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Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components

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ABSTRACT

Linear double-stranded (ds)DNA, obtained by excision of the cloned A and B components of tomato golden mosaic virus (TGMV) from recombinant plasmids, was found to infect plants and to elicit symptoms identical to those obtained with TGMV or TGMV DNA. Progeny virus isolated from plants infected with cloned DNA was infective and indistinguishable from TGMV on the basis of (a) its circular single-stranded (ss)DNA genome, (b) its capsid polypeptide, (c) its particle morphology and (d) serological identity. Southern blot analysis of DNA extracted from cells infected with cloned DNA, or TGMV DNA, revealed the same intracellular ss and dsDNA species, represented in both A and B components, except for a subgenomic, possibly defective, DNA, which was not detected in infections with cloned DNA.

Infection with cloned DNA was achieved when cloned A and B components were both present, but not with either cloned A or B components separately. TGMV is the first DNA virus for which unequivocal proof of a bipartite genome has been obtained.

INTRODUCTION

The geminiviruses, which include tomato golden mosaic virus (TGMV), bean golden mosaic virus (BGMV) and cassava latent virus (CLV), are characterised by the geminate structure of their virions, a capsid polypeptide species of molecular weight ca. 28 000 and a genome of covalently closed circular single-stranded (ss)DNA. Restriction endonuclease cleavage of virus-specific double-stranded (ds)DNA obtained from plants infected with BGMV and TGMV suggested that the DNA of these viruses may consist of two components (2, 3). For TGMV physical separation of the two components was achieved by cloning Eco RI-cut dsDNA into the Eco RI site of plasmid pAT 153 in Escherichia coli (4). The two components were of similar size (ca. 2 600 nucleotides) but had very different restriction maps and only a small amount of sequence homology. Recently nucleotide sequence analysis has shown that CLV DNA also consists of two components of similar size

but unrelated sequence except for a highly homologous region of ca. 200 nucleotides (5). However, until now, unequivocal evidence that both DNA components are essential parts of the virus genome has been lacking for all these geminiviruses. In the present communication we show that cloned TGMV dsDNA is infectious in plants, giving rise to progeny infectious virus indistinguishable from native TGMV, except for the presence in the latter of a subgenomic, possibly defective, DNA. We further demonstrate that both cloned DNA components are required for infectivity, thereby providing unequivocal proof for the bipartite nature of the TGMV genome.

MATERIALS AND METHODS

Cloning of TGMV DNA component B in pAT 153

Covalently closed circular TGMV dsDNA was isolated from agarose gel fractionated crude TGMV DNA extracts by a modification (3) of the procedure of Dretzen et al. (6). The isolated DNA was digested with endonuclease Cla I and cloned into Cla I cut, phosphatase treated plasmid pAT 153 (7) as previously described (4). Transformants were selected on agar plates containing ampicillin. Potential recombinants from eight transformants were isolated from 1 ml "mini-prep" cultures (8) and screened by Bam HI, Eco RI and Cla I endonuclease digestion (4). Plasmids pBH 602 and 604 were selected for further use as they appeared to contain full-length component B inserts in opposite orientations. Bulk preparations of the plasmids were obtained as described (4).

Inoculation and growth of plants

DNA for inoculation was prepared by digesting recombinant plasmids with endonuclease Eco RI or Cla I depending on the site of cloning. Bulk digests were incubated at 37°C overnight with DNA concentrations of 1.6 mg/ml and enzyme concentrations of 500 units/mg DNA in 20 mM Tris, 7 mM Tris, 7 mM $MgCl_2$, 100 mM KCl, 100 mg/ℓ gelatin, 1mM dithiothreitol, pH 7.5. Digests were extracted with 1 volume of neutralised phenol, containing 0.1% 8-hydroxyquinoline, followed by extraction with 1 volume of chloroform/isoamyl alcohol (24:1) and ethanol precipitation. DNA was resuspended in TAE buffer (40 mM Tris, 5 mM acetic acid, 1 mM EDTA, pH 8.2) to a concentration of 2 mg/ml with respect to the excised insert DNA. Inoculation of Nicotiana benthamiana plants, grown to the six to eight leaf stage (9), was carried out by rubbing DNA suspension onto carborundum-dusted leaves. 50 micrograms of insert DNA per component was applied to each plant, split between the two to three youngest leaves i.e. a total of 100 micrograms of insert DNA was used when both components were inoculated simultaneously. Uninoculated

