Mutational analysis of a conserved motif of *Agrobacterium tumefaciens* VirD2

Ann M. Vogel¹, Joonhyun Yoon¹ and Anath Das^{1,2*}

¹Department of Biochemistry and ²Plant Molecular Genetics Institute, University of Minnesota, 1479 Gortner Avenue, St Paul, MN 55108, USA

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ABSTRACT

The VirD2 polypeptide from *Agrobacterium tumefaciens*, in the presence of VirD1, introduces a site- and strand-specific nick at the T-DNA borders. A similar reaction at the origin of transfer (*oriT*) of plasmids is essential for plasmid transfer by bacterial conjugation. A comparison of protein sequences of VirD2 and its functional homologs in bacterial conjugation and in rolling circle replication revealed that they share a conserved 14 residue segment, HxDxxx(P/u)HuHuuux [residues 126–139 of VirD2; Ilyina, T.V. and Koonin, E.V. (1992) *Nucleic Acids Res.* 20, 3279–3285]. A mutational approach was used to test the role of these residues in the endonuclease activity of VirD2. The results demonstrated that the two invariant histidine residues (H133 and H135) are essential for activity. Mutations at three sites, histidine 126, aspartic acid 128 and aspartic acid 130, that are conserved in a subfamily of the plasmid mobilization proteins, led to the loss of VirD2 activity. Aspartic acid at position 130, could be substituted with glutamic acid and to a much lesser extent, with tyrosine. In contrast, another conserved residue, asparagine 139, tolerated many different amino acid substitutions. The non-conserved residues, arginine 129, proline 132 and leucine 134, were also found to be important for function. Isolation of null mutations that map throughout this conserved domain confirm the hypothesis that this region is essential for function.

INTRODUCTION

Agrobacterium tumefaciens, a gram negative soil bacterium, transfers DNA to most plants causing crown gall disease (2,3). The transferred DNA (T-DNA) resides on a large plasmid, is processed within the bacterium and is exported to the plant where it is ultimately integrated into the chromosome. DNA transfer requires plasmid encoded virulence (*vir*) genes as well as several chromosomal genes. Of the six major *vir* loci, the *virD* operon encodes three essential virulence genes, *virD1*, *virD2* and *virD4* (4–7). VirD1 and VirD2 recognize 24 base pair imperfect direct repeat sequences called the border sequences. These *cis*-acting sequences define the T-DNA and are the only T-DNA encoded

element essential for DNA transfer (8). In the presence of VirD1, VirD2 introduces a nick in the border sequence in a site- and strand-specific manner and subsequently becomes covalently attached to the 5' end of the nicked DNA (9–13). The nicked strand is then displaced from the plasmid by an unknown mechanism producing single-stranded T-strand DNA. In addition to the covalently attached VirD2 molecule at the 5' terminus, the T-strand DNA is coated with the single-stranded DNA-binding protein, VirE2 (14,15). This T-strand DNA–protein complex is believed to be transferred to the plant through a pore structure which is thought to contain VirD4 and the VirB proteins (16,17).

The transfer of genetic material from bacteria to plants appears similar to plasmid transfer in bacterial conjugation. In fact, the VirB proteins and VirD4 allow conjugal transfer of plasmid DNA between two *A.tumefaciens* strains in a *vir*-dependent manner (16). Several of these proteins are homologous to the proteins that are essential for conjugal transfer of plasmids, for example, the Tra proteins of plasmid pRP4 (18–20). In addition, the DNA sequence of the initial DNA processing sites of the Ti plasmids and plasmid pRP4, the T-DNA border sequences and the *oriT*, are also conserved (21,22). In both systems, two proteins, VirD1–VirD2 of the Ti plasmid and TraJ–TraI of plasmid pRP4, are the enzymes that initiate DNA processing by introducing a site- and strand-specific nick in the target DNA (10,23). *In vitro*, VirD2 and its equivalents in bacterial conjugation catalyze nicking of a single-stranded DNA substrate (24–26) and VirD1–VirD2 and their equivalents together catalyze nicking of supercoiled plasmid DNA (27,28). The reactions catalyzed by the proteins in both systems are very similar, however, only limited homology is found in the primary sequence of the proteins. The VirD1 group of proteins share a 15 residue segment that probably constitutes a DNA-binding domain (29,30). In the VirD2 group of proteins, a tyrosine residue near the N-terminus participates in a covalent protein–DNA complex (24,31). Significant homology is found only in a 14 residue segment that has the consensus sequence HxDxxx(P/u)HuHuuux (x, any amino acid; u, hydrophobic residue; 1). Since DNA processing in T-DNA transfer and in bacterial conjugation appears to be similar, it is plausible that the reaction mechanism may have been conserved in these systems. In that event, the conserved residues are likely to be functionally important. To test this hypothesis we used a targeted random mutagenesis approach to determine the functional requirement of these residues. Our results demonstrate that this

