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Characterisation of cauliflower mosaic virus DNA forms isolated from infected turnip leaves

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ABSTRACT

Several different forms of cauliflower mosaic virus (CaMV) DNA were detected in nucleic acid preparations from CaMV-infected turnip leaves. As well as supercoiled and open-circular molecules, various linear DNA structures were identified. The relative amounts of these DNA forms varied in plants infected with different CaMV isolates. Restriction enzyme mapping and one- and two-dimensional gel electrophoresis revealed the presence of linear molecules apparently formed by breaks in the second strand at each of the three discontinuities. Two major linear DNA forms are double-stranded over part of their length and appear to have single-stranded extensions of the -strand of variable length. Since these DNA forms are not produced during extraction and probably exist as unencapsidated or partially encapsidated molecules, they may represent intermediates either in DNA replication or in virion assembly.

INTRODUCTION

Cauliflower mosaic virus (CaMV) contains double-stranded DNA [for review see (1)] of about 8Kbp (2,3). The encapsidated DNA exists in several conformations ranging from full-length linear molecules to open circles and circles with varying degrees of twistedness (4,5); the cause of the twistedness is unknown. The encapsidated CaMV DNA also has three discontinuities (except CM4-184 which has two) at specific sites (Fig. 1), one strand (the -strand) having one discontinuity (gap 1 or G1) and the other having two discontinuities (G2 and G3) delimiting the - and -strands (4, 6,7). Sequencing has shown that, at least for one CaMV isolate, the discontinuities are in fact overlaps (2,8).

Studies of CaMV transcription (9) revealed that one transcript traversed G1. This led us to search infected plants for unencapsidated 'supercoiled' forms of CaMV DNA which could be the template for such transcripts. While this work was in progress, supercoiled CaMV DNA was reported by Menissier et al. (10) and by Olszewski et al. (11). In addition to supercoiled forms, we found other forms of unencapsidated CaMV DNA.These are described in this paper.

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MATERIALS AND METHODS

The CaMV isolates used have been previously described by Hull (12). They were propagated in turnip [Brassica rapa cv. Just Right] grown under glasshouse conditions; daylight was supplemented to 16h when necessary. Virus was purified as described by Hull et al. (13) and DNA was extracted from purified virus by the method of Hull and Howell (4).

Total cellular nucleic acid was extracted from systemically infected leaves, usually 18-25 days after inoculation, as described by Covey & Hull (14). The supernatant from the 3M sodium acetate precipitation stage contained RNA smaller than 5S and DNA. The nucleic acid from this was precipitated with 2.5 vol ethanol at -20°C overnight and, if necessary, the RNA was removed by RNase treatment (1 µg pancreatic RNase A/ml in 10mM Tris-HCl, 1mM EDTA pH 7.5 for 30 min at 37°C) followed by phenol extraction and further ethanol precipitation.

Cellular DNA (100µg per tube) was centrifuged through 5-20% sucrose gradients (in 50mM Tris-HCl, 5mM EDTA, 50mM NaCl pH 8.0) in a Beckman SW40 or SW41 rotor at 25,000 rpm for 18h at 4°C. The gradients were fractionated (0.4 ml fractions) using an Isco UA2 density gradient analyser. Plant and Escherichia coli ribosomal RNAs were used as markers.

Restriction endonuclease digests were as described by Hull (12). The sites of the restriction endonucleases on the CaMV genome are shown in Fig. 1. Gel electrophoresis was in 1.0% or 1.5% agarose slab gels. Neutral gels were in the Tris-acetate buffer described by Hull & Howell (4) either in a vertical apparatus run at 7V/cm for 3h or a flat-bed apparatus run at 1.3V/cm for 18h. DNA, denatured by alkali, was electrophoresed as described by McDonnell et al. (15). Two-dimensional gel electrophoresis was by the method of Favoloro et al. (16). DNA fragments were blotted onto nitrocellulose, using the conditions described by Thomas (17) and probed with CaMV DNA labelled with ^{32}P by nick-translation. Cabb B-JI DNA was usually used as a probe for all isolates as preliminary experiments showed that it gave the same results as homologous hybridizations with NZ1 and CM4-184 DNAs. Molecular weight markers were full length CaMV DNA (8Kbp) excised from a recombinant plasmid (pMD324), BglII fragments -a to -e (3328, 1861, 1149, 1085 and 452 bp) also excised from recombinant plasmids, EcoRI fragment -d excised from a recombinant plasmid and further cut with HindIII (257 and 201 bp); for single-strand markers the single-strand fragments from encapsidated CaMV DNA (Cabb B-JI) were used.

