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DNA modifying enzymes of *Agrobacterium tumefaciens*: Effect of DNA topoisomerase, restriction endonuclease, and unique DNA endonuclease on plasmid and plant DNA

(sequence specific endonuclease/superhelical DNA/plant)

JEANNE M. LEBON*, CLARENCE I. KADO†, LEONARD J. ROSENTHAL‡, AND JACK G. CHIRIKJIAN*§

* Department of Biochemistry,‡ Department of Microbiology, Georgetown University Medical Center, Washington, D.C. 20007; and † Department of Plant Pathology, University of California, Davis, California 95616

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ABSTRACT Extracts from *Agrobacterium tumefaciens* strain 1D135 contain three enzymes that have been characterized and partially purified. The first enzyme, a DNA topoisomerase, appeared to relax only negatively twisted DNA. The second enzyme, *Atu* I, a type II restriction endonuclease, generated the identical DNA digestion pattern as *Eco*RII when several DNAs were used. The third enzyme, endonuclease A, showed a preference for superhelical DNAs as substrates. When plasmid pCK135 DNA, obtained from the virulent strain 1D135 of *A. tumefaciens*, or plant DNA was exposed to the three enzymes, changes in DNA patterns were observed due to either conformational changes or digestion of the DNAs. These enzymes may function *in vivo* in the processing and incorporation of bacterial DNA in plant cells.

For some time *Agrobacterium tumefaciens* has been known to induce tumors in various dicotyledonous plants (1). Virulent strains of *A. tumefaciens* are known to harbor large plasmids, and it was suspected that virulent genes were contained in these plasmids. The infective process resulting in the formation of crown gall tumors in plants appears, at least in part, to involve the presence of a DNA segment obtained from virulent bacterial plasmids (2). Our interest has been in the enzymology of bacterial DNA insertion into host plant DNA. In this report we wish to describe the purification and characteristics of three enzymes¶: (*i*) *Atu* DNA topoisomerase which acts on superhelical DNAs to generate a number of intermediates with variable amounts of superhelicity; (*ii*) a type II restriction DNA endonuclease (*Atu* I) that generates DNA digestion patterns similar to *Eco*RII with several DNA substrates; and (*iii*) a third activity, described as endonuclease A, that preferentially catalyzes the conversion of superhelical DNAs to their corresponding linear forms. Mobility of plasmid and plant DNAs were altered on polyacrylamide electrophoretic gels after treatment with combinations of these enzymes, suggesting a possible role for these enzymes *in vivo*. A preliminary report of this work has been made (3).

METHODS AND MATERIALS

Growth of Bacterial Cells. Cells of *A. tumefaciens* 1D135 were obtained from 1.2 liters of medium inoculated with a 1% (vol/vol) overnight inoculum and grown at 30° for 16 hr. The medium contained, in 1 liter, 10 g of sucrose, 8 g of casein hydrolysate, 4 g of yeast extract (Difco), 2 g of K_2HPO_4, and 0.3 g of $MgSO_4$. Cells, 7 g/liter (wet wt), were harvested, washed with 0.9% saline, and stored at −90° until further use.

DNA Substrates. Simian virus 40 (SV40) was prepared from purified virions by standard published methods (4). Radioactive SV40 DNA was obtained by labeling with [^{3}H]thymidine. Other DNAs were prepared by modification of published procedures (5, 6). Restriction endonucleases and T7 and adenovirus DNAs were obtained from Bethesda Research Laboratories, Inc., Rockville, MD.

Leaf Vinca Rosea DNA. Samples were prepared as described (7). We purified nuclei first in order to avoid the presence of contaminating mitochondrial DNA. The homogenated leaf extract was filtered and incubated in 2% (vol/vol) Triton X-100 for 10 min at 23°. Nuclei were collected by centrifugation, resuspended in the buffer (7), incubated again in 2% Triton X-100 (10 min), centrifuged, resuspended in 50 ml of the buffer, and incubated in 4% (wt/vol) sodium dodecyl sulfate for 3 hr at 23°. $NaClO_4$ was added to a final concentration of 1 M, and the crude DNA was deproteinized by extraction with chloroform. The DNA was precipitated with cold 95% ethanol, spooled onto a glass rod, and then dissolved in 10 mM NaCl. The DNA was treated with pancreatic RNase (50 μg/ml, 30 min, 37°) and then digested with pronase (50 μg/ml, 90 min, 37°). Sodium dodecyl sulfate was added to 0.5% (final concentration), and the incubation was continued for 30 min. NaCl was added to 0.5 M, and the DNA was extracted with neutralized, water-saturated, redistilled phenol and then with chloroform. The DNA was again precipitated with ethanol and dissolved in 10 mM NaCl.

