Association of the VirD2 Protein with the 5' End of T Strands in Agrobacterium tumefaciens

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The soil bacterium Agrobacterium tumefaciens can incite tumors in many dicotyledonous plants by transferring a portion (T-DNA) of its Ti plasmid into susceptible plant cells. The T-DNA is flanked by border sequences that serve as recognition sites for specific cleavage by an endonuclease that comprises two virD-encoded proteins (VirD1 and VirD2). After cleavage, both double-stranded, nicked T-DNA molecules and single-stranded T-DNA molecules (T strands) were present. We have determined that a protein is tightly associated with, and probably covalently attached to, the 5' end of the T strands. Analysis of deletion derivatives in Escherichia coli, immunoprecipitation, and a procedure combining immunoblot and nucleic acid hybridization data identified this protein as the gene product of virD2.

Agrobacterium tumefaciens species containing a Ti (tumor-inducing) plasmid can incite tumors on a number of dicotyledonous plants. A specific segment of the Ti plasmid (the T-DNA) is transferred to the plant cell and integrated into the plant nuclear genome (for review, see references 29, 38, and 50). Loci responsible for the transfer and, possibly, the integration steps are located both on the Ti plasmid (vir) and on the bacterial chromosome (chv) (8, 16, 18, 23, 27, 47, 52). The vir loci are induced in Agrobacterium by signal molecules produced by wounded plant cells (46). Induction also occurs when Agrobacterium cells are cocultivated with tobacco suspension cells (1, 63) or by addition of certain plant phenolic compounds to the growth medium (46).

The T-DNA is flanked by border sequences consisting of 24-base-pair (bp) imperfect direct repeats (3, 62). Deletion of the right border abolishes tumorigenicity, whereas deletion of the left border has no significant effect (41, 57). An early event in T-DNA transfer is the generation of single-stranded nicks in the bottom strand (as defined by Barker et al. [3]) of the T-DNA within the border sequences (1, 48, 49, 58). Two proteins encoded by the virD operon (VirD1 and VirD2) mediate cleavage (24, 63), which occurs with equal frequency at one or both borders. Both double-stranded and single-stranded linear DNA molecules (T strands) with termini located within the borders have been observed (1, 24, 48, 49, 55, 56). In the case of the octopine-type pTiA6 plasmid, which contains four borders delimiting three T-DNA regions (T1, Tc, and TR), all borders are utilized as termini, yielding six distinct T strands (T1L, TcL, TR, T1R, TcR, and TL+Cr) (49, 56). While it has been reported that the relative amounts of single-stranded and double-stranded T-DNA molecules are comparable (24), we and others have identified the single-stranded molecules as the predominant species in induced bacteria (1, 48, 49). The roles of the two types of T-DNA molecules in transfer are uncertain.

T strands have 5' ends derived from cleavage at the right border and 3' ends derived from cleavage at the left border. It has been suggested that these single-stranded T-DNA molecules are the intermediates transferred to plant cells (1, 48, 49). If this is the case, the requirement for a right border implies that the 5' end of the T strand is necessary for transfer. The data presented here indicate that the VirD2 protein, one of the two proteins responsible for cleavage at the borders, remains associated with the 5' end of the T strand after cleavage. The possible role of this protein-DNA association transfer is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and vir gene inductions. A. tumefaciens A348 is strain A136 containing pTiA6 (Fig. 1) (19). pVK225 is a derivative of pVK102 encoding virG, virC, virD, and virE and resistance to kanamycin (28, 47). The A348(pVK225) strain was maintained on AB minimal agar with kanamycin (100 μg/ml). Agrobacterium cells were grown at 28°C to an A600 of 0.1 in MG/L (a 1:1 mixture of L broth [37] and mannitol-glutamate broth [5]) and transferred to MS plant medium (14), and acetylsyringone (46) was added to a final concentration of 100 μM. After 18 h of growth, DNA was extracted as described below. LBA4404 (40) harbors a Ti plasmid that contains an intact vir region but lacks the T-DNA; A136(pTi304) (47) is A136 containing a Ti plasmid with a Tn3HoHo1 insertion into the 3' end of the virD2 gene.

The Escherichia coli host strain was JM101 (36). Plasmids pMY1153 and pMY1125 have been described (63). pMY1153 contains the entire virD operon under control of the tac promoter (2). pMY1125 contains the Smal-BamHI fragment of the virD operon under control of the tac promoter. This Smal-BamHI fragment contains the virD1 and approximately two-thirds of the virD2 genes. E. coli strains were grown at 37°C in L broth to an A600 of 0.5 before isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. After 2 h of further growth, DNA was isolated. The border-containing plasmid pE2 has been described (Fig. 1) (63). It contains the left and right borders of the T1-DNA of pTiA6 (borders A and B) cloned into a broad-host-range vector.

