Production of cDNA Clones from the MAV Isolate of Barley Yellow Dwarf Virus

By D. J. BARBARA,† E. E. KAWATA, P. P. UENG, R. M. LISTER AND B. A. LARKINS*

Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907, U.S.A.

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SUMMARY

A library of cDNA clones was produced from the approximately 6 kb RNA of the MAV isolate of barley yellow dwarf virus (BYDV) in bacteriophage λgt11, by a method that involved random priming and cloning ds cDNAs of between 1.0 and 2.5 kbp. Screening with antiserum to the dissociated coat protein of the MAV isolate showed that approximately 2.5% of the recombinants were capable of expressing this protein. After subcloning some inserts into the plasmid pUC18, restriction endonuclease mapping showed that they collectively represented at least 85% (a total of 5.1 kbp) of the BYDV genome. We did not attempt to determine which, if any, of the immunologically positive clones expressed the entire coat protein, but of the nine examined, all shared a region of approximately 1000 bp, located between 750 bp and 1750 bp from the 3' terminus of the restriction map. Sequence homology among different isolates of BYDV was examined by using selected MAV cDNA clones as probes in viral nucleic acid hybridization studies. Hybridization specificity varied according to the origin of the clones within the BYDV genome. Those from the putative coat protein-coding region hybridized well only to the homologous MAV isolate; those from elsewhere hybridized also with another isolate from the same subgroup (P-PAV). No clones hybridized significantly to a third isolate (RPV), which is in another subgroup of BYDV. The sensitivity of detection was related to probe size; the larger clones detected as little as 70 pg of purified virus (1.4 ng/ml in a 50 μl sample), and with these the sensitivity of virus detection in plant extracts by dot-blot hybridization was greater than that of ELISA.

INTRODUCTION

Barley yellow dwarf virus (BYDV), the type member of the luteovirus group (Matthews, 1982), comprises a cluster of interrelated viruses or viral strains that are obligately aphid-transmitted in the circulative manner. These viruses infect a very wide range of monocotyledonous hosts and collectively are regarded as the most economically important viral pathogens of small grains world-wide (Jones & Clifford, 1983). Isolates have been divided into five major types, with vector specificity as the major distinguishing characteristic (Rochow, 1970, 1979). This division is exemplified by the five isolates described by Rochow (Rochow, 1970, 1979), namely MAV specifically transmitted by Macrosiphum (=Sitobion) avenae, RPV specifically transmitted by Rhopalosiphum padi, PAV transmitted by both these vectors, RMV specifically transmitted by R. maidis, and SGV specifically transmitted by Schizaphis graminum. Furthermore, based on serology and other criteria, isolates may be grouped into two subgroups, with the isolates MAV, PAV and SGV in one and isolates RMV and RPV in the other (Aapola & Rochow, 1971; Gill & Chong, 1979; Rochow & Carmichael, 1979).

† Present address: East Mailing Research Station, East Mailing, Maidstone, Kent ME19 6BJ, U.K.
Currently, ELISA is the most widely used diagnostic method for BYDV. However, specific antisera are not widely available, and they reflect only a proportion of the viral genome although, presumably, the same part that appears to determine vector specificity (Rochow et al., 1975), i.e. that which codes for the coat protein. The use of labelled, cloned cDNAs as probes for the detection of plant viruses by dot-blot hybridization provides a sensitive alternative to serology and is, moreover, capable of detecting not only the coat protein-coding region of the genome, but also other parts. Here we describe the production of cDNA clones to the MAV isolate, and assess their usefulness as probes for the detection of homologous and heterologous isolates of the virus. Some of these results have been published as an abstract (Barbara et al., 1986). Since this work was begun, similar work with PAV-like and RPV-like isolates has been reported by Waterhouse et al. (1986).

