The Plant Cell, Vol. 4, 799–809, July 1992 © 1992 American Society of Plant Physiologists

Sequence-Specific Interaction with the Viral AL1 Protein Identifies a Geminivirus DNA Replication Origin

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The bipartite geminiviruses such as tomato golden mosaic virus (TGMV) and squash leaf curl virus (SqLCV) have two single-stranded circular genomic DNAs, the A and B components, thought to be replicated from double-stranded circular DNA intermediates. Although it has been presumed that the origin sequences for viral replication are located in the highly conserved 200-nucleotide common region (CR) present in both genomic components and that the viral-encoded AL1 protein interacts with these sequences to effect replication, there has been no evidence that this is in fact so. We have investigated these questions, demonstrating selectivity and sequence specificity in this protein-DNA interaction. Simple component switching between the DNAs of TGMV and SqLCV and analysis of replication in leaf discs showed that whereas the A components of both TGMV and SqLCV promote their own replication and that of their cognate B component, neither replicates the noncognate B component. Furthermore, using an in vivo functional replication assay, we found that cloned viral CR sequences function as a replication origin and direct the replication of nonviral sequences in the presence of AL1, with both circular single-stranded and double-stranded DNA being synthesized. Finally, by the creation of chimeric viral CRs and specific subfragments of the viral CR, we demonstrated sequence-specific recognition of the replication origin by the AL1 protein, thereby localizing the origin to an ~90-nucleotide segment in the AL1 proximal side of the CR that includes the conserved geminiviral stem-loop structure and ~60 nucleotides of 5′ upstream sequence. By deletional analysis, we further demonstrated that the conserved stem-loop structure is essential for replication. These studies identify the functional viral origin of replication within the CR, demonstrating that sequence-specific recognition of this origin by the AL1 protein is required for replication.

INTRODUCTION

The bipartite geminiviruses such as tomato golden mosaic virus (TGMV) and the squash leaf curl viruses (SqLCV-E and SqLCV-R) contain two ~2.7-kb circular single-stranded DNA genomic components designated A and B (Hamilton et al., 1983, 1984; Lazarowitz and Lazdins, 1991). Together, these two components encode six genes that are named according to the component (A or B) and location on the virion (rightward) or complementary (leftward) DNA strand. Those genes essential for replication are located on the A component, whereas the B component is required for systemic movement in the plant. The two B-encoded genes (BR1, BL1) and AL2 are needed for viral movement in the plant, the latter possibly having an indirect role (Brough et al., 1988; Etessami et al., 1988; Sunter and Bisaro, 1991). On the A component, in addition to the coat protein gene, are two genes involved in viral replication—AL1 and AL3. Mutational analyses and expression of viral genes in transgenic plants have shown that the AL1 protein is the only virus-encoded product required for replication, and it is essential for the replication of both the A component and its cognate B component (Brough et al., 1988; Elmer et al., 1988a; Hanley-Bowdoin et al., 1990; Etessami et al., 1991). The AL3 gene product increases the efficiency of viral DNA replication but is not essential (Sunter et al., 1990).

Although the genetic maps have been determined for several bipartite geminiviruses and shown to be similar, the viral replication origin has not been identified. The cognate A and B components comprising the genome of a given bipartite virus are distinguished by an ~200-nucleotide intergenic common region (CR) (Davies et al., 1987; Lazarowitz, 1987, 1992). These CR sequences are identical in the two genomic components of any single bipartite geminivirus, but they are completely different among the separate geminiviruses with the exception of a conserved 29- to 32-nucleotide sequence element that can potentially form a hairpin with the loop sequence TAATATTAC present in all viruses. In the geminiviruses infecting dicotyledonous hosts, this conserved element has the consensus sequence GGCCA$^{T}_{A}$CCGN$^{TA}_{AT}$TAATATTACCG-G$^{A}_{T}$TGGCC (Lazarowitz, 1987). The existence of this viral CR

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and the construction of a few insertion and deletion mutations within this region that destroyed viral replication and/or infectivity have led to the presumption that the AL1 protein must interact with sequences (the origin) in the CRs of the two genomic components to effect viral DNA replication (Revington et al., 1989; Ugaki et al., 1991). However, the existence and specificity of this presumed AL1 protein–origin interaction have not been demonstrated.

Recent characterization of SqLCV-E (extended host range) and SqLCV-R (restricted host range) has shown them to be highly homologous. The CRs of these two viruses are nearly identical, differing by a 13-nucleotide deletion and a few nucleotide changes in SqLCV-R, as compared to SqLCV-E. Furthermore, AL1 of SqLCV-E was shown to replicate SqLCV-R, as evidenced by the ability of the SqLCV-E A component to replicate the genomic components of SqLCV-R in certain restrictive hosts (Lazarowitz, 1991). Nucleotide sequence analysis shows that these SqLCVs and TGMV are as distantly related as are all other bipartite geminiviruses characterized to date (Lazarowitz and Lazdins, 1991), with the different individual genes being ~61 to ~84% homologous. Their CRs also display no obvious sequence homology except for the canonical geminivirus sequence element and 18 nucleotides at the beginning of each viral CR. We have therefore used these SqLCVs and TGMV and their ability to infect a common host, namely *Nicotiana benthamiana*, to define the viral replication origin and demonstrate that the AL1 protein interacts with the viral CR in a sequence-specific manner to effect viral DNA replication. As described below, component switching and replication studies in tobacco leaf discs demonstrated the selectivity of each viral A component to replicate its cognate B component CR and inability to replicate a viral B component with a noncognate CR. Furthermore, analysis of the replication of chimeric viral CR constructs in the presence of each viral AL1 demonstrated a sequence-specific interaction of the AL1 protein with the AL1 proximal part of the viral CR. These studies, combined with further deletional analysis, identified the viral origin(s) for both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) replication as being located within an ~90-nucleotide fragment that includes the conserved geminivirus potential stem-loop structure and ~60 nucleotides of 5′ upstream sequence, with the potential stem-loop structure being essential for replication.

RESULTS

Component Exchange

To investigate the specificity of the interaction between the AL1 protein and the replication origin presumed to lie within the viral CRs, we first performed a simple component switching experiment between the DNAs of TGMV and SqLCV-E. Each viral A component was agroinoculated onto tobacco leaf discs together with either its cognate B component or the heterologous B component (e.g., TGMV A + SqLCV-E B) and assayed for the ability of the AL1 protein to replicate the coinoculated B component. Although each viral A component was capable of autonomous replication in these leaf disc studies and could support the replication of the cognate B component, neither A component supported the *trans*-replication of the heterologous B component, as shown in Table 1. For example, TGMV B was replicated in the presence of TGMV A but not in the presence of SqLCV-E A, and in a similar manner SqLCV-E B was replicated by SqLCV-E A but not by TGMV A. SqLCV-E B also did not replicate when agroinoculated onto leaf discs from transgenic tobacco expressing TGMV AL1, although these same leaf discs supported the replication of TGMV B (Table 1). These findings were consistent with the AL1 protein recognizing sequences within the CR to effect replication of the cognate B component and supported the presumption that a specific viral origin was located within this shared region of the genome. More important, these results further demonstrated the strict specificity of the TGMV and SqLCV-E AL1 proteins for each individual viral replication origin, thus establishing the basis for the construction of origin cassette vectors to map the viral origin.

