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Virulence Genes, Borders, and Overdrive Generate Single-Stranded T-DNA Molecules from the A6 Ti Plasmid of *Agrobacterium tumefaciens*

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***Agrobacterium tumefaciens* transfers the T-DNA portion of its Ti plasmid to the nuclear genome of plant cells. Upon cocultivation of *A. tumefaciens* A348 with regenerating tobacco leaf protoplasts, six distinct single-stranded T-DNA molecules (T strands) were generated in addition to double-stranded T-DNA border cleavages which we have previously reported (K. Veluthambi, R. K. Jayaswal, and S. B. Gelvin, Proc. Natl. Acad. Sci. USA 84:1881–1885, 1987). The T region of an octopine-type Ti plasmid has four border repeats delimiting three T-DNA regions defined as T left (TL), T center (TC), and T right (TR). The six T strands generated upon induction corresponded to the TL, TC, TR, TL+TC, TC+TR, and TL+TC+TR regions, suggesting that the initiation and termination of T-strand synthesis can occur at each of the four borders. Most TL+TC+TR T-strand molecules corresponded to the top T-DNA strand, whereas the other five T strands corresponded to the bottom T-DNA strand. Generation of T strands required the *virA*, *virG*, and *virD* operons. Extra copies of *vir* genes, harbored on cosmids within derivatives of *A. tumefaciens* A348, enhanced production of T strands. The presence of right and left border repeats in their native orientation is important for the generation of full-length T strands. When a right border repeat was placed in the opposite orientation, single-stranded T-DNA molecules that corresponded to the top strand were generated. Deletion of overdrive, a sequence that flanks right border repeats and functions as a T-DNA transmission enhancer, reduced the level of T-strand generation. Induction of *A. tumefaciens* cells by regenerating tobacco protoplasts increased the copy number of the Ti plasmid relative to the bacterial chromosome.**

Agrobacterium tumefaciens causes crown gall tumors on a number of dicotyledonous (reviewed in references 4, 9, and 25) and some monocotyledonous (reviewed in reference 30) plants by transferring and expressing the transferred DNA (T-DNA) portion of its tumor-inducing (Ti) plasmid. The T-DNA portion is flanked by similar 25-base-pair (bp) direct repeat sequences designated as borders (3, 31, 48, 51). The T-DNA borders are *cis*-acting components that functionally define the T-DNA region. The rightward border repeat is required in its native orientation together with its flanking sequences (overdrive) for efficient transformation of plant cells (14, 15, 28, 29, 42, 44).

Two sets of bacterial genes play an essential role in tumorigenesis. Transposon mutagenesis of the chromosomal loci *chvA* and *chvB* (8) and other chromosomal loci (6, 22, 40) that specify binding of bacteria to plants greatly reduces virulence. These chromosomal virulence genes are constitutively expressed in the bacterium (8). Virulence (*vir*) genes on the Ti plasmid are organized into six genetic complementation units called *virA*, *virB*, *virG*, *virC*, *virD*, and *virE* (10, 12, 35). *virA* and *virG* are constitutively expressed, whereas the expression of *virB*, *virC*, *virD*, and *virE* is highly induced when *A. tumefaciens* contacts wounded plant cells (33, 38). In addition, the expression of *virG* is inducible both by lowering the pH of the culture medium from 7.0 to 5.6 (43) and by cocultivation with plant cells (33, 38). Phenolic compounds such as acetosyringone and hydroxyacetosyringone are synthesized and secreted by wounded plant cells and act as signal molecules mediating the induction of these *vir* genes (5, 34). *virA* and *virG* mediate the induction of the other *vir* genes (38, 47). *virA* and *virG* share sequence homology with other two-component regulatory systems such as *ntrB-ntrC*, *envZ-ompR*, and *phoR-phoB* (18, 27, 47). *virA* protein, localized on the bacterial membrane, may sense wound-induced phenolic compounds secreted by the plant cells and transduce this signal by transforming an inactive *virG* protein into a functional transcriptional activator of other *vir* genes (47).

The *Agrobacterium*-plant cell interaction offers an unique example of interkingdom DNA transfer. The T-DNA portion of the Ti plasmid is specifically transferred across the bacterial and plant cell walls and membranes and is integrated into the plant genome (41). Several potential intermediates in T-DNA transmission accumulate in *A. tumefaciens* after induction of the *vir* genes. Various groups have reported the generation of circular forms of the T-DNA (2, 17, 20, 49). Induction of *A. tumefaciens* with plant cells (1, 50) or acetosyringone (45) generates site-specific nicks within the border repeats largely on the bottom strand, and linear single-stranded copies of the bottom strand of the T region (called T strands) accumulate (36). Recently, we reported that double-stranded cleavages occur at each of the T-DNA borders upon cocultivation of *A. tumefaciens* with regenerating tobacco protoplasts (43). All of these different reactions require *virA*, *virG*, and the first two open reading frames of the *virD* locus (2, 13, 37, 43, 49, 50).

To understand the molecular mechanism of T-DNA transmission we characterized the complex pattern of T strands generated from the octopine-type Ti plasmid pTiA6 that contains four T-DNA borders (3). We investigated the effect of mutations in different *vir* genes on T-strand generation. We also evaluated the roles of right and left border repeats

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and the overdrive sequence in the generation of T-strands and of double-stranded T-DNA border cleavages. While this manuscript was in preparation, Stachel et al. (37) reported observations on some aspects of T-strand generation in an octopine-type Ti plasmid.

MATERIALS AND METHODS

Bacterial strains. *A. tumefaciens* A348 (16) harboring the Ti plasmid pTiA6 was used in our studies. Stachel et al. (33) generated strains containing mutations in the virulence region by transposon mutagenesis with Tn*3*-HoHo1. The mutant plasmids harbor Tn*3*-HoHo1 insertions in the *virA*(237), *virB*(243), *virC*(364), *virD*(311), *virE*(341), and *virG*(321) loci of pTiA6 (35). Knauf and Nester (16) constructed the cosmids pVK224, pVK225, pVK227, and pVK257, carrying overlapping segments of the pTiA6 *vir* region, by cloning *Sal*I partial digests of pTiA6 into the unique *Sal*I site of the broad-host-range vector pVK102. The construction of *A. tumefaciens* strains with border deletions or border substitutions (WR3095, WR1043, WR1031, WR1032, and WR1105) has been described previously (28, 29). *Escherichia coli* LE392 [F^- *hsdR514* (r_K^- m_K^-) *supE44 supF58 lacY1* or Δ(*lac-1ZY*)*6 galK2 galT22 metB1 trpR55* λ^-] (21) was used as the host for the cloning of riboprobe plasmids. *A. tumefaciens* strains were grown at 30°C on either AB minimal medium supplemented with 0.5% glucose or on YEP medium (19). *E. coli* strains were grown at 37°C on LB (21). The antibiotic concentrations used for *A. tumefaciens* were as follows: rifampin, 10 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; and carbenicillin, 100 μg ml^{-1}. For *E. coli*, ampicillin and kanamycin were used at 40 μg ml^{-1}. The cosmids containing *vir* region segments were introduced into *A. tumefaciens* A348 or into the *vir* mutant strains by triparental mating with pRK2013 as a helper plasmid (7).

