

# Detection *in vivo* of a new gene product (gene *III*) of cauliflower mosaic virus

(synthetic peptides/protein-blotting/radioimmunoassay)

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**ABSTRACT** Cauliflower mosaic virus DNA contains six major open reading frames (ORFs). As only the mRNA corresponding to the transcription of gene *VI* and its translation product have been isolated, the identification in infected plants of products corresponding to the five other putative genes remains to be established. The present paper reports the detection of an ORF *III* product by means of antibodies raised against an NH<sub>2</sub>-terminal synthetic peptide of 19 amino acids corresponding to a sequence predicted from the nucleotide sequence of ORF *III*. The detection of this gene product raises the question of the mechanism of its expression.

Cauliflower mosaic virus (CaMV) contains circular relaxed double-stranded DNA as genetic material (for review, see refs. 1–3). Determination of the CaMV DNA sequence (4–6) revealed the presence of six large (I–VI; putative genes *I–VI*) and two small (VII and VIII; putative genes *VII* and *VIII*) open reading frames (ORFs). The functions of the ORF II (7–10) and the ORF IV (4, 11) have been deduced, but no direct demonstration of such functions has been given. Several RNA species (35S, 19S, and 8S) transcribed from the viral DNA have been detected in extracts from virus-infected turnip leaves (12–14). In an *in vitro* system only the 19S mRNA, transcript of the ORF VI, was translated to give a P66 protein, which corresponded to the main inclusion body protein (15–17).

In 1980, Lebeurier *et al.* (18) demonstrated that the presence of the ORF *III* was necessary for viral multiplication, but up to the present time no mRNA corresponding to ORF *III* has been isolated and, thus, the presence of the corresponding protein in infected plants has remained speculative. However, since the DNA nucleotide sequence for gene *III* is known, it has been possible to use an alternative approach to detect the gene product. Synthetic peptides with the appropriate sequence can be used to elicit the formation of antibodies which also will react specifically with the native protein (19–21).

In the work described here, we used this strategy to detect the protein product of ORF *III* in infected tissue. As far as we are aware, identification of an unknown gene product in plants has not been reported previously.

## MATERIALS AND METHODS

**Virus Isolates and Plants.** The CaMV isolate used in this study was Cabb-S (22). The isolate was propagated in turnip plants (*Brassica rapa* L., cultivar Just Right) grown under greenhouse conditions as described by Xiong *et al.* (23). Infected leaves were taken 30 days after inoculation for the extraction of inclusion bodies and nuclei.

**Extraction of Inclusion Bodies.** Semipurified inclusion bodies were extracted by the method described by Al Ani *et al.* (24).

**Extraction of Nuclei.** Semipurified nuclei were isolated by a modification of the method of Hamilton *et al.* (25) as described by Ansa *et al.* (26).

**Protein Blotting Procedure.** Protein blotting was performed by a modification of the method described by Towbin *et al.* (27). Briefly, the semipurified inclusion bodies or nuclei were treated by boiling for 2 min in 62.5 mM Tris-HCl, pH 6.8/2% NaDodSO<sub>4</sub>/2% 2-mercaptoethanol/10% glycerol, subjected to electrophoresis in a NaDodSO<sub>4</sub>/12.6% polyacrylamide slab gel, and then electrophoretically transferred to nitrocellulose sheets (0.45- $\mu$ m pore size; Schleicher & Schüll BA-85) prewetted with 150 mM NaCl/10 mM Tris-HCl, pH 7.4 (NaCl/Tris). A voltage gradient of 7 V/cm was applied for 1.5 hr. The blot was either stained with amido black or used directly for immunological testing.

**Peptide Synthesis.** Solid-phase peptide synthesis was carried out by the method of Merrifield (28, 29).

**Purification of the Peptide.** The peptide was purified by high-performance liquid chromatography (HPLC). The peptide was dissolved in water and filtered (HVLP 01300 Millipore 0.45  $\mu$ m) prior to injection. The HPLC separation was performed on a Beckman 344 instrument with a flow rate of 1 ml/min at ambient temperature. Peptides were eluted with a linear gradient of acetonitrile (20–70% in water containing 0.05% trifluoroacetic acid) for 20 min. Peptide-containing fractions were dried under vacuum and used directly for amino acid analysis or coupling with rabbit immunoglobulin G (IgG) or bovine serum albumin.