Viral Replication Origin(s) within the CR

To more precisely delimit the viral replication origin as well as demonstrate sequence-specific interactions with the AL1 protein, we devised an assay to identify functional elements within

Table 1. Specificity of AL1 Protein for Replication of CR Constructs

Cloned DNA	Replication in the Presence of AL1 from TGMV[a]	Replication in the Presence of AL1 from SqLCV-E[b]
TGMV B (intact)[c,d]	+[e]	−[e]
SqLCV-E B (intact)[c,d]	−	+
TGMV-CR/CAT (*pMON1603*)	+	−
SqLCV-E-CR/CAT (*pSQE-33*)	−	+
SqLCV-R-CR/CAT (*pSQR-48*)	−	+
TGMV:SqLCV-E/CAT (*pMON1714R* and derivatives)	+	−
SqLCV-E:TGMV/CAT (*pMON1716* and derivatives)	−	+

[a] Expression cassette contained TGMV AL1-2-3.
[b] Results the same with SqLCV-E AL1-2, AL1-2-3, AL1-2⁻, and AL1-2⁻-3 expression cassettes.
[c] Dimeric construct of intact viral genomic component.
[d] AL1 supplied either by coinoculation with an intact A component (TGMV or SqLCV-E) or inoculation into leaf discs from transgenic *N. benthamiana* expressing TGMV AL1 (Hanley-Bowdoin et al., 1990).
[e] +, replication based on detection of CAT mini-circle DNA of the expected size; −, no replication detected.

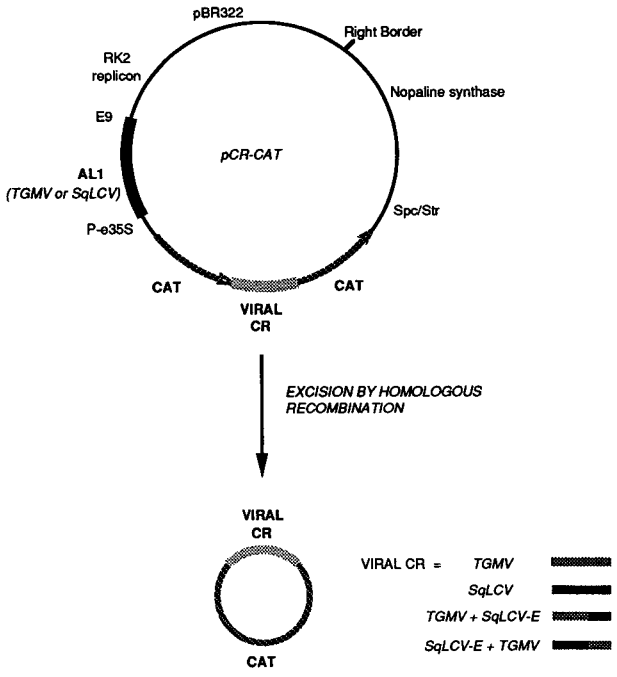

Figure 1. Schematic Diagram of AL1-Dependent Replication Assay.

Binary vector *pCR-CAT* contains the CR construct to be tested (▒▒▒), cloned between two copies of the CAT coding sequence, and a cassette (■■■) expressing the AL1 of TGMV or SqLCV-E under the control of the enhanced 35S CaMV promoter (P-e35S). Upon introduction into a leaf disc, a copy of the CR::CAT mini-circle DNA is excised by homologous recombination within the CAT sequences. If the AL1 protein being expressed recognizes the CR, the CR::CAT mini-circle is amplified and can be detected on DNA gel blots.

the CR as judged by their ability to direct replication when present rather than by loss of replication when absent. We directly demonstrated that viral replication origins are completely within the CR by testing the ability of cloned CR sequences to replicate nonviral DNA segments. TGMV or SqLCV-E CRs were cloned between direct duplications of the bacterial chloramphenicol acetyltransferase (CAT) coding sequence contained within an Agrobacterium plant transformation vector (see Methods), as shown in Figure 1. Similar vectors had been used successfully in earlier studies of the excision and replication of maize streak virus genomes (Lazarowitz et al., 1989). In these previous studies, circular replicating chimeric DNAs containing viral sequences fused to CAT sequences were generated by homologous recombination within the CAT duplications following their introduction into plant cells. For the present assay, the appropriate AL1 protein was provided in *trans* from an expression cassette located on the same plant transformation vector as the CR to drive replication of the excised CR-CAT circular DNAs (Figure 1). Thus, different combinations of CR constructs and AL1 proteins could be easily tested. Following introduction into leaf discs by agroinoculation, mini-circle chimeric DNA containing a single copy of CAT and the cloned CR construct would be excised from the vector (Figure 1). If the cloned CR contains a functional origin, this will be detected on DNA gel blots as amplification of the CR::CAT mini-circle using a CAT-specific probe. The design of the vector assures that each cell containing an excised chimeric mini-circle will also be expressing the viral AL1 protein, thus increasing the potential sensitivity of this assay.

As expected, each intact viral CR was recognized and replicated by its cognate AL1 protein. Thus, as shown in Figures 2A and 3, following agroinoculation of tobacco leaf discs with *pMON1603* (containing the intact TGMV CR and TGMV AL1-2-3 expression cassette) or with *pSQE33-116* (containing the intact SqLCV-E CR and SqLCV AL1-2-3 expression cassette), small circular DNAs of the expected sizes (TGMV::CAT = 1.2 kb; SqLCV-E::CAT = 943 b) were detected when blots were probed with CAT-specific sequences (Figure 2A, TGMV lane, and Figure 3, SqLCV-E lane). No small DNAs were detected if the expression cassette was omitted from the construct (data not shown). Both replicating ssDNA and dsDNA were detected. The identity of the slower migrating small DNA as circular dsDNA of the correct structure was confirmed by its resistance to digestion by *S1* nuclease and susceptibility to digestion with restriction enzymes that uniquely cut within the different viral CRs to yield distinct fragments from the integrated or excised DNA forms (data not shown). That the faster migrating small replicated DNA was indeed ssDNA was demonstrated by its susceptibility to digestion with mung bean nuclease (Figure 2B, TGMV lanes). These studies clearly localize the viral replication origin(s) to sequences completely within the viral CR and further suggest that origins for both viral ssDNA and dsDNA replication are within this region.

AL1 Recognition Maps to Specific CR Sequences That Include the Functional Origin(s)

To more precisely delineate the origin sequences within the CR and define those elements recognized by the AL1 protein, we used our mini-circle replication assay to test chimeric CR constructs containing sequences from the CR of each virus constructed in such a manner as to preserve potentially critical structural features. Thus, as shown in Figure 4, chimeric viral CR clones joined at the unique SspI site (see also Methods) were tested for their ability to be replicated by the AL1 protein of either TGMV or SqLCV-E. We reasoned that not only would such constructs allow us to define precisely the viral origin, they would do so in the context of the specificity of interactions with either the TGMV or SqLCV-E AL1 protein. Thus, if a particular construct was not replicated by one of the viral AL1 proteins, we could determine that this resulted from lack of recognition of the origin by the particular AL1 protein expressed rather than a structural defect within the cloned CR since that same chimeric CR should be replicated by the alternative viral AL1 protein.

