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High levels of double-stranded transferred DNA (T-DNA) processing from an intact nopaline Ti plasmid

(*Agrobacterium tumefaciens*/crown gall disease)

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ABSTRACT **To obtain bacterial-mediated oncogenic transformation of plants, the transferred DNA (T-DNA) of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* is transferred to its plant host cells during infection. The initial phases of transformation involve the processing of the T-DNA in the bacterial cell after induction of the *vir* genes located on the Ti plasmid. The kinetics and conditions of this processing were examined and upon induction with acetosyringone up to 40% of the left and right borders of the T-DNA were cleaved. This cleavage was dependent upon *virA*, *virG*, and *VirD* and was *rec*-independent. Processed T-DNA was observed within 30 min after induction and was delayed by an increased concentration of phosphate in the induction medium. When DNA was isolated in the absence of protease treatment, the DNA fragment corresponding to the left side of the cut at both the left and right border region exhibited gel retardation, suggesting one or more "pilot" proteins may be involved in T-DNA transfer. Although the relative abundance of a processed product does not necessarily imply relative importance, the preponderance of double-stranded cleavage products suggests that double-stranded T-DNA should be considered as a possible intermediate in T-DNA transfer.**

Agrobacterium tumefaciens, a Gram-negative bacterium that infects a wide variety of wounded plants, causes a neoplastic growth disorder known as crown-gall disease. Essential to tumorigenesis is the ≈200-kilobase (kb) Ti plasmid, of which an ≈25-kb section known as the transferred DNA (T-DNA) is transferred and integrated into the plant nuclear DNA during infection (1, 2). T-DNA transfer is induced in response to phenolic compounds such as acetosyringone (AS). These inducers, released by plant suspension cells (3) or wounded plant tissue (4, 5), stimulate the transcription of genes in the *vir* region located on the Ti plasmid. This region encodes trans-acting proteins required for T-DNA transfer (6, 7). In the nopaline plasmid pTiC58, we have shown that there are six *vir* operons (*virA*, *virB*, *virG*, *virC*, *virD*, and *virE*) containing about 22 genes and spanning ≈25 kb (5, 8). Functions have been determined for some of these genes. *virA* and *virG* are regulatory and thought to be involved, respectively, in sensing inducing compounds of the plant and activating transcription of *virB*, *virC*, *virD*, and *virE* (5, 9). One *virE* gene from both nopaline (10) and octopine (11–13) plasmids encodes a nonspecific single-strand DNA binding protein. And *virD1* and *virD2* from octopine plasmids encode an undefined endonuclease (14–16) that nicks the 25-base-pair imperfect direct repeats (17) at the T-DNA left and right borders (LB and RB, respectively). In addition a non-Ti plasmid locus, *ros*, regulates *virC* and *virD* (18).

To understand T-DNA processing, several laboratories have focused on characterizing the physical properties of processed T-DNA. Bottom-strand-specific nicking at both borders has been observed in response to *vir* gene induction (16, 19, 20). Moreover, single-stranded linear DNA corresponding to the T-DNA bottom strand (T-strand) has been observed by Southern blot analysis after AS induction (9, 19, 21). In addition to the observance of single-stranded molecules, two types of double-stranded processed T-DNA have been reported. (*i*) Double-stranded circular DNA has been recovered from *Escherichia coli* by plasmid rescue (22) and from induced *A. tumefaciens* by phage λ *in vitro* packaging (23) and plasmid rescue (23–25). (*ii*) Double-stranded cleaved borders have occurred at approximately equal rates as T-strands upon cocultivation of *A. tumefaciens* carrying the octopine plasmid pTiA6 with tobacco protoplasts (26, 27). Based on these results, two general models for T-DNA processing have emerged: (*i*) nicking of the bottom strand of the borders followed by strand displacement results in a linear, single-stranded T-DNA intermediate that might be transferred by a conjugative mechanism (the single-stranded model) (21) and (*ii*) double-strand cleavage of (28–30) or recombination between (22–24) the T-DNA borders results in a double-stranded, linear or circular intermediate (the double-stranded model).

To elucidate the nature of the T-DNA intermediate, we have examined the T-DNA processing reaction of a nopaline Ti plasmid in rec^+ and rec^- backgrounds and have determined the kinetics of the reaction after AS induction with cells lysed in the presence and absence of protease. These studies revealed that AS induction leads to a high level of double-stranded cleavage at the T-DNA borders and binding of one or more "pilot" proteins at the cleaved ends.

