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S1-sensitive sites in the supercoiled double-stranded form of tomato golden mosaic virus DNA component B: identification of regions of potential alternative secondary structure and regulatory function

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ABSTRACT

The sensitivity of the supercoiled double-stranded form of the DNA of tomato golden mosaic virus (TGMV), a geminivirus, to the single-strand specific enzyme S1 nuclease has been demonstrated. Specific S1 cleavage sites were identified in TGMV DNA component B by cloning into the single-strand bacteriophage vector M13 mp8 and sequencing of the inserted DNA. Analysis of the DNA sequence at the sites of S1 sensitivity in TGMV DNA component B revealed several possible regions of alternative secondary structure which were clustered in an intergenic region upstream of the starts of the two major open reading frames which are in opposite orientations. This region contains putative transcriptional promoter and modulatory sequences and a possible replication origin. The extreme S1 sensitivity of the supercoiled form of TGMV DNA component A precluded its cloning under the conditions employed for selective cleavage of DNA component B.

INTRODUCTION

Negative supercoiling of DNA is a widespread phenomenon, and is found in the genomic and/or intracellular DNA forms of a number of eukaryotic DNA viruses, e.g. cauliflower mosaic virus (CaMV; 1), tomato golden mosaic virus (TGMV; 2,3), SV40 (4), bacteriophages e.g. ΦX174 (5) and plasmids e.g. ColE1(6).

DNA supercoiling has both structural and functional consequences particularly as the free energy held by supercoiled molecules could be coupled to many structural transitions including localised alterations in secondary structure. It has been known for some time that supercoiled DNA molecules can be cleaved by single-strand-specific enzymes (see 7 for references) and the accessibility of these enzymes to the DNA may be improved by local unwinding (8). Such structural perturbations may be sequence-dependent and three alternative secondary structures have been shown experimentally to be stabilised by negative supercoiling: (a) an alternating purine-pyrimidine sequence which results in the formation of Z-DNA that changes the DNA twist by reversing the handedness of the helix

(9,10,11); (b) a homopurine-homopyrimidine sequence for which a non-Z, left-handed helical structure has been suggested (12,13); (c) inverted repeats, or palindromes which have the potential to form cruciform-like structures (14-19).

Inverted repeats are often found at operator, promoter and transcription termination regions as well as in DNA replication origins, in prokaryotes and eukaryotes and may have functional significance (see 20 for references). Cruciforms have also been implicated in homologous genetic recombination and the similarity to four-way Holliday-type intermediates (21) has increased speculation on this phenomenon (22).

Local DNA sequences can also strongly influence the frequency of mutation and Glickman and Ripley (23) have proposed a model for the role of palindromic DNA as structural intermediates in deletion mutagenesis.

The genome of TGMV consists of two DNA components, A and B, of similar size (ca 2.5 Kb) but unrelated sequence except for a homologous region of about 200 bases (24). S1 cleavage sites in the negatively supercoiled double-stranded form (3) of TGMV DNA component B have now been identified and possible alternative secondary structures and regulatory roles are discussed.

MATERIALS AND METHODS

Isolation of closed circular double-stranded DNA

Cellular extracts from TGMV-infected Nicotiana benthamiana plants were obtained as described by Hamilton et al (2) and suspended in 50 mM tris-HCl, pH 7.8 containing 0.1M NaCl (TNE). Supercoiled TGMV DNA was then purified according to Sunter et al (3) by alkaline denaturation/ neutralisation followed by caesium chloride-ethidium bromide isopycnic centrifugation. Fractions 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 butanol and the DNA was ethanol precipitated.

S1 cleavage of TGMV supercoiled DNA

S1 nuclease (Sigma) reactions were performed in 10 mM sodium acetate, 5mM $MgSO_4$, adjusted to pH 5.0 with acetic acid. The concentration of S1 ranged from 1-100 units/µg DNA and reactions were carried out at 7°C and 37°C.

DNA-DNA hybridisation

DNA samples were analysed by electrophoresis on 1% agarose gels (containing 0.5 µg/ml ethidium bromide) in TAE buffer (40mM Tris, 5mM

acetic acid, 1mM EDTA, pH 8.2). Hybridisation of ^{32}P cloned DNAs with DNA samples, transferred to Genescreen (NEN) after agarose gel electrophoresis (25), was accomplished according to the procedure given in the manufacturer's manual (a modification of the procedure of Wahl et al (26)).

