The Product of the *virB4* Gene of *Agrobacterium tumefaciens* Promotes Accumulation of VirB3 Protein

A. LOUISE JONES, KEN SHIRASU,[†] AND CLARENCE I. KADO*

Department of Plant Pathology, University of California, Davis, California 95616

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The process of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells is thought to involve passage of a DNA-protein complex through a specialized structure in the bacterial membrane. The *virB* operon of *A. tumefaciens* encodes 11 proteins, of which 9 are known to be located in the membranes and 10 have been shown to be essential for virulence. Sequence comparisons between proteins encoded by the *virB* operon and those encoded by operons from conjugative plasmids indicated that VirB proteins may form a structure similar to a conjugative pilus. Here, we examine the effects of mutations in *virB4* on the accumulation and localization of other VirB proteins. VirB4 shares amino acid sequence similarity with the TraC protein of plasmid F, which is essential for pilus formation in *Escherichia coli*, and with the PtlC protein of *Bordetella pertussis*, which is required for toxin secretion. Polar and nonpolar *virB4* mutants were examined, and all were shown to be unable to accumulate VirB3 protein to wild-type levels. A low level of VirB3 protein which was present in induced NT1RE cells harboring *virB4* nonpolar mutant pBM1130 was found to associate with the inner membrane fraction only, whereas in wild-type cells VirB3 associated with both inner and outer membranes. The results indicate that for VirB3 to accumulate in the outer membrane, VirB4 must also be present, and it is possible that one role of VirB4 is in the correct assembly of a VirB protein membrane structure.

Agrobacterium tumefaciens has been the subject of much detailed analysis because of its fascinating natural ability to transform plant cells. This transformation process involves the transfer of a specific portion (T-DNA) of the resident Ti (tumor-inducing) plasmid into the plant cell and subsequent incorporation into the plant nuclear DNA (recently reviewed in references 5, 10, and 43). For this interkingdom DNA transfer process to occur, a set of Ti plasmid-encoded virulence (*vir*) genes is required. These genes are organized in six essential operons, of which the largest is the 9.5-kb *virB* operon composed of 11 genes (*virB1* to *virB11*). The products of *virB2* through *virB11* have been shown to be essential to the process of tumorigenesis (2, 2a, 6, 31, 32, 37).

From detailed sequence analysis, information can be gained as to the potential functions of the *virB* gene products (18, 33, 34, 36). The VirB1, VirB2, VirB5, VirB7, and VirB9 proteins each contain a signal peptide-like sequence which suggests that they are membrane components. VirB2, VirB3, VirB6, VirB8, and VirB10 proteins all contain stretches of hydrophobic residues which provide potential membrane-spanning domains. Immunological studies have now confirmed the membrane location of several of the VirB proteins. VirB4, VirB10, and VirB11 have been detected exclusively in the inner membrane (2, 31, 32, 38), and VirB2, VirB3, and VirB9 have been detected in the inner and outer membrane fractions (30). On the other hand, Thorstenson et al. (35) recently reported detecting VirB1, VirB4, VirB5, VirB8, VirB10, and VirB11 in both membrane fractions. The apparent discrepancy between the distribution of VirB4, VirB10, and VirB11 as reported by these laboratories may be explained by differences in the fractionation procedures used. Nevertheless, current data suggest that VirB proteins form a structure in the bacterial

membrane to allow passage of the T-DNA complex to the plant cell, and it is likely that there is close association of the inner and outer membranes at the point where the VirB proteins accumulate.

The notion of there being a structure in the bacterial membrane that promotes export of a DNA-protein complex is further strengthened by sequence comparisons with open reading frames from certain conjugative plasmids. The PilW operon of IncW plasmid R388 is required for conjugative transfer of this plasmid between bacteria and, more specifically, for synthesis and assembly of the conjugative pilus (3). Recent sequence analysis has shown there to be on average 50.8% similarity between 10 of the predicted products of the PilW and *virB* operons and that these operons share a similar overall genetic organization (14, 28, 29, 32). Lessl et al. (20) have reported similarities between TrbB, TrbC, TrbD, TrbE, TrbF, and TrbI gene products from the Tra2 region of IncP plasmid RP4 and VirB11, VirB2, VirB3, VirB4, VirB5, and VirB10, respectively. TrbB, TrbC, TrbE, TrbG, and TrbL have been proposed to be membrane components and have been shown to be essential for conjugation and absorbance by donor-specific phage (21).

