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Kinetics of Tomato Golden Mosaic Virus DNA Replication and Coat Protein Promoter Activity in *Nicotiana tabacum* Protoplasts

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We have analyzed the replication kinetics of the DNA A and DNA B genome components of the geminivirus tomato golden mosaic virus (TGMV) in protoplasts derived from *Nicotiana tabacum* suspension culture. In addition, the kinetics of TGMV coat protein promoter activity, as measured by expression of a β-glucuronidase (GUS) reporter, have been examined. In our protoplast system, double-stranded DNA forms of both viral genome components appeared by 18 hr post-transfection, while single-stranded DNA accumulated to detectable levels after 18–24 hr. Expression of GUS from the TGMV coat protein promoter did not require viral DNA replication, nor was it dependent on expression of AL1, the only viral gene necessary for DNA replication. However, maximal expression was achieved following AL1-mediated replication of DNA A. GUS activity from replicating templates exceeded that from nonreplicating templates by 60- to 90-fold. Expression of the GUS reporter gene from nonreplicating viral DNA templates was similar to GUS expression from the 35S promoter of cauliflower mosaic virus in *N. tabacum* protoplasts.

INTRODUCTION

The geminiviruses are a group of plant infectious agents characterized by a circular single-stranded DNA genome and a unique geminate (paired) particle morphology (for review see Lazarowitz, 1987; Davies and Stanley, 1989). Tomato golden mosaic virus (TGMV) is a member of a subgroup of geminiviruses which are whitefly transmitted, restricted to dicotyledonous hosts, and have genomes consisting of two DNA components of approximately the same size. The two DNA components of TGMV (designated DNA A and DNA B) are both required for infectivity (Hamilton *et al.*, 1983), although DNA A encodes all viral functions necessary for the replication and encapsidation of viral DNA (Rogers *et al.*, 1986; Sunter *et al.*, 1987). DNA B encodes functions required for systemic movement in the infected plant.

As illustrated in Fig. 1, the two TGMV genome components contain a total of six open reading frames (ORFs) which can encode proteins greater than 10 kDa (Hamilton *et al.*, 1984). Analysis of DNA A ORF mutants has demonstrated that AL1 is the only viral gene absolutely required for replication (Elmer *et al.*, 1988a). Studies performed with transgenic plants expressing the AL1 protein have confirmed this and have shown that AL1 protein, in conjunction with host proteins, is sufficient for replication of TGMV DNA (Hayes and Buck, 1989; Hanley-Bowdoin *et al.*, 1990). Coat protein (AR1) mutants are infectious but produce attenuated symptoms in inoculated plants (Brough *et al.*, 1988; Gardiner *et al.*, 1988). In protoplasts, AR1 mutants accumulate wild-type levels of dsDNA but only small amounts of ssDNA (Sunter *et al.*, 1990). AL2 mutants are unable to infect plants (Elmer *et al.*, 1988a). However, like coat protein mutants, AL2 mutants accumulate wild-type levels of dsDNA but reduced amounts of ssDNA in protoplasts and do not produce detectable amounts of coat protein (Sunter *et al.*, 1990). A more recent study has shown that the similar phenotype of coat protein and AL2 mutants in protoplasts is due to the fact that the AL2 gene product transactivates expression of the coat protein gene (Sunter and Bisaro, 1991).

The use of protoplasts allows the study of viral DNA replication and gene expression under more or less synchronous conditions, independent of systemic movement requirements (for review, see Motoyoshi, 1985). Transient replication of several geminiviruses has been demonstrated in protoplasts, including bean golden mosaic virus (Haber *et al.*, 1981), African cassava mosaic virus (ACMV, synonym cassava latent virus; Townsend *et al.*, 1986), beet curly top virus (BCTV; Briddon *et al.*, 1989), TGMV (Bisaro *et al.*, 1990; Sunter *et al.*, 1990), and wheat dwarf virus (WDV; Woolston *et al.*, 1989; Matzeit *et al.*, 1991). In the case of ACMV, BCTV, and WDV, replicating viral DNA can be detected 2–3 days after transfection. In *Nicotiana tabacum* protoplasts transfected with constructs con-

