricine-SDS–16.5% polyacrylamide gel for resolution in a second dimension under denaturing conditions. The molecular sizes of the His-VirE1 complexes were estimated by comparison of His-VirE1 migration with migration of the native protein markers BSA (66 kDa), ovalbumin (45 kDa), trypsin inhibitor (20 kDa), and cytochrome *c* (12 kDa); these markers were subjected to the same two-dimensional electrophoresis procedure used for resolution of His-VirE1 complexes. VirE1 was identified by Coomassie blue staining and development of immunoblots with anti-VirE1 antiserum, and the molecular size markers were identified by Coomassie blue staining.

Enzyme assays. *A. tumefaciens* KE1 and At12516 cells expressing the *lacZ* gene fusions were grown to the mid-log phase in MG/L broth and induced for expression of the *vir* genes as described above. β-Galactosidase activities (in Miller units) were determined as previously described (13). At least three independent assays were performed in triplicate for each strain.

Phage immunity assay. Transformants of *E. coli* AG1688 expressing wild-type or chimeric repressors were each cross-streaked against >10⁶ PFU of lytic phage AKH54 spread on one-half of the surface of a Luria-Bertani agar plate. After incubation overnight at 37°C, the streaks were examined visually for bacterial growth in the presence of the phage. Immunity was defined by the ability of strains to grow in the presence of the phage (26, 37).

Virulence assays. Strains of *A. tumefaciens* were tested for virulence on uniformly wounded *Kalanchoe daigremontiana* leaves (51). The controls for the tumorigenesis assays included coinoculation of the same leaf with wild-type A348 and strain A136 lacking plasmid Ti. Virulence was scored in terms of tumor size and time course of tumor appearance. Assays were repeated at least four times for each strain on separate leaves. Tumors were photographed 4 to 5 weeks after inoculation.

RESULTS

Effects of *cis* and *trans* expression of *virE1* on *virE2* expression. In a previous study, we determined that expression of *virE2* from a *P_{virE}* promoter without *cis* coexpression of the upstream *virE1* gene resulted in accumulation of low levels of VirE2 protein (52). To further define the specific contributions of *virE1* to *virE2* expression, we first compared steady-state levels of VirE2 in an *A. tumefaciens* Δ*virE1* mutant expressing various combinations of *virE1* and *virE2* from Ti or IncP plasmids (Fig. 1A).

Δ*virE1* strain PC3001 did not synthesize VirE1 and accumulated very low levels of VirE2 (Fig. 1A, lane 2). PC3001 also was avirulent when it was inoculated onto wounded *K. daigremontiana* leaves and failed to export VirE2, as determined by a coinfection (34) assay (Fig. 1B, #2, and data not shown). We were not able to significantly increase the level of VirE2 or restore virulence of PC3001 cells by (i) expression of *virE2* from an IncP replicon in the absence of *virE1* (pPCB732 [*P_{virE}::virE2*] or pXZB46 [*P_{lac}::virE2*]) (Fig. 1A; Fig. 1B, #3 and #4), (ii) expression of *virE1* from an IncP plasmid (pZZB12 [*P_{virB}::his-virE1*] or pXZ426 [*P_{virE}::virE1*]) (Fig. 1B, #6 and #7), or (iii) expression of both *virE1* and *virE2* from separate promoters on an IncP plasmid (pZZB68 [*P_{virE}::virE1* and *P_{virE}::virE2*]) (Fig. 1B, #8). By contrast, VirE2 abundance and virulence of PC3001 were restored to wild-type levels by coexpression of both *virE1* and *virE2* from the same promoter on an IncP replicon (pPCB731 [*P_{virE}::virE1 virE2*]) (Fig. 1A; Fig. 1B, #5). Together, these results indicate that *virE1* must be coexpressed in *cis* with *virE2* for synthesis of functional VirE2 when gene expression is controlled by the native *P_{virE}* promoter.