As shown in Figures 2 and 3, the chimeric CR in which the left half was derived from TGMV and the right half from

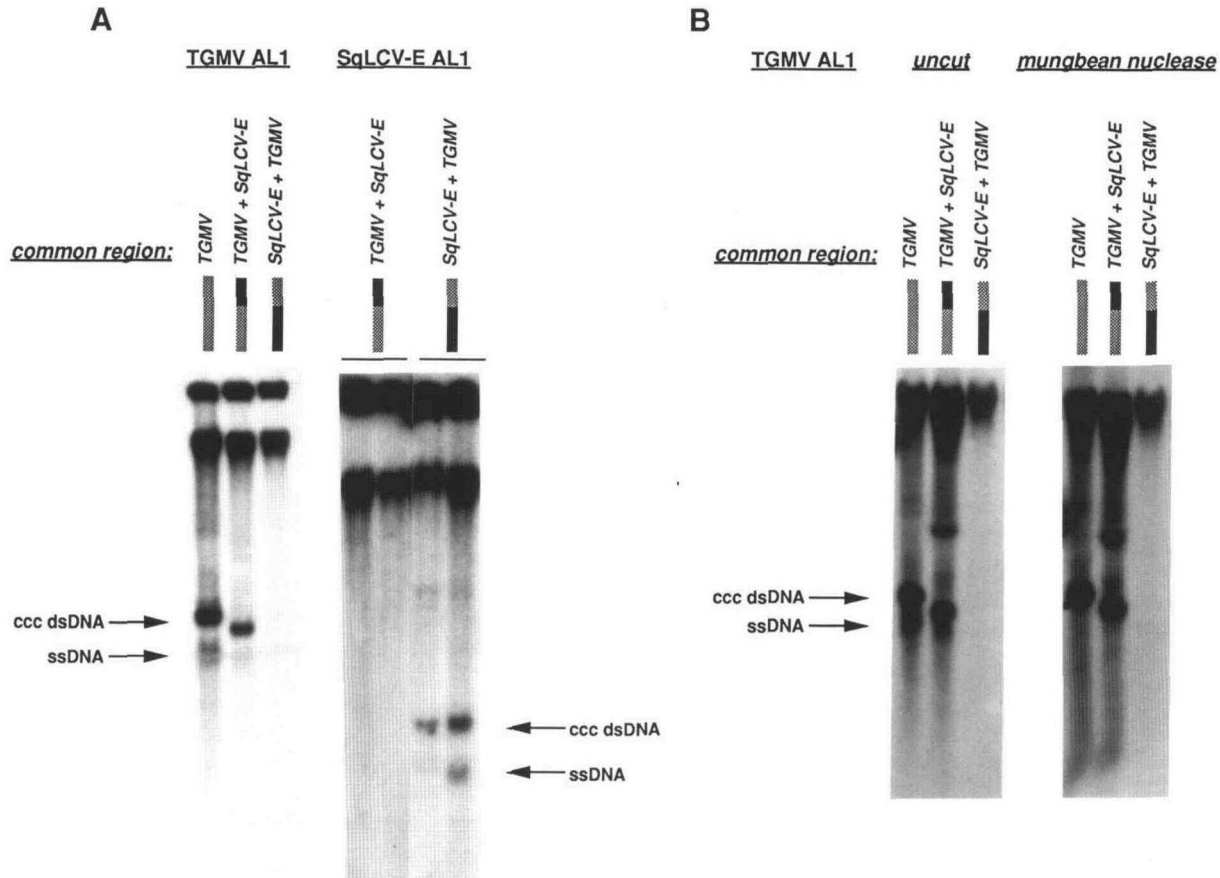

Figure 2. Replication of CR::CAT Constructs in the Presence of AL1 from TGMV or SqLCV-E.

DNA gel blots of extracts from agroinoculated leaf discs were hybridized with ^{32}P-labeled CAT coding sequence probe.

(A) Replication in the presence of the AL1 protein from TGMV or SqLCV-E as indicated above the gel blots. Plasmid constructs tested are (left gel) *pMON1603* (TGMV CR), *pMON1714* ($TGMV_{left}$ + $SqLCV\text{-}E_{right}$ CR ▒▒▒), *pMON1716* ($SqLCV\text{-}E_{left}$ + $TGMV_{right}$ CR ▒▒▒); and (right gel) *pMON1714-14*, *pMON1714-29* ($TGMV_{left}$ + $SqLCV\text{-}E_{right}$ CR lanes ▒▒▒), *pMON1716-243*, *pMON1716-255* ($SqLCV\text{-}E_{left}$ + $TGMV_{right}$ CR lanes ▒▒▒). See Table 2 for details of each CR plasmid construct.

(B) Sensitivity to digestion with mung bean nuclease. Replication is in the presence of AL1 protein from TGMV. Extracts were incubated without or with addition of mung bean nuclease as indicated. Plasmids are *pMON1603* (TGMV CR), *pMON1714* ($TGMV_{left}$ + $SqLCV\text{-}E_{right}$ CR ▒▒▒), *pMON1716* ($SqLCV\text{-}E_{left}$ + $TGMV_{right}$ CR ▒▒▒).

Marked are the positions of covalently closed circular CR::CAT double-stranded (ccc dsDNA) and single-stranded DNA (ssDNA).

SqLCV-E (TGMV + SqLCV-E lanes) was replicated by the TGMV AL1 protein (*pMON1714R*) but not by the AL1 protein from SqLCV-E (*pMON1714-14*, *pMON1714-29*, and *pMON1714-3*). In a similar manner, the chimeric CR consisting of the left half of the SqLCV-E CR and the right half of the TGMV CR (SqLCV-E + TGMV lanes) was replicated by the AL1 protein of SqLCV-E (*pMON1716-243*, *pMON1716-44*) but not by the AL1 protein of TGMV (*pMON1716*). Furthermore, Figures 2 and 3 and Table 2 show that ssDNA and dsDNA of the expected sizes were found to be replicated (1714R::CAT = 1.1 kb; 1716::CAT = 1.3 kb), as was the case for the individual viral CRs. These results strongly suggested that the viral replication origin(s) for at least both dsDNA-to-ssDNA and dsDNA-to-dsDNA synthesis were located within a fragment that includes the left half (AL1 proximal side) of the viral CR and the potential stem-loop structure. The only TGMV AL1 protein expression cassette tested contains the intact AL1-AL2-AL3 region (hereafter AL1-2-3) from the viral A component. For SqLCV-E, AL1, AL1-AL2, and AL1-AL2-AL3 expression cassettes (hereafter AL1, AL1-2, or AL1-2-3) were all tested (see Methods and Table 2). Both the SqLCV-E AL1-2 and AL1-2-3 expression cassettes gave identical results in terms of specificity, the sizes of the replicated CR::CAT constructs, and the presence of replicated ssDNA and dsDNA (see above, and Figures 2 and 3; data not shown).

