The infectivity of several monomeric clones of pepper huasteco virus was investigated. All clones were infectious when inoculated excised from the plasmid DNA. However, only certain clones were infectious when inoculated in the non-excised form. Constructs in which the cloning site lies inside regions or genes involved in replication (e.g. Rep-binding site, rep and AC2–AC3 genes) were not infectious, whereas constructs in which the site was located inside the CP or BC1 genes were infectious. A clone that interrupts the BV1 gene was not infectious suggesting an early role of BV1 during the establishment of the infection. Linear viral clones containing different DNA fragments at both extremes were also infectious although with a lower efficiency. Analysis of the progeny suggested a precise excision mechanism since in most cases only wild type virus was recovered. The results suggest that excision could be linked to replication through a very specific recombinant process.

Geminiviruses are a group of plant virus characterized by their twinned isometric particles (20 × 30 nm) and small circular ssDNA genomes. They are classified in three subgroups on the basis of their insect vector, host range and genomic organization (Murphy et al., 1995). Pepper huasteco virus (PHV) is a member of subgroup III since it possesses a bipartite genome, infects dicotyledonous plants and is transmitted by the whitefly Bemisia tabaci (Garzón-Tiznado et al., 1993; Torres-Pacheco et al., 1993). Geminiviruses replicate in the nucleus of the infected cell by a rolling circle mechanism.

Experimental evidence obtained with dimeric clones of beet curly top virus (BCTV) and wheat dwarf virus (WDV) suggests that the sequence between the two hairpin structures is preferentially released as a monomeric unit (Heyraud et al., 1993; Stenger et al., 1991). However, in the case of BCTV, monomeric circular units can also arise from random intramolecular recombination events, although with a lower efficiency and a higher variability (Stenger et al., 1991). To initiate replication, viral Rep protein recognizes the origin of replication by interacting with iterative elements found upstream of the hairpin structure (Argüello-Astorga et al., 1994; Fontes et al., 1994). Then, Rep performs a cleavage between the 7th and 8th nucleotides of the conserved nonanucleotide (TAATTT*AC) found in all geminiviruses and corresponding to the loop of the hairpin structure. After the nick, Rep binds to the generated 5’ end. DNA synthesis begins, using a host DNA polymerase, from the free 3’ end replacing/releasing a ssDNA molecule until a new nicking site is found. Rep is also believed to be involved in the circularization process (Heyraud-Nitschke et al., 1995; Laufs et al., 1995).

Like most geminiviruses, PHV is non-mechanically transmissible; however, a good inoculation efficiency is achieved using cloned viral DNA delivered by a biolistic procedure (Garzón-Tiznado et al., 1993). This inoculation method also facilitates the genetic analysis of geminiviruses since it does not require elaborate manipulation of viral DNA before introducing it into the plant tissue. Although the procedure is relatively simple and reproducible, some contradictory results have been reported in terms of the infectivity of cloned viral DNA. For example, PHV monomeric clones were infectious when inoculated as either circular (non-excised from the plasmid) or linear (excised) forms. In other cases, however, it was reported that successful inoculation (either agroinoculation or biolistics) was achieved only by using either dimeric or excised monomeric constructs (Elmer et al., 1988; Gilbertson et al., 1991; Morris et al., 1988; Sung & Coutts, 1995). In the case of African cassava mosaic virus (then called cassava latent virus), Stanley & Townsend (1986) reported that monomeric clones were infectious by mechanical inoculation. However,
Fig. 1. Location of the restriction sites used to produce monomeric clones of pepper huasteco virus. (A) Tandem genomic repeats (dimers) were used to obtain the monomers which were ligated in the respective cloning site of the pBluescript plasmid. When two enzymes are shown, the first one corresponds to the plasmid restriction site whereas the second one corresponds to the site located in the viral genome. (B) Location of the SacI site in the intergenic region of PHV B. The SacI site is located between the putative Rep recognition sites (small arrows). Other overlapping inverted repeats are also indicated (thick arrowheads).

the elimination of plasmid DNA was not accurate and several rearrangements were found in the viral progeny.

To investigate whether the infectivity of the original clones of PHV was a unique characteristic, or whether any monomeric constructions were infectious, a series of monomeric versions of PHV was produced and their infectivity was analysed by biolistic inoculation. The enzymes used for the monomeric clones were selected according to the position of their unique site on the viral genome (Fig. 1). The resulting clones were named after the viral component, the enzyme used and the interrupted gene. For PHV A the following monomers were obtained: Aeco-rep (EcoRI, which lies inside the rep gene), Akpn-ac23 (KpnI, AC2 and AC3 genes), Axba-ac3 (XbaI, AC3 gene), and Amsc-cp (MscI, coat protein gene). The original infectious clone for PHV A was Ahin-ir (HindIII, located in the intergenic region). For PHV B: Bbam-bv1 (BglII, BC1 gene), Bbam-bv1 (BamHI, BV1 gene), Bsac-ir (SacI, intergenic region). The original clone for PHV B was Bhin-bc1 (HindIII, BC1 gene). Considering the viral DNA as the ‘active’ unit inside the plant cell, the plasmid DNA in each construct can therefore be defined as an ‘insertion’ into the viral genome. Thus, the set of monomeric clones can also be considered as a series of ‘insertion mutants’ (Fig. 1A).