Enzymes and reagents. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Pharmacia, Inc., and New England Biolabs and used according to the recommendations of the suppliers. T3 RNA polymerase was from Bethesda Research Laboratories. T7 RNA polymerase, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were from Pharmacia. Lysozyme (grade I, from chicken egg white), diethylpyrocarbonate, S1 nuclease (from *Aspergillus oryzae*), and ribonucleoside triphosphates were from Sigma Chemical Co. [α-^{32}P]dCTP (800 Ci/mmol) and the nick translation kit were purchased from Amersham Corp. [α-^{32}P]UTP (600 Ci/mmol) was from ICN Radiochemicals.

Plasmid constructions. Figure 1 shows an *Eco*RI restriction endonuclease map of the pTiA6 T region. The T-DNA of this octopine-type Ti plasmid has two noncontiguous sections termed T left (TL) and T right (TR) (41). The region of the Ti plasmid between TL and TR is not generally found in plant tumors and is designated as T center (TC). Figure 1 also shows the locations of border repeats (designated as A, B, C, and D), fragments expected from double-stranded border cleavages (in kilobase pairs), possible T strands (in kilobases), and the sequences used as probes. We have used the coordinates from the numbering system of Barker et al. (3) in the following description of plasmid constructions. We used the vector pBSM13$^+$ obtained from Stratagene to construct plasmids employed for the synthesis of strand-specific RNA probes (riboprobes). In pBSM13$^+$ the T3 and T7 RNA polymerase promoters are individually present on either side of the multiple cloning site. We monitored insertion of fragments into the multiple cloning site of this vector by the

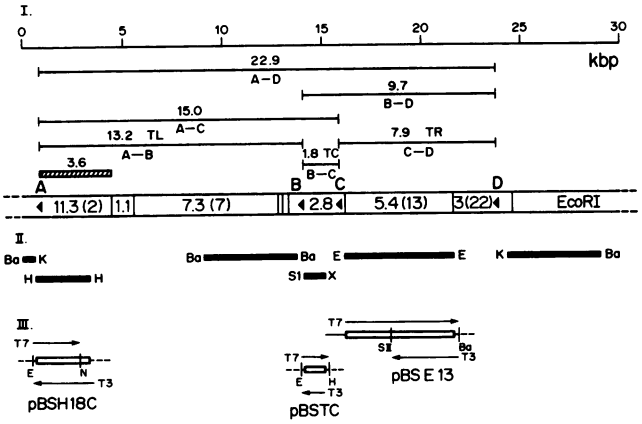

FIG. 1. *Eco*RI restriction endonuclease map of the T region of pTiA6 (section I). The sizes of these fragments (in kilobase pairs) are indicated. Numbers within parentheses represent the *Eco*RI fragment numbers. The coordinate system of Barker et al. (3) is indicated at the top in kilobase pairs. The border repeats designated as A, B, C, and D delimit the T region into three T-DNA elements depicted as TL, TC, and TR. The T-DNA segments between different borders are designated by using border repeats as the endpoints. These represent the six discrete T strands that could possibly be generated from pTiA6. The length of each segment between the borders is indicated on top of each fragment (in kilobases for single-stranded fragments). A 3.6-kbp *Eco*RI fragment expected from double-stranded cleavage by the *virD* endonuclease at border A and *Eco*RI cleavage at coordinate 4494 (3) is shown as a hatched bar. In section II the restriction endonuclease fragments used as nick-translated, double-stranded probes are shown as solid bars. The open bars in section III represent the regions that were cloned into pBSM13$^+$ for preparing strand-specific RNA probes. The strategy for making strand-specific probes is outlined. For example, pBSH18C was linearized with *Nco*I and transcribed with T7 RNA polymerase to prepare a top-strand probe. Similarly, pBSH18C was linearized with *Eco*RI and transcribed with T3 RNA polymerase to make the bottom-strand probe. The arrows indicate the directions of transcription. Abbreviations for restriction endonucleases: Ba, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; SI, *Sst*I; SII, *Sst*II; X, *Xho*I.

loss of β-galactosidase activity upon transformation of *E. coli* LE392. We cloned *Hin*dIII fragment 18C (between bp 602 and 3390) into the *Hin*dIII site of the vector, generating pBSH18C (Fig. 1). We cloned a *Sst*I-to-*Xho*I fragment from TC (between bp 14089 and 15208) between the *Sst*I and *Sal*I sites to construct pBSTC. We cloned the *Eco*RI fragment 13 (between bp 16202 and 21631) into the *Eco*RI site to construct plasmid pBSE13.

We constructed *A. tumefaciens* WR3079 (see Fig. 5A) with a TL left border deletion as follows. We first constructed a deletion in pWR26, a pHK17 derivative (16) containing *Eco*RI fragment 2 with a Tn*3* insertion (at approximate coordinate 3400 in the T-DNA region) to generate plasmid pWR27. pWR26 contains two *Bam*HI sites, one in *Eco*RI fragment 2 (coordinate 1 in reference 3) left of the TL left border and another in Tn*3* (on the opposite side of the border). By digesting pWR26 with *Bam*HI and ligating the resulting fragments into circles we deleted the *Bam*HI fragment that contains the TL left border; the *bla*$^+$ (carbenicillin resistance) portion of Tn*3* remained intact, constituting the left end of the T-DNA. We translocated the *bla*$^+$ deletion into pTiA6 by selecting carbenicillin-resistant recombinants.

A. tumefaciens WR1120 was constructed from shuttle vector pWR16, which contains *Eco*RI fragment 2 in the cosmid vector pHK17. pWR16 contains an unique *Bam*HI

site flanked by 6.8 kbp of EcoRI fragment 2 to the left and 4.7 kbp to the right, including the TL left border repeat (border A). We cloned a 1,125-bp *Acc*I TL right border fragment (bp 13911 through 15116; 3) into the *Hin*cII site of pUC8. We linearized this plasmid with *Bam*HI and ligated it to pWR16 digested with *Bam*HI. We identified a clone that contained the right border fragment in the orientation opposite to that of the border A repeat in *Eco*RI fragment 2 and translocated the construction by homologous recombination into the wild-type strain A348. The resulting strain (WR1120) has an additional right border in the opposite orientation and to the left of border A (see Fig. 5A).

