Etiology

Horseradish Latent Virus, a New Member of the Caulimovirus Group

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This research was supported by National Science Foundation grant PCM-8342878, USDA CRGO grant CRCR-1-1501, and grant 4E031 from the Kentucky Tobacco Research Board.
Accepted for publication 26 February 1986 (submitted for electronic processing).

ABSTRACT

Richins, R. D., and Shepherd, R. J. 1986. Horseradish latent virus, a new member of the caulimovirus group. Phytopathology 76: 749-754.

A virus isolated from horseradish in Europe had properties similar to those of cauliflower mosaic virus (CaMV). It reacted weakly with CaMV antiserum, however, and in further reciprocal tests with antiserum to both viruses, it was found serologically distinct from CaMV. The horseradish virus had a DNA genome with the same conformational forms of CaMV DNA during agarose gel electrophoresis. However, it was found to have site-specific discontinuities at different locations from those of CaMV DNA when the polarity of the genome was determined by polynucleotide kinase labeling of the 5′-termini of the DNA. In addition, the restriction endonuclease map of horseradish latent virus DNA is completely different from that of any reported strain of CaMV. These differences indicate the horseradish virus is a new member of the caulimovirus group. We propose the name "horseradish latent virus" for this new member of the group.

The caulimoviruses are a group of small isometric plant viruses containing double-stranded DNA. Most are aphid-transmissible and infect a fairly limited range of plants (11). The group now comprises about 10 viruses (17). Some members of the group also have caused economically important diseases. One of these viruses, cauliflower mosaic virus (CaMV), has been the object of much attention recently because it provides a simple genetic system for the study of gene expression in plants (13).

The caulimoviruses induce prominent amorphous inclusion bodies in infected cells (6) that have been characterized biochemically and structurally (2,5,28,31,33). During one of these investigations in which a comparative study was made of the inclusion bodies of different strains (31), a serological discrepancy was observed with one strain of the virus. When samples were taken from leaves infected with a strain of CaMV from horseradish (*Armoracia rusticana* Gaertn., May., & Scherb.), unusually low values were obtained in an enzyme-linked immunosorbent assay using CaMV antibody. Further investigation has revealed that the horseradish virus has a different coat protein than CaMV.

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Consequently, it is a distinctively different caulimovirus. We propose to name it "horseradish latent virus" (HRLV). Its properties are described herein.

MATERIALS AND METHODS

The virus was obtained from N. Paludan of the State Plant Pathology Institute, Lyngley, Denmark, in April 1973. It had been isolated from horseradish during a study of the effects of turnip mosaic virus on that crop (29). The virus was inoculated to *Brassica campestris* L. and maintained as lyophilized tissue stored over a desiccating agent.

Virus and host manipulations. The virus was propagated in *B. campestris* 'Just Right' (turnip). For host range tests, inoculum was prepared by grinding infected turnip tissue in 50 mM phosphate, pH 7.5, at a tissue-to-buffer ratio of about 1:10. Test plants were grown in a peat-sand-vermiculite mixture in 12.5-cm plastic pots in a greenhouse at 22–24 C. All test plants were inoculated in the three- to four-leaf stage after dusting with 600-mesh Carborundum. Plants were maintained for symptom development for at least 1 mo after inoculation.

Aphid transmission tests were done with green peach aphids (*Myzus persicae* Sulz.) after they had been starved for about 3 hr. Five aphids per plant were used after a 5-min feeding period on an infected source plant. Aphids were left on test plants overnight before removal by an application of nicotine sulfate.

Virus was purified from turnips infected for 3–4 wk by the CaMV isolation procedure of Hull et al (21), except 1.5 M urea was added to the resuspension buffer. One to 4 mg of purified virus was obtained per kilogram of starting material. Purified virus was usually stored in 50% (v/v) glycerol at −20 C.