METHODS AND MATERIALS

Bacterial Strains and Media. *A. tumefaciens* strains LBA4011 Rm^r, LBA4301 Rm^r, rec^- (31), and LBA4301 *ros*1 (18) (where Rm is rifampicin) were maintained in medium 523 (32) containing (per liter) 10 g of sucrose, 8 g of casein hydrolysate, 4 g of yeast extract, 3 g of K_2HPO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$ (pH 7.0), and rifampicin at 25 μg/ml at 29°C. Murashige–Skoog (MS) medium (33) was supplemented with 12.5 mM sodium phosphate buffer (pH 5.7), or with 2 mM sodium phosphate (pH 5.7) and 20 mM Mes buffer (Sigma) as described in *Results*. pGV3850 (34) was kindly provided by Patricia Zambryski (University of California, Berkeley) and Hans Bohnert (University of Arizona).

AS Induction. Cells of overnight cultures of *A. tumefaciens* strains were grown at 29°C in medium 523 containing rifampicin at 25 μg/ml and neomycin at 25 μg/ml (where appropriate) and diluted 1:6 into buffered MS medium containing

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Abbreviations: AS, acetosyringone; T-DNA, transferred DNA; LB and RB, left and right borders, respectively.

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neomycin at 5 μg/ml. The cells were grown at 29°C for 24 hr and then diluted ≈1:50 typically into 10 ml of buffered MS containing 0 or 10% (vol/vol) medium 523 and neomycin at 5 μg/ml (where appropriate). AS (3',5'-dimethoxy-4'-hydroxyacetophenone; Aldrich), at a stock concentration of 10 mg/ml in 70% (vol/vol) ethanol, was added to early exponential stage cells to a final concentration of 100 or 200 μM. After addition of AS, cells were harvested by centrifugation as indicated in the text.

T-DNA Intermediate Isolation. One milliliter of the above cell preparation was resuspended in 50 μl of TEN buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/10 mM NaCl); 25 μl of proteinase K at 600 μg/ml (Sigma) was added, followed by 25 μl of 10% (wt/vol) $NaDodSO_4$ in TEN buffer. Cells were incubated at 37°C for 60 min, followed by multiple pipeting through a 1-ml tip to dissociate clumps. Sodium chloride was added to a final concentration of 150 mM, and the lysate was incubated at 65°C for 30 min. The lysate was extracted twice with redistilled phenol/chloroform, 1:1 (vol/vol), and then twice with chloroform, followed by precipitation with an equal volume of isopropyl alcohol. The precipitated DNA was then redissolved overnight on ice in TEN buffer containing RNase A at 0.1 μg/ml (Sigma).

Southern Blot Analysis. Equal amounts of DNA (based on A_{260}), from 1 to 5 μg per experiment, were subjected to electrophoresis at 3–5 V/cm in 0.6–0.8% agarose gels in 1× TBE buffer (35). Transfer of DNA to Zeta-Probe nylon membranes with (36) or without (37) denaturation, hybridization, and washing was performed as described by Bio-Rad. DNA fragments to be labeled as probes were gel-purified and radioactively labeled using the random-primer sequencing method of Feinberg and Vogelstein (38). S1 nuclease digestion of DNA was performed prior to electrophoresis according to Stachel *et al.* (21). The relative intensity of hybridized DNA was quantitated using a Zeineh SL-504-XL scanning densitometer (Biomed Instruments, Fullerton, CA).

