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Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation

(plant transformation/T-DNA transfer/DNA–protein complex/Ti plasmid)

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ABSTRACT **We show that upon induction of *Agrobacterium tumefaciens*, free linear double-stranded T-DNA molecules as well as the previously described T-strands are generated from the Ti plasmid. A majority of these molecules are bound to a protein. We show that this protein is the product of the virulence gene *virD2*. This protein was found to be attached to the 5′ terminus of processed T-DNA at the right border and to the rest of the Ti plasmid at the left border. The protein remnant after Pronase digestion rendered the right end of the double-stranded T-DNA resistant to 5′ → 3′ exonucleolytic attack *in vitro*. The protein–DNA association was resistant to SDS, mercaptoethanol, mild alkali, piperidine, and hydroxylamine, indicating that it involves a covalent linkage. The possible involvement of this T-DNA–protein complex in replication, transduction to the plant, nuclear targeting, and integration into the plant nuclear DNA is discussed.**

DNA delivery from *Agrobacterium tumefaciens* to plants is the only system known in which a stretch of DNA is transferred from one kingdom to another. This stretch of DNA, the T-DNA (transferred DNA), serves as a "messenger" from the bacterium to the plant which, following integration into the nuclear DNA, commands the synthesis of novel metabolites (opines) useful only for the bacterium. Plant hormone synthesis, also directed by bacterial T-DNA genes, ensures proliferation of the transformed plant tissue resulting in a tumor (reviews in refs. 1–4).

T-DNA as an integral part of the Ti plasmid in vegetatively grown agrobacterial cells is not mobile as such. It becomes transferable only upon exposure of the cells to certain metabolites (such as acetosyringone, AS) excreted by wounded plants. This induction results in the expression of the Ti plasmid-encoded virulence genes. As a consequence, processing occurs at the border sequences, the two 25-base-pair (bp) imperfect direct repeats bordering the T-DNA: reported were double-stranded cuts (5, 6) and lower-strand nicks (7–9) within the 25-bp repeats, single-stranded T-DNA molecules of lower-strand polarity (the so-called T-strands; refs. 7 and 8), and circular double-stranded T-DNA molecules (10–12). The products of virulence gene *virD1*, a DNA topoisomerase (13), and of gene *virD2* were shown to be responsible for the endonucleolytic cleavage events involved (14–16).

In nature, infectious agents traveling from one organism to another as a virus are nucleic acids protected by coats. T-DNA on its move from bacterium to plant may also have to be shielded from nuclease attack. While a *virE2*-encoded single-stranded DNA-binding protein has been identified that might protect a single-stranded T-DNA molecule (17–20), VirD2 protein has been shown to bind to nicked double-stranded and single-stranded T-DNA inside the bacterium (21–23). Here we confirm these findings and demonstrate that (*i*) free double- and single-stranded T-DNA molecules are bound to the protein at the right border, (*ii*) the protein also binds to the rest of the Ti plasmid at the cleaved left border, and (*iii*) the covalent DNA-amino acid bond protects the 5′ terminus of the right T-DNA end from exonucleolytic degradation *in vitro*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and *vir* Gene Induction. *A. tumefaciens* nopaline strains C58C1(pGV3850) and C58C1(pTiCos7) have been described (7, 10). The C58 Ti plasmid pJK270 (24) was conjugated to a C58C1 strain lacking a Ti plasmid, resulting in C58C1(pJK270). Growth and induction with AS were as described (7) but using 0.2 mM AS and Murashige–Skoog medium buffered with 20 mM Mes (pH 5.5).

DNA Isolation. Total DNA was isolated using different procedures for cell lysis: (*i*) Pronase/sarkosyl (*N*-lauroylsarcosine) (25); (*ii*) French pressure cell; (*iii*) lysozyme (5).