There are also sequence similarities between VirB2, VirB3, and VirB4 and plasmid F-encoded products TraA, TraL, and TraC, respectively (15, 30, 32). TraA is the F-pilin protein that is the major constituent of the conjugative pilus. F pilin is first synthesized as a 121-amino-acid pilin precursor protein (propilin) from which a 51-amino-acid signal sequence is removed to form a 7.2-kDa pilin structural protein. VirB2 protein is also thought to be cleaved, since a signal peptide peptidase I cleavage site like that of TraA propilin is present and the predicted 12-kDa size of the protein is found to be 7.2 kDa by immunoblotting (30). TraL and TraC proteins are believed to have a role in pilus assembly, since F derivatives that carry mutations in these genes do not produce F pili (42). Thus, it remains a possibility that the *virB* gene products form a structure, similar in both physical characteristics and function to the pilus of conjugative plasmids to allow passage of DNA.

* Corresponding author. Mailing address: Department of Plant Pathology, University of California, Davis, CA 95616. Phone: (916) 752-0325. Fax: (916) 752-5674.

[†] Present address: Plant Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA 92037.

Indeed recent studies have shown that the *vir* region can function to promote the conjugative transfer of certain broad-host-range plasmids between *Agrobacterium* cells and that the *virB* operon is essential for this process (1).

Other sequence comparisons have shown that some VirB proteins may be involved in protein secretion. VirB7 is similar to lysis proteins encoded by plasmids CloDf3, ColA-Ca31, ColE3, and ColE1 (33). These lysis proteins facilitate the release of colicins, which lack a secretory signal sequence from the bacterial cell. There is also sequence similarity between VirB2, VirB3, VirB4, VirB6, VirB8, VirB9, VirB10, and VirB11 and *Bordetella pertussis* proteins PtlA, PtlB, PtlC, PtlD, PtlE, PtlF, PtlG, and PtlH, respectively (reviewed in references 14 and 15). The *ptl* operon is believed to be involved in pertussis toxin secretion, since a mutation in *ptlC* renders the bacteria unable to secrete the toxin (41).

Despite significant advances in our knowledge of the *virB*-encoded proteins and ideas concerning the type of structure that may be formed, there is as yet no direct evidence to show that these proteins do interact. Proteins that share sequence similarity with VirB4, such as PtlC and TraC, have roles in protein secretion and pili assembly, respectively. This suggests that VirB4 may have an important and essential role in directing protein localization or assembly of a membrane structure. Thus, in this study we have examined the requirement of *virB4* for the presence and localization of other *virB*-encoded proteins. The long-term aim of this work is to substantiate the hypothesis that *vir*-encoded proteins indeed interact with each other. In this study we show that an intact *virB4* gene (and thus its product) is critical for the accumulation of VirB3 and also affects its localization. The level or localization of other VirB proteins such as VirB2 and VirB9 remained unaffected.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. NT1RE is a spontaneous rifampin- and erythromycin-resistant mutant of *A. tumefaciens* NT1, which lacks the Ti plasmid (40). The pJK series of Ti plasmid Tn5 mutants (22) and the pBM series of pUCD2619 Tn5*pvirB* mutants (32) have previously been described. *A. tumefaciens* cells were routinely grown in medium 523 (16) at 28°C with the following antibiotic concentrations when required: rifampin, 50 µg/ml; neomycin, 20 µg/ml; gentamicin, 20 µg/ml; and tetracycline, 3 µg/ml. *A. tumefaciens* cells were induced by using mid-log-phase cells in AB induction medium (4). After 4-h incubation at 28°C, acetosyringone (Aldrich Chemical Co., Milwaukee, Wis.) was added to the culture to the final concentration of 200 µM. Cells were harvested by centrifugation (7,000 × g, 10 min, 4°C) after further incubation of 16 h at the same temperature.