They each directed the replication of CR clones containing the left half of the SqLCV-E CR regardless of the viral sequences comprising the right half of the CR and did not support the replication of the CR construct containing the left half of the TGMV CR (Figures 2 and 3, and Tables 1 and 2).

Rather unexpectedly, SqLCV-E AL1 expression cassette clones did not appear to support the replication of either chimeric viral CR within the level of sensitivity of our assay system. However, precisely the same CR clones were replicated by either the SqLCV-E AL1-2 and AL1-2-3 or TGMV AL1-2-3 expression cassettes, depending on their left halves being from

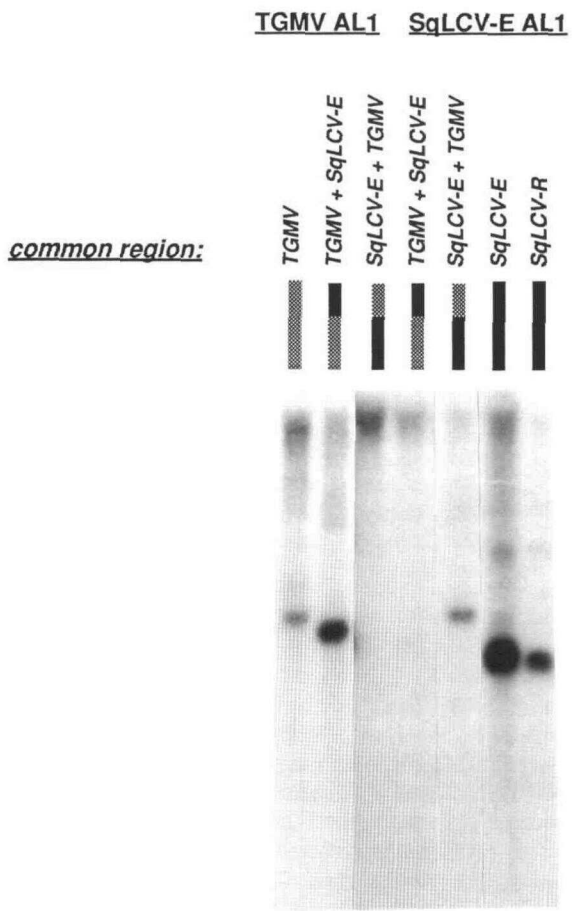

Figure 3. Replication of CR::CAT Constructs in the Presence of the AL1 Protein from TGMV or SqLCV-E.

The TGMV (lanes 1 to 3) or SqLCV-E (lanes 4 to 7) AL1 expression cassette is indicated above each DNA gel blot. Detected are covalently closed dsDNA mini-circle forms. Plasmid constructs are as follows (left to right): *pMON1603* (1220 nucleotides) (TGMV CR), *pMON1714R* (1090 nucleotides) ($TGMV_{left}$ + $SqLCV\text{-}E_{right}$ CR ▒▒▒▒), *pMON1716* ($SqLCV\text{-}E_{left}$ + $TGMV_{right}$ CR ▒▒▒▒), *pMON1714-3* ($TGMV_{left}$ + $SqLCV\text{-}E_{right}$ CR ▒▒▒▒), *pMON1716-243* (1290 nucleotides) ($SqLCV\text{-}E_{left}$ + $TGMV_{right}$ CR ▒▒▒▒), *pSQE33-113* (943 nucleotides) (SqLCV-E CR), and *pSQR-48* (930 nucleotides) (SqLCV-R CR).

the SqLCV-E or TGMV CR, respectively. Hence, it was formally possible that the AL2 protein which could be expressed by those SqLCV cassettes that did support replication could be functioning in excision and/or replication in our assay. The AL2 protein of TGMV, a transactivator of coat protein transcription, is not essential for viral DNA replication (Sunter et al., 1990; Sunter and Bisaro, 1991), and AL1 and AL3 of TGMV are the only viral genes needed for viral replication, with the latter not being essential but appearing to increase the efficiency of viral DNA replication. Thus, to draw conclusions concerning the interaction of AL1 with the CR, we directly demonstrated that the AL2 protein of SqLCV-E was not affecting the results of our assay. AL1-2 and AL1-2-3 expression cassettes containing a frameshift mutation that eliminated AL2 function (see Methods) were tested for their ability to replicate both chimeric and SqLCV CRs in our assay. Although they did not replicate the chimeric CR containing the left half of the TGMV CR, both of these expression cassettes replicated the chimeric CR containing the left half of the SqLCV-E CR (data not shown) and subregions of the SqLCV-E CR (see below) to the same extent as the expression cassettes containing an intact SqLCV-E AL2 gene. Hence, we concluded that the geminivirus AL1 protein interacts with the viral CR in a sequence-specific manner, recognizing sequences located within the AL1 proximal half of the CR and possibly including the stem-loop structure. Although this sequence-specific recognition may involve host-encoded proteins, it does not require the participation of any other virus-encoded proteins.

The above results with intact as well as chimeric TGMV and SqLCV-E CRs suggested that the viral replication origin(s) was located within a 123-nucleotide sequence within the CR that includes the canonical geminivirus sequence element (potential stem-loop structure) and ~90 nucleotides of 5′ upstream sequence (Figure 4). To further delimit the viral origin sequences, as well as determine the requirement for the conserved potential stem-loop structure, we analyzed the replication of specific cloned subregions of the SqLCV-E CR (see Methods). Previous leaf disc analyses using intact A and B components of SqLCV-E and the highly homologous SqLCV-R have demonstrated that the A component of SqLCV-E could direct the replication of SqLCV-R DNA components (Lazarowitz, 1991). The CRs of SqLCV-E and SqLCV-R are nearly identical, the major difference being a 13-nucleotide deletion in the CR of SqLCV-R located 1 nucleotide upstream of the apparent TATA element for AL1. Previous studies predicted that this deletion should not affect viral DNA replication (Lazarowitz, 1991). This is indeed so, as demonstrated by the ability of the SqLCV-E AL1-2-3 expression cassette to replicate *pSQR-48*, which contains the cloned SqLCV-R CR (Figure 3, SqLCV-R lane, and Figure 4). This 13-nucleotide deletion has also been directly introduced into the cloned SqLCV-E CR along with a deletion that removes the 5′ end of the viral CR to within 25 nucleotides of the TATA box for AL1. This mutant SqLCV-E CR, which begins at nucleotide 62 in the viral CR (*pSQ-dl62*), was also replicated by the SqLCV-E AL1-2-3 expression cassette