**METHODS**

**Virus isolates.** The isolates used in this study were the MAV and RPV isolates of Rochow (1970) and the P-PAV isolate of Hammond et al. (1983), which is similar to the PAV isolate of Rochow (1970). All were maintained in oats, cv. Clintland 64, in constant environment chambers at 18 °C, and transferred as required with the calf thymus DNA (Taylor myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories; BRL) and [32p]dCTP (New England Nuclear). A second strand of cDNA was then produced by using a mixture of RNase H and DNA polymerase I as described by Huynh et al., 1985). Twenty ~tg of MAV RNA was primed with 5 ~tg of fragments of calf thymus DNA (Taylor et al., 1976) and a complementary DNA strand was synthesized by using avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories; BRL) and [32P]dCTP (New England Nuclear). A second strand of cDNA was then produced by using a mixture of RNase H and DNA polymerase I as described by Gubler & Hoffman (1983). The cDNA was then treated with S1 nuclease to remove any possible hairpin loop structures, and the cDNA was methylated using EcoRI methylase. EcoRI-compatible linkers were attached to both termini and digested with EcoRI. The tagged cDNA was then cloned into the unique EcoRI site in the bacteriophage λgt11 (Young & Davis, 1983). This vector was chosen as the initial cloning vehicle primarily because of its high cloning efficiency. It has the additional advantage of allowing expression of the cloned sequences as a chimeric protein (composed of encoded foreign protein and the λgt11 β-galactosidase protein), so that clones containing those sequences coding for viral coat protein can be identified serologically.

**Preparation and cloning of cDNA.** Except where noted below, cDNA synthesis and cloning into λgt11 were carried out as described by Huynh et al. (1985). Twenty μg of MAV RNA was primed with 5 μg of fragments of calf thymus DNA (Taylor et al., 1976) and a complementary DNA strand was synthesized by using avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories; BRL) and [32P]dCTP (New England Nuclear). A second strand of cDNA was then produced by using a mixture of RNase H and DNA polymerase I as described by Gubler & Hoffman (1983). The cDNA was then treated with S1 nuclease (to remove any possible hairpin loop structures), and the cDNA was methylated using EcoRI methylase. EcoRI-compatible linkers (dodecamers; BRL) were then ligated to the termini with T4 DNA ligase, and the linkers were digested with EcoRI. To prevent self-igation during cloning, the 5' termini of one-half of the material were dephosphorylated with calf intestinal alkaline phosphatase. Both lots of cDNA were size-selected on Bio-Gel A-50 (Bio-Rad). The first fractions containing significant amounts of cDNA (i.e. the largest available cDNA as estimated by agarose gel electrophoresis) were ligated into bacteriophage λgt11, which was digested to completion with EcoRI prior to use.

After ligation, the DNA was assembled into bacteriophage (Packagene, Promega Biotec. Inc.) and the resulting library stored at 4 °C until used. Aliquots were plated with Escherichia coli on agar plates containing 160 μg/ml isopropyl β-D-thiogalactopyranoside and 400 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as described by Huynh et al. (1985). Colourless plaques were presumed to contain recombinant bacteriophage; blue plaques were assumed to be non-recombinant.

**Screening of the library.** Approximately 600 recombinant plaques were grown on bacterial lawns and screened in two ways: (i) by hybridization to end-labelled RNA from the three BYDV isolates and (ii) by serologically screening for expression of the viral coat protein. For nucleic acid hybridization, RNA from each of the three isolates was partially hydrolysed by heating to 90 °C in 0·05 M-Tris–HCl pH 9·5, for 20 min. Fragments were then
cDNA clones of the MAV isolate of BYDV

labelled with $^{32}$P at the 5' terminus with T4 polynucleotide kinase (BRL) (Rezaian et al., 1983). An antiserum (F. Fattouh, D. J. Barbara & R. M. Lister, unpublished results), raised against MAV-BYDV dissociated by alkali treatment (Disco et al., 1986), was used for serological screening as described by Huynh et al. (1985), except that the protein blocking step was omitted. Non-specific binding to nitrocellulose was prevented by carrying out all steps after transfer of proteins from the plaques, other than the incubation with substrate, in the presence of 0:1 to 0:15% Tween-20 (C. Burkitt, personal communication).

Subeloning. Selected inserts prepared from plate stocks of λgt11 (Maniatis et al., 1982) were subcloned into pUC18 (Vieira & Messing, 1982) for further study and use as probes.

Preparation and labelling of plasmids. Plasmids were normally prepared from overnight liquid cultures by a combination of standard procedures involving SDS/NaOH lysis, salt precipitation of contaminating RNA and precipitation of the plasmid with polyethylene glycol (Maniatis et al., 1982). DNA preparations were pure enough to be cleaved with all restriction endonucleases tested. Hybridization probes were made by nick translation of plasmid DNA (Maniatis et al., 1982), usually to asp. act. of approx. 2 × 10$^8$ c.p.m./µg DNA.