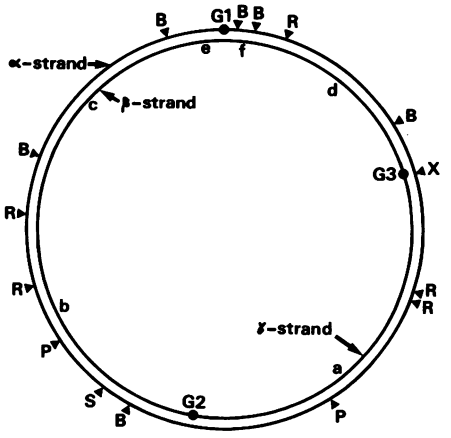

FIG. 1. Features of CaMV DNA referred to in this paper. The three discontinuities, G1 in one strand and G2 and G3 in the other, yield three single-strand fragments, α, β and γ on denaturation; a double-strand break at G1 divides β into β' and β''. The restriction endonuclease sites marked are those of BglII=B, EcoRI=R, PstI=P, SalGI=S and XhoI=X. The lower case letters refer to the BglII fragments.

RESULTS

Cellular DNA from CaMV isolates

CaMV specific DNA in total cellular DNA migrated in agarose gels as at least eight bands (Fig. 2B-D, Figs. 3,4). The banding pattern differed somewhat between isolates (Fig. 2B-D and other data, not shown) but the banding pattern of the cellular CaMV DNA of all the isolates differed markedly from that of encapsidated Cabb B-JI viral DNA (Fig. 2A); the encapsidated DNA of the other isolates gives similar patterns to that of Cabb B-JI (data not shown).

The RNase treatment described in Materials and Methods did not remove any of the CaMV bands; DNase treatment removed them all. RNA was removed from the cellular DNA preparations when quantitation was needed. From the intensity of labelling of virus-specific cellular DNA compared with known amounts of encapsidated DNA (Fig. 2) it was estimated that CaMV DNA comprises about 0.1% of total DNA and the unencapsidated DNA about 10% total CaMV DNA.

Addition of DNA, which had been extracted from virus particles, to extracts from healthy leaves from which DNA was subsequently prepared, showed that the cellular CaMV DNA forms did not result from the degradation of the encapsidated forms during extraction. Furthermore, other methods of extraction, including ones similar to those of Zimmerman and Goldberg (18), Bedbrook (19), Murray & Thompson (20) and Kislev and Rubenstein (21) gave results similar to those produced by the technique described in Materials and Methods.

The cellular DNA preparation method appeared to release little of the

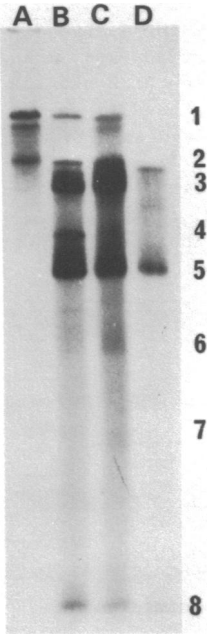

FIG. 2. Electrophoresis in 1% agarose gel of A. 5ng CaMV Cabb B-JI encapsidated DNA. B-D 5ug cellular DNA from plants infected with CaMV isolates. B. NZ1, C. Cabb B-JI, D. CM4-184. The DNA was transferred onto nitrocellulose and probed with ^{32}P-labelled CaMV Cabb B-JI DNA. The numbers on the right hand side identify bands referred to in the text.

encapsidated DNA. In three out of four purified virus preparations, the method released less than 1% of the encapsidated DNA; significant amounts of CaMV DNA were released from the fourth preparation.

The profile of CaMV-specific DNAs extracted from Cabb B-JI systemically infected leaves 8 days after inoculation (two days before symptom appearance) did not differ much from that of DNA extracted 21 and 25 days after inoculation (data not shown). There was no significant difference between DNA isolated from plants inoculated with cloned (ungapped) DNA and those inoculated with gapped virion DNA.

The cellular DNAs from two isolates were chosen for the more detailed studies on bands 1-7 described below, NZ1 because it contained all the DNA bands in reasonable proportions and Cabb B-JI because it is the 'type' isolate for studies in our laboratory. The nucleic acid in band 8 is described in another publication (22).

