REGULATION OF
TOBAMOVIRUS GENE EXPRESSION

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I. Introduction

The tobamoviruses make up a group of plant viruses whose type member is tobacco mosaic virus (TMV) (see Gibbs, 1977). The group is characterized by virions, which are straight tubes of approximately 300 × 18 nm with a 4-nm-diameter hollow canal, made up of about 2000 units of a single structural protein surrounding one molecule of single-stranded RNA of approximately 2×10^6 Da. Tobamoviruses are widespread throughout the world, generally have wide host ranges, and cause substantial crop losses. No natural vectors have been identified, but these viruses are easily transmitted mechanically and move quickly through crops that are handled; sometimes they are also spread in the soil.

Tobamoviruses infect almost all cells within the plant, reaching high titers. They replicate in the cytoplasm, but virions are often found in chloroplasts and other organelles. The viruses usually cause disease by preventing proper chloroplast development, resulting in leaves with a mosaic pattern of light and dark green on stunted plants. For detailed reviews of the history, members, virion structure, replication, epidemiology, and cytopathological effects of this virus group, see Volume 2 of

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The Plant Viruses, edited by Van Regenmortel and Frankel-Conrat (1986).

There are numerous viruses within the tobamovirus group (Gibbs, 1986). Although this is one of the best-studied virus groups, the taxonomic relationships among viral strains or distinct viruses are not always clear. The best-examined tobamoviruses often are referred to as strains of TMV, but they are sufficiently different that in other virus groups they would be considered distinct viruses. This situation is compounded by the historical tendency of plant virologists to consider all 300-nm viruses that are unusually stable and have a wide host range to be strains of TMV.

In this chapter we discuss TMV strains U1, OM, L, CGMMV, O, and Cc. The U1 and OM strains are the American and Japanese isolates of what is referred to as the common, *vulgare,* or type strain of TMV. Their sequences differ by only a few nucleotides (Meshi *et al.,* 1982b). The L strain, tomato strain, or tomato mosaic virus (Brunt, 1986) is a closely related virus that is approximately 80% similar to TMV-U1 at the nucleotide level (Ohno *et al.,* 1984). This virus is characterized by having a distinct host range and symptomatology, although most of its host range overlaps that of the common strain. Thus, TMV-L should be considered a strain of the type virus.

Cucumber green mottle mosaic virus (CGMMV) (Okada, 1986) has a host range consisting primarily of cucurbits and differs enough to be considered a separate tobamovirus. Strain O is a field isolate that we (M. E. Hilf and W. O. Dawson, unpublished observations) obtained from orchids. Its characteristics overlap those described for *Odontoglossum* ringspot virus and TMV-O (Edwardson and Zettler, 1986). It has a host range that includes monocotyledonous species and is sufficiently different from the type strain to be considered a separate tobamovirus. The cowpea (Cc) strain, or sunn-hemp mosaic virus (Varma, 1986), is the most distantly related virus in this group (Gibbs, 1986). However, because of custom we refer here to these viruses as strains of TMV.

A. Genome Organization

The genomes of tobamoviruses consist of one molecule of plus-sense RNA of approximately 6400 nucleotides. The entire sequences of the genomes of two tobamoviruses (i.e., TMV-U1, Goelet *et al.,* 1982; TMV-L, Ohno *et al.,* 1984) and parts of several others have been determined: Cc (Meshi *et al.,* 1981, 1982a), OM (Meshi *et al.,* 1982b), CGMMV (Meshi *et al.,* 1983; Saito *et al.,* 1988), and TMV-O (M. E. Hilf and W. O. Dawson, unpublished observations). The tobamovirus genomes ex-

amined have four large open reading frames (ORFs) (Fig. 1). The first, which begins near nucleotide 70 and is terminated near nucleotide 3420, with an amber stop codon (UAG), encodes an approximately 126K protein. Read-through of this amber stop codon and termination with a stop codon (UAA) near residue 4920 results in a 183K protein.

