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Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: Implications for the T-DNA transfer process

(single-stranded DNA-binding protein/T-DNA transfer complex/VirE2 protein)

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ABSTRACT **Induction of *Agrobacterium tumefaciens vir* gene expression by wounded plant cells results in production of a free transferable single-stranded (ss) copy of T-DNA, the T-strand. One of the Vir proteins, the VirE2 polypeptide, is a ssDNA-binding protein. In the present work, interaction of nopaline-specific VirE2 protein (M_r 69,000) with ssDNA was studied by using nitrocellulose filter binding, gel retardation, and electron microscopy techniques. The VirE2 protein was found to bind to ssDNA molecules with strong cooperativity, forming VirE2–ssDNA complexes with a binding site of 28–30 nucleotides. The VirE2–ssDNA complexes are stable at high salt concentrations and resistant to exonucleolytic activity. When examined under the electron microscope, the VirE2 protein converted collapsed free ssDNA molecules into unfolded and extended structures. The structure and properties of VirE2–ssDNA complexes predict possible functions in *Agrobacterium* virulence to (*i*) protect the T-strands from cellular nucleases and (*ii*) facilitate transfer of the T-strands through bacterial membranes possibly by specific interaction with putative membrane pores formed in plant-induced *Agrobacterium* cells.**

Proteins capable of preferential binding to single-stranded DNA (ssDNA) sequences play an essential role in DNA metabolism. One class of ssDNA-binding proteins (SSBs) is those with enzymatic activities, such as RNA polymerase and RecA protein. The latter, for example, is a DNA-dependent ATPase that regulates an inducible pathway of DNA repair via its proteolytic activity (1). RecA also promotes pairing of ssDNA (or partially single stranded) with homologous duplex DNA (2). Homologous recombination in *Escherichia coli* is dependent on another SSB, *E. coli* SSB, which serves as an assembly site for RecA protein in its polymerization along ssDNA (3). *E. coli* SSB belongs to the class of so-called "true" SSBs, known also as helix-destabilizing proteins (4). The most-studied SSBs are *E. coli* SSB, T4 bacteriophage gene32 protein, and fd bacteriophage gene5 protein. Common properties of these proteins are (*i*) lack of enzymatic activity, (*ii*) binding to ssDNA without sequence specificity and in preference to double-stranded DNA (dsDNA), (*iii*) requirement in stoichiometric rather than catalytic amounts, and (*iv*) cooperative binding to ssDNA.

E. coli SSB protects ssDNA from nucleolytic digestion, is required for DNA recombination and repair, and stimulates *E. coli* DNA polymerases (4). The ssDNA-binding properties of *E. coli* SSB are complex. This protein exists as a stable tetramer over a wide range of salt concentrations (5) and has been shown to possess two binding modes that differ by the number of nucleotides occluded upon DNA binding. At high binding densities—i.e., high protein–ssDNA mass ratios—and low salt concentrations a smooth-contoured complex is formed with a binding site of 33–36 nucleotides per SSB tetramer (5). Under other conditions, low binding densities and/or high salt concentrations, a complex with beaded morphology and a binding site of 60–65 nucleotides per SSB tetramer is observed (5, 6). Each "bead" is composed of two SSB tetramers, and beads are interspersed by 30 nucleotides of protein-free ssDNA (6).

Gene32 protein of T4 bacteriophage is also essential for DNA replication, recombination, and repair processes (4). Binding of T4 gene32 protein to ssDNA is highly cooperative and always results in formation of smoothly contoured protein–ssDNA complexes with a binding site of 6–8 nucleotides per protein molecule (4, 7, 8). Though *E. coli* SSB and T4 gene32 proteins are required for DNA replication, gene5 protein of filamentous bacteriophages has been proposed to act as a repressor of complementary strand synthesis (9). Tight and cooperative binding (10) of gene5 protein to newly synthesized ssDNA phage molecules prevents conversion to replicative form template and initiates phage assembly (9).

