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In vivo recombination of cauliflower mosaic virus DNA

(infective DNA/DNA ligation/DNA cloning)

GENEVIÈVE LEBEURIER*, LÉON HIRTH*, BARBARA HOHN†, AND THOMAS HOHN†

*Laboratoire des Virus des Plantes, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, 15 rue Descartes, 67000 Strasbourg, France; and †Friedrich-Miescher-Institut, P.O. Box 273, CH4002 Basel, Switzerland

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ABSTRACT **Ligation and recombination of the DNA of cauliflower mosaic virus (CaMV) is demonstrated by the following experiments: (*i*) Ligation: Different noninfectious fragments of the CaMV genome (obtained after insertion into plasmid pBR322 followed by enzymatic excision) regained infectivity when mixtures of them were used to inoculate their host. The symptom appearance was delayed by comparison with a typical CaMV infection, and only the newly formed leaves were affected. (*ii*) Recombination: Pairs of noninfectious recombinant full-length CaMV genomes (integrated into pBR322 at different restriction endonuclease sites) regained infectivity upon simultaneous inoculation of a sensitive host. The symptomatology of the resulting infection was indistinguishable from that of a typical CaMV infection. We show that progeny DNA had the same characteristics (size, structure, restriction endonuclease digestion pattern) as *bona fide* CaMV DNA, and that the vector pBR322 had been completely eliminated. A cloned tandem dimer of CaMV DNA with a partial deletion similarly was infectious in the plant assays. This system should be useful to study the expression of mutant genomes, thus allowing characterization of the CaMV genes.**

Recombination of eukaryotic DNA molecules has been observed both during meiosis and as a result of biological processes unrelated to sexual reproduction. This phenomenon has not yet been explained at the molecular level. The approach to this problem has been the development of model systems based on animal DNA viral recombination [e.g., simian virus 40 (SV40)] (1). With plants such a system can be studied by using cauliflower mosaic virus (CaMV). The genome of this virus is a double-stranded, relaxed circular DNA molecule, 8,024 base pairs (bp) long (2). Three single-stranded discontinuities ("gaps") occur in most strains (3, 4). Our previous work (5, 6) and that of Howell *et al.* (7) have shown that double-stranded, full-length, nongapped (5, 6), cloned CaMV DNA, when released from the bacterial vector, is infectious and yields virions with all the characteristics of typical CaMV. This demonstrates that the plant cells are able to perform at least one of the functions needed for recombination, namely ligation. In this paper we demonstrate that the plant cell promotes also the ligation of CaMV inoculated as two separately cloned restriction fragments, yielding fully infectious virions.

It was also interesting to find out whether complete recombination is possible. For this purpose the double-stranded circular genome of CaMV was integrated into bacterial plasmids at different unique restriction sites. The resulting hybrids each contained full-length CaMV DNA but were not infectious (5) unless released from the vector. We now show that upon simultaneous inoculation of a host of CaMV with pairs of these hybrids infectious virions are released. Infectivity was also recovered upon inoculation with a cloned partially deleted tandem dimer of CaMV (6).

While this paper was being written, a paper by Howell *et al.* (8) appeared using somewhat different methods to show that CaMV DNA molecules can undergo recombination in plants. A comparison of these results and ours will be presented in *Discussion*.

MATERIALS AND METHODS

Virus Purification. CaMV Cabb-S (9) and its cloned derivatives were grown in turnips (*Brassica rapa* Linnaeus, cv. Just Right) and purified according to Hull *et al.* (10).

Viral DNA Extraction and Cloning. DNA was isolated from purified virus, cloned, and analyzed as described by Hohn *et al.* (11).

Plasmids and CaMV Clones Used. The following were used: pHC79 (12); pHA10 (13); Ca4, pBR322 *Pst*CaMV; Ca6, pBR322 *Pst*CaMV *Pst*CaMV (Δ1900–3100); Ca8, pBR322 *Bam*CaMV; Ca10, pBR322 *Bam*CaMV A; Ca11, pBR322 *Bam*CaMV B; Ca37, pBR322 *Sal*CaMV; Ca39, pHC79 *Bst*CaMV (all B. Hohn and T. Hohn); Ca80, pHA10 *Bam/Bgl* CaMV A (A. Honigman and T. Hohn) (cloning sites: *Pst*, *Pst* I; *Bam*, *Bam*HI; *Sal*, *Sal* I; *Bst*, *Bst*EII; *Bgl*, *Bgl* II). Arrows indicate the orientation of CaMV DNA relative to the plasmid according to the conventional CaMV map (11).