Additionally, within this read-through region in the same ORF, there is a start codon that could encode a 54K protein. The 183K ORF is followed by the 30K (nucleotides 4900–5700) and 17.5K (nucleotides 5700–6200) ORFs. The 183K/54K ORF overlaps the 30K ORF by eight to 23 nucleotides in the different tobamoviruses. The tobamoviruses differ by whether the 30K and 17.5K (i.e., coat protein) ORFs overlap. These ORFs do not overlap in the U1, OM, L, and O strains, having two or three nucleotides between the ORFs, while those of strains Cc and CGMMV overlap by 26 nucleotides. In all tobamoviruses the ORFs are followed by approximately 200 nucleotides that are not translated.

The 126K/183K proteins are thought to be translated from genomic RNAs. These proteins are produced by *in vitro* translation of virion RNA, whereas the other proteins are not (Knowland, 1974). The 30K and coat proteins are translated from subgenomic mRNAs that are
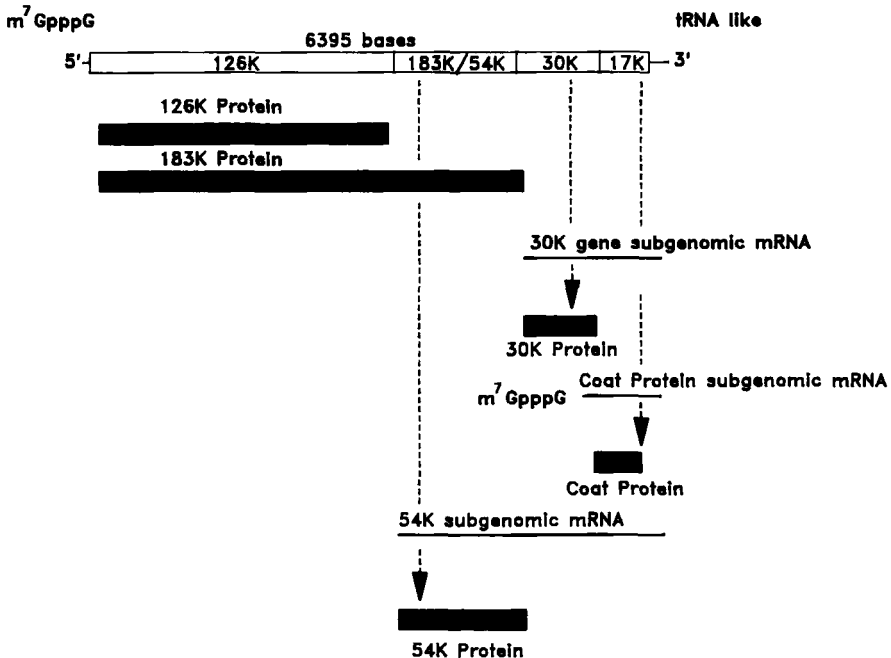

FIG. 1. Tobamovirus genomes.

produced during replication (Hunter *et al.*, 1976; Siegel *et al.*, 1976; Bruening *et al.*, 1976; Higgins *et al.*, 1976; Beachy and Zaitlin, 1977). These mRNAs initiate upstream from the ORFs and continue to the 3′ terminus of the genome, but, as with the genomic RNA, only the 5′-most ORF is translated. An mRNA for the 54K protein has been found in infected cells associated with polyribosomes (Sulzinski *et al.*, 1985), but this protein has not been found *in vivo*.

The coat protein mRNA (corresponding to nucleotides 5703–6395 of TMV-U1) has an eight- or nine-nucleotide leader that is AU rich and has a 7-methylguanosine (m⁷G) cap. The 30K mRNA (nucleotides 4828–6395 of TMV-U1) also contains the coat protein ORF. The 30K mRNA leader is reported to be 65 nucleotides for the OM strain (Watanabe *et al.*, 1984b) and 75 nucleotides for the U1 strain (Lehto *et al.*, 1990b). Available evidence suggests that this mRNA is not capped (Hunter *et al.*, 1983; Joshi *et al.*, 1983; Lehto *et al.*, 1990b). The putative 54K mRNA initiates at nucleotide 3405 of TMV-U1 and continues to the 3′ terminus (Sulzinski *et al.*, 1985). This mRNA has 90 nucleotides upstream from the probable ORF. There is no information concerning whether it is capped.