Recently a ssDNA-binding protein was also shown to be involved in a unique process of transfer of genetic information from *Agrobacterium tumefaciens* to plant cells (11–14). *Agrobacterium* genetically transforms plants by transferring a specific segment, the T-DNA, from its Ti plasmid to plant genome. The T-DNA is a cis-acting element that does not itself encode products essential for the transfer (reviewed in ref. 15). Only the ends of the T-DNA segment—i.e., 25-base-pair (bp) direct repeat border sequences—are recognized during its mobilization from the Ti plasmid. The trans-acting functions that mediate transfer are encoded by the Ti plasmid virulence (*vir*) region. This region is organized into six loci, *virA*, *virB*, *virC*, *virD*, *virE*, and *virG* (16), and its transcription is specifically activated by phenolic compounds secreted from wounded plant cells (17). Following plant-induced transcriptional activation of the *vir* region, a free transferable copy of the T-DNA, identified as a ssDNA molecule—the T-strand, is mobilized from the Ti plasmid (18, 19). We (12) and others (11, 13, 14) have shown that the largest open reading frame of the *virE* locus, *virE2*, encodes a ssDNA-binding protein. Earlier studies suggested that protein products of *virE* locus may be secreted extracellularly by plant-induced *Agrobacterium*, since insertional mutants of this locus can be complemented by coinoculation with bacteria harboring a wild-type Ti plasmid (20). These observations indicate that the VirE2 protein may function outside the bacterial cell potentially forming complexes with the ssDNA transfer intermediate, the T-strand. In fact, it is conceivable that VirE2 participates directly in transfer of T-strands from *Agrobacterium* into plant cells. Thus, characterization of VirE2–ssDNA complexes is likely to provide new insights regarding this process.

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, ssDNA-binding protein.

In the present work, filter binding and gel retardation assays were utilized to characterize the mode of VirE2 binding to ssDNA molecules. Electron microscopy studies also revealed that VirE2 protein converts collapsed free ssDNA molecules into thin and extended structures. Such VirE2–ssDNA complexes are insensitive to exonuclease VII and S1 nuclease activities. Strong and cooperative binding of VirE2 to ssDNA suggests that in *Agrobacterium* the T-strands exist as VirE2 protein-coated rather than free molecules. Based on the properties of VirE2–ssDNA complexes, we propose that the T-strands are transferred from *Agrobacterium* into plant cells as protein–ssDNA complexes and that VirE2 molecules are a part of this transfer structure.

MATERIALS AND METHODS

Purification of VirE2 Protein. The nopaline VirE2 coding sequence, cloned in the T7 RNA polymerase expression vector pET3b (12), was transformed into *E. coli* BL21(DE3) cells that carry the T7 RNA polymerase gene (under the *lac*UV5 promoter) integrated into the bacterial chromosome (21). For overexpression of VirE2 protein, 500 ml of cells (OD = 0.6) was induced with 0.4 mM isopropyl β-D-thiogalactoside for 1 hr at 37°C, centrifuged (10,000 × *g* for 5 min at 4°C), resuspended in 3 ml of lysis buffer (10 mM Tris·HCl, pH 8.0/200 mM NaCl/10 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride/10% glycerol), and disrupted in a French press minicell at 20,000 psi (1 psi = 6.89 kPa). The lysate was passed through a 26-gauge needle to shear DNA and centrifuged at 10,000 × *g* for 5 min at 4°C. The overproduced VirE2 protein occurred mainly in an insoluble fraction after lysis. The final pellet fraction was washed four times in lysis buffer containing 1 M NaCl, resuspended in the same buffer with 2.5 M urea, and centrifuged (25,000 × *g* for 15 min at 4°C), and the supernatant was dialyzed against lysis buffer. This procedure solubilizes about 50% of the overproduced protein and yields 100–200 μg of soluble VirE2 per 10^9 *E. coli* cells. The partly purified VirE2 preparation was diluted 1:1 with Laemmli sample buffer and electrophoresed (without preboiling) on a preparative 7.5% polyacrylamide/SDS gel. The M_r 69,000 band of VirE2 protein was excised from the gel, electroeluted in TBE buffer (90 mM Tris base/90 mM boric acid/20 mM EDTA), dialyzed in lysis buffer, and stored at −70°C until use.

