Mutational Analysis of *Agrobacterium tumefaciens virD2*: Tyrosine 29 Is Essential for Endonuclease Activity

ANN M. VOGEL[1] AND ANATH DAS[1,2]*

*Department of Biochemistry[1] and Plant Molecular Genetics Institute,[2] University of Minnesota,
1479 Gortner Avenue, St. Paul, Minnesota 55108*

Agrobacterium tumefaciens VirD2 polypeptide, in the presence of VirD1, catalyzes a site- and strand-specific nicking reaction at the T-DNA border sequences. VirD2 is found tightly attached to the 5' end of the nicked DNA. The protein-DNA complex is presumably formed via a tyrosine residue of VirD2 (F. Durrenberger, A. Crameri, B. Hohn, and Z. Koukolikova-Nicola, Proc. Natl. Acad. Sci. USA 86:9154–9158, 1989). A mutational approach was used to study whether a tyrosine residue(s) of VirD2 is required for its activity. By site-specific mutagenesis, a tyrosine (Y) residue at position 29, 68, 99, 119, 121, 160, or 195 of the octopine Ti plasmid pTiA6 VirD2 was altered to phenylalanine (F). The Y-29–F or Y-121–F mutation completely abolished nicking activity of VirD2 in vivo in *Escherichia coli*. Two other substitutions, Y-68–F and Y-160–F, drastically reduced VirD2 activity. A substitution at position 99, 119, or 195 had no effect on VirD2 activity. Additional mutagenesis experiments showed that at position 29, no other amino acid could substitute for tyrosine without destroying VirD2 activity. At position 121, only a tryptophan (W) residue could be substituted. This, however, yielded a mutant protein with significantly reduced VirD2 activity. The nicked DNA from strains bearing a Y-68–F, Y-99–F, Y-119–F, Y-160–F, Y-195–F, or Y-121–W mutation in VirD2 was always found to contain a tightly linked protein.

Agrobacterium tumefaciens incites crown gall tumor disease on most dicotyledonous plants by transferring a segment of its tumor-inducing (Ti)-plasmid-borne DNA to plant cells. The transferred (T) DNA is found stably integrated into the plant nuclear genome. A 24-bp direct repeat sequence, the border sequence, is found conserved at the two termini and functions in *cis* by defining the boundary of the T-DNA. This is the only element within the T-DNA that is required for DNA transfer to plant cells. *trans*-acting factors for DNA transfer are encoded within the bacterial chromosome and the virulence (*vir*) region of the Ti plasmid (for reviews, see references 17 and 31).

The *vir* region consists of at least eight complementation groups, *virA* through *virH* (6, 12, 16, 22). Most of these genes, except *virA* and *virG*, are transcriptionally silent in the bacterium. Expression of these genes is induced when a bacterium encounters a susceptible host plant and is positively controlled by two regulatory loci, *virA* and *virG* (24).

Upon encountering a plant cell, the bacterium activates a series of events leading to the formation of a single-stranded (ss) T-strand DNA, composed of the bottom strand of the T-DNA (1, 23, 27). This DNA contains a tightly, perhaps covalently, linked VirD2 molecule at its 5' end (8, 10, 28, 30) and is completely coated by an ssDNA-binding protein, VirE2 (4, 19). This DNA-protein complex, the T complex, is believed to be an intermediate in DNA transfer. The initial reactions for T-strand DNA synthesis are catalyzed by the enzymes encoded within the *virD* locus (29). *virD* can potentially encode four polypeptides, of which the first two, VirD1 and VirD2, catalyze a site- and strand-specific nick at the T-DNA borders (11, 29). VirD1 possesses a DNA-relaxing activity (7), and VirD2 presumably catalyzes the endonucleolytic nick. On the basis of chemical-stability analysis of the VirD2-DNA covalent complex, Durrenberger

et al. (5) suggested that a tyrosine residue of the protein may be involved in this complex formation. We speculated that such a residue may be required for VirD2 activity. To test this hypothesis, we used site-specific mutagenesis procedures to change individual tyrosine residues of VirD2 to phenylalanine, a close homolog, and studied the effects of these mutations on VirD2 activity.