SDS-PAGE. Glycine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (glycine-SDS-PAGE) (19) was performed by using gels containing 10% (wt/vol) acrylamide (monomer/dimer ratio, 30:0.8) for VirB4 or VirB9 analysis. For VirB2 and VirB3 analyses, tricine-SDS-PAGE (27) using 10% (wt/vol) acrylamide (monomer/dimer ratio, 48:1.5) was employed. Whole-cell extracts were prepared from 30-ml cultures of NT1RE cells, induced by acetosyringone for 16 h as described above. Washed cells were resuspended in 1-ml portions of H₂O, and 50 µl of this suspension was then mixed with 50 µl of sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 3% SDS, 10% glycerol, 5% β-mercaptoethanol), boiled for 5 min, and centrifuged at 10,000 × g for 1 min. Ten microliters of supernatant was loaded per track unless specified otherwise. Prestained molecular size markers (Amersham, Arlington

Heights, Ill.) were phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), and insulin (b and a) chains (3.4 and 2.35 kDa).

Subcellular fractionation of *A. tumefaciens* cells. The separation of *A. tumefaciens* membranes was based on the method of De Maagd and Lugtenberg (7) with minor modifications (30). NADH oxidase activity was used as a marker for identifying and verifying the inner membrane fraction (23).

Immunoblot analysis. The production of rabbit-borne antibodies to VirB2, VirB3, VirB4, and VirB9 proteins were described previously (30, 32). The presence of VirB2, VirB3, VirB4, and VirB9 proteins in whole-cell extracts and subcellular fractions were determined by immunoblotting (Western blotting). Specific antiserum reactions were visualized with the ECL chemiluminescence system involving donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham).

Construction of pUCD4546. A 2.5-kb *NdeI*-*HpaI* fragment from pUCD3471 was inserted into *NdeI*-*HincII*-digested pUCD3421. The construction of pUCD3421 and pUCD3471 were described previously (32). The resulting construct, pUCD4545, carries *virB4* downstream of the *virB* promoter in a vector derived from pBluescript SK⁻ (Stratagene Cloning Systems, La Jolla, Calif.). In order to express *virB4* in *A. tumefaciens* for complementation studies, a 2.9-kb *NotI* fragment containing the *virB4* region was excised from pUCD4545 and inserted into the unique *NotI* site of pUCD2001 (9). This construct, which carries the entire *virB4* gene under control of the *virB* promoter, was shown to be biologically active by complementation of a *virB4* mutation in assays of tumor formation on *Datura stramonium*.

RESULTS

Accumulation of VirB3 protein is dependent on *virB4*. Different Tn5 insertions within the *virB* operon of the Ti plasmid were analyzed by examining whole-cell extracts prepared from acetosyringone-induced NT1RE cells. The Tn5 transposon is located in *virB3* of pJK502, in *virB4* of pJK190, in *virB5* of pJK104, in *virB10* of pJK210, and outside of the virulence region in pJK270 (22). Samples prepared from equal numbers of cells were separated by SDS-PAGE followed by electroblotting and initially analyzed by using VirB2-specific antisera. Figure 1A shows the presence of the 7.2-kDa VirB2 protein in extracts of NT1RE harboring pJK270, pJK502, pJK190, pJK104, or pJK210 (lane 2, 3, 4, 5, or 6, respectively). Equivalent quantities of VirB2 were detected in each of the test strains. As expected, no VirB2 was detected in induced Ti plasmid-free NT1RE cells (lane 1). These results indicate that mutations downstream of *virB2* do not affect the accumulation of VirB2 protein and since equal amounts of protein were loaded per lane, the level of *vir* gene induction is similar for each culture. The gels were then examined for the presence of VirB3 by using VirB3-specific antisera. Figure 1B shows that the level of detectable VirB3 depended on the site of transposon insertion within the *virB* operon. For instance, VirB3 did not accumulate in NT1RE strains harboring pJK502 (lane 3), and its presence was severely diminished in those harboring pJK190 (lane 4). Plasmid pJK502 carries a Tn5 insertion in the *virB3* gene, and therefore the absence of VirB3 protein is expected. On the other hand, in pJK190 the transposon is located in *virB4*. VirB3 is readily detected in extracts of NT1RE harboring either pJK104, pJK210, or pJK270 (lane 5, 6, or 2, respectively). Figure 1C shows that intact VirB4 is