***In Vivo* Labeling of M13mp7 DNA.** *E. coli* JM107 cells (OD = 0.6) were infected with M13mp7 (multiplicity of infection = 10) and grown for 12 hr at 37°C in 50 ml of low phosphate medium (22) in the presence of 2.5 mCi (1 Ci = 37 GBq) of $^{32}P_i$. The cells were centrifuged (10,000 × *g* for 10 min at 4°C), the phage particles were precipitated with polyethylene glycol, and ^{32}P-labeled ssDNA of M13mp7 (specific activity of 5 × 10^6 cpm/μg of DNA) was isolated (23, 24). Circular M13mp7 DNA was linearized following digestion with *Bam*HI.

Nitrocellulose Filter Binding Assay. Nitrocellulose filters (0.45 μm, Whatman) were pretreated by consecutive 20-min washes in each of the following: 0.5 M NaOH, distilled water, and 0.5 M Tris·HCl (pH 8.0). For binding experiments, the indicated amounts of either VirE2 protein or *E. coli* SSB (United States Biochemical) were incubated at 0°C for 15 min (or as indicated) with 10 ng of ^{32}P-labeled M13mp7 ssDNA in 20 μl of buffer B (10 mM Tris·HCl, pH 8.0/10% glycerol and either 200 mM NaCl for experiments with VirE2 or 50 mM NaCl for experiments with *E. coli* SSB). After incubation, the samples were applied to the pretreated filters and washed with 20 ml of buffer B at a flow rate of 5 ml/min.

Retardation of Gel Electrophoresis. VirE2 or *E. coli* SSB was incubated in 20 μl of buffer B at 0°C for 15 min with 1.0 ng of heat-denatured probe DNA [587-bp *Hae* III fragment of pUC18 blunt end-labeled (23) to a specific activity of 2 × 10^7 cpm/μg]. At the end of the incubation period the samples were loaded onto a 4% native polyacrylamide gel and electrophoresed as described (12).

Electron Microscopy. M13mp7 ssDNA and M13mp7 ssDNA–VirE2 complexes were prepared for electron microscopic visualization using two basic techniques. For direct platinum shadowing, samples in buffer B were applied for 1 min to a glow discharged carbon-coated grid and shadowed with platinum at an angle of 8°. For spreading on cytochrome films, the protein–ssDNA binding was performed in buffer B with Tris·HCl substituted for sodium phosphate (10 mM, pH 8.0). The samples were fixed with 1% glutaraldehyde for 10 min at 37°C and spread with cytochrome *c* as described (24), and the grid was shadowed with platinum as above. Pictures were taken with a magnification of 33,000× at 80 kV.

RESULTS

Binding of VirE2 Protein to ssDNA Is Highly Cooperative. VirE2 protein has been shown to preferentially bind ssDNA without sequence specificity (11–14). ssDNA of M13mp7 (25), which is readily available and can be easily linearized by restriction endonucleases, was therefore utilized as a probe for VirE2–ssDNA interactions. A filter binding assay was used to determine the maximal amount of VirE2 protein capable of binding to M13mp7 ssDNA. Nopaline Ti plasmid-specific VirE2 polypeptide was titrated in the presence of a constant limiting concentration of either circular or linearized radioactively labeled M13mp7 ssDNA. Binding curves for both DNAs (Fig. 1*A*) showed similar weight ratios of 7:1 for VirE2:ssDNA at saturation, corresponding to an apparent stoichiometry of 30 nucleotides per VirE2 monomer. Control experiments using SSB of *E. coli* (Fig. 1*B*) revealed a 36 nucleotides/SSB tetramer stoichiometry corresponding to a protein:ssDNA weight ratio of 6:1 at saturation. These latter results agree with existing data, since under the conditions used here—i.e., saturating amounts of SSB, low salt concentration (50 mM NaCl), and absence of Mg^{2+} ions—a