MATERIALS AND METHODS

Plasmids. Plasmid pAD1201 was constructed by cloning a 3.5-kb *Hind*III fragment from plasmid pEnd1 (13) into the *Hind*III site of the plasmid vector pACYC184 (3). The pEnd1 fragment contains the left border and right border (RB) sequences of the octopine Ti plasmid pTiA6 left T- (T_L)-DNA. Plasmid pAD1201 was used as a substrate for VirD2 endonuclease assays in vivo in *Escherichia coli*.

Plasmid pAD1244 was constructed by cloning a 2.6-kb *Eco*RI fragment from plasmid pJG1 (7) into the plasmid vector pUC118 (25). To introduce a stop codon for the *lacZ'* polypeptide encoded within pUC118, both vector and insert were filled in with DNA polymerase I Klenow enzyme and deoxynucleoside triphosphates before cloning. pAD1244 contains pTiA6 *virD1 virD2 virD3* coding regions under the control of the *E. coli* lactose (*lac*) operon promoter and was used for overproduction of VirD1 and VirD2 for endonuclease assays and for site-specific mutagenesis.

Other plasmids used in this study are pAV1 (*tac virD1*), pAV8 (*tac virD1 virD2 virD3*), and pAD1190 (*tac virD2 virD3*). All plasmids were introduced into *E. coli* AD256 (7).

Site-specific mutagenesis. Seven of the tyrosine (Y) residues of VirD2 at positions 29, 68, 99, 119, 121, 160, and 195 were replaced with phenylalanine (F) by the site-specific mutagenesis procedure of Kunkel (14). To introduce these mutations, uracil containing ss pAD1244 DNA was mutagenized with synthetic deoxyoligonucleotide primers. Whenever feasible, an additional mutation(s) was introduced into

---

* Corresponding author.

the adjacent codon to create a new restriction enzyme site to facilitate analysis of potential mutants. In no case did these additional mutations alter the amino acid in question. The mutations (underlined) and the restriction endonuclease sites they created are as follows: Y-29–F, GAGTAC→GAATTC, EcoRI; Y-68–F, Y-99–F, and Y-160–F, TATGA→TTCGA, TaqI; and Y-119–F, TATAA→TTTAA, MseI.

Uracil containing ss pAD1244Y29F DNA (which contains a unique EcoRI site at the site of mutation) was mutagenized with a degenerate 33-residue deoxyoligonucleotide (dTCCC TTACGGGACAGCNNTTCCAACTGATTGAT, sequence complementary to the coding strand) to obtain all possible amino acid substitutions at the tyrosine 29 position. Transformants were first screened for the loss of the EcoRI site, and the DNA sequences of these clones were determined to identify the amino acid substitution.

For substitution of Y-121, ss pAD1244 DNA was first mutagenized to introduce a PvuII site (TATCTG→CAG CTG), which created a Y-121–Q mutation. ss pAD1244 Y121Q DNA was then mutagenized with a degenerate deoxyoligonucleotide (dGTGGTAGGCTGTCAGCNNGTTATAG CGGCCACC) to introduce all possible amino acid substitutions at position 121.

All mutations were confirmed by DNA sequence analysis on double-stranded DNA templates by using Sequenase (U.S. Biochemical Corp.) (18).

VirD2 endonuclease assay. VirD2 activity was assayed in E. coli in the presence of VirD1. E. coli AD256 was transformed with plasmids pAD1201 (chloramphenicol-resistant substrate plasmid) and pAD1244 (ampicillin-resistant VirD1-VirD2–overproducing plasmid) or its derivatives. A 5-ml culture was grown in Luria broth containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) at 37°C until an A_{600} of 0.6 to 0.8 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 0.1 mM) was added to the culture, and cells were grown for an additional 4 h. Cells were harvested in 1-ml aliquots by centrifugation and stored frozen at −70°C.

DNA isolation. Total DNA was isolated from an aliquot by resuspending the cell pellet in 300 μl of 10× TE (1× TE is 10 mM Tris-HCl, pH 8.0, plus 1 mM EDTA). Proteinase K and sodium dodecyl sulfate (SDS) were added to the suspension at final concentrations of 50 μg/ml and 0.5%, respectively. After a 30-min incubation at 65°C, the mixture was extracted three times with an equal volume of phenol-chloroform mixture and once with an equal volume of chloroform. The aqueous layer was removed, sodium chloride was added to 0.2 M, and 2 volumes of 95% ethyl alcohol were added to precipitate all nucleic acids. After centrifugation, the pellet was washed with 75% ethyl alcohol, dried, and resuspended in 50 μl of TE. Aliquots of 4 μl were used in the primer extension assay for VirD2 activity.