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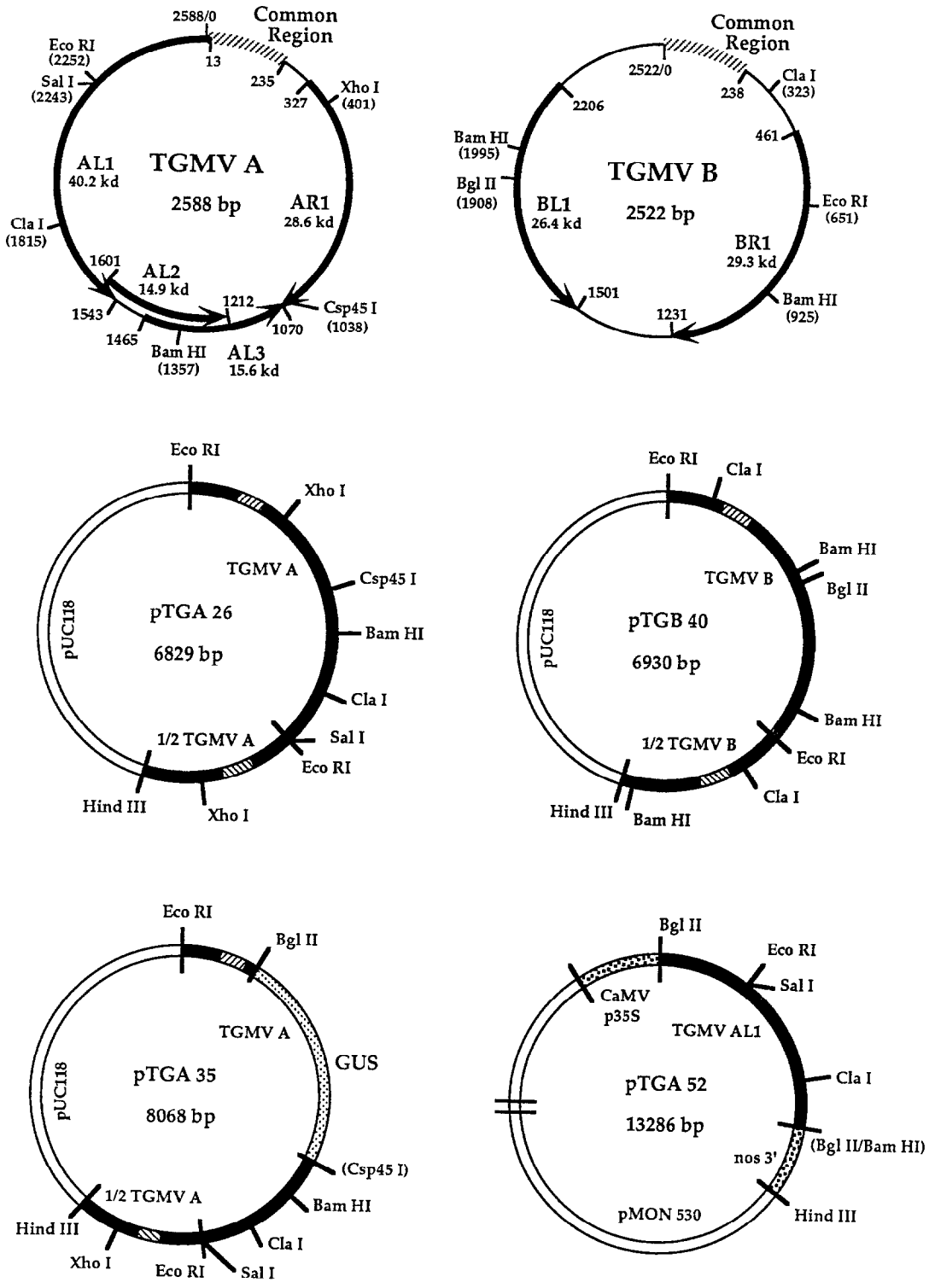

FIG. 1. Diagrams of TGMV DNA A, DNA B, and derived vectors. The two genome components of TGMV are represented as circular, double-stranded replicative forms. The solid arrows show the positions of open reading frames, and in all diagrams the hatched boxes represent the common region. The capsid protein is encoded by ORF AR1. The vectors pTGA26, pTGB40, and pTGA35 contain one and one-half copies of the TGMV DNA A or B (shaded) in pUC118 (unshaded). In the diagram of pTGA35, the GUS coding sequence is indicated as a dotted box. Plasmid pTGA52 contains the TGMV AL1 ORF (shaded) cloned into the CaMV 35S-nos 3' cassette (dotted) of pMON530 (unshaded). The parallel lines through the pMON530 sequence indicate that it is not drawn to scale. In all diagrams the positions of restriction sites are indicated outside of the circles.

taining one and one-half copies of the TGMV A and B genomes, freely replicating viral DNA forms identical to those found in systemically infected plants can be detected as early as 18 hr post-transfection (Bisaro *et al.,* 1990).

In this communication, we report the kinetics of TGMV DNA A and DNA B replication in *N. tabacum* protoplasts. The kinetics of viral coat protein promoter activity, as measured by the expression of a β-glucuronidase (GUS) reporter gene, is also examined and related to the replication of viral DNA. We demonstrate that expression from the coat protein promoter does not require functional AL1 protein and is independent of TGMV DNA A replication, although maximal expression is correlated with AL1-mediated amplification of this genome component.

