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Molecular Cloning of the Double-stranded RNA of Beet Cryptic Viruses

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SUMMARY

Three of the four dsRNA components of purified beet cryptic virus (BCV) were copied into cDNA and cloned into pUC9. Clones corresponding to RNAs 1, 3 and 4 did not hybridize to each other or to RNA 2, suggesting that there is no significant sequence homology between the four dsRNA components. RNA extracted from 15 BCV-infected beet plants was analysed by Northern blotting using the cDNA clones as probes. Nine plants were found to contain RNAs 1, 3 and 4 whereas in six plants only RNAs 3 and 4 were detectable. The results are compatible with the occurrence of two different viruses. The sensitivity and specificity of the cDNA hybridization assay was greater than that of immunosorbent electron microscopy in the detection of BCVs.

Beet cryptic virus (BCV) has isometric particles about 30 nm in diameter and is widespread in different cultivars of *Beta vulgaris* but induces no apparent symptoms (Kassanis *et al.*, 1977). Furthermore, it is transmitted only through seed and pollen, and cannot be transmitted by mechanical inoculation (Kassanis *et al.*, 1977, 1978). BCV purified from seedlings of beet cv. Sharpes Klein E contains two proteins (mol. wt. 52 500 and 54 500) and four dsRNA components (mol. wt. 1.36×10^6 , 1.15×10^6 , 0.94×10^6 and 0.87×10^6) (Accotto & Boccardo, 1986). Beet temperate virus (BTV) found in Japan is serologically related to BCV but contains only two dsRNA components which are the same size as the two largest BCV dsRNAs (Natsuaki *et al.*, 1986). It was suggested that BCV may consist of a mixture of two viruses, tentatively named BCV1 and BVC2, and that BTV might be identical to BCV1 (Accotto & Boccardo, 1986; Natsuaki *et al.*, 1986).

The aim of this work was to obtain cDNA clones of BCV RNAs which could be used in hybridization assays to detect virus nucleic acid and to compare the sensitivity and specificity of the hybridization assay with that of immunosorbent electron microscopy (ISEM) as a means of detecting infected plants.

Sugar beet plants cv. Sharpes Klein E and cv. Regina were grown from seed in a heated glasshouse and were used as sources of the virus when they had 10 to 12 fully developed leaves. BCV was purified from batches of 200 g leaf by the method of Kassanis *et al.* (1977). RNA was extracted from purified virus by sequential extraction once with bentonite, SDS and phenol-chloroform (1:1), twice with phenol-chloroform, and twice with ether. The RNA was recovered by precipitation with ethanol, redissolved in $2 \times$ SSC (0.3 M-sodium chloride, 0.03 M-sodium citrate pH 7.2), and gel-filtered through Sephadex G-50 (fine) equilibrated in $2 \times$ SSC. Purified BCV RNA was separated into three bands by electrophoresis in 1.5% agarose (Fig. 1*a*) and the fastest migrating band was further resolved into two bands by electrophoresis in 5% polyacrylamide (Fig. 1*b*). The four bands were numbered 1 to 4 in order of increasing electrophoretic mobility. The dsRNA nature of these bands was confirmed by their resistance to DNase I digestion and resistance to RNase (T1 and A) in high salt ($2 \times$ SSC) but complete degradation by RNase in low salt ($0.1 \times$ SSC). These are the same four dsRNA components of BCV described by Accotto & Boccardo (1986), with mol. wt. corresponding to lengths of about 2060, 1740, 1420 and 1320 base pairs.

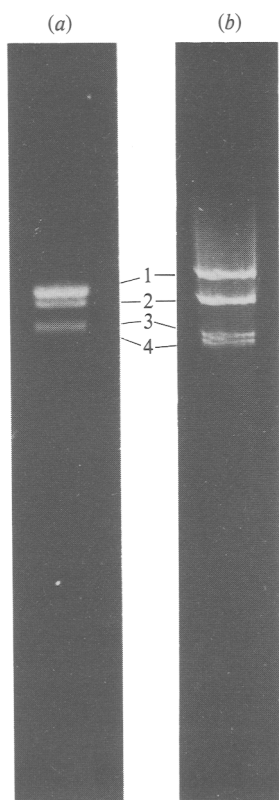


Fig. 1. Gel electrophoresis of BCV RNA. BCV RNA was electrophoresed in a 1.5% agarose gel (a) or in a 5% polyacrylamide gel (b) and visualized by staining with ethidium bromide. RNAs are numbered 1 to 4 in order of increasing mobility.