Protoplast isolation and cocultivation with *A. tumefaciens*. We isolated protoplasts from the leaves of aseptically grown *Nicotiana tabacum* cv. Wisconsin 38 plants (43). We diluted the protoplasts to 10^5 protoplasts ml^{-1} in supplemented K_3 medium (24) and allowed them to regenerate for 3 1/2 days. The growth conditions for *A. tumefaciens* and the conditions for the cocultivation of the bacteria and protoplasts have been described (43). We performed cocultivation for 24 h at a concentration of 10^5 protoplasts ml^{-1} and 10^8 bacteria ml^{-1}. We refer to *A. tumefaciens* cells cocultivated with protoplasts as induced bacteria (also as P) and the control bacteria grown in K_3 medium for 24 h as uninduced bacteria (also as K). We separated the bacteria from the plant cells (43) and extracted the bacterial DNA. We induced *A. tumefaciens* with acetosyringone (10 μM) for 24 h (43). We evaluated *vir* gene induction during cocultivation by determining the levels of β-galactosidase activity in strains harboring Tn*3*-HoHo1 insertions in *vir* genes.

Analysis of DNA. We extracted DNA from *A. tumefaciens* by using lysozyme and sodium dodecyl sulfate (43) and determined the concentration fluorimetrically by using diaminobenzoic acid (11). We digested the DNA with various restriction endonucleases by using 10 U of enzyme per μg of DNA under the conditions recommended by the suppliers. We digested the DNA with S1 nuclease as described previously (21). Routinely, we fractionated 500 ng of each DNA sample by electrophoresis through 0.7% agarose gels in TBE buffer (21). We performed denatured Southern blotting as described previously (32). For nondenatured transfer (36), we soaked the gel in H_2O for 20 min and then in 20× SSC (21) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min before performing capillary blotting. We nick translated gel-purified restriction endonuclease fragments with Amersham nick translation kits. Southern hybridization conditions have been described previously (43).

Synthesis of RNA probes. We synthesized radioactive RNA probes (riboprobes) by in vitro runoff transcription (23). The strategy used for linearizing the plasmids is outlined in Fig. 1. In all of the clones shown in Fig. 1, the use of T7 RNA polymerase resulted in the transcription of the top T-DNA strand, and the use of T3 RNA polymerase resulted in the transcription of the bottom T-DNA strand. We prepared plasmid DNA by alkaline lysis and two cycles of CsCl-ethidium bromide centrifugation (21). We digested the plasmid DNA (20 μg) with the indicated restriction enzyme (Fig. 1), extracted the solution with neutral phenol-chloroform (1:1), precipitated the DNA with ethanol, and dissolved it in 1 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA.

We treated all glassware and solutions with diethylpyrocarbonate to minimize RNase contamination (21). We transcribed a 0.5-μg sample of each linearized plasmid with 20 U of T3 or T7 RNA polymerase for 1 h at 37°C in a 20-μl reaction mixture containing 40 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 25 mM NaCl, 2 mM spermidine hydrochloride, 5 mM dithiothreitol, 500 μM each ATP, CTP, and GTP, 12 μM UTP, and 3.6 μM [α-^{32}P]UTP (600 Ci/mmol). We added all of the reagents at room temperature to avoid precipitation of DNA in the cold in the presence of spermidine. The linearization of pBSE13 with *Sst*II results in the generation of a 3′ protruding end that might cause a significant amount of extraneous transcription in the opposite direction (Promega Notes, no. 1, March 1985; Promega Biotech, Madison, Wis.). To avoid this, we converted the 3′ protruding end into a blunt end by treating the template with 5 U of Klenow fragment at 37°C for 15 min. We performed this reaction in the transcription buffer described above, except that we added the ribonucleoside triphosphates and the RNA polymerase later at the start of transcription. We separated the labeled RNA from unincorporated nucleoside triphosphates by using a spin-column procedure (21). We routinely achieved specific activities of 20×10^7 to 60×10^7 cpm of RNA per μg of DNA template. We used these riboprobes directly in hybridization reactions. We conducted recombinant DNA experiments under P1 containment conditions as specified by the National Institutes of Health guidelines.

RESULTS

Efficiency of T-strand production in induced bacteria. To study whether T strands are generated efficiently in *A. tumefaciens* upon cocultivation with protoplasts, we subjected the DNA from induced (P) and uninduced (K) bacteria to nondenaturing Southern blot analysis. When the DNA is not denatured, only single-stranded DNA will bind upon transfer to nitrocellulose (36). We probed the blots with nick-translated *Hin*dIII fragment 18C. Two fragments equivalent to 8.5 and 9.5 kbp hybridized to the probe (Fig. 2, lane 2). These fragments were present only in the induced bacteria. The size of the T strand derived from TL is 13.2 kilobases, with an expected mobility of a 6.6-kbp double-stranded fragment. A single-stranded fragment may migrate through a gel differently from a double-stranded molecule of half the nucleotide chain length. Because the bottom fragment (8.5-kbp equivalent mobility) hybridizes only to probes from within TL (Fig. 3 and data not shown), it most likely represents the T strand derived from the TL region between borders A and B. We shall define the T strands by using the border repeats as the expected endpoints and refer to this fragment as the A-B strand. Similarly, the top 9.5-kbp equivalent fragment represents the T strand generated between borders A and C, the A-C strand. S1 nuclease treatment eliminated the A-B and A-C strands, indicating that they are single-stranded molecules (data not shown). When we introduced either the cosmid pVK224 (containing *virG*, *virC*, *virD*, and *virE*) or the cosmid pVK257 (containing *virA*, *virB*, and *virG*) into strain A348 and cocultivated the bacteria with protoplasts, the number of T strands greatly increased (Fig. 2, lanes 3 and 4). These results indicate that *vir* genes play a role in T-strand generation and that some of the *vir* gene products may be at limiting concentrations in the wild-type strain A348. Border-specific nicking activity has been observed to increase upon introducing cosmids containing *vir* genes into *A. tumefaciens* (50). Introduction of additional copies of these *vir* genes quantitatively increased the levels of T strands without causing any change in the size of the T strands. Therefore, we used *A. tumefaciens* A348 harboring the cosmid pVK224 [A348(pVK224)] in most of the following cocultivation experiments.

We compared the level of T strands generated in strain A348(pVK224) induced either by cocultivation with proto-

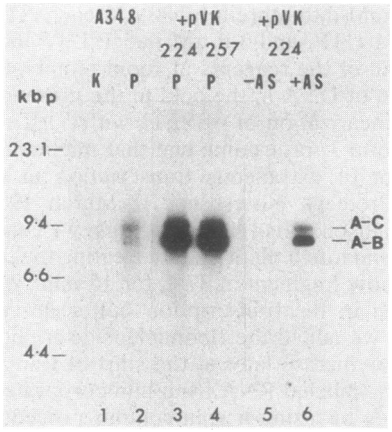

FIG. 2. T strands generated in bacteria induced either by cocultivation with protoplasts or by acetosyringone treatment. The wild-type *A. tumefaciens* (strain A348) and its transconjugants harboring either pVK224 (containing *virG*, *virC*, *virD*, and *virE*) or pVK257 (containing *virA*, *virB*, and *virG*) were used for analysis. The bacteria were incubated for 24 h either in K_3 medium (K) or in K_3 medium with protoplasts (P). In acetosyringone induction experiments, the control (−AS) and induced (+AS) bacteria were grown for 24 h in K_3 medium and K_3 medium with 10 μM acetosyringone, respectively. A 500-ng sample of bacterial DNA was separated on a 0.7% agarose gel, transferred to nitrocellulose under nondenaturing conditions, and probed with nick-translated *Hin*dIII fragment 18C. The T strands expected between borders A and B and between A and C are designated as A-B and A-C, respectively. Because of the relatively short exposure of this blot, the expected A-D strand cannot be observed.

plasts or by acetosyringone treatment. We treated the bacteria for 24 h with 10 μM acetosyringone, a concentration at which the *vir* genes are maximally induced (34). Acetosyringone treatment generated T strands at much lower levels than did cocultivation with protoplasts (Fig. 2, lane 6).