MATERIALS AND METHODS

DNA constructions

All restriction endonucleases and other DNA modifying enzymes were obtained from Promega and used according to the manufacturer's protocols, unless otherwise specified. Other techniques were performed as described by Ausubel *et al.* (1987).

Plasmids containing one and one-half copies of DNA A (pTGA26) or DNA B (pTGB40) have been described previously (Sunter *et al.,* 1990). A derivative of pTGA26 in which the coat protein coding sequence (AR1) has been replaced by the GUS coding sequence (pTGA35) has also been described (Sunter and Bisaro, 1991). Plasmid pBI221, a derivative of pBI121 (Jefferson *et al.,* 1987) was obtained from Clonetech and contains a CaMV 35S-GUS-nopaline synthetase (nos) 3′ end cassette in pUC19.

Frameshift mutations in the AL1 ORF were constructed by restricting TGMV A DNA with either *Cla*I at nucleotide 1815 or *Sal*I at nucleotide 2243, followed by end-filling with the Klenow fragment of DNA polymerase I and subsequent ligation. The mutation at the *Cla*I site results in the formation of a new *Nru*I site and is predicted to produce a truncated AL1 protein consisting of the 270 N-terminal amino acids terminating at nucleotide 1789. The *Sal*I mutation results in the formation of a new *Pvu*I site and is predicted to produce a truncated AL1 protein consisting of the 125 N-terminal amino acids terminating at nucleotide 2224. The unit-length TGMV A DNAs containing the introduced *Nru*I (pTGA70) or *Pvu*I (pTGA72) sites were cloned into pTGA20 (Sunter *et al.,* 1990) to give a tandemly repeated genome with a single copy of the mutated region.

Plasmid pTGA52 was constructed by cloning the wild-type AL1 ORF from pMON434 (Elmer *et al.,* 1988a) into the expression vector pMON530 (Rogers *et al.,* 1987) to create a CaMV 35S-AL1-nos cassette for expression of AL1 protein in protoplasts. The plasmid pGUSBE, used for the preparation of GUS-specific riboprobes, was constructed by cloning the 1920-bp *Bgl*II–*Eco*RI fragment from pMON9749 (Hinchee *et al.,* 1988) containing the GUS gene into pGEM4 (Promega).

Preparation and transfection of protoplasts

Suspension cells of *N. tabacum* var. Wisconsin 38 were maintained at 26° with continuous light and shaking at 150 rpm. Serial transfers were performed weekly (1:5 dilution) in modified Murashige and Skoog medium containing MS salts (GIBCO), 3% (w/v) sucrose, 100 mg/liter myo-inositol, 0.5 mg/liter 2,4-D, pH 5.0. Three days following subculture, suspension cells (typically 15 ml) were sedimented by centrifugation at 60 *g* (5 min) to obtain a packed cell volume of 2–4 ml. The cells were then washed in 10 ml 400 m*M* mannitol, recovered by centrifugation, and resuspended in 7 ml of enzyme solution (2.5% driselase [Karlan], 0.25% macerozyme R10 [Karlan] in 0.5% MES-KOH [2-*N*-morphilinoethanesulfonic acid], 0.38 *M* mannitol, 8 m*M* $CaCl_2$, pH 5.6). The cells were agitated gently at 50 rpm at 28° for 30 min and incubated overnight (12–16 hr) at room temperature in the dark without agitation. Protoplasts were then isolated as described by Potrykus and Shillito (1986) for *Lolium,* except that final washes and resuspension were done in W5 medium which is suitable for polyethylene glycol (PEG)-mediated DNA delivery (Negrutiu *et al.,* 1987). Protoplasts were counted using a hemocytometer.

Time course experiments were performed by transfecting 2–2.5 × 10^6 protoplasts with 10 μg of pTGA26 (or derivatives thereof) alone or with 10 μg of pTGB40, and aliquots containing 2–2.5 × 10^5 protoplasts were incubated for each time point. Protoplasts were sedimented and the appropriate DNA samples (10–20 μg/ml) in 250 μl of MaMg solution (0.4 *M* mannitol, 15 m*M* $MgCl_2$, 5 m*M* MES-KOH, pH 5.6, containing 50 μg/ml calf thymus carrier DNA) were added, followed by the addition of 250 μl of PEG solution (30% PEG 6000 in 0.4 *M* mannitol, 15 m*M* $Ca(NO_3)_2$, 5 m*M* MES-KOH, pH 8.3) 30 sec later. After incubation in PEG for 30 min, the solution was serially diluted five times with 1 vol of MKC (0.5% MES-KOH, 200 m*M* $CaCl_2$, pH 5.8) and the protoplasts were sedimented by centrifugation at 60 *g* for 5 min. The transfected protoplasts were then resuspended in K3A culture medium (Potrykus and Shillito,

1986), except that 450 m*M* sucrose was substituted for 400 m*M* sucrose. Transfected protoplasts were incubated without agitation in the dark for varying time periods.

