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Genetics

Role of the overdrive sequence in T-DNA border cleavage in *Agrobacterium*

(T-DNA processing/endonuclease/*vir* genes)

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ABSTRACT **The T-DNA of the Ti plasmid of *Agrobacterium* is flanked by 25-base-pair imperfect direct repeats that are required in cis for transfer to the genome of the plant host. Another sequence, designated overdrive, is located adjacent to the right-border repeats and functions in cis to enhance tumor formation. We have examined the effect of the overdrive sequence on the early steps in T-DNA processing. We report here that overdrive greatly enhances cleavage by the site-specific endonuclease in *Agrobacterium*, perhaps by directing the endonuclease to the adjacent border sequences. We also show by a gel mobility-shift assay that overdrive affinity-purified proteins from acetosyringone-induced *Agrobacterium* cells interact with T-DNA border and overdrive sequences. Further, we show that *in vivo* the *virC* operon enhances cleavage at the T-DNA borders, most likely by interaction between the VirC1 protein and the overdrive sequence.**

Tumor formation by *Agrobacterium tumefaciens* involves the transfer and integration of a defined segment of DNA (T-DNA) into the plant genome. The T-DNA is flanked by 25-base-pair (bp) direct repeats, termed border sequences (1–4), which are required in cis for transfer (5, 6). Immediately adjacent to the right borders (borders B and D) is another conserved sequence (5′-TAARTYNCTGTRTNTGTTTGT-TTG-3′), called "overdrive," that enhances the efficiency of tumor formation (7, 8) even when located several thousand base pairs away from the border (9). Other studies have indicated that the right border is intrinsically more active than the left in promoting DNA transfer (8, 10, 11) and that transfer is a polar process proceeding from right to left (7, 12, 13).

An early step in T-DNA processing is a site-specific cleavage within the bottom strand of each of the border sequences, which is mediated by products encoded within the 5′ end of the *virD* operon (14–16). Although the *virD* operon contains at least five open reading frames (17, 18), only *virD1* and *virD2* have been shown to be involved in the endonuclease activity (14, 15, 18). It has been proposed that after nicking, DNA replicates from one nicked border to the next and displaces the bottom strand from 5′ to 3′, thereby generating linear single-stranded molecules called T-strands (13, 19). Overdrive most likely functions during the first steps of T-DNA processing because, after T-strand formation, it would be separated from the possible T-DNA intermediate during transfer.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Plant Cell Culture. *Agrobacterium* strains LBA4404 (20), A348 and A1034 (21), and 379MX, 365MX, and 364MX (22) were maintained on AB minimal agar (23) supplemented with the appropriate antibiotics (carbenicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml). Plasmids pBL20, pBL37, and pBL18 (19), pVK102 (24), pMY938 (25), and pTVK79M31 (26) have been described. pTB1 and pTB2 are described below. *Nicotiana tabacum* suspension cultures were maintained as described (27, 28).

Enzymes and Reagents. [α-^{32}P]CTP and [α-^{32}P]dATP were purchased from New England Nuclear. RNase A (type III-A) was from Sigma. Klenow DNA polymerase; RNase T1, and nuclease S1 were from Bethesda Research Laboratories. RNase-free DNase and the RNase inhibitor RNasin were purchased from Promega Biotec. Unlabeled rNTPs were from P-L Biochemicals.

Induction of *vir* Genes. *vir* genes were induced either by cocultivation (19) or by acetosyringone (13).

Plasmid Constructions. The numbering system of Barker *et al.* (5) is used in describing plasmid constructions. For pTB1 (Fig. 1), the *Hin*dIII–*Xho* I fragment was removed from pBL18 [pGEM-2 vector (Promega Biotec) containing the *Bam*HI–*Eco*RI fragment spanning bp 13,774–16,202 (19)] and inserted into the same sites of pVK102 (24). The *Bam*HI (13,774)–*Xho* I (15,208) fragment comes from Ti plasmid sequences (see legend to Fig. 1), and the small *Hin*dIII–*Bam*HI fragment of pTB1 is derived from the polylinker region from pGEM-2 vector sequences (Fig. 1). pTB2 (Fig. 1) was constructed by deleting the *Sac* I–*Xho* I fragment of pTB1 and replacing it with the *Sac* I–*Sal* I fragment from the polylinker of pUC18 (29).