Serological reactions. Virus antiserum against the horseradish virus was produced by subcutaneously injecting New Zealand white rabbits biweekly for 3 wk with 0.5 mg of purified virus emulsified in Freund's incomplete adjuvant. Six weeks after the injections began, the antibody titer was 1/128 in microprecipitin tests. Two intravenous injections of purified virus were then administered. During the next several weeks, the antibody titer reached 1/512. Antiserum to CaMV Cabbage B strain was prepared as described previously (10). A modification of the enzyme-linked immunosorbent assay of Clark and Adams (3) was used in which a horseradish peroxidase-IgG conjugate (37) was substituted for the alkaline phosphatase-IgG conjugate. The substrate used to produce the colorimetric reaction was *o*-phenylenediamine (0.7 mg/ml) in a buffer of 50 mM sodium phosphate, 24 mM citric acid, pH 5.0, containing 0.012% (v/v) hydrogen peroxide.

The serological relationship between the horseradish virus and CaMV was investigated by comparing the homologous and heterologous dilution end points of the sera in the immunosorbent assay procedure. CaMV or the horseradish virus γ-globulin was applied to an Immulon 1 (Dynatech, Alexandria, VA) plate at concentrations of 0, 0.0043, 0.02, 0.093, 0.43, and 2 μg/ml. After incubating for 90 min at 37 C and thorough washing, purified CaMV or horesradish virus was added to specified wells at a concentration of 2 μg/ml. The plate was incubated and washed as before. CaMV or horseradish virus conjugate (2 μg/ml) was then added to selected wells and again incubated. After a final washing, freshly prepared substrate was added to the plate. Absorbance values (450 nm) were determined using a Titretek Multiscan spectrophotometer (Flow Laboratories, McLean, VA). The data presented represent the mean of triplicate determinations.

Protein analyses. The separation and molecular weight analysis of viral coat protein was done by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) as described by Laemmli et al (22). SDS-denatured virus was electrophoresed into 12% (w/v) slab gels, then stained with Coomassie Blue.

Nucleic acid analyses. Viral DNA was extracted from purified virions as described by Shepherd et al (32), except proteinase K (E. Merck, Dormstadt, West Germany) was substituted for pronase. Viral DNA was also isolated directly from infected leaves by the procedure of Gardner and Shepherd (9). The purified viral DNA was digested with a variety of restriction endonucleases (New England Biolabs, Beverly, MA) to determine which would cut the DNA uniquely. *Pvu*I was one enzyme found to do so. A recombinant plasmid consisting of full-length viral DNA and pBR327 (34) was prepared by cutting both DNAs at their unique *Pvu*I sites. The linearized DNAs were then ligated using T4 DNA ligase. *Escherichia coli* K12 strain 71-18 (26) served as the recipient host for transformations. The cells were rendered competent by the procedure of Cohen et al (4). The ampicillin-sensitive, tetracycline-resistant recombinant, designated pHRLV-P1, was isolated from transformed bacterial cultures by the boiling procedure of Holmes and Quigley (14). When necessary, the DNA was further purified on ethidium bromide-cesium chloride density gradients (15). The infectivity of pHRLV-P1 was determined by mechanically inoculating 20 μg of *Pvu*I-cut DNA onto a Carborundum-dusted seedling of Just Right turnip.

Restriction endonuclease mapping was performed by running single, double, and triple restriction digests of pHRLV-P1 on agarose and/or polyacrylamide gels (24). Restriction digests of pCaMV10, an infectious clone of CaMV (strain CM1841), which has been sequenced (7), were used as molecular weight standards. Mapping results were confirmed with single restriction enzyme digests of viral DNA. Single-stranded interruptions were analyzed both by S1 nuclease digestion and by heat-denaturation (36).

The polarity of HRLV was determined by labeling the 5′ ends of viral DNA with [γ^{32}P] ATP by the T4 polynucleotide kinase exchange reaction of Maniatis et al (24) followed by restriction enzyme cleavage, heat denaturation, and gel electrophoresis for separation and analysis of the fragments. The labeled DNA fragments were detected by autoradiography of the dried gel. Dupont Cronex Lightning-Plus intensifying screens were used to enhance development of the Kodak X-Omat AR-5 film. A similar method has been used to determine the polarity of CaMV (18).