Assay in Plants and Leaf Inoculation. These procedures were described by Lebeurier *et al.* (5).

Infectivity Assay. The infectivity of each sample of cloned DNA was tested at 2, 5, or 10 μg of CaMV DNA per ml of sterilized double-distilled water. A batch of 10, 15, or 30 plants was used for each DNA sample. Three leaves of each plant (5 weeks old) were inoculated with 30 μl of the DNA solution. Batches of control plants were spaced between the batches of inoculated plants. The control plants remained healthy throughout the experiments.

Purification of Total DNA from Infected Plants. The method used is based on that described by Zimmerman and Goldberg (14). Twenty grams of fresh leaves, without midribs, were frozen and ground to a fine powder in liquid nitrogen. Cells were lysed at 60°C for 15 min by addition of 20 ml of 200 mM Tris·HCl (pH 9)/200 mM EDTA/4% $NaDodSO_4$. Proteins were extracted with a 1:1 (vol/vol) mixture of phenol and chloroform. The aqueous phase was washed three times with chloroform. Nucleic acids were then precipitated from the aqueous phase with ethanol and resuspended in a buffer consisting of 50 mM Tris·HCl, pH 7.9/50 mM NaCl/5 mM EDTA. The contaminating RNA and proteins were removed by successive treatment with RNase A at 0.2 mg/ml for 1 hr at 37°C and proteinase

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Abbreviations: CaMV, cauliflower mosaic virus; SV40, simian virus 40; bp, base pair(s).

K (Merck, Darmstadt) at 1 mg/ml in 0.5% $NaDodSO_4$ for 2 hr at 37°C. Proteins were extracted once more with phenol and chloroform and DNA was precipitated with ethanol and solubilized in 50 mM Tris·HCl, pH 7.9/50 mM NaCl/5 mM EDTA. The concentration of each preparation was estimated by spectrophotometry: for $A_{1\ cm}^{0.1\%}$ (260 nm) a value of 20 was assumed.

DNA·DNA Hybridization. Either labeled CaMV DNA or pBR322 was used as a hybridization probe (see *Results*). Five micrograms of total DNA (either intact or digested with a restriction endonuclease) was fractionated by gel electrophoresis, denatured *in situ*, and transferred onto nitrocellulose sheets (Schleicher & Schüll 85 BA) according to the method of Southern (15) as modified by Breathnach *et al.* (16). DNA was labeled to 2×10^7 cpm/μg with [α-^{32}P]dATP (2,000 Ci/mmol, Amersham; 1 Ci = 3.7×10^{10} becquerels) using *Escherichia coli* DNA polymerase (Boehringer) according to the nick-translation method of Rigby *et al.* (17).

RESULTS

Ligation. The ability of the cell to ligate a fragmented viral DNA genome was demonstrated by cleaving the CaMV genome into two fragments with restriction endonuclease *Bam*HI (see Fig. 4 and more details ref. 11). Fragments *Bam* A (7,800 bp) and *Bam* B (230 bp) were cloned separately. After purification of the hybrids and fragment excision by *Bam*HI cleaving, the two fragments were mixed and used to inoculate a batch of 30 plants (batch I). Fragment *Bam* A alone was treated similarly (batch II). A third batch of plants was inoculated with full-length CaMV DNA (batch III). Twenty days later, plants of batch I but not of batch II showed the first symptoms of CaMV infection on newly formed leaves, whereas control plants (batch III) showed symptoms after only 10–12 days (5). Thus fragmentation reduces the infectivity of the DNA but does not abolish it (Table 1). Restriction endonuclease *Bam*HI cleaves twice within the putative coding region of "gene" *III* (2). Still, there was a remote possibility that the *Bam* B fragment with the code of "gene" *III* would act as a helper in the replication of the *Bam* A fragment without being integrated into progeny virion DNA. To evaluate this hypothesis, free intracellular viral DNA from plants infected with the mixture of restriction fragments was compared to the DNA from plants infected with intact viral DNA. Because encapsidated DNA is not extracted by the technique used (18), viral DNA was detected after gel electrophoresis by *in situ* hybridization, as described in *Materials and Methods*. Fig. 1*A* shows that the electrophoretic patterns of viral DNA from batches I (lane b) and III (lane c) are similar. As expected, no viral DNA was detected in batch II (lane d). It is noteworthy that the pattern of intracellular viral DNA is different from that of virion DNA (lane a). Both contain circular and linear full-length DNA (bands 1 and 2), but the infected cells in addition contain supercoiled forms (band 4), as well as two incomplete linear fragments (bands 3 and 5) (18).