In addition, as demonstrated previously (52), we identified one case in which *virE2* expression from a heterologous promoter in the absence of *virE1 cis* coexpression yielded functional VirE2. As shown for PC3001(pZZB46 [*P_{virB}::his-virE1 P_{virB}::virE2*]), expression of *virE2* and *his-virE1* from independent *P_{virB}* promoters resulted in high levels of VirE2 and restoration of virulence (Fig. 1A; Fig. 1B, #9). Note that PC3001 expressing *P_{virB}::virE2* without *cis* or *trans* expression of *virE1* (or *his-virE1*) was avirulent (data not shown). Therefore, the finding that virulence of PC3001 was restored by introduction

FIG. 1. Effect of *virE1* on VirE2 protein accumulation. (A) VirE2 protein abundance as determined by immunoblot analysis. Lane 1, wild-type strain A348; lane 2, $\Delta virE1$ mutant PC3001; lane 3, PC3001(pCB732) merodiploid with $P_{virE}::virE2$ carried on Ti and IncP replicons; lane 4, PC3001(pXZB46) merodiploid with $P_{virE}::virE2$ (Ti) and $P_{lac}::virE2$ (IncP); lane 5, PC3001(pCB731) merodiploid with $P_{virE}::virE2$ (Ti) and $P_{virE}::virE1$ *virE2* (IncP); lane 6, PC3001(pZBB12) with $P_{virE}::virE2$ (Ti) and $P_{virE}::his-virE1$ (IncP); lane 7, PC3001(pXZ426) with $P_{virE}::virE2$ (Ti) and $P_{virE}::virE1$ (IncP); lane 8, PC3001(pZBB68) with $P_{virE}::virE2$ (Ti) and both $P_{virE}::virE1$ and $P_{virE}::virE2$ (IncP); lane 9, PC3001(pZBB46) with $P_{virE}::virE2$ (Ti) and both $P_{virE}::his-virE1$ and $P_{virE}::virE2$ (IncP). (B) Virulence of *virE* mutants as determined by inoculation of wounded *K. daigremontiana* leaves. The numbers refer to the strains in the corresponding lanes in panel A. Ti, leaf wound site inoculated with negative control strain A136.

of plasmid pZBB46 established that His-VirE1 was fully functional. The basis for our finding that *virE2* expression from P_{virB} yields functional VirE2 independent of *virE1* *cis* coexpression is explored further below.

Next, we examined the effects of *cis* and *trans* expression of *virE1* on transcription and translation of *virE2* with the *lacZ* fusions shown in Fig. 2. The β -galactosidase activities of strains were compared to that of A348(pSM358), which expresses a *virE2*'-*lacZ* translational fusion from an IncP plasmid. $\Delta virE$

FIG. 2. β -Galactosidase (β -Gal) activities of the *lacZ* fusion strains expressed as percentages of the activity of strain A348(pSM358). Experiments were done in triplicate, and standard deviations are indicated by error bars. The *virE* genes expressed from the P_{virE} promoter on Ti and IncP plasmids are shown on the right.

operon strain KE1 carrying pZBB14 or pZBB15 had β -galactosidase activity comparable to that of A348(pSM358). Plasmids pZBB14 and pZBB15 express *virE2*'-*lacZ* transcriptional fusions with and without the upstream *virE1* gene, respectively. Similarly, $\Delta virE2$ mutant At12516 carrying pZBB15 exhibited a high level of β -galactosidase activity (Fig. 2). These data show that *cis* expression of *virE1* is not required for efficient *virE2* transcription. Furthermore, production of VirE1 does not influence transcription from the native P_{virE} promoter positively or negatively.

Plasmids pZBB16 and pZBB17 express *virE2*'-*lacZ* translational fusions with and without the upstream *virE1* gene, respectively. These constructs encode the N-terminal 12 residues of VirE2 fused to β -galactosidase. Strain KE1 carrying pZBB16 exhibited β -galactosidase levels similar to those of the isogenic strain expressing the *virE2*' *lacZ* transcriptional fusion. However, both KE1 and At12516 carrying pZBB17 exhibited appreciably lower β -galactosidase activities; these activities were approximately six- and threefold less, respectively, than the KE1(pZBB16) activity (Fig. 2). Expression of *virE1* and *virE2*'-*lacZ* from independent P_{virE} promoters on an IncP plasmid in KE1 cells yielded β -galactosidase activities comparable to that of strain At12516(pZBB17) (data not shown). Together, these findings demonstrate that the presence of the upstream *virE1* coding sequence strongly influences the efficiency of *virE2* translation. The *virE2* start codon is separated by only 4 bp from the *virE1* stop codon; therefore, one explanation for these findings is that translation through *virE1* facilitates ribosomal loading at the *virE2* Shine-Dalgarno sequence. The slightly elevated (about twofold-higher) β -galactosidase activity of At12516(pZBB17) cells compared to KE1(pZBB17) cells could reflect a possible stabilizing activity of VirE1 protein for the VirE2:: β -galactosidase fusion, although this seems unlikely in view of findings described below which indicated that VirE1 stabilizes VirE2 via an interaction with a domain located between residues 39 and 84. Alternatively, VirE1 might slightly enhance VirE2 translation through a direct interaction with mRNA or the ribosome.

