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In Vitro Translation of the Full-Length RNA Transcript of Figwort Mosaic Virus (Caulimovirus)

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The circular DNA genome of FMV consists of seven tandemly arranged genes placed successively on a full-length RNA transcript that spans the entire circular viral genome. This transcript is a tentative mRNA for at least five of the six major conserved genes of this virus (genes I–V) that are positioned on this transcript. The sixth major gene (gene VI) is expressed as a separate monocistronic transcript. A long 5′-nontranslated leader (598 nucleotides), a small nonconserved gene (VII), and a short intergenic region (57 nucleotides) precede the five major conserved genes (I through V) on the full-length transcript. A reporter gene (CAT), as a separate cistron or fused in-frame, to viral cistrons in various downstream positions in cloned versions of the viral genome was used in a transcription vector to generate artificial full-length transcripts of FMV. When these mRNAs were translated in vitro (rabbit reticulocyte lysate system), the reporter gene was translated efficiently in all positions. Translation of internal native viral gene positioned on the full-length transcript of FMV was also determined (the gene VI product). These observations suggest that the full-length FMV transcript functions as a polycistronic mRNA in plants. Results are best explained on the basis of translational coupling/relay race model.

Figwort mosaic virus (FMV) In vitro translation Reticulocyte lysates Polycistronic mRNA

THE figwort mosaic virus (FMV) is a member of the caulimoviruses (15), a group with genomes of circular double-stranded DNA of about 8000 base pairs (bp). The virus is very similar to the cauliflower mosaic virus (CaMV), the type member of the group, except in host range.

The genetic organization of some members of the caulimovirus group has been revealed by nucleotide sequencing and transcriptional mapping. Of the seven to nine genes coding for tentative proteins of more than 10 kDa, five are conserved to varying extent in the five viruses that have been sequenced. Some members share a sixth region (gene II) involved in insect transmission; other members lack this gene. Each virus has one to three small open reading regions that are not conserved between members of the group (6,8, 16,18,34).

The strategy for gene expression and replication of the caulimoviruses has been emerging for the past several years. For those members for which transcriptional mapping has been done (CaMV and FMV), two major RNA transcripts are produced. One transcript, which spans the entire circular genome, is initiated from a promoter in the large intergenic region (Fig. 1A). The full-length transcript may function as both a template for reverse transcription during replication of the DNA genome and as a messenger RNA for the translation of five to six closely spaced genes ap-

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pearing on this RNA [for a review see (37)]. The second major transcript is a subgenomic RNA that spans only gene VI. This transcript arises, in the case of CaMV and FMV, from a promoter in the small intergenic region between genes V and VI. Gene VI is the only gene that appears in a 5′ proximal position on a subgenomic monocistronic transcript (1,4,23,35) (Fig. 1A). Both major transcripts terminate at the same point in the large intergenic region.

The caulimoviruses may be exceptions to the conventional model for translation in eukaryotes. Several observations suggest that the full-length transcript functions as a polycistronic mRNA for translation of several closely spaced genes of these viruses. The arrangement and spacing of these genes in the viral genomes, the behavior of mutants of CaMV in nonessential regions of the genome, and viral *trans*activator for the enhanced expression of these internal genes all suggest that the downstream genes of the full-length transcript have an unconventional mode of expression. According to the tenets of the scanning model for translation in eukaryotes [for an updated review see (20)], initiation occurs by binding of 40S ribosomal subunit to the capped 5′ terminus of mRNA followed by lateral migration of the ribosome along the RNA to the first 5′ proximal AUG in a favorable context. At this point the 40S subunit couples with a 60S subunit and protein synthesis is initiated. The initiation complex is believed to assemble at the 5′ end of the mRNA before ribosomes scan inward in a 3′ direction to seek out the proper start codon. Ribosomal subunits may scan past upstream AUG codons in less favorable context until the proper AUG triplet is reached. Although in some exceptions to the rule, two proteins are synthesized from overlapping reading frames by a leaky scanning mechanism [(19) and references therein], generally initiation or reinitiation at other downstream AUG codons is inefficient. The translational activity of many restructured model genes supports the scanning behavior of eukaryotic ribosomes (20), but several well-documented exceptions are known [(17,28, 38) and references therein].

The full-length transcripts of the caulimoviruses may also be exceptions to the 5′ proximal rule for translational initiation. The most frequent transition sequence at the junction of genes I through V of the caulimoviruses is characterized by a striking economy between successive stop and start codons. Frequently, only one to two nucleotides intervene between the two codons, or there are short overlaps of one or a few nucleotides. For example, the transition sequences . . . TAATG . . . with a one nucleotide overlap or . . . ATGA . . . with a two nucleotide overlap are common (Table 1). Gronenborn (13) has pointed out that this is a feature of procaryotic genes, which exhibit translational coupling. These observations have offered credence to the suggestion that ribosomes may not dissociate from the mRNA at a stop codon but may scan for short distances to reinitiate translation at nearby start codons (13, 25,26). Although unusual for eukaryotes, this mechanism may account for translation of the closely spaced genes of the caulimoviruses.