Pattern of T-strand generation. The use of all possible combinations of the four T-DNA border repeats of pTiA6 would produce six different T strands (Fig. 1). To analyze the pattern of T strands generated, we used a number of probes derived from different regions of the T-DNA (Fig. 1). We transferred DNA from uninduced and induced A348 (pVK224) cells to nitrocellulose under nondenaturing conditions. We used nick-translated *Hin*dIII fragment 18C and *Bam*HI fragment 19 as probes for the TL region. We used an *Sst*I-to-*Xho*I fragment and *Eco*RI fragment 13 as probes for the TC and TR regions, respectively. We nick translated a *Bam*HI-to-*Kpn*I fragment to the left of border A and a *Kpn*I-to-*Bam*HI fragment to the right of border D and used them as probes to analyze the regions of the Ti plasmid just outside the T-DNA region. The TL probes, *Hin*dIII fragment 18C and *Bam*HI fragment 19, hybridized to three fragments (Fig. 3A, lanes 2 and 4). The 8.5- and 9.5-kbp equivalent fragments correspond to the A-B strand (TL) and the A-C strand (TL+TC), respectively. The 25-kbp equivalent fragment represents the A-D strand (TL+TC+TR). The nick-translated TC probe (*Sst*I-to-*Xho*I fragment) hybridized to four different fragments (lane 6). As expected, the A-D strand and the A-C strand that hybridized to the TL probes also hybridized to the TC probe. The 1.3- and 5.3-kbp

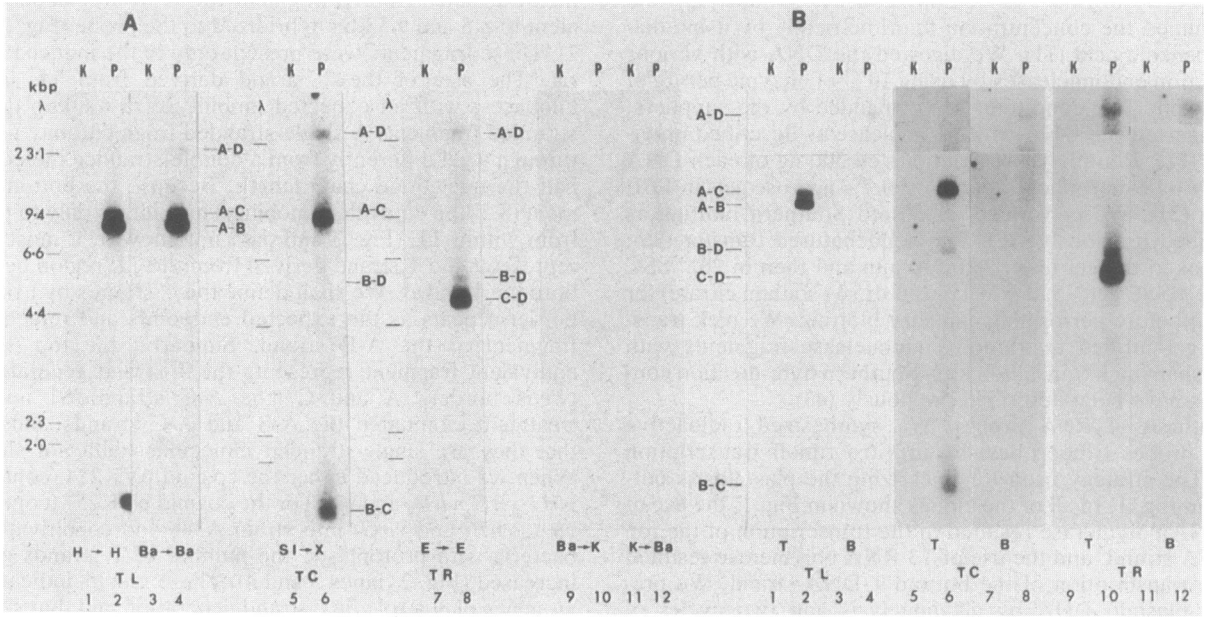

FIG. 3. Characterization of T strands generated from different regions of pTiA6 by using double-stranded DNA probes (A) and strand-specific RNA probes (B). Uninduced (K) and induced (P) *A. tumefaciens* A348(pVK224) DNA was subjected to nondenaturing Southern analysis. The T strands that could be generated between all combinations of the four borders are indicated in Fig. 1 (A-B, B-C, C-D, A-C, B-D, A-D) and are defined by their flanking border repeats. The numbers on top of each strand (Fig. 1) depict their expected sizes in kilobases. The following fragments shown as solid bars in Fig. 1 (section II) were nick translated and used as double-stranded probes: H→H, *Hin*dIII fragment 18C (coordinates 602 to 3390) (3); Ba→Ba, *Bam*HI fragment 19 (9062 to 13774) (3); Sl→X, *Sst*I-to-*Xho*I fragment (14089 to 15208) (3); E→E, *Eco*RI fragment 13 (16202 to 21631) (3); Ba→K, *Bam*HI-to-*Kpn*I fragment to the left of border A; K→Ba, *Kpn*I-to-*Bam*HI fragment to right of border D. The strategy for making strand-specific RNA probes is outlined in Fig. 1 (section III). The top- and bottom-strand RNA probes are designated as T and B, respectively. The regions of the T-DNA (TL, TC, TR) from which the probes were derived are also indicated at the bottom of the figure. Because the intensity of the A-D strand was very weak in comparison to other T strands, the top section of Fig. 3B is presented with pictures obtained from autoradiographs exposed for two to three times longer than the bottom section.

equivalent fragments represent the B-C strand (TC) and the B-D strand (TC+TR), respectively. The nick-translated TR probe (*Eco*RI fragment 13) hybridized to three fragments (lane 8). Two of these are the A-D and B-D fragments, and the third is a 4.8-kbp equivalent fragment that corresponds to the C-D strand (TR). The probes from outside the T-DNA region did not hybridize to any of the T strands (lanes 9 through 12). These results show that all four borders are used to delimit the various T strands.