Isolation of Plasmid pCK135 from *A. tumefaciens*. Cells were grown in Vogel–Bonner medium (8) at 30° to a density of 200 Klett units (green filter). They were harvested, washed twice with 150 mM NaCl/0.1 M Na_2EDTA, pH 8.0, and resuspended in 50 mM Tris-HCl, pH 8.0/50 mM Na_2EDTA. The cells were incubated with lysozyme at 200 μg/ml for 30 min at 23°. Pancreatic ribonuclease I (pretreated at 90° for 30 min with 20 mM Na acetate, pH 6.0, to remove any DNase activity) was added to 50 μg/ml and the cells were lysed with Sarkosyl NL-97 [1% (wt/vol) final concentration]. The lysate was incubated with proteinase-K (100 μg/ml) for 45 min at 37°. The viscosity of the lysate was decreased by pipetting the sample 10 times with a 5-ml serological pipette and by the dropwise addition of 2 M NaOH to pH 12.0 with slow mechanical stirring. While being stirred, the lysate was adjusted to pH 9.0 by the dropwise addition of 2 M Tris-HCl, pH 7.0, and then Na perchlorate was added to 0.5 M. The solution was extracted with

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Abbreviations: SV40, simian virus 40; buffer A, 10 mM Tris-HCl, pH 7.8/5 mM 2-mercaptoethanol/1 mM $MgCl_2$.

§ To whom reprint requests should be addressed.

¶ DNA topoisomerases have also been referred to as nicking closing enzymes, DNA relaxing enzymes, and superhelical DNA unwinding enzymes. Sequence specific endonucleases are commonly referred to as type II restriction endonucleases. The enzyme *Atu* I is such an activity.

an equal volume of redistilled phenol and the aqueous phase, separated by centrifugation (10,000 rpm, 10 min, 4°), was extracted with chloroform. The DNA was precipitated from the aqueous phase by the addition of $MgCl_2$ (to 15 mM), Na phosphate (to 5 mM), and 0.7 volume of 95% ethanol according to the procedure of Dessev and Grancharov (9) as modified by Currier and Nester (10). The precipitated DNA was dissolved in 0.1 M Na_2EDTA, pH 8.0, and dialyzed against 50 mM Tris·HCl, pH 8.0/50 mM NaCl/5 mM Na_2EDTA. Covalently closed circular plasmid DNA was purified by dye buoyant density gradient centrifugation in cesium chloride containing ethidium bromide (11).

Incubation Conditions for Enzymes. (*i*) DNA topoisomerase: The reaction mixture contained, in 0.05 ml, 20 mM Tris·HCl (pH 7.8), 2 mM $MgCl_2$, 0.01 A_{260} unit of SV40 component I, and enzyme. Mixtures were incubated at 37° for 30 min. (*ii*) Endonuclease A and the restriction endonuclease *Atu* I: The reaction mixture contained, in 0.05 ml, 20 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 0.01 A_{260} unit of DNA, and enzyme. Mixtures were incubated at 37° for 1 hr.

Conditions for Displaying DNA on Agarose and Polyacrylamide Gels. All assay reactions were stopped by the addition of 10 μl of denaturing buffer [0.02% bromophenol blue, 1% sodium dodecyl sulfate, and 50% (vol/vol) glycerol] and analyzed by gel electrophoresis. For routine assays, gels (1.4% agarose), were prepared and electrophoresis was carried out in 40 mM Tris/12 mM Na acetate, pH 7.8/2 mM EDTA for 2 hr at 100 V. Polyacrylamide gels were run for 2 hr at 200 V in Tris borate/EDTA buffer (12). Gels were stained with ethidium bromide (0.5 μg/ml) and DNA bands were visualized by using a short wavelength UV-light plate. Photographs were obtained by the use of a red filter (Kodak 23A) and Polaroid type 57 film.