RESULTS

Double-Stranded Cleavage of Wild-Type Nopaline T-DNA. Processing of the T-DNA of the nopaline pTiC58 plasmid containing Tn*5* (insertion pJK270) in the T-DNA (39) in strain LBA4301 was first induced with AS and then assayed by Southern blot analysis. A restriction map of the T-DNA region is presented in Fig. 1*A* indicating the LB and RB in the restriction fragments examined and the expected size of fragments if cleavage occurred at the borders. Total bacterial DNA was isolated and digested with *Asp*718 (an isoschizomer of *Kpn* I) and *Pst* I and hybridized with *Kpn* I fragment 13a (40), which spans the LB of T-DNA, to examine LB processing events (Fig. 1*B*). Similarly, *Eco*RI-, *Sma* I-, and *Xba* I-digested DNA was hybridized with labeled *Eco*RI restriction fragment 1 (40) that spans the RB of T-DNA, to examine RB processing (Fig. 1*C*). The results of these studies show that the fragments newly arising after AS induction are of the size expected from double-stranded DNA cleavage at both the T-DNA LB and RB. For example, in the absence of AS treatment, digestion with *Asp*718 resulted in an unprocessed 3.2-kb fragment and, with AS treatment, *Asp*718 digestion resulted in a 3.2-kb fragment as well as 2.0- and 1.2-kb processed fragments. The intensity of these bands as judged by densitometry varied with the various restriction endonuclease treatments between 3 and 25% of the corresponding unprocessed border fragments. As can be seen in Fig. 1*C*, *Xba* I- (lanes 3 and 4) and *Sma* I- (lanes 5 and 6) digested DNA results in hybridization to two unprocessed bands as expected (Fig. 1*A*) when hybridized with *Eco*RI fragment 1. No single-stranded or intact double-stranded T-DNA was observed in the absence of restriction endonuclease treatment.

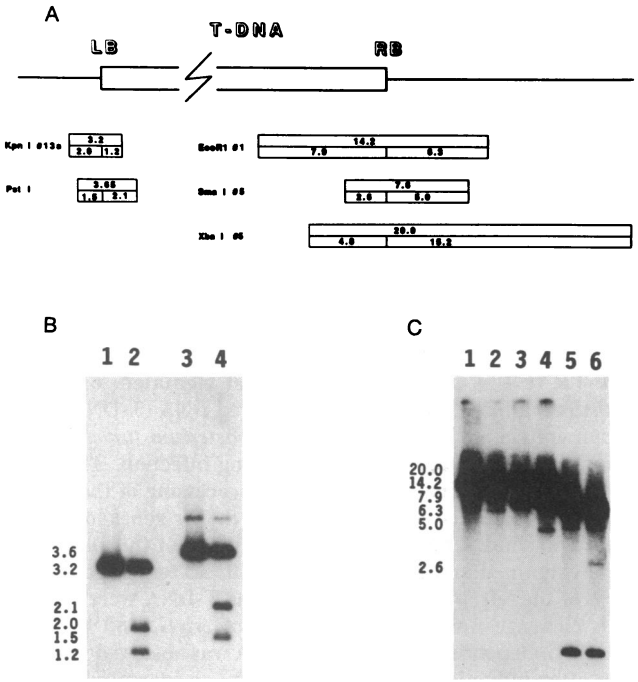

FIG. 1. T-DNA processing of pTiC58. (*A*) Restriction endonuclease map of T-DNA border regions from pTiC58. The number and size of fragments spanning the LB and RB generated by restriction endonucleases (40) are indicated in the upper section of boxes diagrammed below the T-DNA. The lower half of each box represents the size of fragments expected with double-stranded cleavage at the T-DNA borders. Numbers in the boxes represent fragment sizes in kb. (*B*) Total cellular DNA was digested with a restriction endonuclease as indicated, Southern blotted, and hybridized with *Kpn* I fragment 13a (36). Lanes: 1 and 2, *Asp*718 digest; 3 and 4, *Pst* I digest; 2 and 4, cells induced with AS; 1 and 3, uninduced cells. (*C*) As in *B* except hybridized with *Eco*RI fragment 1 (36). Lanes: 1 and 2, *Eco*RI digest; 3 and 4, *Xba* I digest; 5 and 6, *Sma* I digest; 2, 4, and 6, induced with AS; 1, 3, and 5, uninduced. Fragment sizes are given in kb.

To confirm that the processed bands corresponded to double-stranded DNA, transfer of DNA from an agarose gel to a nylon membrane was performed without denaturation—conditions in which only single-stranded DNA binds. No DNA was seen to bind by Southern blot analysis (data not shown). Furthermore, nuclease S1 treatment did not degrade the processed bands (data not shown). These results suggest that double-stranded cleavage at the nopaline T-DNA borders is the predominant form of T-DNA processing.

Because no single-stranded T-DNA was observed with the wild-type length nopaline Ti plasmid, we felt it necessary to confirm that our lysis procedure was not selectively altering or removing single-stranded DNA. Addition of 2 μg of exogenous 5-kb single- and double-stranded DNA to the lysate during the first step in lysis did not lead to degradation of either DNA form as determined by Southern blot analysis (data not shown).