T strands are derived from the bottom strand of the T-DNA. In induced bacteria single-stranded nicks occur at the border sequences predominantly on the bottom strands as the Ti plasmid map is conventionally drawn (1, 45, 50). It has been proposed that the initiation of synthesis of a new strand at these nicks and displacement of the previous strand results in the generation of the bottom strand as the T strand (1, 36). To characterize the strand(s) of the T-DNA from which the T strands of the octopine-type Ti plasmid are derived, we used strand-specific riboprobes. The strategy for making top- and bottom-strand riboprobes from the TL, TC, and TR regions is shown at the bottom of Fig. 1. The results of the analysis of T strands with strand-specific riboprobes are shown in Fig. 3B. A top-strand RNA from pBSH18C (TL) hybridized to the A-B and A-C strands (Fig. 3B, lane 2), whereas the bottom-strand RNA did not hybridize to these fragments (lane 4). Surprisingly, the bottom-strand RNA hybridized to the A-D strand (lane 4). These results indicate that the A-B and A-C strands correspond to the bottom strand of the T-DNA, whereas the A-D strand corresponds to the top strand of the T-DNA. Similarly, the top-strand RNA from pBSTC (TC) hybridized to the A-C, B-C, and B-D strands (lane 6), whereas the bottom-strand RNA did not hybridize to these T strands (lane 8). However, the bottom-strand RNA hybridized to the A-D strand (lane 8). The top-strand RNA derived from pBSE13 (TR) hybridized to the C-D and B-D strands (lane 10), whereas the bottom-strand RNA did not hybridize to these fragments (lane 12). Both top- and bottom-strand RNA probes hybridized to the A-D fragment (lanes 10 and 12). These results suggest that the A-B, B-C, C-D, A-C, and B-D strands correspond to the bottom strand. Apparently, both top and bottom strands can produce T strands that migrate at the A-D position. Longer exposures of Fig. 3B, lanes 2 and 6, did not detect the A-D T strand (data not shown). These results would suggest that the band migrating at the A-D position corresponds to the top strand between borders A and C and that it is possibly double stranded in the TR region. Alternatively, full-length A-D T strands may be derived from either T-DNA strand, but for technical reasons we may not have detected the bottom strand with TL and TC top-strand riboprobes.

Role of *vir* genes in the generation of T strands. Introduction of additional copies of *vir* genes into *A. tumefaciens* strain A348 resulted in the generation of higher levels of T strands (Fig. 2), suggesting that the *vir* genes may play a role in T-strand generation. We examined the effects of mutations in individual *vir* genes on the generation of T strands. These mutations (Fig. 4A) were made by Tn*3*-HoHo1 insertions (33). We cocultivated each mutant strain with protoplasts and analyzed the DNA for T strands by using nick-translated *Hin*dIII fragment 18C as the probe. The wild-type strain (A348) generated the A-B and A-C strands upon induction (Fig. 4B, lane 2). These T strands were also generated in the *virB*, *virC*, and *virE* mutants (lanes 6, 10, and 14). These T strands were not generated in *virA*, *virG*, and *virD* mutants (lanes 4, 8, and 12). These results suggest that *virA*, *virG*, and *virD* mediate the generation of T strands.

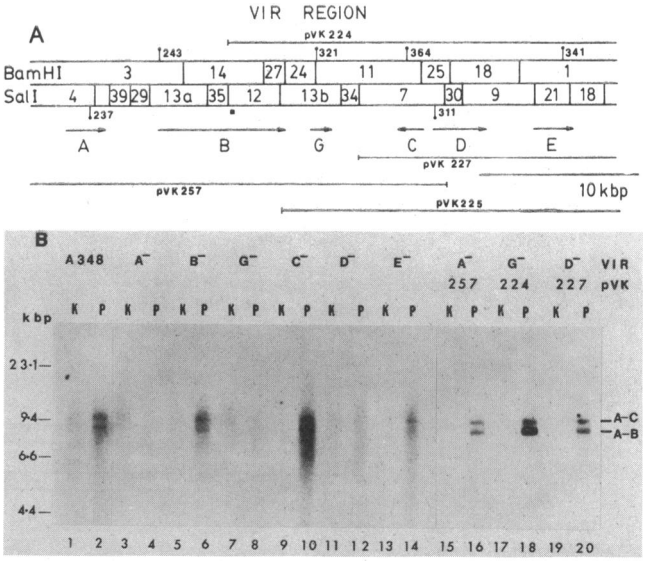

FIG. 4. Effect of mutations in different *vir* genes on T-strand generation. (A) Restriction endonuclease map of the *vir* region (35), positions of the Tn*3* HoHo1 insertions (33), and the regions covered by the cosmids pVK224, pVK225, pVK227, and pVK257 (16) used for complementation analysis. (B) Analysis of T strands in *A. tumefaciens* A348, in *vir* mutants, and in *vir* mutants complemented with cosmids carrying wild-type *vir* genes. The DNA from uninduced (K) and induced (P) bacteria was subjected to nondenatured Southern analysis and hybridized with nick-translated *Hin*dIII fragment 18C.

To confirm these findings, we restored the normal activity of these genes by complementation in *trans* with cosmids containing the wild-type *vir* genes. We used the cosmids pVK257, pVK224, and pVK227 to complement the *virA*, *virG*, and *virD* mutations, respectively (Fig. 4A). We transferred the cosmids to *A. tumefaciens* harboring the respective mutation on the Ti plasmid, cocultivated the transconjugants with protoplasts, and analyzed the DNA for T strands by using the nick-translated *Hin*dIII fragment 18C as the probe. Complementation of each of the mutations with the appropriate cosmids restored T-strand production (Fig. 4B, lanes 16, 18, and 20). These results indicate that *virA*, *virG*, and *virD* are required for the generation of T strands.

Role of border repeats and the overdrive sequence in the generation of T strands. Several investigators have proposed a model for T-DNA processing and transfer to plants based upon the presence of T strands and the occurrence of strand-specific nicks in the T-DNA of induced bacteria (1, 36, 50). This model parallels conjugal transfer of bacterial plasmids (46). Processing presumably begins with a site-specific nick in the bottom strand at each of the 25-bp repeats. Initiation of replication at the nick site and subsequent synthesis of a new strand could displace the old bottom strand in the 5′-to-3′ direction as a T strand. The nick at the second border could define the other end of the T strand. This model requires both right and left border repeats in their native orientation and position to generate full-length T strands. We tested this model by analyzing T-strand generation in a number of strains having deletions of the right or left borders or of the overdrive sequence (28, 29).