Complementary DNA copies of the BCV genome were synthesized and cloned into pUC9 essentially as described for wound tumour virus by Asamizu *et al.* (1985) but with some modifications. After polyadenylation, BCV dsRNA was denatured by incubation with oligo(dT)₁₂₋₁₈ primer in 90% DMSO at 65 °C for 30 min. The solution was cooled rapidly on ice, and the RNA and primer were co-precipitated with ethanol in the presence of 0.15 M-sodium chloride. The first-strand cDNA synthesis reaction was catalysed by reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) in the presence of 4 mM-sodium pyrophosphate. Second-strand cDNA was synthesized using RNase H, *Escherichia coli* DNA polymerase I and *E. coli* ligase (all from P-L Biochemicals) (Gubler & Hoffman, 1983). The cDNA was tailed with dCTP using terminal transferase, annealed to *Pst*I-cut, oligo(dG)-tailed pUC9 and then used to transform *E. coli* as described by Dagert & Ehrlich (1979). Plasmid DNA was isolated as described by Birnboim & Doly (1979) and characterized by Southern hybridization (Maniatis *et al.*, 1982).

pUC9 clones with inserts of more than 500 bp were analysed by hybridization techniques. The clones were grouped into three classes (A, B and C); within one class the inserts cross-hybridized, but no hybridization was observed between inserts from different classes. The clones with the largest cDNA inserts were chosen from each class (A, 1100 bp; B, 1800 bp; C, 1100 bp) and used for subsequent experiments. To determine the relationships of the clones to the different segments of the BCV genome, the four dsRNA components of the BCV preparation were separated by electrophoresis in a 5% polyacrylamide gel (Fig. 1b). The ethidium bromide-stained bands were cut from the gel and the RNA was eluted in buffer (0.5 M-ammonium acetate, 1 mM-EDTA, 0.1% SDS, pH 6.5) and precipitated with ethanol, then

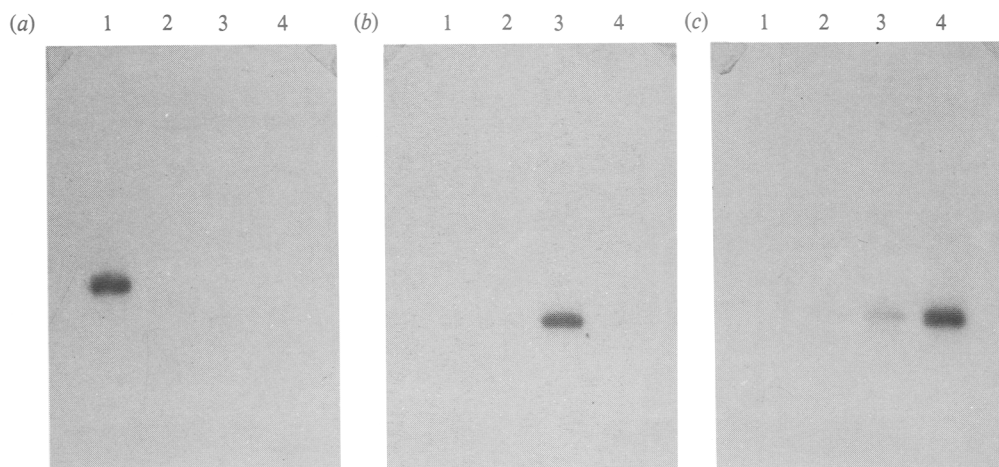


Fig. 2. Northern blots of individual BCV RNAs probed with different BCV clones. The four RNA bands shown in Fig. 1(b) were sliced from the gel and the RNA was extracted, glyoxalated and electrophoresed on 1.5% agarose gels. These were capillary-blotted onto Genescreen membranes and hybridized with ^{32}P -labelled inserts from the plasmids of three different BCV clones (A, B and C; a to c). Lanes 1 to 4 contain RNAs 1 to 4.