Dot-blot hybridization. Purified virus of known concentration (estimated spectrophotometrically), tRNA and pUC18 were diluted as required in 0:1 M-sodium phosphate pH 6:5, prior to immobilization on nitrocellulose. Samples (50 µl) were applied with the aid of a Hybri-Dot manifold (BRL) onto nitrocellulose that had been wetted in 2 × SSC (1 × SSC is 0:015 M-sodium citrate, 0:15 M-sodium chloride). Blots were air-dried, baked under vacuum for 2 h at 80 °C and then prehybridized for 20 h at 68 °C in 5 × SSC, 50 mM-sodium phosphate pH 6:5, 250 µg/ml calf thymus DNA, 0:1% SDS and 5 × Denhardt’s solution (Thomas, 1980). Hybridization was in 5 × SSC, 20 mM-sodium phosphate pH 6:5, 100 µg/ml calf thymus DNA, 0:1% SDS, 1 × Denhardt’s solution and 5% dextran sulphate at 68 °C for 24 h. Blots were washed twice at room temperature for 10 min and twice at 68 °C for 10 min in 1 × SSC, 0:1% SDS. After washing, blots were exposed to film with intensifying screens at −80 °C.

Leaf tissue was ground with the aid of carborundum in 0:1 M-sodium phosphate pH 6:5 (1 g tissue/2 ml of buffer). The extract was expressed through cheesecloth and clarified by a brief (2 min) spin in a microfuge. Hybridization was essentially as described above except the hybridization buffer contained 1 × SSC instead of 5 × SSC and the blots were washed twice for 20 min at room temperature, and then twice at 68 °C for 30 min in 0:1 × SSC, 0:1% SDS.

ELISA. Standard methods as described elsewhere (Lister & Rochow, 1979) were used for double antibody sandwich ELISA.

RESULTS

Production of library

After size-fractionation of the cDNAs by chromatography through Bio-Gel A-50 and electrophoresis through 0-6% agarose, autoradiography indicated that the total size range of the cDNA was from 0-1 kbp to more than 3-0 kbp. The fractions cloned into λgt11 ranged in size from 1 to 3 kbp and contained roughly 30 ng of cDNA. Trial platings suggested that the total library contained 5 × 10$^4$ recombinant bacteriophages from the material which was not dephosphorylated, and 5 × 10$^3$ from that which was dephosphorylated.

Screening of library

Eighty percent of λgt11 clones hybridized to labelled RNA from both the MAV and P-PAV isolates. Approximately 15% of the clones hybridized only to MAV RNA; 5% hybridized with RNA from both MAV and P-PAV and also appeared to show very slight hybridization with RNA from the RPV isolate. By serological methods, 15 of 600 (2:5%) clones were found to express immunologically recognizable coat protein.

Size estimation of inserts and subeloning into pUC18

The sizes of the insert in nine of the clones capable of expressing the coat protein, and in 22 other clones chosen to represent the three different classes of hybridization response (MAV only; MAV and P-PAV; MAV, P-PAV and minimally RPV), were estimated by digesting bacteriophage DNA from plate lysate preparations (Maniatis et al., 1982) to completion with EcoRI, followed by electrophoresis through a 0-8% agarose gel.

Internal EcoRI sites were present in some clones, but no more than two in any single clone. With one exception, which was 0-5 kbp in length, the clones ranged from 1-0 to 2-5 kbp (average 1-62 kbp) with no differences among the three hybridization classes. Inserts from immunologically positive clones ranged in size from 1-0 to 2-0 kbp. In the initial immunological screening,
clones varied in apparent levels of antigen production but this was not correlated with the size of the viral DNA insert.

All of the immunologically positive clones and nine others (three from each hybridization class) were subcloned into pUC18. Where EcoRI excised inserts from the bacteriophage DNA in more than one piece, all recognizable fragments were subcloned. This, therefore, produced clones containing inserts ranging in size from 0.35 to 2.4 kbp.