FIG. 1. Immunoblots of VirB2 (A), VirB3 (B), VirB4 (C), and VirB9 (D) proteins derived from strain NT1RE bearing polar *virB* mutations in the Ti plasmid. Whole acetosyringone-induced cell extracts were separated either by 10% tricine-SDS-PAGE for VirB2 and VirB3 detection or by 12% glycine-SDS-PAGE for VirB4 and VirB9 detection. Proteins were transferred to nitrocellulose, analyzed by Western blotting, and visualized by using specific antiserum and anti-rabbit horseradish peroxidase-conjugated secondary antibody. Lanes 1, NT1RE (no Ti plasmid); lanes 2, NT1RE(pJK270) (*vir*⁺); lanes 3, NT1RE(pJK502) (*virB3*::Tn5); lanes 4, NT1RE(pJK190) (*virB4*::Tn5); lanes 5, NT1RE(pJK104) (*virB5*::Tn5); lanes 6, NT1RE(pJK210) (*virB10*::Tn5). Each lane was loaded with an extract prepared from equivalent numbers of induced cells, and the results shown are representative of at least four induction experiments. The markers on the left indicate the positions of prestained proteins and their molecular masses (in kilodaltons), and the arrows on the right indicate the VirB protein identified by their respective antiserum.

present in NT1RE cells harboring either pJK104, pJK210, or pJK270, but is absent in strains containing pJK502 or pJK190. These results are consistent with the location of the transposon in these plasmids and confirm the polar effect of Tn5 on the expression of genes downstream of the Tn5 insertion as well as the presence or absence of VirB4 protein. The polar effect of Tn5 was further verified by the presence or absence of VirB9 in the extracts. Figure 1D shows that VirB9 is present in NT1RE cells when Tn5 was in *virB10* (pJK210) or outside of the *vir* region (pJK270) but is absent when Tn5 was inserted in genes upstream of *virB9* (pJK502, pJK190, and pJK104).

Since VirB3 was severely diminished in pJK190 (*virB4*::Tn5) but not in pJK104 (*virB5*::Tn5), it appears that for VirB3 to accumulate, VirB4 must also be present.

Confirmation that the product of the *virB4* gene is required for VirB3 accumulation. The above initial experiments using

FIG. 2. Immunoblots of VirB2 (A), VirB3 (B), and VirB4 (C) proteins identified by specific antisera. Lanes show whole-cell extracts of acetosyringone-induced *A. tumefaciens* cells, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described in the legend to Fig. 1. Lanes 1, NT1RE (no Ti plasmid); lanes 2, NT1RE(pJK270) (*vir*⁺); lanes 3, NT1RE(pJK190) (*virB4*::Tn5); lanes 4, NT1RE(pJK190, pUCD4546) (*virB4*::Tn5, *virB4*); lanes 5, NT1RE(pJK104) (*virB5*::Tn5). Each lane was loaded with an extract prepared from equivalent numbers of induced cells, and the results shown are representative of four independent induction experiments. The markers on the left indicate the positions of prestained protein molecular mass markers and give their sizes (in kilodaltons). The respective VirB proteins are identified by the arrows on the right.

