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The *Agrobacterium tumefaciens virC1* Gene Product Binds to Overdrive, a T-DNA Transfer Enhancer

NICOLAS TORO,[1]† ASIS DATTA,[1]‡ OFER A. CARMI,[1] CALVIN YOUNG,[1]§ RABI K. PRUSTI,[2] AND EUGENE W. NESTER[1]*

Department of Microbiology[1] and Department of Pharmacology,[2] University of Washington, Seattle, Washington 98195

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In *Agrobacterium tumefaciens*, a *cis*-active 24-base-pair sequence adjacent to the right border of the T-DNA, called overdrive, stimulates tumor formation by increasing the level of T-DNA processing. Recent results from our laboratory have suggested that the *virC* operon which enhances T-DNA processing probably does so because the VirC1 protein interacts with overdrive (N. Toro, A. Datta, M. Yanofsky, and E. W. Nester, Proc. Natl. Acad. Sci. USA 85:8558–8562, 1988). We report here the purification of the VirC1 protein from cells of *Escherichia coli* harboring a plasmid containing the coding sequences of the *virC* locus of the octopine Ti plasmid. By gel mobility shift and DNase I footprinting assays, we showed that this purified *virC1* gene product binds to overdrive but not to the right border of T-DNA.

During crown gall tumor formation, *Agrobacterium tumefaciens* transfers a specific segment of its tumor-inducing (Ti) plasmid, the T-DNA, to the plant cell nucleus. The T-DNA becomes covalently integrated into the plant genome and is maintained stably thereafter (7, 12). The transfer of T-DNA depends on the function of a 35-kilobase *vir* region of the Ti plasmid which includes six well-defined transcriptional units: *virA*, *virB*, *virG*, *virC*, *virD*, and *virE*. Mutations in these loci result in either avirulence (*virA*, *-B*, *-D*, and *-G*) or attenuated virulence (*virC* and *-E*) (15, 16). Certain strains of *A. tumefaciens* that have a limited host range have no functional *virC* locus (23, 25). All *vir* loci are transcriptionally activated by phenolic signal molecules produced by wounded plant cells (17, 21).

The T-DNA is bounded by 25-base-pair (bp) imperfect direct repeats termed border sequences (2, 22). A *virD*-encoded, site-specific endonuclease cleaves at unique and identical locations in the bottom strand of these border sequences (1, 25). Another sequence adjacent to the right border, designated overdrive, stimulates tumor formation (13, 14), probably by increasing the level of single-strand formation (18). The *virC* operon also enhances T-DNA processing through an interaction between the VirC1 protein and the overdrive sequence (18). DeVos and Zambryski (3) have also shown that the nopaline VirC1 protein promotes single-strand T-DNA formation in *Escherichia coli*. In this report, we present evidence that the *virC1* gene product specifically binds to the overdrive, but not to the border sequence. These results provide clues as to how the *virC1* gene product functions along with the VirD endonuclease to stimulate T-DNA processing.

Recently, we reported that both the overdrive and the *virC* operon enhance T-DNA processing by the *virD*-encoded endonuclease (18). Moreover, our results from DNA-affinity chromatography, using the right T-DNA border and overdrive sequences, suggested that the VirD2 protein interacts with both the T-DNA border and overdrive sequences, whereas the VirC1 protein specifically interacts with the overdrive sequence (18). However, it was not clear whether other proteins from *Agrobacterium* spp. (including other *vir* gene or Ti plasmid-encoded non-*vir* gene products) are also involved in the binding of the VirC1 protein to overdrive. To exclude the possibility that other proteins are involved and also to determine whether the VirC1 protein binds specifically to overdrive, the VirC1 protein was purified from an *E. coli* strain harboring pMY929. Plasmid pMY929 carries the *SmaI-Hin*dIII fragment which contains the *virC* locus inserted into the high-copy vector pUC18 (24). The strain harboring pMY929 produces the VirC1 protein, identified as a unique band by both Coomassie blue staining and immunostaining (Fig. 1A). Furthermore, in *E. coli* the *virC1* gene was expressed from its own promoter and not from vector sequences, since the same restriction fragment in the opposite orientation yielded the same level of VirC1 protein (24). Cell-free extracts of *E. coli*(pMY929) which contained the VirC1 protein could retard the DNA probe containing overdrive, but not that containing the right border (Fig. 1B). As a control, we used *E. coli*(pUC18) cell extracts which did not contain the VirC1 protein (Fig. 1A). These extracts did not retard DNA probes containing either overdrive or right border (data not shown). Moreover, high-pressure liquid chromatography (HPLC) fractions isolated from pMY929 cell extracts (Fig. 2A, peaks 1 to 8) which did not contain the VirC1 protein did not retard either one of these DNA probes (data not shown). These results suggested that the VirC1 protein interacted with a sequence containing overdrive, but not with the right border sequence.

