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Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: Analogy to bacterial conjugation

(sequence-specific recognition of single-stranded DNA/covalent protein–DNA complex/T-DNA integration)

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ABSTRACT **As an early stage of plant transformation by *Agrobacterium tumefaciens*, the Ti plasmid is nicked at the border sequences that delimit the T-DNA. Cleavage results in covalent attachment of VirD2 to the 5′ termini of the nicked strand by a process resembling initiation of DNA transfer that occurs in the donor cell during bacterial conjugation. We demonstrate that this cleavage can be reproduced *in vitro*: VirD2 protein, the border-cleaving enzyme, was overproduced and purified. Cleavage assays were performed with single-stranded oligodeoxyribonucleotides encompassing the Ti plasmid border region or the transfer origin's nick region of the conjugative plasmid RP4. VirD2 of pTiC58 cleaves both border- and nick region-containing oligonucleotides. However, the relaxase TraI of RP4 can cut only the cognate nick regions. The respective proteins remain covalently bound to the 5′ end of the cleavage sites, leaving the 3′ termini unmodified. VirD2-mediated oligonucleotide cleavage was demonstrated to be an equilibrium reaction that allows specific joining of cleavage products restoring border and nick regions, respectively. The possible role of VirD2 in T-DNA integration into the plant cell's genome is discussed in terms of less stringent target-sequence requirements.**

T-DNA transfer from agrobacteria to plant cells is proposed to share mechanistic analogies with bacterial conjugation (1). Most of the components needed to direct this process are plasmid-encoded as is the case for conjugative DNA transfer between bacteria (recently reviewed in refs. 2 and 3). Recent reports on sequence similarities between several bacterial conjugation systems and the agrobacterial Ti (tumor-inducing) plasmid document the relationships impressively (4–11). Since more sequence data of transfer systems have become available, it turns out that these relationships extend to several other systems—i.e., F (9), IncI (12, 13), the *Thiobacillus ferrooxidans* plasmid pTF-FC2 (14), and mobilizable and conjugative plasmids of Gram-positive bacteria (4, 11, 15). Interestingly, the Ti VirB region is analogous not only to the Tra2 region of RP4 responsible for recipient contact and the Pil_W region of the IncW plasmid R388 (F. de la Cruz, personal communication) but also to the chromosomally encoded Ptl region of *Bordetella pertussis* thought to be involved in toxin secretion (16, 17). Thus, it is most likely that the transfer systems listed above encode a widespread family of accessory proteins involved in the passage of macromolecules through bacterial cell membranes.

The studies presented here are based on the functional analogy of the DNA transfer machineries of plasmids RP4 and Ti. According to the mechanistic models, components of the relaxase operon (RP4) or the VirD operon (pTi) interact with the transfer origin (*oriT*) or T-DNA border sequences, respectively, to generate the single-stranded (ss) DNA destined for transfer to the recipient (3, 18). Site- and strand-specific nicking of T-DNA border sequences *in vivo* requires the presence of both the *virD1* and *virD2* gene products. Since VirD1-containing extracts exhibit topoisomerase I activity (19), VirD1 was suggested to prepare the DNA for T-border-specific cleavage by VirD2. Upon cleavage, VirD2 attaches tightly to the 5′ terminus of the T-DNA and probably heads the T-DNA complex exported to the plant cell (20–24), where it is thought to target the T-DNA to the nucleus (25, 26).

In vitro reconstitution studies of RP4 relaxosomes with purified proteins revealed that the plasmid-encoded proteins TraJ, a specific *oriT*-binding protein (27), and TraI, the relaxase, form a nucleoprotein complex with the supercoiled transfer origin. The complex is stabilized by the chaperone-like TraH protein, which does not bind to DNA by itself but interacts specifically with TraI and TraJ (28). Relaxosomes exist in a thermodynamic equilibrium of covalently closed form I DNA associated noncovalently with proteins and DNA cleaved at the *nic* site of *oriT*. The latter is kept in the superhelical state by TraI, which attaches covalently to the 5′ terminus at the cleavage site and interacts tightly with a 6- to 8-nt sequence on the 3′ side of *nic* (6, 11, 29, 30). Attachment of TraI to the cleaved DNA occurs via formation of a phosphodiester between Tyr^{22} of TraI and the DNA 5′ terminus (30). Recently, it has been demonstrated that the RP4 TraI protein catalyzes site-specific cleaving–joining reactions on ssDNA containing the *nic* region of the RP4 *oriT* (30).