For analysis of the tight association of VirD2 and DNA, total DNA was isolated as described above, except that the cells were lysed with lysozyme and proteinase K was omitted from the reactions. Both the aqueous layer and the aqueous-organic interphase were recovered. Nucleic acids in the aqueous layer were recovered by ethanol precipitation. The interphase was dialyzed against TE for 12 to 16 h (30), and the dialysate was treated with SDS and proteinase K. After extraction with phenol-chloroform, nucleic acids in the dialysate were precipitated with ethyl alcohol as described above. The DNA was resuspended in 40 μl of TE and used for VirD2 assay by the primer extension procedure.

Primer extension procedure. Total DNA isolated from E. coli was digested with the restriction endonuclease HindIII,
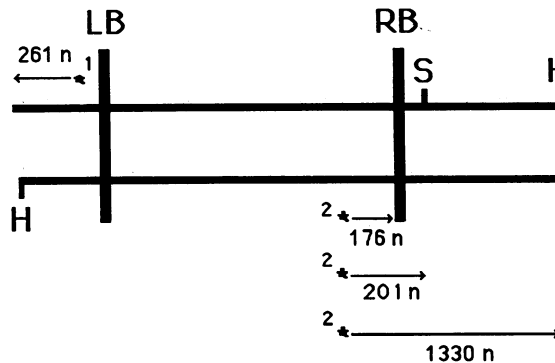

FIG. 1. Physical map of pTiA6 T_L-DNA in pAD1201. Sites of right border (RB), left border (LB), and restriction endonucleases HindIII (H) and SacI (S) are shown. Arrows indicate primer extension products (sizes are in nucleotides [n]) expected to be generated from primer 1 and primer 2.

and Sequenase buffer (U.S. Biochemical Corp.) (final concentration, 1×) and two 5′-³²P-labeled deoxynucleotide primers (1 × 10⁵ cpm; specific activity, 1 × 10⁸ to 3 × 10⁸ cpm/μg) were added (see below and Fig. 1). The DNA was boiled for 3 min, and the primers were annealed for 15 min at 37°C. Deoxynucleoside triphosphates (final concentration, 0.5 mM) and Sequenase (1 to 2 U) were added, and the mixture was incubated at 37°C for 15 min. Samples were denatured and analyzed on a 6% polyacrylamide-urea sequencing gel by standard procedures (15). Extension of primer 1, 5′dCGCAGAACTGAGCCGGTTAGGC (sequence complementary to residues 863 to 842 of Barker et al. [2]), terminates at a HindIII site, producing a 261-nucleotide fragment. This primer is used as an internal control of plasmid DNA content of the sample and can be used for quantitative analysis. Primer 2, 5′dGTGGGCCTGTGGTC TCA (residues 13886 to 13902 of Barker et al. [2]), is used to monitor nicking at the T_L-DNA RB. If the DNA is nicked at the RB sequence, a 176-nucleotide product will be synthesized. Otherwise, the primer will extend to the next HindIII site, yielding an approximately 1,330-nucleotide product.