The VirC1 protein was purified from *E. coli*(pMY929) cells. Most of the VirC1 protein appeared in the pellet after centrifugation of extracts from French press-disrupted cells. The pellet was first solubilized with guanidine-HCl, and the VirC1 protein was separated from other proteins by reverse-phase HPLC on a C4 column (Fig. 2A). The protein was then eluted with a linear gradient of acetonitrile, and 10 major absorbance peaks were observed; UV spectrum analysis indicated that all major peaks were proteins. Immunoblot analyses of fractions from the HPLC column (Fig. 2A) suggested that only peaks 9 and 10 contained VirC1 protein

* Corresponding author.
† Present address: Departamento de Microbiologia, Estacíon Experimental del Zaidin, C.S.I.C., 18008 Granada, Spain.
‡ Present address: School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India.
§ Present address: Department of Biological Science, California State University at Fullerton, Fullerton, CA 92634.

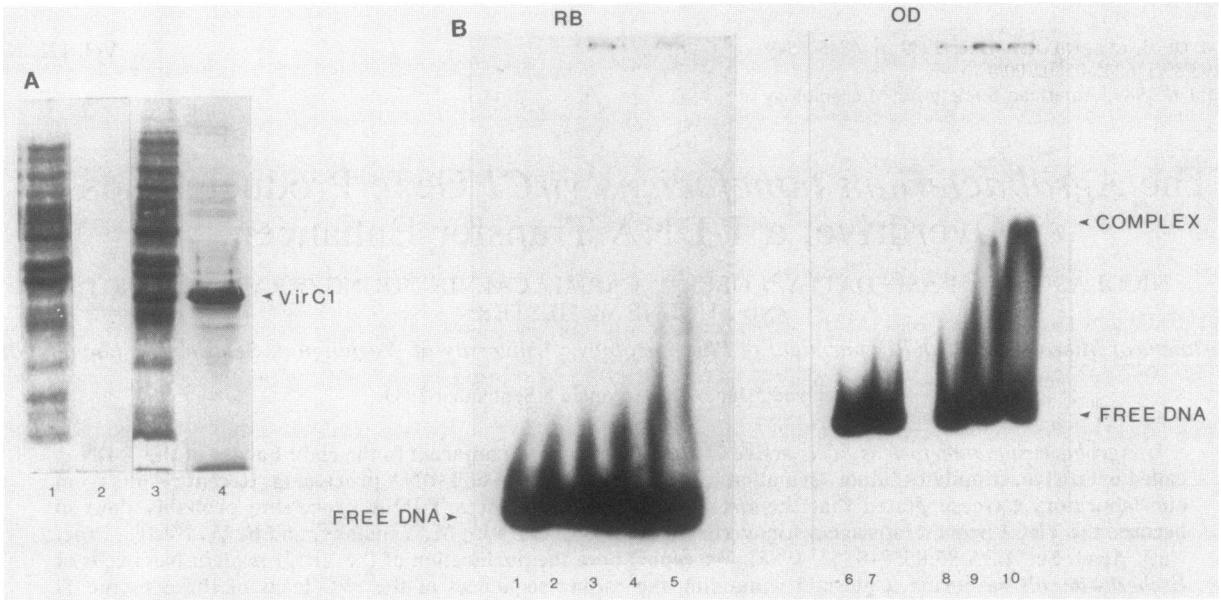
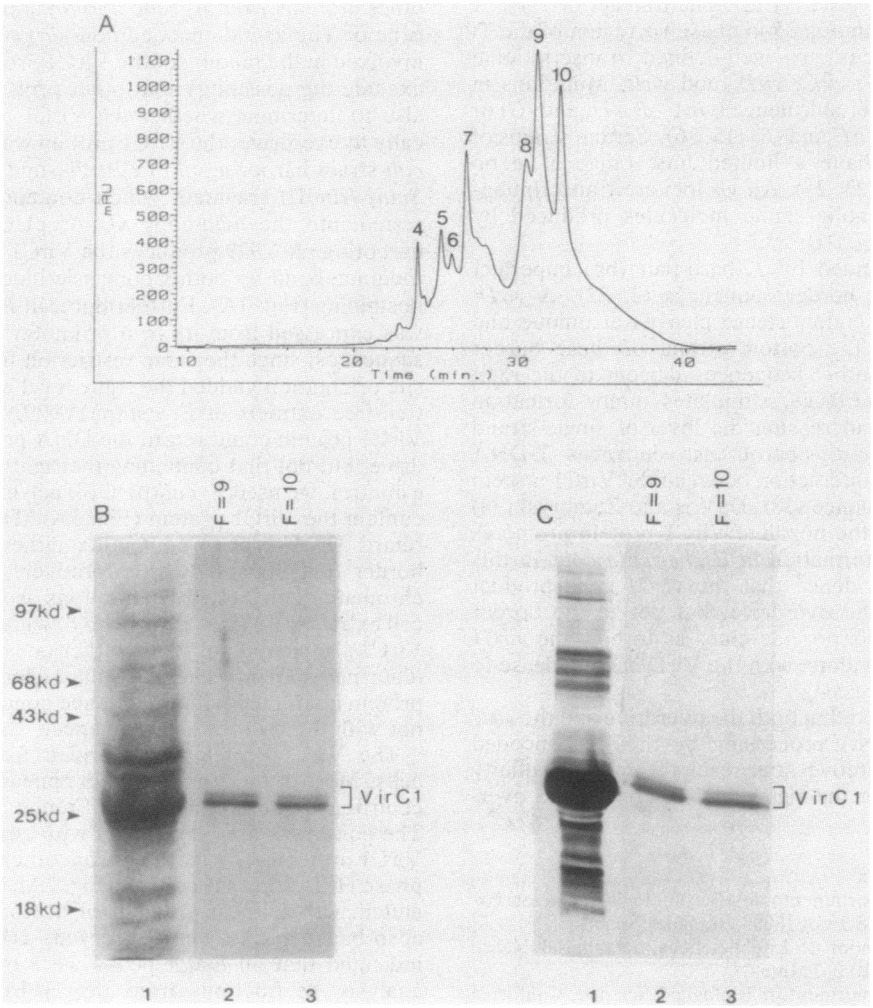


FIG. 1. Expression of the VirC1 protein from a VirC1-producing *E. coli* strain (A) and gel retardation assays with total proteins from *E. coli*(pMY929) cells (B). (A) Proteins were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then immunostained with polyclonal anti-VirC1 antisera (18). Profile of total protein stained with Coomassie brillant blue G (lanes 1 and 3); protein immunostained with anti-VirC1 antisera (lanes 2 and 4). Lanes: 1 and 2, JM101(pUC18); 3 and 4, JM101(pMY929). (B) To isolate a probe containing either overdrive (OD) or right border (RB), the 285-bp fragment (as described in the legend to Fig. 3A) was digested with *Sst*I and then the *Acc*I (13991)-*Sst*I (14089) restriction fragment containing right border or the *Sst*I (14089)-*Eco*RI (14276) fragment containing overdrive was isolated. Gel retardation assays were carried out essentially as described by Fried and Crothers (4). Lanes: 1 and 6, Control no protein added; 2 and 7, 3 μg of protein; 3 and 8, 6 μg of protein; 4 and 9, 9 μg of protein; 5 and 10, 12 μg of protein.