Here we demonstrate that the VirD2 protein of pTiC58 catalyzes a similar ssDNA cleaving–joining reaction *in vitro*. Following efficient overproduction in *Escherichia coli*, VirD2 was purified to near homogeneity. In an *in vitro* assay we found that VirD2 catalyzes a site-specific cleaving–joining reaction with single-stranded oligonucleotides containing homologous or heterologous T-DNA border sequences or the RP4 nick region. The mechanistic implications of these reactions for generating the single-stranded T-DNA molecule in the bacterial cell and the fate of T-DNA in the plant nucleus are discussed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Medium. *E. coli* SCS1, a high-transformation variant of DH1 (31), was used as host for plasmids. Plasmid pMS119HE has been described (32). Plasmids pT7-7 (33) and pVIR97.89 (34) were kindly provided by S. Tabor (Harvard Medical School) and J. Alt-Moerbe (Institut für Biologie III, Universität Freiburg, F.R.G.), respec-

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Abbreviations: ss DNA, single-stranded DNA; IPTG, isopropyl β-D thiogalactopyranoside.

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tively. Cells were grown in YT medium supplemented with Mops (100 mM, pH 8.0), thiamin hydrochloride (20 μg/ml), and glucose (0.1%) (28). When appropriate, ampicillin, sodium salt, was added (100 μg/ml).

Proteins and DNA. TraI protein was purified as described (28). Oligodeoxyribonucleotides (200 pmol) were labeled either at their 3′ ends by using [α-^{32}P]ddATP (370 kBq, 110 TBq/mmol) and terminal deoxynucleotidyltransferase (Amersham) or at their 5′ ends by using [γ-^{32}P]ATP (370 kBq, 110 TBq/mmol) and phage T4 polynucleotide kinase (35). Standard molecular cloning techniques were performed as described (35).

Cleavage and Site-Specific Recombination of Oligodeoxyribonucleotides by VirD2. Oligodeoxyribonucleotides (10 pmol) were incubated with VirD2 (2.4 μg, 100 pmol) in 30 μl of 20 mM Tris·HCl, pH 8.8/50 mM NaCl/5 mM $MgCl_2$ for 3 hr at 37°C. The reaction was stopped by the addition of SDS (0.1%) and formamide [30% (wt/vol)]. Products were electrophoresed in 20% (wt/vol) polyacrylamide gels containing 8 M urea (30). Reaction products were quantified by autoradiography of gels with the storage phosphor technology (36).

RESULTS

Overproduction of VirD2. To obtain VirD2 protein in amounts suitable for biochemical analysis the original translational initiation signal preceding the *virD2* structural gene of pTiC58 was replaced by that of phage T7 gene *10*. The gene was placed under control of the *lacI*-regulated *tac* promoter of expression vector pMS119HE, resulting in the VirD2-overproducing plasmid pPS11 (Fig. 1). Following induction of transcription with IPTG (see legend to Table 1), nearly 10% of SDS-soluble *E. coli* cell extracts consisted of VirD2 (Fig. 2*A*, lanes a and b).

Purification of VirD2. VirD2 was purified by a four-step procedure to near homogeneity (Fig. 2*A*, lanes c–f; Table 1). Initially, the protein was found in the insoluble pellet obtained by high-speed centrifugation of lysed cells. After extensive washing of the insoluble protein with high-salt buffer, VirD2 was solubilized in 6 M urea. Stepwise dialysis against buffers of decreasing urea concentration resulted in a VirD2 fraction soluble under physiological conditions. The following purification steps involved chromatography on heparin-Sepharose and DEAE-Sephacel. A final gel filtration on Superose 12 by FPLC (Pharmacia) removed VirD2 degradation products, resulting in a 93% pure VirD2 fraction (Table 1). N-terminal microsequencing (38) of the overproduced and purified VirD2 protein revealed an amino acid sequence in agreement with the proposed start of the pTiC58 *virD2* gene (Fig. 1; ref. 38). A solid-phase immunoassay using VirD2-specific antiserum revealed identical sizes for *virD2* gene products encoded by a gene under its original translational control (Fig. 2*B*, lanes d and e) and those obtained from cells containing pPS11 (lanes a–c).