Table 1. Infectivity of complementary cloned fragments of the CaMV genome

	Batch I*: *Bam* A + *Bam* B fragments mixed	Batch II*: *Bam* A fragment	Batch III†: Control viral DNA
Infected plants/ inoculated plants	15/30	0/30	30/30

* *Bam* A and B fragments were integrated into pBR322, amplified, excised by restriction endonuclease *Bam*HI, mixed in equimolar concentrations to a final concentration of 10 μg/ml in water, and used to inoculate plants. The excised *Bam* A cloned fragment was inoculated alone at the same concentration.

† The control viral DNA was inoculated at 1 μg/ml.

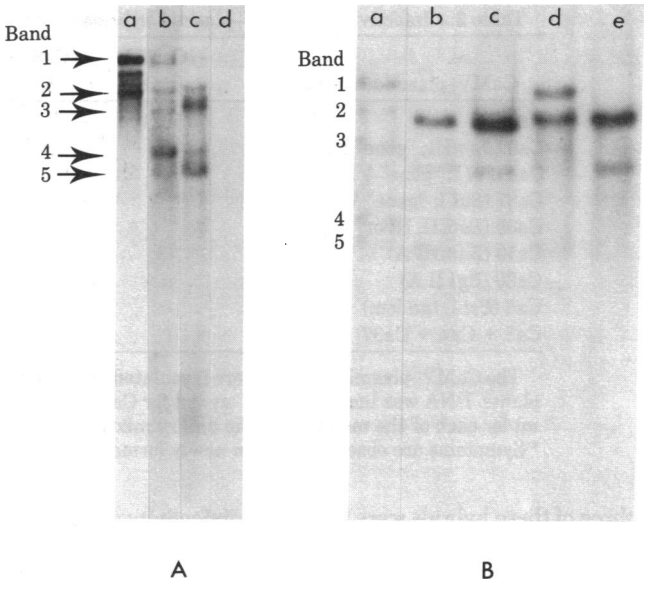

FIG. 1. Southern blot analysis of total DNA extracted from plants inoculated with cloned *Bam*HI fragments of the CaMV genome. (*A*) Intact total DNA. The samples described in Table 1 were analyzed by hybridization with labeled CaMV-Cabb S virion DNA. Lane a, control CaMV virion DNA (0.02 μg); lane b, total DNA from batch I (5 μg); lane c, total DNA from batch III (5 μg); lane d, total DNA from batch II (5 μg). (*B*) DNA treated with restriction enzymes *Bam*HI and *Kpn* I. Lane a, total DNA from batch I; lane b, *Bam*HI-treated DNA from batch I; lane c, *Bam*HI-treated virion DNA; lane d, *Kpn* I-treated DNA from batch I; lane e, *Kpn* I-treated virion DNA. The numbered bands correspond to the species of DNA found in cells infected with intact virions: band 1 is full-length circular relaxed DNA; band 2 is linear full-length DNA; bands 3 and 5 are fragmented linear genome pieces (18); band 4 corresponds to supercoiled DNA. Note that after *Kpn* I treatment of DNA from batch I two populations of molecules are obtained: the linear full-length species expected from cleavage of virion circular DNA and some residual circular DNA that apparently does not contain the *Kpn* I site.

To demonstrate the integrity of progeny DNA, the total DNA from infected plants was cleaved separately by endonuclease *Kpn* I [which cleaves the CaMV genome at a unique site located in the *Bam* B fragment (11)] and by endonuclease *Bam*HI. The restriction products were analyzed as described above. It appears from Fig. 1*B* that viral DNA from batch I (lane b) and control viral DNA (lane c) were completely cleaved with restriction enzyme *Bam*HI; in contrast, only part of the sample from batch I (lane d) was cleaved by restriction endonuclease *Kpn* I under reaction conditions in which control DNA is totally cleaved (lane e). The proportion of circular to linear full-length DNA after *Kpn* I digestion was similar in two independent experiments, and did not depend on enzyme concentration or incubation time. We therefore assume that progeny DNA obtained after a mixed infection by two complementary cloned fragments *Bam* A and B includes two populations of DNAs: one of them identical with the original CaMV genome and the other lacking the small *Bam*HI fragment with the *Kpn* I restriction site.