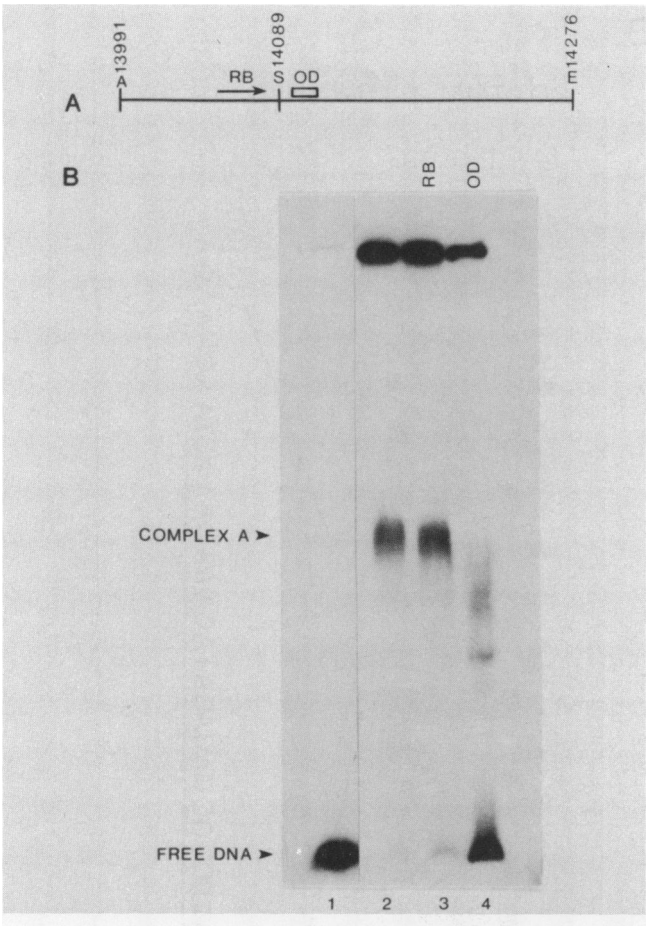


FIG. 3. Binding of VirC1 protein to overdrive sequences. The probe containing right border and overdrive (A) was prepared by digesting pBL20 (1) with *Eco*RI and end labeling with DNA polymerase I (Klenow fragment) and [α-^{32}P]dATP (10). The probe was then digested with *Acc*I, and the 285-bp fragment was purified by polyacrylamide gel electrophoresis. A gel retardation assay with 2 ng of the end-labeled DNA probe was performed as described in the legend to Fig. 1B. For the competition studies, the synthetic oligonucleotides for overdrive (5′ TAAGTCGCTGTATGTGTTTG TTTG3′) and right border (5′ GACTGGCAGGATATATACCGT TGTA3′) sequences, made on a BioSearch 8600 DNA synthesizer, were annealed as described by Kadonaga and Tjian (6). A 0.8-μg portion of the annealed right border or overdrive oligonucleotides was added to the reaction mixture before the addition of the protein sample. Lanes: 1, Control, no protein added; 2, 3, and 4, purified VirC1 protein (10 μg) added; 3, competition with right border; 4, competition with overdrive sequence.

(Fig. 2C). Although these fractions have different chromatographic behavior on a C4 reverse-phase column, they are identical by such criteria as Coomassie blue staining or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2B), immunoblot analysis (Fig. 2C), and absorption ratios at 206 and 275 nm (data not shown). The heterogeneity of the VirC1 protein observed on reverse-phase chromatography could be due to the formation of distinct conformational states of the protein in acetonitrile-trifluoroacetic acid (TFA) that interact differently with the column stationary phase (5, 20). This hypothesis is consistent with the observation that rechromatography of either fraction 9 or 10 with different mobile phases always gave rise to the same two distinct peaks (data not shown). Since fractions 9 and 10 gave identical results in gel retardation and DNase I footprinting assays, the data obtained with fraction 10 only are shown.