* To whom correspondence should be addressed

Figure 1. Physical map of pTiA6 T_L-DNA from pAD1201. RB, right border; S, *SstI* restriction site; *, primer 13886 binding site; arrows, the direction of primer extension; numbers, size of products expected in nucleotides.

domain is essential for the endonuclease function of VirD2. The invariant residues as well as the majority of partially conserved and non-conserved residues within this region were found to be important.

MATERIALS AND METHODS

Mutations in *virD2* of plasmid pAD1244 (31) were introduced by site-directed mutagenesis according to Kunkel (32). Plasmid pAD1244 contains VirD1 and VirD2 under the control of the *Escherichia coli lac* promoter. Mutagenic oligonucleotides were synthesized under two conditions. 'Doped' oligonucleotides were synthesized (Microchemical Facility, University of Minnesota) with a small percentage of each of the non-template bases (at all positions of the oligonucleotide) to create on average, a single base alteration per primer (33,34). A second set of oligonucleotides (25mer) containing 'NNC/G' as the central codon was used to alter a specific amino acid to a random one. In the latter, the use of only two bases at the third position reduces the total population of oligonucleotides in half but still maintains the potential to generate all possible amino acids at the site of mutation. Following the mutagenesis reaction, the DNA was transformed into *E. coli* strain AD256 (35) harboring the plasmid pAD1201. Plasmid pAD1201 contains a T-DNA border sequence which is the substrate for the nicking reaction catalyzed by VirD1 and VirD2 (31).

Escherichia coli cells were grown in LB media (Bio101, Inc.; Vista, CA) to an OD₆₀₀ of ~0.5, induced for 4 h with 0.1 mM IPTG, chilled and harvested. Total DNA was isolated and analyzed by primer extension assays as previously described (31). Briefly, total DNA was digested with the restriction endonuclease *SstI*, mixed with a ³²P-labeled primer (RB13886; 0.5 ng), denatured by boiling for 3 min and annealed for 15 min at 37°C. Following the addition of accessory components and Sequenase (USBC), the extension reaction was incubated at 37°C for 20 min. The reactions were terminated by the addition of formamide and denatured by boiling for 2 min. The samples were electrophoresed on a 6% denaturing polyacrylamide-urea gel, the gel was dried and exposed to X-ray film. When the T_L-DNA right border is nicked, the extension reaction would terminate at the nick site generating a product of 176 nucleotides. When the T-DNA border is intact, termination would occur at the *SstI* site yielding a 201 nucleotide product (Fig. 1).

The level of VirD2 was monitored by Western blot assays. Total proteins were separated by SDS-PAGE, transferred to Immobilon P membrane (Millipore), and the blot was probed with a rabbit polyclonal antibody raised against a TrpE-VirD2 fusion protein.

Consensus			H x D x x x P/u H u H u u u x
1.	pTiA6 (residues 126-139)	VirD2	H V D R D H P H L H V V V N
2.	pTic58	VirD2	H I D R D H P H L H V V V N
3.	pRiA4	VirD2	H I D R D H P H L H V V V N
4.	pRP4	TraI	H N D T D N L H I H I A I N
5.	R751	TraI	H H D T D N L H I H I A I N
6.	R64	NikB	H T D T D N L H V H V A V N
7.	pTF-FC2	MobA	H A D T D N L H L H L A I N
8.	pS194	Rlx	H T D K D H Y H N H I I I N
9.	pC223	Rlx	H N D T D H V H N H I V I N
10.	pC221	Rlx	H T D K D H Y H N H I V I N
11.	pMV158	Mob	H L D E S T P H M H M G V V
12.	pGI2	Mob	H M D E A T P H M H I G V M
13.	pE194	Mob	H L D E R V P H M H F G F V
14.	pLAB1000	Mob	H M D E K T P H M H M G I V
15.	pT181	Mob	H M D E K T P H M H Y G V V
16.	pT913	Mob	H V D E K T P H M H L G V V
17.	pUB110	Mob	H N D E Q T P H M H L G V V
18.	pTX14-3	Mob	H D D E A N P H L H I N Y V

Figure 2. A conserved motif in *Agrobacterium* VirD2s, conjugation and mobilization proteins. Primary sequence of a conserved region in *Agrobacterium* VirD2s and their functional homologs involved in conjugal transfer of plasmids and plasmid mobilization is shown. Lines 1-3, *Agrobacterium* VirD2s; 4-7, Mob proteins of IncP and IncI plasmids; 8-10, relaxase proteins of pT181 family; 11-18, Mob proteins of gram positive bacterial ssDNA plasmids. Data shown is adapted from Ilyina and Koonin (1).