^{32}P -labelled probes were prepared according to the nick-translation procedure of Rigby et al (27). Probes were constructed separately, either to cloned TGMV DNA component A (930 bp EcoR1/Bam H1 fragment; (2)) or to cloned TGMV DNA component B (1080 bp EcoR1/Bam H1 fragment; (2)). These fragments lacked the 200 base pair homologous region (24) to ensure no cross-hybridisation between the two DNA components. Dot blots were used to determine the presence or otherwise of TGMV DNA inserts in S1 cut and cloned DNA. M13 DNA clones of TGMV DNA components A and B (1µl) were dotted onto strips of Pall Biodyne A transfer membrane (Pall Process Filtration) according to the manufacturer's instructions. Dot-blots were probed with labelled probes for either DNA components A or B , prepared from recombinant plasmids missing the 200 base pair homologous DNA region as above. Hybridisations were carried out according to the procedures of Wahl et al (26).

Re-hybridisation of blots was performed following exhaustive washing of blots in 1xSSC, 50% formamide for 1-2 hr at 65°C followed by prolonged exposure to X-ray film to detect any residual radioactivity. Blots were then ready for re-hybridisation which was carried out as above.

Cloning of S1-cleaved TGMV cccDNA

TGMV supercoiled DNA cleaved with S1 nuclease was blunt-ended using the Klenow fragment of DNA polymerase I (Amersham). End-filling reactions containing up to 1µg DNA were performed in 50mM Tris-HCl, pH 7.2, 10mM $MgCl_2$, 0.1mM DTT, 50 ug/ml BSA (BRL), in the presence of all four dNTPs each at a final concentration of 0.1mM and 2-3 units Klenow fragment at 37°C for 45 min. Full-length TGMV linear dsDNA was extracted from 1% agarose gels by the method of Dretzen et al (28) as previously modified (2). Kinated, synthetic oligonucleotide DNA linkers, containing a Sal 1 restriction site (NEN) were annealed to the blunt-ended TGMV DNA molecules for 24 hr at 4°C in the presence of T4 DNA ligase (kindly provided by W. Fiers). Kinase rections were carried out in 66mM Tris-HCl,pH 7.6, 10mM $MgCl_2$, 15 mM DTT, 1mM spermidine, 1mM rATP, 200 µg ml^{-1} BSA, 5 units T4 polynucleotide kinase (BRL) at 37°C for 1 hr. Kination and ligation reactions were performed sequentially by adding T4 DNA ligase to the kinase reaction mixture after 1 hr incubation using a ratio of 2 ug of kinated linkers to ca 0.4 µg TGMV

DNA. Following phenol extraction and ethanol precipitation the linkered DNA was restricted (3 hr at 37°C) with Sal 1 or Sma I (45 units) in 2mM KCl, 1mM, Tris-HCl, pH 8.0, 1mM $MgCl_2$, 0.1mM DTT, or with Sal I and Eco R1 (45 units each) in a double-digest, in 5 mM Tris-HCl, pH 7.5, 1mM $MgCl_2$, 0.1 mM DTT, 100 mM NaCl. All restriction enzymes were purchased from BRL and digestions were terminated by phenol extraction and ethanol precipitation.

Restricted TGMV DNA was annealed to an M13 mp8 vector (29) cleaved either with Sma I, Sal 1 or Sal I and Eco R1 using 3-4 ul of TGMV DNA and 10 ng vector for 24 hr at 4°C in the presence of T4 DNA ligase in the ligation buffer described earlier. E. coli JM101 cells were transformed and single-stranded (ss) template DNA was isolated as described by Messing et al. (30).

Sequencing

Sequencing was carried out using the dideoxy chain-termination method of Sanger et al (31) with ^{32}P dCTP (3000 Ci/mmol; Amersham) and pentadecamer M13 primer (NEB). The sequencing products were electro- phoresed on 6% polyacrylamide gels (32) which were dried directly onto one of the electrophoresis plates prior to autoradiography (33).

Initially the clones were subject to a sequencing reaction involving only the dTTP/ddTTP reaction and following autoradiography the T-pattern of each of the clones was compared and those exhibiting an identical pattern grouped together in classes. Autoradiography was with Kodak S1 or RR3 film at room temperature or at -70°C.