DNA Isolation and RNase Protection. Plasmid (30) and genomic (19) DNA were isolated as described. *Agrobacterium* cocultivations and the RNase protection assay were performed as described (19). To distinguish between processed duplex or single-stranded DNA, the hybridizations were performed in aqueous conditions, about 30°C below the melting temperature of the DNA, to stabilize DNA duplexes (19).

Sequence-Specific DNA Affinity Chromatography. Affinity resins were prepared according to the procedure of Kadonaga and Tjian (31), with synthetic oligodeoxynucleotide sequences, 5′-TAAGTCGCTGTATGTGTTTGTTTG-3′ for overdrive and 5′-GACTGGCAGGATATATACCGTTGTA-3′ for the T-DNA right border from the octopine plasmid pTiA6. Protein extracts were prepared from 100-ml cultures of *Agrobacterium* strain A348(pTVK79M31) or 364MX-(pTVK79M31) that were uninduced or induced with acetosyringone (13). *Agrobacterium* cells were lysed by one passage through a French pressure cell. The lysate was centrifuged at low speed to remove cell debris and incubated for 10 min with

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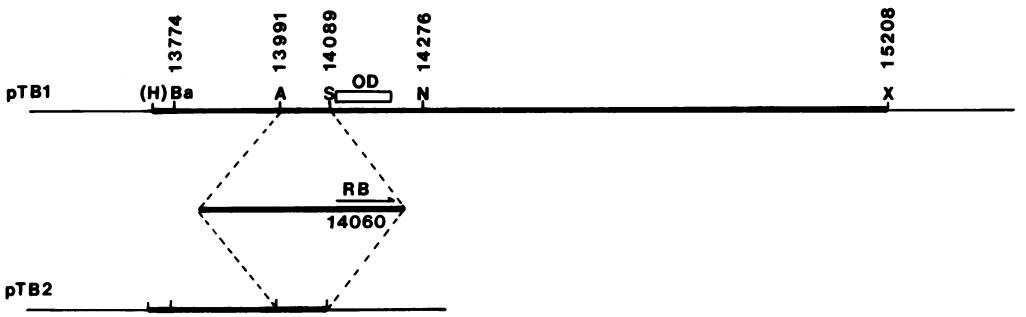

FIG. 1. Plasmids used to analyze cleavage. Nucleotide numbers refer to base pairs defined from the sequence data of Barker *et al.* (5); restriction sites are *Bam*HI (Ba), *Acc* I (A), *Sac* I (S), *Nru* I (N), *Xho* I (X), and *Hin*dIII (H). Positions of the T-DNA right border B (RB) and overdrive sequence (OD) are indicated; thin lines represent pVK102 vector DNA.

500 μg of sonicated calf thymus DNA (20 μg/ml), prior to passage over 1 ml of either the overdrive or the right-border affinity resin. All steps were carried out at 4°C. Columns were washed extensively with buffer Z [25 mM Hepes (K^+), pH 7.5/12.5 mM $MgCl_2$/1 mM dithiothreitol/20% (vol/vol) glycerol/0.1% (vol/vol) Nonidet P-40] containing 100 mM KCl. Proteins were eluted in two steps with buffer Z (0.5 M KCl). First-pass eluates (volume, 2 ml) were diluted to 0.1 M KCl with buffer Z, incubated again with calf thymus DNA or poly[d(I-C)], applied to 1-ml affinity columns, and eluted as before (second pass). The process was repeated, generating the third-pass eluates. Proteins were detected by NaDodSO$_4$/polyacrylamide gel electrophoresis (32) and immunoblotting (33) using antiserum against VirD2 (17) or other Vir proteins.