The noncoding areas of the virus are prime candidates for involvement in the regulation of replication and gene expression. The 5′-nontranslated region consists of about 70 nucleotides that compose the leader of the 126K/183K mRNA. It contains a m⁷G cap (Keith and Fraenkel-Conrat, 1975) and is AU rich. These sequences are thought to be involved in regulation of the expression of the 5′ genes. The 3′-nontranslated region contains a tRNA-like sequence that specifically accepts histidine (or valine for the Cc strain) *in vitro* (Oberg and Philipson, 1972; Beachy *et al.*, 1976).

Considerable sequence similarity exists among the different strains, including a 30-nucleotide sequence that is almost identical in all strains examined and is also contained in the 3′ region of the satellite of TMV (Mirkov *et al.*, 1989). Both terminal regions are presumed to be required for recognition by the replicase to initiate minus- and plus-sense RNA syntheses, as has been demonstrated for bromoviruses (Bujarski *et al.*, 1985; Miller, *et al.*, 1986; French and Ahlquist, 1987). The TMV replicase is assumed to recognize specific viral sequences and to replicate only TMV RNAs. However, tobamovirus hybrids with 3′ termini and replicase genes from different viruses replicate efficiently (Ishikawa *et al.*, 1988; M. E. Hilf and W. O. Dawson, unpublished observations), demonstrating that this specificity must extend to other tobamoviruses.

In contrast to bromoviruses, no internal sequences appear to be required for TMV RNA replication. Viral mutants with the 30K and/or coat protein genes deleted are able to replicate. Mutants with the 30K

and/or coat protein ORFs deleted can replicate in protoplasts, but not in intact plants (Meshi *et al.*, 1987). Mutants with the coat protein ORF removed replicate in plants and protoplasts as free-RNA viruses (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988), while those with most of the 126K/183K ORFs deleted replicate if the wild-type virus is present as a helper (A. J. Raffo and W. O. Dawson, unpublished observations). The latter mutants consist only of the 5' 250 nucleotides and the 1500 3' nucleotides of the U1 genome.

One type of internal regulatory sequence is the subgenomic RNA promoters. The RNA species produced by *in vitro* replicase preparations (Watanabe and Okada, 1986) and the apparent similarities with bromoviruses (Marsh *et al.*, 1987, 1988) indicate that the virus replication complex recognizes a specific sequence of the genomic-length minus-sense RNA to initiate synthesis of plus-sense subgenomic RNAs, which continues to the genomic 3' terminus. With tobamoviruses three different subgenomic RNA promoters should exist, one each for the 54K, 30K, and coat protein mRNAs (Fig. 1). The bromovirus RNA 4 promoter consists of the 60–70 nucleotides upstream from the coat protein ORF (Marsh *et al.*, 1988; French and Ahlquist, 1988). The tobamovirus subgenomic RNA promoters have not been defined, but based on sequence similarities to a proposed RNA virus core promoter sequence and the results of experiments using deletion mutants (Meshi *et al.*, 1987) and hybrid–virus constructs (Dawson *et al.*, 1989), the coat protein promoter of TMV appears to be within the 100 nucleotides upstream from the ORF. Since each promoter probably is within the preceding ORF, sequences comprising the promoter must be bifunctional.

There is no obvious similarity in primary sequence in these three putative subgenomic RNA promoter regions, which might be due to protein coding constraints on the sequence; alternatively, the replicase complex might recognize three-dimensional features that are dictated by more than one primary sequence. An additional possibility is that the three promoters are different to allow differential regulation.

Another potential internal regulatory region is the origin of assembly. This sequence initiates virion assembly, which begins at an internal area of the RNA, proceeds to the 5' terminus, and then continues to the 3' terminus (Butler *et al.*, 1977; Lebeurier *et al.*, 1977; see Bloomer and Butler, 1986). The origin of assembly is within the coding sequences of the 30K ORF of some strains (i.e., U1, OM, L, and O), but within the coat protein ORF of others (i.e., Cc and CGMMV). Subgenomic RNAs that contain the origin of assembly are also encapsidated and make up a minor component of the virion population. It is possible that assembly might function in the regulation of gene expression by making mRNAs unavailable for translation. Not only

would this remove genomic RNA, which is the message for the 126K and 183K proteins, but also the 54K and 30K mRNAs in all tobamoviruses and the coat protein mRNAs of the Cc and CGMMV strains.