Southern Transfer and Hybridization. Denaturing Southern transfers onto Zeta-Probe nylon membranes with 0.4 M NaOH were done as recommended (Bio-Rad). Hybridization to random-primed (Boehringer Mannheim kit) probes was as described (26). Single-stranded oligonucleotide probes were ^{32}P-labeled by using T4 polynucleotide kinase (Boehringer Mannheim) (26). Hybridization to oligonucleotide probes was as described (27).

Immunoprecipitation of the Covalent T-DNA–Protein Complex. Anti-VirD2 serum (gift of J. Schroeder, University of Freiburg; 50 μl of antiserum per ml) was preadsorbed to protein A-Sepharose beads (40 mg of beads per ml; Pharmacia) in PBS (10 mM sodium phosphate/150 mM NaCl, pH 7.1). Beads were washed with PBS and resuspended in immunoadsorption buffer (PBS/1% Triton X-100/0.2% sodium deoxycholate/0.5% bovine serum albumin/5 mM EDTA). Restricted total DNA was added to 2.5 volumes of slurry and incubated overnight at 4°C. The complex was dissociated from washed beads by boiling for 5 min in SDS sample buffer (28). Recovery of the T-DNA–VirD2 complex was 1–10%, depending on the experiment.

Oligodeoxynucleotide Probes. Probe A (5′-TATCGAGTGGTGATTTTGTGCCGAG-3′) is specific for the left border. It hybridizes to the lower strand, 33–58 bases left (according to the conventional T-DNA map) of the lower-strand nick (see Fig. 2). Probes B–D are right-border-specific. Probes B (5′-TCTCCGCTCATGATCAGATTGTCGT-3′) and D (5′-CAGATTGTCGTTTCCC-3′) hybridize to the lower strand, 33–58 and 28–44 bases left of the lower-strand nick. Probe C (5′-GAGGCGAGTACTAGTCTAACAGC-3′) hybridizes to the upper strand, 34–57 bases left of the nick. The sequences were derived from published pTiT37 sequence (29).

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Abbreviation: AS, acetosyringone.

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RESULTS

Free Double- and Single-Stranded T-DNA Molecules Are Detected in Induced *Agrobacterium*. The Ti plasmid pGV3850, which has a T-DNA region of 9.5 kilobases (kb) was used for Southern blot analysis of induced T-DNA molecules. In addition to the band representing the Ti-plasmid-linked T-DNA, two new bands at about 5 and 9 kb were detected with a probe spanning the right T-DNA border (Fig. 1, lanes 1 and 3). The 5-kb band corresponds to free single-stranded T-DNA by size and its sensitivity to single-strand-specific endonuclease S1, whereas the 9-kb band, corresponding to the predicted size of free linear double-stranded T-DNA, is S1-resistant (lanes 4 and 6). Thus, in addition to the T-strands, free linear double-stranded T-DNA molecules are detected in induced bacteria. The relative amounts of single- and double-stranded molecules were found to vary from experiment to experiment and to depend on conditions of transfer onto filter membranes (data not shown).

The lower- and upper-strand nicks that might release the T-DNA from the Ti plasmid were mapped. Primer extension analysis localized the lower-strand nicks to the bond between the third and fourth base of both the left and the right 25-bp border sequences (Fig. 2; but see *Discussion*). This confirms earlier findings (8, 9) within the precision of the methods used. Although the upper-strand nicks were located in the vicinity of the lower-strand ones, they could not be mapped precisely, indicating that different upper strands are nicked at different positions (Z.K.-N., Carolyn J. Meduski, and B.H., unpublished data).