VirD2 Specifically Cleaves Synthetic T-Border Oligonucleotides. The TraI protein of the conjugative IncPα plasmid RP4 catalyzes a specific cleaving–joining reaction on single-stranded oligonucleotides containing the nick region of the RP4 *oriT* (30). It is known that TraI and VirD2 as well as their targets—the RP4 *oriT* nick region and T-border repeats, respectively—share significant sequence similarity (4, 10, 11). Therefore, we used purified VirD2 to investigate whether the protein can exert analogous reactions on synthetic oligonucleotides containing the cleavage site of pTi border sequences or the RP4 nick region. Similar experiments using VirD2 with form I plasmid DNA containing the right border of pTiC58 revealed that under the conditions applied the

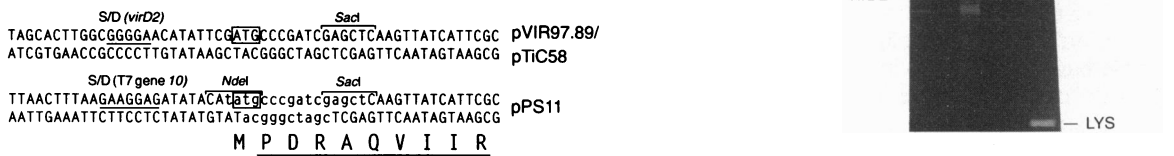

FIG. 1. Construction of a *virD2* overexpression plasmid. (*Upper*) Nucleotide sequence flanking the *virD2* initiation codon in pTiC58/pVIR97.89 (34, 37). The Shine–Dalgarno sequence (S/D) is indicated by a horizontal line; the initiation codon is boxed. Recognition site for *Sac* I restriction enzyme is marked by a bracket above the sequence. (*Lower*) To construct the *virD2* overexpression plasmid, the 1410-bp *Sac* I–*Bam*HI fragment of pVIR97.89 carrying the *virD2* reading frame except for the first five codons was inserted between the *Nde* I and *Bam*HI sites of the polylinker sequence of the T7 promoter φ*10*/gene *10* S/D expression plasmid pT7-7. Synthetic oligodeoxyribonucleotides, printed in lowercase letters, were applied to restore the original 5′ end of the gene and to link the *Sac* I cohesive end of the *virD2* fragment to the *Nde* I end of the vector molecule. To place the manipulated *virD2* gene under control of the more convenient isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter, the manipulated gene, together with the T7 gene *10* S/D sequence, was inserted as an *Xba* I–*Hin*dIII fragment in the multicloning site of pMS119HE, resulting in pPS11. The N-terminal amino acid sequence of VirD2 is shown below the nucleotide sequence. The underlined region was verified by N-terminal microsequencing of the overproduced and purified protein.