The infectivity of all clones was analysed inoculating them in excised or non-excised form. Table 1 summarizes the results of the inoculation assays and shows that all monomeric constructs were infectious when inoculated as excised DNA. Although the degree of efficiency varied, in all cases the symptoms appearance and severity were similar and viral DNA was recovered from young newly developed leaves in equivalent amounts (data not shown). This suggests that linear

Table 1. Infectivity assays of different PHV monomeric constructs inoculated into pepper plants

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inoculation efficiency</th>
<th>Interrupted region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excised</td>
<td>Non-excised</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ahin-ir</td>
<td>10/12</td>
<td>10/20</td>
</tr>
<tr>
<td>Aeco-rep</td>
<td>5/8</td>
<td>0/16</td>
</tr>
<tr>
<td>Akpn-ac23</td>
<td>4/19</td>
<td>0/19</td>
</tr>
<tr>
<td>Axba-ac3</td>
<td>3/11</td>
<td>0/16</td>
</tr>
<tr>
<td>Amsc-cp</td>
<td>*</td>
<td>4/18</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bbam-bv1</td>
<td>4/10</td>
<td>0/24</td>
</tr>
<tr>
<td>Bhin-bc1</td>
<td>10/12</td>
<td>10/20</td>
</tr>
<tr>
<td>Bbg1-bc1</td>
<td>*</td>
<td>6/12</td>
</tr>
<tr>
<td>Bsac-ir</td>
<td>9/15</td>
<td>1/15</td>
</tr>
</tbody>
</table>

* Monomers Amsc-cp and Bbg1-bc1 were cloned at the EcoRV and BamHI sites of pBluescript, respectively; thus, it was not possible to recover the viral DNA for inoculation.
viral DNA can be ligated upon entry into the plant cell to generate a circular dsDNA capable of establishing the infection.

When the monomeric constructs were inoculated as non-excised DNA, the results varied. All monomeric clones that have plasmid DNA interrupting genes involved in replication (Aeco-rep, Akpn-ac23, and Axba-ac3) were not infectious. Monomeric constructs with the cloning site in the intergenic region (Ahin-ir) or the CP gene (Amsc-cp) were infectious, although with variable efficiency. In the case of component B, constructs with the cloning site in the BC1 gene (Bhin-bc1 and Bbgl-bc1) were infectious. Surprisingly, the monomer Bbam-bv1, which interrupts the BV1 gene, was not infectious. Whether the lack of infectivity of this monomeric clone is a consequence of the impairment of BV1 as nuclear shuttle protein (Pascal et al., 1994; Sanderson et al., 1996) or another unreported function, remains to be determined. A fourth monomeric clone obtained using the SacI site located in the common region of PHV B (Bsac-ir) presented a very low inoculation efficiency with only one out of 15 plants developing symptoms. The SacI site is located between the iterative elements suggested to be the binding site for Rep (Fig. 1B) (Argüello-Astorga et al., 1994). Thus, the replication of PHV B might be impaired by the insertion of plasmid DNA between the Rep binding sites. The appearance of symptoms in that plant was delayed several weeks and their severity was also reduced.

These results also help to explain some previous reports. Gilbertson et al. (1993) reported that monomeric clones of the GA and DR isolates of bean golden mosaic virus (BGMV) were not infectious when inoculated as non-excised monomers. In both cases, component A was cloned using the EcoRI site that lies in the overlapping AC2–AC3 genes. Sung & Coutts (1995) also reported that non-excised monomeric clones of potato yellow mosaic virus (PYMV) were not infectious. In this case, the monomeric clone of PYMV component A was cloned using the HindIII site that lies inside the rep gene. Similar observations have also been made with other geminiviruses, including Texas pepper geminivirus (I. Torres-Pacheco & R. F. Rivera-Bustamante, unpublished).