RESULTS

VirD2 assay. VirD2 endonuclease activity was assayed in vivo in a binary assay in E. coli. One plasmid, pAD1201, serves as the substrate for VirD1-VirD2 endonuclease produced from a second plasmid, pAD1244, or its derivatives. Primer extension procedure was used to monitor the nicking activity of VirD2 (see Materials and Methods for details). When VirD1 or VirD2 was absent from the host cell, no 176-nucleotide product (characteristic of pAD1201 DNA nicked at the T-DNA RB) was observed (Fig. 2, lanes 1 and 2). When both proteins were produced simultaneously, this product was synthesized (Fig. 2, lane 3). In the presence of primer 1 (a primer used as an internal control) alone, synthesis of the characteristic 261-nucleotide product from total plasmid DNA was observed (Fig. 2, lane 4). No 176-nucleotide product was observed with this primer. In the presence of primer 2 (a primer used to monitor nicking at the T-DNA RB), a single 176-nucleotide band expected from a nick at the border was synthesized (Fig. 2, lane 5). Additional bands with sizes greater than 261 nucleotides were also observed in this reaction (data not shown). These bands presumably resulted from the extension of DNA past the RB
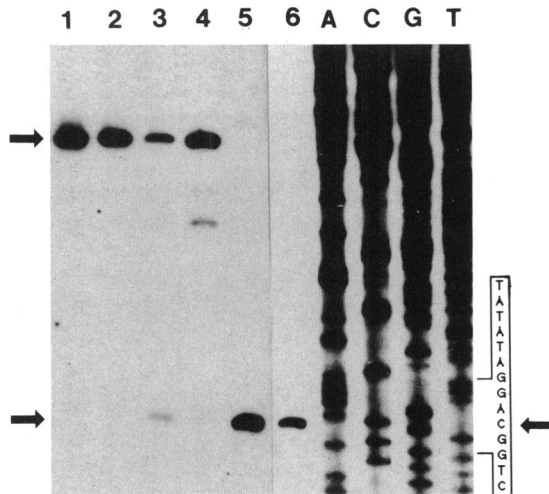
FIG. 2. VirD2 endonuclease assay. Total DNA was isolated from *E. coli* expressing VirD1 (lane 1), VirD2 (lane 2), or VirD1 and VirD2 (lanes 3 to 5). After digestion with *Hind*III, the DNAs were analyzed by primer extension assays in the presence of primer 1 (lane 4), primer 2 (lane 5), or both primers (lanes 1 to 3). Lane 6 is a lighter exposure of lane 5. Extension from primer 1 results in the top band (261 nucleotides) and extension from primer 2 terminated at the RB nick site results in the lower band (176 nucleotides) indicated by arrows. The DNA sequence around the nick site is also shown. A, C, G, and T represent dideoxy DNA sequencing reactions (18) with pAD1201 DNA as the template and primer 2 as the sequencing primer.
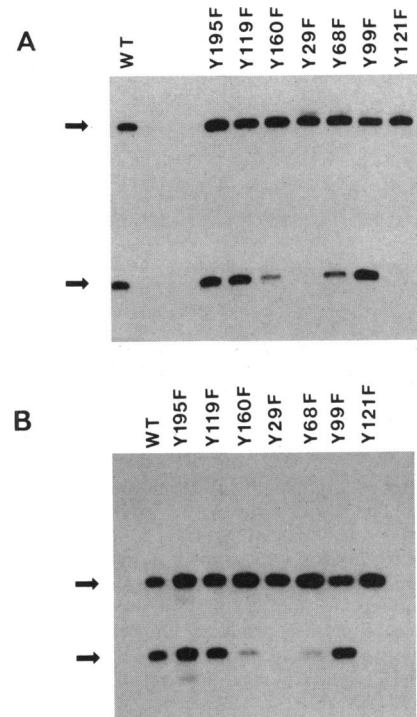

FIG. 3. Assay of VirD2 mutants. VirD2 activity was assayed by the primer extension procedure following digestion of DNA with the restriction endonuclease *Hind*III (A) or *Sac*I (B). Extension reactions were performed as described in Materials and Methods in the presence of primers 1 and 2 (A) or primer 2 (B). WT, wild-type VirD2; Y*n*Fs, tyrosine-to-phenylalanine substitutions at the *n*th residue of VirD2. The 176-nucleotide product, characteristic of nicking at the RB, is indicated by an arrow. The other bands indicated by arrows are the 261-nucleotide product from primer 1 (A) and the 201-nucleotide product from primer 2 (B).

nick site. The identity of the expected 1,330-nucleotide band (product of extension of primer 2 to the *Hind*III site) could not be confirmed in this assay because this large product barely enters the gel and because other nonspecific bands in similar positions were present in all samples. To ensure that primer 2 extended beyond the nick site, separate experiments were performed (see below). When both primers were used, the band pattern was a composite of reactions with individual primers (Fig. 2, lane 3). All bands shown, except for the 176-nucleotide product, were synthesized from primer 1. The additional products obtained with primer 1 presumably result from premature termination (Fig. 2, lane 4). Size determination of the extension product of primer 2 indicates that the product is 176 nucleotides in length. This result would imply that the nick site lies between a C and an A (residues 14062 and 14063 of Barker et al. [2]). This is in total agreement with previous reports of Albright et al. (1).