Most Double- and Single-Stranded T-DNA Molecules Are Bound to Protein. Total cellular DNA was isolated from the interface of phenol extraction of cells lysed without using protease. Lane 3 of Fig. 1 shows induced T-DNA molecules after digestion of the sample with Pronase. The signals corresponding to free double- and single-stranded T-DNA molecules as well as to Ti-plasmid-linked T-DNA molecules decreased if the Pronase step was omitted (Fig. 1, compare lanes 2 and 3). The difference in the amounts of the detected free T-DNA molecules and of the presumably nicked Ti-plasmid-linked T-DNA sequences in the Pronase-treated and untreated samples is at least a factor of 10. Attached proteins

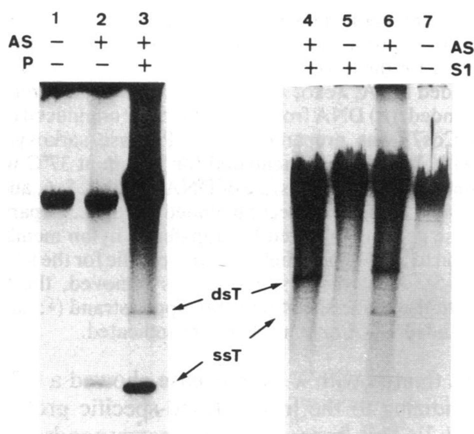

FIG. 1. Free double- and single-stranded T-DNA molecules are bound to protein(s). DNA isolated (with the lysozyme method, from the water/phenol interface) from AS-induced or uninduced cultures of C58C1(pGV3850) (lanes 1–3) was digested with Pronase (P) or not digested. Total DNA isolated by the Pronase/sarkosyl method (lanes 4–7) was digested with S1 nuclease or not digested (ref. 7). Samples of 2 μg (lanes 1–2), 4 μg (lane 3), or 1 μg (lanes 4–7) were electrophoresed in a 0.7% agarose gel, transferred to a nylon membrane, and hybridized to a random-primed probe spanning the right border (see Fig. 3*A*). dsT, free double-stranded T-DNA (9 kb); ssT, free single-stranded T-DNA (apparent size of 5 kb, compared to linear double-stranded size standards).

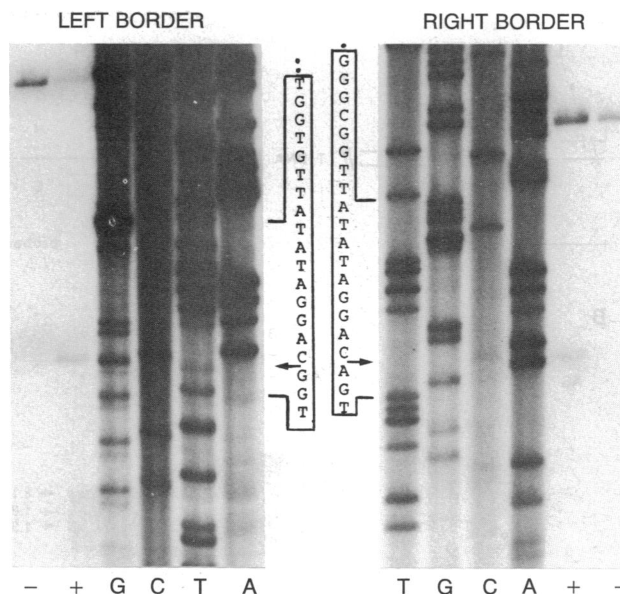

FIG. 2. Precise mapping of the lower-strand nicks. DNA isolated from AS-induced or uninduced cultures of C58C1(pTiCos7) was digested with *Dde* I and subjected to primer extension analysis (8) using ^{32}P-labeled oligonucleotide primers hybridizing to the lower strand either left of the left 25-bp sequence (probe A) or left of the right 25-bp repeat (probe B). The adjacent four lanes show the products of sequencing reactions primed with the corresponding phosphorylated oligonucleotides. The relevant sequences of the newly synthesized (upper) strands are shown in the 5′ → 3′ direction from bottom to top of the figure. The nicks (arrows) are within the complementary lower strand. The complete sequence of the left nopaline 25-bp repeat is 5′-TGGCAGGATATATTGTGGTG-TAAAC-3′; underlined bases represent the sequence fully conserved between all identified nopaline and octopine border repeats (2).

might reduce the efficiency of entry of the DNA–protein complex into the gel (30), of alkaline transfer to nylon membrane, and/or of hybridization to the radioactive probe. The fact that induced T-DNA molecules partition to the phenol/water interface, together with the "Pronase effect," suggests that the processed T-DNA molecules are tightly bound to protein(s).