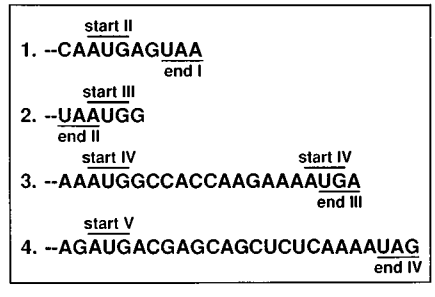
In this article, we describe the results of experiments on the in vitro translation of transcripts of FMV that support the translation coupling model. The experimental approach consisted of introducing a reporter gene, chloramphenicol acetyltransferase (CAT) into various downstream positions (gene II through gene VI) into the cloned genome of FMV. The reconstructed viral genomes were then excised and ligated into an expression vector (pGEM-3Z or pGEM-4Z) that contains a T7 promoter recognized by T7 RNA polymerase. The CAT-containing viral clones were cleaved at restriction sites distal to the CAT gene and the linearized DNA used as a template for mRNA synthesis with T7 RNA polymerase (22,39). The artificial transcripts were then translated in a reticulocyte lysate system (27,31,33). CAT expression with mRNAs with the gene in various downstream positions was obtained, suggesting that the full-length transcript of FMV may serve as a polycistronic mRNA. A preliminary account of this work has been presented recently (30). Moreover, it should be noted that these results are largely corroborated by the in vivo studies of Scholthof et al. (35), with DNA constructs of the FMV genome.

MATERIALS AND METHODS

Materials

The materials were obtained from the following sources: [^{35}S]methionine (1146 Ci/mmol) from

TABLE 1
CLOSE PACKING OF FMV GENES



Dupont NEN Products; ATP GTP, creatine phosphate, and creatine phosphokinase from Sigma Chemical Co.; m^7GpppG cap analogue and restriction enzymes from New England Biolab and US Biochemical Corp.; RNasin, T7 RNA polymerase, and expression plasmids pGEM-3Z and pGEM-4Z from Promega; and Pansorbin (*Staphylococcus aureus* cells coated with protein A) were from Calbiochem.

Preparation of Rabbit Reticulocyte Lysates and Protein Synthesis Assay

Details of the preparation of high-efficiency rabbit reticulocyte lysates and in vitro protein synthesis assay have been described except that [^{35}S]methionine (18–20 μCi/100 μl of reaction mixture) was substituted for [^{14}C]leucine (31,33). Lysates were treated with micrococcal nuclease (27). Samples (20–30 μl) were incubated at 30°C for 1 h. Aliquots (5 μl) were removed to assay for CAT enzymatic activity (10) and part of the reaction mixture (10–15 μl) was subjected to electrophoresis in sodium dodecyl sulfate-polyacrylamide gel (12%) along with prestained standards (Bio-Rad). The resolved polypeptide components were then transferred to a nitrocellulose filter (Millipore Corp.), which was then reacted with antibodies specific for the protein of gene VI and/or autoradiographed if the translations were carried out with radioactive methionine. In other assays the in vitro-synthesized product(s) was specifically immunoprecipitated. The antigen-antibody complexes were absorbed to *S. aureus* cell protein A (Pansorbin) and washed with buffered saline (three times). The complexes were dissociated from *S. aureus* cells with SDS in the presence of β-mercaptoethanol and then subjected to SDS-polyacrylamide gel electrophoresis (21,32). The protein bands were transferred to a cellulose nitrate filter and autoradiographed.

Plasmid Constructs for mRNA Synthesis

The FMV genome used for most of the CAT gene insertions was that of a clone of a naturally occurring deletion mutant of the FMV clone (36). In this deletion mutant 1237 bp encompassing most of gene IV, the coat protein gene, and the 5′ end of gene V (the reverse transcriptase gene), between positions 2173 and 3410 of the FMV genome [see (34)], were missing. The deleted segment resulted in the fusion of genes IV and V to give a product consisting of about one-fourth the amino-terminus of the coat protein fused to viral polymerase devoid of 54 amino acids at its amino-terminus (36). This deleted FMV genome was cloned into pUC119 at the *Sac I* site in gene IV to give a viral clone designated pFHS (36).

For evaluating the translation of genes in downstream positions in the FMV genome, a reporter gene (CAT) was inserted into the viral genome in a variety of internal positions. These CAT-containing viral genomes were then cloned into a transcription vector for preparation of the artificial transcripts for translation in vitro. The pGEM transcription vectors of Promega were used for this purpose.

The series of CAT-FMV recombinants shown in Fig. 1B are derived from plasmids of this deletion mutant FMV genome used for gene expression studies in plant protoplasts (35). In these plasmids the CAT gene is inserted at the nucleotide positions of the FMV genome defined by Richins et al. (34). The CAT-FMV plasmid series consists of:

a) pH52—a *Sca I* site at position 1286 of the FMV genome was converted to an *Xho I* site by insertional mutagenesis (*Xho I* linker) and the CAT gene with *Sal I* ends inserted by its matching extensions. Thus, gene II of FMV is interrupted by CAT gene.
b) pH53(8)—the CAT gene was inserted at a *Sac I* site in gene IV of FMV at position 1910 (Fig. 1B). In this clone the *Pst I* fragment spanning positions 2392 to just beyond the redundant gene VII, in the distal end of the FMV genome in this plasmid, has been deleted (see Fig. 1B). The gene IV-CAT fusion gene contains the first 107 bp of the coat protein gene of FMV ligated in-frame to the CAT gene. The fused open reading region is 813 bp in length and is estimated to give a fusion product of about 32,000 Da.
c) pH54—a *Sca I* site at position 4683 of FMV gene V was converted to an *Xho I* site and its cohesive extensions used for insertion of the CAT gene with *Sal I* ends. The CAT coding region of 208 codons is not in-frame with gene V in this case.
d) pH55—a *Stu I* site at position 5379 of FMV gene VI was converted to an *Xho I* site and used for insertion of the CAT gene fragment. Consequently, the CAT gene is fused in-frame to the first 16 nucleotides of FMV gene VI to give an open reading region of 237 codons (Fig. 1B).
e) pH75—this construct was similar to pH55 except that it contained a *Sac I* (position 1910) to *Sna BI* (position 4795) fragment of wild-type FMV strain DxS (37), which was used to restore the missing portion of the deletion mutant. Hence, pH75 is a wild-type FMV genome with the CAT gene fused in-frame with gene VI near its 5′ end (position 5379 of the FMV genome) (see Fig. 1B).