VirE1 chaperone stabilizes VirE2 in vivo. Next, we examined the contribution of the VirE1 chaperone to the stability of VirE2. As shown above, PC3001 expressing *virE1* or *his-virE1* from an IncP replicon accumulated higher levels of VirE2 than parental PC3001 cells. Similar results were obtained when *virE1* and *virE2* were expressed from separate P_{tac} promoters (15). Furthermore, even though cells lacking *virE1* accumulated high levels of VirE2 when *virE2* was expressed from the P_{virB} promoter, the VirE2 levels were still appreciably higher in isogenic cells expressing *virE1* (see below). A previous study showed that upon expression of *virE2* from P_{lac} , detection of VirE2 in gels was enhanced by treatment of cell extracts with urea prior to electrophoresis (15). In our studies, urea treatment did not appreciably alter the VirE2 protein content upon electrophoresis of extracts from cells expressing *virE2* from the P_{virE} or P_{virB} promoters (data not shown).

To test directly whether VirE1 influences VirE2 turnover, we monitored the stability of radiolabeled VirE2 in wild-type A348 and *virE* mutant strains. As shown in Fig. 3, labeled VirE2 was stable in induced wild-type cells. We were able to detect some protein degradation only after 6 h following the pulse-labeling period (data not shown). VirE2 was less stable,

FIG. 3. Effect of *virE1* expression on VirE2 protein turnover in *A. tumefaciens*. (A) Autoradiographs showing VirE2 precipitated from extracts of radioactively labeled *A. tumefaciens* cells incubated for the chase times indicated. The strains expressing *virE* genes are indicated below the autoradiographs. (B) Graphic representation of the results expressed as the percentage of VirE2 detected at zero time for each strain. See text for experimental details.

with a half-life of about 2 h in KE1(pZZB67 [*P_{virE}::virE1 P_{virB}::virE2*]) cells expressing the two genes from separate promoters. In striking contrast, VirE2 was degraded with half-lives of less than 10 min in two strains lacking *virE1*, PC3001 and KE1(pXZB27 [*P_{virB}::virE2*]). These findings clearly show that the VirE1 chaperone contributes to stabilization of VirE2. Furthermore, the stabilizing effect appears to be optimal when *virE1* and *virE2* are coexpressed from the same promoter. Finally, the presence of low levels of VirE2 at zero time following pulse-labeling of PC3001 cells is further evidence that *virE1* contributes to efficient *virE2* translation.

VirE1 stabilization is mediated by an interaction with the N terminus of VirE2. VirE1 interacts with a domain in the C-terminal half of VirE2, as shown by yeast two-hybrid screens (15, 45, 52). It has been proposed that a second interaction domain is localized in the N-terminal half of VirE2, but the boundaries of this domain could not be delineated because the N terminus of VirE2 self-activates transcription at the GAL4 promoter when it is fused to the GAL4 DNA-binding domain (45). To localize the region of VirE2 necessary for VirE1-mediated stabilization, we monitored steady-state accumulation of VirE2 truncation and insertion derivatives in isogenic *virE1*⁺ and *virE1* strains as an indicator of intrinsic resistance to endogenous proteases. In the following experiments, we expressed the *virE2* derivatives from the *P_{virB}* promoter. This is because initial studies showed that native VirE2 accumulates at appreciably higher levels when *virE2* is expressed from *P_{virB}* than when *virE2* is expressed from *P_{virE}* in the absence of *virE1* *cis* coexpression (Fig. 1A and 4). Moreover, VirE2 protein levels approach wild-type levels in strains expressing *virE2* from *P_{virB}* and *virE1* from a separate promoter (Fig. 4, bottom). Use of the *P_{virB}* promoter therefore enabled us to uncouple translational effects of the *virE1* gene from stabilizing effects of the VirE1 chaperone.