Computer analysis

The sequence of the recombinant M13 clones were mapped onto the sequence of TGMV component B (24) using the SEQFIT program (34) and the DNA was searched for inverted repeat sequences with the potential to form hairpin or cruciform structures using the HAIRPN program which recognises between 2 and 15 nucleotides in a loop and a minimum of 5 bases in a stem.

RESULTS

S1-sensitivity of TGMV supercoiled DNA

Supercoiled TGMV DNA was incubated for 2 hr at 37°C with 1, 10 and 50 units S1 nuclease/μg DNA. Gel electrophoresis of the products indicated that cleavage occurred in a two-step reaction, first on one strand to form the open circular DNA (Fig. 1A) and then on the other strand to produce linear DNA (Fig. 1B and C). At the highest enzyme concentration used approximately equal amounts of open circular and linear DNAs had been formed

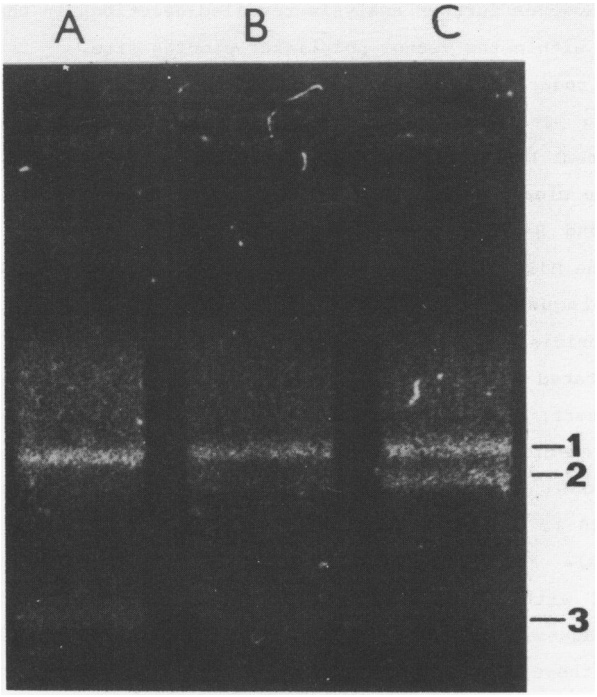

Figure 1
Agarose gel electrophoresis of the products of cleavage of supercoiled TGMV DNA with S1 nuclease for 2 hr at 37°C.
Units of enzyme/μg DNA were (A) 1, (B) 10 and (C) 50. Electrophoresis was from top to bottom and, after staining with ethidium bromide, the gel was photographed on an ultra-violet transilluminator. The bands, in order of increasing mobility are the (1) open circular, (2) linear and (3) supercoiled forms of TGMV DNA.

(Fig. 1C). When incubation was carried out at 4°C cleavage was extremely slow, even using 100 units S1 nuclease/μg DNA; after a 20 hr incubation the extent of cleavage was similar to that achieved with 1 unit S1 nuclease/μg DNA for 2 hr at 37°C (Fig. 1A).

Cloning of S1-cleaved TGMV DNA component B

Supercoiled TGMV DNA was cleaved with S1 nuclease (50 units S1/μg DNA) for 2 hr at 37°C and, after gel electrophoresis, the approximately full length DNA was extracted and blunt-ended using the Klenow fragment of DNA polymerase I. Cloning of this DNA either by direct blunt-end ligation to an M13 mp8 vector cleaved with Sma I or by cohesive-end ligation, using Sal I oligonucleotide linkers, to an M13 mp8 vector cleaved with Sal I, resulted in a number of recombinant M13 DNA molecules, all of which contained TGMV B

DNA inserts. However further analysis revealed deletions in the inserts and rearrangements within the vector polylinker cloning site.

In order to reduce the length of the DNA to be cloned into the vector, a forced cloning strategy was used in which S1-cleaved approximately full-length linear DNA molecules (produced as above) after attachment of Sal I linkers, were cleaved with EcoR1, for which there is one site in TGMV DNA components A and B (35), and Sal I. The doubly-cut molecules were then cloned using the M13 mp8 vector cut with the same enzymes. Of 401 potential recombinant plaques obtained, 144 were isolated and template DNA was prepared. Hybridisation of dot-blots to TGMV component A and B-specific probes demonstrated that all but three of these clones contained a TGMV DNA component B insert; the remaining clones contained M13 vector DNA only.