A map of the T-DNA region of the mutant strains used in this analysis is shown in Fig. 5A. All of the strains harbored either the cosmid pVK224 or pVK225 (Fig. 4A), so that the *vir* gene products did not limit T-strand generation. We

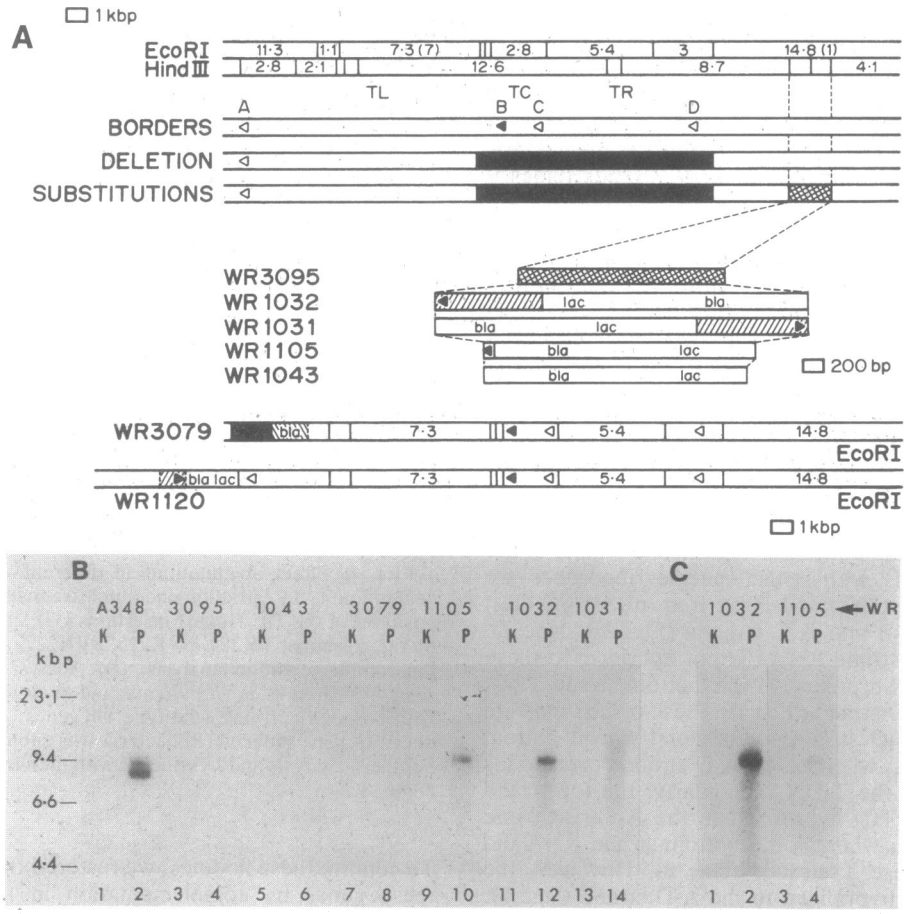

FIG. 5. Effect of border mutations on T-strand generation. (A) Restriction endonuclease map of the T-DNA border mutants. The details regarding the construction of various border and overdrive mutants have been described (28, 29). Briefly, the T region between *Eco*RI fragments 7 and 1 that contains borders B, C, and D was deleted. Sequences containing the right border or overdrive or both were cloned into pUC8 and substituted in the position of the two *Hin*dIII fragments indicated in the figure. Symbols: ■, deletion; ▨, substitution; ▨, T-DNA sequences flanking border B; ◀, TL right border; ◁, other borders; lac bla pUC8 plasmid-*bla* is β-lactamase, and *lac* is β-galactosidase. (B) Analysis of *A. tumefaciens* A348 and the border and overdrive mutants harboring pVK224 or pVK225 (both containing *virG*, *virC*, *virD*, and *virE*). Uncut DNA was subjected to nondenaturing Southern analysis and hybridized with nick-translated *Eco*RI fragment 7. The T strand generated in the border mutants migrated slower than the A-B strand because the T-DNA of these mutants is 2.8 kbp longer than the TL portion of the wild-type strain A348. (C) WR1032 and WR1105 used in this experiment did not contain *vir* gene cosmids. The DNA was analyzed as in panel B.

subjected the DNA from uninduced and induced bacteria to nondenaturing Southern analysis and probed it with nick-translated *Eco*RI fragment 7 from the TL region (Fig. 1). The T strands (A-B and A-C strands) generated in the wild-type strain A348(pVK224) (Fig. 5B, lane 2) were not generated in WR3095(pVK225) (lane 4), which lacks borders B, C, and D. Introduction of pUC sequences after the deletion of the right border [WR1043(pVK224)] did not restore T-strand generation (lane 6). However, when pUC sequences carrying the right border and the overdrive sequence were reintroduced in their native orientation [WR1032(pVK224)], a T strand was generated (lane 12). This strain contains only two borders and therefore produced only one T strand. This T strand was larger than the A-B strand since the T-DNA of this strain was longer by 2.8 kbp. A strain containing a synthetic right border repeat without the normal flanking sequences [WR1105(pVK225)] also generated a T strand (lane 10). However, when the right border was inverted [WR1031(pVK225)], the T strand was not generated (lane 14). Similarly, deletion of the TL left border in a strain harboring a normal TL right border and TR region [WR3079(pVK224)] prevented generation of A-B and A-C strands (lane 8). The B-D and C-D strands were, however, generated at normal levels in this TL left border deletion mutant (data not shown). Thus, both right and left border repeats are required in their native orientations to generate a specific T strand.

The overdrive sequence was not strictly needed for the generation of T strands. Overdrive enhances T-DNA transmission (28, 42), but it plays a nonessential role in this process. It seemed plausible that extra copies of specific *vir* genes generated abnormally abundant amounts of *vir* proteins and rendered the overdrive sequence unnecessary. To investigate this possibility, we compared the levels of T strands generated in WR1032 (with overdrive) and WR1105 (without overdrive) in a wild-type situation without *vir* gene cosmids. WR1105 (without overdrive; Fig. 5C, lane 4) generated much less T strand than did WR1032 (with overdrive, lane 2). Therefore, overdrive seems to function as a quantitative factor enhancing T-strand generation when *vir* gene products are at limiting concentrations.

Effect of right border and overdrive deletions on the double-

stranded cleavage at the left border. We have previously shown that, upon induction of *A. tumefaciens* by cocultivation with tobacco protoplasts, double-stranded cleavage occurs at or near each of the T-DNA border repeats (43). We tested whether the right border repeat and the overdrive sequence influence the double-stranded cleavage at the left border repeat by using the strains shown in Fig. 5A. We introduced cosmid pVK224 or pVK225 into these strains to increase the frequency of cleavage at border A (43). We digested the DNA from control and induced bacteria with *Eco*RI, subjected it to denaturing Southern analysis, and probed it with nick-translated *Hin*dIII fragment 18C. Double-stranded cleavage at border A followed by *Eco*RI cleavage at position 4494 (3) yields a 3.6-kbp fragment (43). Induction of strain A348(pVK224) and all the mutants of the right border and overdrive sequence generated the 3.6-kbp fragment (Fig. 6). Thus, double-stranded cleavage occurred at the left border independent of the right border and overdrive sequences. Additional 2.6- and 2.2-kbp equivalent fragments, generated upon induction in strain A348 (pVK224) and to a lesser extent in all right border and/or overdrive deletion mutants, appear to be single stranded because they are digested by S1 nuclease and hybridize specifically to top-strand riboprobes (Fig. 7 and data not shown).