The foregoing series of FMV genomes containing the CAT gene in various positions were cloned into the multiple cloning site of pGEM-3Z or pGEM-4Z using the *Eco RI* site at position 6886 of the FMV genome. Under control of the T7 promoter of the pGEM vectors the transcripts of FMV started near the native start site for the full-length transcript.

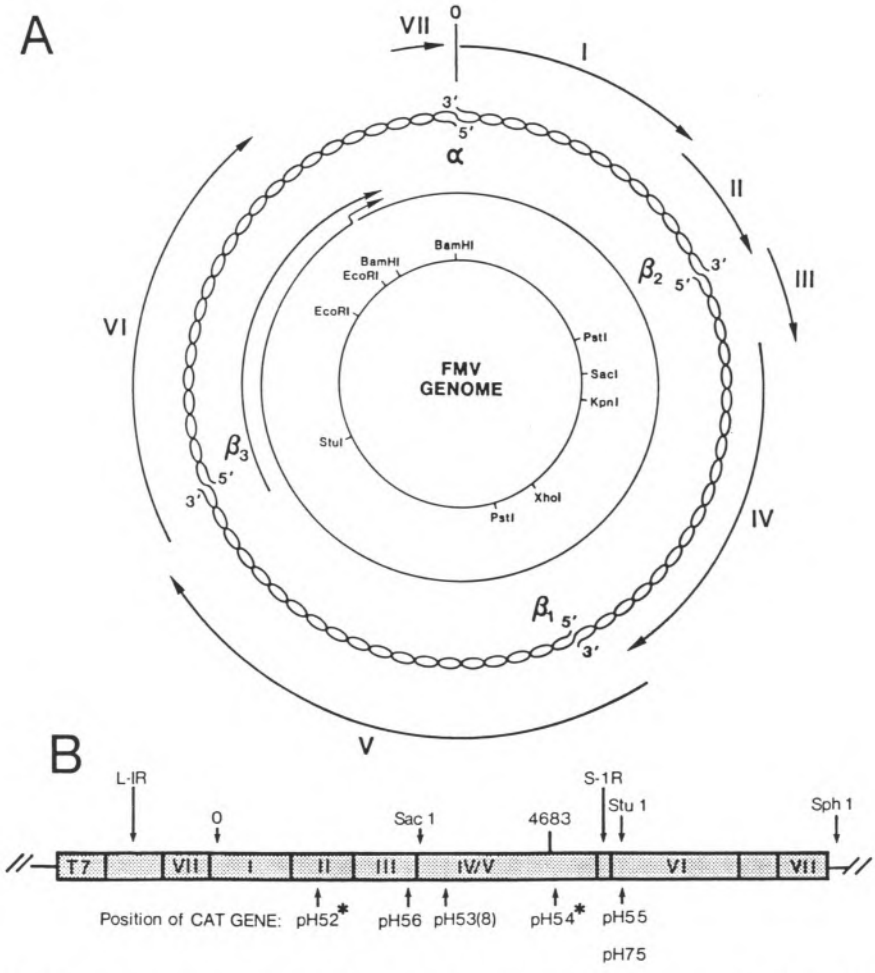


FIG. 1. (A) Genome organization of figwort mosaic virus (FMV). The circular double-stranded DNA of 7743 base pairs is indicated by the interwoven lines. The inner circle shows the position of selected restriction sites. The two RNA transcripts are indicated by lighter inner lines. The single interruption in the minus strand (designated α) and three in the complementary strand (designated β_1, β_2, and β_3) are indicated. The peripheral arrows and Roman numerals indicate the location of the open reading frames. The two intergenic regions, the smaller one between genes V and VI [shown as S-IR in (B)] and the larger one between genes VI and VII [shown as L-IR in (B)], contain promoters for gene VI mRNA and the larger full-length transcript, respectively. (B) Positions of the chloramphenicol acetyltransferase (CAT) gene insertions into the cloned genome of figwort mosaic virus. These DNAs were inserted into a transcription vector (pGEM-3Z or pGEM-4Z) and used for making artificial transcripts of the viral genome. The T7 promoter and the viral genome are shown as the hatched retangular bar. The position of unique restriction sites (*Sac I*, *Stu I*, and *Sph I*) used to linearize the plasmid constructs for the preparation of runoff transcripts driven from the T7 promoter are indicated. Constructs with asterisks indicate that the CAT gene is positioned as a separate cistron with its own start codon.