Alkaline phosphatase or horseradish peroxidase conjugated secondary antibody was used for detection.

RESULTS

Agrobacterium tumefaciens VirD2, as well as its functional homologs involved in mobilization and conjugation, contain a 14 amino acid segment that contains a HxDxxx(P/u)HuHuuux motif (x, any residue; u, bulky hydrophobic) (Fig. 2; ref. 1). Invariant are the latter two histidines that are conserved in over 82 proteins which are involved in initiation of rolling circle replication, plasmid conjugation and T-DNA transfer (1). Because of the strong conservation of the histidine residues and of the sequence motif, it is very likely that this motif is important for function.

To better define this region, we undertook a targeted random mutagenesis approach on this domain of VirD2 (residues 126-139) to theoretically generate any amino acid substitution at each position. Mutagenic primers were synthesized in the presence of a small percentage of wrong bases at each position ('doped' oligonucleotides; 33,34). The percentage of contaminating bases was calculated using the binomial distribution equation. This calculation predicts the probability of incorporating one base change within the oligonucleotide as a function of the size of the oligonucleotide as well as the percentage of wrong

bases in the synthesis reaction. While this method has been successfully used before without a bias in the position of mutations generated within the oligonucleotide (end versus middle; 34), we found a bias for mutations within the middle of the oligonucleotide. Additionally, we found the theoretically predicted percentage of wrong bases was insufficient to generate the desired frequency of mutations. To circumvent this problem, we used oligonucleotides 'doped' at 12 positions and anchored on both sides by 10 wild type residues to stabilize annealing. To generate a single mutation within a 12 bp sequence, the theoretical calculation predicted that the use of 90% wild type nucleotides at each position would give maximal percentages of mutants with only one change. However, we needed to use 75% wild type nucleotides with the remainder composed of equal percentages of the wrong bases. Additionally, to reduce the total number of wild type plasmids recovered, a restriction modification screen (addition or deletion of a restriction site that did not affect the amino acid sequence) was incorporated into the oligonucleotide to facilitate the identification of a potential mutant by restriction enzyme digestion.

Following an initial screening by restriction endonuclease digestion, DNA sequences of the putative mutants were analyzed to identify the site(s) of mutation, if any. The sequence results showed that 20% of the plasmids encoded wild type VirD2 (including degenerate changes), 40% had one codon change and 20% had two or more mutations. The remaining 20% were duplicates of previous mutations. Mutants that contained only a single codon change were saved for further analysis. These mutagenesis reactions still resulted in a higher frequency of mutation at some positions than at others. Therefore, other primers were designed to change a specific amino acid in order to increase the number of positions tested. All strains were checked for stable protein production by Western blot analyses using an anti-VirD2 antibody (data not shown). All the mutants studied encoded protein at least 75–100% of wild type levels, except where noted. The endonuclease activity of the mutant VirD2 proteins was quantified by using a primer extension assay as described earlier (31).

Within the conserved motif of the DNA replication and relaxation proteins, two histidines (at positions 133 and 135 of VirD2) are invariant. Of the other residues in this motif, histidine 126 and aspartic acid 128 are conserved in a number of mobilization proteins, aspartic acid 130 and asparagine 139 are conserved in a smaller subset of the mobilization proteins, proline 132 is conserved in a different subset of these proteins and in all cases, hydrophobic residues occupy positions 136–138 (Fig. 2). Of these residues, a mutation of histidine 133 to glutamine, leucine or proline destroyed VirD2's nicking activity indicating the indispensable nature of this residue (Fig. 3). While the levels of protein in VirD2 H133Q strain were comparable to that in wild type, the levels of protein in VirD2 H133L and VirD2 H133P strains were lower than that in wild type (~10–20%). At histidine 135, a change to proline, glutamine, arginine or valine all destroyed the activity of the protein.