Induced double-stranded T-DNA molecules were initially detected by λ *in vitro* packaging and plasmid rescue (10). The rescued T-DNA molecules detected by packaging have more recently been shown to be linear, the ends being held together by complementary single-stranded protrusions (Z.K.-N., Carolyn J. Meduski, and B.H., unpublished data). Also, the packaging approach has suggested that double-stranded T-DNA molecules are bound to protein. In two separate experiments, the number of colonies rescued after λ *in vitro* packaging and transduction into *Escherichia coli* decreased from 717 to 25 and from 262 to 38, respectively, if the DNA was not Pronase-treated (see ref. 10 for methods; DNA from a sample not treated with Pronase was shown not to inhibit packaging *per se*).

Both the Left and the Right T-DNA Borders Carry an Attached Protein. In Pronase-untreated samples isolated from the aqueous phase, the 2.2-kb *Hin*dIII and 0.5-kb *Cla* I fragments characteristic for double-stranded cuts at the right border were missing from Southern blots of DNA preparations from AS-induced cells, although the corresponding 1.1-kb *Hin*dIII and 1.6-kb *Cla* I fragments were detected (Fig. 3*B*, lanes 5 and 8; map in Fig. 3*A*). In the DNA sample treated with Pronase, however, the missing fragments were detected (lanes 4 and 7), suggesting that these fragments were attached to protein. The protein part promotes partitioning of the

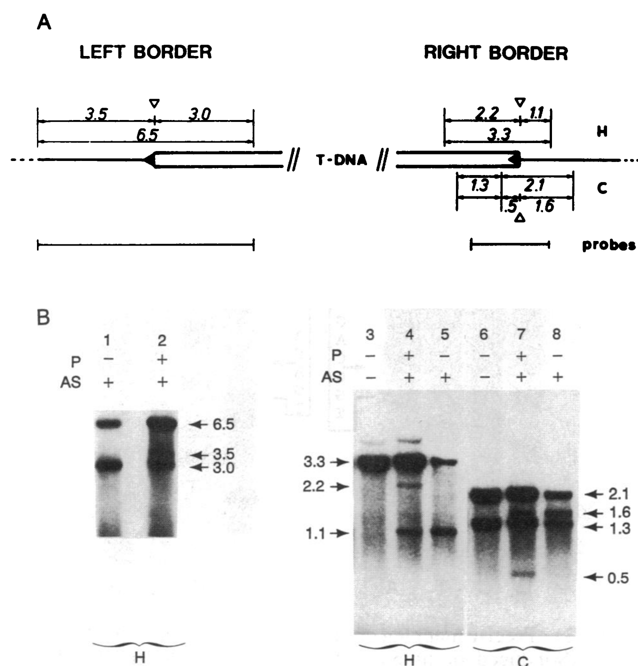

FIG. 3. Upon double-stranded cleavage both the left and the right border carry attached protein(s). (*A*) *Hin*dIII (H) and *Cla* I (C) restriction map of the T-DNA border regions of nopaline pTiC58. Sizes (kb), left and right 25-bp sequences (◄), the site of double-stranded cleavage (▽), and hybridization probes are indicated. (*B*) DNA from AS-induced or uninduced cultures of C58C1(pTiCos7) was isolated from the aqueous phase after lysis in a French press and either treated with Pronase (P) or not treated. Samples were digested with *Hin*dIII (H, lanes 1–5) or *Cla* I (C, lanes 6–8), electrophoresed in a 0.9% agarose gel, transferred to a nylon membrane, and hybridized to the probe spanning the left (lanes 1 and 2) or right (lanes 3–8) border.

complex to the interface and probably interferes with detection by Southern analysis.