In addition, plasmid pGV3850 (34), which gives rise to a 9.6-kb single-stranded T-DNA (T-strand) upon AS induction (21), was examined as above using three lysis procedures (Fig. 2). pGV3850 contains an intact nopaline *vir* region and nopaline border fragments with the internal T-DNA replaced with pBR322. When strain C58C1 containing pGV3850 was hybridized with pBR322, a 4.2-kb band hybridized in response to AS treatment (Fig. 2*A*). When the same DNA was blotted under nondenaturing transfer conditions, a 5-kb band hybridized to pBR322 (Fig. 2*B*). Because single-strand DNA migrates at a different position relative to double-stranded DNA in gels subjected to electrophoresis for various lengths

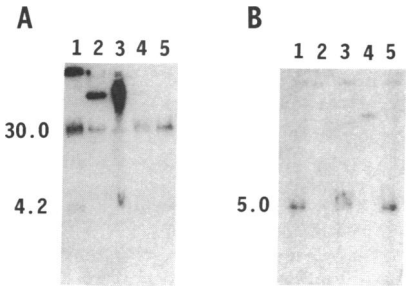

FIG. 2. Processing of pGV3850. DNA was isolated from *A. tumefaciens* C58C1 containing pGV3850. Undigested DNA was Southern blotted under denaturing (*A*) or nondenaturing (*B*) conditions and hybridized with pBR322. Lanes: 1, 3, and 5, cells induced with AS; 2 and 4, uninduced cells; 1, cells lysed as described herein; 2 and 3, cells lysed as described in ref. 41; 4 and 5, cells lysed as described in ref. 21.

of time, it is likely that the 4.2-kb and 5-kb bands represent the same single-stranded DNA fragment. Thus, only T-strands were observed by Southern blot analysis, in agreement with published results (21).

VirA, VirG, and VirD Are Required for T-DNA Processing. To determine if the double-stranded T-DNA processing was dependent upon *vir* region gene products, Southern blot analysis was performed on DNA isolated from 14 *vir* region Tn*5* or NEO-cartridge insertion mutants (indicated in Fig. 3*A* and described in ref. 5). Mutations in *virA*, *virG*, and *virD1* prevented processing at both the LB (Fig. 3*B*) and the RB (Fig. 3*C*). The amount of processing observed in the *virB*, *virC*, *virD4*, and *virE* mutants and pJK270 varied between 5 and 40% of the total amount of border-region T-DNA loaded in each lane. The absolute amount of processing observed for a given mutant varied between experiments within this range.

Processing Products Are Observed 60 Min After Induction. To determine if T-DNA border nicking or cleavage was dependent on time after AS induction and if processing of the LB and RB were sequential or simultaneous events, LBA4301 (pJK270) was harvested at various times after addition of AS and assayed for T-DNA processing. Double-stranded cleavage of the T-DNA borders was observed within 2 hr after induction (as shown for the LB in Fig. 4*A*). The amount of cleavage increased with time and reached a maximum at ≈48 hr. No difference in kinetics between the LB and RB processing was observed (data not shown).

T-DNA cleavage of pJK270 within 2 hr of addition of AS is faster than border processing observed with the mini T-DNA plasmid pGV3850. Nicking in pGV3850 occurs 4 hr after AS induction, reaching a maximum by 12 hr (20), whereas T-strand production is first observed 6–8 hr after induction (21). Because pH is known to affect the basal levels of *virG* expression (27, 29), it was possible that the 24-hr period of cell conditioning in low pH medium led to higher basal VirG levels than would exist with a cell conditioning period of 5–6 hr with pGV3850 (20, 21) and hence led to a faster response to AS induction. Therefore, we examined the kinetics of processing in strains grown with a conditioning period of 5–6 hr at low pH. The shorter conditioning did not have any appreciable effect on T-DNA processing (data not shown).