Analyses of steady-state levels of VirE2 truncation and insertion derivatives synthesized in *virE1* and *virE1*⁺ cells resulted in identification of several mutant classes (Fig. 4). One

class resembled the native protein by showing *virE1*-enhanced accumulation. Mutants in this class included truncation derivatives composed of the first 227, 391, 406, 438, or 469 residues, mutants from which 9 N-terminal residues or 10 or 38 C-terminal residues had been deleted, internal deletion mutant Δ 189–319, and all but one of a collection of mutants carrying an i31 peptide insertion (Fig. 4) (52). Class II mutants were undetectable even by chemiluminescence in *virE1*⁺ and *virE1* strains; this class included truncation derivatives lacking 84 or more N-terminal residues. A class III mutant, VirE2 Δ 39–84, accumulated at comparable low levels detectable only by chemiluminescence in *virE1* and *virE1*⁺ strains. A class IV mutant, VirE2.39::i31, accumulated at comparable high levels in both *virE1*⁺ and *virE1* strains.

The phenotypes of the first two mutant classes together indicate that VirE1 exerts its stabilizing effect via an interaction with an N-terminal domain of VirE2. Conversely, the VirE1 interaction with the C-terminal domain identified by the yeast two-hybrid screening method is insufficient for VirE2 stabilization. Residues 11 to 38 appear to be important but are not essential for VirE1-mediated stabilization of VirE2. The phenotype of the class III mutation (*virE1*-independent accumulation of VirE2 Δ 39–84 at a low level) suggests that residues 39 to 84 are absolutely essential for VirE1-mediated stabilization of VirE2. The phenotype of the class IV mutation (*virE1*-independent accumulation of VirE2.39::i31 at a high level) is of considerable interest and is discussed further below.

VirE1 self-associates in vivo. VirE1 self-activates transcription from a yeast GAL4 promoter when it is fused to the GAL4 DNA-binding domain, which prevents the use of the yeast two-hybrid screening method to assay for VirE1 self-association (45). To test for VirE1 self-association, we used λ cI repressor fusion and pull-down assays. cI repressor protein binds as a dimer to operator sites in λ early lytic gene promoters, and each monomer consists of an N-terminal DNA-binding domain and a C-terminal dimerization domain. The N-terminal domain binds DNA efficiently and represses transcription only when it is fused to the C-terminal dimerization domain or a heterologous dimerizing protein or peptide (26, 27).

The binding of full-length cI to target operator sites was demonstrated by growing AG1688(pZ157) cells in the presence of λ phage (Fig. 5A). By contrast, AG1688(pZ150) vector-only cells and AG1688(pKH101) cells expressing the N terminus of cI did not confer immunity to λ superinfection. AG1688(pZZ2) expressing cI'-*virE1* grew in the presence of phage, and the level of immunity was approximately that of cells expressing full-length cI repressor protein (Fig. 5A). A fusion of VirE1 to cI' was essential for operator binding because independent production of VirE1 and cI' from separate promoters did not confer immunity (Fig. 5A). AG1688(pZZ1) cells expressing the corresponding fusion between the N-terminal DNA-binding domain of cI and full-length VirE2 also conferred phage immunity, which is consistent with previous reports of VirE2 self-association determined by yeast two-hybrid and immunoprecipitation assays (15, 45, 52). However, the cI':VirE2 fusion protein seems to inefficiently dimerize or forms nonproductive aggregates in *E. coli*, as judged by the reduced growth of AG166(pZZ1) compared to that of unexposed cells or phage-exposed AG1688(pZZ2) cells (Fig. 5A).