Moreover, comparison of lanes 4 and 5 and of lanes 7 and 8 reveals that the intensity of the bands corresponding to the fragments spanning the border (i.e., the 3.3-kb *Hin*dIII and 2.1-kb *Cla* I fragments) is strongly increased in Pronase-treated samples. This may be due to proteolysis of proteins bound to DNA that is nicked at the border (see *Discussion* and Fig. 6).

In corresponding experiments with probes spanning the left border, the 3.5-kb DNA fragment was seen only in the Pronase-treated sample (Fig. 3, lanes 1 and 2). Thus, upon double-stranded cleavage at the borders, proteins are attached to the right end of the T-DNA and to the right end of the non-T-DNA part of the Ti plasmid.

The Protein Bound to the 5′ Right End of the Processed T-DNA Protects it from Exonucleolytic Degradation *in vitro*. Since even extensive Pronase digestion leaves an oligopeptide attached to DNA (31), we analyzed whether a remnant of the protein might inhibit λ exonuclease activity (32). We used this approach to test whether the protein is bound to the 5′ or 3′ terminus—i.e., to the lower or upper strand of the right end of the processed double-stranded T-DNA. Double-stranded cuts at the right nopaline border give rise to a 1.2-kb *Bam*HI fragment corresponding to the right T-DNA part from the *Bam*HI site up to the cut border (Fig. 4*A*). Phage λ exonuclease, known to act 10–100 times faster on double- than on single-stranded DNA (33), and only in the 5′ → 3′ direction, should reduce this fragment to a single-stranded one of lower-strand polarity if the protein is attached at the 5′ end. If the protein is attached to the 3′ end or if no protein is attached, λ exonuclease should degrade the whole fragment.