polar Tn5 insertions in the *virB* operon suggested that an intact *virB4* gene may be required for VirB3 to accumulate. To confirm these results and to demonstrate that the *virB3* gene of pJK190 is functional, NT1RE(pJK190) was transformed with pUCD4546 which carries the intact *virB4* gene under the control of the native *virB* promoter. NT1RE(pJK270), NT1RE(pJK104), NT1RE(pJK190), and NT1RE(pJK190, pUCD4546) were induced by acetosyringone, and whole-cell extracts of these strains were separated by SDS-PAGE, electroblotted, and analyzed by using VirB2-, VirB3-, and VirB4-specific antisera. Figure 2A shows that the amount of VirB2 protein detectable in each of the test strains is similar in each sample, indicating that the level of induction in each culture was equivalent. Figure 2B shows that VirB3 was only very weakly visible in an extract of NT1RE cells harboring pJK190 (lane 3) compared with that of NT1RE harboring pJK270 (lane 2) or pJK104 (lane 5); however, introduction of pUCD4546 into NT1RE(pJK190) had the effect of substantially increasing the level of detectable VirB3 protein (lane 4). Like NT1RE(pJK104), this strain should still be lacking the products of *virB5* through *virB11*. Thus, the proteins encoded by *virB* genes downstream of *virB4* appear to be unnecessary for VirB3 accumulation. The intact VirB4 protein is detectable in the NT1RE(pJK270), NT1RE(pJK104), and NT1RE(pJK190, pUCD4546) extracts but not in NT1RE(pJK190) bearing an insertional mutation in *virB4*, indicating that the intact *virB4* gene carried by pUCD4546 is expressed. Plasmid pUCD4546 is able to complement a nonpolar *virB4* mutant, as determined by tumor assay on *Datura* plants (data not shown), which confirms that the cloned *virB4* gene is biologically active.

Analysis of VirB3 accumulation in Tn5*SpvirB* *virB4* mutants.

FIG. 3. Position of the *Tn5pvirB* insertion within *virB4* of each of the five pBM mutants studied. The precise site of insertion was determined by nucleotide sequencing, and in each mutant, the *virB* promoter faces downstream. The hatched box represents the *virB4* gene of pUCD2619. The numbers in parentheses refer to the insertion site in base pairs from the 5' end of *virB4*. Open circles represent nonpolar insertions and closed circles are polar insertions, as determined previously by genetic analysis (32).

Five additional mutations in *virB4* were tested for their effect on the accumulation of VirB3. These mutations were generated in pUCD2619 (26), which contains the entire virulence region, by introduction of a *Tn5pvirB* transposon (31, 32). This transposon has the *virB* promoter facing outward such that expression of the downstream genes is maintained. The *virB4* mutants were designated pBM1110, pBM1123, pBM1125, pBM1130, and pBM1159 (32). All five mutants resulted in an avirulent phenotype, as demonstrated by tests of both tumor formation and agroinfection (32). pBM1123, pBM1125, and pBM1130 were shown to be nonpolar and pBM1110 and pBM1159 were shown to be polar by genetic tests involving complementation of pJK104, which carries *Tn5* within *virB5* of the Ti plasmid (32). The position of the *Tn5pvirB* insertion within *virB4* of each of these mutants was determined by sequencing and is shown in Fig. 3. Each insertion has the *virB* promoter facing downstream. The reason for the genetic polarity of pBM1110 and pBM1159 is not known but may be due to local sequence effects at the point of insertion.

Whole-cell extracts from acetosyringone-induced NT1RE cells harboring the various pBM mutants were prepared and analyzed by Western blotting using VirB2- and VirB3-specific antisera. NT1RE(pBM1070) was included as a control, since this plasmid carries the *Tn5pvirB* transposon inserted into pUCD2619 outside of the virulence region. Figure 4A shows that the control and mutant samples contained equivalent levels of VirB2 protein. However, all of the *virB4* mutants had levels of detectable VirB3 that were less than that of the NT1RE(pBM1070) control (Fig. 4B). The level of VirB3 was also examined in NT1RE cells harboring pUCD2619 with *Tn5pvirB* inserted into *virB6* and was shown to be similar to the wild-type control (data not shown). The insertions in plasmids pBM1123 and pBM1125 result in the synthesis of VirB4 proteins lacking the first 13 amino-terminal amino acids and lacking the last 34 amino acids at the carboxy terminus, respectively. Even though the VirB4 protein produced from pBM1123 and pBM1125 is almost of the wild-type length, these insertions are known to result in an avirulent phenotype and are shown here to affect levels of VirB3 accumulation. However, the level of VirB3 accumulation observed in extracts of pBM1110, pBM1130, or pBM1159 (lanes 4, 6, and 7) was always lower than that of pBM1123 and pBM1125 (lanes 3 and 5), which may reflect the less severe disruption of the *virB4* gene in the latter two plasmids. The pattern of VirB3 accumulation presented here was consistently obtained over four independent induction experiments.