The inductions yielded varying amounts of nicking. We have found a range of 10 to 70% of T-DNA molecules nicked.

DNA isolations. DNA was isolated by one of two procedures. (i) The bacteria were lysed in 150 μg of proteinase K–1% sodium dodecyl sulfate (SDS) per ml for 1 h at 37°C, followed by the addition of NaCl to 0.5 M and incubation at 68°C for 30 min. After phenol-chloroform extraction and ethanol precipitation, DNA was suspended in TE (10 mM
Tris hydrochloride [pH 7.0], 0.1 mM EDTA). (ii) The bacteria were treated with 0.67 mg of lysozyme per ml in 10 mM Tris hydrochloride (pH 8.0)-10 mM EDTA for 5 min on ice. SDS was added to 1.5% final concentration, and the sample was incubated for 5 min at room temperature. The lysate was extracted with phenol-chloroform four times, and the DNA was precipitated with ethanol and suspended as described. The interphase, which is more viscous than either the aqueous or phenol phase, was collected from the first two phenol-chloroform extractions and dialyzed against TE for 12 to 16 h at 4°C, and nucleic acids were precipitated with ethanol and suspended as described.

Synthesis of RNA probes and RNase protection assay. RNA probes were synthesized by in vitro runoff transcription of restriction enzyme-digested plasmid DNA as described previously (1, 35). The probe used throughout this paper was synthesized from pBL20 (1) and is complementary to the bottom strand at border B. This plasmid contains sequences from bp 13774 to bp 14277 (3) inserted into pGEM2 (Fig. 1). This plasmid was linearized with EcoRI and transcribed with T7 RNA polymerase. The resulting 544-nucleotide transcript shares 502 nucleotides of homology with the border B region. Since the position of border B is bp 14060, cleavage within the border protected RNA fragments of 285 nucleotides corresponding to the left side of the nick (5' side on the bottom strand) and 215 nucleotides corresponding to the right side (3' side on the bottom strand).

The RNase protection assay has been described (1, 65). Briefly, 10 fmol of RNA probe was mixed with the various samples in 30 μl of 80% formamide-40 mM PIPES [pipera- zine-N,N'-bis(2-ethanesulfonic acid)] (pH 6.5)-0.4 M NaCl-1 mM EDTA. Nucleic acids were then denatured and allowed to anneal overnight at 47°C. The aqueous hybridization mixture contained 20 mM Tris hydrochloride (pH 8.0), 0.3 M NaCl, and 0.1 mM EDTA; hybridization was done at 65°C. A 220-μl portion of cold 10 mM Tris hydrochloride (pH 7.0)-0.4 M NaCl-5 mM EDTA was added, followed by RNases A and T1 to 1 μg/ml and 800 U/ml, respectively, and incubation for 30 min at 20°C. The digestions were stopped by addition of SDS and proteinase K to 0.5% and 50 μg/ml, respectively, with incubation at 37°C for 15 min. The nucleic acid was revealed by autoradiography after being extracted with phenol-chloroform, precipitated, suspended in 80% formamide, and subjected to polyacrylamide gel electrophoresis in 90 mM TBE-7 M urea. The relative intensities of the bands were determined by a densitometer.

Electrophoretic transfer of nucleic acids. A sample of the interphase fraction was treated with proteinase K. This sample and an untreated sample were subjected to polyacrylamide gel electrophoresis in 90 mM TBE-0.1% SDS. Nucleic acids were then transferred to nylon membranes electrophoretically in 45 mM TBE at 80 V for 2.5 h as described previously (43). The nucleic acids were fixed by soaking the membrane in 0.4 NaOH for 10 min. The filter was prehybridized and hybridized as described previously (45) with 5 x 10^6 cpm of the RNA probe. The left border probe was from bp 602 to 1161; the right border probe was from bp 13774 to 14277. Both RNA probes were complementary to the bottom strand.