Generation of T strands from the top strand when a border is placed in the opposite orientation. When border repeats are in their native orientations, border nicks largely occur in the bottom strand, leading to the generation of T strands corresponding to the bottom strand (1, 45, 50). Therefore, when a border is placed in the opposite orientation, we expected that nicks would occur in the top strand, thereby releasing the top strand as a T strand. To analyze this possibility, we used WR1120(pVK224) (Fig. 5A) in which we placed an additional TL right border (border B) in an inverted orientation to the left of the TL left border (border A) in the A348 background. In this strain, a T strand may initiate at the introduced border from the top strand but terminate at unknown points. The top-strand T strands generated may therefore be heterogeneous in length and would not be detectable as discrete fragments. Therefore, we used *Eco*RI to cleave the DNA and possibly generate discrete-sized T-strand-derived fragments. *Eco*RI can cut single-stranded DNA (26). We analyzed the DNA isolated from induced and uninduced bacteria either uncut or after digestion with *Eco*RI, followed by nondenaturing Southern analysis. We digested the DNA isolated from A348(pVK224) with *Eco*RI and analyzed it similarly. We used nick-translated *Hin*dIII fragment 18C or its top- or bottom-strand riboprobes for hybridization. Uncut DNA from induced WR1120(pVK224) (Fig. 7, lane 4) and A348(pVK224) (Fig. 2, lane 3) yielded identical A-B and A-C strands when hybridized with the nick-translated probe. These strands in WR1120(pVK224) hybridized only to the top-strand riboprobe (Fig. 7, lane 10), indicating that these two T strands correspond to the bottom strand as in the wild-type strain A348(pVK224) (Fig. 3B, lane 2). Thus, WR1120(pVK224) generated normal T strands mediated by the native border repeats. In the uncut DNA we did not detect new T strands that correspond to the top strand, possibly because of the expected heterogeneity in their size. Analysis of *Eco*RI-digested DNA with the nick-translated probe revealed a more complex pattern of fragments in WR1120(pVK224) (Fig. 7, lane 6) as compared with the DNA from strain A348(pVK224) (lane 2). The newly observed 3.5-, 5.8-, 9.0-, and 11-kbp equivalent fragments in WR1120(pVK224) hybridized only to the bottom-strand riboprobe (lane 18). These additional fragments that hybridized to the bottom-strand riboprobe were not detected in the wild-type strain A348(pVK224) (lanes 2 and 14). Evidently, placing a border in the opposite orientation resulted in the generation of new single-stranded DNA structures that correspond to the top T-DNA strand.

Increase of Ti-plasmid sequences upon cocultivation with protoplasts. In addition to the structural changes occurring in the T-DNA region upon cocultivation, we have generally observed that the DNA isolated from bacteria cocultivated with protoplasts showed higher levels of hybridization with probes from the T-DNA region than did DNA isolated from control bacteria (43). Given that we used the same amount of DNA from uninduced and induced bacteria for analysis, this could be explained either by a selective increase in the T-DNA sequences alone or by an increase in all of the Ti plasmid sequences relative to the bacterial chromosomal DNA. To analyze these possibilities, we isolated DNA from bacteria grown in AB minimal medium (B) or in K_3 (plant) medium for 24 h (K) or from bacteria cocultivated with protoplasts in K_3 medium for 24 h (P). We digested equal amounts of DNA (500 ng) with *Eco*RI and subjected the samples to Southern analysis. We also digested purified pTiA6 plasmid with *Eco*RI and analyzed it as a control. We mixed nick-translated probes from the T-DNA region (*Eco*RI fragment 7), the Ti plasmid *vir* region (*Bam*HI fragment 24), and the *A. tumefaciens* chromosome (a 3.7-kbp *Eco*RI fragment) for this analysis. Hybridization with the chromosomal probe was the same in DNA isolated from bacteria grown under all three conditions (Fig. 8). However, hybridization to the T-DNA and the *vir* region probes was much higher in bacteria induced by cocultivation with protoplasts. Evidently, the copy number of the entire Ti plasmid increased with respect to the bacterial chromosomal DNA upon cocultivation of the bacteria with protoplasts. The substantial increase seen in Fig. 8 occurred under cocultivation conditions that induced the *vir* genes to high levels. In strains that harbor cosmids carrying *vir* genes, a major portion of the T-DNA is processed into various intermediates, and this increase in hybridization to intact Ti plasmid sequences is therefore not evident (Fig. 6).

DISCUSSION

We have characterized many of the complex reactions that occur in the T region upon cocultivation of *A. tumefaciens* with regenerating protoplasts. Induction of the *vir* genes by cocultivation generated linear single-stranded DNA molecules corresponding to the bottom strand of the T region (T strands) and double-stranded border cleavages. Stachel et al. first reported that T strands accumulate in acetosyringone-treated *A. tumefaciens* harboring a nopaline-type Ti plasmid that contains one T-DNA region flanked by two border repeats (36). Site-specific nicks occur in the bottom DNA strand within the border repeats in octopine-type (1, 50) and nopaline-type (45) Ti plasmids. In this study, we characterized the complex pattern of T strands generated in the octopine-type Ti plasmid pTiA6 that contains four border sequences in direct repeat. While this manuscript was in preparation, Stachel et al. (37) reported similar findings on T-strand generation in an octopine-type Ti plasmid. The use of all possible combinations of the four borders for initiation and termination of T strands produced at least six different T strands, and their production required the *virA*, *virD*, and *virG* loci. In addition, we observed specific single-stranded

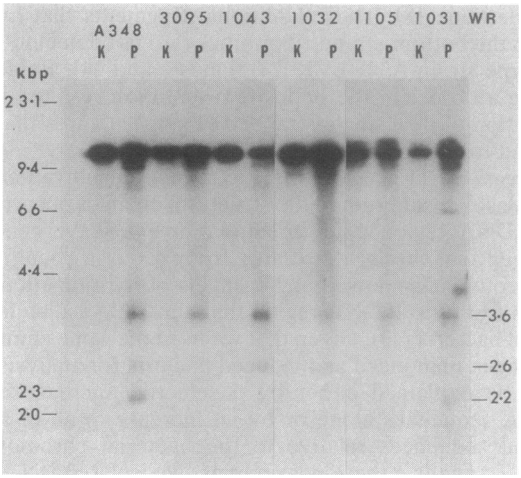

FIG. 6. Effect of right border and overdrive mutations on the double-stranded T-DNA cleavage at border A. All strains harbored the *vir* region cosmid pVK224 or pVK225. The DNA was digested with *Eco*RI, subjected to denaturing Southern analysis, and hybridized with nick-translated *Hin*dIII fragment 18C.

fragments corresponding to the top T-DNA strand. These DNA molecules migrated to the same position as a 25-kbp double-stranded linear DNA fragment, and they exhibited a complex hybridization pattern. DNA molecules migrating at this position hybridized detectably only to bottom-strand RNA transcribed from the TL and TC regions, but in the TR region riboprobes complementary to either strand hybridized to DNA molecules with the same mobility (25 kbp). Several hypotheses may explain these results. First, distinct A-D T strands corresponding to both top and bottom strands may migrate to the same position, but for technical reasons we may not have detected hybridization of the bottom A-D strand with top-strand RNA transcribed from TL and TC. Alternatively, the band migrating at the A-D position may correspond to the top strand between borders A and C, and it is possibly double stranded in the TR region. Although further work is necessary to characterize fully the DNA species that comprise the 25-kbp T-DNA-derived band, single-stranded T-DNA structures corresponding to the top T-DNA strand as well as the bottom strand accumulated upon cocultivation of *A. tumefaciens* with tobacco protoplasts.

