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NEGATIVELY SUPERCOILED DNA FROM PLANTS INFECTED WITH A SINGLE-STRANDED DNA VIRUS

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A method for isolating covalently closed circular double-stranded DNA from plants infected with the geminivirus, tomato golden mosaic virus, is described. Ethidium bromide titration showed this DNA to be negatively supercoiled with a superhelical density of - 0.062. The presence of S1 nuclease-sensitive secondary structure in the supercoiled DNA was demonstrated by its conversion to the open circular and linear DNA forms on treatment with this enzyme.

Tomato golden mosaic virus belongs to the geminivirus group of plant viruses which are characterised by their geminate morphology and genome of circular single-stranded DNA (1, 2). Several virus-specific DNA forms, in addition to viral DNA, have been detected in DNA isolated from TGMV-infected plants. These included covalently closed circular, open circular and linear forms of double-stranded DNA of genome length, double-stranded DNAs of greater than genome length, possibly concatemers, and a subgenomic, possibly defective, single-stranded DNA (3). In the present communication we report a method for isolation of closed circular double-stranded DNA from TGMV-infected plants, show that the DNA is negatively supercoiled and sensitive to S1 nuclease and determine its superhelical density. This is the first demonstration of negatively supercoiled DNA from plants infected with a single-stranded DNA virus.

MATERIALS AND METHODS

Isolation of closed circular double-stranded DNA. Cellular extracts from Nicotiana benthamiana plants infected with TGMV were obtained as described by Hamilton et al. (3) and suspended in 50 mM-tris-HCl buffer, pH 7.8 containing 0.1 M NaCl (TNE). 2 volumes of 0.2 M NaOH/1% SDS were added, followed, after 5 min at 0°C, by 1.5 volumes of potassium acetate, pH 4.8. After incubation at 0°C for 60 min the precipitate of plant DNA was removed by centrifugation

Abbreviations: TGMV, tomato golden mosaic virus; RF, replicative form.

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(10,000 g, 10 min). Viral DNA forms were precipitated from the supernatant by addition of 2.5 volumes of ethanol, recovered by centrifugation and resuspended in TNE. The density was then adjusted to ca. 1.55 g/cm^3 with solid CsCl and ethidium bromide was added to give a final concentration of 600 mg/l. The solution was then centrifuged at 32,000 rev/min at 15°C for 65 h in a Beckman SW 50.1 rotor. Fractions (0.05 ml) were collected and those containing closed circular double-stranded DNA were located by agarose gel electrophoresis and pooled. Ethidium bromide was removed by exhaustive extraction with 1-butanol saturated with water and DNA was precipitated from CsCl solution by addition of 2 volumes of water followed by 6 volumes of ethanol, collected by centrifugation, resuspended in 40 mM tris, 5 mM sodium acetate, 1 mM EDTA, 100 mM NaCl, pH 8.2 (TAE) and stored at - 20°C. The closed circular double-stranded RF I DNA of phage M 13 mp 9 was a gift from Dr. W.D.O. Hamilton.

Determination of superhelical density. Closed circular double-stranded DNA in 20 mM tris-HCl pH 7.5, containing 7 mM-$MgCl_2$, 100 mM KCl, 100 mg/l gelatin, 1 mM dithiothreitol was incubated with deoxyribonuclease I (50 mg/l) at 0°C for 45 min to convert a small proportion to the open circular form. The reaction was terminated by addition of EDTA to 10 mM. DNA was electrophoresed through 1% gels in glass tubes (80 x 6 mm) in TAE buffer and ethidium bromide concentrations ranging from 0 to 2 mg/l for 2 h at 3 volts/cm. Bands were visualised on an ultraviolet transilluminator. Superhelical density was calculated from the concentration of ethidium bromide at which the closed circular and open circular forms of double-stranded DNA comigrated as described by Johnson and Grossman (4), except that 26° was used for the DNA duplex winding change that results from the binding of ethidium bromide and the values were corrected to standard conditions as described by Bauer (5).

S1 nuclease digestions were carried out in 10 mM sodium acetate, 5 mM $MgSO_4$, 0.5 mM $ZnSO_4$, adjusted to pH 5.0 with acetic acid with DNA concentrations ranging from 10 mg/l to 150 mg/l.

RESULTS

Isolation of covalently closed circular double-stranded DNA

A DNA preparation, enriched in the intracellular forms of viral single-stranded and double-stranded DNA, was obtained from Nicotiana benthamiana plants as described by Hamilton et al. (3). A variety of methods was investigated for separating the closed circular double-stranded DNA from plant DNA and from the other virus-specific DNA forms. These included various combinations of the following: selective denaturation of plant DNA with alkali or heat, followed by renaturation by neutralisation or cooling to precipitate the plant DNA as a network (6, 7), hydroxyapatite/urea chromatography to separate single-stranded DNA and high and low molecular weight double-stranded DNA (8) and caesium chloride/ethidium bromide isopycnic centrifugation to separate closed circular from open circular and linear double-stranded DNA and single-stranded DNA (9). The procedure finally adopted consisted of selective alkaline denaturation/neutralisation followed

by caesium chloride/ethidium bromide sedimentation. Closed circular double-stranded DNA, obtained by this procedure, migrated as a single band when electrophoresed in an agarose gel containing excess ethidium bromide. Under these conditions, the DNA becomes positively supercoiled and migrates faster than linear or open circular double-stranded DNA (Fig. 1).