As with pH, the possibility of low phosphate levels affecting T-DNA processing was also examined using medium containing low levels of sodium phosphate. Incubation of bacteria in MS medium supplemented with 20 mM MES and 2 mM sodium phosphate instead of 12.5 mM sodium phosphate led to processed products being produced within 30–60 min after induction with either 24 hr (Fig. 4*B*) or 5 hr (data not shown) of conditioning in low pH medium. Additionally, the maximal level of processed T-DNA occurred 3–6 hr after induction, and

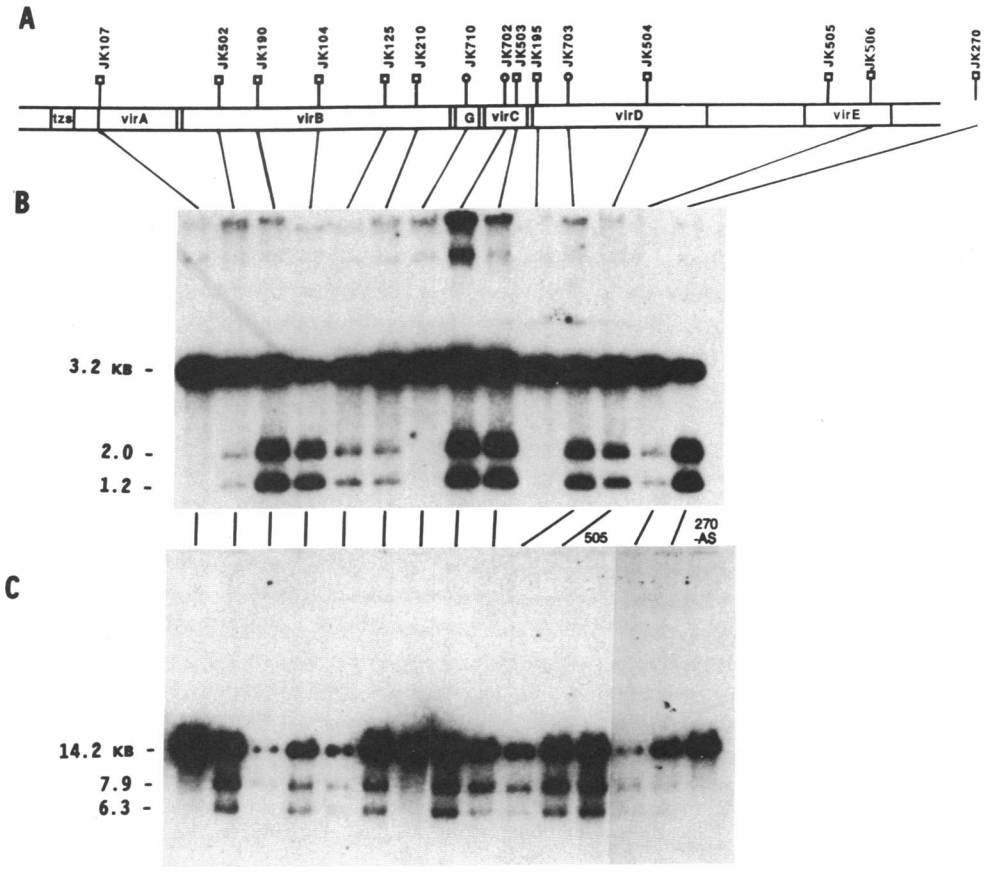

FIG. 3. T-DNA processing in *vir* mutant strains. (*A*) Locations of insertion sites of Tn*5* (♀) and NEO cartridges (♀) (5) are shown above a genetic map of pTiC58 (6). (*B*) DNA isolated from the corresponding insertional mutants in pTiC58, digested with *Asp*718, Southern blotted, and hybridized with *Kpn* I fragment 13a. (*C*) DNA from a different cell lysate than in *B*, isolated from the corresponding mutants, digested with *Eco*RI, Southern blotted, and hybridized with *Eco*RI fragment 1. Gel fragment sizes are given in kb.