leaves were pinched off. After standing in a glasshouse overnight, plants were transferred to a Fi-totron 600 H growth cabinet (Fisons) maintained at 18°C and 75% relative humidity with a 16h photoperiod for 21 days. Inoculated leaves were removed from the plants which were stored at -70°C prior to further processing.

Preparation of cellular extracts, virus and viral DNA

Virus was isolated from infected plant material and purified by sucrose density gradient centrifugation according to the procedure of Stein et al. (10), modified to include a DNase I digestion step (11) to remove non-encapsidated DNA which cosedimented with the virus. Alternatively final purification was achieved by centrifugation in Cs_2SO_4 gradients. DNA was isolated from purified virus as described (9). Cellular extracts enriched in viral DNA (crude TGMV DNA) were prepared as described (3).

TGMV dsDNAs were isolated from crude TGMV DNA using RPC-5 analogue chromatography by a scaled down version of the manufacturer's (Bethesda Research Laboratories) 'batch method'. The dsDNA forms were found to elute from the resin with 0.6 M NaCl.

Nuclease digestions using DNase 1 (Worthington), RNase A (Sigma) and nuclease S1 (Sigma) were carried out as described previously (3).

Gel transfer hybridisation

DNA samples, electrophoresed in 1% agarose gels containing 0.5 mg/ℓ ethidium bromide in TAE buffer, were transferred onto GeneScreen membranes (New England Nuclear). Transfer with phosphate buffer, hybridisation of ^{32}P-labelled probes in the presence of dextran sulphate, subsequent probe stripping and rehybridisation were all carried out as recommended by the manufacturer. Probes were made by nick-translating recombinant plasmids pBH 604 and pBH 404 (4) with DNase 1 (Worthington) and DNA polymerase 1 (Sigma) as described (12).

Polyacrylamide gel electrophoresis

Electrophoresis of viral DNA in 4% polyacrylamide gels containing 8 M urea and 0.1% sodium dodecyl sulphate (SDS) was as described (9). Electrophoresis of denatured virus polypeptides was done in 10% SDS-polyacrylamide gels according to Laemmli (13). Marker proteins included bovine serum albumin, ovalbumin, carbonic anhydrase, trypsinogen and lysozyme.

Serology and electron microscopy

The rabbit anti-TGMV serum and method for gel immunodiffusion analysis have been described (10). Antiserum was used at $1/8$ dilution in 0.15 M NaCl. Purified virus samples were negatively stained with 1% sodium phos-

photungstate, pH 7.0 or 1% methylamine tungstate prior to electron microscopy using a Phillips EM 301 or 613 microscope.

RESULTS

Attempted infection with TGMV dsDNA components A and B cloned in the Eco RI site of pAT 153.

Nicotiana benthamiana plants were inoculated with mixtures of recombinant plasmids containing the A or B components of TGMV (4). Twelve different A and B clones were tested (three clones with each of the two possible orientations of TGMV inserts), both as intact plasmids and after excision of TGMV DNA with Eco RI, in a total of 12 pairwise A and B combinations. In no case was infection produced as judged by the failure of plants to develop symptoms 21 days post-inoculation, nor was any virus-specific DNA detected in cell extracts which were subjected to Southern blot analysis (data not shown).