**Restriction mapping**

All of the subcloned inserts were mapped using a set of restriction enzymes, and a composite representation approximately 5.1 kbp long, equivalent to 85% of the genome, was constructed (Fig. 1). The relative orientations of the two major segments of our map were deduced from the λgt11 clone 6−, from which the three pUC18 clones 6−, 6s1− and 6s2− were all derived, and which spanned the two internal EcoRI sites near the centre of our map (Fig. 1). Correct placement and orientation of viral sequences in the λgt11 vector are necessary to obtain the synthesis of a chimeric protein composed of λgt11 β-galactosidase and immunologically reactive viral coat protein. Since the 3' to 5' orientation of λgt11 is known, the 3' to 5' orientation of the viral genome was deduced by digestion of an immunologically positive λgt11 clone with the restriction enzymes BamHI and SacI. These enzymes digest the insert and the vector so as to give recognizable unequal fragments from the ends of the inserts and adjoining vector sequences.

Because random priming was used during the initial cDNA synthesis, the 3' terminus of the genome would not be represented in our library. It is not known whether any of the clones contain the 5' terminal sequence of the genome.

Those clones found capable of expressing the coat protein (Fig. 2) shared a common region of 1000 bp, lying between 750 and 1750 bp from the 3' end of the restriction map. We did not attempt to determine which (if any) of the clones were expressing the complete coat protein, but as only about 650 bp would be required for a protein of this size (mol. wt. 23500; Scalla & Rochow, 1977) most of the coat protein sequence probably occurs within this common region. On the basis of the available data, we cannot precisely ascribe a position on the genome to the coat protein gene.
Probes for the detection of BYDV

The ability to detect and distinguish different BYDV isolates in dot-blot hybridization was assessed with clones collectively representing the entire length of the genome (Fig. 3). The sensitivity of detection for purified virus in dot-blot hybridization was proportional to the size of cDNA insert up to 1.5 kbp for all probes tested. Usually a total of 200 pg of homologous purified virus (50 μl of 4 ng/ml) was readily detected, and at best, 70 pg (50 μl of 1.4 ng/ml) of virus gave a clearly visible reaction. The ability to detect the P-PAV isolate varied with the clone used as a probe. Clones that originated from the putative coat protein-coding area and bordering region (Fig. 2) were most specific, hybridizing well only to the homologous isolate, whereas clones from the remainder of the genome reacted equally well to both MAV and P-PAV isolates (Fig. 3, 4). A single small clone, 6s 1-, which originated away from the coat protein region (Fig. 1) reacted more strongly with MAV than with P-PAV, but was not as specific as clones from the coat protein region (Fig. 3). This has been omitted from Fig. 4 for clarity. No clones hybridized to the RPV isolate even when a virus concentration of 1 μg/ml (50 μl) was used. This result appeared to contradict the earlier results (see above) that indicated that labelled RPV RNA hybridized slightly to some λgt11 clones in plaque replicas, in the reactions used as the basis for selecting clones for further studies. We assume that these weak reactions were artefacts, eliminated in the reverse procedure of hybridizing these clones (now in pUC18) to immobilized RPV RNA as the target species.

Clone specificities were not significantly affected when the stringency of hybridization was reduced by lowering reaction temperatures by as much as 25 °C (data not shown). In addition, hybridization in 50% formamide at 42 °C did not alter hybridization patterns (data not shown). No clones hybridized to E. coli tRNA at concentrations equivalent to those of the viral RNA, nor did non-recombinant pUC18 DNA hybridize to either viral RNA or tRNA (data not shown).

The detection of virus in infected plant tissue was investigated by dot-blot hybridization, using an immunologically negative clone, 17– (2.2 kbp), as the probe. Virus was detected in extracts of infected plants to a dilution endpoint of 1/256. In comparison, ELISA detected virus to a dilution endpoint of about 1/64 (Fig. 5). It thus appeared that the dot-blot hybridizations were about four times more sensitive than ELISA, but 200 μl of extract was used for ELISA tests and only 50 μl for dot-blot hybridizations. It is likely that the sensitivity of hybridization could be further increased by using larger volumes of extract. A point of interest was that in dot-blot
**Fig. 3.** Dot-blot hybridization of BYDV isolates with various MAV cDNA clones. The viruses and control nucleic acids indicated were probed with cDNA clones representing different parts of the MAV genome (see Fig. 1, 2) (a) MAV 6s1 - (a 0.5 kbp cDNA insert in pUC18), (b) MAV 7+ (a 1.2 kbp cDNA insert in pUC18) and (c) MAV 17- (a 2.2 kbp cDNA insert in pUC18). For the blots probed with MAV 17- and MAV 7+, the viruses were used in a twofold dilution series from 1 μg/ml. For the blots probed with MAV 6s1- (which was done in a separate experiment) the viruses were used in a threefold dilution series from 1 μg/ml. Transfer RNA at 1 μg/ml and 0.5 μg/ml, and pUC18 DNA at 0.5 μg/ml and 0.25 μg/ml, were used as negative and positive controls, respectively. Five × 10^5 c.p.m. of probe was used per ml of hybridization buffer. Lanes 1, tRNA; lanes 2, pUC18; lanes 3, RPV; lanes 4, MAV; lanes 5, PAV.