FIG. 4. Abundance of VirE2 truncation and i31 peptide insertion derivatives upon introduction of expression constructs into wild-type strain A348 (*virE1*⁺ *virE2*⁺), At12516 (*virE1*⁺), and KE1 (*virE*). All derivatives were expressed from *P_{virB}*. The line at the top represents wild-type *virE2* encoding the 533-residue SSB. The two nuclear localization sequences are indicated by white boxes; the VirE1 interaction domain was identified previously by yeast two-hybrid analyses (Y2H), and the second proposed interaction domain was identified by stabilization analyses (ST). Protein contents relative to wild-type VirE2 levels in A348(pXZB27 [*P_{virB}::virE2*]) cells are indicated by symbols ranging from +++ (wild-type levels) to – (no detectable protein); the content of the Δ39–84 mutant was detected by chemiluminescence and not by histochemical staining. At the bottom the numbers below the *virE2* line representation correspond to the sites of the i31 peptide insertions. The level of each VirE2::i31 mutant in the KE1 host strain is shown in the lane below each number in the immunoblot. WT, pXZB27 [wild-type *virE2* expressed from *P_{virB}*]. The immunoblots at the bottom show the protein levels in VirE2 and i31 derivatives. Lane 1, A348(pXZB27); lane 2, PC3001(pXZB27); lane 3, At12516(pXZB27); lane 4, KE1(pXZB27); lane 5, A348; lane 6, KE1(pXZB761 [VirE2.39::i31]); lane 7, At12516(pXZB761); lane 8, KE1(pXZB762 [VirE2.84::i31]); lane 9, At12516(pXZB762); lane 10, KE1(pXZB768 [VirE2.147::i31]); lane 11, At12516(pXZB768); lane 12, KE1(pXZB763 [VirE2.184::i31]); lane 13, At12516(pXZB763). See reference 52 for levels of additional i31 mutant proteins in *virE1*⁺ strains.

For the pull-down assay, VirE1 and His-VirE1 were cosynthesized in the Δ*virE* operon mutant KE1. Initially studies established that His-VirE1 is a functional protein (Fig. 1) and that both native VirE1 and His-VirE1 partition with the soluble fraction of *A. tumefaciens* cells, which is consistent with the predicted cytoplasmic localization of this chaperone (Fig. 5B, lanes 1 to 3). His-VirE1 was retained by Co²⁺ columns upon fractionation of extracts from KE1(pZZB12) cells expressing *his-virE1* or from KE1(pZZB13) cells coexpressing *his-virE1* and *virE1* (lanes 5 and 6). In the latter case, native VirE1 also was retained by the column (lane 6). By contrast, VirE1 was not retained by the Co²⁺ column upon fractionation of extracts from KE1(pXZ426) cells expressing only *virE1* (lane 4). These findings show that retention of VirE1 by the Co²⁺ column was dependent on the presence of His-VirE1, supporting the proposal that VirE1 self-associates in *A. tumefaciens*.

To determine the molecular size(s) of the VirE1 complex(es) in *A. tumefaciens*, we purified His-VirE1 from KE1(pZZB12) cells (Fig. 6A) for size fractionation by two-dimensional native denaturing gel electrophoresis and by gel filtration chromatography. Several native protein markers were

similarly fractionated either independent of or together with His-VirE1. As shown in Fig. 6B, two VirE1-containing species were detected upon native denaturing gel electrophoresis, and the two species migrated close together between the 20-kDa trypsin inhibitor and 45-kDa ovalbumin size standards. The predicted molecular weight of His-VirE1 is 9,500, suggesting that at least one of the species is a homodimer. The larger complex might correspond to a form of the homodimer that migrates aberrantly or to a higher-order homomultimer.

His-VirE1 eluted from a gel filtration column with a predicted molecular mass of 20 to 30 kDa, as judged by comparisons with elution profiles for the 29-kDa BSA and 12-kDa cytochrome *c* size markers (Fig. 6C). Very small amounts of His-VirE1 were present in fractions whose sizes corresponded to the sizes expected for the monomer or complexes larger than a homodimer. These findings further suggest that His-VirE1 assembles as a homodimer in *A. tumefaciens*, at least when it is synthesized in the absence of VirE2 (see below).