Several groups of plant RNA viruses, including the tobamoviruses, have a series of "pseudoknots" in the 3'-nontranslated region, generally between the tRNA-like region and the 3' ORF (Pleij *et al.*, 1985, 1987). Pseudoknots are involved in translational regulation (Tang and Draper, 1989) and in ribosomal frameshifting in coronaviruses (Brierley *et al.*, 1989). TMV has three pseudoknots immediately 3' from the coat protein ORF. There is no information concerning whether they are involved in the regulation of TMV gene expression or replication. Coat protein deletion mutant *cp35* has the first pseudoknot deleted, but retains the ability to replicate, demonstrating that all three are not absolutely necessary for infectivity (Dawson *et al.*, 1988).

B. Ability to Manipulate Tobamovirus Genomes

The life cycles of two tobamoviruses have been artificially extended through a DNA phase that allows manipulation by recombinant DNA techniques. The complete genomes of TMV-U1 (Dawson *et al.*, 1986) and TMV-L (Meshi *et al.*, 1986) were cloned as cDNAs behind a λ phage promoter (Ahlquist and Janda, 1984) so that precise replicas of virion RNA could be produced *in vitro*. The m⁷G cap is added to the 5' end by initiating transcription with the capped dinucleotide, m⁷Gpp-pG. The cap is required for infectivity when RNA is used as the inoculum (Dawson *et al.*, 1986), but a low level of infectivity is achieved when the uncapped RNA is assembled into virions prior to inoculation (Meshi *et al.*, 1986). RNA with a nonmethylated cap is essentially as infectious as that with the methylated cap. A nearly precise 5' sequence is required for infectivity. In contrast, the addition of up to 10 nucleotides to the 3' end has little effect on infectivity. On replication initiated by RNAs with additional nucleotides on either end, the progeny viral RNA does not contain the extra nucleotides.

The development of infectious cDNA clones of tobamoviruses has greatly increased the scope of possible experiments with which to examine the molecular genetics of these viruses. Entire genomes, even those of variants carrying lethal mutations, can be maintained in bacterial plasmids and later transcribed into full-genomic RNAs, providing a uniformity of inoculum not previously available. This has allowed the examination of primary and secondary functions of specific genes (Ishikawa *et al.*, 1986; Meshi *et al.*, 1987; Dawson, *et al.*, 1988), mapping of specific mutant phenotypes to specific nucleotides (Meshi

et al., 1988; Saito *et al.,* 1987; Knorr and Dawson, 1988; Watanabe *et al.,* 1987; Culver and Dawson, 1989a,b), or the manipulation of genome organization (Dawson *et al.,* 1989; Lehto *et al.,* 1990b; Lehto and Dawson, 1990b; Beck and Dawson, 1989).

C. Replication

Tobamoviruses replicate through double-stranded intermediates in a manner similar to that of other plus-sense RNA viruses. RF molecules, which consist of intact plus and minus strands, and larger RI molecules, which are partially double stranded and partially single stranded, are found in infected tissues (Nilsson-Tillgren, 1970; Jackson *et al.,* 1971). Labeling kinetics under numerous different conditions show that RF and RI appear to interconvert rapidly, each being different transient states of the same replicating unit. From short to long labeling periods, the amount of label in each of these double-stranded RNAs is always approximately equal (Kielland-Brandt, 1974; W. O. Dawson, unpublished observations). Incorporation during longer labeling periods (i.e., 2–4 hours) results in about 90% incorporation into genomic-length single-stranded RNA and about 4–6% each into RF and RI. Free minus-sense strands have not been detected. Genomic RNAs are quickly assembled into virions, and the concentration of free plus-sense strands is never more than a small percentage of the final amount of viral RNA produced (Dawson and Schlegel, 1976b).

The first step of replication is assumed to be the infection process, which requires entry of the viral RNA into the cell and its translation into proteins required for the initial events of replication. Whether virions or viral RNA enters cells and how the virions disassemble have been controversial (de Zoeten, 1981). Recently, it has been shown *in vitro* that virions can disassemble in association with ribosomes as the genomic RNA is translated (Wilson, 1984a,b). This cotranslation/disassembly process might occur as the first step in replication. Structures that resemble those created *in vitro* by virions associated with ribosomes have been isolated from newly infected cells, supporting this concept (Shaw *et al.,* 1986).