The gel retardation assay (4) was used to assess the interaction of the VirC1 protein with the overdrive sequence. A 285-bp *Acc*I-*Eco*RI DNA restriction fragment containing both right border (25 bp) and overdrive (24 bp) sequences was used as a probe (Fig. 3A). The probe was incubated with the VirC1 protein purified by HPLC and excess poly(dI-dC), a nonspecific competitor DNA. The mixtures were loaded onto a nondenaturing polyacrylamide gel, and the migration pattern of free and complexed DNA was analyzed by autoradiography. A distinct retarded complex (A) was observed (Fig. 3B) in addition to a complex which did not enter into the gel, most likely as a result of protein aggregation. The affinity of the VirC1 protein for specific DNA sequences was assessed by competition experiments with either double-stranded right border or overdrive synthetic oligonucleotides. The DNA-protein complex A could be successfully inhibited with the overdrive-oligonucleotide (Fig. 3B), but not with the same quantity of the right border-oligonucleotide. Interestingly, both complex A and the complex which did not enter the gel were efficiently inhibited by overdrive, suggesting that these complexes are related. These experiments suggest that the VirC1 protein binds specifically to the overdrive sequence. Other HPLC fractions (Fig. 2A, peaks 1 to 8) did not retard the DNA probe (data not shown). In all of these DNA-binding assays, protein samples were tested directly after HPLC fractionation, without undergoing any specific renaturation step. Since the exposure of VirC1 protein to guanidine-HCl, acetonitrile, and TFA during purification may have reduced its ability to bind DNA, a high ratio of protein to DNA was used.

To demonstrate more definitively that the VirC1 protein binds specifically to overdrive, DNase I footprinting experiments were performed. The specific DNase I protection of VirC1 on the DNA probe extended from nucleotides 14093 to 14133 on the top strand and 14085 to 14130 on the bottom strand (Fig. 4). This region contains the overdrive sequence, but not the right border sequence (Fig. 4). Specific DNase I protection by the VirC1 protein on the DNA probe depended on an increasing amount of protein. A variety of binding conditions that were tried did not modify the ability of the VirC1 protein to bind to the DNA probe. Nevertheless, the mobility shift and DNase I footprinting assays indicate that

FIG. 2. Purification of VirC1 protein by reverse-phase HPLC. Proteins from a guanidine-solubilized pellet harvested from 500 ml of *E. coli*(pMY929) were separated on a C4 reverse-phase column (Aquapore butyl, BU-300, 2.1-mm inside diameter by 10 cm; Brownlee), using a gradient of acetonitrile in TFA. A 30-min gradient of 0 to 80% buffer B contained 0.1% TFA (Pierce Chemical Co.) (0%), whereas buffer B (80%) contained 0.08% TFA in 80% aqueous acetonitrile (HPLC grade; Pierce). Individual peaks were collected, lyophilized, suspended in water, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie blue staining (8), and immunostaining (19). Absorption was monitored at both 206 (A) and 275 (data not shown) nm. (B) Coomassie blue-stained sodium dodecyl sulfate–12.5% polyacrylamide gel. (C) Corresponding immunoblot developed with antiserum raised against VirC1 protein (18). Lanes: 1, Pellet suspended in 8 M guanidine-HCl; 2, fraction 9; 3, fraction 10. Numbers indicate molecular weights (10^3).