glyoxalated (McMaster & Carmichael, 1977), electrophoresed in agarose gels, and blotted onto Genescreen (Thomas, 1980). When these Northern blots were probed with the cDNA inserts from the three clones (Fig. 2) clone A hybridized with band 1, clone B hybridized mostly with band 3, and clone C hybridized mostly with band 4. The low hybridization of clone B with band 4 and of clone C with band 3 was probably due to cross-contamination of these bands after excision from the gel. The absence of cross-hybridization between clones A, B and C indicates that there is little homology between the nucleotide sequences of BCV RNAs 1, 3 and 4. Clones A and C correspond to 55% and 85% respectively of the estimated lengths of RNAs 1 and 4. The estimated length of clone B exceeds that of RNA 3. This may be due to the tailing procedure or to the introduction of repeated sequences during the cDNA synthesis.

Plants of two different cultivars of beet (cv. Regina and cv. Sharpes Klein E) were analysed for the presence of BCV by ISEM. The antiserum used was that prepared by Kassanis *et al.* (1977), and had a titre of 1:243. Carbon-coated EM grids were floated on 50 μl drops of BCV antiserum diluted 1:1000 in 0.067 M-phosphate buffer pH 6.5 for 30 min. The grids were washed twice by floating them on 20 ml buffer, blotted dry, and left overnight at 4 $^{\circ}\text{C}$ on 50 μl drops of sap extracted by grinding 0.1 g beet leaf material with 1 ml phosphate buffer. The grids were washed and stained with 2% phosphotungstic acid pH 6.8, before examination under the electron microscope. Five infected and five apparently healthy plants of each cultivar were found.

Total nucleic acid was extracted from leaves of the 20 different beet plants. Samples of leaf (about 0.5 g) were powdered in liquid nitrogen and homogenized in extraction buffer (176 mM-glycine, 24 mM-NaOH, 176 mM-NaCl, 20 mM-EDTA, 2% SDS, pH 9.0), extracted twice with phenol-chloroform, and twice with ether. The total nucleic acid was recovered, by precipitation with ethanol in the presence of 0.15 M-sodium acetate, and redissolved in TE buffer (10 mM-Tris-HCl pH 7.6, 0.1 mM-EDTA).

Samples of beet leaf nucleic acid (approx. 10 μg) were denatured by glyoxal (McMaster & Carmichael, 1977), electrophoresed in 1.5% agarose gels, transferred to Genescreen (Thomas, 1980) and hybridized to cDNA inserts from the three classes of clones (Fig. 3) previously labelled by nick translation (Maniatis *et al.*, 1982). Extracts from 15 of the 20 plants were found to hybridize to two or three different clones, whereas only five plants were now judged virus-free (numbers 2, 3, 5, 12 and 13 in Fig. 3). As judged from their hybridization to clones B and C, the