Combined immunoblotting and nucleic acid hybridization. The protein-DNA complex was electrophoresed through 10% SDS-polyacrylamide gels (30) and transferred to nitrocellulose filters as described previously (53). The filter was then cut in half. One half was used for immunoblotting with antisera raised against VirD2 (42). The other half was baked at 80°C to fix any nucleic acids. This filter was prehybridized and then hybridized with 5 x 10^6 cpm of kinase-treated oligonucleotides as described previously except that both procedures were carried out at room temperature. The filter was washed in 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature. The two oligonucleotides were from bp 13992 to 14067 (internal) and bp 14047 to 14062 (terminal), with the cleavage site between bp 14062 and 14063 (1).

Immunoprecipitation. Immunoprecipitation was performed in buffer Z (25) with bovine serum albumin (5 mg/ml), 0.5% SDS, and 10 μl of appropriate dilutions of antisera at room temperature for 15 min. Protein A-Sepharose (4 mg) in buffer Z was added, and the mixture was incubated at room temperature for 5 min and then centrifuged for 10 s in a microfuge. The pellet was washed twice with buffer Z containing 200 mM KCl at room temperature. Immunoprecipitated nucleic acids were extracted from the final pellet by addition of proteinase K and SDS to 0.25 mg/ml and 2%.
VirD2-T-DNA COMPLEX

RESULTS

Proteinase treatment enhances recovery of nicked T-DNA molecules. We previously used an RNase protection assay to show single-stranded cleavage at the borders of the T-DNA (1, 63). In these studies, total DNA was isolated from A. tumefaciens or E. coli after treatment of the bacterial cells with proteinase and SDS. The lysates were then extracted with phenol, and the DNA was precipitated from the aqueous phase with ethanol. We compared this procedure with a second one which did not require proteinase treatment. In this procedure, the cells are lysed with lysozyme and SDS, the lysate is extracted extensively with phenol, and the DNA in the aqueous phase is precipitated with ethanol. When DNA was isolated by these two methods, very different results were obtained in an RNase protection assay (Fig. 2). The radioactively labeled RNA probe was complementary to the bottom strand of the T-DNA and spanned border B. The band corresponding to 502 nucleotides represented full-length protection of the probe by DNA molecules that were intact throughout the region homologous with the probe. The presence of the smaller RNA fragments of 215 and 285 nucleotides indicated the presence of a nick within the border sequences in the bottom strand of the T-DNA (1). When the DNA was isolated by treatment with proteinase (lane 3), the relative intensities of the signals corresponding to nicked molecules were comparable to the signal corresponding to intact DNA molecules within the sample. Therefore, a substantial amount (approximately 70% in this case) of the DNA isolated by the proteinase treatment was nicked. In sharp contrast, when the DNA was isolated without proteinase digestion (lane 4), very little of the DNA appeared to be nicked (less than 10%). Since the samples were prepared from the same culture, and since the RNase protection assay provides an indirect measure of the frequency of recovery of both nicked and unnicked DNA molecules, a greater percentage of nicked than of unnicked molecules were recovered when proteinase was used in the DNA isolation procedure.

T-DNA molecules can be recovered from the phenol-aqueous interphase. The decreased recovery of nicked molecules without proteinase treatment could be explained if these molecules had protein associated with them and were thus removed from the aqueous phase during the subsequent phenol extractions. If this were true, these DNA-protein complexes should be recoverable from the interphase between the aqueous and phenol phases after phenol extraction. To test this hypothesis, induced Agrobacterium cells were treated with lysozyme and SDS, the lysates were extracted with phenol, the interphase was collected, and nucleic acids were precipitated as described in Materials and Methods. The nucleic acids were pelleted, suspended, and used in an RNase protection assay to determine whether nicked DNA molecules were present. DNA molecules able to protect the entire RNA probe were present in this sample (Fig. 2, lane 5). In addition, nicked DNA molecules were also present, with the relative percentage of nicked to unnicked molecules being comparable to that found when the DNA was prepared with proteinase (approximately 70%). This indicates that when DNA was prepared without using proteinase, nicked molecules could be recovered from the interphase after phenol extraction, suggesting that these nicked DNA molecules are associated with protein. In support of this conclusion, prior treatment with proteinase prevented recovery of T-DNA from the interphase (data not shown).