Probe for Gel Electrophoresis DNA-Binding Assays. DNA probe (Fig. 6A) for gel electrophoresis DNA-binding assays was prepared by end-labeling the *Eco*RI site (bp 14,276) of pBL20 with Klenow polymerase and [α-^{32}P]dATP. The probe was then digested with *Acc* I (bp 13,991) and the 285-bp fragment was purified by polyacrylamide gel electrophoresis.

Gel Electrophoresis DNA-Binding Assays. Protein–DNA binding was assayed by mobility shift, essentially as described by Fried and Crothers (34). Binding was carried out in 50 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/5% (vol/vol) glycerol/1 mM dithiothreitol containing 2 μg of poly[d(I-C)] and ≈2 ng of the end-labeled DNA probe. For competition experiments, 0.8 μg of the annealed right-border or overdrive oligonucleotide was added to the reaction mixture before addition of protein samples. Excess nonspecific competitor DNA, poly[d(I-C)], was always present.

RESULTS

Overdrive Sequence Enhances the Generation of Processed T-DNA Molecules in *Agrobacterium*. Initial studies on overdrive demonstrated that it promoted tumor formation (7, 8). To determine whether this stimulation resulted from an enhancement in the amount of processed T-DNA molecules in *Agrobacterium*, plasmids pTB1 and pTB2 (Fig. 1) were mobilized into strain LBA4404. Total DNA was assayed for processed T-DNA molecules at border B by the technique of RNase protection (19).

Processed T-DNA molecules at border B (bottom strand) were observed for pTB1, as indicated by the protection of 327- and 215-nucleotide-long fragments (Fig. 2, lane 5), and for pTB2, as indicated by the protection of a 327-nucleotide-long fragment (lane 7). However, whereas substantial numbers of processed T-DNA molecules were observed for pTB1 (Fig. 2, lane 5), comparatively few were observed for pTB2 (lane 7). These data indicate that overdrive enhances processing of T-DNA molecules in *Agrobacterium*.

Overdrive Primarily Enhances Cleavage in *Agrobacterium*. The RNase protection procedure assays processed T-DNA molecules in both duplex and single-stranded form (19). To distinguish between the possibilities that overdrive enhances either nicking or T-strand formation, we modified the conditions used in the RNase protection assay (*Materials and Methods* and ref. 19). Under aqueous hybridization conditions, the double-stranded DNA control (pBL20-EcoRI) did not protect the RNA probe unless it had first been denatured (Fig. 3, lanes 4 and 5). However, the single-stranded DNA control (pBL37) protected the RNA probe equally well under aqueous and formamide conditions, and this protection did not require prior denaturation of the DNA (Fig. 3, lanes 7–9). Thus, these control reactions indicated that single-stranded DNA could be differentiated from double-stranded DNA under these aqueous hybridization conditions. Under aqueous hybridization conditions (Fig. 3, lanes 2 and 3), the 327- and 215-nucleotide signals generated from DNA nicked at border B of pTB1 were detected at very low levels that did not depend on denaturation before hybridization. This indicates that some of the molecules nicked at border B were single-stranded. However, since the signals generated by hybridization under aqueous conditions without denaturation were strongly reduced compared with signals generated under formamide conditions of hybridization (Fig. 3, lanes 1 and 3), most of the DNA nicked at border B of pTB1 must have remained in duplex form. Thus, we conclude that the nicked DNA remained largely in the duplex form.