Sucrose gradient analysis

Cellular DNA from leaves infected with CaMV isolate NZ1 was centrifuged through a neutral sucrose gradient and fractionated. The DNA from various fractions was electrophoresed, blotted onto nitrocellulose and probed for CaMV specific sequences (Fig. 3). It can be seen from Fig. 3

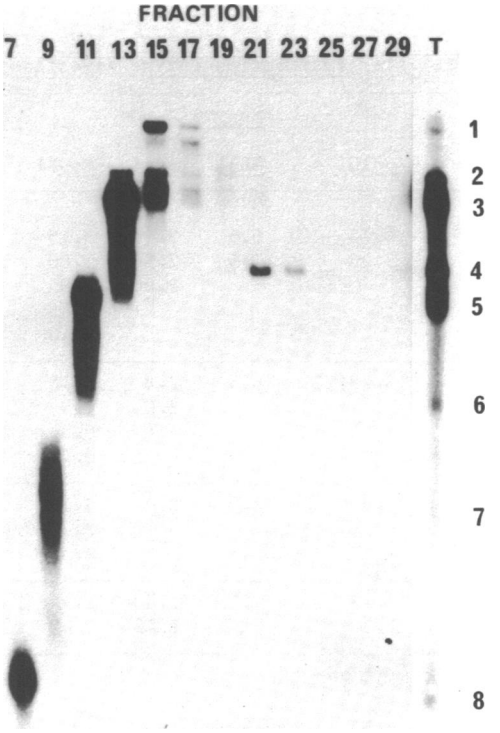

FIG. 3. Electrophoresis in 1% agrarose gel of sucrose gradient fractions of CaMV NZ1 cellular DNA. The gradient and fractionation are described in Materials and Methods. DNA was transferred to nitrocellulose and probed with ^{32}P-labelled CaMV DNA. The track T is unfractionated CaMV NZ1 cellular DNA. The numbers on the right hand side identify bands referred to in the text.

that all the CaMV-specific DNAs except band 4 DNA sediment in a manner expected for their size based on gel electrophoretic mobility. The DNA in band 4 sediments much more rapidly than might be expected.

Mapping of CaMV specific DNAs

The CaMV-specific DNAs in bands 1-7 were characterised by electrophoresis in neutral and denaturing gels. To facilitate understanding the structure of these DNAs, a map of the pertinent features of CaMV DNA (both Cabb B-JI and NZ1) is shown in Fig. 1.

1-dimensional gel electrophoresis. DNA (NZ1 isolate) from fractions 11 (predominantly band 5), 13 (predominantly band 3), 16 (predominantly band 2) and 21 (DNA band 4) of the gradient shown in Fig. 3 was collected by ethanol-precipitation and treated with various restriction endonucleases.

Table 1

Estimated number of Kbp of fragments of CaMV (NZ1) specific DNAs after restriction enzyme digestion

DNA Band	Enzyme SalG1	XhoI	PstI	EcoRI
2	4.75 3.25	6.5 1.5	3.20 2.80 2.00	3.15 2.00 1.9 0.45 0.40
3	3.25(x2)	6.5	2.8 2.0 1.6	3.15 1.9 0.8 0.45
4	8.0	8.0	8.0	3.15 2.35 0.45 2.00
5	3.25 1.0	4.25	2.80 1.45	1.9 x 2 0.45

The products were separated by electrophoresis in 1.0% neutral agarose gels and after transfer onto nitrocellulose, the CaMV specific fragments were detected by hybridisation (Table 1). The data in Table 1 suggest that CaMV DNA band 2 comprises molecules linearised at G1 and that DNA band 3 is linear molecules from G3 clockwise to G1. DNA band 4 contains all the restriction endonuclease sites found in full-length circular CaMV DNA and single-cutting enzymes convert it to full-length linear molecules. This, together with its anomalous sedimentation behaviour (Fig. 3) and its migration in alkaline gels, (see below) suggests that it is the supercoiled form of CaMV DNA described by Menissier et al. (10) and Olszewski et al. (11). The CaMV DNA in band 5 appears to be mainly linear molecules from G2 clockwise to G1. In the band 5 fraction there was also a small amount of CaMV DNA which mapped anticlockwise from G2 to G1 (data not shown).