Mutational analysis of VirD2 tyrosine residues. DNA sequences of VirD2 coding regions of the octopine Ti plasmid pTiA6, the nopaline Ti plasmid pTiC58, and the *Agrobacterium rhizogenes* root-inducing (Ri) plasmid pRiA4b have been determined (9, 26, 29). The pTiA6 VirD2 protein contains 10 tyrosine residues at positions 29, 68, 99, 114, 119, 121, 125, 160, 195, and 304 (29). Of these, seven tyrosines at positions 29, 68, 99, 119, 121, 160, and 195 are conserved in VirD2 of the three Ti and Ri plasmids (Fig. 2; 26). If a tyrosine residue of VirD2 is functionally important, one (or more) of the conserved tyrosine residues would be expected to be an essential residue. Since a tyrosine residue is suspected to form a protein-DNA covalent complex (5) and the hydroxyl group of the tyrosine is known to participate in protein-DNA covalent complexes, we decided to mutagenize the seven conserved tyrosine residues to phenylalanine, an amino acid equivalent to tyrosine minus the

hydroxyl group. Analysis of these mutations showed that substitution of phenylalanine for tyrosine 99, 119, or 195 had little or no effect on VirD2 activity (Fig. 3A). A substitution at position 68 or 160 drastically reduced VirD2 activity, and a substitution at position 29 or 121 completely abolished VirD2 nicking activity (Fig. 3A).

In this assay, the identity of the 1,330-nucleotide product could not be established unequivocally because of low resolution of large products in the gel system used in this work. To determine that primer 2 does extend beyond the nick site, we digested the DNA samples with the restriction endonuclease *Sac*I. A *Sac*I recognition sequence is located 25 bp 3' of the RB nick site. Therefore, extension with primer 2 would yield the same 176-nucleotide product when the DNA is nicked at the RB and would yield a 201-nucleotide product when extension continues past the nick site and terminates at the *Sac*I restriction site. Results presented in Fig. 3B demonstrate that all DNA samples analyzed support the synthesis of a 201-nucleotide product. However, as shown in Fig. 3A, the 176-nucleotide product was synthesized only in a subset of the samples. These results indicate that four tyrosine residues, at positions 29, 68, 121, and 160, are required for VirD2 activity. Of these, tyrosine 68 and 160 are not essential, as they could be replaced with phenylalanine without destroying VirD2 activity.
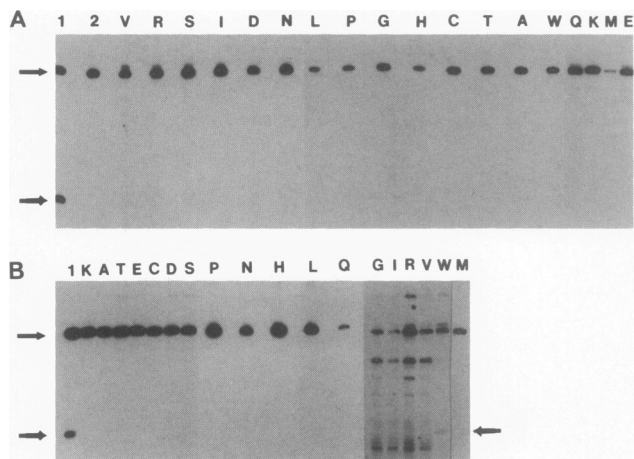
FIG. 4. Effect of amino acid substitution at position 29 or 121 on VirD2 activity. The tyrosine residue of VirD2 at position 29 (A) or position 121 (B) was replaced with each possible amino acid by site-directed mutagenesis (14). Substitutions are indicated by the single-letter amino acid code at the top. Both the 261- and 176-nucleotide products are indicated by arrows. Additional bands in some of the reactions are products of premature termination of primer 1. Lanes 1, positive control from cells producing wild-type VirD1 and VirD2; lane 2, negative control from cells producing wild-type VirD1 (A).