Nicked T-DNA molecules are associated with protein. If the nicked DNA molecules isolated from the phenol-aqueous interphase are associated with protein, these DNA molecules should migrate more slowly in an acrylamide gel than the same molecules treated with proteinase. The T-DNA from the border-containing target plasmid pE2 (Fig. 1) is approximately 2 kilobases long. If pE2 were cleaved with a restriction endonuclease which recognized a site within the T-DNA, the right end of the T-DNA could be analyzed separately from the left end. Therefore, induced Agrobacterium cells containing pE2 were lysed with lysozyme and SDS, and the lysate was extracted with phenol. DNA isolated from the phenol-aqueous interphase was then treated with EcoRI, which can cleave single-stranded (39) as well as double-stranded DNA (see below). Digestion with EcoRI cleaved the T-DNA into two fragments of approximately 1.3 and 0.7 kilobase, corresponding to the left and right side of the T-DNA, respectively. Part of this EcoRI-digested sample was treated with proteinase; another was left untreated. The DNA was denatured by boiling in loading buffer containing 0.1% SDS, size-fractionated by electrophoresis in a polyacrylamide gel, transferred electrophoretically to nylon membranes, and hybridized with probes specific for either border A or border B. With a probe specific for border B (Fig. 3, lanes 1 to 3), DNA fragments which had been treated with proteinase (lane 3) displayed a greater mobility than the corresponding fragments which had not been treated (lane 2). As an internal control, intact pE2 was
cleaved with HaeIII and added to each sample. This control DNA displayed the same mobility whether or not it had been subjected to proteinase treatment. DNA fragments containing the left border (lanes 4 and 5) displayed the same mobility irrespective of proteinase treatment. This strongly indicates that the differences seen with DNA isolated via the two methods result from the association of protein to the right end of the T-DNA. Since this association was resistant to boiling in SDS and phenol treatment, it is most likely a covalent attachment.

Nicked molecules are both double-stranded and single-stranded. When total DNA is isolated from Agrobacterium, many of the nicked T-DNA molecules are single-stranded (1, 48, 49). To characterize the nicked molecules isolated here, hybridizations for the RNase protection assay were carried out under conditions in which only single-stranded DNA molecules could protect the RNA probe (Fig. 4) (1). As a control, the percentage of single-stranded T-DNA molecules present in total Agrobacterium DNA isolated after proteinase digestion was determined (Fig. 4, lanes 3 to 5). By comparing the intensities of the 285- and 215-nucleotide bands between lanes, the amount of single-stranded, nicked T-DNA molecules (lanes 4 and 5) was found to be approximately 20% of the total amount of nicked molecules (lane 3). When analyzing the DNA isolated from the phenol-aqueous interphase (lanes 6 to 8), the percentage of single-stranded, nicked T-DNA molecules (lanes 7 and 8) relative to total nicked molecules (lane 6) was virtually the same as when the DNA was isolated with proteinase. In each case, the intensities of the signals in aqueous conditions were not affected by previous denaturation of the samples (lanes 4 and 7). This indicates that both double-stranded and single-stranded nicked T-DNA molecules were recovered by this method. Therefore, both types of molecules are associated with protein.

VirD1 and VirD2 proteins are responsible for the protein-DNA association. In E. coli, when the entire virD operon (virD1 and virD2) is expressed, the proteins are produced in large amounts and are associated with the T-DNA. However, when the entire operon is deleted, no association is observed. This suggests that VirD1 and VirD2 are necessary and sufficient for the association of the T-DNA with the bacterial cell. The association of VirD1 and VirD2 is specific, as other proteins do not associate with the T-DNA. This specificity is demonstrated by the fact that only VirD1 and VirD2 are capable of protecting the T-DNA from digestion by RNase. Therefore, VirD1 and VirD2 are responsible for the association of the T-DNA with the bacterial cell.
VirD2 protein was present in the fractions isolated from the phenol-aqueous interphase, the VirD1 protein was not (data not shown). To determine whether VirD2 is associated with the nicked T-DNA molecules, we used the techniques of Western transfer (immunoblotting) of proteins and nucleic acid hybridization (Fig. 6). Interphase samples isolated from A348(pVK225) and A136(pTi304) were treated with DNase I, mixed with loading buffer, boiled, and subjected to electrophoresis on SDS-polyacrylamide gels. The proteins were electrophoretically transferred to a nitrocellulose filter (53). The filter was cut in half; one half was probed with antiserum to VirD2 (42), and the other probed with a $^{32P}$-labeled oligonucleotide complementary to the 16 nucleotides at the 5' end of the T strand (based on the cleavage site determined by Albright et al. [1]). Since DNase I cleaves DNA into oligonucleotides (31), we reasoned that if nicked T-DNA molecules were tightly associated with VirD2, DNase treatment would leave an oligonucleotide associated which could be transferred to nitrocellulose with the protein. We assumed that this oligonucleotide would be of sufficient length to hybridize with the radiolabeled probe. The VirD2 protein was present in this sample (Fig. 6, lane 4). With A136(pTi304) (lane 5), the wild-type VirD2 protein was not present, and instead a larger protein, the fusion between part of VirD2 and β-galactosidase, reacted with the antisera. When the same samples were probed with the radiolabeled oligonucleotide, a band was seen that corresponded to the size of VirD2 with A348(pVK225) (lane 8). With the A136(pTi304) strain, no band was detected at the position of native VirD2 (lane 9). Instead, a band was seen that corresponded to the size of the fusion protein. In order to show that the signals were due to hybridization between nucleic acids rather than binding of the probe by VirD2 and the fusion protein, we included protein samples from VirD2-producing strains which lack any T-DNA. Proteins isolated from a VirD2-overproducing E. coli strain (lacking a border-containing plasmid; S. Porter, personal communication) (Fig. 6, lanes 2 and 6) and from LBA4404 (strain containing vir genes but no T-DNA) (lanes 3 and 7) did not show any bands when probed with the radiolabeled oligonucleotide. This indicates that the VirD2 protein did not bind to the probe under these conditions. In addition, the association was to the 5' terminus of the T strand, since under the same conditions, an oligonucleotide complementary to an internal T-DNA sequence (located within 70 nucleotides of the 5' terminus) did not hybridize to the filter (data not shown). Also, no signal was apparent at the position to which VirD1 migrated (16 kilodaltons [kDa] [63]). Therefore, the VirD1 protein is not associated with nicked T-DNA molecules. These results suggest that the VirD2 protein is tightly associated with nicked T-DNA molecules at or very near the 5' terminus.