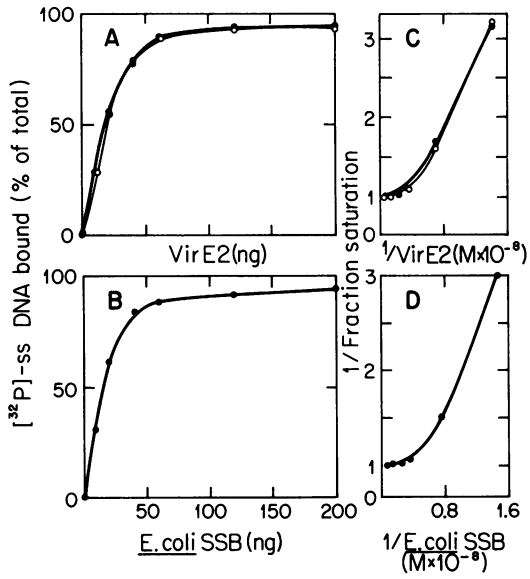

FIG. 1. Saturation of limiting ssDNA by increasing amounts of VirE2 protein or *E. coli* SSB. Ten nanograms of radioactively labeled circular (○) or linearized (●) M13mp7 ssDNA was incubated with the indicated amounts of VirE2 protein (*A* and *C*) or *E. coli* SSB (*B* and *D*) and the extent of protein–ssDNA binding was determined by using nitrocellulose filter binding. (*A* and *B*) Saturation binding curves. (*C* and *D*) Double reciprocal plots derived from the binding data shown in *A* and *B*, respectively. Each point represents the average from three different experiments.

smooth contoured binding mode with 33–36 nucleotides/SSB tetramer binding site has been observed for the *E. coli* SSB (5). Further precise analysis of VirE2 binding parameters requires direct measurements of free ligand (protein) concentrations. The filter binding assay is not suitable for this purpose, since this technique is based on irreversible binding of proteins to nitrocellulose. However, a general mode of VirE2–ssDNA interaction can be evaluated from double reciprocal plots of the binding data from Fig. 1 *A* and *B*.

SSBs can bind any ssDNA with equal affinity (4, 26). As such, ssDNA presents a continuous lattice of potential protein-binding sites, rather than individual discrete and isolated binding sequences. Since every nucleotide can initiate attachment of SSB to DNA, noncooperatively bound SSB molecules will be randomly distributed on the DNA lattice (26). Due to steric interference, randomly bound SSB will impair attachment of additional molecules to some of the still unoccupied regions of the ssDNA. This type of binding behavior is characteristic for negative cooperativity and results in nonlinear concave double reciprocal plots of binding data (26, 27). Positive SSB–SSB protein cooperativity antagonizes this nonlinearity and leads to plots of the opposite convex curvature (26, 27). The results in Fig. 1*C* show the double reciprocal plot for VirE2–ssDNA binding. The plot curvature is convex, indicating a positively cooperative mode of binding. Similar results were obtained in control experiments for binding of *E. coli* SSB (see Fig. 1*D*), which exhibits positive cooperativity at saturation binding densities (4, 5).

Further support to a cooperative mode of VirE2–ssDNA binding is derived from gel retardation experiments. The results in Fig. 2*A* show that increasing the VirE2–ssDNA weight ratio results in a sharp transition from free, nonretarded ssDNA to protein bound, strongly retarded probe. This change from zero to essentially complete protein–ssDNA binding occurs over a narrow range of VirE2 concentration and indicates a highly cooperative mode of protein binding. VirE2 binding cooperativity appears to be even stronger—namely, to exhibit a closer to "all or none" behavior—than *E. coli* SSB (Fig. 2*B*). Distinct bands of partially retarded ssDNA, which appear at subsaturating concentrations of *E. coli* SSB (Fig. 2*B*), represent partly coated ssDNA probe molecules. This result may reflect different binding modes for *E. coli* SSB depending on the protein-binding density (5, 6).