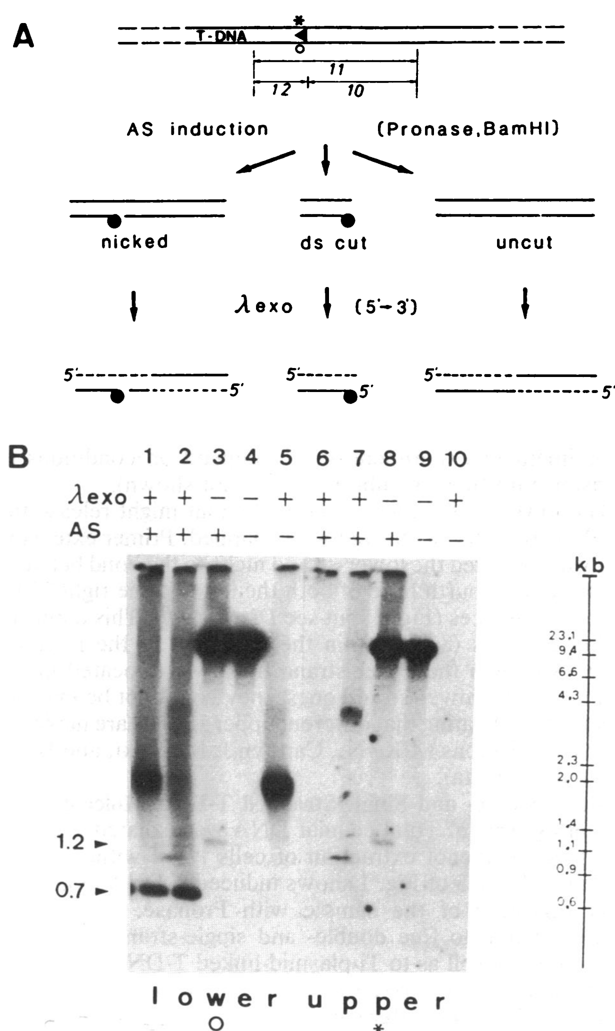

FIG. 4. Protection of 5′ right end of T-DNA against exonuclease. (*A*) Scheme of the experiment. Size (kb) and position of the *Bam*HI restriction fragment spanning the right border and of the predicted fragments arising upon cleavage at the right border are indicated. ◄, 25-bp repeat; *, probe specific for the upper strand (probe C); ○, probe specific for the lower strand (probe B); broken lines, degraded single-stranded DNA; λexo, λ exonuclease; ●, protein remnant; ds, double-stranded. (*B*) DNA from AS-induced or uninduced cultures of C58C1(pTiCos7) was prepared by the Pronase/sarkosyl method, digested with *Bam*HI, and incubated for 30 min at 37°C without or with λ exonuclease (12.5 units/μg of DNA, lanes 1, 5, 6, and 10; 1.25 units/μg, lanes 2 and 7) as recommended by BRL. Separation in a 1.2% agarose gel was followed by transfer to nylon membrane and hybridization to the 5′-end-labeled probe specific for the lower strand (○; lanes 1–5). After the first probe was removed, the filter was hybridized to the probe specific for the upper strand (*; lanes 6–10). Double-stranded DNA size markers are indicated.

Samples treated with λ exonuclease showed a 0.7-kb fragment hybridizing to the lower-strand-specific probe (Fig. 4, lanes 1 and 2). This fragment, which corresponds to the fragment expected for a protected 5′ end, was not detected with the upper-strand probe (lanes 6 and 7). Both probes hybridized to the 1.2-kb fragment of the sample untreated with exonuclease (lanes 3 and 8). The intensity of the 0.7-kb fragment was greater than that of the 1.2-kb fragment, which can be explained if the nicked double-stranded border fragments released the protected single-stranded fragment upon the action of the enzyme.

The complementary experiment employing the exonuclease activity of T4 DNA polymerase, a double-strand-specific 3′ → 5′ exonuclease that accepts 3′ protruding as well

as recessed ends, yielded the expected results: both 3′ ends of the 1.2-kb *Bam*HI border fragment were degraded (data not shown).

The Protective Protein is Bound Covalently to T-DNA and is Encoded by *virD2*. Since the VirD1 and VirD2 proteins are responsible for border processing, they were the most likely candidates for binding to T-DNA molecules. However, only the two bands of 56 and 43 kDa corresponding to free VirD2 protein (34) were detected by antibodies against VirD2 (Fig. 5, lane 2). To visualize a minor species that may have been present as a DNA–protein complex, *Sau*3A-digested DNA was boiled in sample buffer (28), cooled on ice, subjected to SDS/PAGE, electroblotted onto nitrocellulose, and hybridized to a radioactive oligonucleotide specific for the lower strand of the right T-DNA end. A band was detected at ≈70 kDa (lane 4). After Pronase digestion the 70-kDa band disappeared, showing that only DNA bound to proteins was retained on the nitrocellulose membrane (data not shown). This DNA–protein complex was specifically iummunoprecipitated by anti-VirD2 serum (Fig. 5, lane 8), showing that VirD2 is attached to the T-DNA. We estimate that <5% of the total VirD2 is attached to DNA (data not shown).

The size of the complex is consistent with the size predicted for VirD2 attached to the single-stranded DNA fragment from the *Sau*3A site up to the lower-strand nick (43 bases, corresponding to about 14 kDa, assuming that migration of DNA and proteins is comparable with respect to molecular mass). The larger size (≈110 kDa) of the complex detected in *Sac* II-digested DNA, in which the attached *Sac* II fragment has a predicted molecular mass of about 52 kDa, is consistent with the above assumption (data not shown). The T-DNA–VirD2 complex must originate from double-stranded T-DNA molecules, since *Sau*3A does not digest single-stranded DNA (New England Biolabs catalogue).

Since the DNA–protein complexes were detected in samples boiled in the presence of 2% SDS and 5% 2-mercaptoethanol, a covalent bond might be responsible. The chemical nature was analyzed with the following criterion: if the covalent nucleotide–amino acid bond of DNA–protein complex is specifically cleaved by a chemical treatment, a free DNA fragment of defined length will be released. The observed resistance to mild alkali (0.1 M NaOH, 3 hr, 37°C), piperidine (5 M, 4 hr, 50°C), and hydroxylamine (4 M, 1 hr, 37°C) suggests tyrosine (35, 36) to be the partner in the chemical bond (data not shown). However, since the strength of a protein–nucleic acid linkage may be strongly affected by neighboring amino acids of the polypeptide chain (36), more direct methods will have to be employed for definite identification of the bond.