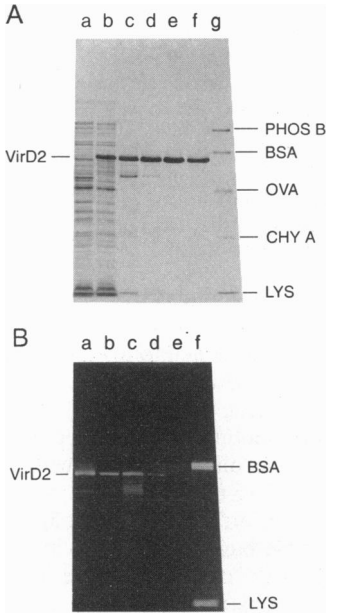

FIG. 2. Purification of VirD2 protein. (*A*) Cell extracts were electrophoresed in a 15% polyacrylamide gel containing 0.1% SDS. The gel was stained with Serva Blue R. Lanes: a, SCS1(pPS11), no IPTG was added; b, SCS1(pPS11), cells were induced for 5 hr with 1 mM IPTG; c, fraction I (16.2 μg); d, fraction II (14.2 μg); e, fraction III (11.8 μg); f, fraction IV (11.0 μg); g, molecular mass standards: phosphorylase B (PHOS B, 97.4 kDa), bovine serum albumin (BSA, 68 kDa), ovalbumin (OVA, 46 kDa), chymotrypsinogen A (CHY A, 25.7 kDa), and lysozyme, (LYS, 14.3 kDa). (*B*) Immunoblot of overproduced and native VirD2 protein. Extracts of SCS1 cells carrying various plasmids were electrophoresed in a 15% polyacrylamide gel containing 0.1% SDS. Proteins were transferred electrophoretically to a nitrocellulose membrane and incubated with rabbit anti-VirD2 serum (diluted 1:1000) and dichlorotriazinyl aminofluorescein-conjugated goat anti-rabbit IgGs (28). A photograph (360-nm UV light) is shown. Lanes: a, purified VirD2 (20 ng); b, pPS11 (3×10^{-2} OD_{600} unit of cells; no IPTG was added to the culture); c, pPS11 (7×10^{-4} OD_{600} unit of cells; cells were induced for 5 hr with 1 mM IPTG); d, pVIR97.89 (3×10^{-2} OD_{600} unit; 1 mM IPTG); e, pMS119HE (3×10^{-2} OD_{600} unit; 1 mM IPTG); f, molecular mass standards: bovine serum albumin (BSA, 68 kDa) and lysozyme (LYS, 14.3 kDa) labeled with fluorescein isothiocyanate.

Table 1. Purification of VirD2 protein

Fraction	Step	Protein, mg	Recovery, %	Purity, %	Specific activity, units/mg
I	Crude extract	1941	100	24.7	—
II	Heparin-Sepharose CL-6B	847	86	48.9	—
III	DEAE-Sephacel	14.7	19	61.3	664
IV	Superose 12	6.6	13	93.0	748