**Amino Acid Analysis.** Peptides were hydrolyzed under nitrogen in 6 M HCl containing 0.02% 2-mercaptoethanol for 20 hr at 110°C. The hydrolysates were dried and redissolved in citrate-HCl buffer (pH 2.2) and then were analyzed on a Beckman Durrum D500 analyzer.

**Coupling.** The synthetic peptides were coupled to carrier proteins with glutaraldehyde or water-soluble carbodiimide.

For coupling with glutaraldehyde, a 10-fold molar excess of peptide was coupled to bovine serum albumin by using 2.5% glutaraldehyde in a 0.1 M potassium phosphate buffer (0.1 M, pH 7.35) at 0°C for 3 hr. The reaction was stopped by addition of NaBH<sub>4</sub>. After overnight standing, the mixture was dialyzed against several changes of phosphate-buffered saline (pH 7.4) (P<sub>i</sub>/NaCl) at 4°C for a period of 24 hr.

For coupling with water-soluble carbodiimide, 8.8 mg of peptide was dissolved in 1.5 ml of sodium phosphate buffer (0.1 M, pH 5.8) and 3 mg of IgG prepared from the preinoculated rabbit in 0.32 ml of water was added. Then a solution of

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Abbreviations: CaMV, cauliflower mosaic virus; ELISA, enzyme-linked immunosorbent assay; ORF, open reading frame.

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16 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 0.18 ml of the same buffer was added. The reaction mixture was shaken overnight and dialyzed against several changes of  $P_i$ /NaCl. This preparation was used for six immunizing injections.

**Immunization of the Rabbits.** Anti-peptide antisera were raised in rabbits by intramuscular injections of peptide coupled to IgG with carbodiimide emulsified in Freund's incomplete adjuvant. Further booster doses were given under the same conditions every 2 weeks.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Wells of polystyrene microtiter plates (Nunc) were coated for 2 hr at 37°C with peptide (0–0.8 μg) coupled to bovine serum albumin with glutaraldehyde in 0.05 M sodium carbonate (pH 9.6). After the wells were rinsed three times in  $P_i$ /NaCl (pH 7.4) containing 0.05% Tween 20 ( $P_i$ /NaCl/Tween), they were saturated with  $P_i$ /NaCl/Tween containing bovine serum albumin at 10 mg/ml overnight at 4°C. After the cells were rinsed, they were incubated for 2 hr at 37°C with antiserum diluted in  $P_i$ /NaCl/Tween. After further rinsing, the anti-rabbit globulin conjugate diluted 1:1000 was allowed to react at 37°C for 2 hr with bound antibodies. After a final rinsing, the bound enzyme conjugate was detected by adding substrate *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer (pH 9.8). After hydrolysis for 3 hr at 37°C, the absorbance at 405 nm was directly read in a Titertek Multiskam RS-232C.

**Immunological Detection of Proteins on Nitrocellulose.** Proteins were detected by a modification of the immunological method of Towbin *et al.* (27). Unless otherwise indicated, all steps in the procedure were performed at room temperature. The electrophoretic blots were rinsed three times for 5 min in NaCl/Tris. Additional protein binding sites were saturated with 3% bovine serum albumin in NaCl/Tris for 6 hr. They then were incubated overnight with antiserum appropriately diluted in 3% bovine serum albumin NaCl/Tris. For inhibition assays, 20 μl of serum was preabsorbed for 8 hr with 200 μg of peptide diluted in 20 ml of NaCl/Tris. After the sheets were washed five times for 30 min in NaCl/Tris, the sheets were incubated with 1.5 μCi (1 Ci = 37 GBq) of <sup>125</sup>I-labeled protein A (Amersham) diluted to 15 ml with NaCl/Tris at 37°C for 1 hr and washed as described above. Processed sheets were air-dried and exposed for autoradiography.

**RESULTS**

**Selection of Potentially Immunogenic Regions.** The amino acid sequence of P15 translation product of ORF III predicted by the CaMV DNA sequence was used to select suitable peptide sequences for chemical synthesis. To select the potentially immunogenic regions, we applied two different methods.