The gel retardation experiments shown in Fig. 2 also define the minimum protein:DNA weight ratios needed for complete binding: 7.5:1 for VirE2–ssDNA and 6.5:1 for *E. coli* SSB–ssDNA complexes. The size of binding sites expected from these ratios will average to 28 nucleotides for nopaline VirE2 and 34 nucleotides for *E. coli* SSB tetramer. This confirms the sizes of the binding sites derived from filter binding experiments (Fig. 1).

Visualization of VirE2–ssDNA Complexes. When VirE2 binding to ssDNA was examined under the electron microscope, dramatic changes in DNA conformation were observed. Fig. 3*A* shows that free ssDNA molecules of M13mp7 appear as folded and collapsed structures. These disappear upon addition of VirE2 polypeptide, indicating formation of protein–ssDNA complexes. These putative complexes, however, could not be readily visualized by using direct platinum shadowing, and only careful examination reveals contours of unfolded DNA molecules (Fig. 3*B*) in an otherwise seemingly

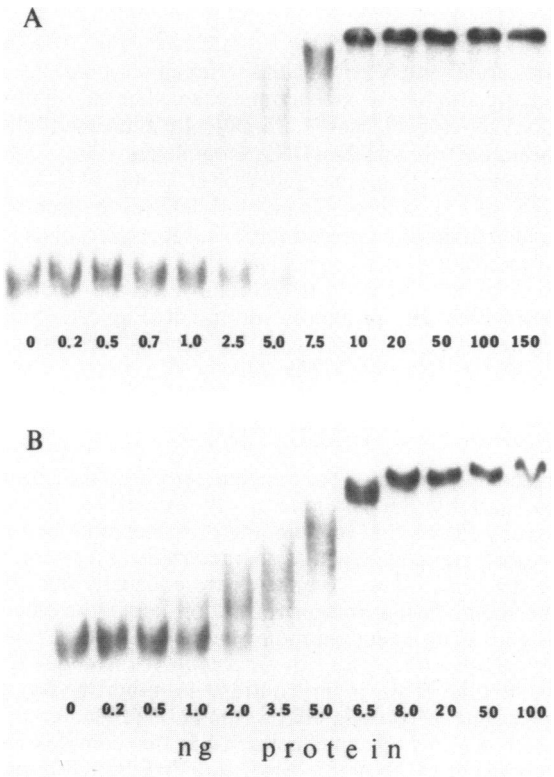

FIG. 2. Cooperative binding of ssDNA probe by VirE2 protein or *E. coli* SSB as assayed by gel retardation. Increasing amounts of protein were incubated with 1.0 ng of ^{32}P-labeled heat-denatured probe DNA and protein–ssDNA complexes were resolved by using gel retardation. (*A*) Binding of VirE2 protein. (*B*) Binding of *E. coli* SSB.