Subcellular localization of VirB3 in NT1RE(pJK104) and NT1RE(pBM1130). Although the quantity of VirB3 detected was diminished in the above *virB4* mutants, it was never entirely absent and appeared to vary, depending on the site of insertion within the *virB4* gene. VirB3 is a highly hydrophobic

FIG. 4. Immunoblots of VirB2 (A) and VirB3 (B) proteins derived from *Tn5pvirB* mutants of *virB4* on pUCD2619. Lanes show whole-cell extracts of acetosyringone-induced *A. tumefaciens* cells, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting as described in the legend to Fig. 1. Lanes 1, NT1RE; lanes 2, NT1RE(pBM1070) (*Tn5pvirB* in vector portion of pUCD2619); lanes 3, NT1RE(pBM1123); lanes 4, NT1RE(pBM1110); lanes 5, NT1RE(pBM1125); lanes 6, NT1RE(pBM1130); lanes 7, NT1RE(pBM1159). Each lane was loaded with a protein extract prepared from an equal number of induced cells, and the results shown are representative of at least four independent induction experiments. The markers on the left indicate the positions of prestained protein molecular mass markers and give their sizes (in kilodaltons). The arrows on the right show the positions of the respective VirB proteins identified by specific antisera.

protein with no apparent signal sequence and a predominantly outer membrane location (30). It was thought possible that the effect we were observing may be due to the dependence of VirB3 on VirB4 for localization and that VirB3 is subject to degradation if not correctly localized. Thus, cellular fractionation experiments were performed as follows: acetosyringone-induced NT1RE(pJK270) and NT1RE(pJK104) cells were separated into inner membrane, outer membrane, cytoplasmic, and periplasmic fractions. Plasmid pJK104 has *Tn5* located within *virB5*, which has been shown to have a polar effect on downstream genes (Fig. 1). Figure 5A shows that VirB3 is located in the inner and outer membrane fractions of both the wild-type strain (pJK270) and the polar *virB5* mutant (pJK104) strains, indicating that the localization of VirB3 is not dependent on the products of *virB5* through *virB11*. The distribution of VirB2 was also examined in these fractions and was found to be in the inner and outer membrane in both mutant and wild-type strains (data not shown). No VirB2 or VirB3 protein was detected in the cytoplasmic or periplasmic fractions (data not shown).

NT1RE(pBM1130) was chosen for the second fractionation experiment, since it is still possible to detect VirB3 protein in this strain, even though the level is significantly lower than the wild-type strain, and we were interested in the location of the VirB3 protein in this mutant. A preliminary experiment in which cells were fractionated into cell envelopes and soluble proteins showed that the VirB3 protein present in NT1RE(pBM1130) was all located in the cell envelope fraction, as was the case for the wild-type strain. The membrane-containing fraction was further separated into cytoplasmic and outer membranes and analyzed by Western blotting using VirB3- and VirB9-specific antisera. Figure 5B shows that the localization of VirB9 is unaffected by the nonpolar *virB4* mutation in pBM1130, since the analysis shows the same protein localization profile for both mutant (pBM1130) and wild-type (pBM1070) strains. However, VirB3 was detected only in the inner membrane fraction of induced NT1RE(pBM1130) cells,