Assays for 3′ and 5′ Exonuclease. The 3′-exonuclease assay is based on solubilization of ^{32}P-labeled XMP from the 3′ end of labeled T7 DNA. The 3′ end is specifically labeled enzymatically by using T4 DNA polymerase in the presence of a single ^{32}P-labeled deoxynucleoside triphosphate as described by Englund (13). Similarly, assays for the 5′ exonuclease are based on solubilization of ^{32}P from the 5′-end-labeled DNA that was prepared by dephosphorylating with alkaline phosphatase and subsequent labeling with polynucleotide kinase, using [γ-^{32}P]ATP as the donor (14).

RESULTS

Purification of DNA Modifying Enzymes from *A. tumefaciens*. All steps were carried out at 4°, and centrifugations were done at 10,000 × *g* for 15 min unless otherwise stated. Frozen *A. tumefaciens* cells (20 g) were broken in buffer A (10 mM Tris·HCl, pH 7.8/5 mM 2-mercaptoethanol/1 mM $MgCl_2$) by sonication for 15 min. Unbroken cells and cell debris were then removed by centrifugation and the supernatant was submitted to ultracentrifugation at 100,000 × *g* for 2 hr. Streptomycin sulfate was added slowly to the clear supernatant in the ratio of 1800 A_{260} units to 1 ml of a 10% solution of the antibiotic. The supernatant was stirred for 30 min and then centrifuged. Solid NaCl was added to the resulting supernatant to a final concentration of 1.0 M and this mixture was fractionated on a Sephadex G-150 column (2.5 × 80 cm) equilibrated with buffer A/1.0 M NaCl. Assays were performed with SV40 component I, and fractions (40 ml) that contained *Atu* DNA topoisomerase or the restriction enzyme were pooled separately and dialyzed for 16 hr against buffer A/10% glycerol. Desalted protein pools containing enzyme activity were further fractionated on a DEAE-cellulose column (2.6 × 15 cm) equilibrated with buffer A/10% glycerol and developed with a 500-ml linear KCl gradient (0–1.0 M). From the pool containing the DNA unwinding activity, endonuclease A eluted between 0.05 and 0.10 M KCl, the *Atu* DNA topoisomerase eluted between 0.10 and 0.20 M KCl, and the restriction enzyme (*Atu* I) eluted between 0.12 and 0.25 M KCl. Active *Atu* I fractions were further purified on a phosphocellulose column from which it eluted between 0.5 and 0.6 M KCl. The pooled fractions were then dialyzed against buffer A/50% glycerol. At this state of purity, the restriction endonuclease was free from interfering exonucleases and nonspecific endonucleases, as well as from *Atu* DNA topoisomerase activity. Unless otherwise stated, most of the characterization of the restriction endonuclease was undertaken with the phosphocellulose fractions, whereas DEAE-cellulose fractions were used for characterization of endonuclease A and the *Atu* DNA topoisomerase.

Properties of the *Atu* DNA Topoisomerase. The topoisomerase obtained from the DEAE-cellulose step appeared to be free of any interfering nonspecific nuclease and of the other two DNA enzyme activities. In certain preparations, the enzyme was further fractionated on a phosphocellulose column. Unlike DNA topoisomerases from eukaryotic sources and in agreement with the property of the *Escherichia coli* enzyme, positive superhelical turns appeared to be resistant to the unwinding activity. The pH optimum of *Atu* DNA topoisomerase was 7.5–8.0 in Tris·HCl and the optimal temperature was 37°. The activity of the enzyme was tested (data not shown) with SV40, ØX174 replicative factor (RF), and polyoma superhelical DNAs. In all cases DNAs were found to be relaxed, and transitional intermediate forms could be detected based on mobility differences on agarose gels. The yield of enzyme obtained from 20 g of cells was approximately 1×10^5 units. A unit is defined as the amount of enzyme that will completely relax 1 μg of superhelical SV40 DNA at 37° in 1 hr. A time course assay using SV40 Component I displayed on 1.4% agarose is shown in Fig. 1.