The plasmid, pGEM VI, from which FMV gene VI-specific transcripts were generated, was prepared by removing the *Hind III* to *Bam HI* fragment of gene VI from pGSI RVI [described by Gowda (11,12)] and cloning it into the *Hind III-Bam HI* window of the pGEM-4Z polylinker. The *Hind III* to *Bam HI* fragment extends from position 5310 to position 7083 of the FMV genome. Gene VI of FMV starts at position 5364 and ends at position 6902. This plasmid was linearized with *Bam HI* before generation of artificial transcripts.

The plasmid designated pGEM-4-3 contained the *Eco RI* (position 6886) to *Sal I* (position 7482, created by oligonucleotide mutagenesis) FMV fragment in the *Eco RI-Sal I* site of pGEM-4Z. This construct was used to prepare antisense RNA of the FMV leader sequence. For this purpose, the plasmid was linearized with *Sal I* (see Hybrid Arrest section). The CAT gene with *Sal I* termini was introduced at *Sal I* site of plasmid pGEM-3Z to

generate a CAT enzyme expression plasmid, pGEM CAT. The pGEM CAT plasmid was used to synthesize CAT enzyme standard by the translation of transcript in rabbit reticulocyte lysates (Fig. 3, lane 2). The plasmid DNA was linearized with *Sph I* to generate transcripts in vitro.

Preparation of Transcripts From Plasmid Constructs

DNAs from the various plasmid constructs were purified by CsCl density gradient centrifugation and linearized at unique *Sac I*, *Stu I*, or *Sph I* sites before use in the T7 polymerase transcription reactions. The transcription reaction mixture (100 μl) contained Tris-HCl (pH 7.9), 40 mM; $MgCl_2$, 6 mM; DTT, 10 mM; spermidine, 2 mM; m^7GpppG, 1 mM; ATP, CTP, and UTP, 0.5 mM each; GTP, 25 μM; RNasin, 120 units; DNA template, 1–2 μg; and T7 RNA polymerase 50 units (22,39). Samples were incubated at 37°C for 20 min after which the GTP concentration in each mixture was raised to 0.5 mM. The incubation was continued for an additional hour. Aliquots (3–5 μl) were removed and subjected to agarose gel (1.2%) electrophoresis to determine the quality and efficiency of transcript synthesis.

Northern Blot Analysis

For Northern blot analysis, pH52 DNA construct was linearized with *Sac I* (Fig. 1B) and used to prepare the pH52 transcript (expected size 2551 nucleotides). Lysate protein synthesis reaction mixtures (100 μl) were incubated at 30° or 0°C with or without pH52 transcript (1 μl). After 30 min of incubation RNA was extracted using the method of Chomczynski and Sacchi (3). Dried RNA pellets were dissolved in 15 μl of denaturing solution containing formamide and formaldehyde according to Davis et al. (5). It was heated at 90°C for 5 min and subjected to formaldehyde-agarose gel electrophoresis (5). RNA in the gel was transferred to a Hybond N+ positively charged nylon membrane (Amersham Corp.) by capillary transfer method. Prehybridzation was carried out at 65°C in Rapid-hyb buffer (Amersham Inc.) for 30 min and then subjected to hybridization with a ^{32}P-labeled CAT gene probe (prepared by random priming) at 55° for 2.5 h. After subsequent washings to remove the unhybridized probe, the filter was autoradiographed.

Hybrid Arrest of Protein Synthesis

Transcripts from the pGEM-4-3 construct representing antisense RNA of the FMV leader sequence was prepared as described in the previous section. The antisense leader sequence transcript (2 μl) was mixed with transcripts (1–2 μl) prepared from pH52 or pH54 constructs in buffer (12 μl) containing Tris-HCl (pH 7.8), 15 mM; NaCl, 90 mM; and $MgCl_2$, 0.06 mM. The samples were heated at 90°C for 1 min and allowed to cool slowly to room temperature, thus permitting hybridization to occur between the antisense RNA leader sequence and the pH52 and pH54 transcripts. It should be noted the antisense RNA is about two to three times in molar excess in the incubation mixture. Controls were treated in the same manner except that the leader antisense RNA was excluded from the reaction mixture. Aliquots (4 μl) of the controls and samples (allowed to hybridize with pH52 or pH54 transcripts) were assayed in 20 μl of in vitro protein synthesis reaction mixture. Following incubation for 60 min at 30°C, aliquots (10 μl) were removed and assayed for the CAT activity.

Antibody Preparation

A synthetic peptide corresponding to the sequence of amino acids 78–103 of a hydrophilic region of FMV gene VI near its 5′ end (inferred from the nucleotide sequence of FMV gene VI) was used to prepare antibody in rabbits (9). Rabbit antibodies to the polypeptide of CAT were purchased from 5-Prime-3-Prime, Inc. (Boulder, CO).

Immunological Selection of Translation Products

At the completion of the translation reactions, antibodies to CAT or the viral gene VI protein was added. After incubation at 37°C for formation of specific antigen-antibody complexes, Pansorbin was added and the absorbed antigen precipitates recovered by low speed centrifugation. The sediment was washed three times, treated with SDS-sample buffer (40–50 μl), and heated at 90°C for 1–2 min (32). The bacterial cells were removed by centrifugation and the clear supernate was analyzed by SDS-polyacrylamide gel electrophoresis. The protein bands in the gel were transferred to cellulose nitrate filters by electroblotting and then autoradiographed.