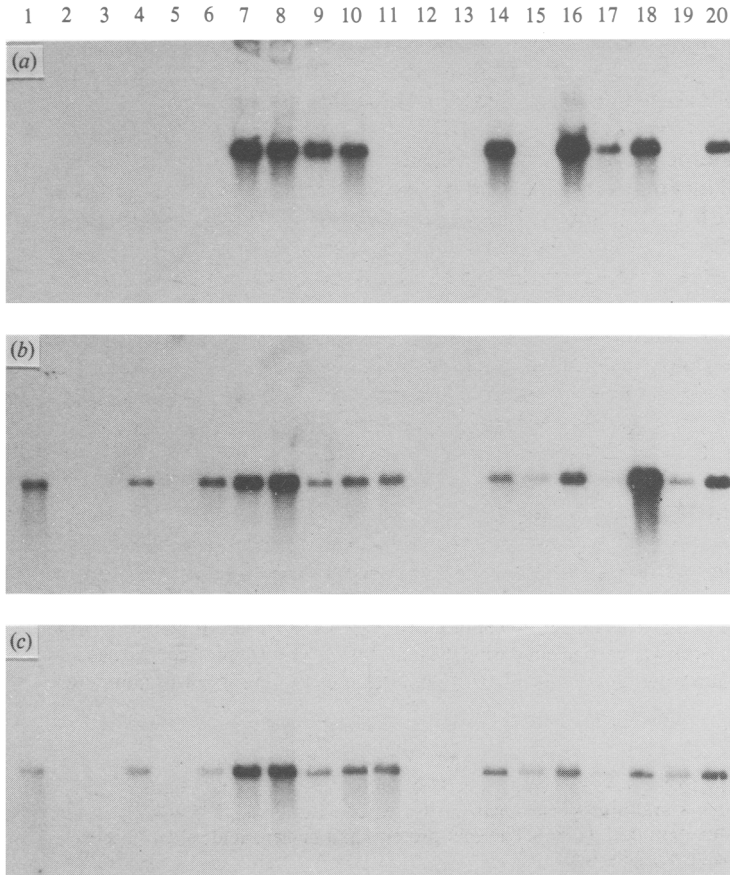


Fig. 3. Northern blots of total nucleic acid extracted from beet leaves. Total nucleic acid extracted from beet leaves was glyoxalated and electrophoresed in three 1.5% agarose gels and subsequently blotted onto Genescreen membranes. The blots were hybridized with ^{32}P -labelled inserts from the plasmids of three different clones (A, B and C, *a* to *c*). Lanes 1 to 10, extracts from beet cv. Regina; lanes 11 to 20, extracts from cv. Sharpes Klein E.

15 infected plants all contained BCV RNAs 3 and 4. In addition, extracts of nine of the 15 infected plants hybridized to the RNA 1-specific probe (clone A, numbers 7, 8, 9, 10, 14, 16, 17, 18 and 20 in Fig. 3*a*). The finding of two different patterns of infection demonstrates that RNA 1 is not required for the replication of RNAs 3 and 4 and suggests that at least two different viruses are present. Our results do not reveal whether or not RNA 1 is able to replicate independently of RNAs 3 and 4.

The results obtained are consistent with the suggestion by Accotto & Boccardo (1986) that band 1 is part of the dsRNA genome of BCV1, whereas bands 3 and 4 represent the dsRNA genome of BCV2. Since no clones have so far been identified which hybridize with band 2 it cannot as yet be definitely assigned to either virus. However, until now no cryptic viruses have been found with less than two dsRNA components (Abou-Elnasr *et al.*, 1985), and if BCV1 is identical to BTV (Natsuaki *et al.*, 1986) then band 2 would represent the second dsRNA component of BCV1. Abou-Elnasr *et al.* (1985) showed that vicia cryptic virus (VCV) contains three dsRNA components of total mol. wt. 3.8×10^6 (approx. 5820 bp) which is probably too large to be encapsidated in a single 30 nm diameter particle and they suggest that each RNA component may be encapsidated separately. BCV has a similar density (1.36 to 1.38 g/ml) and particle size (30 nm diam.) to VCV (Accotto & Boccardo, 1986), but each BCV has only two

dsRNA genome segments. The total genomic mol. wt. (BCV1 2.5×10^6 ; BCV2 1.8×10^6) are therefore probably small enough for the two dsRNA genomic segments of each BCV to be encapsidated together. The relative intensities of the bands (Fig. 1) are consistent with the view that BCV1 contains bands 1 and 2 in equal amounts whereas BCV2 contains equal amounts of bands 3 and 4. If this is correct then BCV2 has one of the smallest genomes of all RNA plant viruses, containing a total of only 2700 base pairs in the two genome segments.

Of the 20 plants that were analysed by the hybridization assay, nine were infected with both viruses, six with BCV2 alone, none with BCV1 alone, and five were not infected with either virus. Both BCVs were found in each cultivar. All of the plants infected with BCV1 were also infected with BCV2. However, the sample size is too small to suggest that this provides evidence for the dependence of BCV1 replication on the presence of BCV2.

Originally, five plants (numbered 1, 4, 11, 14 and 15 in Fig. 3) were identified as virus-free by ISEM but were found by cDNA hybridization to contain BCV2 alone or BCV1 and BCV2 (one plant, number 14). A more extended re-analysis by ISEM showed the presence of virus particles in all of these plants. In this second analysis, no virus particles were found in plants that were scored as virus-free in the cDNA hybridization assay. This indicates that detection of BCV by cDNA hybridization is more reliable than detection by ISEM.

Attempts are continuing to find clones to the fourth dsRNA segment and the clones are being sequenced to investigate the genome structure of this new group of viruses. The clones will be used to look for homologies with cryptic viruses in other plants.

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