Properties of the Restriction Endonuclease *Atu* I. Enzyme fractions obtained from the phosphocellulose step were used for most of the studies to be described. The enzyme was found to be free of *Atu* DNA topoisomerase and endonuclease A. Specific fractions of the enzyme were found to be free of any detectable nonspecific 3′- or 5′-exonuclease activity as well as

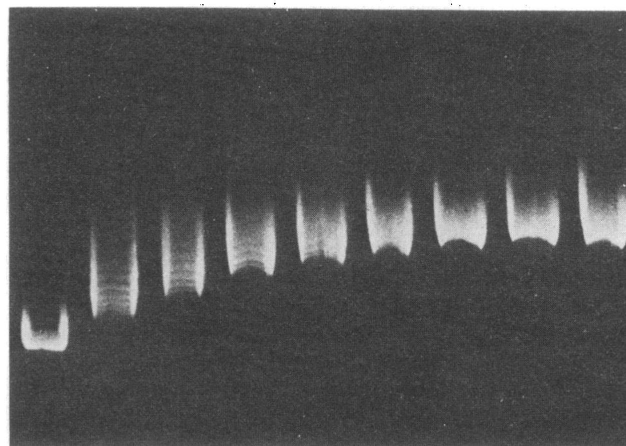

FIG. 1. A time course assay of DNA topoisomerase with superhelical SV40 DNA as substrate. The results are displayed on a 1.4% agarose gel (7.5 V/cm). From left to right: superhelical SV40 DNA (control) in slot 1 and 0.5, 1, 3, 6, 12, 30, 60, and 80 min in slots 2–9. Fixed amounts of SV40 DNA (1 μg) and *Atu* DNA topoisomerase (1 unit) were used. Complete conversion to closed circular form was obtained in 30 min.

other contaminating endonuclease activities. In subsequent experiments in which the aim was to selectively obtain small amounts of *Atu* I free of other contaminants, the supernatant, after the 100,000 × *g* step, was passed over a phosphocellulose column (2.6 × 12 cm) equilibrated in 50 mM phosphate (K^+), pH 7.0/1 mM EDTA/7 mM 2-mercaptoethanol. The column was developed with a 600-ml salt gradient (0–1.0 M KCl), and the enzyme was eluted between 0.5 and 0.6 M KCl.

Atu I digestion patterns of several DNAs were similar to the patterns obtained from endonuclease *Eco*RII from *E. coli* strain 245. As shown in Fig. 2, single digestions of λ, SV40, adenovirus 2, and ØX174 RF DNAs with *Eco*RII or *Atu* I yielded identical digestion patterns; in addition, the double digestion of SV40 DNA with *Atu* I and *Eco*RII did not generate additional DNA bands. Because *Atu* I is present in *A. tumefaciens* cells in relatively large yields and is more stable than *Eco*RII, it clearly presents advantages over *Eco*RII. The yield of *Atu* I from 20 g of cells was 2–6 × 10^3 units (a unit is defined as the amount of enzyme that will digest 1 μg of SV40 DNA at 37° in 1 hr).

Properties of the Endonuclease A. Endonuclease A shows a preference for the conversion of superhelical DNAs to their corresponding linear forms and will not cut linear DNAs. Superhelical forms of SV40, ØX174, polyoma, and PM2 DNAs, as well as of plasmids pBR322 and pMB9, were converted by endonuclease A to their linear forms. We checked the effect of increasing levels of enzyme on a fixed amount of SV40 DNA. As shown in Fig. 3, an intermediate nicked circular form was generated prior to the appearance of the 30% linear form. When superhelical SV40 DNA was digested with excess enzyme, a symmetrical peak was obtained on alkaline sucrose gradients with a sedimentation value of 16S which corresponds to the linear form (data not shown). In most preparations