Recombination. It has been demonstrated (5) that pBR322–CaMV hybrids are not infectious when the plasmid is integrated into CaMV "gene" *V* (*Sal* I or *Pst* I sites) or "gene" *III* (*Bam*HI sites) (Table 2, Ca37, Ca4, and Ca8). The same result was obtained after inoculating plants with a pHC79–CaMV hybrid integrated in the *Bst*EII site (intergenic region or "gene" *VII*) (Table 2, Ca39; refs. 2 and 6). If, however, mixtures of two or

Table 2. Infectivity of CaMV–plasmid hybrids

CaMV–plasmid hybrids	CaMV (*a*)	Ca8 (*b*)	Ca4 (*c*)	Ca37 (*d*)	Ca39 (*e*)	Ca10 (*f*)	Ca80 (*g*)	Ca6 (*h*)
CaMV	10/10							
Ca8 (*Bam*HI; "gene" *III*)		0						
Ca4 (*Pst* I; "gene" *V*)			0					
Ca37 (*Sal* I; "gene" *V*)			4/15	0				
Ca39 (*Bst*EII; IR or "gene" *VII*)					0			
Ca10 (*Bam*HI A)				3/15		0		
Ca80 (*Bgl* II A)						5*/15	0	
Ca6 (*Pst* I, tandem)								5/15
Ca8 + Ca4 + Ca37			30/40					

The CaMV–plasmid hybrids were inoculated alone and in combinations. Results are expressed as infected plants/inoculated plants. DNA was inoculated at 1 μg/ml for CaMV DNA, 2 μg/ml for each of the members of the ternary mixture, and 5 μg/ml for each of the members of the binary mixtures and the single inoculations. IR, intergenic region.
* Symptoms are observed only on newly formed leaves and late in infection.

three of these hybrids were inoculated, infectivity was partially restored (Table 2, column *c*, and Ca8 + Ca4 + Ca37). Restoration of infectivity was greater with a ternary than with the binary mixtures. Moreover, we observed infectivity originating from a full-length CaMV clone mixed with a fragment clone (Table 2, column *d*), as well as from a mixture of two overlapping cloned CaMV restriction fragments (Table 2, column *f*). Column *h* of Table 2, finally, gives the results of an experiment with a partially deleted tandem dimer CaMV clone (1.2 kilobases deleted from one of the repeating CaMV units).

Symptoms in plants infected with the different samples were similar to those in plants infected with the original CaMV DNA but appeared later and on the newly formed leaves earlier than on the inoculated leaves. In contrast, with a mixture of two overlapping cloned CaMV restriction fragments (Table 2, column *f*), symptoms were also observed later, but only on the newly formed leaves.

Plants infected with the various mixtures of CaMV clones were analyzed for the presence of free viral DNA as well as for the presence of pBR322-specific sequences by DNA hybridization. Results obtained with the ternary mixture (Fig. 2, lane b), and those with the binary mixtures (not shown) were identical. A comparison of Fig. 2, lane b, and Fig. 1, lane c (control infection by full-length CaMV genome), shows the close similarity of the two types of progeny DNAs. The supercoiled DNA band in Fig. 2, lane b, is noticeably stronger than in Fig. 1, lane c; however, this difference may reflect variations in efficiency of transfer or hybridization. No hybridization was detected with pBR322 DNA, suggesting that DNA stretches containing the