One of the earliest events observed after infection is a proliferation of membranes in the cytoplasm (Nilsson-Tillgren *et al.,* 1969). Most evidence suggests that replication occurs in this region, in membranous vesicles within the cytoplasm (Hills *et al.,* 1987; Okamoto *et al.,* 1988).

Synthesis of viral proteins and viral RNA are first detected at approximately the same time: 3–6 hours after infection (Sakai and Take-

be, 1974; Paterson and Knight, 1975; Aoki and Takebe, 1975; Dawson and Schlegel, 1976b; Siegel *et al.*, 1978). Each initially increases exponentially, but then assumes a linear rate of synthesis at approximately 12–16 hours. Synthesis continues at or near this maximal rate for a number of hours, which varies among studies. The duration of synthesis in protoplasts often depends on the longevity of the protoplasts, in which growth curves have continued longer when better methods were developed to maintain protoplasts. Overall, it appears that replication continues for about 3–4 days, after which synthesis decreases to 1–2% of the maximal rate (W. O. Dawson, unpublished observations).

Other undefined steps of replication have been identified indirectly by measuring the times that an infection is sensitive to inhibition by specific chemicals. One of the earliest steps is an actinomycin D-sensitive step that occurs prior to viral protein or RNA synthesis (Lockhart and Semancik, 1969; Dawson, 1978). 2-Thiouracil inhibits a step that occurs later than the actinomycin D-sensitive step, but earlier than viral protein or RNA synthesis (Dawson and Schlegel, 1976a; Dawson and Grantham, 1983). Ribovirin and low concentrations of guanidine inhibit with the same kinetics and might inhibit the same process (Dawson, 1975; Dawson and Lozoya-Saldana, 1984). Cycloheximide and arabinofuranosyladenine inhibit a later step that coincides with protein and RNA syntheses (Dawson and Schiegel, 1976a; Dawson and Lozoya-Saldana, 1986). When added to an infection in which virus replication is at the maximal rate, arabinofaranosyladenine inhibits the syntheses of single-stranded, but not double-stranded, RNA and viral proteins. These experiments emphasize that there must be viral functions for which we have not been able to associate biochemical events and that at least two inhibitor-sensitive functions occur before viral protein and RNA syntheses.

The above discussions describe the progression of events in individual cells. In young rapidly growing plants the virus infection spreads from individual cells to almost all other cells. Within an inoculated leaf the infection spreads from initially infected cells to other cells of the leaf both by cell-to-cell movement through plasmodesmata and by long-distance movement in vascular tissues. Free-RNA mutants can move from cell to cell as well as wild-type virus (Dawson *et al.*, 1988). However, the coat protein greatly facilitates long-distance movement. This can be seen clearly by examining free-RNA mutants in inoculated leaves. Infection by the free-RNA mutants proceeds radially at the same rate as by wild-type virus, but wild-type virus spreads quickly to distant parts of the inoculated leaf and fully infects the leaf within 10–12 days, whereas the free-RNA mutant will have moved only 2–3 cm. After about 2 days infectious virus begins moving via

phloem cells into the rest of the plant, utilizing both cell-to-cell and long-distance movement.

D. Gene Functions

1. Coat Protein Gene

The primary function of the coat protein is as a structural unit of virions. Additionally, several secondary characteristics have been associated with this protein. In plants of the genus *Nicotiana* with a specific gene (*N'*) for resistance to most strains of TMV, the host specifically recognizes the coat protein, and a hypersensitive resistance response is actuated (Saito *et al.*, 1987; Knorr and Dawson, 1988; Culver and Dawson, 1989a,b). In addition, the coat protein has been implicated in altering chloroplast structure and preventing normal photosynthesis in susceptible plants (Dawson *et al.*, 1988; Reinero and Beachy, 1989; Hodgson *et al.*, 1989). There is evidence that the coat protein is involved in long-distance movement within the plant in some manner other than encapsidation (Dawson *et al.*, 1988; W. O. Dawson, unpublished observations). Additionally, the coat protein is involved in cross-protection (Sherwood and Fulton, 1982; Register and Beachy, 1988).