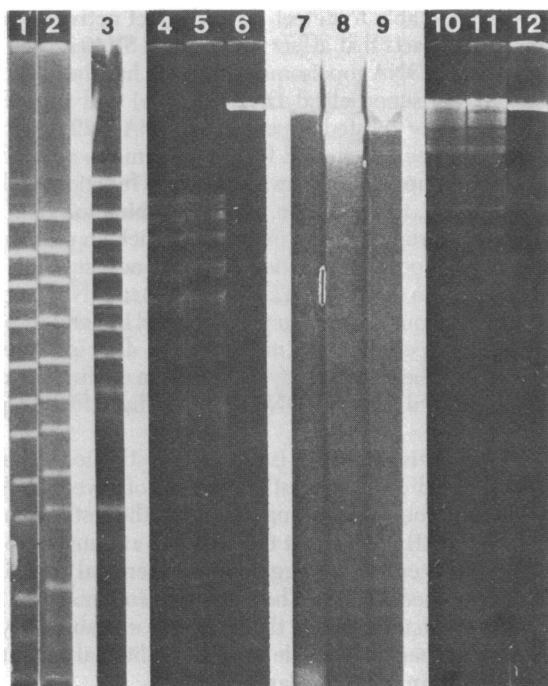

FIG. 2. Digestion characteristics of DNAs by the restriction endonuclease *Atu* I. Patterns are displayed on 4% polyacrylamide gels. Slots 1 and 2, digests of SV40 DNA with *Atu* I and *Eco*RII, respectively; slot 3, a codigestion of SV40 DNA by *Atu* I and *Eco*RII; slots 4–6, digestion patterns of adenovirus-2 with *Atu* I and *Eco*RII and undigested adenovirus DNA, respectively; slots 7–9, ØX174 RF DNA and its digestion pattern by *Atu* I and *Eco*RII, respectively; slots 10–12, digestion patterns of lambda DNA by *Atu* I and *Eco*RII and undigested lambda DNA.

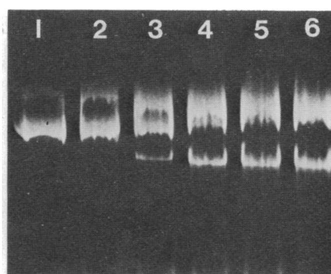

FIG. 3. Effect of endonuclease A on superhelical SV40 DNA. A time course assay using excess substrate is displayed on a 1.4% agarose gel run in 0.2 M glycine/NaOH at 15 V/cm. Slot 1 represents control SV40 DNA; slots 2–6, representing DNA treated with enzyme for 0, 15, 30, 45 and 60 min, show the formation of nicked circular intermediates prior to conversion to linear forms. A similar effect is also seen with other superhelical DNAs.

complete conversion of the superhelical form to the linear form could not be obtained. At this time we cannot exclude the possibility that the isolated activity consists of more than one enzyme. Superhelical SV40 DNA first unwound by the DNA relaxing enzyme was not as good a substrate as untreated SV40 DNA for endonuclease A. It is not clear at this time whether endonuclease A selected molecules which may have some superhelicity or, alternatively, double-stranded circular DNAs may be catalyzed at a slower rate. By contrast, when linear DNAs such as lambda, T7, and adenovirus-2 were used, no additional bands could be seen.

Superhelical SV40 DNA converted to linear (70%) and nicked circular (30%) forms by endonuclease A and recovered by phenol extraction was sequentially digested by one of several known restriction endonucleases. When endonuclease A-digested DNA was exposed to *Hpa* I, *Bam* HI, and *Hind*III, no modification of patterns obtained from SV40 DNA digested only by these restriction endonucleases was observed (data not shown). This suggests either that endonuclease A introduces a cut near one of the known restriction endonuclease cutting sites or that the new fragment generated is lost during separation on gels. Alternatively, endonuclease A catalyzes the conversion of superhelical DNAs to their linear forms in a similar fashion to S_1 nuclease (15); however, the endonuclease A, unlike S_1 nuclease, is inhibited by Zn^{2+} and it requires Mg^{2+} as a cofactor. In addition, unlike S_1 nuclease, it does not hydrolize tRNA and exhibits a broad pH optimum (pH 7.5–8.3). At the purity we can obtain currently, the activity is stable for several months in 50% glycerol and is stable to thermal denaturation at 45°.

Effects of the *Atu* DNA Topoisomerase, the Restriction Endonuclease *Atu* I, and DNA Endonuclease A on Plasmid and Plant DNAs. The effect of the three enzymes singularly and in different combinations were tested on plant and *A. tumefaciens* plasmid pCK135 DNAs. We carried out electrophoresis on 4% polyacrylamide gels in order to obtain well defined bands from untreated DNAs. As shown in Fig. 4 *left*, slot 1, not all of the plant DNA penetrated the gel. Essentially no effect was seen when plant DNA was exposed to endonuclease A (shown in slot 2). When plant DNA was exposed to *Atu* I, limited degradation, which generated a smear in addition to slowing the DNA mobility, was seen (slot 3); this suggests a less compact structure. *Atu* DNA topoisomerase had little effect on plant DNA (slot 4). The small amount of the intermediate band could be due to slight contamination by *Atu* I. The effect of different combinations of the three enzymes were tested. Total digestion of plant DNA was obtained (slot 6) when it was exposed to endonuclease A and *Atu* I simultaneously. A second interesting observation is the generation of several faint bands (slot 8) when plant DNA was exposed to the three enzymes.