It has been suggested that viral monomeric clones, since they have only one hairpin structure, favour a recombination event as the main mechanism for the excision of viral DNA. This event, however, has been reported as inaccurate and can produce aberrant molecules (Stanley & Townsend, 1986; Stenger et al., 1991). To determine the level of accuracy in this case, viral progeny obtained from several plants inoculated with the non-excised form of Ahin-ir (+Bhin-bc1) clone was analysed. The HindIII site is located in a region that theoretically could tolerate changes. DNA from symptomatic plants was extracted individually since the viral progeny recovered in each plant originated from independent recombination events. The intergenic region of component A of the viral progeny was analysed by PCR amplification and sequencing using oligonucleotides and conditions previously described (Torres-Pacheco et al., 1996). Sequence analysis of the amplified fragments showed that in five independent events, the sequence of the progeny was identical to the wild-type virus. This suggests that the excision can be precise even in a context with low selection pressure. A similar analysis was carried out with the only plant that developed symptoms after inoculation with the non-excised monomer Bsac-ir (see Table 1). Viral progeny was recovered from the plant, which showed delayed and mild, atypical symptoms, and most of the intergenic region of component B was amplified by PCR using the oligonucleotide primers described by Rojas et al. (1993). Interestingly, the fragment amplified from the viral progeny was smaller than the expected size. Sequence analysis showed that a 148 nt fragment was deleted (nucleotides 2318–2465 of PHV B). However, the SacI site and the iterative elements were maintained since the deletion started 28 nt to the left of the SacI cloning site.

The rolling circle mechanism requires a circular molecule for the replication process. To investigate whether the excision process also required a circular molecule, plants were inoculated with several linear monomeric clones in which viral DNA was flanked by plasmid DNA of various sizes (Fig. 2). In certain cases, to reduce the possibility of a religation event, non-compatible restriction enzymes were used to generate the linear molecules. For PHV A, linear molecules were obtained by digesting the Ahin-rep circular molecule with PvuII (cohesive ends), PvuII (blunt ends), and BamHI–ClaI (non-compatible cohesive ends). For PHV B, linear Bhin-bc1 was linearized by digestion with XbaI (cohesive ends) and XbaI–XhoI (non-compatible cohesive ends) (see Fig. 2). Table 1 shows that the deletion was achieved in all cases. However, the efficiency was lower than that obtained with either non-excised or excised viral molecules without additional plasmid DNA. Because of the low efficiency obtained in these experiments, it was not possible to correlate efficiency of inoculation with neither the size of the additional plasmid DNA nor the type of molecule end (cohesive, blunt or non-compatible).

Viral progeny from plants inoculated with linearized Ahin-rep (PvuII, PvuII and BamHI–ClaI) was also analysed by PCR amplification and sequencing (as mentioned before). Although in most cases the sequence of the progeny was identical to wild-type PHV, in one case the viral progeny recovered from
a plant inoculated with Ahin-Poull showed a couple of nucleotide changes detected near the HindIII site. One of the changes was localized in a hexanucleotide (AAGCGT) almost identical to the HindIII sequence (AAGCTT) and located a few nucleotides upstream.

There are examples of replication of viral molecules with a size smaller than that of the wild-type virus (Azzam et al., 1994; Etessami et al., 1989; Gardiner et al., 1988; Stanley & Townsend, 1985). However, there are no reports on the replication of viral variants with a larger size. Thus, a larger size seems to be a limitation for replication and/or movement within the plant. Therefore, the difference between an infectious and a non-infectious monomeric clone could be the efficiency (or achievement) of the excision of plasmid DNA by a recombination event. The possibility that several independent mechanisms with varying precision and efficiency could be acting simultaneously cannot be rejected. Nevertheless, similar questions have to be addressed. Are viral factors required for the recombination event? If no viral factors are needed, recombination could take place before viral gene expression and replication begins. The results reported here do not seem to support this hypothesis. If the expression of viral genes is a requirement for recombination, then, the excision will take place during or after replication. It is not possible, however, to completely differentiate at this point whether only expression itself is necessary (e.g. viral proteins) or also the process of replication.

The results obtained with the Amsc-cp clone (a rather low inoculation efficiency) were interesting since the CP gene is not essential for PHV infectivity in pepper (R. G. Guevara-Gonzalez & R. F. Rivera-Bustamante, unpublished). Since Mscl is a blunt-end cutter, the monomeric unit was cloned in the EcoRV site of pBluescript. This resulted in the absence of the six-base redundancy found when the virus genome and the plasmid DNA are digested with the same enzyme and ligated. It is possible that this type of redundancy could be important during the recombination event needed for the excision (hot spots?) and its absence might reduce the efficiency of the process. The changes found in the plant inoculated with Ahin-rep (Poull) support this hypothesis.

We would like to thank Pedro L. Ramos for helpful discussions and technical assistance, and Dr. June Simpson for critical reading of the manuscript. This research was supported by a grant from CONACYT-México (4769-N9406) and ICGEB (CRP/MEX94–03; 95/005) to R. F. R.-B.G.M. B.-R. J.T.-P. and R. G. G.-G. also acknowledge fellowship support from CONACYT.

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Received 29 October 1996; Accepted 19 December 1996