2. 30K Gene

The 30K protein is required for cell-to-cell movement of the virus (Nishiguchi *et al.*, 1978; Meshi *et al.*, 1987). The protein becomes tightly bound to cell wall membrane fractions, but its precise mode of action is not understood. The movement function can be provided by other unrelated viruses (Dodds and Hamilton, 1972; Malyshenko *et al.*, 1988), and the tobamovirus 30K protein can mediate movement of other viruses. Transgenic plants expressing the 30K protein allow the movement of both TMV mutants with a defective 30K gene (Deom *et al.*, 1987) and some other unrelated viruses (C. Holt and R. N. Beachy, personal communication). The ability of the movement protein to function in association with the host component appears to be a determinant of the viral host range. TMV has been shown to replicate in inoculated cells of several hosts in which it cannot move from cell to cell (Sulzinski and Zaitlin, 1982). This suggests that the 30K protein must specifically interact with a host component(s) to facilitate cell-to-cell movement.

3. 126K/183K Genes

Most evidence indicates that the 126K and 183K proteins are required for replication. A protein approximately the size of the 126K

protein is found in partially purified replicase preparations (Zaitlin *et al.*, 1973). Virus deletion mutants with only the 126K/183K ORFs are capable of replicating in protoplasts (Meshi *et al.*, 1987). Viral deletion mutants without the 126K or 183K ORF replicate only in association with a helper virus that contains these genes (A. J. Raffo and W. O. Dawson, unpublished observations). When mutants are engineered to prevent production of the 183K protein, no replication occurs (Ishikawa *et al.*, 1986). When the stop codon is removed, so that only the 183K protein is produced, replication occurs at a low level until a stop codon is regenerated. This result implies that the 183K protein alone, which contains all of the functional domains of this region, can replicate viral RNA, but that the production of both proteins together results in much more efficient replication.

Another line of evidence suggesting that the 126K and 183K proteins are replicase proteins comes from comparison of the amino acid sequences of different viruses. Members of a supergroup, including tobamoviruses, alphaviruses, bromoviruses, cucumoviruses, ilarviruses, and tobraviruses (Haseloff *et al.*, 1984; Ahlquist *et al.*, 1985; Cornelissen and Bol, 1984; Hamilton *et al.*, 1987), have similarities in three domains, all within similar regions of the genomes (Fig. 2).

The D3 domain is thought to function as an RNA polymerase, based on the GDD motif (Kamer and Agros, 1984). Recent work with alphaviruses suggests that the D1 domain is required for the initiation of minus-sense RNA synthesis (Hahn *et al.*, 1989) and has a methyltransferase activity (Mi *et al.*, 1989). D2 appears to be involved in subgenomic RNA synthesis, and D3 appears to function as the viral polymerase (Hahn *et al.*, 1989).

Some evidence suggests that the tobamovirus replicase complex contains host proteins, as shown for other plant virus replicases (Mouches *et al.*, 1984; Dorssers *et al.*, 1984). Tomato plants with the *Tm-1* gene for resistance to TMV allow only minimal levels of replication of wild-type TMV-L, even in protoplasts. Reduction of the ability to replicate is greater in *Tm-1/Tm-1* plants than in *Tm-1/+* (heterozygous) plants.