Four 1.2-liter cultures of SCS1(pPS11) were grown in YT medium containing ampicillin, sodium salt (100 μg/ml), at 37°C with shaking. At an OD_{600} of 0.7, transcription of *virD2* was induced by addition of IPTG (1 mM). Shaking was continued for 5 hr. The final OD_{600} reached 3.8. Cells were harvested by centrifugation (4000 × *g*, 10 min), and 1 g of wet weight was resuspended in 5 ml of 0.1 M spermidine tris(hydrochloride)/0.2 M NaCl/2 mM EDTA and frozen in liquid nitrogen. All subsequent procedures were performed at 0–4°C and contact of protein solutions with metal surfaces was strictly avoided. *Fraction I, crude extract.* Frozen cells (24.4 g in 120 ml) were thawed and lysed by addition of 10% (wt/vol) sucrose, 100 mM Tris·HCl (pH 7.6), 5 M NaCl, 10% (wt/vol) Brij-58, and lysozyme (100 mg/ml) to final concentrations of 4.5%, 45 mM, 1.3 M, 0.225%, 0.67 mg/ml, respectively. The total volume of the lysis mixture was 600 ml. After 1 hr at 0°C the highly viscous lysate was centrifuged at 100,000 × *g* for 90 min. The pellets, containing VirD2 protein in the form of inclusion bodies, were washed thoroughly with 80 ml 1 M NaCl/100 mM Tris·HCl, pH 7.6, and centrifuged at 100,000 × *g* for 60 min. To solubilize VirD2, pellets were eluted for 2 hr with 90 ml of buffer A [20 mM Tris·HCl, pH 8.5/1 mM EDTA/0.1% Brij-58/10% (wt/vol) glycerol]/6 M urea/1 M NaCl and centrifuged again at 100,000 × *g* for 60 min. To the supernatant (90 ml), 33.6 g of $(NH_4)_2SO_4$ was added (60% saturation) and precipitated proteins were collected by centrifugation at 100,000 × *g* for 30 min. The sediment was solubilized in 100 ml of buffer A/6 M urea/0.5 M NaCl. The protein solution was dialyzed for 10 hr each against six changes of buffer A/0.5 M NaCl (1 liter) containing 5, 4, 3, 2, 1, and 0.5 M urea. In a final step proteins were dialyzed for 10 hr against 1 liter of buffer A/0.2 M NaCl (fraction I, 140 ml). *Fraction II, heparin-Sepharose CL-6B.* Fraction I was diluted with buffer A/0.1 M NaCl to a final volume of 300 ml and applied at 90 ml/hr to a heparin-Sepharose CL-6B column (2.6 × 24 cm) equilibrated with buffer B [20 mM Tris·HCl, pH 7.6/1 mM dithiothreitol/1 mM EDTA/0.01% Brij-58/10% (wt/vol) glycerol]/0.1 M NaCl. The column was washed with 250 ml of buffer B/0.1 M NaCl and proteins were eluted with a 1.9-liter gradient from 0.1 to 1 M NaCl in buffer B at a flow rate of 90 ml/hr. VirD2 eluted at about 0.375 M NaCl (fraction II, 175 ml). *Fraction III, DEAE-Sephacel.* Fraction II was applied at a flow rate of 60 ml/hr to a DEAE-Sephacel column (2.6 × 10 cm) equilibrated with buffer B/50 mM NaCl. The column was washed with 250 ml of the same buffer. Fractions of the flowthrough containing VirD2 essentially free from degradation products were concentrated by dialysis against 20% polyethylene glycol 20,000 in buffer B/100 mM NaCl (fraction III, 2.5 ml). *Fraction IV, Superose 12.* Aliquots (0.1 ml) of fraction III were applied to a Superose 12 gel filtration column (1 × 30 cm) equilibrated with buffer B/0.5 M NaCl (Pharmacia FPLC equipment). The column was run with the same buffer at a flow rate of 0.5 ml/min. VirD2-containing fractions were pooled, concentrated by dialysis against 20% polyethylene glycol 20,000 in buffer B/0.1 M NaCl and finally dialyzed against 50% glycerol in buffer B/0.25 M NaCl (fraction IV, 1.8 ml). The protein was stored at −20°C without detectable loss of activity for at least 8 months. One unit of activity is defined as the amount of enzyme that cleaves a 17-mer oligonucleotide [d(p*CCAATATATCCTG$^{\nabla}$TCAA), 5 pmol] to 10% in total volume of 20 μl under the conditions described in *Materials and Methods.*

protein does not catalyze the conversion to form II (data not shown).

Cleavage products obtained following incubation of VirD2 with 17-mer oligonucleotides ^{32}P-labeled at the 5′ end were separated in a denaturing polyacrylamide gel. A product with the electrophoretic mobility of a 13-mer was observed irrespective of whether an oligonucleotide with a pTiC58 (Fig. 3, lanes a and b), a pTiA6 (lanes f and g), or an RP4 sequence (lanes i and j) was offered. However, oligonucleotides with certain defined single-base substitutions in the conserved 6-nt region on the 3′ side of the cleavage site were not cut, demonstrating the substrate specificity of the reaction (data not shown). We could not detect any significant difference in cleavage efficiency between oligonucleotides with the sequence of the left or of the right border of pTiC58 (data not shown). Occurrence of a 13-mer indicates that VirD2 mediates cleavage at the same site within the pTiC58 border sequences that was found to be cleaved *in vivo* (23). Cleavage at identical sites was also found for a VirD2 protein obtained from pTiA6 (data not shown). This result is in conflict to the results of Albright *et al.* (39), who mapped the pTiA6 border cleavage sites by primer extension 1 nt upstream of the cleavage site found in our assay. Incubation of the reaction products with trypsin did not alter the electrophoretic mobility of the 5′-end-labeled product, indicating that the 3′ end produced by VirD2-catalyzed cleavage is not covalently associated with protein (lane c). The reaction is dependent on the presence of Mg^{2+} (lane d). In contrast to the broad spectrum of substrates that are accepted by VirD2, the RP4 TraI protein's catalytic activity is confined to oligonucleotides with the sequences of the cognate RP4 or R751 nick regions (Fig. 3, lanes e, h, and k and data not shown).