It was considered possible that lack of infection with the cloned DNA might be due to the following. (a) TGMV dsDNA might not be infective. Previously only virus and virus ssDNA were shown to produce infection in N. benthamiana (9). (b) Circular, as opposed to linear, dsDNA might be required. (c) One or both of the cloned DNA components could be defective. To test (a) TGMV dsDNA, consisting mainly of the covalently closed circular form with some open circular form (3) was prepared by RPC-5 analogue chromatography and shown to be free from ssDNA by Southern blotting. When three plants were inoculated with this DNA, all developed the symptoms typical of TGMV infection within 21 days. TGMV dsDNA is therefore infective. To test (b) TGMV dsDNA was excised from recombinant A and B containing plasmids, and circularised with DNA ligase. Agarose gel electrophoresis indicated that the product contained about 90% of closed circular dsDNA (3). However no infection was obtained with this material. It was concluded that (c) was probably correct i.e. one or both of the Eco RI-cloned A and B DNA components were defective. Although 66 Eco RI DNA clones (31 A and 35 B) obtained previously appeared to be full length copies of TGMV dsDNA as judged by agarose gel electrophoresis of Eco RI/Bam Hl double digests (4), the possibility remained that component A and/or component B might carry two Eco RI sites in close proximity, resulting in a small deletion on cloning into the Eco RI site of pAT 153. A comparison of nucleotide sequence data for Eco RI-cloned TGMV component A (14) with that of CLV DNA component 1 (5) has shown that these two DNAs are partially homologous and it has been

possible to align a region of 391 nucleotides around the single Eco RI site in TGMV DNA A with the corresponding region of CLV DNA 1. Within this region TGMV DNA contained only 3 fewer nucleotides than CLV DNA. Hence it was considered unlikely that a deletion around the Eco RI site of TGMV DNA A had occurred during cloning. Sequence data for TGMV B component is not yet available and so it was decided to resolve this problem by recloning this component at a different restriction site.

Infection with TGMV Eco RI-cloned DNA component A and Cla I-cloned DNA component B

Recombinant plasmids, consisting of TGMV DNA component B linearised at its unique Cla I site (4) and inserted into the corresponding site in plasmid pAT 153, were obtained and characterised by procedures analogous to those described for the Eco RI clones (4).

When N. benthamiana plants were inoculated with mixtures of Eco RI-cloned DNA A and Cla I-cloned DNA B, excised from recombinant plasmids with Eco RI and Cla I respectively, symptoms typical of TGMV infections (leaf curl, yellow mosaic, stunting) developed after 10 days and were particularly striking after 14 days. In three independent experiments a total of 15 out of 16 plants developed symptoms. The efficiency of infection and time course of development of symptoms for cloned DNA and native TGMV DNA were the same. In contrast none of 16 plants developed symptoms when inoculated separately with Eco RI-cloned DNA A or Cla I-cloned DNA B. Similarly none of 7 plants developed symptoms when inoculated with mixtures of the recombinant plasmids from which DNA A and DNA B had not been excised, irrespective of their orientations with the plasmids.

DNA extracted from plants 21 days after inoculation with (a) a mixture of cloned DNAs A and B, (b) cloned DNA A only, (c) cloned DNA B only and (d) TGMV DNA were electrophoresed on agarose gel alongside markers of DNA from purified native TGMV and TGMV linear dsDNAs A and B, excised from recombinant plasmids. Following transfer to GeneScreen membrane the DNA was tested for homology with ^{32}P-labelled dsDNA A and dsDNA B (Fig. 1). The DNA components from TGMV-infected plants (lane 6) have been described (4) and correspond to open circular and linear dsDNA (ca. 2 600 base pairs), virus genomic ssDNA (ca. 2 600 nucleotides) and a subgenomic ssDNA (ca. 2 000 nucleotides), previously designated bands 1, 2, 4 and 5 respectively. Only the latter two components are encapsidated in virus particles (lane 8). The subgenomic DNA hybridised more strongly with DNA B than DNA A probes.