FIG. 5. Analysis of covalent attachment of VirD2 to nicked DNA. DNA samples were prepared from VirD2 tyrosine-to-phenylalanine (YnF, where n is the position) and tyrosine 121-to-tryptophan (Y121W) mutants as described in Materials and Methods. The DNAs from the aqueous layer and aqueous-organic interface were isolated and analyzed by primer extension assays with both primers. The primer extension products are denoted with arrows. WT, wild-type VirD2.

Effects of amino acid substitutions at positions 29 and 121.

We reasoned that if a specific tyrosine residue is essential at the active site for VirD2 activity, no amino acid substitution would be tolerated at this particular position. To test this hypothesis, we used oligonucleotide-directed site-specific mutagenesis to change tyrosine 29 or tyrosine 121 to each possible amino acid. Analyses of the mutant VirD2 proteins indicate that a tyrosine residue at position 29 is essential for VirD2 activity and no substitution can be tolerated at this site (Fig. 3 and 4A). At position 121, a tryptophan residue could functionally substitute for tyrosine, although no other amino acid could be tolerated at this position (Fig. 3 and 4B). The Y-121–W mutation led to significant reduction in VirD2 activity. Analysis of the VirD2 polypeptide by Western blot (immunoblot) hybridization with rabbit anti-VirD2 antibody showed that all mutants produced stable VirD2 in quantities comparable to that produced with the wild-type strain (data not shown). These results suggest that tyrosine 29 is the tyrosine residue required at the active site for VirD2 activity and is probably the residue involved in covalent protein-DNA complex formation (see below). Since tyrosine 121 is also required for VirD2 activity but could be replaced with a tryptophan residue, this residue must lie in an essential region of the protein. A close scrutiny of the protein sequence around the region shows that it lies close to a Gly-X-Gly-X-X-Gly (X = any amino acid) sequence. This sequence is found conserved in many proteins and is presumed to form a nucleotide-binding domain (for a review, see reference 20). This region is also conserved among the three VirD2 sequences of pTiA6, pTiC58, and pRiA4b plasmids. It is possible that tyrosine 121 has an effect on the nucleotide-binding pocket of VirD2.

Covalent attachment of VirD2 to nicked DNA. Reports from several laboratories indicate that VirD2 is very tightly (and perhaps covalently) attached to the 5' end of the T-strand DNA (8,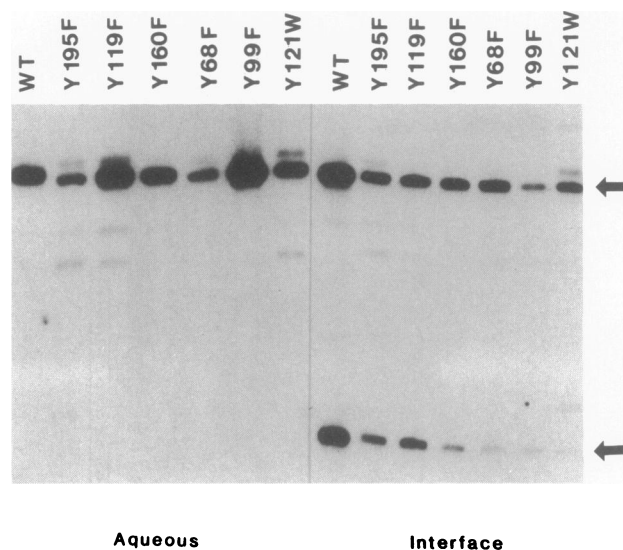 10, 28, 30). The covalent attachment and nicking reaction may be a one-step process in which the reactions are coupled. Alternatively, the covalent attachment may take place as a second reaction in a two-step process. In the former case, it is likely that the tyrosine residue that is found attached to DNA is a part of the active site of the enzyme. In this case, alteration of the required tyrosine residue will yield a nonfunctional protein. In the latter case, the active site may be composed of a completely different region of the protein which may or may not include a tyrosine residue. Following nicking, a nearby tyrosine residue might become attached to the nicked DNA to prevent reclosure of the nick and/or another, undetermined function(s). If this is true, it may be feasible to obtain a mutation that is competent in nicking at the T-DNA borders but does not yield a covalent DNA-protein complex.