VirD2 Covalently Attaches to the 5′ End of Cleaved Oligonucleotides. VirD2 has been found to be covalently associated with the 5′ ends of the T-DNA single strands that are produced in agrobacteria after induction of Ti plasmid *vir* gene expression by plant phenolic compounds such as ace-

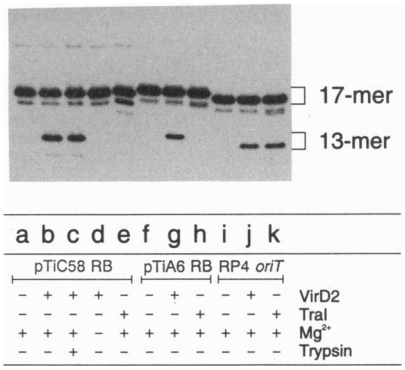

FIG. 3. Site-specific cleavage of oligonucleotides by VirD2 and TraI. Oligonucleotides labeled at their 5′ ends were incubated with proteins and reactions were stopped and analyzed as described under *Materials and Methods.* Presence or absence of proteins and Mg^{2+} is shown. Where indicated, the cleavage reaction was followed by trypsin digestion in presence of 0.1% SDS. Positions of bands corresponding to the input oligonucleotides (17-mers) and cleavage products (13-mers) are indicated at right. The following oligonucleotides were used as substrates: pTiC58 RB (right border), d(p*CCAATATATCCTG$^{\nabla}$TCAA);pTiA6RB,d(p*GGTATATATCCTG$^{\nabla}$CCAG); RP4 oriT, d(p*TTCACCTATCCTG$^{\nabla}$CCCG). Positions of the cleavage sites are indicated by wedges. The radioactive label is symbolized by an asterisk.

tosyringone (for a review see ref. 2). To find out whether covalent attachment of VirD2 to the DNA 5′ end can be mimicked by the oligonucleotide cleavage reaction *in vitro*, 3′-labeled oligonucleotide was incubated with VirD2 and the products were separated by gel electrophoresis. Oligonucleotide cleavage under these conditions resulted in a labeled species that did not enter the gel, indicating that the 3′-terminal oligonucleotide moiety was tightly associated with high molecular weight material (Fig. 4*A*, lane b). Treatment of the material with a variety of proteases (lanes c–f) resulted in labeled species with distinct electrophoretic mobilities reflecting the covalent association of the 5′ end of cleaved oligonucleotides with different peptide species generated by proteases of different specificity. Inspection of the VirD2 sequence revealed that the observed pattern is consistent only with the interpretation given in Fig. 4*B* placing the active-site tyrosine at position 28 of the VirD2 sequence. This assignment agrees with a mutational analysis of *virD2* demonstrating that Tyr^{28} cannot be substituted without loss of T-border cleavage activity *in vivo* (40). Furthermore, amino acid sequence comparison with TraI of RP4 shows that Tyr^{28} of VirD2 and the active-site Tyr^{22} of TraI are located at equivalent positions within a conserved motif (10, 11).