Structural Analysis of band 3 DNA. The analysis of band 3 DNA from Cabb B-JI was extended by restriction endonuclease mapping of a sucrose gradient fraction enriched in it. This fraction contained all of band 3 and a small amount of band 2; this contrasts with the NZ1 band 3 fraction described above which was predominantly the faster migrating portion of

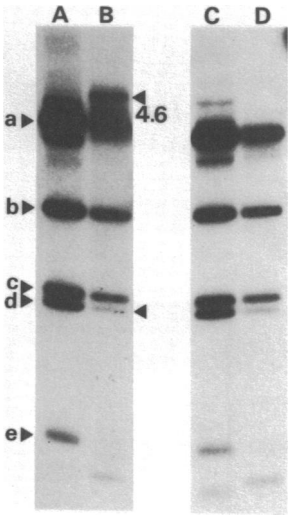

FIG. 4. Mapping of CaMV Cabb B-JI cellular DNA band 3. Track A. encapsidated DNA cut with BglII, B. band 3 DNA cut with BglII, C. encapsidated DNA cut with BglII + XhoI, D. band 3 DNA cut with BglII + XhoI. Electrophoresis in a neutral 1.2% agarose gel. The BglII fragments are designated on the left hand side; BglII-f has migrated off the gel. The arrows in the middle indicate the anomolous 4.6Kbp fragment and the diminished BglII-d fragment.

band 3. Digestion of virion DNA with BglII produces 6 major fragments (Fig. 4 track A). In contrast, band 3 DNA showed several deviations from that expected for virion DNA (Fig. 4 track B). BglII-e (0.45Kbp) of virion DNA was absent from the linear form although a small fragment of 0.36Kbp was present. Since G1 resides in BglII-e of virion DNA, the reduction in its size in the linear form would be produced by a break in the β-strand at the G1 position. BglII-d (1Kbp) is much reduced in the linear form and the faint band that is observed (Fig. 4.) is probably due to contamination of the gradient fraction by virion type DNA.

An additional BglII fragment of 4.6Kbp, which is 1.6Kbp longer than virion DNA BglII-a, was present in the linear (band 3) form (Fig. 4). This fragment is sufficiently long to accomodate BglII-a plus those fragments anticlockwise between G3 and G1 including the much reduced BglII-d. Since a XhoI site is located very close to G3 in virion DNA (see Fig. 1), double-digestion of band 3 DNA with BglII and XhoI should remove the 4.6Kbp fragment, assuming it has a XhoI site. Fig. 4 track D shows that the 4.6Kbp BglII fragment of band 3 DNA was removed by XhoI digestion with a concomitant increase in amount of the XhoI-truncated BglII-a fragment, thus confirming the origin of the 4.6Kbp fragment. We have not been able to detect the 1.6Kbp fragment from the XhoI site anticlockwise to G1 which should also be produced following digestion of the band 3 4.6Kbp fragment.

2-dimensional gel electrophoresis. In 2-dimensional gel electrophoresis, we

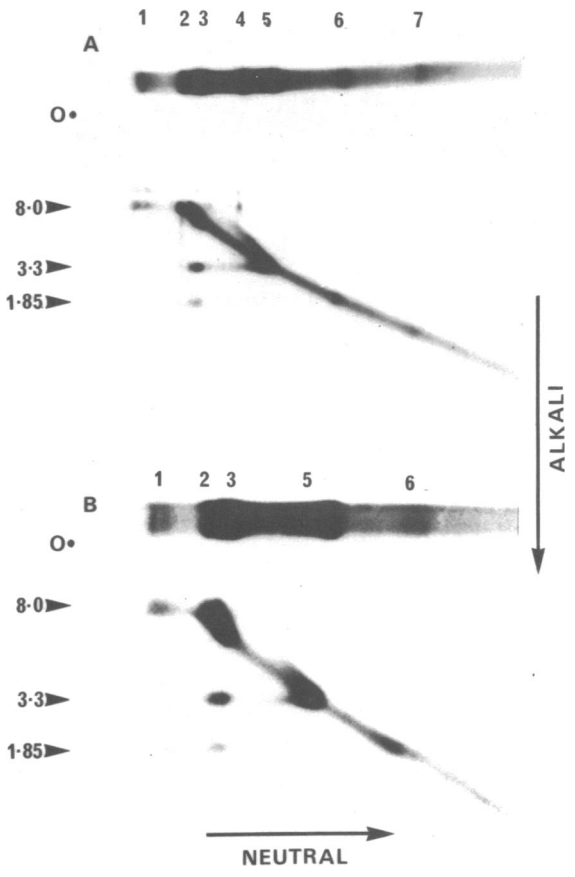

FIG. 5. Two dimensional gel electrophoresis in 1% agarose gel of CaMV cellular DNAs A. NZ1, B. Cabb B-JI. Along the top is a track run in parallel with the neutral dimension showing the positions of the double-stranded DNA bands which are numbered. The arrows on the left hand side indicate the origin (o) of the 2-D gel and the positions of the single-strand markers.

separate the double-stranded molecules in the neutral dimension; these are then denatured and the single-stranded (ss) molecules from each double-stranded form are separated in the alkali dimension. The 2-dimensional gel electrophoresis of NZ1 and Cabb B-JI specific DNAs in cellular DNA are shown in Figs 5A and B.