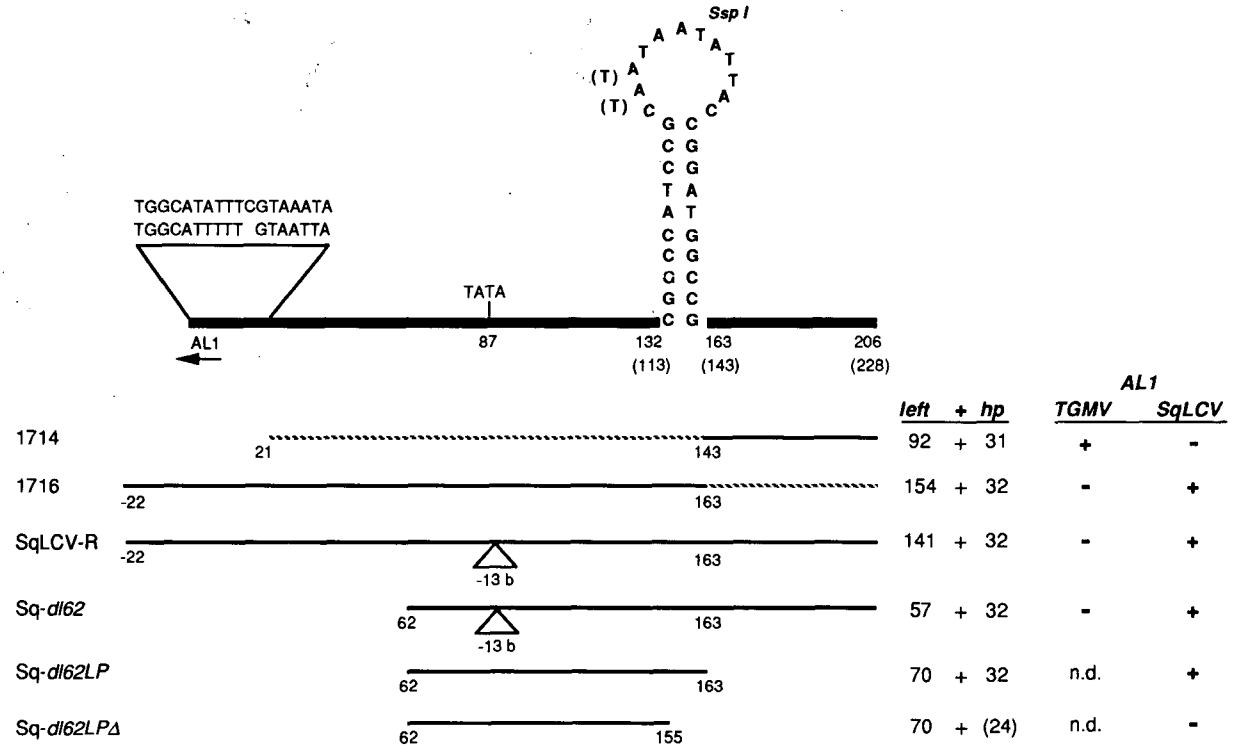

Figure 4. Diagrammatic Representation of the CRs (Virion Strand 5′-to-3′) of TGMV and SqLCV-E.

CRs are aligned beginning at the second codon of AL1 (left) and showing the near identical sequences at the beginning of the CR (top, TGMV; bottom, SqLCV-E) and the sequence of the potential stem-loop structure from SqLCV-E with the changes in TGMV (TT) in the loop as indicated. Numbers underneath are the nucleotide positions within the CR of SqLCV-E or TGMV (in parentheses), with the position of the apparent TATA box for AL1 marked. Aligned beneath are the fragments containing the replication origin(s) based on their sequence-specific interaction with AL1 and a summary of their replication in the presence of AL1 from TGMV or SqLCV-E. "left + hp" indicates the size of the stem-loop structure ("hp") and 5′ upstream sequences ("left"). SqLCV CR sequences, ——; TGMV CR sequences, ∿∿∿; n.d., not determined; +, construct replicated in the presence of the AL1 indicated; −, construct did not replicate.

when tested in our CAT reporter gene assay (Figure 4). Together with our analysis of the chimeric CRs, these results are consistent with the viral replication origin(s), as defined by the sequence-specific interaction with the AL1 protein, being located within an ∿90-nucleotide segment of the viral CR that contains the conserved viral stem-loop structure and ∿60 nucleotides of 5′ upstream sequence.

To more precisely demonstrate this result, we cloned the regions of the SqLCV-E CR located between nucleotides 62 and 163 (*pSQ-dl62LP*) or 62 and 155 (*pSQ-dl62LPΔ*) (see Methods) and tested these for their ability to direct the replication of the CAT gene in our assay. The 3′ end of the former clone is located precisely at the last nucleotide in the stem of the potential stem-loop structure (Figure 4) and thus the clone contains no viral sequences from the right half of the CR. The latter clone terminates two nucleotides past the loop sequence (Figure 4) and hence is not capable of forming the potential stem-loop structure. As shown in Figures 4 and 5, *pSQ-dl62LP* is replicated equally well by the cassette expressing SqLCV-E AL1-2-3 (*pSQ-dl62LP-125*) and by those expressing SqLCV-E AL1-2$^-$ and AL1-2$^-$-3 in which AL2 function has been eliminated by a frameshift mutation (*pSQ-dl62LP-23* and *pSQ-dl62LP-34*). *pSQ-dl62LPΔ*, in which the potential stem-loop structure cannot be formed, was not replicated by the SqLCV AL1-2-3 expression cassette. These results, together with those described above, clearly identify the ∿90-nucleotide segment of the viral CR encompassing the conserved viral stem-loop structure and ∿60 nucleotides of 5′ upstream sequence as both containing the viral replication origin(s) and being specifically recognized by the AL1 protein to effect DNA replication. Our findings further demonstrate that the conserved viral stem-loop structure is essential for replication to occur.

DISCUSSION

The studies described here directly demonstrate that the geminivirus AL1 protein interacts with the viral CR in a sequence-specific manner to effect viral DNA replication.

Additionally, our results also map the viral origin of replication to within an ~90-nucleotide region that includes the conserved sequence element (potential stem-loop structure) and ~60 nucleotides of 5′ upstream sequence in the AL1 proximal half of the CR, with the stem-loop structure being essential for replication. An in vivo origin cassette vector system in which the replication of different chimeric viral CRs could be directly assayed in the presence of the AL1 protein from either TGMV or SqLCV-E (Figure 1) allowed us to identify sequence elements that when present would direct replication, rather than infer their existence by loss of replication in their absence. Thus, we could directly evaluate the recognition of the relevant sequences by the AL1 protein knowing that if a particular CR construct did not work it was not because that construct was defective. When a particular CR clone was not replicated by one of the viral AL1 proteins, we knew that it was not because we had destroyed important structural features such as the potential stem-loop structure in making the clone, but rather that it was not being recognized by the AL1 protein being tested. Previous studies based on the negative findings that particular constructs did not replicate or were not infectious had supported the assumption that the AL1 protein interacted with the CR (Revington et al., 1989; Ugaki et al., 1991). However, by their very nature these negative studies did not permit an evaluation of the direct or indirect role of these sequences in replication, nor did they demonstrate sequence-specific interactions with AL1 protein or define the origin. The advantage of our assay is in permitting us to obtain positive as well as negative results, thus directly testing the recognition of the relevant sequences by the AL1 protein and defining the replication origin.