DNA analysis and GUS assays

DNA was isolated from protoplasts by the method of Mettler (1987) and quantified by spectrophotometry. Samples containing 0.5–2 μg DNA were electrophoresed through agarose gel, transferred to nitrocellulose or Nytran membranes (Schleicher and Schuell), and analyzed by hybridization with ^{32}P-labeled riboprobes prepared from pGEM vectors (Promega) containing TGMV DNA or GUS DNA inserts. GUS assays were performed according to Jefferson (1987), and protein concentrations were estimated by the procedure of Bradford (1976).

RESULTS

Time course of TGMV DNA replication in transfected protoplasts

Protoplasts were transfected with pTGA26, which contains one and one-half copies of TGMV DNA A, or cotransfected with pTGA26 and pTGB40, which contains one and one-half copies of TGMV DNA B (Fig. 1). In plant cells, unit-length viral DNAs are released from tandem viral genome repeats both by homologous recombination and by a replicative mechanism (Elmer *et al.,* 1988b; Stenger *et al.,* 1991). Southern hybridization analysis of DNA extracted from protoplasts at various times post-transfection showed that unit-length, supercoiled TGMV A and B DNA could be observed as early as 18 hr post-transfection (Fig. 2). Viral ssDNA appeared after 18–24 hr, and an increase in the amount of ssDNA relative to that of the supercoiled dsDNA form was observed between 18 and 48 hr (Fig. 2). Both supercoiled dsDNA and ssDNA levels reached a maximum at 48–72 hr post-transfection. TGMV DNA was still present 96 hr after transfection and in other experiments has been detected after 7 days (data not shown). The kinetics of dsDNA and ssDNA accumulation demonstrated in Fig. 2 are reproducible in this protoplast system; similar results have been obtained in seven independent experiments with DNA A alone or DNA A and DNA B (data not shown). High-molecular-weight DNAs corresponding to closed circular, open circular, and linear forms of the pTGA26 and pTGB40 inoculum were present at all time points (Fig. 2), although the amount of this DNA decreased over time. The inoculum-sized DNAs are resistant to DNase I treatment prior to extraction of DNA from the protoplasts (Sunter *et al.,* 1990). Further, the inoculum-sized DNAs were completely digested by the *dam* methylation-requiring enzyme *Dpn*I and were resistant to digestion by the methylation-sensitive isoschizomer *Mbo*I (Fig. 3), indicating that these DNAs are residual inoculum which has not replicated. Unit-length TGMV DNAs were resistant to *Dpn*I but were cleaved by *Mbo*I, demonstrating that these forms were replicated in the protoplasts.

Time course of GUS expression from the TGMV coat protein promoter

Protoplasts transfected with pTGA35, in which the coat protein coding region has been replaced by GUS, were harvested at various times post-transfection and extracts containing equal amounts of protein were assayed for GUS activity as described by Jefferson (1987). As shown in Fig. 4, a relatively low level of GUS activity was detected under the assay conditions used until 18 hr post-transfection, at which time the activity increased dramatically and continued to increase until 72 hr post-transfection. GUS activity reached a maximum at 72 hr, but was still detectable after 6 days (data not shown). Total DNA isolated from the same extracts, when hybridized with a GUS-specific riboprobe, showed unit-length supercoiled and open circular forms of dsDNA corresponding to the GUS-containing TGMV genome. These replicating DNAs appeared 18 hr post-transfection and accumulated with the same kinetics as wild-type TGMV DNA (data not shown). We conclude from these experiments that high level expression of the GUS reporter gene, and therefore the viral coat protein, correlates with the onset of viral DNA replication.

GUS expression from replicating and nonreplicating DNA templates

The expression of GUS from the replicating pTGA35 was compared with GUS expression from nonreplicating TGMV genomes and from a 35S-GUS-nos reporter cassette (pBI221). Two different pTGA35 derivatives, pTGA70 and pTGA72, which are unable to replicate as a result of lesions introduced into the AL1 ORF, were used in these experiments. The use of more than one mutant was considered important since it was possible that the lesions also might affect transcription of AL2, which is required to transactivate expression from the coat protein promoter (Sunter and Bisaro, 1991). Figure 5 summarizes the results of experiments comparing the levels of GUS activity detected 3 days post-transfection. GUS activity was detected from both replicating (pTGA35) and nonreplicating (pTGA70 and