The DNA in NZ1 DNA band 4 gives ss molecules of two sizes (Fig. 5A), one of unit length (8Kb) and one apparently of greater than unit length. NZ1 band 1 consists of two components resolved in the neutral dimension, both of which subsequently denature to give ss molecules of unit length and

apparently greater than unit length. Cabb B-JI DNA band 1 also consists of two components in the neutral dimension. It denatures to give unit length molecules together with smaller amounts of ss molecules of the sizes of β- and γ- (Fig.5B); these are only detectable after longer exposures of the autoradiograph. Cabb B-JI cellular DNA does not contain detectable amounts of band 4 DNA.

It was suggested above that band 4 DNA is supercoiled and so the spots on 2-D gels probably arise from nicking of one strand giving unit length linear (faster-migrating) and circular (slower-migrating) ss molecules.

The DNA in band 2 of both NZ1 and Cabb B-JI cellular DNA denatures to give mainly molecules of 8Kb. There are also a series of smaller molecules derived from this band. These show in the 2-D analysis of NZ1 (Fig. 5A) and on longer exposures of the Cabb B-JI gel. The minor spots include molecules of the sizes expected for β- (5.4Kb), β- broken at G1 [3.8Kb (β') and 1.6Kb (β")] and γ- (2.6Kb). The DNAs in bands 6 and 7 give ss molecules of the sizes expected for γ- and β"- respectively.

It can be seen from Figs 2,3 and 5 that bands 3 and 5 appear in neutral gels to be more diffuse than the other bands. In 2-D gels these bands denature to give complex spots (Fig. 5A and B). Band 3 DNA comprises a range of ss molecules from 8.0-6.5Kb (elongated spot on diagonal) and elongated spots (horizontal) of molecules of the sizes expected for β'- and γ-. DNA in band 5 is of the size expected for β'- but this spot is really the point of a chevron shape made up of a diagonal line steeper than the normal diagonal and a horizontal line of β'-sized molecules. Longer exposures of autoradiographs of 2-D gels revealed other very minor spots and diagonals. Most of these have not yet been characterized but, associated with band 5 DNA are very minor spots of the sizes expected for γ- and β"- (data not shown).

To analyse the complex bands 3 and 5 further, blots of 2-D gels in which Cabb B-JI DNA had been electrophoresed were hybridised with two M13 clones which contained the α- and γ-strands of the Sau 3A fragment from nucleotide 3445-3639 (2) (kindly supplied by J.Stanley and M.Gay); these were therefore probes for the γ- and α-strands respectively. These hybridizations showed that the α-strand probe (Fig. 6A) revealed the elongated spot on the diagonal derived from band 3 and the diagonal part of the chevron associated with band 5; the horizontal part of the chevron was revealed on reprobing with complete CaMV DNA. The γ-probe lights up the γ-spot (band 6) on the diagonal,the γ-spot derived from band 3 and much of

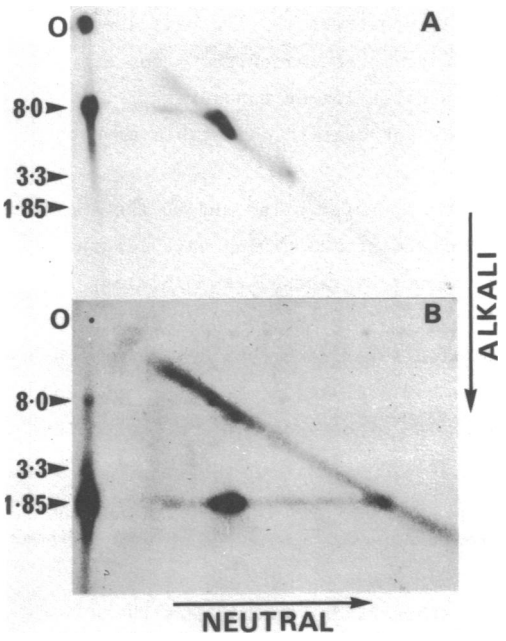

FIG. 6. Two dimensional gel electrophoresis of Cabb B-JI cellular DNA hybridized with A. a probe for α-strand, B. a probe for γ-strand; other conditions as in Fig.5.

the diagonal. The latter feature is probably due to the presence of small amounts of variable lengths of ungapped DNA complementary to the α-strand which are found in CaMV DNA preparations (S. Covey, unpublished observation).