Recently, it was successfully demonstrated that a surface helix in a protein could be considered as an antigenic determinant (30). In searching for any potential helix in P15, we

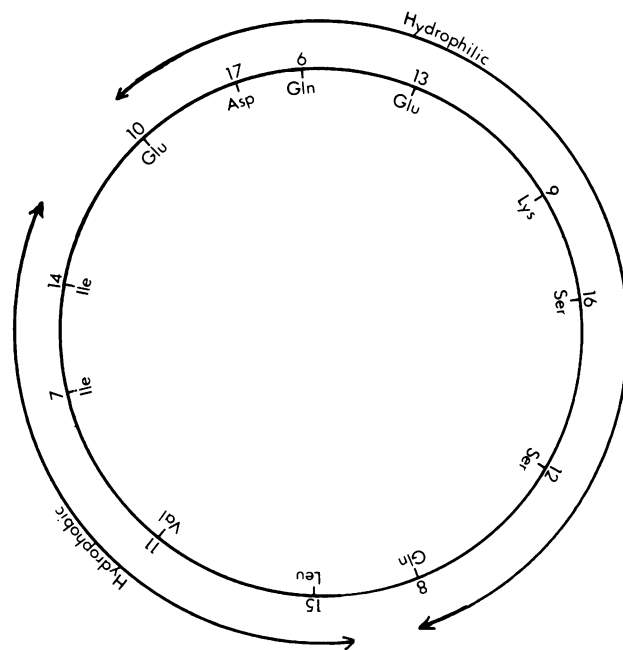


FIG. 2. Helix wheel of the peptide in PV-A containing residues 6–17. All side chains are considered to be in a regular α helix at their appropriate azimuthal position. This helix is then projected along its axis onto the plane of the paper, revealing that the lower left side of the presumed helix is hydrophobic, whereas the upper right side is hydrophilic.

applied the method of Chou and Fasman (31). Among the several regions thus assigned as helical in P15, an NH<sub>2</sub>-terminal peptide PV-A (Fig. 1), which was expected to contain an α-helical region covering residues 6–17, was selected as a candidate antigenic determinant for two reasons. First, when we used the method of Shiffer and Edmundson (32) to detect whether the helix is exposed at the surface of the protein, we found that the helix wheel representation, which is a projection of all side chains along the helix axis, was split into a purely hydrophobic and a purely hydrophilic half (Fig. 2), suggesting that the α helix might lie on the surface of the protein. Second, it has often been observed that the terminal regions of a protein are conformationally more free than other parts, so that under conditions otherwise favorable, such a region is more likely to be an antigenic determinant.

**Synthesis of PV-A and Antisera Raised Against the Synthetic Peptide.** Using solid-phase methods, we synthesized the 19-amino-acid peptide PV-A with a molecular weight of 22,000. Amino acid analysis showed the purified synthetic peptide to be of the correct composition (Table 1). Its purity was confirmed by HPLC.

The peptide that had been coupled to IgG with carbodi-

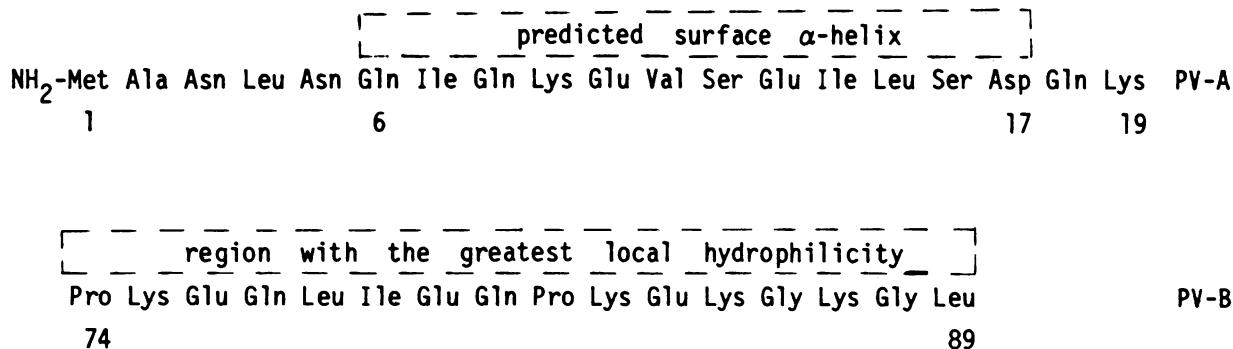


FIG. 1. Amino acid sequences selected from the putative product of ORF III for the synthesis of PV-A and PV-B peptides.