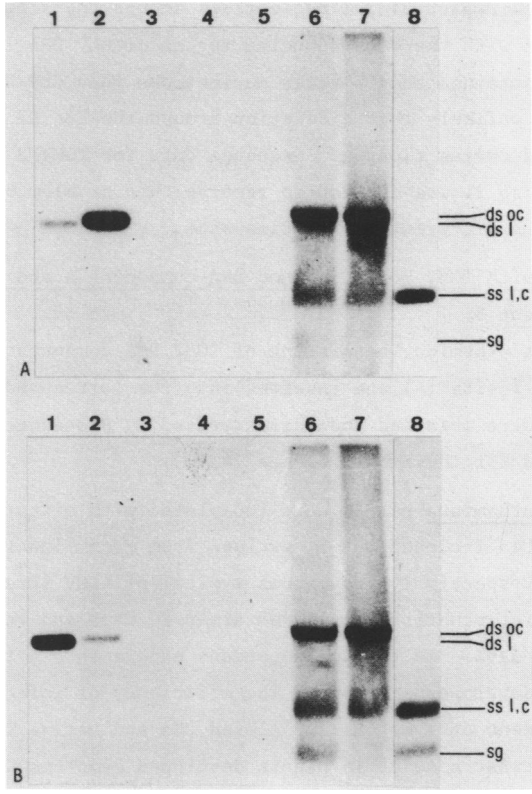

Figure 1. Autoradiograms of a blot probed with ^{32}P-recombinant plasmids containing: (A) TGMV DNA component A; (B) TGMV DNA component B. The blot was probed with component B, stripped and then probed with component A. Lanes. (1) Isolated component B insert DNA. (2) Isolated component A insert DNA. (3) Cellular extract of uninoculated healthy plants. Cellular extracts of plants inoculated with: (4) A component only; (5) B component only; (6) native TGMV; (7) mixture of A and B components; (8) DNA from purified native TGMV. DNA in tracks 6 and 7 was obtained from plants inoculated at the same time with native and cloned TGMV DNA respectively and harvested and processed concurrently. Key: (ds), dsDNA; (ss), ssDNA; (l), linear; (c) circular; (sg), subgenomic species.

Closed circular dsDNA (previously designated band 3) was not detected. It is clear that TGMV dsDNA and genomic ssDNA are also found in DNA extracts from plants inoculated with cloned DNA (lane 7) and hybridise with both DNA A and DNA B probes; however the 2 000 nucleotide subgenomic DNA could not be detected. No species homologous to the probes were detected in extracts from plants inoculated with cloned DNA A only (lane 3) or cloned DNA B only (lane 4), or in extracts from healthy plants (lane 5).

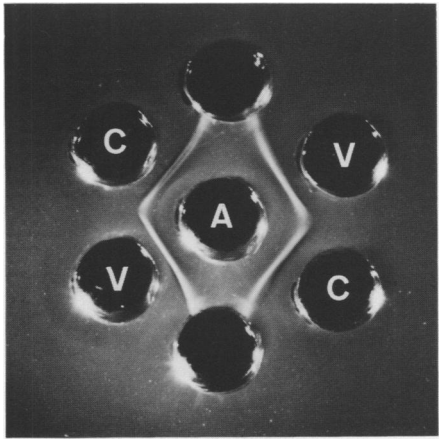

Figure 2. Gel immunodiffusion test. Wells contained: (A), antiserum to native TGMV; (V), native TGMV; (C), virus produced from infection with cloned A and B components.

Virus particles were isolated from plants infected with cloned DNA components A and B or with native TGMV DNA and purified by sucrose density gradient centrifugation. In each case a light scattering band detected about 3/4 way down the gradient, was collected and shown by electron microscopy to contain geminate particles identical in morphology to native TGMV. Analysis of the polypeptide composition of the purified virus by SDS-polyacrylamide gel electrophoresis revealed in each case a major component with a mobility identical to that of TGMV capsid polypeptide and corresponding to a molecular weight of ca. 28 000.