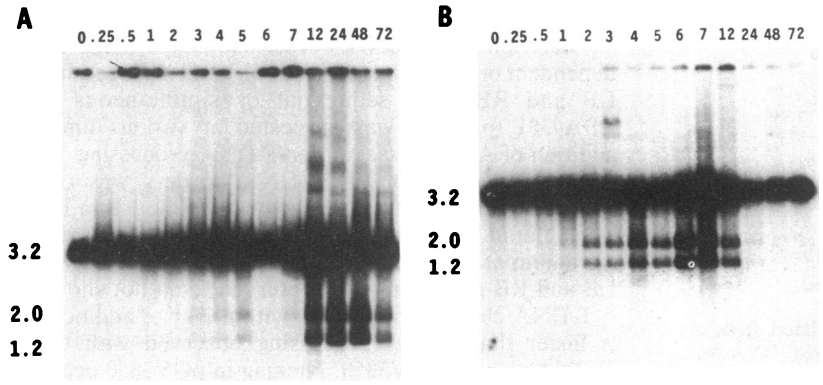

FIG. 4. Kinetics of T-DNA processing. Cells were induced with AS. At various times after induction, total DNA was isolated, digested with *Asp*718, Southern blotted, and hybridized with *Kpn* I fragment 13a. (*A*) Cells were grown as described herein. (*B*) Cells were grown as described herein except using MS medium buffered with 20 mM Mes (pH 5.7) and 2 mM sodium phosphate. Harvest time is given in hours after induction above each well. Gel fragment sizes are given in kb.

there was a striking decrease in levels of processed T-DNA by 24 hr after induction. These results are consistent with phosphate levels affecting the kinetics of T-DNA processing but not the type of T-DNA processing.

Homologous Recombination Is Not Involved in Processing. In the experiments described above, the *rec*$^-$ strain LBA4301 was used as a host. Because recombination has been proposed to be involved in T-DNA intermediate formation (19, 25), T-DNA processing of various *vir* region mutant plasmids in the recombination proficient parental strain LBA4011 was examined. No difference between strains LBA4301 and LBA4011 was observed; processing occurred only in response to AS treatment and was dependent upon *virA* and *virG* genes.

Protein Bound to T-DNA Border Fragments. In induced strains a *vir* region encoded protein is covalently bound to the 5′ side of bottom-strand T-DNA border nicks (42). To determine if a similar effect could be observed with double-stranded processed T-DNA, cell lysis was performed excluding protease treatment. The result of Southern blot analysis of DNA isolated from *vir* region mutant plasmids is given in Fig. 5*A* (for LB fragments) and Fig. 5*B* (for RB fragments). One of the two processed bands from both borders exhibits decreased intensity and mobility. This shifted band seen with the LB probe corresponds to the non-T-DNA fragment (compare to Fig. 3*B*), whereas the shifted band observed with the RB probe corresponds to the T-DNA fragment (compare to Fig. 3*C*). Hence these shifted bands correspond to those fragments that would contain the 5′ end of a nick in the bottom strand. That band retardation is due to a bound protein was confirmed by protease treatment resulting in restoration of normal band migration (Fig. 5*C*).

DISCUSSION

We have investigated the initial steps in T-DNA processing of a nopaline Ti plasmid. In response to AS induction, up to 40% of both T-DNA borders were cleaved. This double-stranded processing was dependent upon the products of *virA*, *virG*, and *virD* and occurred within 60 min of AS treatment. Neither virulence (unpublished results) nor T-DNA processing was affected in a *rec*$^+$ or *rec*$^-$ background, suggesting that homologous recombination mediated by this recombination system is not necessary for T-DNA processing.

These results can be contrasted with those of Stachel *et al.* (21), in which only border-specific nicking of pGV3850 in strain C58C1 after AS addition was reported by Southern blotting. Both pTiC58 and pGV3850 contain the same *vir* region genes. That investigators using similar experimental systems to determine T-DNA processing events have different observations suggests additional factors affecting processing may play a role. However, with octopine plasmid pTiA6, both double-stranded border cleavage and single-stranded T-DNA structures corresponding to the bottom strand have been observed in *A. tumefaciens* (26, 27) and in *E. coli* containing the *virD* locus from pTiA6 and a mini-Ti plasmid derived from pTiAch5 (16). The amount of cleaved borders varied between 1 and 10% of total hybridization signal (27).