2-dimensional gel electrophoresis of encapsidated DNA

To compare encapsidated DNA with the cellular DNA, DNA extracted from a preparation of purified CaMV Cabb B-JI particles was analysed by 2-D gel electrophoresis. In the neutral dimension of 1% agarose gels (Fig. 7A) there are many bands which are resolved on shorter exposures. In the alkali dimension, most of these bands, with the exception of some around the size expected for full length linear molecules, resolve into sizes expected for α-, β- and γ- ss fragments. Some of the double-stranded molecules of full-length linear size or slightly less also denature into a range of fragment sizes similar to that described above for cellular DNA band 3 (α 8.0-6.5Kb) and into sizes expected for β', and γ. An unusual feature of the electrophoresis of encapsidated DNA in 1% gels is that a proportion of molecules containing α-, β-and γ-fragments (and hence presumably circular) migrate faster than full-length linear molecules in the neutral dimension. This is not so apparent in 1.5% gels (Fig. 7B) in which the multiple fine banding

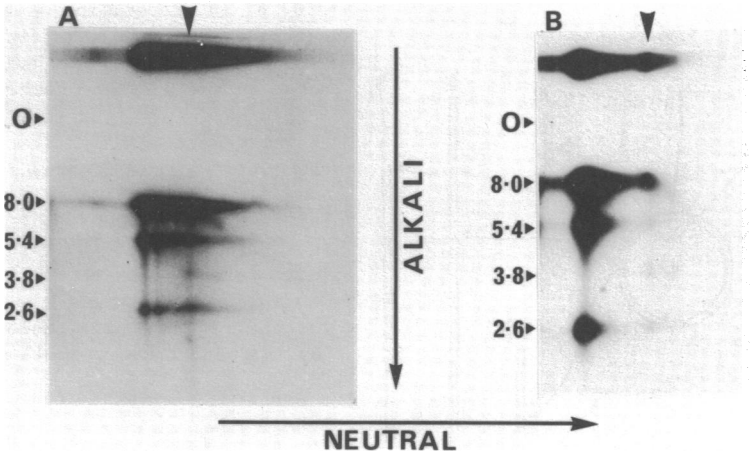

FIG. 7. Two dimensional gel electrophoresis of CaMV Cabb B-JI encapsidated DNA in A. 1% and B. 1.5% agarose gel. Along the top of each is a track run in parallel with the neutral dimension. Arrows indicate positions of 8Kbp linear molecules. The other markings are as in Fig. 4.

is also not revealed.

The 2-dimensional analysis presumably shows the different conformational forms of virion DNA. In 1% gels, the conformers of circular DNA move both slower and faster than linear molecules in the neutral dimension; in 1.5% gels they move slower. This is in accord with the previously reported behaviour of CaMV virion DNA in different gel systems (23).

DISCUSSION

There is a range of forms of CaMV specific DNA in infected cells. We recognised eight different types (bands) and report on the characterisation of seven of them in this paper. Our interpretation of how the seven relate to the full size CaMV genome is given in Fig. 8. These structures can be subdivided into three categories.

In the first category are band 1 and band 4 DNAs. There are various lines of evidence suggesting that band 4 DNA is the supercoiled CaMV DNA described by Menissier *et al.* (10) and Olszewski *et al.* (11). These include the disparity between gel electrophoretic and sedimentation behaviour (Figs. 2,3) and the results of restriction endonuclease mapping (Table 1). In alkali gels NZ1 band 4 DNA gives two spots (Fig. 5A) which it is suggested arise from the nicking of one strand of the supercoiled molecule giving unit length linear and circular molecules. It is not known if the

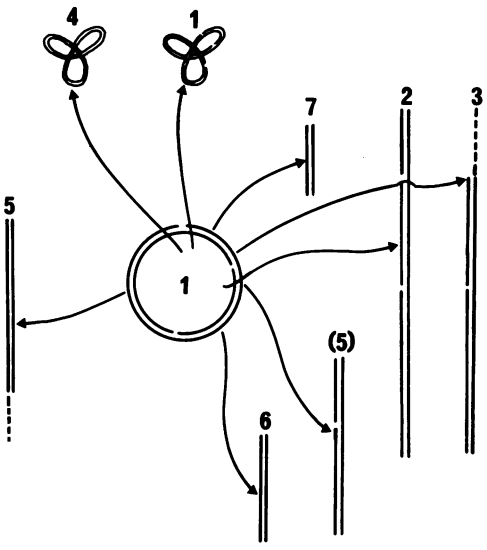

FIG. 8. Interpretation of the nature of the CaMV specific cellular DNA bands. The numbers refer to the DNA band numbers mentioned in previous Figs. and in the text. Arrows which start inside the centre circle (representing open circular encapsidated DNA) indicate full-size DNA molecules; those from outside the circle indicate the parts of the full-size DNA molecule which are the same as the smaller molecules. Molecule (5) is found in very small amounts.