Table 1. Amino acid analysis of the synthetic peptide PV-A

Amino acid	Molar ratio	
	Theoretical	Calculated*
Asp	3	2.96
Ser	2	1.93
Glx	5	5.23
Ala	1	0.86
Val	1	1.09
Met	1	0.83
Ile	2	2.02
Leu	2	2.04
Lys	2	2.05

\*Molar ratio based on the average of 19 residues.

mide was used to immunize rabbits. The level of peptide antibodies was measured by ELISA with peptide coupled to bovine serum albumin with glutaraldehyde as antigen. Fig. 3 illustrates the antibody response to the synthetic peptide: 50 ng of peptide per ml could be detected readily by ELISA with antiserum diluted 1:20,000. There was no response to normal rabbit serum.

**Recognition of Virus-Specific Polypeptide P15 by the Anti-Peptide Antisera.** A virus-specific peptide of molecular weight 15,000 could be discerned by NaDodSO<sub>4</sub>/PAGE (Fig. 4A, lane 3), which was not detectable in uninfected plants (Fig. 4A, lane 4). Its mobility in NaDodSO<sub>4</sub>/PAGE corresponds to the molecular weight of the translation product of ORF III predicted by the CaMV DNA sequence. Because this band migrates very close to an intense band that also could be found in healthy plants, it was difficult to determine whether it was in fact virus-specific or whether it simply resulted from diffusion of the band found in healthy material. On the other hand, if this band is virus specific, the

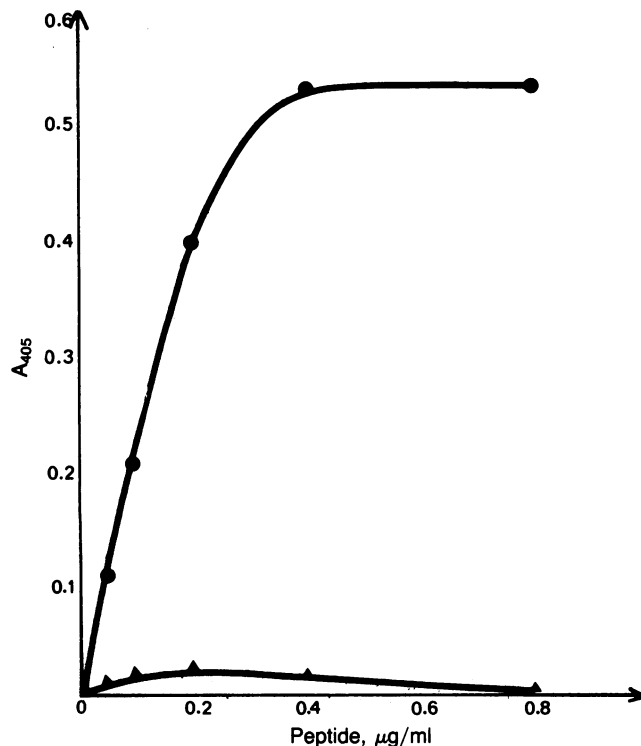


FIG. 3. Activity of anti-PV-A peptide antibodies measured by ELISA. Anti-peptide antisera were raised by peptide coupled to IgG with carbodiimide. Peptide coupled to bovine serum albumin with glutaraldehyde was used as antigen (abscissa) for the ELISA assay. ●, Peptide antiserum diluted 1:20,000; ▲, normal serum diluted 1:20,000.