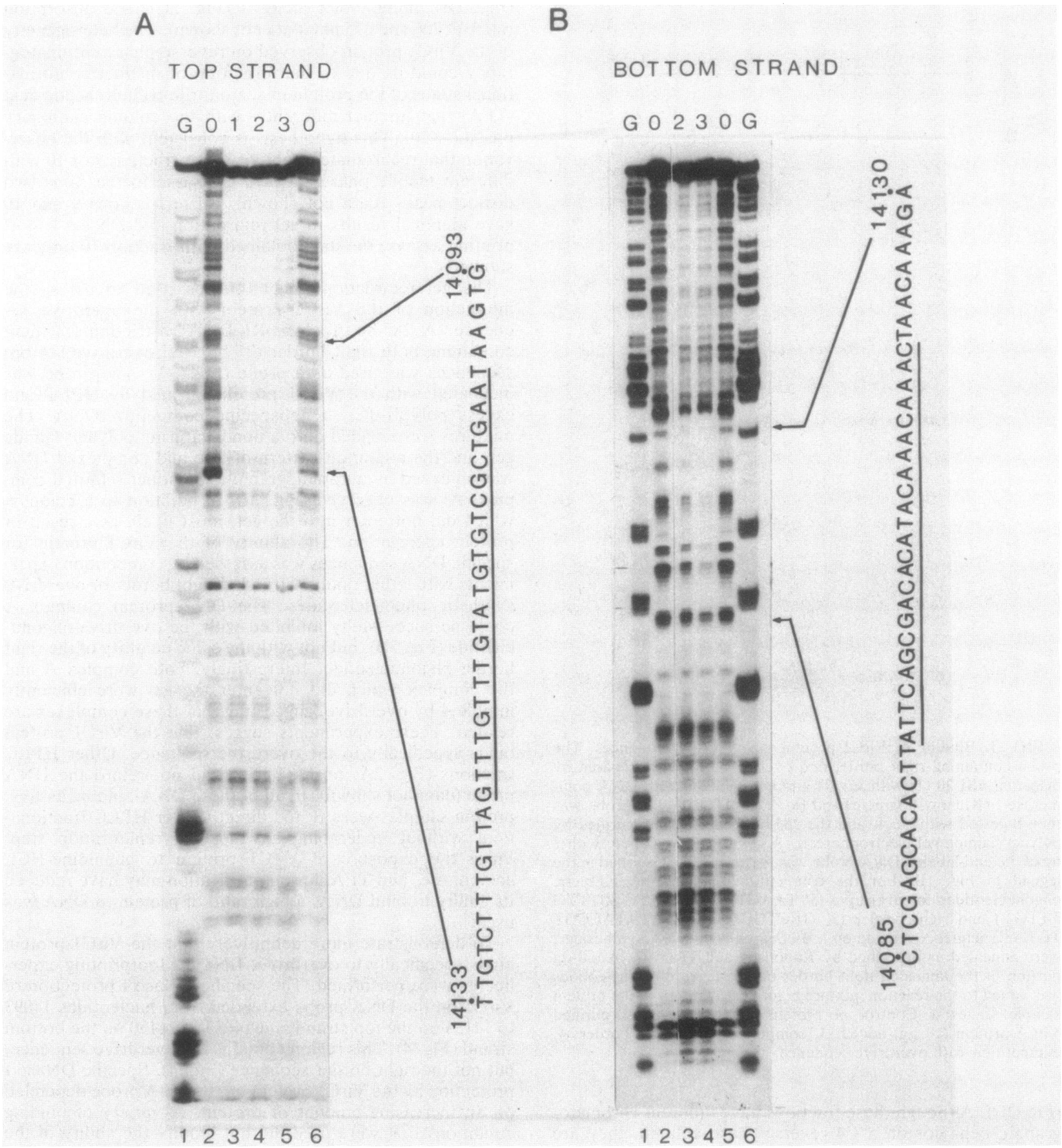


FIG. 4. DNase I footprint analysis. DNase I protection experiments were performed by the method of Lee et al. (9). The 285-bp *Acc*I-*Eco*RI DNA fragment used in the mobility shift assays was used as a probe for the DNase I footprint analysis. The DNA probe was end labeled with DNA polymerase I (Klenow fragment) and [α-^{32}P]dATP at the *Eco*RI site (top strand) or with [α-^{32}P]dCTP at the *Acc*I site (bottom strand). The same probe was used for Maxam-Gilbert G-cleavage reactions (11). Numbers at the top of each panel indicate the relative concentration of VirC1 protein used; G indicates the Maxam-Gilbert G-cleavage reaction. The protected sequences are indicated at the right of each panel, with nucleotide positions those of Barker et al. (2). The corresponding overdrive sequence is underlined.

the VirC1 protein interacts with the overdrive sequence but not with the right border sequence. These results are consistent with our earlier observations in which the VirC1 protein did not bind to affinity columns containing right border sequences (18). It is also clear from these data that the binding of the VirC1 protein to the overdrive sequence does not require other *Agrobacterium* proteins. Since we observed earlier that the VirD2 protein was retained in

overdrive as well as right border affinity columns (18), it is possible that the interaction of the VirD2 protein with overdrive could be through the VirC1 protein. Further experiments are needed to elucidate how the interaction of the VirC1 protein with overdrive brings the VirD endonuclease to the adjacent border sequence.

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