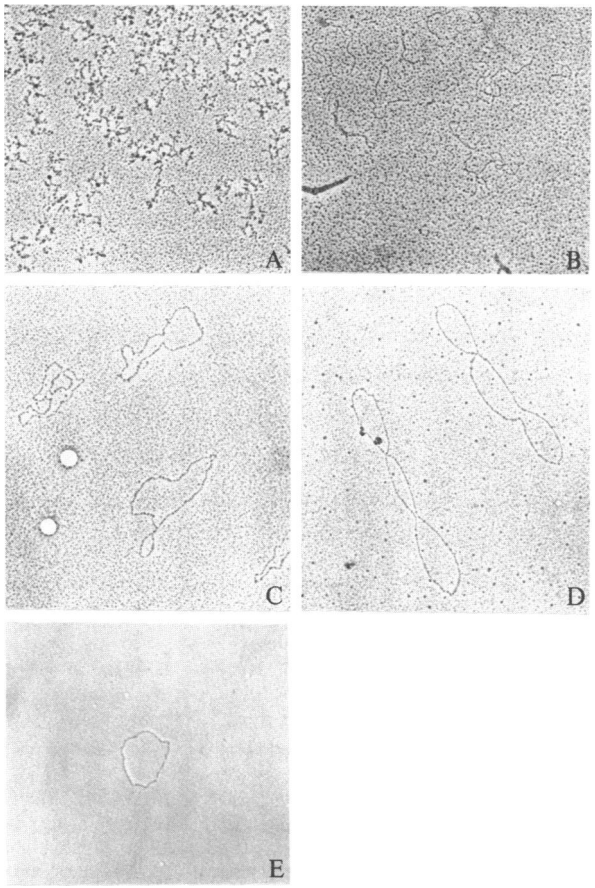

FIG. 3. Visualization of VirE2 or *E. coli* SSB complexes with ssDNA. M13mp7 ssDNA (0.03 μg) was incubated for 15 min at 4°C alone (*A* and *C*) or in the presence of 0.6 μg of either VirE2 protein (*B* and *D*) or *E. coli* SSB (*E*). Samples were processed for direct platinum shadowing (*A* and *B*) or fixed and spread on cytochrome films (*C–E*).

empty field. Since the potential function of VirE2 protein is to facilitate transfer of ssDNA molecules through cell membranes, one might expect it to form very thin extended structures with ssDNA. To better visualize these VirE2–M13mp7 ssDNA complexes we used the technique of spreading nucleic acids on a cytochrome film (24). A similar approach has been successfully used for visualization of gene 32 protein complexes with fd DNA (8). When spread on cytochrome film, free M13mp7 ssDNA appears as partially unfolded circles (Fig. 3*C*) with an average length (mean ± SD) of 0.84 ± 0.12 μm. Binding of the VirE2 protein leads to an increase in length of about 55% to 1.3 ± 0.19 μm. In this respect binding of VirE2 to ssDNA differs from that of another SSB, *E. coli* SSB, which, under the same conditions, condenses ssDNA molecules by one-third (Fig. 3*E*).

Previous studies using gel retardation (11–14) showed that VirE2 protein does not bind to dsDNA. The probe DNA used in this assay is necessarily very short (usually 500 bp), and thus the possibility of potential affinity to long native dsDNA molecules could not be excluded. Indeed, T4 bacteriophage gene32 protein has been shown to bind and locally denature dsDNA of simian virus 40 and of bacteriophage λ when prepared for electron microscopy (8). Such structures, however, were not found following incubation of VirE2 protein with dsDNA of λ bacteriophage (data not shown).

Kinetics and Salt Dependence of VirE2 Binding to ssDNA. The strength of protein binding to DNA is often reflected by the concentration of salt required to inhibit formation of the protein–DNA complexes. By using the filter binding technique and saturating amounts of VirE2 protein we found that 50% inhibition of formation of VirE2–ssDNA complexes is achieved at 0.6 M NaCl (Fig. 4*A*). Concentrations greater than 1 M NaCl almost completely prevented binding of VirE2 to ssDNA. These data suggest that VirE2–ssDNA complexes are very stable under physiological (0.1–0.2 M) salt concentrations. Binding of VirE2 to ssDNA is a rapid process. The results in Fig. 4*B* demonstrate that at 0°C VirE2 forms complexes instantaneously with both linear and circular ssDNA molecules. These observations indicate a strong affinity of VirE2 for ssDNA, a characteristic feature of SSBs (4).