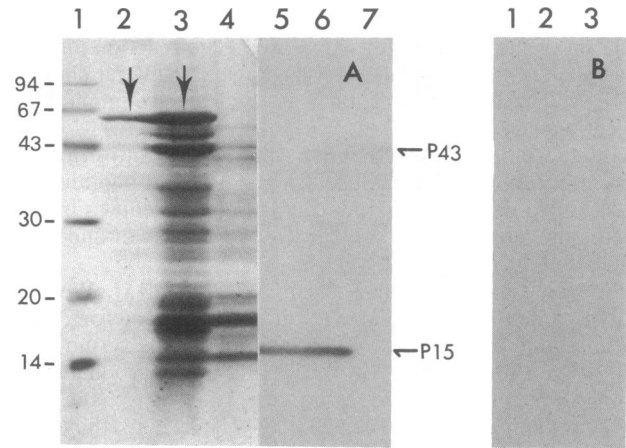


FIG. 4. Immunological detection of P15 on nitrocellulose sheets. Plant extracts were fractionated by electrophoresis on a 12.6% NaDodSO<sub>4</sub>/PAGE prior to electrophoretic transfer to nitrocellulose sheets. In each lane was loaded 15  $\mu$ l of inclusion bodies or nuclear fraction (corresponding to 1 ml/5 g of leaf tissue) pretreated by boiling for 2 min in 62.5 mM Tris-HCl, pH 6.8/2% NaDodSO<sub>4</sub>/2% 2-mercaptoethanol/10% glycerol. (A) Molecular weight standards are shown  $\times 10^{-3}$  in lane 1: phosphorylase b (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (30); trypsin inhibitor (20); and  $\alpha$ -lactalbumin (14). Other lanes contain semipurified nuclei (A, lanes 2 and 5; B, lane 1), semipurified inclusion bodies (A, lanes 3 and 6; B, lane 2), and a fraction from healthy plants made by the procedure used to isolate inclusion bodies (A, lanes 4 and 7; B, lane 3). The nitrocellulose sheets were stained with amido black (lanes 1-4 in A), or the transferred nitrocellulose sheets were incubated with anti-peptide antiserum diluted 1:1000. After being treated with <sup>125</sup>I-labeled protein A (0.1  $\mu$ Ci/ml), processed sheets were exposed for autoradiography (A, lanes 5-7) or the transferred nitrocellulose sheets were incubated with normal serum diluted 1:1000 (B, lanes 1-3).

question arises as to whether it is a newly identified gene product or merely a degradation product of viral-coat protein or inclusion-body protein. For these reasons, the gels were electroblotted to nitrocellulose papers. The papers were then incubated with either PV-A antiserum or normal serum. Fig. 4A shows that the anti-peptide antiserum revealed the presence of P15 in both the preparation of semi-purified inclusion bodies (lane 6) and semipurified nuclei from infected plants (lane 5), but no reaction could be detected in the same region in the extract from uninfected plants (lane 7). It must be pointed out that the total amount of protein loaded onto different lanes in the gel of Fig. 4 are different. In particular there is substantially more total protein in lane 3 (semi-purified inclusion bodies from infected leaf) than in lane 4 (an extract from the same initial quantity of healthy leaf, made by the procedure used to isolate inclusion bodies). The main reason for this difference is that all of the viral proteins and their enzymatic degradation products are absent in the sample from healthy leaves. The normal serum did not react with either the extract of infected plants (Fig. 4B, lanes 1 and 2) or that from uninfected plants (Fig. 4B, lane 3). A faint band of approximately molecular weight 43,000 could be seen after reaction with <sup>125</sup>I-labeled protein A, both in the extract of infected plants (Fig. 4A, lanes 5 and 6) and in uninfected plants (Fig. 4A, lane 7).

To determine whether the faint band of molecular weight 43,000 was also peptide specific, we carried out the following experiment. Extracts from healthy and infected plants were divided into two aliquots. After being subjected to electrophoresis and electroblotted, one was allowed to react with anti-peptide antiserum, while the other was allowed to react with anti-peptide antiserum preabsorbed with an excess of peptide. The specific reaction of the anti-peptide