FIG. 5. Membrane localization of VirB proteins in NT1RE cells harboring either pJK270 (*vir*⁺) or pJK104 (*virB5*::Tn5) (A) or pBM1070 (*vir*⁺) or pBM1130 (*virB4*::Tn5*pvirB*) (B). Membranes were separated by sucrose density gradient centrifugation. NADH oxidase activity was used to assess the efficiency of separation of inner and outer membranes and was found to be predominantly located in the inner membrane fraction (96%). Equal amounts of protein were loaded per lane, and immunoblot analysis was performed as described in the legend to Fig. 1. The markers on the left indicate the positions of prestained protein molecular mass markers and give their sizes (in kilodaltons). The arrows on the right indicate the positions of the respective VirB proteins. Lanes 1, inner membranes from uninduced cells; lanes 2, inner membranes from acetosyringone-induced cells; lanes 3, outer membranes from uninduced cells; lanes 4, outer membranes from acetosyringone-induced cells. The results shown are representative of three independent fractionation experiments per strain and three immunoblots per fractionation.

whereas in induced NT1RE(pBM1070) cells, it can be detected in both fractions, with its predominant location being in the outer membrane.

DISCUSSION

The *virB* operon encodes 11 proteins which are thought to form a structure in the bacterial membrane to allow passage of the T-DNA complex to the plant cell. Nine of these proteins have been localized to membrane fractions of induced *A. tumefaciens* cells containing wild-type Ti plasmid-borne virulence genes. From sequence comparisons, it has been shown that the *virB* operon shares similarities with operons from conjugative plasmids and with protein secretion systems (14, 15, 20, 28, 29, 41). The conjugative plasmid operons (such as those from R388, RP4, and F plasmids) with which *virB* shares similarity are believed to be involved with synthesis and

assembly of conjugative pili, suggesting that *virB*-encoded proteins may form a similar structure.

In the case of plasmid F, the genes required for synthesis and assembly of the conjugative pilus are well documented (reviewed in reference 8), and there is sequence similarity between the F plasmid TraA, TraL, and TraC proteins and VirB2, VirB3, and VirB4, respectively (30, 32). TraA is the major component of the pilus, and TraL and TraC are known to be essential for assembly of the structure. VirB4 is the largest of the VirB proteins, is known to be required for tumorigenesis and T-DNA transfer, and has ATPase activity (32). VirB4 lacks properties typical of a membrane protein but has a cytoplasmic membrane location that is not dependent on the presence of other virulence genes (2, 31, 32). The similarity between VirB4 and TraC of F plasmid indicates that VirB4 may have a role in assembly of a structure in the membrane. Indeed, VirB4 also shares similarity with PtlC of *B. pertussis*, a protein which is required for secretion of the pertussis toxin into the extracellular medium (41).

A polar Tn5 insertion within *virB4* on the Ti plasmid was shown herein to have the effect of severely reducing levels of VirB3 accumulation, whereas an insertion within the *virB5* or *virB10* gene has no effect. Introduction of a cloned *virB4* gene into the *virB4* mutant strain had the effect of increasing levels of VirB3 toward that produced by the wild-type plasmid. The levels of VirB2 protein were similar in each of the test strains, indicating that the observed effect was not due to differences in the level of virulence gene induction, and since the *virB3* gene is immediately downstream of *virB2*, transcription should not have been interrupted. It appeared from these results that the accumulation of VirB3 was dependent on the presence of an intact *virB4* gene and not on the products of the *virB* genes downstream of *virB4*. To further substantiate this observation, we analyzed five different Tn5*pvirB* insertions within *virB4* of pUCD2619. All of these insertions were shown previously to result in an avirulent phenotype in assays of tumor formation and agroinfection (32). All five mutants had levels of VirB3 that were substantially lower than that of the wild-type control. pBM1123 and pBM1125, two mutants, which had insertions that resulted in production of nearly full-length VirB4 protein had levels of VirB3 protein lower than that of the wild-type strain but higher than that of pBM1110, pBM1130, and pBM1159, all of which have a more severe disruption of the *virB4* gene. Thus, for wild-type levels of VirB3 to accumulate, it appeared that a full-length *virB4* gene is important, since all the *virB4* mutants examined showed diminished levels of VirB3 protein.