**Nicked T-DNA molecules can be immunoprecipitated with antisera to VirD2.** To confirm that the VirD2 protein was associated with nicked T-DNA molecules, we used antisera to VirD2 to try to immunoprecipitate these DNA molecules. The DNA-protein complexes isolated from the phenol-aqueous interphase were subjected to immunoprecipitation with antisera raised to VirD2. The samples were then treated with proteinase, and the DNA was precipitated and used in an RNase protection assay to determine whether nicked T-DNA molecules had been immunoprecipitated. Nicked T-DNA molecules were recovered when antisera against VirD2 was used (Fig. 7, lanes 4 and 5). As controls, samples of the interphase fraction were immunoprecipitated with nonimmune sera (lane 6), preimmune sera (lane 7), and antisera raised against either virG (S. Jin, personal communication) or virE (11a). These treatments did not immunoprecipitate any T-DNA molecules (Fig. 7; data not shown). These results further indicate that the VirD2 protein is associated with nicked T-DNA molecules.

**DISCUSSION**

The results presented here indicate that the VirD2 protein is associated with the 5' terminus of nicked T-DNA molecules after cleavage at the border sequences. The association is resistant to phenol extraction and boiling in SDS, suggesting that it is a covalent attachment. Treatment of the complex with DNase I left VirD2 associated with an oligonucleotide which corresponded to the 5' terminus of T.
strands. The discovery of a tight association between VirD2 and nicked T-DNA molecules is important in understanding the transfer mechanism.

Both double-stranded nicked and single-stranded T-DNA molecules were recovered from the phenol-aqueous interphase. The ratios of single-stranded to double-stranded molecules present in the interphase fraction and in total DNA samples were comparable. It is therefore clear that VirD2 is associated with T strands as well as double-stranded, nicked T-DNA molecules. These double-stranded molecules were only nicked in the bottom strand (data not shown). It is possible that they are actually precursors to T strands.

The finding that the 5′ termini of T strands are associated with the VirD2 protein may explain the polarity of transfer of the T-DNA and strengthens the argument that T-DNA processing is analogous to the DNA processing seen in bacterial conjugation (1, 48–50, 57). When supercoiled DNA is isolated from E. coli harboring the mobilizable plasmid CoIE1, a DNA-protein complex is obtained (6, 12, 13, 20, 33, 59). This is referred to as a relaxation complex and consists of the supercoiled CoIE1 DNA associated with three proteins of 11, 16, and 60 kDa. Treatment of this complex with protein denaturants can generate a single-stranded nick within the origin of transfer (oriT). After nicking (relaxation) of the supercoiled DNA, the 11- and 16-kDa proteins are no longer associated with the DNA, but the 60-kDa protein remains covalently attached to the 5′ end of the nick site. It has been proposed that this protein may act as a pilot protein to lead the single-stranded DNA out of the donor cell and into the recipient.