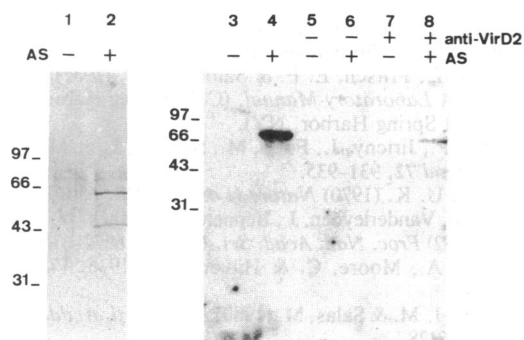

FIG. 5. Free VirD2 and covalent T-DNA–VirD2 complexes. AS-induced or uninduced cultures of C58C1(pJK270) were used. Samples were subjected to SDS/10% PAGE (28) and electroblotted to nitrocellulose. Lanes 1 and 2, total bacterial proteins reacted (34) with rabbit anti-VirD2 serum and visualized with goat anti-rabbit antibodies coupled to alkaline phosphatase (Bio-Rad). Lanes 3–8, covalent T-DNA–VirD2 complexes. Bacteria were opened with the French pressure cell without use of Pronase, and DNA was isolated from the aqueous phase (since DNA isolated from the interface was degraded during digestion with restriction enzyme), and restricted with *Sau*3A. Samples were either directly loaded on the gel (lanes 3 and 4) or first subjected to immunoprecipitation with anti-VirD2 serum (lanes 7 and 8) or incubated with protein A-Sepharose as a control (lanes 5 and 6). Ten times more restricted DNA was used for the immunoprecipitation (lanes 7 and 8) than for direct loading (lanes 3 and 4). The DNA–protein complex was identified by hybridization to probe D after the DNA was affixed to the filter by baking for 2–3 hr at 80°C in a vacuum oven. The transfer conditions allowed very little binding of DNA without attached proteins (as tested by transfer of ^{32}P-labeled, denatured pBR322 restriction fragments). Protein molecular size markers (Bio-Rad) are indicated in kilodaltons.

DISCUSSION

We have presented evidence that free linear double-stranded as well as single-stranded T-DNA molecules are produced upon AS induction and are bound to proteins. On double-stranded cuts at the borders and double-stranded fragments nicked at the borders the bond was identified as a covalent linkage between the 5′ end left of both cut or nicked borders and the VirD2 protein.

Although the involvement of a single-stranded T-DNA molecule seems certain, its direct participation in transfer to the plant, in a conjugationlike manner, was suggested but not proven (7, 8). We have shown that free linear double-stranded T-DNA molecules are also detected in induced bacteria. Thus both forms might be part of the same transfer pathway or, alternatively, one form might be a side-product or *in vitro* artifact of the other. In any case, the quantity of a given T-DNA structure cannot be used as an argument for its biological relevance. In addition, both processed T-DNA molecules are identical with respect to the covalently attached protein. Fig. 6 shows a schematic representation of the various discussed T-DNA molecules and suggests their possible relationship and involvement in T-DNA transfer.

Both borders behaved identically, i.e., in both cases the protein was found attached to the 5′ ends of the introduced lower-strand nicks (Fig. 6). As a consequence, there is no formal distinction between T-DNA and non-T-DNA on the Ti plasmid. The terms left border and right border are meaningful only when defined with respect to the marker that is to be transferred to the plant.