A mutant virus was found that was able to overcome resistance, multiply to a high titer, and cause a mosaic-type disease. This mutant has two nucleotide substitutions, which result in amino acid changes Gln-979 to Glu and His-984 to Tyr within the D2 domain of the 126K and 183K proteins (Meshi *et al.*, 1988). One possibility is that this viral protein must interact with a host protein to form the replicase complex and the *Tm-1* allele encodes that host protein. The host protein in plants without the *Tm-1* allele might interact with the wild-type replicase, but the replicase must be altered to function with the Tm-1 protein. In heterozygous (*Tm-1/+*) plants there might be reduced
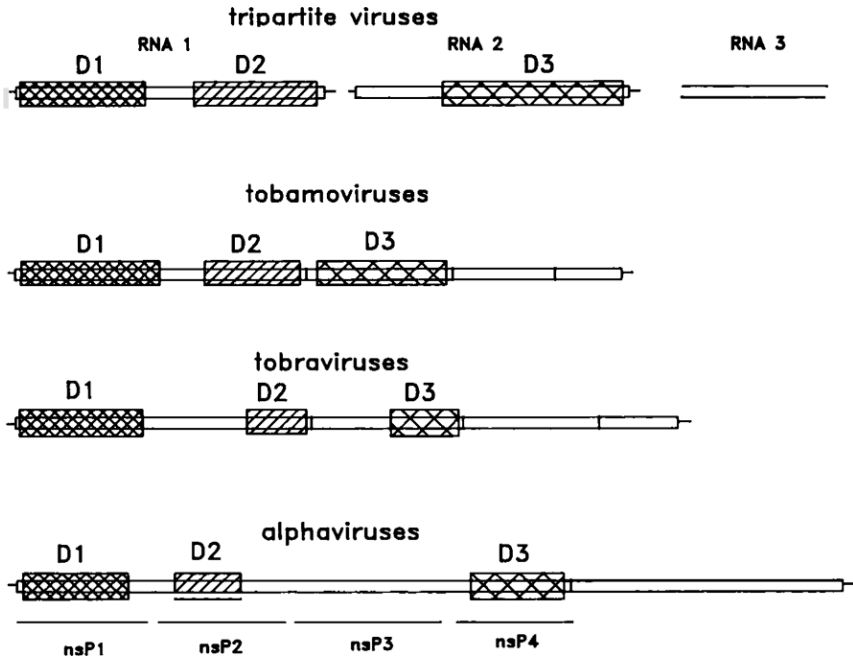
FIG. 2. Viral genomes showing regions having amino acid sequence similarities (Haseloff *et al.*, 1984; Cornelissen and Bol, 1984; Ahlquist *et al.*, 1985; Hamilton *et al.*, 1987). The three similar domains are labeled D1, D2, and D3 and are diagrammed as hatched boxes.

amounts of host protein with which the wild-type virus protein can interact.

Other features of TMV replication have been identified by examining replication-deficient temperature-sensitive mutants (Dawson and Jones, 1976). One group of mutants was deficient in the synthesis of all viral RNAs on shift to the restrictive temperature (Dawson and White, 1978); in the other group of mutants double-stranded RNA synthesis continued, but single-stranded RNA synthesis ceased after the temperature shift (Dawson and White, 1979). These results suggest that the first set of mutants, at the restrictive temperature, lost a function required for the synthesis of all RNAs. It is possible that these mutants had a defect in the D3 domain for polymerization. The second set of mutants was capable of synthesizing double-stranded, but not single-stranded, RNA; perhaps these mutants were defective, at the nonpermissive temperature, in a function that regulates plus-sense–minus-sense RNA ratios or is required to initiate plus-sense strands at the 3′ terminus of minus-sense strands. Arabinofuranosyla-

denine, when added to an ongoing infection, inhibits similarly (Dawson and Lozoya-Saldana, 1986).

Other functions are known to be involved in viral replication. Genomic and coat protein subgenomic RNAs are capped. This process, which apparently occurs in the cytoplasm, might require a virus-encoded protein. Some function must control the ratio of double-stranded RNA produced relative to single-stranded RNA and ratios of plus-sense to minus-sense molecules. Finally, some mechanism must exist for shutting off replication after a specific amount of virus accumulates within the infected cell.

II. REGULATION OF GENE EXPRESSION

A. Synthesis of Tobamovirus Proteins Is Individually Regulated

The production of each TMV protein is regulated differently, both in amounts and times of production. Theoretically, after infection the 126K and 183K proteins should be produced first to provide the replicase complexes required to produce subgenomic RNAs for the syntheses of 30K and coat proteins. However, because RNA viruses replicate by self-saturation kinetics, in which initial progeny RNAs become templates for new replication centers, even within a single cell, replication does not occur synchronously and transcription of the infecting RNA producing only 126K and 183K proteins has not been detected. In practice, all of the viral proteins are detected initially at about the same time, usually between 3 (Watanabe et al., 1984a) and 7 (Siegel et al., 1978) hours after infection. The syntheses of 126K, 183K, and coat proteins reach maximal rates at 16–24 hours. After a sharp peak of maximal synthesis that lasts for only a few hours, the rates of synthesis of 126K and 183K proteins begin declining and reach a low level by 72–96 hours. In contrast, synthesis of the coat protein continues at maximal rates for more than 40 hours. However, during the course of infection, the relative proportions of these proteins vary. Initially, the coat protein is produced at approximately one-half the rate of the 126K protein, but at 70 hours coat protein synthesis exceeds that of the 126K protein by 20-fold (Siegel et al., 1978; Ogawa and Sakai, 1984).