VirE1-VirE2 complex formation in *A. tumefaciens*. To further characterize VirE1 complex formation in *A. tumefaciens*, total soluble proteins from wild-type strain A348 and strains

FIG. 5. VirE1 self-association. (A) cI repressor fusion assay. Cells were grown in the absence (left side) or presence (right side) of λ KH54 phage. Dimerization of cI derivatives was assessed on the basis of immunity to phage infection. AG1688 cells expressing the proteins indicated carried the following plasmids: cI'::VirE2, pZZ1; cI'::VirE1, pZZ2; cI' only and His-VirE1, pKH101 and pZZB4; cI' only, pKH101; cI full length, pZ157; and vector only, pZ150. (B) His-tag pull-down assay. The first three lanes contained the total soluble proteins loaded onto the Co^{2+} columns; the other three lanes contained the proteins eluted from the columns. His-E1, KE1(pZZB12); E1, KE1(pXZ426); His-E1, E1, KE1(pZZB13). The arrow and the arrowhead indicate the positions of His-VirE1 and VirE1, respectively. The positions of molecular mass markers (in kilodaltons) are indicated on the right.

expressing either *virE1* or *virE2* were fractionated by gel filtration chromatography (Fig. 7). VirE1 from strain At12516 (Δ *virE2*) eluted in fractions whose sizes corresponded to molecular masses of ~ 30 to 40 kDa, which were slightly larger

than the masses expected for a homodimer. This species might correspond to an aberrantly migrating homodimeric species or to a higher-order homo- or heteromultimer.

The distribution of VirE1 shifted upon cosynthesis with VirE2 in wild-type A348 cells (Fig. 7). VirE1 and VirE2 coeluted as a putative complex at about 70 to 80 kDa. Given the molecular masses of native VirE1 (7.5 kDa) and VirE2 (60 kDa), these findings suggest there is only one molecule of VirE2 in the presumed VirE1-VirE2 complex. The molecular mass of the complex and our evidence for VirE1 self-association further suggest that there are two molecules of VirE1 in the complex, although further studies are needed to confirm this proposed stoichiometry. VirE2 synthesized in the absence of VirE1 eluted in many fractions with sizes ranging from the size of the monomeric protein to more than 443 kDa. Therefore, VirE1-VirE2 complex formation probably is critical for preventing VirE2 aggregation or assembly as higher-order multimers. Finally, the functional VirE2.39::i31 mutant cofractionated with VirE2 upon chromatography of extracts from merodiploid strain A348(pXZB761) (Fig. 7) and from a Δ *virE2* strain expressing the i31 insertion mutant (data not shown). Thus, even though the Tyr.39::i31 mutation confers VirE1-independent stability, the mutant protein still apparently multimerizes with VirE1.

DISCUSSION

VirE2 contributes several functions to the *A. tumefaciens* infection process upon export from the bacterium (9, 46, 48). In a plant cell, VirE2 multimerizes with itself, the T-DNA-VirD2 transfer intermediate, and, presumably, plant proteins

FIG. 6. Formation of purified His-VirE1. (A) His-VirE1 purification from *A. tumefaciens* KE1(pZZB12) by IMAC. Lane 1, total soluble proteins loaded onto the Co^{2+} columns; lane 2, His-VirE1 eluted from the column and visualized by Coomassie blue staining of the gel (arrow). The positions of molecular mass markers (in kilodaltons) are indicated on the left. (B) His-VirE1 complexes analyzed by native denaturing PAGE as described in the text. His-VirE1 complexes were detected by immunoblot analysis (arrows); the positions of molecular mass markers used for electrophoresis in the first dimension (in kilodaltons) are indicated at the top, and the positions of the molecular mass markers used for the second dimension (in kilodaltons) are indicated on the right. (C) His-VirE1 complexes analyzed by gel filtration chromatography. Purified His-VirE1 (1 mg) was fractionated as described in the text, and fractions were analyzed for the presence of His-VirE1 by immunoblot analysis (arrow). The positions of fractions corresponding to elution peaks for molecular mass markers (in kilodaltons) (see Materials and Methods) are indicated at the top.

FIG. 7. VirE1 and VirE2 complex formation in *A. tumefaciens* as analyzed by gel filtration chromatography. Total soluble proteins (5 mg) from the strains indicated on the right were applied to a gel filtration column and fractionated under identical conditions. The VirE proteins indicated on the left were detected by immunoblot analysis. The positions of elution peaks for molecular mass markers (in kilodaltons) are indicated at the bottom.

to yield a complex that is competent for intracellular trafficking to the site of T-DNA integration, the plant nuclear genome (9–11, 14, 22, 38). Of considerable further interest, purified VirE2 recently was shown to self-assemble as single-stranded DNA-specific channels in artificial membranes; it has been proposed that these channels facilitate translocation of substrates such as the T-DNA across the plant plasma membrane or through the plant cell (18). In a bacterium, VirE2 must be processed as a secretion-competent molecule. Minimally, this processing pathway must (i) configure VirE2 as a substrate recognizable by the type IV T-DNA transfer machine and (ii) prevent formation of VirE2 aggregates or pore-forming channels and nonproductive interactions with other exported effector molecules. In this study, we demonstrated that *virE1* and its product play central roles in the second of these requisite processing activities.