A substitution of histidine 126 to proline or arginine, as well as that of aspartic acid 128 to alanine or glycine, abolished nicking activity. Of the other two conserved residues, aspartic acid 130 could be altered to glutamic acid but not to arginine, asparagine or valine. A tyrosine at position 130 was tolerated, but led to a dramatic reduction in activity. The only mutation which retained wild type nicking activity at this position was the substitution to

Figure 3. Assay of VirD2 mutants. VirD2 activity was assayed using a primer extension analysis on *SsrI* digested DNA templates. WT, wild type VirD2; letters, single letter amino acid abbreviations; numbers indicate position of residue within VirD2 polypeptide; letters below are wild type amino acids at that position, letters above are amino acid substitutions at that position. The extension product from a nick at the T-DNA border is indicated by an arrowhead.

another acidic residue, glutamic acid, indicating that the carboxyl side chain of residue 130 is essential for VirD2 activity. The last conserved residue, asparagine 139, was found to be the most amenable to substitution. Alteration of asparagine 139 to glycine, histidine, leucine, serine, threonine or valine had little or no effect on enzyme activity. A change to proline substantially reduced activity and only a change to lysine destroyed VirD2 activity.

Three residues following the HuH sequence (at positions 136–138 in VirD2) are not conserved in the DNA-relaxing proteins, but are predominantly hydrophobic ones (valine, isoleucine, leucine, alanine and glycine). In VirD2, positions 136–138 contain valine residues. A substitution of valine 136 to alanine, glycine or serine and that of valine 138 to alanine, cysteine, isoleucine or serine did not affect nicking activity. However, a change to asparagine or proline at position 136 led to a severe reduction or a complete loss of activity, respectively. At position 138, substitution of valine to proline led to a reduction in border nicking activity, whereas that to a leucine or asparagine led to a complete loss of nicking activity. The loss of activity in VirD2 V138L is unexpected since a leucine at this position fits the consensus sequence. However, none of the proteins in the T-DNA transfer and plasmid mobilization subfamily contain a leucine at that position.

The use of random mutagenesis allowed us to isolate mutations in four other positions of VirD2: valine 127, arginine 129, proline 132 and leucine 134. Of these positions, valine 127 and arginine 129 are not conserved; proline 132 is conserved in a subset of mobilization proteins and position 134 contains a hydrophobic residue. One mutant at position 127, VirD2 V127A, was obtained in this study and the protein was found to be enzymatically active, although less active than wild type, indicating that this position can tolerate a substitution. However, mutations at the other three positions had a drastic negative effect. Alteration of arginine 129 to leucine or another basic amino acid, histidine, led to almost complete loss of activity. Substitution of proline 132 to glutamine destroyed the nicking activity. This loss of activity may be due to a significant change in secondary structure resulting from the removal of the structure breaking proline residue. Leucine at position 134 lies between the two invariant histidines (positions 133 and 135 of VirD2). Changes at this position to glutamic acid, isoleucine, arginine or serine resulted in an inactive protein. However, strains with the latter three mutations (VirD2 L134I,R,S) contained less VirD2 (~10–20% of wild type) and therefore, the loss of activity could be due to either the inability of the protein to function and/or a lower level of the protein.

DISCUSSION

DNA transfer from *A.tumefaciens* to plants as well as that between two bacteria appears to have several similar characteristics. The *vir*, *tra* and *mob* regions all encode two proteins that are involved in the initiation of production of a single-stranded DNA molecule, the transfer intermediate. Additional proteins contribute to the stability and/or efficiency of this step, and many other proteins are involved in the subsequent steps of DNA transfer. Functional similarities are obvious in the proteins that carry out the nucleophilic attack on the DNA backbone. For example, VirD2 and TraI both utilize a tyrosine residue as the nucleophile and remain covalently attached to the 5' end of the nicked DNA (reviewed in ref. 20).

A comparison of the primary sequences of these proteins revealed that one domain is conserved in many replication initiation and mobilization proteins (1). Our mutational analysis of this region allows the identification of important amino acid residues. Since the mutations created are random, some will be more or less similar to the naturally occurring amino acid. Therefore inactivation of function could be due to the loss of a functional group, incorrect interactions generated from the new group or changes in higher order structure resultant from the change in the side chain of the new amino acid.

The results revealed that the majority of residues in this domain are extremely important for the endonuclease activity of VirD2. Mutations in all of the conserved residues except for asparagine 139 led to a loss of function. Additionally, mutations at three non-conserved residues, arginine 129, proline 132 and leucine 134, led to a drastic reduction or a complete loss of the nicking function. Therefore, the region encompassing residues 126–139 is necessary for VirD2 activity and provides an essential function(s).