To determine whether any of the single-site mutations used in our study is defective in covalent protein-DNA complex formation, we determined the nature of the nicked DNA. Young and Nester (30) demonstrated that when DNA is extracted with phenol, the VirD2-DNA complex migrates to the aqueous-organic interface while deproteinized DNA is partitioned into the aqueous layer. Using this criterion, we observed that the nicked DNA in all Y→F mutant strains that yielded functional VirD2 and in Y-121–W partitioned exclusively into the aqueous-organic interface, indicating that the nicked DNA contained a tightly attached protein (Fig. 5). Only DNA recovered from the interface led to the synthesis of the 176-nucleotide product. The 261-nucleotide product, resulting from total plasmid DNA, was synthesized from DNA isolated from both the interface and the aqueous layer. These results indicate that Y-68–F, Y-99–F, Y-119–F, Y-160–F, Y-195–F, and Y-121–W yield functional VirD2 polypeptides that nick T-DNA at the RB sequence and attach tightly to the nicked DNA.

DISCUSSION

The VirD2 protein of *A. tumefaciens*, in the presence of VirD1, nicks the Ti plasmid at the T-DNA borders and becomes covalently attached to the 5' end of the nicked DNA. A tyrosine residue of the protein has been implicated in the protein-DNA complex formation (5). This residue is expected to be conserved in VirD2 from different sources and may be required for VirD2 activity. We used a mutational approach to determine the functional requirement of the conserved tyrosine residues of pTiA6 VirD2. By individually altering each tyrosine residue that is conserved in VirD2 of three Ti and Ri plasmids, we demonstrated that a substitution for the tyrosine residue at position 68 or 160 significantly reduced VirD2 activity and that two other substitutions, at position 29 or 121, completely abolished VirD2 activity (Fig. 3). Of the tyrosine residues, tyrosine 29 is essential, as no other amino acid could be substituted at this position without destroying enzyme activity (Fig. 4A). The tyrosine residues at positions 68, 121, and 160 are required but could be replaced with at least one other amino acid, producing mutant proteins with greatly reduced nicking activity (Fig. 4B and 5). Since tyrosines 68, 121, and 160 could be replaced with a different amino acid but tyrosine 29 could not, these observations suggest that tyrosine 29 is the most likely candidate to participate in the covalent protein-DNA complex formation. Final confirmation of this will come from protein sequence analysis of the isolated complex.

To speculate on the requirement of tyrosine 121 for VirD2 activity, we reexamined the primary sequence of the protein. We noted that a Gly-X-Gly-X-X-Gly sequence is conserved at positions 111 to 116 of VirD2 from all three Ti and Ri plasmids (Fig. 2) (26). This conserved sequence has been implicated as a nucleotide-binding domain of protein kinases and other proteins (20). We have observed that VirD2 requires ATP for its enzymatic activity in vitro (21). Therefore, it is likely that tyrosine 121, close to the conserved glycine-rich sequence, may affect the nucleotide-binding ability of VirD2 and thereby regulate its activity.

Several laboratories reported the formation of a tightly attached VirD2-DNA complex (8, 10, 28, 30). Results presented in this work confirm this observation (Fig. 5). Our studies also demonstrate that mutations that did not affect VirD2 nicking activity allowed formation of a covalent protein-DNA complex, suggesting that the nicking reaction and attachment of protein to DNA may happen concurrently. A covalent complex was observed with Y-68-F, Y-99-F, Y-119-F, Y-160-F, Y-195-F, and Y-121-W mutations, again suggesting that the only remaining conserved tyrosine residue at position 29 is likely to participate in covalent DNA-protein complex formation. The chemical-stability studies of Durrenberger et al. (5) suggest that residues other than tyrosine are not likely candidates for this complex formation.