To verify this conclusion we treated the nicked DNA with S1 nuclease prior to the RNase protection assay. Such treatment before hybridization in formamide conditions did not change the protection profile (Fig. 3, lanes 10 and 11), in support of our conclusion that most of the processed T-DNA

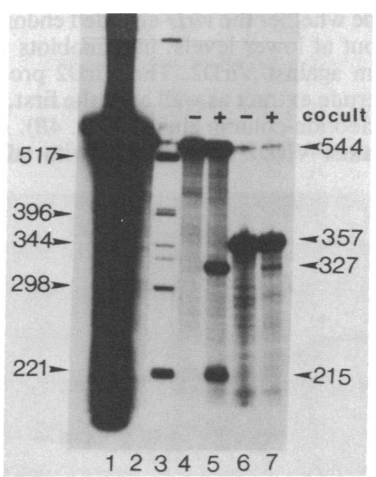

FIG. 2. Analysis of cleavage at border B in *Agrobacterium*. RNase protection was carried out with a probe derived from pBL20 (19) digested with *Eco*RI. This probe contains *Agrobacterium* sequences from bp 13,774 to bp 14,276 (Fig. 1). *Agrobacterium* strains were cultured in the presence (+) or absence (−) of tobacco suspension cells for 30 hr, and DNA was isolated (19). *Agrobacterium* strain LBA4404 harbored pTB1 (lanes 4 and 5) or pTB2 (lanes 6 and 7). Lane 3, size markers (*Hin*fI-digested pBR322; fragment lengths in nucleotides at left); lanes 1 and 2, no added DNA to protect the RNA probe; RNase was not added to the sample in lane 1.

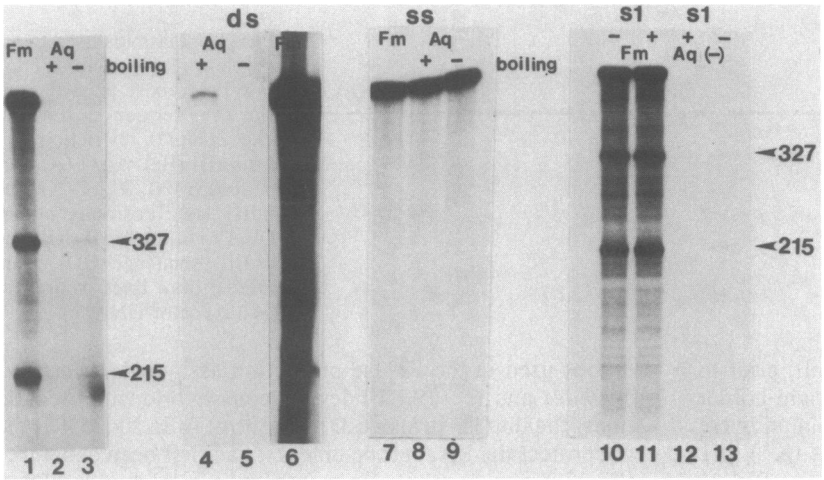

FIG. 3. Analysis of single- vs. double-stranded DNA after induction. RNA probe pBL20-EcoRI was hybridized to pBL37 single-stranded (ss) DNA (lanes 7–9) or to pBL20-EcoRI double-stranded (ds) DNA (lanes 4–6) under aqueous (lanes 4, 5, 8, and 9; Aq) or formamide (lanes 6 and 7; Fm) conditions with (+) or without (−) heating the reaction mixture first in a boiling water bath. Under formamide conditions the reaction mixture was always boiled prior to hybridization. Total DNA from strain LBA4404 harboring pTB1 was hybridized with RNA probe pBL20-EcoRI under formamide (lanes 1 and 10) or aqueous conditions (lanes 2, 3, and 13) with (+) or without (−) prior boiling. The same DNA was treated with nuclease S1 and hybridized with the RNA probe in formamide (lane 11) or aqueous conditions without prior denaturation (lane 12).

molecules at border B of pTB1 remained in duplex form. No signals were detected after S2 treatment in aqueous hybridization conditions without prior denaturation (Fig. 3, compare lanes 12 and 13), which further confirmed the single-stranded nature of the molecule detected under these conditions. Single-stranded molecules generated from border B were not detected with pTB2 (data not shown). This would be predicted if the ratio of single-stranded molecules to duplex nicked molecules was the same in pTB2 and pTB1. From these experiments we conclude that overdrive enhances primarily nicking and not T-strand formation.