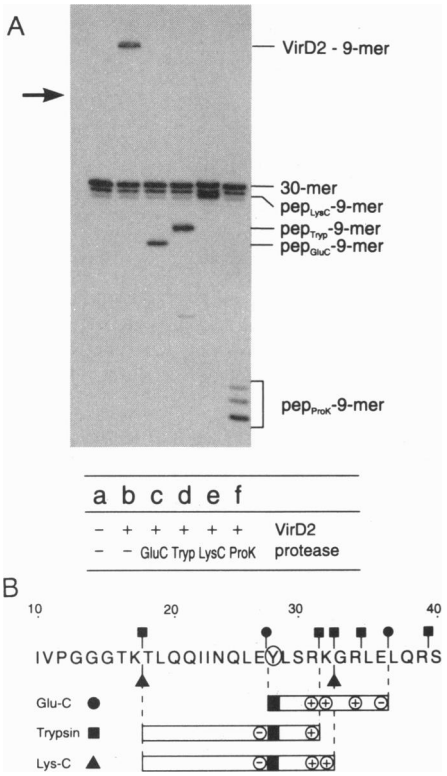

FIG. 4. Covalent attachment of VirD2 to the 5′ end of cleaved oligonucleotides. (*A*) Oligonucleotide [30-mer, d(TTTACCCGCCAATATATCCTG^{V}TCAAACAC)ddA*], labeled at the 3′ end, was incubated with VirD2 and reactions were stopped by addition of SDS (0.1%) and $CaCl_2$ (2 mM). The following protease concentrations were used: endoproteinase Glu-C, 200 μg/μl; trypsin (Tryp), 500 μg/μl; Lys-C, 20 μg/μl; proteinase K (ProK), 100 μg/μl. Following incubation for an additional 120 min at 37°C, formamide [30% (wt/vol)] was added and the products were analyzed. Presence or absence of VirD2 in the reactions and protease treatment is indicated. Positions of bands corresponding to the input oligonucleotides (30-mers) and main cleavage products (9-mers with covalently attached VirD2 peptides) are indicated on the right. At the position of the arrow about a quarter of the total length of the gel, a part which contained no radioactivity, was cut out. (*B*) Partial amino acid sequence of VirD2. The proposed active-site Tyr^{28} is circled. Cleavage sites for endoproteinases Glu-C, trypsin, and Lys-C are indicated (●, ■, and ▲, respectively). Open boxes mark the extension of protease-generated peptides containing Tyr^{28}, represented by a shaded box. Acidic and basic amino acid residues are indicated by ⊖ and ⊕, respectively.

VirD2 Transfers a Covalently Attached Oligonucleotide Moiety to a Preformed Border-Sequence 3′ End. Integration of the T-DNA into the plant genome requires the reverse of the cleavage reaction (see *Discussion*). To detect specific joining of oligonucleotides by VirD2 *in vitro*, a preformed border 3′ terminus was synthesized as a 13-mer oligonucleotide and incubated in various amounts together with VirD2 and a 3′-end labeled 30-mer oligonucleotide carrying the pTiC58 right-border cleavage site. Specific transfer of the 3′-terminal moiety of the 30-mer to the 13-mer by VirD2 resulted in a 22-mer detectable on polyacrylamide gels by the 3′ label (Fig. 5). This demonstrates that the 13-mer participates in the oligonucleotide cleavage reaction, presumably by reaction with free VirD2–d(pTCAAACAC)ddA*. Oligonucleotides (13-mers) with defined single-base substitutions in the conserved 6-nt region on the 3′ side of the cleavage site did not function as acceptors. This control demonstrates the specificity of the joining reaction (data not shown). Quantification of reaction products revealed that (*i*) the labeled 3′-terminal moiety was efficiently transferred to the preformed 3′ terminus even at moderate molar ratios of 13-mer to 30-mer; (*ii*) recovery of the cleavage product pep_{Tryp}–d(pTCAAACAC)ddA* drops with increasing concentration of 13-mer, indicating that the thermodynamic equilibrium can be shifted by addition of one reaction product to the joined oligonucleotide form (Fig. 5*B*).

DISCUSSION

The relaxosome of the conjugative plasmid RP4 has become a paradigm for the generation of a covalent ssDNA–protein