Titration of closed circular double-stranded DNA with ethidium bromide

Closed circular double-stranded DNA isolated from TGMV-infected plants was treated with a low concentration of DNase I to generate a small amount of the open circular form in the preparation. The electrophoretic mobility of the closed circular DNA with respect to that of the open circular form in agarose gel in the presence of different concentrations of ethidium bromide was then determined. In the absence of ethidium bromide closed circular DNA migrated faster than open circular DNA. With increasing concentrations of the dye the mobility of closed circular DNA decreased until eventually it became the same as that of the open circular form. At

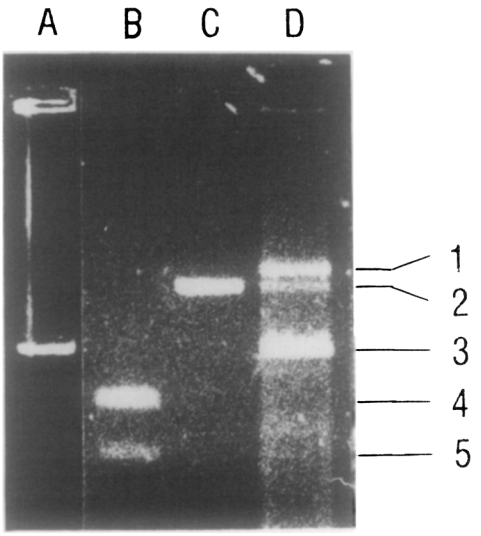

Fig. 1. Agarose gel electrophoresis of TGMV-specific DNAs. Lane A, purified closed circular double-stranded DNA (band 3); lane B, viral single-stranded DNA (band 4), containing a subgenomic single-stranded DNA (band 5)(ref. 3); lane C, linear double-stranded DNA (band 2)(refs 3, 17); lane D, closed circular double-stranded DNA digested with S1 nuclease showing the formation of open circular (band 1) and linear (band 2) double-stranded DNAs.

higher ethidium bromide concentrations the mobility of closed circular DNA relative to that of open circular DNA again increased. This behaviour is characteristic of negatively supercoiled DNA. Ethidium bromide intercalates between base pairs causing a reduction in the number of negative superhelical turns. With increasing dye concentration the DNA eventually becomes relaxed and at this equivalence point the closed circular and open circular forms have the same mobility. Further increases in dye concentration cause the DNA to become positively supercoiled and closed circular DNA again has a higher mobility than the open circular form (4).

The superhelical density of TGMV closed circular double-stranded DNA, calculated from the concentration of ethidium bromide at the equivalence point, was - 0.062 ± 0.002 (stranded error, 6 determinations). As a check of the method the superhelical density of phage M 13 RF I DNA was determined. The value found, - 0.072 ± 0.004 (5 determinations) was in good agreement with the value of - 0.071 determined by Espejo and Lebowitz (10) and corrected to standard conditions by Bauer (5).

Sensitivity of closed circular DNA to S1 nuclease

TGMV closed circular double-stranded DNA was incubated with S1 nuclease over a range of concentrations (1 to 50 units per microgram of DNA) and temperatures (4°C to 37°C) and the products were analysed by agarose gel electrophoresis at time intervals from 10 min to 100 h. In all cases disappearance of closed circular DNA was accompanied by appearance of the open circular and linear forms; prolonged digestion resulted in further degradation to give fragments not visible on the gel. The results of a digestion for 2 h at 37°C with 50 units of S1 nuclease per microgram of DNA are shown in Fig. 1.

DISCUSSION

The negatively supercoiled, double-stranded DNA isolated from TGMV-infected plants is probably an intermediate in the replication of TGMV

single-stranded DNA, analogous to the RFI molecules found in bacteria infected with single-stranded DNA phages (11). It may also be the template for virus transcription. The superhelical density, of - 0.062 is within the range of values (- 0.35 to - 0.074) reported for eukaryotic supercoiled DNAs and corresponds to ca. 16 superhelical turns in a molecule of 2540 base pairs (3). However, in vivo the DNA may well exist complexed with histones in minichromosomes analogous to those found in cells infected with eukaryotic double-stranded DNA viruses, such as SV40 (12) and cauliflower mosaic virus (13).

Negatively supercoiled DNA molecules are under constraint due to a net negative linkage relative to their relaxed topisomeric counterparts. Any process which reduces the superhelical density, e.g. cruciforms (14), transitions from B to Z DNA in the same molecule (15) and single-stranded loop structures derived from short direct repeats (16), has a favourable free energy. Such structures have been suggested as possible determinants of transcriptional regulation and all contain regions which are preferentially sensitive to S1 nuclease. The genome of TGMV consists of two DNA components of similar size, but different nucleotide sequence, except for a small region of close homology. Both components are represented in all the intracellular DNA forms found in TGMV-infected plants (17, 18). The finding that the negatively supercoiled DNA can be completely cleaved by S1 nuclease indicates that both DNA components have S1-sensitive sites. It will be interesting to determine if these sites have regulatory functions.

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