The analysis of the ability of our TGMV::SqLCV-E chimeric CR constructs to replicate strongly suggested that specificity for recognition by the viral AL1 protein resides in the AL1 proximal half of the viral CR (Figures 2 and 3, and Table 1). All CR constructs containing the left half of the TGMV CR (up to the unique SspI site) were replicated in the presence of the TGMV AL1 protein and not in the presence of the SqLCV-E AL1 protein. In a similar manner, all constructs containing the left half of the SqLCV-E CR were replicated in the presence of the SqLCV-E AL1 protein and not in the presence of the TGMV AL1 protein (Figure 4 and Table 1). The choice of these two viruses and the construction of the chimeric viral CRs were guided by several findings. First, both TGMV and SqLCV-E have a common host, *N. benthamiana*, thus providing a convenient in vivo leaf disc system in which to develop a replication assay (Elmer et al., 1988b; Lazarowitz, 1991). Second, the CRs of TGMV and SqLCV-E are completely different in sequence except for 18 nucleotides at the beginning of each region and the conserved geminivirus sequence element (stem-loop

Table 2. CR Fragments from TGMV and SqLCV

CR (left/right)	Size (bp) (left/right)	Source	Plasmid with AL1 from	
			TGMV[a]	SqLCV[a]
TGMV	303 (112/191)	*pMON477*[b]	*pMON1603*	–
TGMV/SqLCV-E	176 (112/64)	*pMON477*[b]/*PCR*[c]	*pMON1714R*	*pMON1714-14* *pMON1714-29* *pMON1714-3*
SqLCV-E/TGMV	370 (179[d]/191)	PCR[c]/*pMON477*[b]	*pMON1716*	*pMON1716-67* *pMON1716-243* *pMON1716-44*
SqLCV-E CR	243 (179[d]/64)	*pCRSQE-33*[e]	–	*pSQE33-113*
SqLCV-R CR	230 (166[d]/64)	*pCRSQR-4*[e]	–	*pSQR-48*

[a] Expression cassette from TGMV consisted of the entire AL1-2-3 region. Expression cassettes tested derived from SqLCV-E were as follows: *pMON1716-67* = AL1; *pMON1714-14*, *pMON1714-29*, *pMON1716-243*, and *pMON1716-255* = AL1-2; *pMON1714-3*, *pMON1716-44*, *pSQE33-113*, *pSQE29-16*, and *pSQR-48* = AL1-2-3.

[b] Elmer et al. (1988a). Right half utilizes a BglII site within *pMON477* outside of the TGMV CR. This right half includes the 126-nucleotide right half of TGMV CR plus 65 nucleotides of sequence from the AR1 side of the CR.

[c] PCR primers for the extreme 5′ and 3′ ends of the SqLCV-E CR were based on Lazarowitz and Lazdins (1991) and constructed to add a BglII site at each end for cloning purposes. Internal primer was derived from the virion or complementary sequence of the stem-loop structure to include the unique SspI site for cloning with the TGMV counterpart CR half. Extreme left PCR primer = GATAGATCTAACGAAAGGAATTAGGG; right PCR primer = GATAGATCTGGGCCTGCCCCG (underlined in new BglII sites); corresponding internal primers were CCGCAATAATATTACCGG and GGCCATCCGGTAATATTATTG (underlined in viral SspI site).

[d] CRs of SqLCV-E and SqLCV-R as defined by sequence identity begin at nucleotide 28 within the AL1 coding region (Lazarowitz and Lazdins, 1991).

[e] Cloned from PCR products amplified from infectious clones of SqLCV-E or SqLCV-R using the same 5′ and 3′ extreme primers as noted in "c" to create BglII sites at each end of CR. These were cloned into *pMON469*, which differs from *pMON477* in lacking ~200 nucleotides of bacterial 3′ untranslated sequences in the upstream copy of CAT. Hence, the excised CR::CAT circular DNAs are 200 nucleotides shorter than their *pMON477* counterparts. All listed constructs were independently derived and tested the same in terms of replication with the SqLCV-E or TGMV AL1 proteins. These clones were constructed independently of the SqLCV halves used in the 1714 and 1716 plasmids.

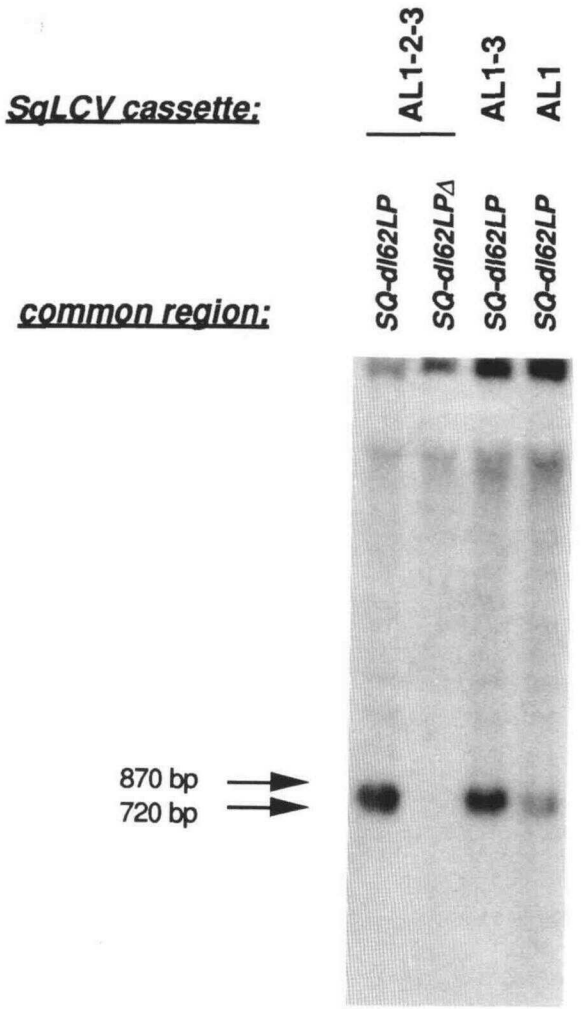

Figure 5. Replication of *pSQ-dl62LP* and *pSQ-dl62LPΔ* CR Clones in the Presence of SqLCV Expression Cassettes.

AL1-2-3 (lanes 1 and 2), AL1-2^{-}-3 (lane 3), or AL1-2^{-} (lane 4) expression cassettes are indicated. DNA gel blots of extracts from agroinoculated leaf discs hybridized with the ^{32}P-labeled CAT coding sequence probe. Detected are covalently closed dsDNA mini-circle forms. Arrows indicate the position of linear dsDNA size markers in base pairs. Specific plasmid constructs used are as follows: *pSQ-dl62LP-125* (lane 1), *pSQ-dl62LPΔ-2432* (lane 2), *pSQ-dl62LP-23* (lane 3), and *pSQ-dl62LP-34* (lane 4). Size of the SQ-dl62LP::CAT mini-circle is 821 nucleotides and that of the SQ-DL62LPΔ::CAT is 813 nucleotides.

structure) (Lazarowitz, 1987, 1991) (Figure 4). Thus, we anticipated that the AL1 proteins would show strict specificity for their cognate CRs. Finally, in constructing the chimeric viral CRs we chose to use the unique SspI site, recognizing that the conserved sequence elements in TGMV and in SqLCV-E are identical in sequence except for the few nucleotides at the beginning of the loop sequence where TGMV contains TT and SqLCV-E contains CAA (Figure 4). Hence, we reasoned that if the potential stem-loop structure was important for replication, it would be present in our chimeric CR constructs and these clones should be functional.