nicking is random during electrophoresis or is due to the presence of alkali-labile sequences. The intensity of labelling in the two spots formed by band 4 DNA on 2-D electrophoresis is considerably less than that of the native DNA. It appears that denatured supercoiled CaMV DNA is not easily detected by this method. From its behaviour following denaturation and its migration in the neutral dimension it seems likely that NZ1 band 1 DNA is mainly a relaxed circular form with just one strand nicked; that of Cabb B-JI appears to have both strands nicked. A small proportion of Cabb B-JI band 1 DNA appears to comprise the α-, β- and γ-strands found in encapsidated DNA.

In the second category are band 2,6 and 7 DNAs which appear to be double-stranded linear molecules delimited by the gaps (Fig. 8). Band 2 DNA maps as a molecule linearized at G1. However on denaturation there are more fragments than those expected from encapsidated DNA linearized at G1. These minor fragments have not yet been mapped. Band 6 DNA is of the size expected from G2 to G3 and band 7 DNA from G3 to G1. The small proportion of band 5 DNA which maps anti-clockwise from G2 to G1 and denatures to give γ- and β"-sized fragments is probably also in this category; this is marked as (5) in Fig. 8.

The third category comprises band 3 and 5 DNAs which show anomalous behaviour on 2-D electrophoresis. The mapping of band 3 DNA suggests that it extends clockwise from G1 to G3; the presence of β' and γ supports this.

However, the 2-D analysis of both isolates showed that the α-strand in band 3 DNA ranged in length from 6.5Kb (the expected length) to 8.0Kb (Fig. 5) but, the β"-strand complementary to the variable part of the α-strand was not found. Furthermore, the restriction endonuclease mapping of band 3 DNA from Cabb B-JI isolate showed that a BglII site expected in this region (Fig. 1)was not present. These results suggest that the α-strand might be a single-strand extension of variable length anticlockwise from G3 to G1. However, in a significant proportion of band 3 molecules from Cabb B-JI, this extension of the α-strand must be completely from G3 to G1 since on digestion with BglII (Fig 4B) a discrete sized band was observed. This DNA is also encapsidated to a small extent(Fig. 7). Band 5 DNA forms an unusual chevron-like spot on 2-D analysis which can be interpreted as arising from a molecule which is partly double-stranded and partly single-stranded. The double-stranded portion is anticlockwise from G1 to G2 (giving the β'-horizontal in the chevron) and the α-strand extends from G2 towards G3 (giving the steep diagonal in the chevron).

It is obvious from Fig. 2 that the isolates differ in the relative prevalence of the various forms of CaMV DNA and that in both NZ1 and Cabb B-JI isolates bands 3 and 5 predominate. NZ1 has a relatively large amount of band 4 (supercoiled DNA) which was not detected in Cabb B-JI. A preliminary experiment reported above suggests that there is not much variation in the proportions of the various DNAs with time of infection, at least in the case of Cabb B-JI isolate. One further feature in Fig. 2 is that isolate CM4-184, which has a deletion of 421bp around G2 (and lacks G2) (4,24) does not have band 3 DNA. The DNA of CM4-184 band 2 migrates faster than that of other isolates; band 5 DNA comigrates with that of other isolates. This is consistent with the mapping of these DNA forms.

It is most likely that our DNA extraction technique, like that of Menisser *et al*. (10) does not release significant amounts of DNA from CaMV particles in purified virus preparations; the reason for one virus preparation giving much DNA is unknown. This does not preclude the possibility of the cellular CaMV-specific DNA existing as a nucleoprotein either with CaMV coat protein or with other proteins. Some preliminary evidence suggests that this might be so. The other experimental control of co-extracting purified encapsidated DNA with healthy leaf DNA indicated that the linear forms of CaMV cellular DNA did not result from cleavage of circular forms during DNA preparation. However, the possibility of the degradation of forms other than encapsidated ones cannot entirely be ruled

out.