RESULTS

Initial experiments on the translation of the transcript prepared from cloned gene VI (pGEM VI) showed synthesis of a single radioactive poly-

peptide of 58,000 Da (Fig. 2, lanes 2). This polypeptide migrated in gels at a rate identical to that of authentic inclusion body protein (Fig. 2, lane 3) isolated from FMV-infected plants and was specifically immunoprecipitated by antibody raised against a gene VI peptide (Fig. 2, lane 6). The level of synthesis of gene VI polypeptide in lysates is significant and comparable to amounts obtained in inclusion body preparations from infected plants, as indicated by its reactivity on Western blots (Fig. 2, lanes 2 and 3). Analysis of translation products synthesized in lysates from the full-length viral transcript of pH54 (Fig. 1B), linearized with *Sph I* distal to gene VI, also shows a polypeptide that migrates in gels at the same rate as a protein isolated from FMV-inclusion bodies (Fig. 2, lane 7). By this criterion and others used to characterize gene VI product, the newly synthesized in vitro product consists of an authentic gene VI polypeptide. These initial observations were not expected because gene VI is in an extreme downstream position near the 3′ end of the full-length transcript that contains seven tandem genes (Fig. 1A).

A series of plasmids containing the entire deletion mutant viral genome with the CAT gene inserted into various downstream positions was used to prepare mRNA transcripts otherwise equivalent to the native full-length transcript of FMV. When these transcripts were used to program the in vitro translation with lysates, CAT enzymatic assays showed that in each case, regardless of the position of CAT in the full-length transcript, synthesis of CAT activity was observed (Fig. 3). The downstream positions of CAT in this series of constructs were as follows: in pH52 the CAT gene is inserted in gene II in an out-of-frame position (Fig. 3, lane 8); in pH53(8) the CAT gene is positioned in gene IV and fused in-frame to the first 107 nucleotides of this region (Fig. 3, lane 4); in pH54 the CAT gene is located in the downstream end of gene V (Fig. 3, lane 6); in pH55 the CAT gene is inserted in the 5′ end of gene VI in an in-frame position (Fig. 3, lane 5); in pH75 the location of CAT is the same as that in pH55 but is inserted into the wild-type viral genome instead of the deletion mutant of FMV (Fig. 3, lane 3). Results of a separate experiment (not shown) with transcripts made from pH56, in which the CAT gene is inserted in-frame in gene III, showed CAT activity comparable to that obtained with pH75. These results showed that the CAT gene was expressed in the lysate system regardless of its position in the full-length transcript of FMV.

The possibility that RNA fragmentation during translation of polycistronic mRNA may account for the observed results was investigated by Northern blot analysis. pH52 transcript isolated from lysate protein synthesis mixtures shows that the integrity of the RNA remained unchanged when compared with the unincubated control (Fig. 4, lane 2 vs. lane 3). Although the RNA band is broad, most of the RNA migrates close to the *E. coli* 23S rRNA marker rather than the 16S rRNA marker and is, therefore, more than 2000 nucleotides long. Therefore, CAT synthesis from this RNA is likely to be due to coupled translation from ribosomes that started at gene VII rather than due to fragmentation of RNA resulting in the CAT initiation codon now being the 5′ proximal AUG codon. Results of hybrid arrest of translation described below with this RNA also support this conclusion.

The translation of viral gene VI polypeptide was also detected in lysates programmed with the transcripts from these various constructs (see Fig. 2, lane 7); most importantly, linearization of these constructs with *Stu I* or *Sac I* before transcription removed gene VI. Transcripts from these linear DNAs showed no synthesis of gene VI protein (results not shown).

These experiments showed that synthesis of both reporter gene protein and native viral polypeptide (gene VI) occurred in lysates by translation of the full-length transcript as a polycistronic mRNA. Consequently, this raised the question as to where entry of ribosomes was occurring during the in vitro translation of the mRNA. Information on this point was examined by hybrid arrest translation experiments (24) using antisense transcripts corresponding to the 5′ untranslated leader of the full-length RNA. These leader antisense transcripts were generated using pGEM-4-3. These negative sense RNAs extended from position 6886 to position 7482 of the FMV genome. The cap site of these transcripts is about 30 bases upstream of the transcription start site for the full-length transcript, and then extends to a point about 18 bases upstream of the gene VII start codon (Fig. 1A). Antisense transcripts from pGEM-4-3 were allowed to hybridize to transcripts of pH52 and pH54. The prehybridized transcripts were then used as mRNA to program the in vitro protein synthesis reactions.

In these experiments CAT protein synthesis was dramatically reduced by hybrid arrest in the case of pH52 transcripts (representative results from three different experiments are shown in Fig. 5,