**Fig. 4.** Specificity of hybridizations to BYDV isolates. The relative specificities of ten MAV clones as classified by dot-blot hybridizations were as follows: M = P, detection of MAV and PAV was equivalent; M >> P, detection of MAV was more than 27-fold greater than for PAV. Another clone, 6s1 - (see Fig. 1, 3) was found to have an intermediate specificity of hybridization. No clones reacted to RPV. Enzymes used were as in Fig. 1.
hybridizations, optimum signal density occurred with diluted extracts (Fig. 5), indicating the presence of inhibitory substances in concentrated sap. Attempts were made to improve the sensitivity of detection by treating extracts in various ways, but they were either ineffective or reduced the hybridization signal. Treatments tested included varying the concentration of the phosphate buffer used to extract the virus, extracting the clarified homogenate with chloroform, toluene or phenol/chloroform, and incubating the plant extract in the presence of 0·5% SDS, proteinase K and NaOH.

**DISCUSSION**

The use of cDNA clones as probes for the sensitive detection of plant viruses and viroids is well established (Owens & Diener, 1981; Maule et al., 1983; Baulcombe et al., 1984). ELISA provides a rapid and sensitive method for the detection of BYDV (Lister & Rochow, 1979) but suitable antisera are not always readily available. The results of this study establish that detection by nucleic acid hybridization offers a feasible alternative.

We have used the efficient bacteriophage λgt11 vector to produce a library of cDNA clones from the MAV isolate of BYDV described by Rochow (1970). Prior to insertion into the vector, the ds cDNA was size-selected and, as a result, the clones were of moderate size (1 to 2.5 kbp,
equivalent to from one-sixth to over one-third of the viral genome) and, in general, significantly larger than those produced from other isolates in a similar study (Waterhouse et al., 1986). The clones identified serologically as containing coat protein-related sequence shared a common region of 1000 bp (the coat protein would require about 650 bp of coding potential) and this common area was assigned to a region towards, but not at, the 3' end of our set of overlapping clones.

The fundamental distinguishing characteristic of the isolates of Rochow (1970) is vector specificity, which is apparently determined by the coat protein of the virus (Rochow et al., 1975). Therefore, it is reasonable to assume that the part of the genome coding for this protein will tend to be isolate-specific. It is probable that other parts of the genome are more conserved among BYDV isolates, and possibly even among other luteoviruses. This is consistent with our finding that probes derived from the part of the MAV genome coding for the coat protein hybridized well only to the homologous isolate, whereas those from the remainder of the genome also reacted to the P-PAV isolate (which is in the same subgroup of BYDV). The degree of cross-hybridization between these two isolates was dependent on the probe's position within the BYDV genome. However, using probes that collectively covered 85% of the genome, we did not obtain cross-reaction to a member of the other subgroup of BYDV, RPV. This result differs from those of Waterhouse et al. (1986), some of whose clones derived from isolates resembling PAV and RPV hybridized to viral RNA from the heterologous isolates across the group division, and also with other luteoviruses.

In dot-blot hybridization, our probes could routinely detect 200 pg of purified virus in a 50 µl sample volume (4 ng/ml), and virus was detected in plant extracts more sensitively than with ELISA. However, for individual clones, sensitivity was related to the size of the insert. This size relationship may explain why Waterhouse et al. (1986) could not detect less than 1 ng of purified virus. Further work is in progress to characterize cDNA libraries developed from the P-PAV and RPV isolates of BYDV, and to assess selected cDNAs as probes in diagnosis.

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REFERENCES


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