Overdrive Affinity-Purified Proteins from *Agrobacterium*. To determine whether any proteins from either induced or uninduced cultures interacted with the overdrive sequence, we applied the sequence-specific DNA-affinity chromatography technique of Kadonaga and Tjian (31). The proteins were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis following passage over the overdrive affinity resins (see *Material and Methods*) and were visualized by staining with Coomassie blue. After two passages, the eluate contained two predominant polypeptide species migrating at 43 and 150 kDa. Neither required induction for synthesis (Fig. 4*A*, lanes 1 and 2).

To determine whether the *virD*-encoded endonuclease was also present but at lower levels, immunoblots were probed with antiserum against VirD2. The VirD2 protein was detected in the crude extract as well as in the first, second, and third oligonucleotide-column eluates (Fig. 4*B*). Although the antiserum against VirD2 crossreacted with additional proteins in the crude extract, none of these crossreacting signals appeared after one passage through the overdrive column (Fig. 4*B*). This suggests that the VirD2 protein was specifically retained. As a control we used antiserum against the VirG polypeptide, another protein induced by acetosyringone. This protein was not detected in the eluates from the overdrive columns, although it was detected in the crude extract (data not shown).

Interaction of *virC* Gene Products with Overdrive. By using specific antisera against the VirC1 and VirC2 proteins, it was possible to detect the VirC1 protein in the overdrive affinity-purified proteins (Fig. 5*B*). However, the VirC2 protein was detected only in the flowthrough (data not shown). VirC1 was detected in the overdrive affinity-purified proteins from A348(pTVK79M31) and from 364MX(pTVK79M31), a *virC2* mutant (Fig. 5*B*, lanes 3 and 4). pTVK79M31 does not complement the attenuated tumor phenotype of either *virC1* or *virC2* mutants in A348 (N.T., unpublished observations). These data suggest that VirC2 is not required for the interaction of VirC1 with overdrive in the affinity columns.

When the crude lysate from acetosyringone-induced *Agrobacterium* cells was passed through either an overdrive or a right-border affinity column, VirD2 was detected in the eluate from both columns (Fig. 5*A*), as were the 43- and 150-kDa proteins (data not shown). However, VirC1 was detected only in the eluate from the overdrive column (Fig. 5*B*). These data suggest that VirC1 interacts specifically with the overdrive sequence and not with the border sequence in the affinity columns.