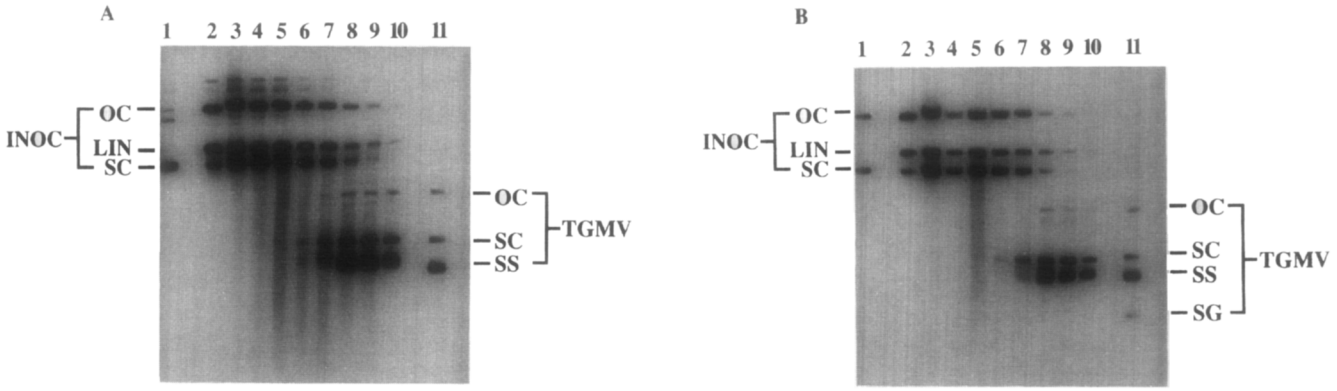

FIG. 2. Time course of TGMV DNA replication. Protoplasts (2.5×10^5 per time point) were cotransfected with TGMV DNA A (pTGA26, 1 μg DNA per time point) and TGMV DNA B (pTGB40, 1 μg per time point), and total protoplast DNA was isolated at various times post-transfection. DNA samples were electrophoresed through agarose gels, transferred to Nytran membranes, and hybridized with ^{32}P-labeled probes specific for TGMV DNA A (A) or DNA B (B). In A, lane 1 contains unrestricted pTGA26 DNA (5 ng). In B, lane 1 contains unrestricted pTGB40 DNA (5 ng). Lanes 2–10 in both A and B contain DNA isolated from protoplasts 0, 6, 10, 12, 18, 24, 48, 72, and 96 hr post-transfection, respectively. In A and B, lane 2 contains 0.2 μg of DNA, lanes 3–5 contain 3 μg of DNA, and lanes 6–10 contain 1 μg of DNA. Lane 11 contains DNA isolated from TGMV-infected plants (60 ng). The open circular (OC), linear (LIN), and supercoiled (SC) forms of the inoculum DNA are indicated to the left and the OC, SC, single-stranded (SS), and subgenomic (SG) forms of the freely replicating TGMV DNA are indicated to the right. Subgenomic DNA is derived from the B genome component.

pTGA72) templates, indicating that AL1 function is not required for expression from the coat protein promoter. However, after 3 days, the level of GUS activity from the replicating template was 60- to 90-fold higher than the activity obtained with the nonreplicating pTGA70 and pTGA72. It should be noted that the level of GUS activity detected in extracts from protoplasts transfected

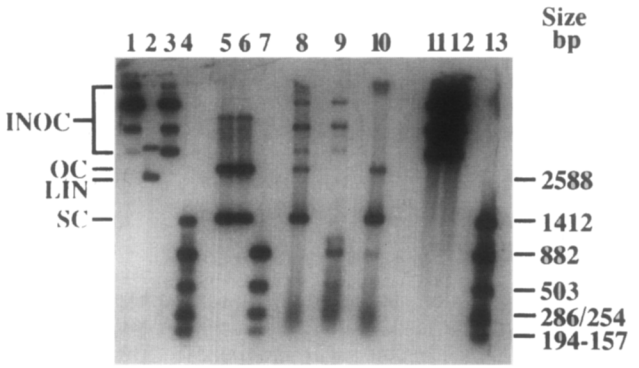

FIG. 3. Analysis of the methylation patterns of TGMV DNA from transfected protoplasts. DNA was isolated from protoplasts transfected with pTGA26 at 0 and 72 hr post-transfection. The DNA was digested with *Mbo*I or *Dpn*I, electrophoresed through an agarose gel, transferred to a nitrocellulose membrane, and hybridized with a viral sense TGMV DNA A riboprobe so that viral ssDNA would not be visible. DNAs isolated from transfected protoplasts at 72 hr (0.5 μg, lanes 8–10) and 0 hr (1 μg, lanes 11–13) post-transfection were compared with pTGA26 DNA (5 ng) purified from *Escherichia coli* (lanes 1–4) and DNA (60 ng) isolated from TGMV-infected plants (lanes 5–7). The DNA in lanes 1, 5, 8, and 11 is unrestricted; lane 2 contains pTGA26 DNA restricted with *Eco*RI; DNA in lanes 3, 7, 9, and 12 is restricted with *Mbo*I; and DNA in lanes 4, 6, 10, and 13 is restricted with *Dpn*I. The positions of pTGA26 inoculum DNA (INOC) and TGMV open circular (OC), linear (LIN), and supercoiled (SC) DNA forms are indicated to the left. Restriction fragment sizes are indicated to the right.