In vitro VirD2 requires magnesium for its activity (25,26,28). It has been proposed that this conserved region provides a metal binding site (1,36). Magnesium ions have been shown to be bound to, in addition to water, carboxylate side chains (aspartic acid and glutamic acid) or nitrogen containing residues (histidine)

and to a lesser extent to asparagine, glutamine, serine or threonine residues (37). Since magnesium binds with a high coordination number, the bound residues are usually located at a break in structure or near the end of a helix. Additionally, a typical feature of a protein that utilizes histidine as a metal ligand is the presence of a proline within two residues of the ligand (38). Consistent with this, an essential proline residue is located in the middle of this domain (position 132). A Chou–Fasman analysis of the VirD2 secondary structure predicts a turn in this region, compatible with the idea that this region could provide the metal binding site (data not shown). Although not all related proteins contain a proline at this position, other combinations of amino acids could provide the secondary structure of a turn.

Ilyina and Koonin (1) hypothesized that the second half of the conserved motif, HuHuuux (x, any amino acid; u, hydrophobic amino acid), functions as a metal binding motif (1). This region is found in the rolling circle replication initiator proteins, cytochrome c oxidase, carbonic anhydrase and other metalloenzymes. The histidines are absolutely conserved in the family of virulence, conjugation and mobilization proteins. In several metalloenzymes the role of one of the two histidines in metal binding has been demonstrated (1). Both histidines (133 and 135) are absolutely necessary for VirD2 function. In contrast, in pRP4 TraI, the first conserved histidine of this domain (histidine 116) is essential for *oriT* nicking, whereas a mutation of the second histidine reduced nicking to 45% (36).

In VirD2, two substitutions at position 133 (H133L and H133P) and all the substitutions of leucine 134, a non-conserved residue flanked by the two conserved histidines, resulted in the loss of nicking activity and led to a low steady state level of the mutant proteins. The low level of protein may be due to instability resulting from structural changes or that resulting from a loss of metal binding. While the leucine would not be a metal ligand, it may contribute to the stability of the bound ligand. This conclusion implies that histidine 133 is a metal ligand and is influenced by leucine 134.

A study on pRP4 TraI proposed that a 'DTD' sequence (equivalent sequence in VirD2 is 'DRD' at positions 128–130) in the conserved motif serves as a ligand for magnesium (36). A similar domain is found in DNA-dependent DNA polymerases (39). The first aspartic acid of DTD is proposed to bind to magnesium. Mutational analysis of TraI showed that an aspartic acid to asparagine substitution of the TraI D111 and TraI D113 had no effect on TraI activity. From these results, the authors suggested that the corresponding amides may allow coordination of Mg^{+2} . However, in the case of VirD2, VirD2 D130N was inactive, although VirD2 D130E was fully functional (Fig. 3) indicating that the carboxylic acid side chain of residue 130 of VirD2 is essential and this residue can not tolerate an amide or a hydrophobic side chain. The role of the carboxylic acid side chain in VirD2 activity is not clear at present.

Overall, our study on VirD2 and a similar study on TraI (36) allow a functional analysis of the proposed conserved motif. Surprisingly, while most positions of VirD2 were found to be important for activity, substitutions of equivalent positions of TraI did not affect enzyme activity. For example, histidine 126, asparagine 128 and aspartic acid 130 are all required for VirD2 activity but the corresponding residues in TraI could be substituted without negatively affecting TraI activity. However, only one of the substitutions in the two studies included the exact same mutation, VirD2 D130N and TraI D113N. A mutation in one of

the invariant histidines, 135 of VirD2 or 118 of TraI, led to a complete loss or an intermediate level of activity, respectively. Mutations in only two residues appeared to have a similar effect in both proteins. While substitutions of histidine 133 of VirD2 and histidine 116 of TraI inactivated both proteins, a number of substitutions of asparagine 139 of VirD2 and asparagine 122 of TraI did not affect the activity of either protein. One explanation for the discrepancy in the two studies is that in most cases, the mutations obtained at the corresponding positions were different. Alternatively, while the DNA substrates that these proteins act upon are similar, neither of these proteins in each system are interchangeable. Therefore, the specific mechanism utilized by the proteins in the two systems may differ which could explain the differences in amino acids tolerated at particular positions. Crystal structures of these proteins would be extremely useful in deciphering the mechanistic aspects of the nicking reaction.

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