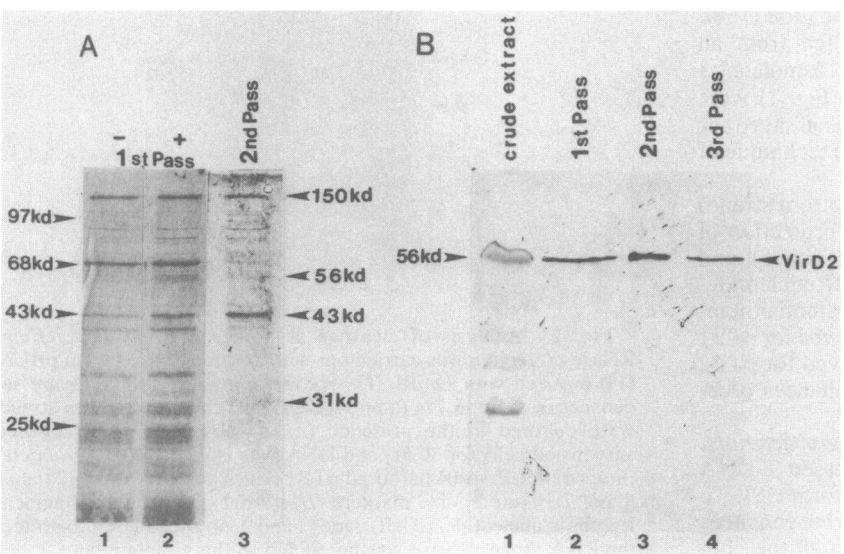

FIG. 4. Affinity-purified proteins from *Agrobacterium* cells. Proteins were obtained from strain A348(pTVK79M31) unless otherwise stated. (*A*) $NaDodSO_4$/polyacrylamide gel electrophoresis of the overdrive affinity-purified proteins after staining with Coomassie blue. Lane 1, first eluate from uninduced cells; lane 2, first eluate from induced cells; lane 3, second eluate from induced cells. (*B*) Immunoblot analysis of the overdrive affinity-purified proteins with antiserum against VirD2. Amount of protein in each lane: crude extract, 350 μg; first eluate, 0.15 μg; second eluate, 0.075 μg; third eluate, 0.06 μg. Markers indicate sizes in kDa (kd).

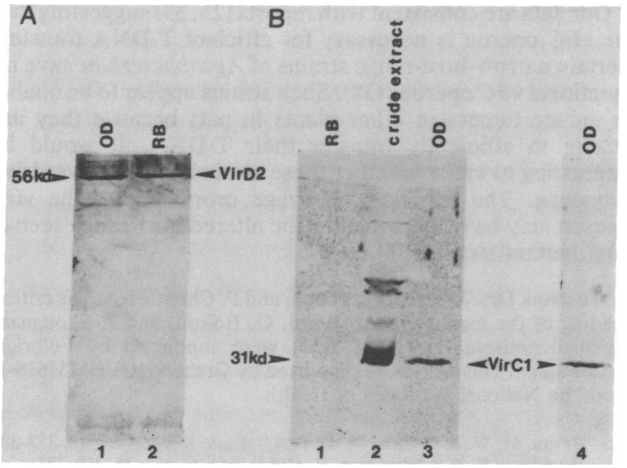

FIG. 5. Overdrive and right-border affinity-purified proteins. Proteins were obtained from *Agrobacterium* strain A348-(pTVK79M31) unless otherwise stated. (*A*) Comparison of VirD2 protein in second-pass eluates from overdrive (OD, lane 1) and right-border (RB, lane 2) columns. Similar amounts of protein were loaded in the two lanes. (*B*) Immunoblot analysis using antiserum against VirC1 of the right-border affinity-purified proteins (lane 1), crude extract (lane 2), overdrive affinity-purified proteins (lane 3), and overdrive affinity-purified proteins from induced 364MX-(pTVK79M31) cells (lane 4). kd, kDa.

Gel Mobility-Shift Assays Using Overdrive Affinity-Purified Proteins. To determine whether the overdrive affinity-purified proteins from uninduced or induced *Agrobacterium* cells could retard the mobility of a radiolabeled DNA fragment containing the right-border and overdrive sequences, we assayed the sequence-specific DNA-binding activity by a gel retardation assay.

By using the labeled 285-bp *Acc* I–*Eco*RI DNA fragment containing both overdrive and right-border sequences (Fig. 6*A*), we observed three DNA–protein complexes (designated A–C in Fig. 6*B*) even in the presence of excess poly [d(I-C)]. Formation of complex A depended on proteins from induced cells (Fig. 6*B*, lane 1), whereas the formation of the other two complexes (B and C) depended on proteins from uninduced cells (lanes 6 and 7). In the control (lane 5) no proteins were added to the DNA probe. To test whether any of these complexes were related to a protein–DNA interaction at the overdrive and/or right-border sequence in the DNA probe, we carried out competition experiments with double-stranded DNA oligomers homologous to the right-border or overdrive sequence. The addition of excess specific unlabeled DNA oligomers (Fig. 6*B*, lanes 2–4) inhibited formation of complex A. However, formation of complexes B and C was not inhibited by any of these oligomer (Fig. 6*B*, lanes 2–4), indicating that they are not related to proteins bound to these specific sequences. Since formation of labeled complex A was efficiently blocked by these double-stranded oligonucleotides, we conclude that this complex contained inducible protein(s) that are involved in binding to the right-border and overdrive sequences.