RESULTS

Symptoms and host range. The horseradish virus produced very mild symptoms on *Brassica* sp. after mechanical inoculation. Occasionally, a few inconspicuous chlorotic lesions were produced on the inoculated leaves. A mild chlorotic mottle with a faint yellow banding of the major veins was produced on systemically infected leaves of *B. campestris* 'Just Right' (Fig. 1). No other local or systemic symptoms developed. The following species also developed mild chlorotic mottling after inoculation: *B. napus* L. (oilseed rape), *B. oleracea* var. *viridis* L. (kale); *B. pekinensis* (Lour.) Rupr. (pe-tsai cabbage), and *Matthiola incana* (L.) R. Br. (annual stock). Although Paludan (29; *personal communication*) found that the virus caused mottling, veinclearing, and stunting when graft-inoculated to Danish cultivars of horseradish, this was not true of American clones. When approach-grafted to infected Just Right turnip, American horseradish cultivars showed no symptoms.

None of the following developed symptoms after mechanical inoculation: *Brassica nigra* (L.) Koch, *B. carinata* Braun., *B. juncea* (L.) Czern., *B. kaber* (DC.) L. C. Wheeler, *Beta vulgaris* L., *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Cucumis sativus* L., *Cucurbita melo* L., *Datura stramonium* L., *D. innoxia* Mill., *Nicotiana tabacum* L., *N. bigelovii* (Torr.) Wats., *N. glutinosa* L., *N. glauca* Graham, *N. plumbaginifolia* Vivian, *Lactuca sativa* L., *Lycopersicon esculentum* Mill., *Phaseolus vulgaris* L., *Pisum sativum* L., *Vigna sinesis* (Torn.) Savi, and *Zinnia elegans* Jacq.

Insect transmission. The virus was transmissible by green peach aphids with low efficiency. When 30 turnip plants were inoculated with five aphids per plant, only two plants became infected in one trial and only one plant in another.

Size and morphology. A single light-scattering band was observed during sucrose density gradient centrifugation. This component sedimented at about the same rate as CaMV and thus had a sedimentation coefficient of about 200 S.

The morphology of the horseradish virus and its inclusion bodies in infected plants has been described by Shalla et al (31). In

that investigation, the virus was assumed to be a strain of CaMV because it had roughly spherical virions 50 nm in diameter that occurred in the cell embedded in an electron dense inclusion body typical of the caulimoviruses.

Viral proteins. Dodecyl sulfate-degraded virus exhibited a series of forms in which the main components had molecular weights ranging from about 34,000 to 44,000 (Fig. 2) when analyzed by SDS-PAGE. The most prominent of these components was about 40,000 Da.

Immunological relationships. Tests with antiserum prepared to the horseradish virus showed that it was related but serologically distinct from CaMV. Immunosorbent assay reactions of antibody of CaMV and the horseradish virus with their homologous and heterologous antigens are shown in Figure 3. In these reactions, a range of concentrations of IgG absorbed to polystyrene plates was reacted with virus antigen at a constant level. The data showed that antibody concentrations of 0.43 μg/ml or higher gave maximum reactions with both antigens, whereas those with antibody at or below about one-fifth this concentration (0.093 μg/ml) gave high homologous reactions but low heterologous reactions (Fig. 3). This large disparity between reactions with the two antigens indicates that reduced antibody availability is limiting binding of the heterologous viruses. At the point at which antibody becomes limiting, the heterologous reactions are roughly one-fourth those of the homologous antigen-antibody pairs, suggesting the viruses have relatively few antigenic determinants in common.

The antigen/antibody reactions shown for CaMV in Figure 3 are given for the strain used to prepare the antiserum (Cabb B) and for another strain of the virus (CM1841) to indicate the kind of variation one might expect between strains of CaMV. In the region in which antibody becomes limiting, e.g., at 0.09 μg/ml IgG, there is about a threefold difference between the reactivities of a heterologous CaMV strain and HRLV with the CaMV antibody. In contrast, a heterologous strain of CaMV shows a reactivity scarcely different from that of its homologous virus antigen (Fig. 3).

Properties of the nucleic acid. When subjected to agarose gel electrophoresis, the native DNA of the horseradish virus exhibited aggregates and closed circular and linear forms much like those of other caulimoviruses (30). Full-length linearized DNA from the horseradish virus showed a single component that migrated in agarose gels at a rate indistinguishable from that of linearized DNA from the CM1841 strain of CaMV, which has 8,031 base pairs (7).

Fig. 1. Symptoms of horseradish latent virus infection on *Brassica campestris* 'Just Right' turnip.