Although for the TGMV AL1 protein only an AL1-2-3 expression cassette was used to provide the viral AL1 protein, we tested AL1, AL1-2, and AL1-2-3 expression cassettes for the SqLCV-E. Both AL1-2 and AL1-2-3 expression cassettes gave identical results, appearing to function equally well in terms of specificity, the replication of ssDNA and dsDNA, and the size of the replicated chimeric CR::CAT mini-circle. Surprisingly, the AL1 expression cassette did not appear to support DNA replication. The AL1 expression clone was constructed to start at precisely the same nucleotide as those for AL1-2 and AL1-2-3, each of these differing only in the position of the 3′ end. Thus, the AL1 clones are capable of being efficiently transcribed from the expression cassette, but perhaps the AL1 transcript from this expression cassette was unstable as constructed. To directly address this point, we tested SqLCV AL1-2 and AL1-2-3 expression cassettes in which AL2 function was eliminated by a specific frameshift mutation that did not affect the coding sequences of either AL1 or AL3. These expression cassettes supported the replication of the chimeric and subcloned CRs containing the left half of the SqLCV CR that included an intact stem-loop sequence and did not support the replication of the chimeric CR containing the left half of the TGMV CR (Figures 4 and 5; data not shown). Hence, our results define the sequence-specific interactions of the AL1 protein and the CR required for viral replication and rule out the involvement of other viral proteins.

Comparison of the ability of the different chimeric and subcloned CR constructs to be replicated by the AL1 protein of either TGMV or SqLCV-E allowed us to specifically localize the viral origin(s) of DNA replication to within a small ~90-nucleotide sequence in the viral CRs. In Figure 4, the TGMV and SqLCV-E CRs are aligned to begin at the start of the AL1 coding sequence. In SqLCV-E and SqLCV-R, the CRs, as defined by homology between the A and B components, begin at nucleotide 28 within the AL1 coding region. This is not true of the other geminiviruses, including TGMV, and thus the alignment has been drawn as shown to facilitate direct comparisons. When viewed this way, we find that all of the viral CRs appear to be constant in distance (~87 nucleotides) from the 5′ end of the CR (defined as the second codon of AL1) to the apparent TATA element for the AL1 gene. Variability in all of the CRs, in terms of spacing, appears to reside between this putative TATA box and the start of the conserved geminivirus sequence element, as a direct comparison of TGMV, SqLCV-E, and SqLCV-R clearly demonstrates (Figure 4). Based on the TGMV and chimeric CR constructs used in this study (*pMON1603*, *pMON1714* and derivatives, and *pMON1716* and derivatives), the sequence-specific interactions between the viral AL1 protein and CR map to a 123-nucleotide fragment located between nucleotides 21 and 143 of the CR. Because both ssDNA and dsDNA were replicated in our assays, this 123-nucleotide region contains the origin(s) for both dsDNA-to-dsDNA and dsDNA-to-ssDNA synthesis. The nature of our assay does not

allow us to draw any conclusions about the origin for ssDNA-to-dsDNA synthesis. This 123-nucleotide region includes the conserved geminivirus sequence element and is consistent with the recent finding for beet curly top virus (Stenger et al., 1991) that rolling circle replication of viral ssDNA appears to initiate within this region.

Replication of the SqLCV-deleted CR *SQ-dl62* further delimits the origin to within an 89-nucleotide region that includes the conserved potential stem-loop structure and 57 nucleotides of 5′ upstream sequence (the analogous TGMV fragment contains 51 nucleotides of 5′ upstream sequence). This deletion to within 25 nucleotides of the apparent TATA element for AL1 removes the entire leader sequence of the AL1 transcript (Lazarowitz, 1991) and suggests a partitioning of functional elements for transcription and replication within the CR. This is also consistent with the constant length of the region between the start of AL1 and the putative TATA element in different geminiviruses. Analysis of the replication of *SQ-dl62LP* and *SQ-dl62LPΔ* more precisely delimits the viral replication origin(s) and its sequence-specific recognition by the AL1 protein. Both of the SqLCV-E CR subclones have the same 5′ end as *SQ-dl62*. *SQ-dl62LP* terminates precisely at the last nucleotide of the stem-loop structure (Figure 4) and thus does not contain any viral sequences beyond this point on the 3′ side. It replicates as well as *SQ-dl62* and thus clearly demonstrates that all of the relevant replication and protein binding sequences are within the ~90-nucleotide fragment consisting of the stem-loop structure and ~60 nucleotides of 5′ upstream sequence. *SQ-dl62LPΔ*, which terminates 2 nucleotides past the loop sequence in the conserved stem-loop structure, does not replicate in the presence of any of the cassettes expressing SqLCV-E AL1 protein (Figure 5; data not shown). Because the deletion in *SQ-dl62LPΔ* precisely removes one of the inverted repeats, these results strongly suggest that formation of the stem-loop structure is essential for replication from the dsDNA templates.

Our results are consistent with two different models concerning the viral replication origin and its recognition by the AL1 protein. Specificity for binding of the AL1 protein could reside in the ~60 nucleotides upstream of the conserved viral stem-loop structure, with the latter structural element functioning to position the nicking site for ssDNA synthesis so as to make it accessible to an AL1 protein–containing complex. This would be analogous to φX174 and is consistent with the observation that the loop sequence TAATATTAC found in all geminiviruses bears a striking similarity to the *geneA* protein recognition and cleavage site in φX174 (Heidekamp et al., 1982). Evidence for this type of model involving cruciform structural elements has also been presented for prokaryotic single-stranded plasmid replication origins (Gruss et al., 1987). Alternatively, specificity for recognition by the AL1 protein to effect replication could involve recognition of sequences in both the conserved stem-loop structure and 5′ upstream region. Since the only sequence difference between TGMV and SqLCV-E in the potential stem-loop structure involves a TT versus a CAA at the start of the loop, experiments in progress to mutate each loop sequence to the other will allow us to directly distinguish between these two possible models. Recent in vitro DNA binding studies by Fontes et al. (1992) are consistent with both of these models and the biological data presented here. Further site-directed mutagenesis will determine whether there is a single origin or separate origins for ssDNA and dsDNA replication within this region, as well as further define the roles of the sequence elements involved and the nature of their interactions with the AL1 protein to initiate DNA replication.