In gel immunodiffusion analysis virus from plants infected with cloned DNA gave a strong precipitin line when allowed to diffuse against rabbit anti-TGMV serum. When this virus and native TGMV were placed in adjacent wells and allowed to diffuse againstthe same antiserum the precipitin lines formed by each virus fused at their point of contact (Fig. 2). It was concluded that virus produced by infection with cloned DNA is serologically identical to native TGMV.

Nucleic acid was extracted from purified virus isolated from plants inoculated with cloned DNA and analysed by electrophoresis in urea-polyacrylamide gels. Bands with mobilities identical to the circular and linear ssDNA components of TGMV were obtained (Fig. 3, lane 1). These bands were resistant to RNase A but could not be detected after digestion of the nucleic

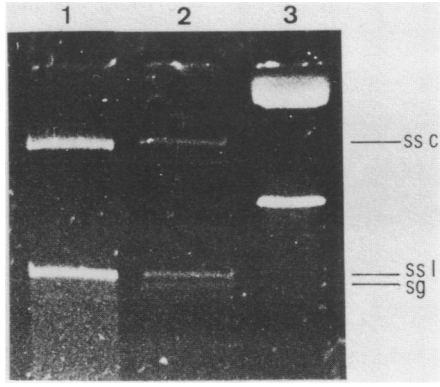

Figure 3. Electrophoresis of viral DNAs in a urea-polyacrylamide gel. Lanes. (1) DNA from purified virus produced from infection with cloned A and B components. (2) DNA from purified native TGMV. (3) Phage fd DNA (Miles). Key: (ss) single-stranded; (c) circular; (l) linear; (sg) subgenomic species. The gel was stained with ethidium bromide.

acid with DNase I or S1 nuclease prior to electrophoresis, confirming that they were ssDNA. The linear band is believed to be derived from nicking of the circular component (9) and is found in preparations of DNA from geminiviruses (15) and other viruses with circular ssDNA genomes e.g. phage fd ssDNA (Fig. 3, lane 3) in which circular and linear components are present. The subgenomic DNA, previously detected in the agarose gel of native TGMV DNA (Fig. 1), was also detected in the polyacrylamide gel (Fig. 3, lane 2).

Virus isolated from plants infected with cloned DNA was found to be infective when inoculated onto N. benthamiana plants. All of six plants inoculated developed symptoms which were indistinguishable from those of plants infected with native TGMV.

DISCUSSION

It is clear that Eco RI-cloned DNA component A together with Cla I-cloned DNA component B are fully infective and give rise to progeny virus which is indistinguishable from native TGMV, except for a subgenomic DNA found only in native TGMV. The demonstration of infection with a mixture of cloned DNA components A and B, and the inability of A or B alone to infect plants, constitutes unequivocal proof of the bipartite nature of the TGMV genome. Although bipartite and multipartite genomes are of common occurrence among RNA viruses, the vast majority of DNA viruses have undivided genomes (1). A possible exception is constituted by the virus-like particles,

found in the ovaries of parasitoid wasps, which have polydisperse DNA (16, 17) and may ultimately be shown to have multipartite genomes. However TGMV is the first example of a DNA virus for which proof of a bipartite genome has been obtained.

The failure to achieve infection with Eco RI-cloned DNA A plus Eco RI-cloned DNA B must have been due to a defect in the Eco RI-DNA B clones, since the Eco RI-DNA A clones have now been shown to be non-defective. Although isolated component B obtained after cloning in the Eco RI or Cla I sites of pAT 153 appear to have the same electrophoretic mobility in agarose gel, a deletion of less than 50 base pairs, possibly as a result of two Eco RI sites in close proximity, would be beyond the resolution of the agarose gel system employed. The nucleotide sequence of Cla I-cloned DNA B, which we are currently determining, will resolve this question.