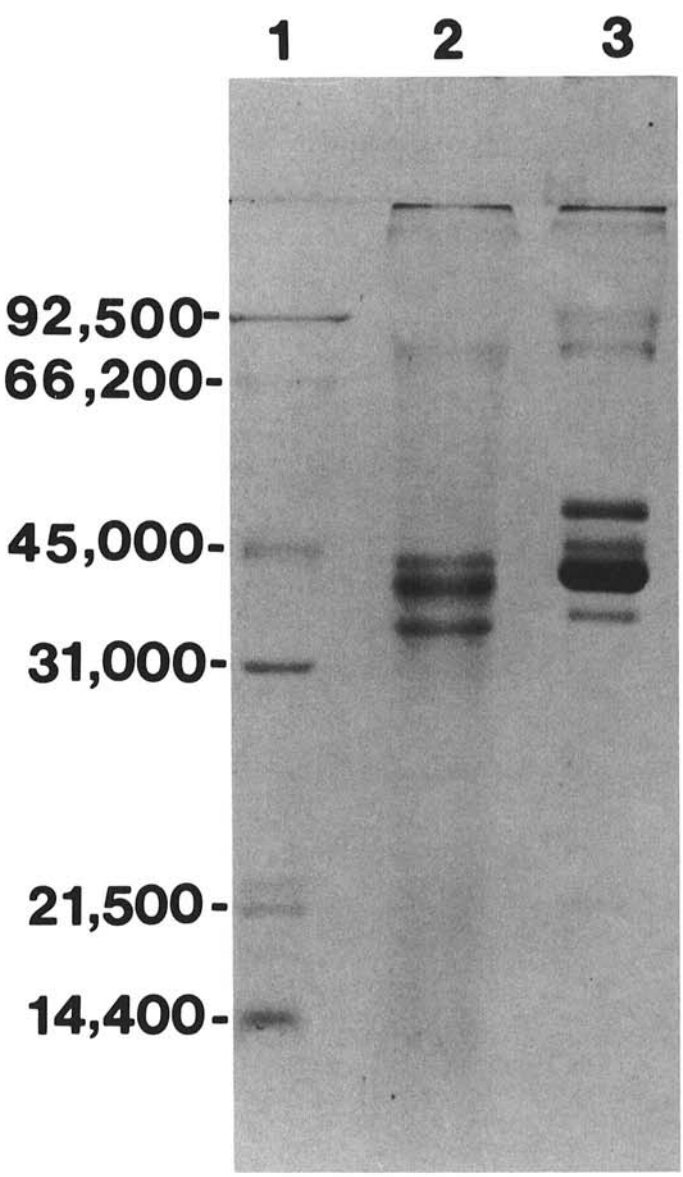

Fig. 2. Protein composition of virions of horseradish latent virus and cauliflower mosaic virus. Samples were as follows: 1 = molecular weight standards (top to bottom) phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with molecular weights as indicated on left; 2 = horseradish latent virus; and 3 = cauliflower mosaic virus. All were separated on a 12% Laemmli gel (22) and stained with Coomassie Blue.

Heat denaturation of the DNA of the horseradish virus resulted in the separation of three single-stranded species during gel electrophoresis. These three fragments were about the same sizes as the three single-stranded denaturation products of CaMV strain CM1841 DNA, except the smallest fragment was slightly smaller than the equivalent fragment of CaMV DNA. The fragments were estimated to be 8,000, 5,500, and 2,500 base pairs long. Therefore, the DNA genome of the horseradish virus appeared to have three single-stranded interruptions at about the same locations as the interruptions in CaMV DNA. However, the polarity experiments indicated that only two of the interruptions were in approximately similar positions to those in CaMV DNA. The third interruption was in a different part of the genome (Fig. 4).

The positions of the three interruptions in horseradish viral DNA are shown in Figure 5. In this physical map of the genome, the zero position at the top of the diagram corresponds to the single interruption in one of the DNA strands, which by analogy with CaMV DNA, is probably the alpha or transcribed strand; the two other interruptions occur in the noncoding complementary strand. The sizes of the single-stranded DNA denaturation products (Fig. 4) indicate the interruptions in the noncoding strand are at positions 0.47 and 0.77 (8,000 bases = 1.00).