A similar situation may occur in F plasmid transfer (for review, see reference 60), in which a single-stranded nick is introduced within the origin of transfer (oriT) (17) and this nicked, single-stranded DNA is transferred out of the donor in the 5′ to 3′ direction. Two plasmid-encoded proteins (TraY and TraZ) are required for the endonuclease activity. Although the 3′ end of the nick is susceptible to end labeling, the 5′ end of the nick is resistant (61). The inability to label the 5′ end may be due to some association with a protein (possibly TraY or TraZ) which could aid in transferring the DNA out of the donor cell and into the recipient.

T-DNA processing may be very similar. In A. tumefaciens, two proteins (VirD1 and VirD2) are required to introduce primarily single-stranded nicks in the bottom strand within T-DNA borders. Present data suggest that at the time of or after cleavage, the VirD2 protein becomes tightly associated with the extreme 5′ end of the T strand. This may then serve to lead the T strand out of the bacterium and into the plant. A further argument linking Agrobacterium transfer to bacterial conjugation is the recent finding that the oriT and mobilization (mob) functions of the broad-host-range plasmid RSF1010 can replace the need for Agrobacterium border sequences for transfer into plant cells (7).

The association of the protein may also constitute an intermediate step in a more complex pathway. Topoisomerases possess both nicking and religating activities. After a topoisomerase molecule has introduced a single-stranded nick, it is found covalently attached at either the 5′ (15, 21, 51, 54) or the 3′ (4, 10, 11) end of the nick. It is believed that this attachment acts to conserve the energy of the bond which has been broken so that it may be used in the subsequent religation reaction. If T-DNA transfer involves the T strand, nicking is one of the steps that leads to production of the transfer intermediate and would therefore not be followed by a subsequent ligation step. However, a covalent linkage of VirD2 after cleavage might still act to conserve the energy of the broken bond for another energy-requiring process. Proteins bound to the 5′ end of DNA molecules can also act to prime synthesis of the complementary strand (32, 34). The VirD2 protein may prime synthesis of a strand complementary to the T strand to give a double-stranded linear molecule. However, the predominance of single-stranded over double-stranded linear T-DNA molecules argues that this is not the case or that the synthesis reaction must be very inefficient. The protein may also serve to protect the 5′ end of the T strand from degradation following transfer to the plant cell. Analysis of the fate of the border sequences in tumor lines (9, 22, 26, 44, 64) supports such a function. Whereas the left border is seldom found in these lines, the right border is often at least partially present. In fact, the protein-DNA association may be essential for a number of these functions. Determination of the actual role will greatly increase our understanding of T-DNA transfer.

We believe that the association of the VirD2 protein with T strands further points to the significant role that these molecules play in transfer. We have found that the VirE2 protein has the ability to bind to single-stranded DNA molecules and that this protein is associated with T-DNA during vir gene induction (11a). It is intriguing to imagine the transfer intermediate as a multiple protein-DNA complex consisting of the single-stranded T-DNA molecule with the VirD2 protein associated to the 5′ end and the VirE2 protein associated with the rest of the DNA molecule. The possible roles of other vir gene products in the formation of this complex are being investigated. If the prevention of nicked T-DNA molecules in the interphase of a phenol extraction results from the VirD2 protein’s being associated to the 5′ end, it is interesting to find DNA molecules capable of protecting the entire RNA probe. These DNA molecules were not nicked at border B. In the case of the DNA isolated from Agrobacterium strains containing pTiA6, the Ti plasmid contained four T-DNA borders and three distinct T-DNA segments. Whereas the results presented focus on border B, there are in fact two border sequences to the right of this border (C and D, Fig. 1). If the
protein-DNA association allows recovery from the
interphase, this suggests that the DNA molecules responsible for
full-length protection of the probe are molecules which are not
nicked at border B but are nicked at either border C or D
and therefore have protein associated to 5′ sides derived from
these borders. Since plasmid pE2 contains only two
borders, molecules which are intact at border B may still
be nicked and have protein associated to the 5′ side of the
nicks at border A and therefore be isolated from the interphase.

The VirD2–β-galactosidase fusion protein produced in the
A136(pT1304) strain possesses several of the activities asso-
ciated with the wild-type VirD2 protein. Both can cause
specific nicking within the border sequences (49, 63). T
strands are produced in the mutant strain, albeit at a lower
frequency than in the wild type (49). This work shows that
the fusion protein can associate with T strands as well. The
Tn33HoHol insertion in virD2 is apparently not polar on
downstream genes essential for transfer (47), yet the
A136(pT1304) strain is avirulent (47). We are currently inves-
tigating possible causes of avirulence in this strain.

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