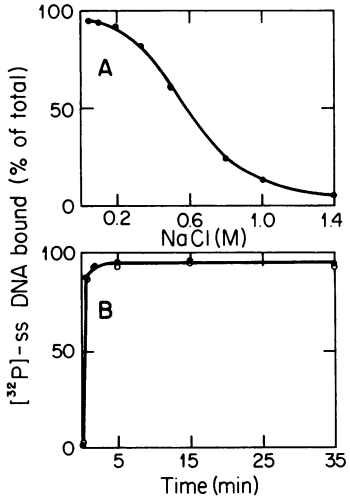

FIG. 4. Binding of VirE2 to linear ssDNA molecules: Effect of salt concentration and kinetics. (*A*) VirE2 protein (100 ng) was incubated at 0°C for 15 min with 10 ng of radioactively labeled linear ssDNA of M13mp7 in the presence of the indicated concentrations of NaCl. After incubation, the samples were applied to nitrocellulose filters, washed with 5 ml of the corresponding incubation buffer, and then washed with 20 ml of buffer B. (*B*) VirE2 protein (80 ng) was incubated at 0°C with 10 ng of radioactively labeled linear (●) or circular (○) M13mp7 ssDNA in 20 μl of buffer B. At the indicated time intervals samples were withdrawn and the extent of protein–ssDNA binding was determined by using nitrocellulose filter binding.

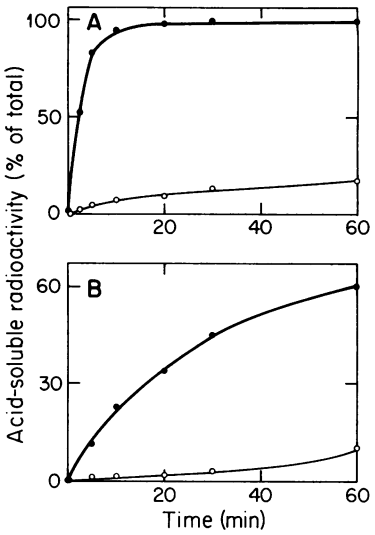

FIG. 5. Effect of VirE2 protein on exonucleolytic degradation of linear ssDNA. ^{32}P-labeled linear ssDNA of M13mp7 (10 ng) was incubated in 20 μl at 0°C for 15 min either alone (●) or with 100 ng of VirE2 protein (○). One-half unit of exonuclease VII (*A*) or 1 unit of S1 nuclease (*B*) was added in 80 μl of the appropriate reaction buffer (prewarmed to 37°C) and the incubation was continued at 37°C. At the indicated times, samples were withdrawn and precipitated with 5% cold trichloroacetic acid in the presence of 10 μg of carrier tRNA per ml. Acid-insoluble DNA was pelleted (12,000 × *g* for 5 min at 4°C), and one-half of the supernatant was assayed for radioactivity in a Tri-Carb Packard 4530 liquid scintillation counter.

VirE2–ssDNA Complexes Are Resistant to Exonucleases. Linear ssDNA of M13mp7 is readily degraded by exonuclease VII (Fig. 5*A*) and by S1 nuclease (Fig. 5*B*) (both enzymes are 3′ and 5′ exonucleases specific for ssDNA and S1 nuclease also acts as an endonuclease). The susceptibility of ssDNA to exonucleases is dramatically decreased following formation of VirE2–ssDNA complexes. Only 5% of protein-bound ssDNA is degraded after 10 min at 37°C with exonuclease VII, whereas as much as 80% of the free DNA is degraded under the same conditions. Moreover, even after 1 hr of incubation with exonuclease VII, when 100% of the free ssDNA has been destroyed, most of the ssDNA in complex with VirE2 protein is still intact (Fig. 5*A*). Similar results were obtained following S1 nuclease treatment of VirE2–ssDNA complexes (Fig. 5*B*).

DISCUSSION

We present evidence that the nopaline-specific VirE2 protein of *Agrobacterium* binds cooperatively to ssDNA. Electron microscopy shows that this binding produces unfolded protein–ssDNA complexes with a characteristic and potentially unique structure. These complexes are unusually thin, their diameter being at the limit of resolution under the electron microscope using direct platinum shadowing—i.e., about 20 Å. The calculated length of these complexes is about 1.8 Å per nucleotide. This is in contrast to other SSBs. For example, gene 32 protein also forms an extended complex with ssDNA (4.6 Å per nucleotide), but this complex has a diameter of up to 70 Å (8). Also, the VirE2-binding mode differs from that of *E. coli* SSB. Upon its binding the latter condenses rather than extends ssDNA molecules (ref. 28 and this work) and possesses only a limited cooperativity (29) in its biologically active beaded binding morphology (3). This difference may explain why the *virE2* gene product does not complement a *ssb* mutation in *E. coli* (14).