The physical map of the horseradish virus genome (Fig. 5) also shows the cleavage sites of nine restriction endonucleases. Two of the enzymes, *Pvu*I and *Xho*I, cut the DNA only once. Several other enzymes that were tested, including *Sal*I, *Pvu*II, *Bst*EII, and *Stu*I, did not cut this DNA.

The full-length clone of the horseradish virus was infectious when cleaved free of its bacterial cloning vector by digestion of the DNA with *Pvu*I. When turnip seedlings were inoculated with *Pvu*I-cleaved DNA, symptoms appeared about 3 wk later. Biological activity indicated the virus genome was probably completely represented in the pHRLV-P1 clone.

DISCUSSION

The horseradish virus has many of the properties of CaMV. It causes diseases of cruciferous plants similar to those caused by CaMV and appears to be largely if not completely confined to the family Cruciferae in its host range. Its inclusion bodies, transmissibility, and virions are also indistinguishable from those of CaMV except for the serological properties of the latter. The virions of the horseradish virus contain several proteins whose molecular weights resemble those of CaMV. The single coat protein of CaMV undergoes proteolysis in vivo to produce several forms of different molecular weights (1,12). The protein analysis indicates the same applies to the horseradish virus coat protein.

Three important differences were found between the horseradish virus and CaMV. The viruses are serologically different, indicating they have different coat proteins; the DNA genome has a radically different restriction endonuclease map, indicating significant sequence differences; and the site-specific discontinuities occur in different positions.

The horseradish virus appears to be related but serologically distinct from CaMV. On the basis of the immunosorbent tests

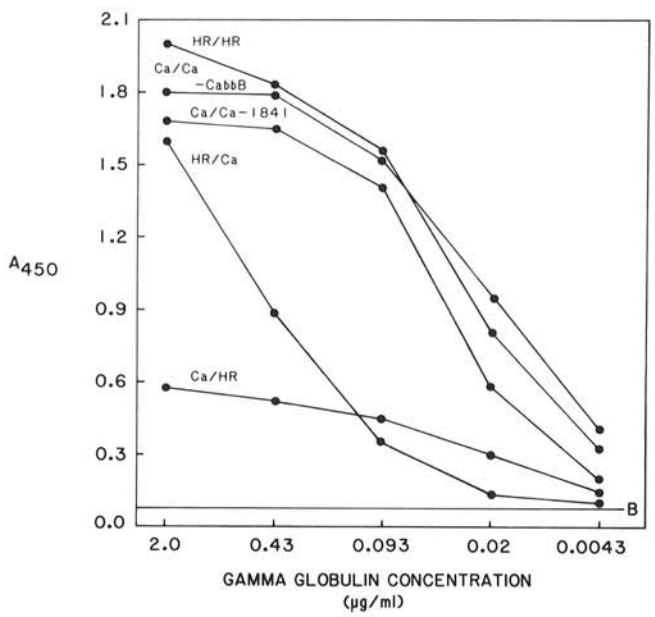

Fig. 3. Serological relationship between horseradish latent virus (HRLV) and cauliflower mosaic virus (CaMV). HR denotes either HRLV coating and conjugate IgG or virus; Ca denotes CaMV coating and conjugate IgG or virus; the second label below the slash mark indicates the virus tested. Data lines Ca/Ca-Cabb B and Ca/Ca-1841 show the reaction of CaMV Cabbage B strain and CaMV CM1841 strain as the purified viruses reacted with antibody prepared to the Cabbage B strain. The horizontal line near the bottom of the graph labeled "B" indicates the background reaction observed (i.e., no coating IgG was applied to the plate).