ACKNOWLEDGMENTS

REFERENCES

1. **Albright, L. M., M. F. Yanofsky, B. Leroux, D. Ma, and E. W. Nester.** 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. J. Bacteriol. **169:**1046–1055.
2. **Barker, R., K. Idler, D. Thompson, and J. Kemp.** 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti-plasmid pTi15955. Plant Mol. Biol. **2:**335–350.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
4. **Citovsky, V., M. Wong, and P. Zambryski.** 1989. Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. Proc. Natl. Acad. Sci. USA **86:**1193–1197.
5. **Durrenberger, F., A. Crameri, B. Hohn, and Z. Koukolikova-Nicola.** 1989. Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. Proc. Natl. Acad. Sci. USA **86:**9154–9158.
6. **Garfinkel, D. J., and E. W. Nester.** 1980. *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J. Bacteriol. **144:**732–743.
7. **Ghai, J., and A. Das.** 1989. The *virD* operon of *Agrobacterium tumefaciens* Ti plasmid encodes a DNA-relaxing enzyme. Proc. Natl. Acad. Sci. USA **86:**3109–3113.
8. **Herrera-Estrella, A., Z.-M. Chen, M. Van Montagu, and K. Wang.** 1988. VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA-protein complex at the 5' terminus of T-strand molecules. EMBO J. **7:**4055–4062.
9. **Hirayama, T., T. Muranaka, H. Ohkawa, and A. Oka.** 1988. Organization and characterization of the *virCD* genes from *Agrobacterium rhizogenes*. Mol. Gen. Genet. **213:**229–237.
10. **Howard, E. A., B. A. Winsor, G. DeVos, and P. Zambryski.** 1989. Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand-protein complex: tight association of VirD2 with the 5' ends of T-strands. Proc. Natl. Acad. Sci. USA **86:**4017–4021.
11. **Jayaswal, R. K., K. Veluthambi, S. B. Gelvin, and J. L. Slightom.** 1987. Double-stranded cleavage of T-DNA and generation of single-stranded T-DNA molecules in *Escherichia coli* by a *virD*-encoded border-specific endonuclease from *Agrobacterium tumefaciens*. J. Bacteriol. **169:**5035–5045.
12. **Kanemoto, R. H., A. T. Powell, D. E. Akiyoshi, D. A. Regier, R. A. Kerstetter, E. W. Nester, M. C. Hawes, and M. P. Gordon.** 1989. Nucleotide sequence and analysis of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens*. J. Bacteriol. **171:**2506–2512.
13. **Klee, H., M. Yanofsky, and E. Nester.** 1985. Vectors for transformation of higher plants. Bio/Technology **3:**637–642.
14. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Ooms, G., P. M. Klapwijk, J. A. Poulis, and R. A. Schilperoort.** 1980. Characterization of Tn*904* insertions in octopine Ti plasmid mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **144:**82–91.
17. **Ream, W.** 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annu. Rev. Phytopathol. **27:**583–618.
18. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
19. **Sen, P., G. J. Pazour, D. Anderson, and A. Das.** 1989. Cooperative binding of *Agrobacterium tumefaciens* VirE2 protein to single-stranded DNA. J. Bacteriol. **171:**2573–2580.
20. **Soderling, T.** 1990. Protein kinases: regulation by autoinhibitory domains. J. Biol. Chem. **265:**1823–1826.
21. **Soliman, M., and A. Das.** Unpublished data.

22. Stachel, S., and E. Nester. 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5:**1445–1454.

23. Stachel, S., B. Timmerman, and P. Zambryski. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature (London) **322:**706–712.

24. Stachel, S., and P. Zambryski. 1986. *vir*A and *vir*G control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46:**325–333.

25. Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.

26. Wang, K., A. Herrera-Estrella, and M. Van Montagu. 1990. Overexpression of *virD1* and *virD2* genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. J. Bacteriol. **172:**4432–4440.

27. Wang, K., S. E. Stachel, B. Timmerman, M. Van Montagu, and P. C. Zambryski. 1987. Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium vir* gene expression. Science **235:**587–591.

28. Ward, E., and W. Barnes. 1988. VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5' end of T-strand DNA. Science **242:**927–930.

29. Yanofsky, M., S. Porter, C. Young, L. Albright, M. Gordon, and E. Nester. 1986. The *vir*D operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. Cell **47:**471–477.

30. Young, C., and E. W. Nester. 1988. Association of the VirD2 protein with the 5' end of T strands in *Agrobacterium tumefaciens*. J. Bacteriol. **170:**3367–3374.

31. Zambryski, P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. **22:**1–30.