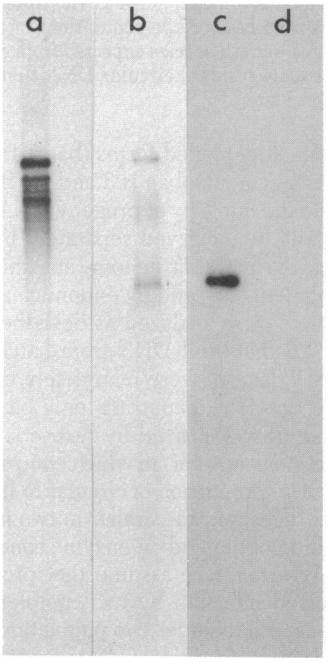

FIG. 2. Southern blot analysis of total DNA from plants infected with a mixture of three full-length recombinant CaMV genomes. Lane a, control CaMV virion DNA (0.02 μg). Lanes b and d, total DNA extracted from plants infected with the ternary mixture of Table 2 (5 μg). Lane c, pBR322 (0.02 μg). Lanes a and b show the hybridization patterns with CaMV virion DNA as a probe. Lanes c and d show the hybridization patterns with pBR322. The pattern of the viral DNA in lane b is very similar to the typical pattern (Fig. 1, lane c). The absence of radioactivity from lane d demonstrates the elimination of pBR322 during the mixed infection.

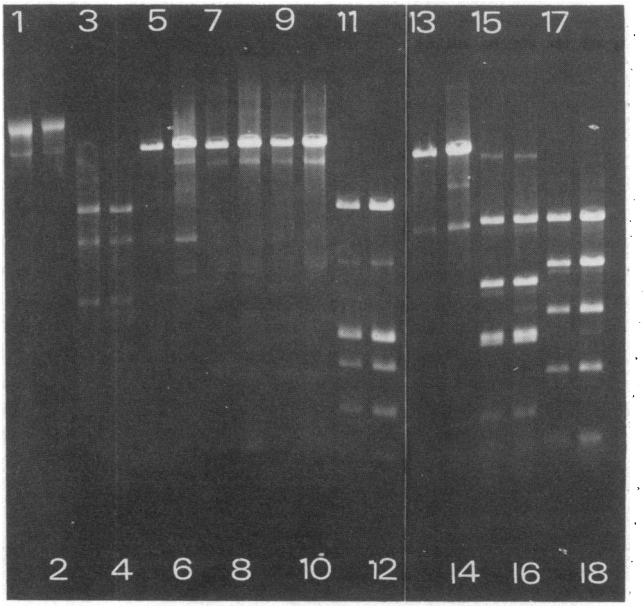

FIG. 3. Electrophoretic pattern of heated and restriction nuclease-cleaved virion DNA extracted from plants infected with the ternary mixture of Table 2. After the indicated treatment, DNA samples (1 μg) were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. Odd-numbered lanes, DNA extracted from plants infected with Cabb-S virions. Even-numbered lanes, DNA extracted from plants inoculated with the ternary mixture. Lanes 1 and 2, untreated DNA; lanes 3 and 4, heated DNA; lanes 5–18, DNA treated with different restriction enzymes: 5 and 6, *Pst* I; 7 and 8, *Bam*HI; 9 and 10, *Kpn* I; 11 and 12, *Hind*III; 13 and 14, *Sal* I; 15 and 16, *Bgl* II; 17 and 18, *Hinc*II.