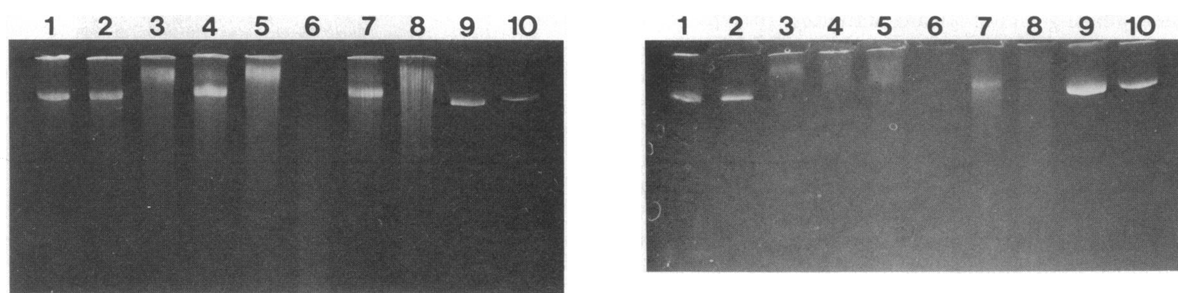

FIG. 4. Effect of three *A. tumefaciens* enzymes on *Vinca rosea* leaf DNA (*Left*) and on plasmid pCK135 DNA (*Right*). Patterns are displayed on 4% polyacrylamide gels that were run in Tris borate/EDTA buffer at 20 V/cm for 2 hr (12). (*Left*) Slot 1, plant control DNA; slots 2–8 represent treatment of plant DNA with enzymes as follows: slot 2, endonuclease A; slot 3, restriction endonuclease *Atu* I; slot 4, *Atu* DNA topoisomerase; slot 5, *Atu* I and *Atu* DNA topoisomerase; slot 6, endonuclease A and *Atu* I; slot 7, endonuclease A and *Atu* DNA topoisomerase; slot 8, *Atu* I, endonuclease A, and *Atu* DNA topoisomerase; slots 9 and 10, ØX174 RF DNA and lambda DNA, respectively. (*Right*) Slot 1, plasmid pCK135 DNA. Slots 2–8 represent treatment of plasmid DNA with enzymes as follows: slot 2, endonuclease A; slot 3, restriction endonuclease *Atu* I; slot 4, *Atu* DNA topoisomerase; slot 5, restriction endonuclease *Atu* I and *Atu* DNA topoisomerase; slot 6, endonuclease A and *Atu* I; slot 7, endonuclease A and *Atu* DNA topoisomerase; slot 8, endonuclease A, *Atu* I, and *Atu* DNA topoisomerase; slots 9 and 10, ØX174 RF DNA and lambda DNA, respectively.

Similar experiments were carried out with pCK135 plasmid. These results are shown in Fig. 4 *right*. Slot 1 displays the untreated plasmid. Plasmid pCK135 is composed of two bands one of which did not penetrate 4% polyacrylamide gels. Endonuclease A catalyzed the conversion of the nonmobile DNA to the mobile form (slot 2). The restriction endonuclease (*Atu* I) did not generate a smear (slot 3) in contrast to the result with plant DNA; however, mobility change was observed. The effect of *Atu* DNA topoisomerase (slot 4) on plasmid DNA was the formation of a smear with decreased mobility suggestive of different degrees of unwound conformations. A surprising result was the combined effect of endonuclease A and *Atu* I (slot 6) or the combined effect of all three enzymes (slot 8). In both cases, plasmid DNA appears to have been digested to a multitude of nondiscrete sizes, which resulted in a smear. Unlike the action of a single enzyme on plant and plasmid DNAs, the effect that was obtained when the two enzymes were used simultaneously may have been due to the alteration of DNA by one of the enzymes to render it as a substrate for the second enzyme. Alternate explanations such as a synergistic effect between the two enzymes, although attractive, remains to be determined.