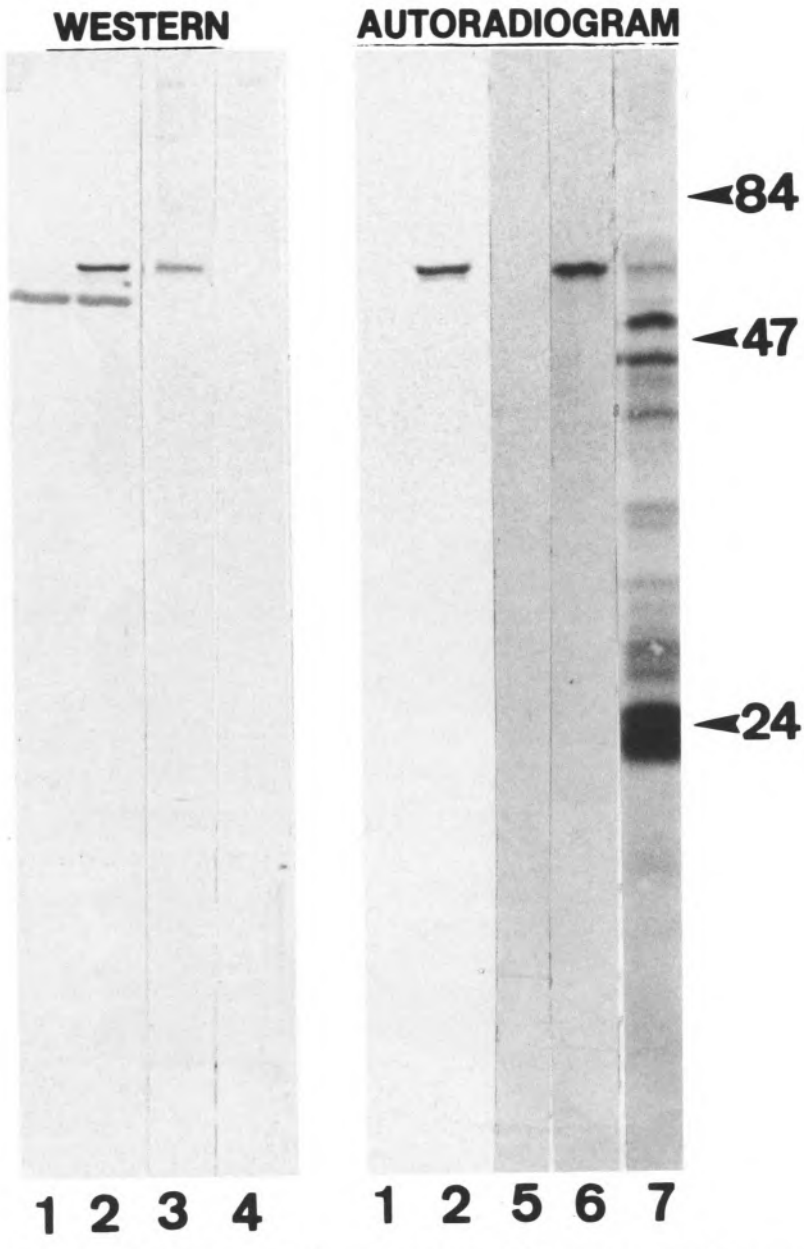


FIG. 2. Proteins synthesized in lysates in response to RNA transcripts containing gene VI of figwort mosaic virus. Reticulocyte lysate reaction mixtures, after treatment with micrococcal nuclease, were incubated for 1 h at 30°C in the absence of added mRNA (lanes 1 and 5, or gene VI transcripts generated from pGEM VI (lanes 2 and 6) or full-length transcripts generated from pH54 (lane 7). Some aliquots were removed and analyzed directly by Western blotting (lanes 1–4) or proteins were immunoselected with antibody to FMV gene VI antibody and Pansorbin, followed by autoradiography (lanes 5 and 6). In the Western blots the filters were treated with gene VI antibody and followed by goat anti-rabbit IgG linked to alkaline phosphatase (14). Lane 3 consists of protein from FMV inclusion bodies isolated from infected plants. Lane 4 had similar extracts from healthy plants. The positions of stained proteins of various molecular weights are indicated on the right margin.