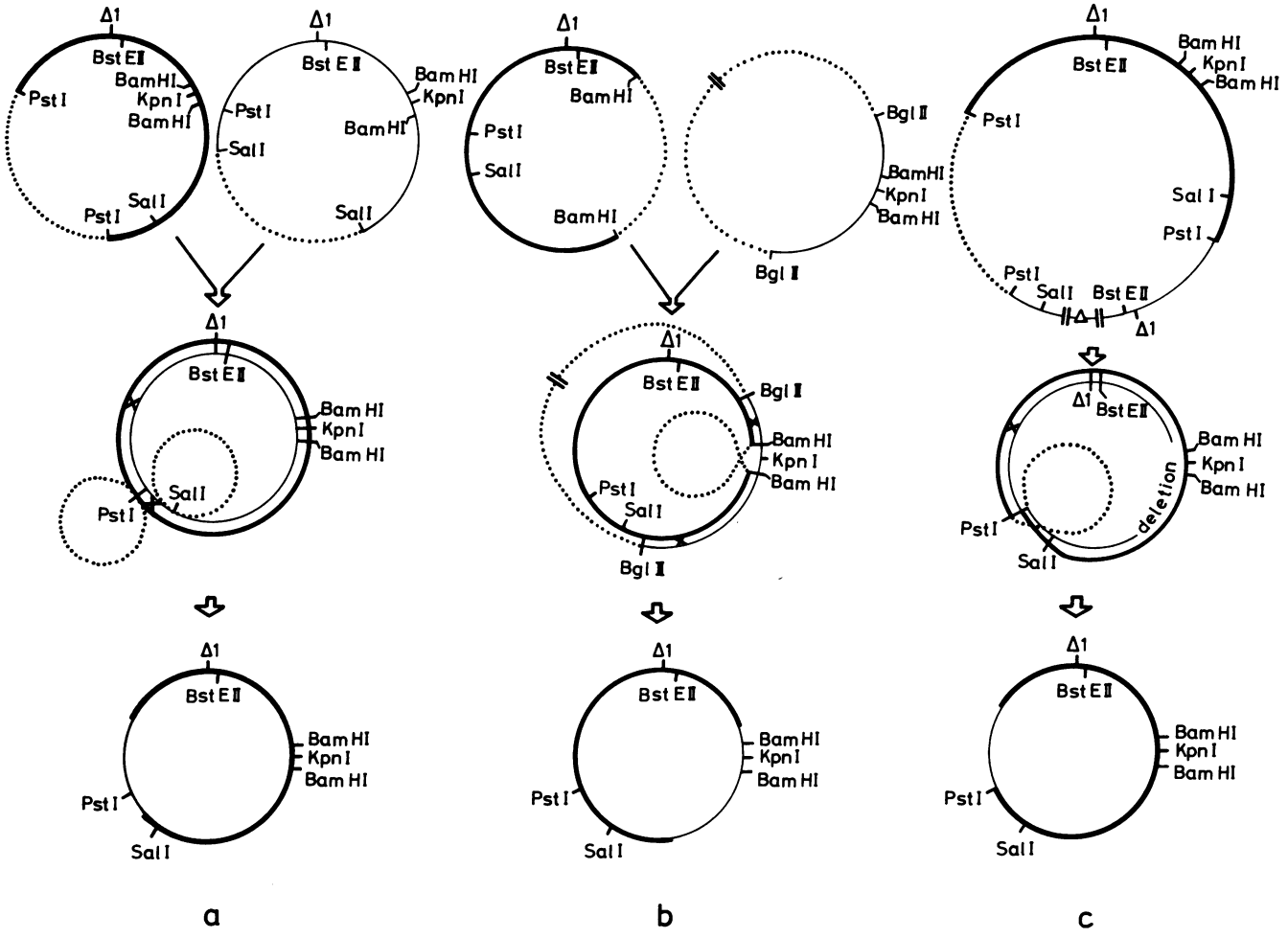

FIG. 4. Suggested scheme for the recombinations observed. (*a*) Recombination between two full-length CaMV DNAs. (*b*) Recombination between two overlapping CaMV DNA fragments. (*c*) Recombination of tandemly dimerized CaMV DNA. One line is a double-stranded DNA. ·····, Plasmid vector sequences; ——, recombination partner 1; ——, recombination partner 2. Δ1, position of gap 1 (see ref. 2) corresponding to origin point of the CaMV DNA map. Δ, Deletion in CaMV DNA genome (*c*).

vector were not amplified (Fig. 2, lane d).

To ascertain sequence homogeneity of progeny DNA from the ternary mixed infection, virions were extracted from infected plants and the DNA was mapped by several restriction enzymes that cleave in the putative recombination regions. The population of encapsidated viral DNA arising after inoculation with the mixture of clones is homogeneous, contains the three gaps (Fig. 3, lane 4), and exhibits the same restriction patterns as control viral DNA (Fig. 3, lanes 5–18).

DISCUSSION

In this paper, we report several findings that demonstrate the occurrence of ligation and recombination of a viral genome (CaMV) in its host.