METHODS

Source of Cloned Viral Genomic Components

Infectious tandem direct repeat ("dimer") clones of tomato golden mosaic virus (TGMV) and squash leaf curl virus (SqLCV) genomic components constructed in the plant transformation vector *pMON505* (Horsch and Klee, 1986) have been described (Elmer et al., 1988b; Lazarowitz, 1991; Lazarowitz and Lazdins, 1991). These were used for infectivity and leaf disc replication assays. Single copy progenitor clones in pBR322-derived plasmids were also used for the constructions described below.

Construction of Chimeric and Deleted Common Region Plasmids

Chimeric viral common regions (CRs) consisting of 5′ sequence ("half") of the CR from TGMV or SqLCV joined to the 3′ "half" from the CR of the other virus were constructed to define the origin and test AL1 specificity. These chimeric clones were readily constructed using the unique SspI site located at the same position in the viral CRs within the conserved loop sequence of the potential stem-loop structure (Figure 1) (Lazarowitz, 1987). Fragments containing the two halves of the TGMV CR were directly subcloned from TGMV recombinant plasmids. *pMON477*, containing the TGMV CR within a BglII fragment, was created by site-directed mutagenesis (Kunkel, 1985) to introduce a second BglII site into *pMON437* (Gardiner et al., 1988) adjacent to nucleotide 28 in the TGMV CR sequence (Hamilton et al., 1984). The intact BglII fragment containing the entire TGMV CR was directly subcloned, or the BglII-SspI fragments containing the two halves of the TGMV CR were isolated and ligated to their counterparts from the SqLCV-E CR (Table 2). Thus, the left half of the TGMV CR extended from nucleotide 28 within the CR at the 5′ end to the SspI site at nucleotide 140, and the right half extended from this SspI site to 65 nucleotides outside of the CR at the 3′ end. The corresponding SqLCV fragments containing the left or right halves of the SqLCV-E CR were isolated following polymerase chain reaction (PCR) amplification (Mullis and Faloona, 1987) using primers designed to create a new BglII site at each end of the viral CR or to utilize the viral SspI site in the potential stem-loop structure (Table 2), and digestion with BglII and SspI. Intact SqLCV-E and SqLCV-R CRs, as well as SqLCV-E subregions and mutants, were also cloned from PCR products designed to have BglII sites at each end of the CR (Table 2). Hence, the left and right halves of the SqLCV CRs began and terminated at precisely the first and last nucleotides of the CR, respectively. The deleted SqLCV-E CR constructs were amplified using synthetic oligonucleotide primers designed to introduce the appropriate deletions and BglII sites for cloning. The

sequences of all PCR-derived constructs were verified by the chain termination reaction (Sanger et al., 1977).

Following ligation of the appropriate CR halves (for chimeric constructs) and redigestion with BglII, the chimeric CRs and subcloned SqLCV-E CR constructs were ligated into the unique BglII site of the *Agrobacterium tumefaciens* plant transformation vectors *pMON469* or *pMON467* (Figure 1). These plasmids are *pMON505* derivatives that contain a direct repeat of the bacterial chloramphenicol acetyltransferase (CAT) coding sequence separated by a unique BglII site (Lazarowitz et al., 1989). The CAT sequence was originally obtained from pBR328 and altered by site-directed mutagenesis to remove internal EcoRI and NcoI sites and add a NcoI site at the ATG initiator codon. *pMON467* and *pMON469* differ by the inclusion of additional bacterial 3′ untranslated sequences adjacent to the upstream copy of the CAT sequence. Previous studies have shown that following introduction into plant cells by way of agroinoculation, circular DNA consisting of the 920-nucleotide CAT sequence and intervening geminivirus sequences is efficiently excised from the plasmid by homologous recombination within the repeated CAT segments and is replicated if the excised fragment includes the viral replication origin and gene(s) essential for replication (Lazarowitz et al., 1989). Following cloning of the different CR constructs into *pMON467* or *pMON469*, an expression cassette containing the AL1 of either TGMV or SqLCV-E was cloned into the unique NotI site of each plasmid (Figure 1). The chimeric expression unit isolated as a NotI fragment from *pMON921* contained the enhanced cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987) and the 3′ untranslated region from the pea small subunit of ribulose bisphosphate carboxylase (Coruzzi et al., 1984) flanking either the entire TGMV AL1-AL2-AL3 region (Hanley-Bowdoin et al., 1989) or the SqLCV-E AL1, AL1-AL2, or AL1-AL2-AL3 region. The TGMV expression fragment began at an introduced BglII site located 14 nucleotides upstream of the start of the AL1 coding sequence. The SqLCV-E expression fragment began at the unique NsiI site located 50 nucleotides upstream of the start of the AL1 coding region. The SqLCV-E fragments terminated at the BamHI site within AL2, the ClaI site within AL3, or the XhoI site within AR1, respectively (Lazarowitz and Lazdins, 1991). The TGMV fragment terminated at the AccI site within AR1. Table 2 lists all of the pertinent constructs.

A frameshift mutation was introduced into the unique AL2 coding sequences through site-directed mutagenesis to create a unique SalI site at nucleotide 60 in the AL2 coding sequence (corresponding to amino acid 27 in the 131–amino acid AL2-encoded protein), which was then digested with SalI and filled in with T4 DNA polymerase (A. Sanderfoot and S. G. Lazarowitz, unpublished data). The resulting frameshift mutant terminates at nucleotide 145 within the AL2 sequence, encoding a 48–amino acid protein consisting of the first 26 amino acids of AL2 followed by 21 amino acids not present in AL2.

DNA Replication Assay

Leaf discs prepared from *Nicotiana benthamiana* were cultured and agroinoculated for viral DNA replication assays as described previously (Elmer et al., 1988a). DNA was extracted from crude nucleoprotein particle preparations (Lazarowitz and Lazdins, 1991) and normalized for the total number of inoculated leaf discs for analysis on DNA gel blots as described previously (Lazarowitz, 1991). The DNA-containing extracts were analyzed on 1.4% agarose gels and transferred to nylon membrane (Hoeffer Scientific) for hybridization (Southern, 1975; Reed and Mann, 1985). Radioactively labeled probe was prepared by oligonucleotide-primed synthesis (Feinberg and Vogelstein, 1984). Replicated double-stranded DNA was detected either as the uncut minicircle of the expected mobility or as the appropriate sized linear fragment following digestion with restriction enzymes that would cleave the small circular DNA to yield unique restriction fragments. Single-stranded DNA was identified by its sensitivity to mung bean nuclease (Laskowski, 1980). All enzymes were from commercially available sources.

ACKNOWLEDGMENTS

We thank Paige Goodlove for her expert technical assistance and Alejandro Martinez for his help in screening and sequencing the *pSQ-dl62*, *pSQ-dl62LP*, and *pSQ-dl62LPΔ* clones. We also thank Deb Crim for excellent editorial assistance, Jack Gladin for expert photographic assistance, and Tony Sanderfoot, Shen-wei Qin, Dave Ingham, and Erica Pascal for lively discussions and helpful comments. This work was supported by National Institutes of Health Grant No. PHS RO1 AI27449 (to S.G.L.) and funds from the Monsanto Company (to J.S.E. and S.G.R.).

Received February 3, 1992; accepted May 18, 1992.

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