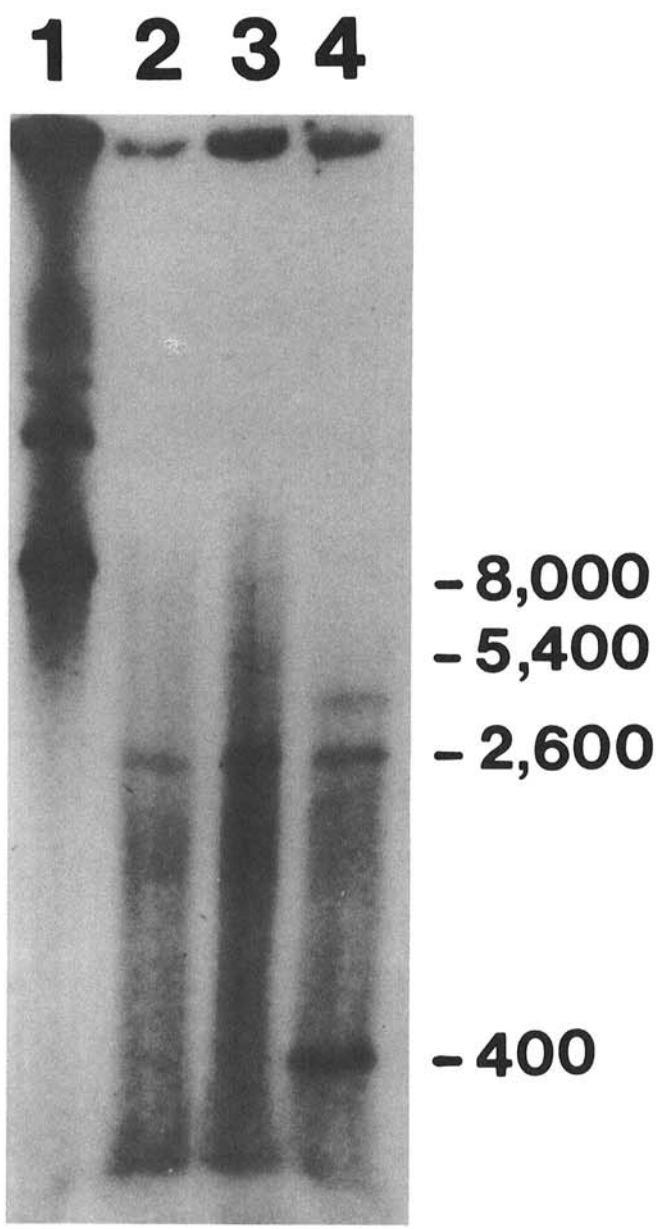

Fig. 4. Autoradiography establishing polarity of the horseradish latent virus genome. The 5′→3′ orientation of horseradish latent viral DNA was determined by end-labeling the DNA using polynucleotide kinase. The labeled DNA was then cut with restriction endonucleases and subjected to agarose gel electrophoresis: 1 = control (uncut/native); 2 = *Pvu*I-cut, then heat-denatured; 3 = heat-denatured (uncut); and 4 = *Xho*I-cut, then heat-denatured. The 1.2% (w/v) agarose gel was dried, then autoradiographed. The scale along the right edge of the figure gives approximate sizes of the DNA fragments in base pairs.

carried out in this investigation, its degree of relatedness appears to be about as different from CaMV as that of dahlia mosaic and strawberry veinbanding viruses. Morris et al (27) showed that these viruses were distantly related immunologically to CaMV. In immunosorbent reactions with reduced levels of CaMV antibody (nonsaturating levels), these viruses showed roughly one-fifth to one-fourth the reactivity of the homologous antigen. In immunosorbent tests done with different levels of IgG as the plate-coating reagent, the heterologous viruses (HRLV vs. CaMV) show a much greater difference in reactivity than heterologous strains of the same virus (Cabbage B vs. CM1841 strains of CaMV) (Fig. 3).