Comparison of the location of the endonucleolytic attack at the borders in the induced *Agrobacterium* with the sequences of the T-DNA ends recovered from plants suggests that VirD2 is attached to the third base of the border, since no border-specific sequence past the third base of the right 25-bp repeat was found in plants (1, 2, 37) except perhaps in one case (37). Hence, the attached protein would be removed

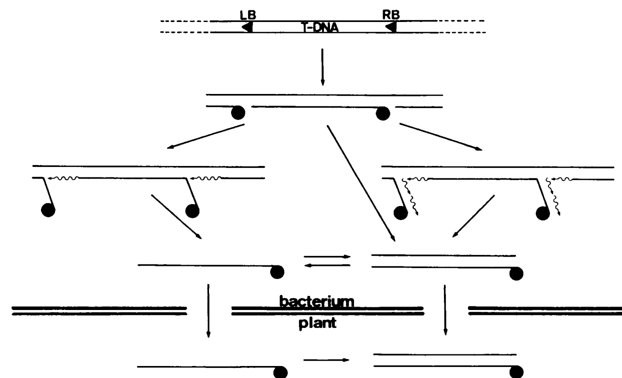

FIG. 6. T-DNA molecules found in induced bacteria: their possible relationship and involvement in T-DNA transfer. LB and RB, left and right border sequences; wavy lines, *de novo* DNA synthesis. •, VirD2.

from the third base in the plant, for example, in the process of integration. Alternatively, the nick could be introduced after the fourth base in the bacterium, and the last base together with the attached protein could be removed in the plant. This would be consistent with our mapping data (Fig. 2) if one assumes that primer extension would proceed to within one residue of the 5′ terminus of the template. Exactly this has been found in a study of cowpea mosaic virus in which the RNA template was also digested by proteinase (38). Such an explanation is attractive because the cleavage of the border by the VirD2 endonuclease would actually be located between bases that are absolutely conserved between the nopaline (see Fig. 2) and octopine borders.

Many prokaryotic and eukaryotic nucleic acids, traveling from one organism to another as DNA or RNA virus, carry terminal proteins covalently linked to the 5′ end(s) of their genome (e.g., adenovirus, bacteriophage ϕ29, and many plant viruses, but also some plant mitochrondrial plasmids). The function of the terminal proteins seems to be primarily in genome replication and possibly packaging (reviewed in refs. 35, 39). In *E. coli* plasmid conjugation, a protein linked to the 5′ end of the single-stranded transferred DNA was suggested, but not proven, to act as a pilot protein to transfer the DNA into the recipient cell. In the case of several plasmids, a protein has in fact been detected attaching covalently to the 5′ terminal nucleotide of the nicked strand of the relaxation complex (40, 41). However, it was not directly demonstrated that the attached protein was actually transferred. Single-stranded DNA of plasmid-unit length is not present within the donor cell, since during conjugation the unwinding of the donor strand is directly coupled to DNA transfer. Thus the fact that induced *Agrobacterium* contains single-stranded T-DNA molecules would reflect a major difference in the mechanism of DNA transfer as compared to plasmid conjugation.

In analogy to viruses and conjugating DNA, and extending beyond, the functions one could envisage for the covalently bound VirD2 protein could be in T-DNA protection, nuclear targeting, priming of DNA replication, and integration.

Does VirD2 enter the plant cell at all? Comparison of excised T-DNA in the bacterium with molecules rescued from plants early after transfer, prior to integration, reveals a better conservation of the right T-DNA end than of the left (37). This may be due to the protective role of the attached virulence protein, as has been shown in this work by *in vitro* experiments. Piloting through cell walls and membranes and into the nucleus of the plant may be a more complex process. In addition to VirD2, other noncovalently attached virulence proteins (for example, VirD1 and VirE2) or host proteins may aid in directing the T-DNA into the nucleus. A function in integration may require bound VirD2 to retain an enzymatic function after having formed the covalent bond. This is the case for ϕX174 phage protein A, which as a covalently bound protein exhibits nicking and ligase activity (reviewed in ref. 39). Alternatively, a plant protein with high affinity for VirD2 may actually carry out the integration step.

Note. Tight binding of VirD2 to T-strands was recently reported by Howard *et al.* (42).

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