Further forced-cloning experiments were carried out as described above using Sal I/Eco R1 double-digests and TGMV supercoiled DNA cleaved with S1 nuclease for 40 hr at $7^{o}C$ (100 units S1/μg DNA) or for 2 hr at $37^{o}C$ (10 units S1/μg DNA). Following transformation 161 and 153 recombinants plaques were detected with DNA cleaved at $7^{o}C$ and $37^{o}C$ respectively and 33 recombinant plaques from each treatment were isolated. Template DNA was prepared from these clones and analysis by dot-hybridisation revealed that 31 clones from the $7^{o}C$ treatment and 30 from the $37^{o}C$ treatment contained TGMV component B DNA inserts; the remaining clones contained vector M13 DNA only. No clones containing DNA component A were obtained in any of the experiments.

T-tracking and sequencing of recombinant DNA

All clones were T-tracked in order to classify them and to determine whether specific or random S1 cleavage had occurred. Ten different classes of banding patterns were obtained. All clones within a class had identical T-tracks, indicating identical sequences. One clone representing each class was sequenced by dideoxy chain-termination procedures and the positions of the S1 cleavage sites are listed in Table 1. The cloning strategy should give rise to two clones in opposite orientations for each S1 cleavage site. These were obtained for 4 of the 6 sites. The production of clones of only one orientation for sites A and F may have been due to failure of the linkers to attach to one end of the linearised DNA; this is also reflected in the inequality of numbers of clones in opposite orientations for some of the sites. For sites B to E the ends of the pairs of clones in opposite orientations are not identical, indicating that S1 had removed a few nucleotides at these sites.

Table 1 S1-cleavage sites in supercoiled TGMV DNA component B

S1 site	Nucleotide number in DNA sequence[a]	Orientation[b]	Number of clones analysed	(Conditions of incubation)[c]
A	2277	C	35	(Z)
B	2333	C	6	(X); 6(Y)
	2344	V	7	(X); 6(Y)
C	140	C	10	(Z)
	145	V	38	(Z)
D	241	C	10	(X); 8(Y)
	248	V	8	(X); 10(Y)
E	320	C	43	(Z)
	326	V	4	(Z)
F	914	V	11	(Z)

[a] Refers to the terminal nucleotide in the cloned DNA at the S1 site

[b] Orientation: V, S1 site to EcoRI site in the virion DNA sense; C, S1 site to EcoRI site in the complementary DNA sense. The EcoRI site is at nucleotide 650

[c] Conditions of incubation: X, 100 units S1/μg DNA, 7°C, 20h; Y, 10 units S1/μg DNA, 37°C, 2h; Z, 50 units S1/μg DNA, 37°C, 2h

Potential secondary structure at the sites of S1 nuclease cleavage in TGMV DNA component B ; a potential regulatory function

To determine whether the S1 cleavage sites identified in TGMV DNA component B contained specific sequence arrangements which might form regions of DNA sensitive to S1 nuclease the TGMV DNA component B sequence was searched for potential hairpin or cruciform structures and for homopurine-homo-pyrimidine stretches in the vicinity of the cleavage sites. The results are shown in Figure 2.

DISCUSSION

Specificity of S1 cleavage

Cleavage of the TGMV cccDNA component B, like that of other negatively supercoiled DNA molecules, was highly specific. Out of 202 clones,