The DNAs in the three categories described above probably arise by different mechanisms. The relaxed circular form (band 1) is most likely derived from the supercoiled form either in vivo or in DNA preparation. Bands 2,6 and 7 appear to arise from double-strand breaks at the gaps. A significant proportion of the DNA of CaMV introduced into protoplasts is broken down in a similar manner (A.Maule, pers.comm.). However, the minor spots found on 2-D analysis of band 2 DNA still require explanation. The structure of the DNAs in bands 3 and 5 is most unusual and suggests that they might be replicative intermediates or the results of defective replication. They are molecules which one might expect from a model of the replication of CaMV DNA involving reverse transcription. There is now considerable evidence to suggest that reverse transcription is involved in CaMV DNA replication (26). The DNAs in bands 3 and 5 are molecules which are predicted from a model of CaMV DNA replication involving reverse transcription.

The only CaMV DNA forms which are encapsidated are the circular molecules with discontinuities and to a small extent full-length and near full-length linear molecules (bands 2 and 3). It is possible that the shorter linear molecules are too small to make stable capsids. There appears to be a maximum size of CaMV DNA which can be encapsidated (25); CM4-184, which has a deletion of about 5%, is also encapsidated. However, the supercoiled form (band 4) is full length but not encapsidated. It might be that the tortional constraints of supercoiling or its association with chromatin (11) might affect encapsidation. Other possible constraints include the lack of discontinuities with their triple-strand structure (2,8) and its occurrence in nuclei (11).

REFERENCES

1. Shepherd,R.J.(1979). Annu. Rev. Plant Physiol 30,405-423.
2. Frank,A.,Guilley,H.,Jonard,G.,Richards,K. and Hirth,L.(1980). Cell 21, 285-294.
3. Gardner,R.C.,Howarth,A.J.,Hahn,P.,Brown-Luedi,M.,Shepherd,R.J.and Messing,J.(1981) Nucl. Acids Res. 9, 2871-2888.
4. Hull,R. and Howell,S.H. (1978). Virology 86, 482-493.
5. Hull,R.(1981).In Genetic Engineering in the Plant Sciences. N.J. Panopoulas Ed., pp. 99-109. Praeger Scientific.
6. Volovitch,M.,Dumas,J.P.,Drugeon,G. and Yot,P.(1976).In Nucleic Acids and Protein Synthesis in Plants, pp.635-641. Colloquium No. 261 CNRS Paris.
7. Volovitch,M.,Drugeon,G. and Yot,P.(1978).Nucleic Acids Res.5, 2913-2925.
8. Richards,K.F.,Guilley,H. and Jonard,G.(1981).FEBS Lett.134, 67-70.

9. Covey,S.N.,Lomonossoff,G.P. and Hull,R.(1981).Nucleic Acids Res. 9, 6735-6747.
10. Menissier,J.,Lebeurier,G. and Hirth,L.(1982).Virology 117, 322-328.
11. Olszewski,N.,Hagen,G. and Guilfoyle,T.J.(1982).Cell 29, 395-402.
12. Hull,R.(1980).Virology 100, 76-90.
13. Hull,R.,Shepherd,R.J. and Harvey,R.D.(1976).J.Gen.Virol. 31, 93-100.
14. Covey,S.N. and Hull,R.(1981).Virology 111, 463-474.
15. McDonnell,M.W.,Simon,M.N. and Studier,F.W.(1977).J.Mol.Biol. 110, 119-146.
16. Favaloro,J.M.,Treisman,R.H. and Kamen,R.I.(1980).Methods Enzymol. 65, 718-749.
17. Thomas,P.S.(1980).Proc.Natl.Acad.Sci.USA. 77, 5201-5205.
18. Zimmerman,J.L. and Goldberg,R.B.(1977).Chromosoma 59,227-252.
19. Bedbrook,J.(1981).Plant Mol.Biol.Newsletter 2, 24.
20. Murray,M.G. and Thompson,W.F.(1980).Nucleic Acids Res. 8,4321-4325.
21. Kislev,N. and Rubenstein,I.(1980).Plant Physiol 66,1140-1143.
22. Covey,S.N.,Turner,D. and Mulder,G.(1983).Nucleic Acids Res.11,251-264.
23 Hull,R.(1979).In Nucleic Acids in Plants T.C.Hall and J.W.Davies Eds., Vol 2, pp. 3-29, CRC Press Inc. Florida.
24. Howarth,A.J.,Gardner,R.C.,Messing,J. and Shepherd, R.J.(1981).Virology 112, 678-685.
25. Gronenborn,B.,Gardner,R.C.,Schaefer,S. and Shepherd,R.J.(1981).Nature, Lond. 294, 773-776.
26. Hull,R. and Covey,S.N.(1983).Trends in Biochem.Sci.(in press).