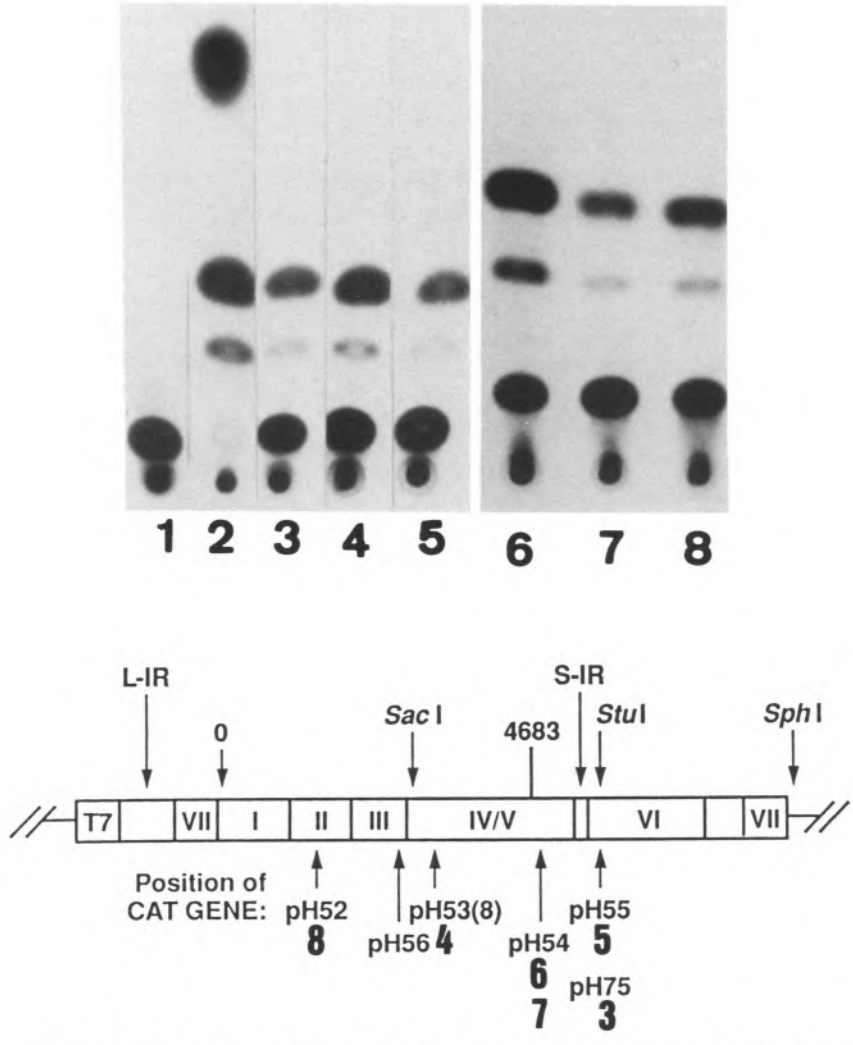


FIG. 3. Assay of CAT enzyme in lysate protein synthesis reaction mixtures incubated with transcripts prepared from various plasmid constructs. Protein synthesis reaction mixture (30 μl) was incubated at 30°C for 1 h with transcripts prepared from various plasmid constructs. Aliquots (5 μl) were diluted with 45 μl of CAT enzyme assay buffer Tris-HCl (pH 7.9), 0.25 M, and then assayed for CAT enzyme as described previously (10). The thin-layer plate was autoradiographed. Lane 1, control without any transcript; lane 2, CAT enzyme standard synthesized in vitro in lysate from a pGEM-CAT construct; lane 3, pH75 transcript; lane 4, pH53(8) transcript; lane 5, pH55 transcript; lane 6, pH54 transcript; lane 7, transcript from pH54 linearized with *Stu I*; and lane 8, pH52 transcript. Data in lanes 6–8 are from a different experiment. Hence, the lanes do not match exactly with lanes 1–5. For convenience, the lane numbers from the autoradiogram are provided under each plasmid construct.

lanes 5 vs. 6) but showed little change with hybrid arrested transcripts from pH54 (Fig. 5, lanes 3 vs. 4). These results with pH52 suggest that region II of the full-length transcript of FMV is translated by ribosomes that enter at the 5′ end of the mRNA and scan inward to AUG codons in the vicinity of gene II. However, the results with pH54 indicate that cistrons situated in distant downstream positions (Fig. 5, lanes 3 and 4) may be translated by internal initiation. Additional work is needed to further elaborate on this point.

DISCUSSION

The translational strategy of FMV is probably reflected in the unique structural features of their RNA transcripts. Although gene VI is translated from a monocistronic transcript (35) that is readily

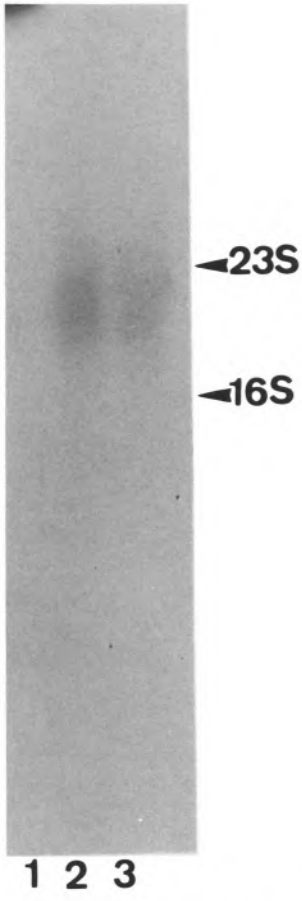


FIG. 4. Northern blot analysis of pH52 transcript incubated in lysate protein synthesis reaction mixture was performed as described in the Materials and Methods section. Lane 1, no transcript added; lane 2, transcript from pH52 but maintained at 0°C; and lane 3, transcript from pH52 incubated at 37°C for 30 min. Positions of 16S and 23S rRNA from *E. coli* are indicated by arrows.

expressed in a cell-free system (Fig. 2), the other major genes of both viruses may be expressed from an unprocessed large transcript that spans the entire circular viral genome. Our results showing translation of the reporter gene from a variety of downstream positions in these various transcripts support this concept. For example, the CAT reporter gene was translated as a part of the full-length transcript whether it was inserted into genes II, IV, V, or VI of the FMV genome including gene III (results not shown). These in vitro results are also corroborated fully by the in vivo studies of Scholthof et al. (35), in which the DNA constructs were electroporated into a plant protoplast-based transient expression system. In addition, native viral protein, a product of gene VI, was also expressed using the full-length transcript as the mRNA. Moreover, and significantly, whenever the CAT gene was fused in-frame with a viral cistron, the resulting fusion products showed a shift to a higher molecular weight [e.g., pH53(8), pH55, and pH75, results not shown].

Several previous observations suggest that gene VI protein is required in vivo for the efficient translation of internal genes of the full-length transcript (2,12,35). However, in the reticulocyte lysate system used for these experiments gene VI in translatable forms gave less conspicuous enhancements of expression, although occasionally considerable increases in translation of downstream cistrons occurred in the presence of gene VI protein (Fig. 3, lane 6 vs. 7). The regulation of translation in this cell-free system is probably less specific than in plant protoplasts, as shown by the efficient expression of CAT in downstream positions. These transcripts were also translated in wheat germ extracts and, other than lower yield of the translation products, results similar to those described here with the lysate system were observed (Scholthof, unpublished).