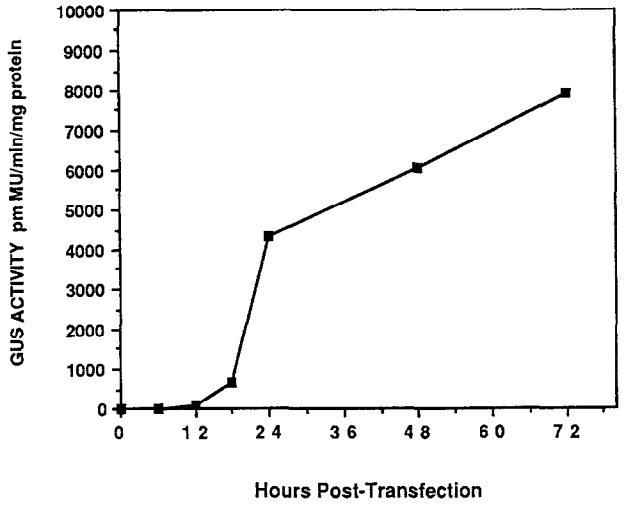

FIG. 4. Time course of GUS expression in protoplasts transfected with a TGMV vector. Protoplasts (2.5×10^5 per time point) were transfected with TGMV A DNA containing the GUS coding sequence (pTGA35; 4 μg per time point) and harvested at 0, 6, 12, 18, 24, 48, and 72 hr post-transfection. GUS assays were performed using 10 μg protein and the calculated GUS activities, expressed as picomoles (pm) of 4-methylumbelliferone (MU) produced per minute per milligram protein, were plotted versus hours post-transfection. Background fluorescence (7–35 pm MU/min/mg protein) was determined using extracts from pTGA26-transfected protoplasts and was subtracted from all time points.

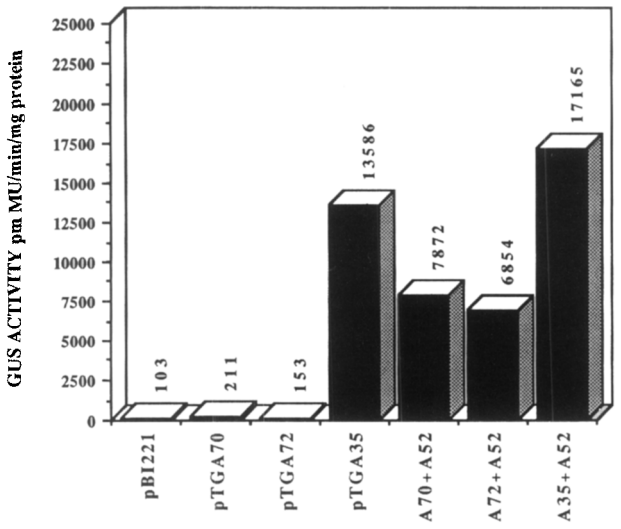

FIG. 5. GUS expression from replicating and nonreplicating TGMV templates. Protoplasts (2.5×10^5) were transfected with a TGMV DNA vector containing the GUS coding sequence (pTGA35) or with mutants derived from pTGA35 which also contain lesions in AL1 (pTGA70 and pTGA72). Plasmids pTGA35, pTGA70, and pTGA72 were transfected alone or were cotransfected with pTGA52, which contains a CaMV 35S-AL1-nos 3′ cassette. Plasmid pBI221, which contains a CaMV 35S-GUS-nos 3′ cassette, was also used to transfect protoplasts. Three days post-transfection, GUS assays were performed using 10–20 μg protein. GUS activities are expressed as picomoles (pm) of 4-methylumbelliferone (MU) produced per minute per milligram protein. The bar chart illustrates average activities determined from several experiments (pBI221, three experiments, range 100–110; pTGA70, two experiments, range 200–220; pTGA72, four experiments, range 90–220; pTGA35, four experiments, range 8100–17,000; pTGA70 + pTGA52, two experiments, range 5200–10,600; pTGA72 + pTGA52, three experiments, range 3100–11,600; pTGA35 + pTGA52, two experiments, range 13,600–24,200). Background fluorescence (four experiments, range 10–40) determined using extracts from pTGA26-transfected protoplasts was subtracted in all experiments.

with pTGA70 or pTGA72 was significant and was approximately twice that detected in extracts from protoplasts transfected with a plasmid containing a 35S-GUS cassette (pBI221).