The *virC* Operon Enhances Cleavage in *Agrobacterium*. The interaction in the affinity-chromatography columns between the VirC1 protein and the overdrive sequence suggested that the *virC* operon might enhance the effect of overdrive on the site-specific cleavage by the VirD endonuclease *in vivo*.

To assess this possibility, total DNA was prepared from acetosyringone-induced cultures of four *A. tumefaciens* mutants with different transposon insertions within the *virC* locus (A1034, 379MX, 365MX, and 364MX). Processed T-DNA molecules at border B (bottom strand) were observed in both wild type (A348) and the *virC* mutant strains (Fig. 7),

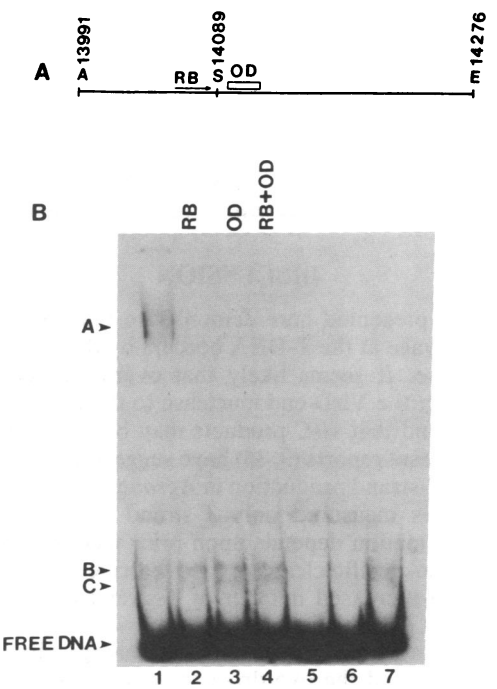

FIG. 6. Interaction of the overdrive affinity-purified proteins with the right-border (RB) and overdrive (OD) sequences. (*A*) The 285-bp *Acc* I–*Eco*RI fragment used as a probe. (*B*) Gel retardation and competition experiments. Lanes 1–4, protein–DNA complexes (A–C) resolved when we used overdrive affinity-purified proteins from induced *Agrobacterium* cells; lanes 6 and 7, complexes resolved when we used overdrive affinity-purified proteins from uninduced cells. For lane 5, proteins were not added to the DNA probe. Lane 2, competition with right-border oligonucleotide; lane 3, with overdrive oligonucleotide; lane 4, with both right-border and overdrive oligonucleotides. Reactions were carried out with proteins from the second-pass eluates (0.5 μg in lanes 1–4 and 6; 1 μg in lane 7).

as indicated by the protection of 285- and 215-nucleotide-long fragments. However, in the *virC* mutants the level of these molecules was consistently lower than that observed in the wild-type cells. Most of the processed molecules in A348, as in LBA4404(pTB1), remained in duplex form; likewise, the ratio of duplex to single-stranded molecules was similar in wild type and *virC* mutants (data not shown). Thus, the *virC*

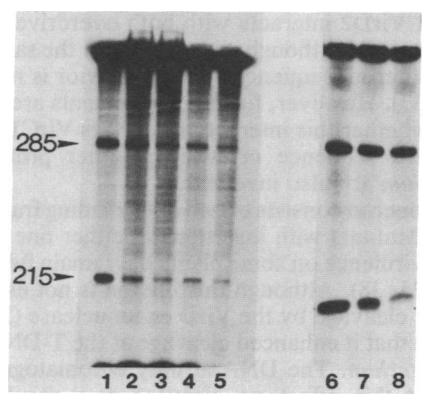

FIG. 7. *virC* operon mutants and cleavage at the T-DNA borders. *Agrobacterium* strains were cultured in MS^- medium in the presence of 100 μM acetosyringone for 24 hr, and DNA was isolated (19). Total DNA was assayed by RNase protection using the same probe described in the legend of Fig. 2. Cleavage at border B is indicated by 215- and 285-nucleotide-long fragments. Lane 1, wild-type strain A348; lane 2, 364MX; lane 3, A1034; lane 4, 379MX; lane 5, 365MX; lane 6, 379MX(pMY938); lane 7, 364MX(pMY938); lane 8, A348. More DNA was loaded in lanes 2 and 3 than in lane 1.