The subgenomic DNA found in native TGMV is probably a defective DNA which arose through repeated passaging of the virus in N. benthamiana plants. Defective-interfering particles of animal viruses are known to arise from multiple passaging at high multiplicity (18). Infection of plants with cloned DNA would not be expected to produce much defective DNA at the first passage and indeed no such DNA could be detected. It is noteworthy that the subgenomic DNA hybridised more strongly with the DNA B probe than with the DNA A probe. It is possible that the subgenomic DNA arose solely from DNA B; the weak hybridisation with the DNA A probe could be due to the small amount of sequence homology between components A and B (4). Hybridisation with probes lacking sequences common to the A and B components would be required to prove this conclusively.

There are still rather few examples where infectivity of full-length viral genomic clones which give rise to a complete virus infection cycle have been demonstrated. Among the plant viruses this had only been achieved previously with cauliflower mosaic virus (CaMV) (19). It is noteworthy that intact recombinant plasmids containing full-length copies of the CaMV genome, like those with full-length copies of the TGMV genome, were not infective. In both cases the genome had to be excised with the appropriate restriction endonuclease to achieve infectivity. This is probably because, within the recombinant plasmids, the viral genomes are interrupted within an essential region. After excision it is likely that circularisation of the dsDNA is required to initiate the infection cycle. Indeed it has been postulated that the circular dsDNA forms detected in extracts from TGMV-infected plants

are intermediates in the replication of viral ssDNA (3). The demonstration that both the circular dsDNA, obtained from TGMV-infected plants, and cloned DNA derived from this intracellular dsDNA, are infective strengthens this view.

The geminiviruses have been considered as potential vectors for plant genetic manipulation (20). Two essential prerequisites for this purpose, separation of the two genome components and infection with cloned dsDNA, have been achieved in the case of TGMV.

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REFERENCES

1. Matthews, R.E.F.(1982) Intervirology 17, 1-199.
2. Ikegami, M., Haber, S. and Goodman, R.M. (1982) Proc. Natl Acad. Sci. U.S.A. 78, 4102-4106.
3. Hamilton, W.D.O., Bisaro, D.M. and Buck, K.W. (1982) Nucl. Acids Res. 10, 4901-4912.
4. Bisaro, D.M., Hamilton, W.D.O., Coutts, R.H.A. and Buck, K.W. (1982) Nucl. Acids Res. 10, 4913-4922.
5. Stanley, J. and Gay, M.R. (1983) Nature 301, 260-262.
6. Dretzen, G., Bellard, M., Sasson-Corsi, P. and Chambon, P. (1981) Anal. Biochem. 112, 295-298.
7. Twigg, A.J. and Sherratt, D. (1980) Nature 283, 216-218.
8. Ish-Horowitz, D. and Burke, J.F. (1981) Nucl. Acids Res. 9, 2989-2998.
9. Hamilton, W.D.O., Saunders, R.C., Coutts, R.H.A. and Buck, K.W. (1981) FEMS Microbiol. Lett. 11, 263-267.
10. Stein, V.E., Coutts, R.H.A. and Buck, K.W. (1983) J. Gen. Virol., in the press.
11. Gardner, R.C. and Shepherd, R.J. (1980) Virology 106, 159-161.
12. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Hamilton, W.D.O. (1983) Ph.D. Thesis, University of London.
15. Harrison, B.D., Barker, H., Bock, K.R., Guthrie, E.J., Meredith, G. and Atkinson, M. (1977) Nature 270, 760-762.
16. Krell, P.J. and Stoltz, D.B. (1979) J. Virol 29, 1118-1130.
17. Krell, P.J. and Stoltz, D.B. (1980) Virology 101, 408-418.
18. Huang, A.S. and Baltimore, D. (1977) In Comprehensive Virology, Fraenkel-Conrat, H. and Wagner, R.R. Eds., Vol. 10, pp. 73-117, Plenum Press, New York.
19. Howell, S.H., Walker, L.L. and Dudley, R.K. (1980) Science 208, 1265-1267.
20. Buck, K.W. and Coutts, R.H.A. (1983) Plant Mol. Biol., in the press.