The translation of the proximal, inner, and most distal cistron from a well-defined polycistronic transcript raises the question as to how these genes are expressed. The genes in FMV polycistronic mRNA are tightly packed in which the termination codon of one cistron either overlaps with the initiation codon of the following cistron or is separated by a few nucleotides (Table 1). These unique tight coupling features of this transcript may allow ribosomes to continue the translation of the next cistron, what has been described by Gronenborn (13) as the relay race model. Our results in vitro and that of Scholthof et al. (35) in vivo can be explained on the basis of this model. Translational coupling is also strongly suggested in translation of CAT cistron in pH52. In this construct out-of-frame insertion of CAT gene in II results in creation of a termination codon 11 nucleotides upstream from the start codon of CAT. It would, therefore, provide for ribosomes to initiate polypeptide from the CAT start codon. Immunoprecipitation of the CAT polypeptide from lysates programmed with pH52 transcript showing synthesis of an authentic CAT polypeptide supports this observation (results not shown). These results again support the translational coupling concept. Alternatively, these sites may contain translational enhancer sequences that may prevent ribosomes from dropping off at the termination signal. Another plausible explanation may be that such sites may contain sequences that allow formation of pseudoknots (29) that serve as

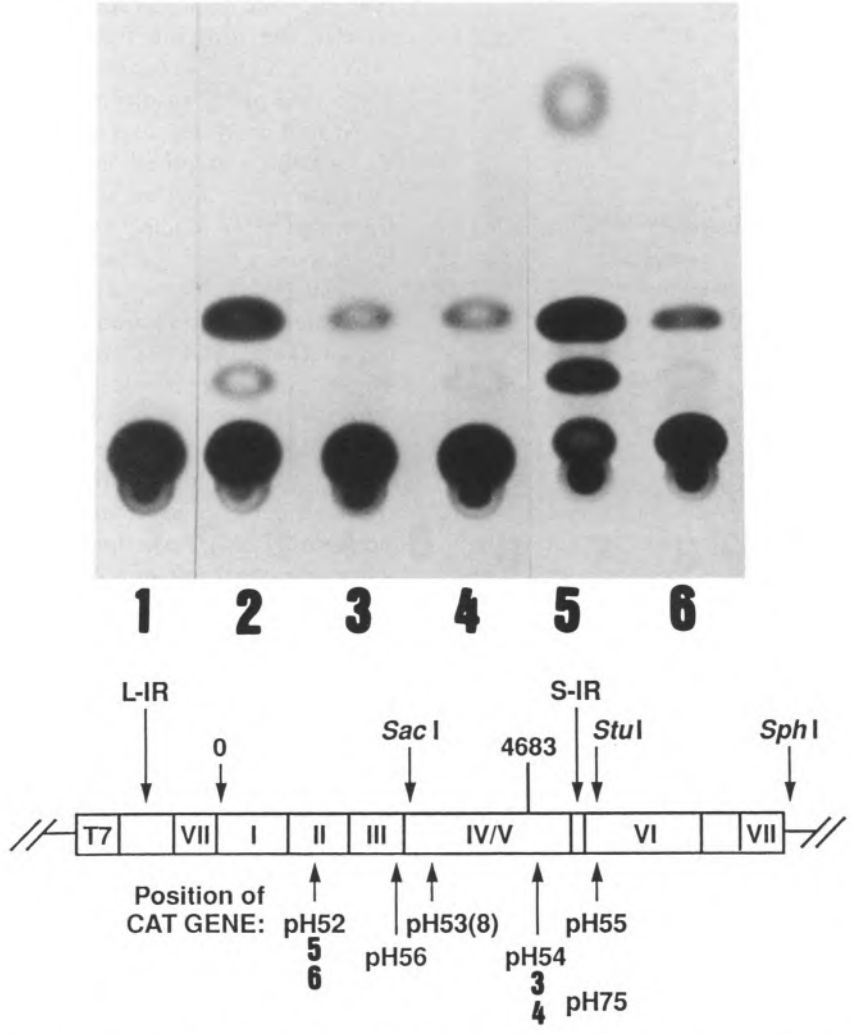


FIG. 5. Hybrid arrest during cell-free synthesis of CAT in lysates in response to FMV transcripts from viral genomes containing CAT gene insertions. Aliquots of lysate reaction mixtures with various transcripts were assayed for CAT enzyme activity. Lane 1, no transcript added; lane 2, CAT enzyme standard; lane 3, transcripts from pH54; lane 4, transcripts from pH54 subjected to hybrid arrest with antisense RNA of the 5′ leader of the full-length transcript; lane 5, transcripts from pH52; lane 6, transcripts to pH52 subjected to hybrid arrest with antisense RNA to the 5′ leader of the full-length transcript. For convenience, lane numbers from autoradiogram are provided under each plasmid construct.

high-affinity ribosome binding element(s) so that ribosomes, instead of dropping off at the termination codon, may be in a position to reinitiate polypeptide chains from the next cistron. Based on their studies with CaMV, Fütterer et al. (7) have proposed a nonlinear ribosomal migration model from the 5′ end of 35S RNA to account for the translation of polycistronic RNA of CaMV. Our results with FMV do not allow us to draw such a conclusion at this point.

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