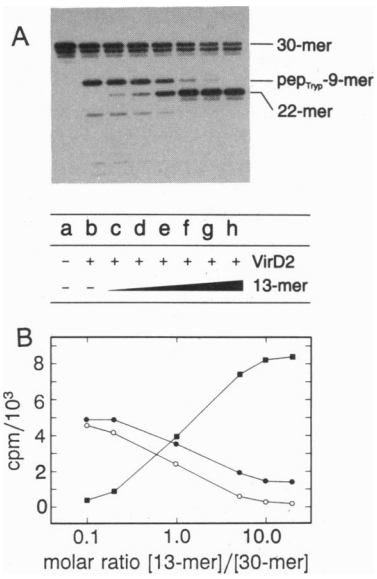

FIG. 5. Site-specific joining of oligonucleotides by VirD2. (*A*) Oligonucleotide (30-mer, see legend of Fig. 4) labeled at the 3′ end was incubated with VirD2 as described under *Materials and Methods*, except that the pH of the reaction mixtures was adjusted to 8.0. Additionally, the mixtures contained various amounts of a 13-mer [d(CCAATATATCCTG)]. Reactions were stopped by addition of SDS and trypsin (0.1% and 200 μg/ml, respectively) and incubation for 2 hr at 37°C. Lanes: a–h, 10 pmol of 30-mer; c–h, 1, 2, 10, 50, 100, and 200 pmol of 13-mer, respectively. Presence or absence of VirD2 and 13-mer in the reaction mixtures is shown. Increasing amounts of 13-mer are symbolized by a wedge. Positions of bands corresponding to the input oligonucleotide (30-mer), the main cleavage product (pep_{Tryp}-9-mer), and the recombination product (22-mer) are indicated on the right. (*B*) Reaction products were quantified by radioactivity and the amounts were plotted against the molar ratio of the reactants: ●, 30-mer; ○, pep_{Tryp}-9-mer; ■, 22-mer.

complex destined for transfer to a recipient cell. Catalytic activity for specific cleaving and joining of DNA has been assigned to the plasmid-encoded TraI protein (29, 30). Here we present evidence that purified VirD2 protein of agrobacterial Ti plasmids *in vitro* catalyzes similar specific cleaving–joining reactions with single-stranded T-border DNA. VirD2-mediated scission of oligonucleotides has several features in common with the ssDNA cleavage exerted by TraI (30): (*i*) site specificity, (*ii*) covalent binding of the protein to the 5′ terminus after cleavage, (*iii*) an equilibrium between nicked and joined forms of DNA molecules, and (*iv*) dependence on Mg^{2+}.

Neither supercoiled nor relaxed double-stranded DNA acts as substrate for cleavage by VirD2 alone under our conditions. Generation of the T-DNA complex *in vivo* requires both VirD1 and VirD2 (ref. 41; for a review see ref. 3). VirD1 could play a role in T-border cleavage analogous to that of TraJ protein in the RP4 system. TraJ functions as a specific *oriT*-binding protein and is essential for TraI-mediated cleavage of supercoiled *oriT* DNA (27, 28). Cleavage of form I RP4 *oriT* DNA is enhanced by a second *oriT*-binding protein, TraK, which locally changes the DNA topology. We suppose that this exposes the *oriT* nick region as a single strand which can be recognized and cleaved by TraI (30, 42). In view of the striking analogy of the reactions catalyzed by TraI and VirD2 on ssDNA, it seems unlikely that the proposed topoisomerase I activity observed in VirD1-containing extracts (19) is related to T-border specific cleavage. Relaxation of double-stranded border sequences by topoisomerase I activity would inhibit local melting of the DNA and therefore prevent access of VirD2 to its target site. Recent *in vitro* experiments demonstrate that purified VirD1 and VirD2 specifically cleave superhelical T-border DNA. However, under the conditions applied (28), topoisomerase I activity of VirD1 alone was never observed (P. Scheiffele, W.P., and E.L., unpublished results).

The main difference between the TraI-catalyzed reaction and that of VirD2 appears to be the wider range of substrates accepted by VirD2. This observation might reflect the requirements that VirD2 has to fulfill upon integration of the T-DNA into the plant cell genome. Sequencing of junctions between integrated T-DNA and plant DNA has revealed that a considerable fraction of integration events are precise with respect to the right end of the T-DNA (43, 44). Precise integration strongly suggests that VirD2, covalently attached to the 5′ terminus of the T-DNA in the bacterium, is directly involved in the illegitimate recombination process in the plant cell nucleus. The *in vitro* experiments presented here demonstrate that ssDNA which is covalently attached to VirD2 can be joined to a preformed 3′ end. Therefore, precise joining of the 5′ end of the right border to the plant DNA is conceivable if covalently bound VirD2 could recognize free 3′ termini (i.e., nicks or gaps) in the plant DNA; this recognition would be followed by joining of the right end of the T-DNA to the plant genome. Relaxed specificity of the ssDNA cleaving–joining reaction observed *in vitro* need not be in conflict with more specific cleavage of border sequences in the donor cell; specificity of double-strand cleavage by VirD2 may be increased by other factors, most probably VirD1. This would be in line with the finding that the sequence of a 5-bp region on the nick site-distal end of the T-border repeat is crucial for tumorigenicity (45) but not for the ssDNA cleaving–joining reaction.

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