To confirm that the relatively low levels of GUS activity observed with nonreplicating DNAs were a consequence of their inability to replicate, pTGA70 and pTGA72 were used in cotransfection experiments with a plasmid designed to express the TGMV AL1 ORF from the 35S promoter (pTGA52). The data presented in Fig. 5 show that after 3 days, GUS activity from the nonreplicating DNAs was stimulated by the presence of pTGA52 to 40–50% of the levels seen in extracts from protoplasts cotransfected with pTGA35 and pTGA52. Stimulation of GUS expression by pTGA52 was observed whether the assays were carried out 1, 2, 3, or 6 days post-transfection (data not shown). While GUS activity was not restored to the level seen with pTGA35, this is probably a consequence of two-hit kinetics inherent in cotransfection experiments. The stimulation of GUS activity by the addition of AL1 function alone suggests that pTGA70 and pTGA72 are not impaired in their expression of AL2.

Total DNA samples were isolated from extracts used to assay GUS activity and hybridized with TGMV and GUS-specific riboprobes (Fig. 6). As expected, replicating unit-length TGMV DNA was detected only in extracts where the inoculum DNA contained an intact AL1 ORF (pTGA26 and pTGA35, lanes 2 and 5) and not in extracts where the inoculum DNA contained a mutation in AL1 (pTGA70 and pTGA72, lanes 3 and 4). Replicating DNA originating from pTGA70 and pTGA72 was detected, however, in extracts from protoplasts cotransfected with pTGA52, which is capable of providing AL1 *in trans* (Fig. 6, lanes 7 and 8). In these extracts, the amount of unit-length pTGA35, pTGA70, and pTGA72 DNA detected appeared to correspond

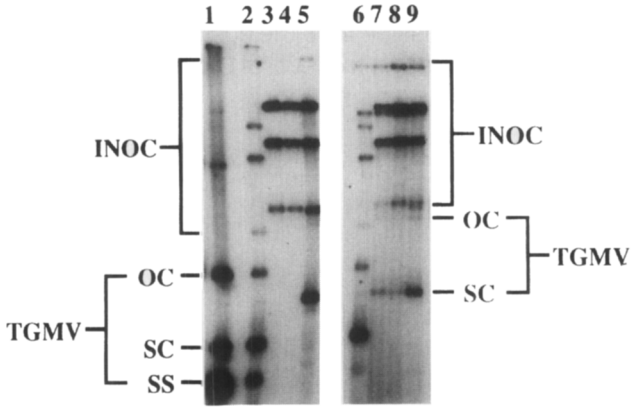

FIG. 6. Southern blot analysis of DNA isolated from protoplasts transfected with replicating and nonreplicating TGMV DNAs. DNA was isolated from the protoplast extracts used for GUS assays, electrophoresed through an agarose gel, transferred to a nitrocellulose membrane, and hybridized to combined ^{32}P-labeled riboprobes specific for both the TGMV AR1 and GUS coding sequences. Lane 1 contains DNA (60 ng) from TGMV-infected plants; lanes 2–5 contain 1 μg DNA from protoplasts transfected with pTGA26, pTGA70, pTGA72, and pTGA35, respectively; lanes 6–9 contain 1 μg of DNA from protoplasts cotransfected with pTGA26 + pTGA52, pTGA70 + pTGA52, pTGA72 + pTGA52, and pTGA35 + pTGA52, respectively. The positions of open circular, linear, and supercoiled inoculum DNA (both the 6.8-kb pTGA26 and the 8.1-kb pTGA35 and derivatives) are indicated to the left and right (INOC). The positions of replicating open circular (OC), supercoiled (SC), and single-stranded (SS) DNAs of genome-sized TGMV A (2.6 kb) are indicated to the left, and the replicating OC and SC DNA forms of the larger GUS-containing derivatives of TGMV A (3.8 kb) are indicated to the right. As expected of coat protein mutants, pTGA35, pTGA70, and pTGA72 do not accumulate ssDNA. All the DNAs shown in this figure were hybridized on the same membrane.

with the relative levels of GUS activity observed (compare Figs. 5 and 6). Restriction endonuclease digestion showed that the replicated pTGA70 and pTGA72 DNAs retained the restriction sites introduced by mutagenesis (data not shown).