Our assay for the existence of ligation is based on the observation that a large subfragment of the CaMV genome, the *Bam* A fragment, is not infectious. This fragment contains 7,800 bp of the 8,024-bp CaMV genome and lacks the central part of "gene" *III* (2). Because the linear full-length CaMV genome is infectious, the lack of infectivity of *Bam* A is due not to its linear structure but rather to the deletion of most of "gene" *III*. Expression of this gene is thus required at some stage of the infection process. [In contrast, a part of gene *II* of strain CM4-184 can be deleted (19) without any loss of infectivity]. If, however, plants are coinoculated with the complementary fragment (*Bam* B, 230 bp), infectivity is partially restored. Progeny DNA consists of full-length circular CaMV DNA as shown by its size and band pattern after cleavage with the enzymes *Bam*HI and *Kpn* I. This shows that ligation of the *Bam* A and *Bam* B fragments has occurred. In addition to finding the full-length genome we have indications (Fig. 1*B*) that progeny DNA contains roughly equimolar amounts of a subgenomic species that lacks the *Kpn* I site (located in the *Bam* B fragment). These results are in favor of a helper relationship between the complete viral genome and the large *Bam* A fragment, with the complete genome providing a function or functions necessary for the replication of the genome that has undergone a deletion.

Our approach to the recombination process makes use of the loss of infectivity of the CaMV genome after integration into pBR322 restriction sites (5–7). This lack of infectivity could have several explanations: (*i*) toxicity of the plasmid sequence for the plant cell [this phenomenon has already been described for SV40–pBR322 hybrids in animal cells (20, 21)]; (*ii*) inhibition of viral functions by the increased size of the DNA [Gronenborn *et al.* (22) have recently demonstrated that the maximal size of a foreign DNA insert that can be successfully propagated through virus particles is about 250 bp]; and (*iii*) inactivation of essential viral genes by the plasmid insert. Concerning this last point it is noteworthy that in our experiments infectivity is lost regardless of the site of integration used. In the case of the *Bam*HI, *Sal* I, and *Pst* I hybrids the integrated plasmid se-

quence interrupts coding regions *II* (*Bam*HI) and *V* (*Sal* I and *Pst* I) and the loss of infectivity may reflect loss of an essential gene function. The *Bst*EII hybrid, however, contains the plasmid insert in the long intergenic region or "gene" *VII* but is nevertheless noninfectious. Howell *et al.* (8) have inserted 8-bp linker sequences into various positions of the CaMV genome and tested the infectivity of the modified DNA. In agreement with our results they found that an 8-bp insertion in coding regions is lethal, but, in contrast to our findings, insertion in the long intergenic region did not affect infectivity. Presumably, this latter difference is related to the difference in the length of the inserted sequence used by us and by Howell *et al.* (8). It should also be considered that a large RNA transcript (23, 24) that covers the whole genome and originates about 600 nucleotides upstream of gap 1 (G. Jonard, personal communication) has been described and it may well be that an interruption near its 5′ extremity by a long foreign DNA sequence is deleterious.

Infectivity of the integrated genomes is partially restored when plants are inoculated with two (or more) different clones simultaneously. Progeny DNA from such infections is identical to original CaMV DNA, indicating that we are indeed dealing with a recombination process. These results are in agreement with those independently obtained by Howell *et al.* (8) who, using another approach, demonstrated that, in some cases, plasmid pairs containing a small linker inserted at different places in the CaMV genome were still infectious upon coinoculation. Further experiments are needed, however, to find out whether the rate of recombination is related to the distance between the plasmid or linker integration sites in two coinoculated hybrid genomes. A hypothetical scheme of the observed recombination processes is depicted in Fig. 4. Also shown is how intramolecular recombination of a (partially deleted) tandem dimer clone of CaMV DNA could form intact DNA. This case constitutes a situation comparable to that of the biologically active cloned SV40 and polyoma virus hybrids (20, 25).

Our system probably depends on positive selection pressure for recombination. Two types of pressure might be exerted: (*i*) pBR322 sequences may be toxic to the plant cell and therefore tend to become eliminated. It should be recalled that pBR322 sequences are only poorly replicated when introduced into animal cells in connection with SV40, polyoma virus, or papilloma virus sequences (1, 20–26). (*ii*) The pBR322 sequences may interfere with CaMV replication and expression as discussed above. Thus molecules from which the foreign sequences are eliminated by recombination are efficiently replicated and expressed. It appears that our method, in contrast to that used by Howell *et al.* (8) in which CaMV DNA is freed from plasmid by restriction endonuclease digestion, allows the recombination process to occur without the need for DNA molecules to have homologous sticky ends and that the plant cell is able to promote the whole recombination process. Such a recombination system should thus permit us to create *in vivo* recombinants containing the genome of two CaMV strains. Appropriate choice of the CaMV fragments of each strain used should enable us to determine the function of each CaMV gene.

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