Our findings indicate the *virE1* and the VirE1 secretion chaperone contribute both translationally and posttranslationally to VirE2 export. A regulatory role for *virE1* at the level of *virE2* translation is supported by analyses of VirE2 abundance and stability in various *A. tumefaciens* mutants and by results of our *lacZ* fusion studies. The first line of investigation showed that in the context of the native *P_{virE}* promoter, *virE1* and *virE2* must be *cis* coexpressed for synthesis of abundant levels of VirE2 and for successful VirE2 export. In the absence of *virE1* or when *virE1* is expressed in *trans* to *virE2*, VirE2 accumulates at low levels and is very unstable. Our *lacZ* fusion studies further showed that *virE1* mediates efficient translation of *virE2* without affecting transcription. Based on our results, we propose that the *virE1* gene plays an important role in production of export-competent VirE2 under native gene expression conditions. The fact that *virE1* and *virE2* are separated by only four nucleotides is compatible with a translational coupling model in which *virE1* translation facilitates ribosomal loading at the *virE2* Shine-Dalgarno sequence. Similar translational regulatory mechanisms have also been proposed for some of the chaperone genes associated with type III trafficking systems (28, 36).

A regulatory role for *virE1* at the level of translation is appealing because a proposed biological consequence of trans-

lational coupling is that it facilitates formation of productive interactions between two or more subunits of a protein complex (17). In support of such an activity for production of export-competent VirE2, our pulse-chase studies showed that *cis* expression of *virE1* and *virE2* significantly favors formation of a stable VirE1-VirE2 complex, resulting in an appreciably longer half-life for VirE2 than when each gene is expressed from a separate promoter (Fig. 4). Furthermore, we present evidence that VirE1 exerts its stabilizing effect via an interaction with the N terminus of VirE2. Translational coupling might therefore serve as a mechanism to facilitate rapid association of newly synthesized VirE1 with the N terminus of VirE2, possibly as soon as this region of the protein emerges from the translational machinery. We propose that this association both stabilizes VirE2 and prevents N-terminally mediated protein misfolding and aggregation.

We identified one nonnative expression system in which *trans* expression of *virE1* and *virE2* can still result in synthesis of functional (i.e., export-competent) VirE2. In this case, *virE2* must be expressed from the *P_{virB}* promoter, whereas *virE1* can be expressed from any functional promoter. We suggested previously that *virE2* expression from *P_{virB}* might simply yield a critical threshold level of VirE2 protein required for export (52). However, VirE2 abundance is not always correlated with virulence, and furthermore, we now have shown that VirE2 produced from *P_{virB}* is very unstable in the absence of VirE1. We therefore suggest an alternative possibility, that the *P_{virB}* promoter provides a *cis*-acting targeting function, perhaps an mRNA signal, that is normally provided by the 5' untranslated region of *P_{virE}* and/or *virE1*. This proposed targeting function would serve to localize the *virE2* translation machinery to the base of the T-DNA transfer channel to temporally and spatially coordinate *virE2* translation and VirE2 translocation. Further comparative studies of the *P_{virE}* and *P_{virB}* promoter activities are needed to explore this and other possibilities.