Purely on the basis of sequence analysis, we may predict VirB3 to have solely an inner membrane location, since it has no typical signal sequence but contains a high proportion of hydrophobic amino acids. However, fractionation of induced NT1RE cells harboring either the wild-type Ti plasmid (30) or a polar Tn5 mutant in *virB5* (pJK104) showed VirB3 to be located in both inner and outer membranes. Fractionation of cells harboring the nonpolar *virB4* mutant pBM1130 showed VirB3 to be exclusively located in the inner membrane and that the total quantity of the protein to be less than wild-type level. However, the amount of VirB3 protein in the inner membrane is higher than in the wild-type strain. In the wild-type cell, VirB4 protein may be actively involved in translocating VirB3 into the outer membrane, since in the absence of full-length VirB4, VirB3 locates only to the inner membrane where it is apparently unstable. The localization of VirB2 and VirB9 in NT1RE(pBM1130) was identical to that in the wild type, indicating that a mutation in *virB4* does not affect localization of these proteins and also that the outer membrane prepara-

tion in which VirB3 cannot be detected does in fact have other *vir*-encoded proteins.

An intact nucleoside triphosphate-binding domain has been shown to be essential for the virulence function of VirB4 (2), and its ATPase activity, as demonstrated by Shirasu et al. (32) may provide the energy for VirB3 protein translocation and assembly of a VirB protein structure. Another possibility is that VirB4 is a critical component of the putative VirB structure and that its effect on the location of VirB3 is an indirect one, with other proteins providing the link between VirB4 and VirB3. Since the localization of VirB3 was not affected by a polar insertion within *virB5*, it is clear that proteins VirB5 through VirB11 are not important for such localization. In order to address the question of what are the components that affect VirB3 localization, further experiments should involve cloned virulence genes that can be expressed in different combinations in the absence of the other *vir* genes such that the precise localization requirements can be determined. The reason for the apparent instability of VirB3 in the absence of full-length VirB4 is unclear at present but may be due to VirB3 forming nonproductive interactions either with itself or with other proteins prior to its delivery to the outer membrane, with VirB4 playing a chaperone-like role in the wild-type system. Instability of outer membrane proteins has been observed in other systems such as chaperone-assisted P-pilus assembly in pathogenic *Escherichia coli* (12, 17) and protein secretion in *Aeromonas hydrophila* (13).

VirB3 has been shown to be essential to the process of tumorigenesis (2a). VirB3 shares 42% similarity with TraL of the F plasmid which is known to be essential for synthesis of the conjugative pilus, so it is possible that correct localization of VirB3 is important for assembly of the putative VirB structure. It is interesting that the VirB proteins share similarities with proteins from both conjugative pilus assembly and protein secretion systems (14, 15, 29). This similarity may reflect the involvement in both systems of passage of macromolecules across the bacterial membranes. Both the processes of T-DNA transfer and conjugation clearly involve passage of a DNA-protein complex through the bacterial membrane, and there is considerable evidence to show an evolutionary relationship between these two systems (20, 25, 29, 39). The first step of conjugation involves cell surface interactions to bring mating cells into close contact, and this process requires conjugative pili to be synthesized by the donor cell. It is generally agreed that after the pilus makes contact with the recipient cell surface, it retracts and brings the cells into closer contact (42). However, two experiments indicate that direct cell-to-cell contact may not be necessary for F-pilus-mediated DNA transfer in *E. coli* and that DNA transfer may occur through an extended pilus (11, 24). Whether the VirB proteins form a structure similar to the conjugative pilus will doubtlessly require electron microscopy studies for clarification. However, even though conjugative pili have been visualized by those studying bacterial conjugation, such a structure does not reveal precisely how the DNA can pass through the bacterial membrane. Studies such as these described herein may begin to answer this question for T-DNA transfer, by indicating how the membrane structure is assembled and which virulence proteins interact with which others.

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