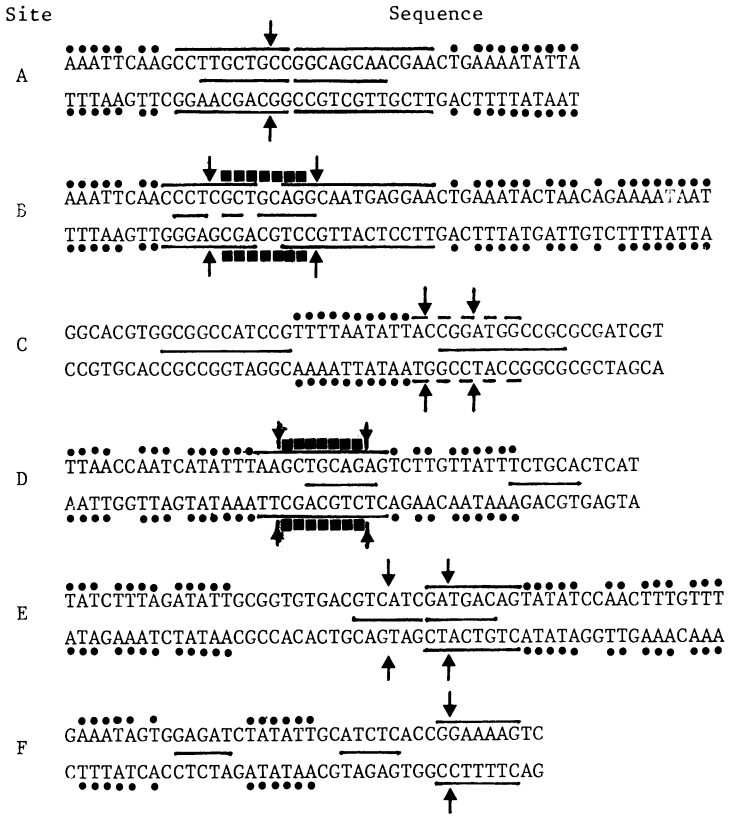

Figure 2
Positions of S1 cleavage sites in relation to inverted repeats, homopurine-homopyrimidine-rich sequences and A-T-rich stretches in the nucleotide sequence of TGMV DNA component B (24). Positions of S1 cleavages (↓) are shown on both strands, although with the cloning strategy employed it was not possible to determine on which strand the primary cleavage occurred. Sequences between the pairs of arrows were not present in the clones. Solid lines between the base pairs indicate the positions of inverted repeats. Solid lines above and below the base pairs indicate the positions of homopurine-homopyrimidine-rich (73-100%) sequences. Broken lines above and below the base pairs at site C indicates a moderately homopurine-homopyrimidine-rich (66%) sequence. Closed circles indicate the positions of A-T-rich regions. Sites A and B occur within 62 base pair imperfect repeats which are aligned. Squares indicate a seven base pair sequence which is conserved between sites B and D.

representing at least 160 independent cleavages, only 6 sites were found. This specificity was significant in relation, not only to the size of the molecule (2508 bp), but also to the reaction conditions which ranged from those in which very small amounts of linear DNA were formed to those in

which open circular and linear DNAs were formed in approximately equal amounts. Only two sites (B and D) were cleaved in reactions carried out at 7°C (or at 37°C with a lower concentration of enzyme). Such sites may be present in the supercoiled DNA, in its conformation as extracted from infected plant cells. Interestingly these sites were not detected under the conditions where the other four sites were found. This suggests that at 37°C in the presence of the higher enzyme concentration, the DNA may undergo a structural transition in which the appearance of the new sites is accompanied by the disappearance of the original ones. It is noteworthy that the original sites are flanked on both sides by the new sites (56 and 304 bases from site B; 72 and 96 bases from site D).

For 4 of the 6 sites clones of both orientations were obtained and the sequences of the ends of these clones indicated that S1 nuclease had removed 4 to 9 nucleotides at the cleavage sites (Table 1 and Figure 2). This could be due to nibbling of the ends of the DNA by the S1 nuclease after the initial cleavage, or could be due to a staggered cut on opposite strands of th DNA by S1 nuclease. The latter possibiity seems more likely since the nucleotide sequences of all the clones on each side of a site were identical indicating two specific cuts at each site. This specificity was maintained for sites B and D in which precisely the same cleavages were observed under two different reaction conditions. Evans et al., (12) recently found evidence for staggered cuts by S1 nuclease in supercoiled DNA from several mammalian sources.

It is unlikely that any S1 sites went undetected as a result of the cloning procedure. Although it has been shown that relatively long inverted repeats cannot be cloned, presumably because they interfere with plasmid DNA replication (36, 37), relatively short inverted repeats with repeat lengths of 9 - 13 bp, which are naturally present in many plasmids, do not result in plasmid instability (36). The longest inverted repeat in the sequence of TGMV DNA component B has a repeat length of only 12 bp (24), previous studies have shown that M13 clones containing this repeat are completely stable (24) and S1 cleavages within this structure (site C) were detected in the present study. Furthermore the entire sequence of TGMV component B, both as long and short fragments, has been cloned into M13 using the same E. coli strain employed in the present study and all recombinant clones were stable (24). Hence TGMV component B does not contain any "poison" sequences which could have resulted in instability of M13 recombinants and consequent selective loss of S1 cleavage products.