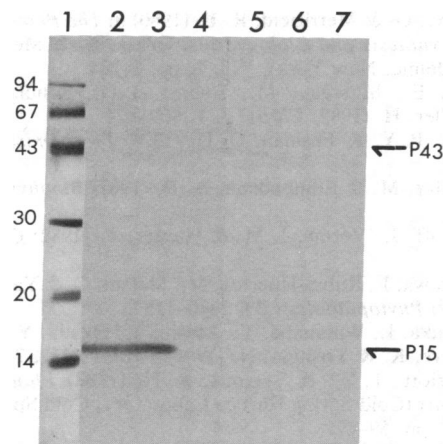


FIG. 5. Inhibition assay for protein P15 reactivity with anti-peptide antibody. Gels were prepared as described in the legend for Fig. 4. Lanes: 1, molecular weight standards (shown  $\times 10^{-3}$ ); 2 and 7, semipurified nuclei; 3 and 6, semipurified inclusion bodies; 4 and 5, a fraction from healthy plants made by the procedure used to isolate inclusion bodies. The transferred nitrocellulose sheets were incubated with anti-peptide antiserum diluted 1:1000 (lanes 2-4). For inhibition assay, 20  $\mu$ l of anti-peptide antiserum was preabsorbed with 200  $\mu$ g of peptide diluted in 20 ml of NaCl/Tris for 8 hr and then incubated directly with the transferred nitrocellulose sheets (lanes 5-7).

antibody with protein should be abolished by preincubation of the antiserum with an excess of peptide (33). Fig. 5 shows that an intense band at P15 (lanes 2 and 3), revealed by anti-peptide antiserum, was abolished by preincubation of the antiserum with excess peptide (lanes 6 and 7). However the band of molecular weight 43,000, in spite of being comparatively faint, was revealed after all treatments (lanes 2-7). This experiment demonstrates that the reaction of the antiserum with a normal host protein does not involve the specificity of the anti-peptide antibodies.

We have met difficulties when we attempted to locate the intracellular site of P15. Cells infected with CaMV contain inclusion bodies, which are essentially proteinaceous and contain most of the virus in the cell (34). When inclusion bodies or nuclei were extracted, they were always found mutually contaminated. For instance, when semipurified nuclei were analyzed by NaDodSO<sub>4</sub>/PAGE, a protein—P66 (arrows in Fig. 4A), the main inclusion body protein (13-15)—was always present. Attempts to extract inclusion bodies free of contaminating nuclei were not successful (data not shown). Since protein P15 was detected in both types of preparation, additional experiments will be required to determine the localization of P15 in infected cells.

## DISCUSSION

In the experiments described in this paper, we have demonstrated with a plant virus that a protein predicted from the nucleotide sequence of a putative gene can be detected *in vivo* although the corresponding mRNA is not known. This approach is similar to that used by Sutcliffe *et al.* (20) for the putative protein R of Moloney leukemia virus. Very recently Ooshika *et al.* (35) showed that the tobacco mosaic virus protein of molecular weight 30,000, which had been known from *in vitro* experiments for some years, could be found in infected cells by using an approach similar to that described here. This technique also could be applied to the geminiviruses whose genomes have been sequenced (36, 37) but whose mRNAs have not been detected yet.

However, it must be pointed out that this technique may be laborious if the peptide first synthesized is not immunogenic. For example we have synthesized two peptides: PV-A

and PV-B (Fig. 1) on the basis of computer programs that predicted their secondary structure (31) and immunogenicity (30, 38). The PV-A peptide gave rise to antibodies that reacted with the ORF III product. However, the 16-amino-acid-long PV-B (Fig. 1) with a molecular weight of 18,000, which was predicted to have the highest local hydrophilic region (38) in P15, did not give rise to such antibodies whether it was coupled to IgG with carbodiimide or to hemocyanin key-hole limpet with glutaraldehyde. This unexpected result remains to be elucidated.

The P15 protein detected *in vivo* has a molecular weight determined on NaDodSO<sub>4</sub> gels that corresponds to that predicted from the nucleotide sequence of ORF III. Therefore, we assume that the P15 protein represents the full translation product of gene III, as it already has been observed for gene VI that codes for the P66 viroplasmic protein.

However, gene VI is the only one for which the messenger RNA has been isolated: the major problem in the expression of the CaMV genome remains the identification of the functional messenger RNA(s) that code for the seven remaining potential genes. Many hypotheses have been put forward to explain the inability to isolate these mRNA(s), but the experimental evidence gathered so far does not favor any of them.

With the anti-P15 antibodies, we have tried to localize this protein in infected cells. Preliminary results (unpublished data) reveal low amounts of P15 product in thin sections of infected tissues embedded in an araldite/Epon mixture. This P15 protein has been shown to be necessary for infectivity (9, 18, 39) and could be implicated in the initial phases of viral multiplication.

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