In general, we found that the VirE1 chaperone stabilized VirE2 derivatives with an intact N terminus. If VirE1 exerts its stabilizing effect via a direct interaction with an N-terminal domain of VirE2, disruption of that domain should eliminate VirE1-mediated stability. This phenotype was observed with

FIG. 8. Alignment of VirE1 with the N terminus of VirE2. The numbers correspond to positions in the VirE2 sequence. Identical residues are indicated by a black background; similar residues are indicated by a gray background. Alignment was performed with the ClustalX Multalign program.

our class III mutation, VirE2 Δ 39–84. In contrast, the Ty39::i31 insertion mutation conferred VirE1-independent stability. We do not know the molecular basis for this finding, but one explanation is that an i31 insertion at this site interferes with formation of an N-terminal structure that targets the protein for degradation. If this is so, the Tyr39::i31 mutation might correspond to a functional mimic of chaperone binding to the N-terminal interaction domain, with the result that VirE2.39::i31 adopts a stable configuration independent of VirE1 binding. Interestingly, VirE1 is still required for VirE2.39::i31 export (52; data not shown). Furthermore, results of our gel filtration (Fig. 7) and yeast two-hybrid studies (52) suggest that a VirE1-VirE2.39::i31 complex is still assembled. Thus, while a VirE1 interaction is not needed for stabilization of this mutant protein, an interaction probably is required to prevent formation of nonproductive complexes or aggregates or to expose a substrate signal.

Intriguingly, VirE1 displays 21% identity and 35% similarity with the N terminus of VirE2 (Fig. 8). This level of sequence relatedness is also evident for the VirE1 and VirE2 proteins encoded by the pTiC58 plasmid of *A. tumefaciens*, as well as the pa megaplasmid of *Rhizobium etli* (data not shown). In addition, VirE1 and this region of VirE2 share a couple of physical features that may have biological importance. The VirE1 chaperones from different Ti or pa plasmid sources are highly acidic, with predicted pIs ranging from 4.7 to 5.2. Similarly, N termini of VirE2 from different Ti or pa plasmids are also highly acidic, with pIs in the range from 4.3 to 5.0, whereas full-length VirE2 proteins have predicted pIs of 6.1 to 6.6. VirE1 and the N terminus of VirE2 also possess hydrophilic N- and C-terminal regions and a hydrophobic central domain. Of further interest is the finding that residues 39 to 84 of VirE2, suggested by our studies to be important for a stabilizing interaction with VirE1, align with the C-terminal half of VirE1. Conversely, the C-terminal half of VirE1, which is predicted to adopt an amphipathic α -helix, was shown previously to mediate interactions with VirE2 (45). Taken together, these observations suggest that the VirE1 interaction with the N terminus of VirE2 might be mediated by conserved structural motifs present on both proteins.

Our studies showed that VirE1 forms at least two types of complexes. VirE1 can self-associate, at least in the absence of VirE2, and VirE1 also assembles as a VirE1-VirE2 complex with an apparent molecular mass of $\sim$ 70 to 80 kDa. The latter finding agrees with results of an early study which identified an $\sim$ 80-kDa complex with single-stranded DNA-binding activity in extracts of *virE*⁺ cells but not *virE* cells (22). Based on the

predicted sizes of VirE1 (7.5 kDa) and VirE2 (60 kDa), the evidence for VirE1 self-association, and the estimated size of the putative VirE1-VirE2 complex, we suggest that the VirE1-VirE2 complex is composed of one molecule of VirE2 and two molecules of VirE1. Formation of such a VirE1-VirE2 complex clearly seems to be important for preventing assembly of higher-order VirE2 complexes or aggregates (Fig. 7) (15). Whether formation of this complex also is a prerequisite for delivery of the secretion substrate to the transfer channel awaits further study. It is intriguing to speculate that the VirE1 dimer interacts dynamically with VirE2, associating with SSB and then dissociating upon successful presentation of the substrate to the transfer channel. Whether a given VirE1 monomer or dimer can repetitively interact with newly synthesized VirE2 monomers destined for export is unknown; what minimally must occur is that VirE1 disengages from VirE2 prior to export.

Of final note, the multiple roles identified for *virE1* and the VirE1 secretion chaperone for VirE2 secretion by this type IV transfer system are analogous to functions ascribed to the secretion chaperones of flagellar and type III secretion pathways (1, 6, 28, 36). Recent work on different members of the type III secretion family has demonstrated that there is considerable variation in the relative importance of mRNA and amino acid targeting signals, the contributions of chaperones for stabilization of effector proteins, and the number and locations of chaperone-binding domains on the effectors (36). Whether secretion chaperones are required for export of all type IV secretion substrates and whether these chaperones (or their cognate genes) provide targeting functions for delivery of the secretion substrates to the cognate type IV translocases are exciting areas for future study.

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