Structural features in the DNA sequences contiguous to the S1 sites

The specific S1 cuts in supercoiled TGMV DNA component B indicated regions of non-B secondary structure. Hence the DNA sequences contiguous to the S1 sites were analysed for their potential to form alternative secondary structures (Figure 2).

Inverted repeat sequences were found close to all the sites. Whether or not these might form hairpin-loop or cruciform structures depends on kinetic and thermodynamic considerations both of which could be influenced by interaction with S1 nuclease. The rate of formation of a cruciform, which is sequence-dependent (38), will depend on the energy of activation, a parameter which is difficult to predict in the absence of knowledge of the mechanisms of cruciform formation or the structure of the transition state. The thermodynamic stability of a cruciform structure in a negatively supercoiled DNA will be dependent inter alia on superhelical density, stem and loop lengths, temperature and ionic strength (39-44). In general stability will be directly proportional to stem plus loop length and inversely proportional to loop length; hence cruciforms with long stems and short loops will be the most stable. Lilley and Hallam (45) concluded that cruciform structures with stem lengths around 9 bp are likely to be the smallest that can be stabilised in supercoiled molecules of native superhelical density (-0.06 to -0.07), which is in agreement with the observed S1 sensitivities of cruciform structures. TGMV supercoiled DNA has a superhelical density of -0.062 (3) and only one potential stem-loop, at site C, fulfils this criterion. This ha a stem-length of 12 residues and although the loop length of 12 residues would have a destablising effect, this would be minimised by its composition of almost all A and T residues. It is surprising, however, that cleavage occurred only between the last two bases of the potential loop and between two bases in the potential stem. Generally cleavage in cruciform structures in supercoiled DNA has been found to take place centrally within the loop (46).

Scanning of the DNA sequence in the regions of the other five S1 cleavage sites revealed homopurine-homopyrimidine-rich stretches of 7 to 13 residues. At sites B and D, which were cleaved at 7^{o}C, both cleavages occurred within such stretches. At site B, referring to the viral strand, a pyrimidine-rich stretch (7/8) is followed by a purine-rich stretch (11/13), one cut being made in each stretch, whereas at site D there is one purine-rich stretch (9/11) in which both cuts are made. All of the remaining sites (A, E and F) have one cut in a homopurine-homopyrimidine-rich stretch. It is noteworthy

that sites A and B occur in homologous regions within two tandem 62 bp imperfect repeats. The one cut identified at site A is 4 nucleotides downstream of the corresponding cut at site B (Figure 2). There were no stretches of alternating purine and pyrimidine residues, characteristic of Z-DNA, in the sequence around any of the S1 cleavage sites.

It is concluded that homopurine-homopyrimidine stretches are an important feature of S1-sensitive sites in TGMV DNA component B. Even at the one site (site C), where extrusion of a cruciform was considered possible, cleavage occurred in a moderately homopurine-homopyrimidine-rich region (6 purines out of 9 residues on the viral strand). These results therefore extend to a DNA of plant origin the observation that S1 sensitivity in DNAs of vertebrate and invertebrate origin is a feature of homopurine-homopyrimidine stretches (12). Cantor et al., (13) proposed that homopurine-homopyrimidine-rich stretches in mammalian DNA adopted a non-Z, left-handed helical structure, but in the absence of supportive evidence it would be premature to conclude that such a structure was characteristic of the S1-sensitive regions in TGMV DNA component B. Since the S1 sites are not the only regions in TGMV DNA component B containing short homopurine-homopyrimidine stretches there must be additional features of the DNA sequence which are important in determining S1 sensitivity. Inspection of the DNA sequence between the pairs of cleavage points at sites B and D (the two sites cleaved at $7^{o}C$) reveals a conserved 7 nucleotide sequence GCTGCAG on the viral strand. Hence the actual sequence of the DNA may be important in determining cleavage. It is also noteworthy that all the regions containing the S1-sensitive sites were flanked on one or both sides by A-T-rich regions (Figure 2), although no cuts occurred within such regions.