operon primarily enhances nicking of the T-DNA borders. To confirm that the *virC* operon was responsible for this effect, pMY938, a plasmid containing the wild-type *virC* locus, was mobilized into the various *virC* mutant strains. The level of processed T-DNA molecules in the complemented *virC* mutant strains was comparable to the level observed in the wild-type strain (Fig. 7). All these data demonstrate that the *virC* operon enhances cleavage in *Agrobacterium*.

DISCUSSION

The results presented here demonstrate that overdrive enhances cleavage at the T-DNA borders by the *virD*-encoded endonuclease. It seems likely that overdrive acts to efficiently direct the VirD endonuclease to the adjacent border sequences and that *virC* products may be important in this process. Recent reports (9, 35) have suggested that overdrive stimulates T-strand production in *Agrobacterium*. However, these studies measured only T-strand production. Since T-strand formation depends upon prior nicking, any stimulation of site-specific cleavage by overdrive could indirectly result in an increased number of T-strand molecules. Our data suggest that this is the case.

Our results from DNA-affinity chromatography using the right T-DNA and the overdrive sequences suggest that the VirD2 protein interacts with both the T-DNA border and overdrive, whereas VirC1 specifically interacts with the overdrive sequence. The observation that VirD2 and VirC1 proteins were barely detectable in Coomassie blue-stained $NaDodSO_4$/polyacrylamide gels could be explained if they are present in relatively low abundance in the crude extract (36). *Agrobacterium* proteins (43 and 150 kDa) that are constitutively synthesized in the absence of acetosyringone were also retained in both right-border and overdrive affinity columns. However, by mobility-shift assays and competition experiments, we found that the specific interaction with the right-border and overdrive sequences depended on inducible proteins (complex A). That both overdrive and right-border oligonucleotide sequences competed equally well in the mobility-shift assays suggests that a similar protein or protein complex was involved in the interaction with right-border and overdrive sequences. Since we have identified at least two acetosyringone-inducible proteins in the overdrive affinity-purified proteins, VirD2 and VirC1, we suggest that at least these two proteins are present in complex A. The data suggest that VirD2 interacts with both overdrive and right-border sequences. Although it is unusual for the same protein to bind to different sequences, such behavior is not without precedent (37). However, further experiments are needed to determine whether this interaction requires VirC1 to bind to the overdrive sequence or whether other proteins from *Agrobacterium* are also involved.

The *virC* operon consists of two open reading frames, *virC1* and *virC2*. Mutants with insertions in either one exhibit an attenuated virulence on some plants but remain fully virulent on others (25, 38). Although this operon is not essential for site-specific cleavage by the VirD endonuclease (14, 15), we have shown that it enhanced cleavage at the T-DNA borders in *Agrobacterium*. The DNA-affinity chromatography data suggest that this effect on cleavage may result from an interaction between the VirC1 protein and the overdrive sequence. This interaction apparently does not require the *virC2* gene product. However, *in vivo*, we found that nicking was decreased in both *virC1* and *virC2* mutants, suggesting that the *virC2* gene also plays a role in enhancing nicking. However, what this role is remains unclear.

Our data are consistent with reports (25, 39) suggesting that the *virC* operon is necessary for efficient T-DNA transfer. Certain narrow-host-range strains of *Agrobacterium* have no functional *virC* operons (38). Such strains appear to be unable to induce tumors in other plants in part because they are unable to efficiently transfer their T-DNA. It would be interesting to know whether these strains have an overdrive sequence. The enhanced cleavage promoted by the *virC* operon may be responsible for the altered host-range seen in *virC* mutant strains (38).

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