The fact that no significant difference in T-DNA processing in cells grown for 5 versus 24 hr in low pH medium prior to AS induction suggests that either 5 hr is sufficient time for low pH effects to occur or pH did not affect processing. In contrast, the faster kinetics of processing observed with

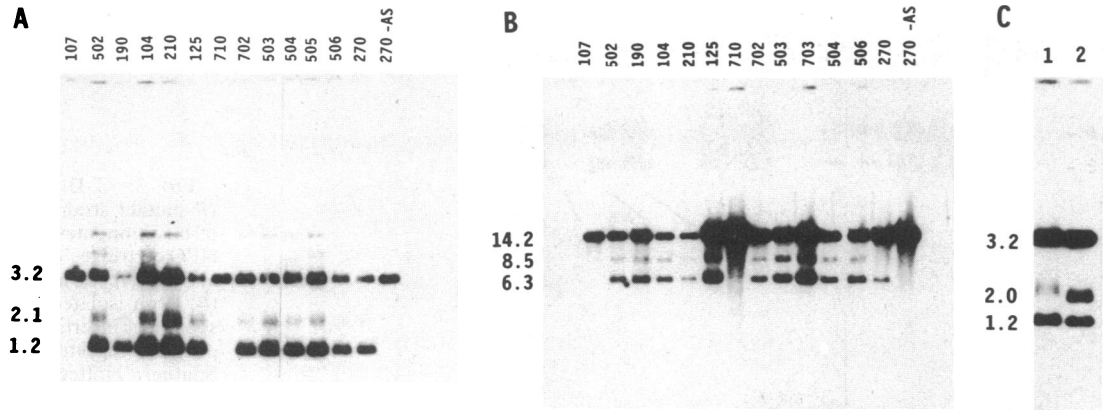

FIG. 5. Protein binding to processed T-DNA borders. Cells induced with AS were lysed as described herein except in the absence of protease. (*A*) DNA isolated from LBA4301 containing plasmids indicated above each lane was digested with *Asp*718 and hybridized with *Kpn* I fragment 13a. (*B*) DNA was digested with *Eco*RI and hybridized with *Eco*RI fragment 1. (*C*) DNA isolated without protease treatment from pJK506 was digested with *Asp*718 and hybridized with *Kpn* I fragment 13a. Lanes: 1, no additional treatment; 2, after treatment with proteinase K. Gel fragment sizes are given in kb.

decreased phosphate levels is consistent with high phosphate levels inhibiting induction responses. Winans *et al.* (29) have reported that induction of *virG* from the octopine plasmid pTiA6NC is reduced with increased phosphate levels. While the initiation of T-DNA cleavage occurred ≈60 min earlier in low phosphate medium, the decrease in cleaved T-DNA occurred at least 24 hr earlier in low versus high phosphate medium. The factors that decrease T-DNA processing at long times after induction are unknown. That phosphate levels affect processing is consistent with phosphorylation being involved in VirG activity.

Alt-Moerbe *et al.* (43) have observed in strain C58C1 containing pGV3850 that *virD2* induction requires only AS, sucrose, and an acidic pH and that incubation temperature, buffer, and amino acid combinations affect the amount of VirD2 produced. From these and other results, it is clear that *vir* induction can be modulated by many factors. For example, we do not know what causes the variation between mutants in the absolute amount of T-DNA processing observed in Fig. 3.

The shifted band observed with T-DNA isolated from induced cells lysed without protease treatment corresponds to the processed band located to the left of each T-DNA border and is the fragment that would contain the 5′ side of a bottom-strand nick. The bound protein is probably covalently attached, because heating the lysate to 65°C followed by phenol extraction should have removed most noncovalently attached proteins. It has been reported that the *virD2* product is bound to the 3′ ends of nicked T-DNA borders (42). All of the processing-proficient *vir* mutants examined here exhibited band retardation in the absence of protease treatment.

Reproducing the results of Stachel *et al.* (21) in detecting only single-stranded T-DNA from pGV3850 (Fig. 2), using both the lysis procedures described herein, in Dhaese *et al.* (41), and in Stachel *et al.* (21), argues against the cleaved forms of T-DNA observed here originating from nicked molecules due to artifacts of lysis. The lack of an intact double-stranded T-DNA fragment in undigested DNA may be due to shearing of the T-DNA (25–30 kb) or to networks being formed between the T-DNA and the Ti plasmid or sheared chromosome. Artifactual T-DNA cleavage at sites opposite single-stranded nicks by a nuclease common to the five restriction endonucleases used in Fig. 1 *B* and *C* seems implausible. The results presented here are consistent with a double-stranded model of T-DNA processing. Although the relative amounts of forms of processed T-DNA found in the bacterium do not imply relative importance in T-DNA transfer to plants, the high frequency of double-stranded T-DNA generated from our nopaline plasmids may implicate double-stranded DNA as a virulent form of T-DNA.

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