Because the orientation of the right border repeat plays an important role in T-DNA transmission and virulence (14, 15, 29, 44), presumably by controlling the polarity of T-strand production (39), we tested the influence of border mutations on T-strand accumulation. Generation of discrete-sized T strands required both a left and a right border sequence in a directly repeated orientation. Inversion of the right border repeat or deletion of either border repeat prevented formation of discrete-sized T strands. Inversion of a right border repeat reversed the direction of T-strand production (from leftward to rightward) and the strand comprising the T strand (from bottom strand to top strand). T strands initiated at the inverted right border apparently terminated at a variety of locations and thereby produced heterogeneous-length molecules. Therefore, we detected specific top-strand fragments only after cleavage of the heterogeneous-length T strands with *Eco*RI. Similarly, in a left border deletion mutant, T strands apparently initiated at the right border but did not terminate preferentially at a few specific sequences. These results confirm the expected association between border repeats and T-strand production, and they indicate that strong pseudo-border sequences did not substitute for missing border repeats.

Wild-type levels of tumorigenesis require the right border repeat and a flanking sequence called overdrive (28, 42). Although it plays a nonessential role, the overdrive sequence enhanced T-DNA transmission. We investigated the influence of overdrive on the formation of T strands and found that overdrive stimulated T-strand production, which presumably represents an early step in T-DNA transmission. Deletion of the overdrive sequence greatly reduced the number of T strands that accumulated upon induction of the *vir* genes. However, introduction of additional copies of specific *vir* genes on a multicopy cosmid restored accumulation of T strands to wild-type levels in strains without overdrive. These cosmids also restored virulence to wild-type levels in overdrive mutants (N. Toro, M. Yanofsky, W. Ream, and E. W. Nester, submitted for publication). There-

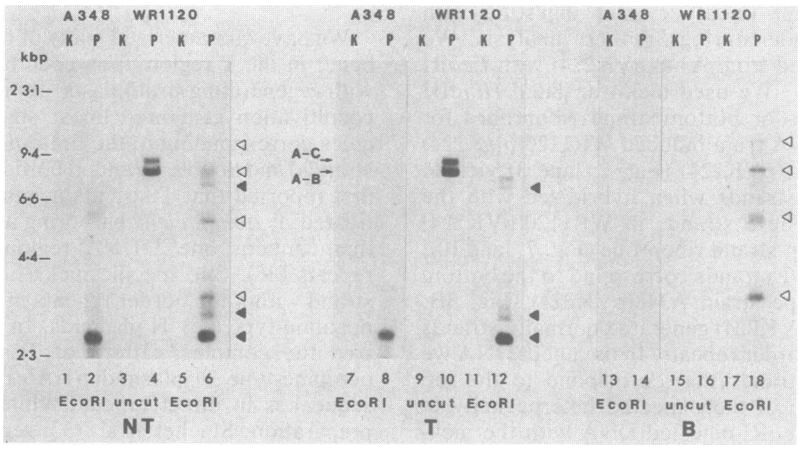

FIG. 7. Border orientation and T-strand generation in strain WR1120(pVK224). This strain (Fig. 5A) contains an additional TL right border to the left of the TL left border in an inverted orientation. The uncut DNA of WR1120(pVK224) and *Eco*RI-digested DNA of WR1120(pVK224) and A348(pVK224) were subjected to nondenaturing Southern analysis. The blots were hybridized with nick-translated *Hin*dIII fragment 18C (NT), or the top-strand (T) or bottom- strand (B) RNA from pBSH18C. Symbols: ◀, *Eco*RI fragments that hybridized to the top-strand RNA; ◁, fragments that hybridized to the bottom-strand RNA.

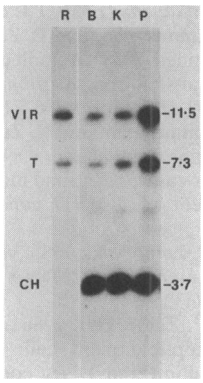

FIG. 8. Effect of cocultivation of bacteria with protoplasts on the Ti plasmid copy number. *A. tumefaciens* A348 was grown in AB minimal medium with 0.5% glucose (B) and incubated for 24 h in K_3 medium (K) or with protoplasts in K_3 medium (P). The DNA was digested with *Eco*RI and subjected to denaturing Southern analysis. As a control, purified pTiA6 was digested with *Eco*RI and subjected to a similar analysis (lane R). Hybridization was performed with a mixture of nick-translated probes derived from the T-DNA region (*Eco*RI fragment 7), the *vir* region (*Bam*HI fragment 24), and the *A. tumefaciens* chromosome (a 3.7-kbp *Eco*RI fragment). The fragments that hybridized to these probes are indicated as T, VIR, and CH, respectively.

fore, the overdrive sequence seems to have a quantitative role in both the generation of T strands and virulence. When the *vir* gene products are limiting (as in the wild-type situation), overdrive serves as an enhancer, possibly by attracting *vir* gene products close to the right border.

Cocultivation of *A. tumefaciens* with tobacco protoplasts or cell suspensions induces *virD*-dependent double-stranded cleavage at or near each border repeat (1, 43), and we monitored the frequency of double-stranded endonuclease activity at the TL left border repeat (border A) in several strains to determine whether the right border repeat influenced cleavage at the left border. In strains lacking the TL right border repeat (border B) and all of TR, double-stranded cleavage at border A occurred at the wild-type frequency. Similarly, inversion of border B did not affect double-stranded cutting at border A. Thus, double-stranded cleavage at the left border repeat did not depend on cleavage at the right border repeat, and presumably nicking by the *virD* endonuclease at border A also remains unaffected by mutations at border B. Other groups reported similar observations while this work was in progress (1, 50).

The analyses presented in this paper reflect the complexity of the reactions occurring in the T region of *A. tumefaciens* upon cocultivation with protoplasts. In this study we have drawn correlations between certain molecular events and physiological aspects of tumorigenesis. The T strands generated by induced *A. tumefaciens* consisted of T-DNA regions between the border repeats. The right and left borders must lie in their wild-type positions and in a directly repeated orientation to generate discrete-length T strands. The overdrive sequence enhanced both T-strand generation and T-DNA transmission. These correlations support the model that T strands act as intermediates in T-DNA transfer. However, because double-stranded border cleavage occurred efficiently in bacteria cocultivated with protoplasts, and because T-DNA circles also form at detectable frequencies (2, 17, 20), future studies must determine whether these other molecular forms also participate in T-DNA transmission.

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