DISCUSSION

Crown gall is a disease of dicotyledonous plants that is caused by *A. tumefaciens* (1). A remarkable property of the disease is the rapid neoplastic growth which ensues when wounds in plants are exposed to this organism. In contrast to nodules induced by rhizobia, crown gall tumors continue to proliferate in the absence of bacteria, and tumors can be maintained in axenic culture. The molecular basis of tumorigenicity for *A. tumefaciens* did not start to evolve until 1974, when several groups reported the presence of large plasmids ($>120 \times 10^6$ daltons) in tumorigenic strains of *A. tumefaciens*; nontumorigenic strains were devoid of such plasmids (16–18). That virulence is lost when the bacteria are cured of plasmids suggests that virulence is associated with the presence of such plasmids (11, 17–19). Recently, it has been hypothesized that part of the plasmid is required to cause the onset of crown gall tumors (2). This is supported by the detection of RNA sequences in tumor cells by hybridization of RNA isolated from normal and tumor cells with restriction endonuclease segments (20).

Our interest in this system has been focused on nucleic acid enzymes that may play a role in the infection process by catalytically activating tumorigenic genes present in the bacterium or, alternatively, by modifying plant host DNA. Apart from the search for abnormal DNA polymerases (21), virtually no information exists on the nucleic acid enzymes that may be involved in the mechanism of transformation. A rationale for concentrating on enzymes that affect plasmids stems from the idea that *Agrobacterium* tumorigenic plasmid or bacterial genes must be processed by either host or plant enzymes to generate the specific segments that are incorporated in plant cells and result in the tumorigenic phenotype.

We set as an initial goal to search first in *A. tumefaciens* and later in plant cells for enzymes that have the ability to interact with plasmid DNA. We monitored the effect of bacterial extracts on SV40 superhelical DNA, because the small size of SV40 DNA in comparison to the 120 Mdalton plasmid present in *Agrobacterium* clearly is an advantage for screening experiments. We were able to detect three distinct activities in *A. tumefaciens* extracts that affect superhelical SV40 DNA. The first activity is a DNA topoisomerase which has the ability to relax negatively superhelical DNAs (Fig. 1) and is inactive towards positively twisted superhelical DNAs (22). Various functions have been suggested for this enzyme in association with changes in superhelical unwinding. Such functions include DNA replication, transcription, and recombination (23, 24). With the recent report of the presence of such an enzyme in vaccinia virus, a possible function in virus encapsulation and assembly has been suggested (25). Because *Atu* DNA topoisomerase brings about changes in plasmid pCK135 and in plant DNA based on electrophoretic mobility (Fig. 4), it is attractive to speculate that the enzyme may play a role in unwinding areas of plant, host, and plasmid DNAs during the infection process.

The second activity, *Atu* I, is a type II restriction endonuclease that, based on the digestion pattern of several DNAs, appears to have the same cutting pattern as the restriction endonuclease *Eco*RII (Fig. 2). At this time we are unable to tell whether a blunt cut or a staggered one identical to that of *Eco*RII is generated (26, 27). The restriction endonuclease *Atu* I from *A. tumefaciens*, unlike the relatively unstable enzyme from *E. coli*, appears to be stable with the additional advantage of being present in relatively large yields.

The third enzyme activity, endonuclease A, appears to be unique in that it cleaves superhelical DNAs to their corresponding linear forms, but does not use as substrates linear double-stranded DNAs. The digestion appears to be a two-step mechanism, in which nicked circular forms are generated and then converted to linear forms (Fig. 3). Analysis of SV40 DNA treated with endonuclease A on alkaline sucrose gradients showed the presence of a single peak at 16 S, suggesting enzyme

action to be limited to a single cut. Codigestion of SV40 component I DNA with endonuclease A and one of several known restriction endonucleases suggested that the endonuclease cut was not introduced at a specific nucleotide sequence. The action of endonuclease A on superhelical DNAs is similar to that of S_1 nuclease, which cuts SV40 DNA resulting in its conversion to linear form (28). However, substrate specificity, optimal reaction conditions, and cation dependency for endonuclease A are different from those for S_1 nuclease.

The effect of the three *A. tumefaciens* enzymes on host pCK135 plasmid and plant DNA points to some interesting results with potential biological implications. As shown in Fig. 4, the *Atu* DNA topoisomerase appears to unwind plasmid pCK135 DNA and has an effect on plant DNA. The restriction endonuclease alters both plant and plasmid DNAs whereas the endonuclease A appears to have no effect on plant DNA but alters plasmid DNA. Combinations of these enzymes were also tested (Fig. 4), and of special interest is the combined effect of *Atu* I and endonuclease A which results in the conversion of plant and plasmid DNAs into a multitude of small fragments.

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