Possible functional significance of the S1 cleavage sites

A striking feature of the S1 sensitive sites in the supercoiled TGMV DNA component B is the clustering of 5 of the 6 sites in a stretch of 600 nucleotides in the large intergenic region upstream of the starts of the two major open reading frames (ORFs)(Figure 3). It has been suggested that transcription of TGMV DNA (24) and that of other geminiviruses (48-50), is bidirectional and the large intergenic region is expected to contain promoters and other transcriptional regulatory sequences. Hence these S1 sites, especially sites B and D which were cleaved at $7^{o}C$, may well have functional significance. Particularly striking is the observation that site D, which is about 170 nucleotides upstream of the consensus promoter sequence closest to the start of ORF BR1 (Figure 3) has a 10 nucleotide

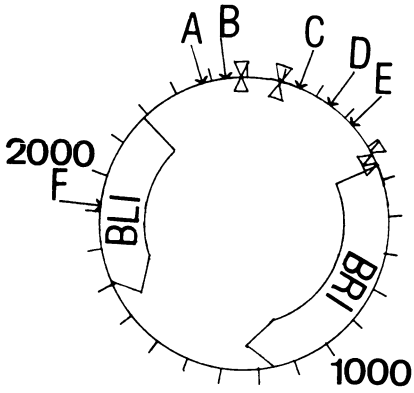

Figure 3
Positions of S1 nuclease sensitive sites in supercoiled TGMV DNA component B in relation to open reading frames (ORFs) and potential transcriptional promoters (▽) conforming to the consensus sequence TATAT/AA (47), shown on the outside and the inside of the circle in the orientations of ORFs BR1 and BL1 respectively.

perfect sequence homology with a possible control region found upstream of the promoter of a legumin gene from pea (Pisum sativum) (51) (Figure 4). Both of these sequences have partial homology to the "AGGA" box, a consensus transcriptional modulatory sequence proposed for plant genes (52). It is noteworthy that the S1-sensitive sites identified in a range of animal DNAs by Evans et al. (12) were located within about 200 nucleotides upstream of transcriptional promoter sequences.

Site C is within a stem-loop structure which has been proposed as a possible origin of DNA replication (24). However the primary role for this structure may be in the viral ssDNA, in which it has a calculated free energy of -31.6 kcal/mole (24). As discussed above, the S1 cutting pattern does not provide unequivocal evidence for a cruciform structure at site C in the supercoiled DNA.

The sequence, GGAAAAG, at site F also occurs in the starting intergenic region of the geminivirus maize streak virus (53) and corresponds closely to the enhancer "core" consensus sequence (G)TGGA/TA/TA/TG for animal viruses (54). However the location of this sequence in TGMV DNA B in the middle of ORF BL1 (Figure 3) suggests that this could be a fortuitous occurrence.

(1) T A A G C T G C A G A G T C T

(2) C A A G C T G C A G A A T G T

Figure 4
Nucleotide sequence homology of S1 nuclease sensitive site D in supercoiled TGMV DNA B (1) and a possible transcriptional modulatory sequence upstream of the legumin A gene (2). The arrows indicate the positions of S1 cleavage at site D. The 7 base sequence GCTGCAG is common also to site B (see Figure 2).

As an extension to the results reported herein it will be of interest to determine whether TGMV supercoiled DNA exists *in vivo* complexed with histones in minichromosomes analogous to those found in cells infected with eukaryotic dsDNA viruses, such as SV40 (55) and cauliflower mosaic virus (56). A comparison of DNase I, micrococcal nuclease and S1 nuclease sensitive sites in actively transcribing TGMV minichromosomes may reveal regions of potential alternative secondary structure similar to those described here.

S1 hypersensitivty of supercoiled TGMV DNA component A

It is noteworthy that no clones corresponding to S1 cleavage products of TGMV DNA component A were obtained in any of the experiments. This has been shown to be due to the much greater S1-sensitivity of DNA component A compared to DNA component B. A time course of the reaction has showed that under the conditions described here to obtain specific cleavage of DNA component B into almost full length linear molecules, DNA component A had been largely degraded into small fragments, even when less than half of supercoiled DNA component B had been cleaved (unpublished results). This indicates significant structural differences between the supercoiled forms of DNA components A and B and could be related to differences in the organisation of genes in the two molecules. Although, like DNA component B, DNA component A has two major ORFs in opposite orientations with a large upstream intergenic region, it also has additional overlapping ORFs with a separate transcriptional control region (24). The hypersensitivity of super-coiled DNA component A to S1 nuclease will form the subject of a further investigation.

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