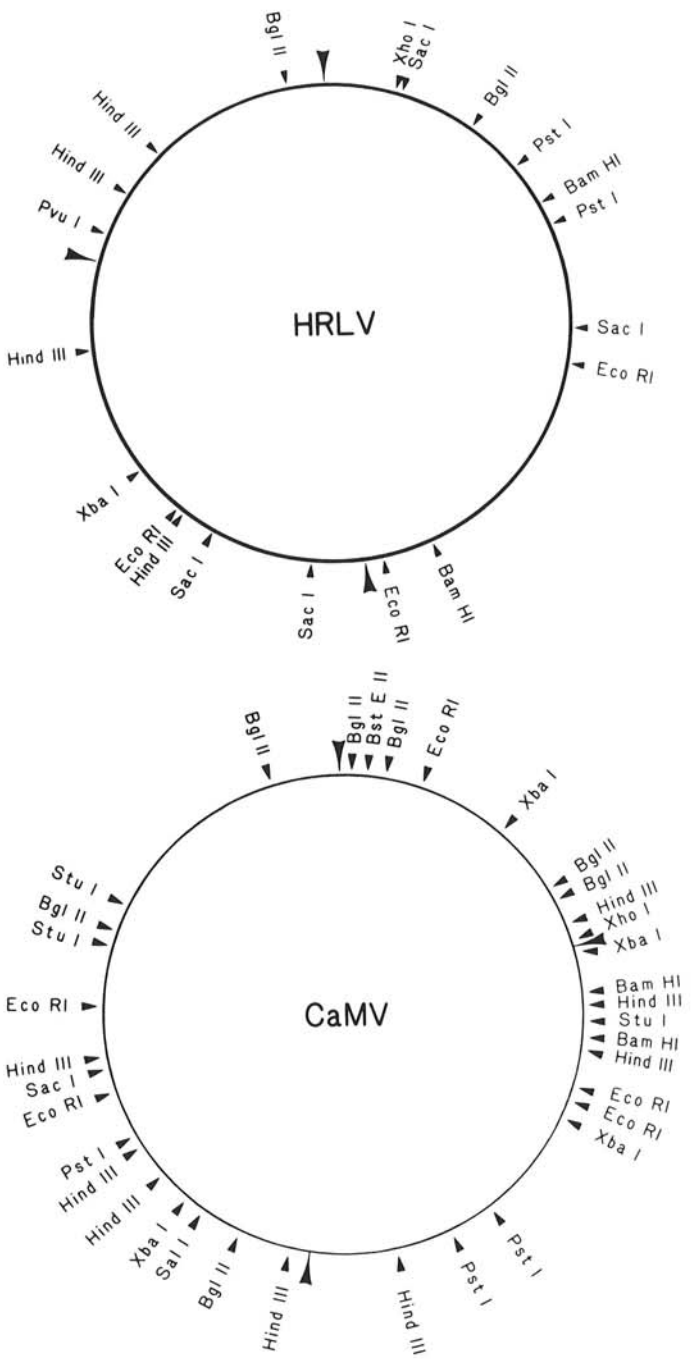

Fig. 5. Physical map of the horseradish latent virus genome (HRLV) compared with that of the cauliflower mosaic virus genome (CaMV). The map is oriented so that the alpha-strand containing only one discontinuity reads 5′→3′ beginning at the alpha interruption (large arrowhead at top of each diagram) as the zero point of the map and continuing clockwise around the genome. Two other single-stranded discontinuities (large arrowheads in the diagram) appear at positions about one-half and three-fourths around the circular genome. The map for CaMV is for strain CM1841 which has been sequenced (7).

Although these tests, or any others for that matter, cannot be used to measure relatedness with any degree of precision, the data do show that the horseradish virus is as serologically different from CaMV as the other viruses that are considered separate.

Another feature of the horseradish virus that differs markedly from CaMV is its restriction endonuclease map. The restriction map of the horseradish virus is markedly different from that of any strain of CaMV that has been reported (8,16,23,25,35). In fact, the restriction map of the horseradish virus does not resemble those of any other caulimoviruses that have been published. A notable feature of the caulimoviruses that are considered different species is that they have markedly different restriction endonuclease maps (19,30). The fact that the restriction endonuclease map of the horseradish virus is unique provides further evidence that the virus is a taxonomically distinct entity.

Another intrinsic feature characteristic of CaMV strains is the location of site-specific interruptions in the DNA genome. All strains of CaMV that have been investigated have discontinuities at positions 0.2–0.22 and 0.5 in the noncoding strand, except for strain CM4-184, which has a deletion at 0.22 (20). Although the horseradish virus has a discontinuity at 0.47, it has none in the 0.2 region of the genome. None of the CaMV strains that have been investigated have an interruption at position 0.77 as does the horseradish virus (Fig. 5). This is further evidence for distinguishing the horseradish virus from CaMV.

The DNA of the horseradish virus shows a greater degree of homology to CaMV DNA in southern hybridization tests than do other caulimoviruses (30). However, no quantitative hybridization tests have yet been done.

The horseradish virus is obviously related to CaMV. However, its serological and sequence differences seem adequate to set this virus apart as a new member of the caulimovirus group. Consequently, we propose that it be called horseradish latent virus.

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