DISCUSSION

In the experiments described in this communication, it was found that replicated forms of both TGMV DNAs accumulate in protoplasts with approximately the same kinetics. Supercoiled dsDNA forms of both DNA A and DNA B appeared by 18 hr post-transfection while ssDNA appeared between 18 and 24 hr after transfection. Viral DNA levels reached a maximum after 2–3 days and have been detected for up to 7 days. This contrasts with the results of transfection experiments with the geminiviruses ACMV, BCTV, and WDV, where replicating viral DNA forms were not observed until 2–5 days post-transfection, coinciding with the onset of protoplast cell division (Townsend *et al.,* 1986; Briddon *et al.,* 1989; Matzeit *et al.,* 1991). Why TGMV DNA replicates more rapidly under the conditions described here is unclear.

The kinetics of expression from the TGMV coat protein promoter was examined using a GUS reporter gene inserted in place of the coat protein coding region. As expected, expression of the GUS reporter was related to replication of the viral genome. A relatively low level of GUS activity was detected in protoplasts until 18 hr post-transfection when TGMV supercoiled DNA, the transcription template, began to accumulate to detectable levels. Between 18 and 24 hr post-transfection a large increase in GUS activity was noted, which paralleled a large increase in the accumulation of replicated TGMV DNA. It is interesting that the dramatic increase in expression from the coat protein promoter observed between 18 and 48 hr after transfection corresponds to an increase in the accumulation of viral ssDNA relative to the ds form which also occurs between 18 and 48 hr. Previous experiments have shown that the coat protein is required for the accumulation of ssDNA in this protoplast system (Sunter *et al.,* 1990).

Further evidence that maximal coat protein expression occurs upon amplification of TGMV DNA was demonstrated by a comparison of replicating and nonreplicating viral DNA templates. Mutants (pTGA70 and pTGA72) containing lesions in the AL1 ORF rendering them incapable of replication were still capable of expressing the GUS gene from the coat protein promoter, indicating that AL1 is not essential for rightward transcription. This is in agreement with previous results of Hayes *et al.* (1989), in which coat protein promoter activity was observed in transgenic plants from monomeric TGMV constructs containing a disrupted AL1 ORF. However, GUS expression from a replicating template (pTGA35) was found to be 60- to 90-fold greater than expression from the nonreplicating mutants pTGA70 and pTGA72. Therefore, while AL1 is not required for AR1 transcription, maximal expression of the coat protein depends upon the AL1-mediated amplification of DNA A. Both DNA replication and high level GUS expression were restored when protoplasts were cotransfected with the nonreplicating mutants and a second DNA capable of providing AL1 function *in trans.*

It is worth noting that while GUS expression from nonreplicating viral DNAs was low relative to a replicating template, it was nevertheless significant. The level of GUS activity detected in protoplasts transfected with the nonreplicating TGMV mutants pTGA70 and pTGA72 was slightly greater (twofold) than that observed in extracts from protoplasts transfected with a CaMV 35S-GUS-nos cassette, suggesting that the activity of the TGMV coat protein promoter is comparable to that of the CaMV 35S promoter in *N. tabacum* protoplasts. Similar results have been obtained in transgenic *N. tabacum* plants (Hayes *et al.,* 1989). In these studies, GUS expression from the 35S promoter was found to be only about twofold greater than expression from the TGMV coat protein promoter. The large increase in GUS expression due to amplification of the template underscores the potential of geminiviruses to serve as vectors for transient expression of foreign genes and for the molecular analysis of heterologous promoters in *N. tabacum* protoplasts. Transient expression of bacterial genes from replicating TGMV DNA vectors has previously been reported in leaf discs (Hanley-Bowdoin *et al.,* 1988), and amplification and expression of foreign genes in protoplasts from replicating TGMV (Sunter and Bisaro, 1991) and WDV vectors have also been described (Matzeit *et al.,* 1991; Ugaki *et al.,* 1991).

Much circumstantial evidence is available to suggest that geminiviruses replicate via a rolling circle mechanism, and some experimental data supporting this have recently been presented (Saunders *et al.,* 1991; Stenger *et al.,* 1991). In the rolling circle model, the ds replicative form serves as template both for transcription and for the synthesis of single-stranded (plus strand) DNA. In TGMV, early gene expression would appear to include the leftward genes AL1, AL2, and AL3, while the coat protein gene is expressed later, subsequent to activation of the AR1 promoter by the AL2 gene product. Amplification of the transcription

template due to the combined activities of AL1, AL3, and the host DNA replication machinery is required for high level coat protein expression, which in turn results in the accumulation of plus strand DNA as it is encapsidated into virus particles. The kinetics of accumulation of TGMV ds and ssDNA, as well as the kinetics of coat protein promoter activity, appear to be consistent with this model.

Apart from the known involvement of TGMV AL1, AL2, and AL3 proteins and some limited information concerning *cis*-acting replication sequences, little is known about the nature of the interactions between viral proteins, host proteins, and viral DNA that govern geminivirus DNA replication and gene expression. Protoplast transfection as described in this communication provides a powerful system for further genetic analysis of these processes.

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