In addition to its high cooperativity, binding of VirE2 to ssDNA is characterized by very rapid, almost instantaneous kinetics resulting in the formation of stable protein–ssDNA complexes. Furthermore, these complexes, when formed with linear ssDNA, appear to be extremely resistant to exonucleolytic digestion. This efficiency of VirE2 binding to ssDNA, as well as its relative abundance in plant-induced *Agrobacterium* (12, 30), makes sense. VirE2 protein is produced following the activation of the T-DNA transfer process. The transfer intermediate, the T-strand, is a ssDNA molecule produced at about one copy per induced bacterial cell (18). The relative abundance of the VirE2 protein and its high affinity for ssDNA would allow this protein to rapidly find and coat a single T-strand molecule before it is destroyed by intracellular and/or extracellular nucleases. Once the VirE2–T-strand complex is formed, it will potentially remain stable during the process of T-DNA transfer. We propose, therefore, that the T-strand is transferred to plant cells not as a free ssDNA molecule but as a protein–ssDNA structure designated the T (transfer)-complex.

Nononcogenic Ti plasmids, where most of the internal sequences of the T-DNA have been deleted and replaced by DNA of interest, have been successfully used as vectors for plant transformation (see ref. 15). This observation implies that any DNA located between 25-bp border sequences can be transferred to plants and function as the T-DNA. Thus, the T-DNA as well as its T-strand derivative and the putative T-complex are sequence nonspecific. Therefore, electron micrographs presented here may actually visualize a potential structure of the T-DNA transfer complexes. DNA transfer from *Agrobacterium* to plant cells was proposed to represent an adaptation of bacterial conjugation (18, 31). By analogy with this process, T-DNA transfer is likely to occur through pores or channels in bacterial and plant membranes (possibly formed by other *vir* proteins) (31, 32). Thus, it is especially interesting that the electron microscopic data show VirE2–ssDNA complexes are very thin and extended, potentially adapted to be transferred through such putative channels. Extracellular complementation of insertional mutants of the *virE* locus (20) implies that its protein products are indeed capable of transmembrane trafficking. Thus, we expect that VirE2, as a part of the T-complex, will specifically recognize the putative membrane pores to facilitate passage of the T-complex.

Results presented in this work indicate that the VirE2 protein plays a direct role in transfer of T-DNA. This idea is further strengthened by observations that intact *virE* operon is required for full expression of *Agrobacterium* virulence. Genetic studies revealed that insertional mutants of the octopine-specific *virE* locus are completely avirulent on *Kalanchoe* spp. and cause attenuated tumors on *Nicotiana tabacum* (16), whereas deletion mutants of nopaline-specific *virE* locus are nononcogenic both on *Kalanchoe* spp. and tomato (33). Why *virE* mutants still possess a residual oncogenic activity on some plant hosts is not clear. By analogy with the F-factor SSB in bacterial conjugation (34), the absence of VirE2 protein might be in part compensated by other bacterial and/or plant ssDNA-binding proteins. Binding of these proteins to the T-strand may protect it sufficiently to allow some T-strand to be transferred. This low efficiency of T-DNA transfer may suffice to cause attenuated tumors in particularly susceptible plants but will be completely avirulent on other, less susceptible host species.

The physiological role of VirE2 protein remains to be demonstrated. Direct interaction of VirE2 with *Agrobacterium* membrane proteins [for example, with plant-inducible *virB* proteins, which were shown to fractionate to the bacterial envelope (30)] should now be sought. Furthermore, demonstrating that VirE2 protein is present on native T-strand–protein complexes isolated from *Agrobacterium* and/or infected plant cells is critical to proving its role in the T-DNA transfer process.

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