The 183K protein is produced in parallel with the 126K protein, but at approximately 10% as much, suggesting that about 10% of the time the stop codon is read through (Siegel et al., 1978). However, syntheses of the 126K and 183K proteins vary somewhat at different periods of the infection. Early in the infection, the 183K–126K ratio is higher than at later times (Siegel et al., 1978; Watanabe et al., 1984a).

In earlier studies of TMV protein synthesis, the 30K protein was not detected, because this protein is tightly bound to a particulate fraction and is not extracted with the soluble proteins during sodium dodecyl sulfate extraction. It was first detected by Ooshika *et al.* (1984) using an antiserum produced against a synthetic polypeptide corresponding to the 16 carboxy-terminal amino acids of the 30K protein.

Although the 30K protein accumulates in the nuclei of infected protoplasts (Watanabe *et al.*, 1986), *in situ* localization demonstrated that in intact leaves the 30K protein accumulates in plasmodesmatal areas of the cell wall (Tomenius *et al.*, 1987). Similar movement proteins of other plant viruses have also been localized in the cell wall by *in situ* labeling (Stussi-Garaud *et al.*, 1987; Linstead *et al.*, 1988). Procedures that have effectively extracted the movement proteins were designed to extract proteins that were tightly associated with the nonsoluble cell wall fraction (Godefroy-Colburn *et al.*, 1986; Lehto *et al.*, 1990a). In a recent subcellular fractionation study Moser *et al.* (1988) confirmed the association of the 30K protein with the nonsoluble cell wall material and, additionally, found that the protein occurs transiently in the cytoplasmic membrane fraction. The localization of the protein in cell walls suggests that in intact tissue it is actively transported out of the cell.

The 30K protein levels detected in protoplasts (Ooshika *et al.*, 1984; Watanabe *et al.*, 1984a; Blum *et al.*, 1989) are substantially lower than in intact leaves (Moser *et al.*, 1988; Lehto *et al.*, 1990a). These data suggest that accumulation and possibly regulation of the 30K protein are different in the cells of intact leaves than in protoplasts. The difference in levels of this protein in intact cells compared to protoplasts could be due to the protein's being transported out of protoplasts (and degraded) during the experiments. Alternatively, the lack of deposition of the protein into cell walls might lead to the shutting down of synthesis earlier in protoplasts than in leaves.

The timing of synthesis of the 30K protein is quite different from that of the other viral proteins. The 30K protein is produced transiently during the early stage of infection (2–10 hours after inoculation) in synchronously infected protoplasts (Watanabe *et al.*, 1984a; Blum *et al.*, 1989). In near-synchronously infected leaves, the production of 30K protein also occurs during the early period of infection, but synthesis continues longer than in protoplasts, until approximately 24 hours (Lehto *et al.*, 1990a). In leaves the 30K protein is stably associated with a particulate fraction and appears to undergo little turnover (Moser *et al.*, 1988; Lehto *et al.*, 1990a). The maximal concentration of the 30K protein produced in leaves appears to be approximately the same as that of the 126K protein, but higher than that of the 183K protein.

The coat protein of TMV is one of the most highly produced proteins in plants. During maximal synthesis coat protein synthesis can constitute up to 70% of the total cellular protein synthesis, even though there is little reduction in the host protein synthesis (Siegel *et al.,* 1978); during the 2- to 3-day period of rapid synthesis, the coat protein can accumulate as much as 10% of the total cellular protein (Fraser, 1987).

B. Regulation of 5' Proximal Genes

Several lines of evidence suggest that in established infections virions or previrion RNA does not function as mRNA for TMV protein synthesis. Polysomes producing the 126K or coat protein contain associated double-stranded RNAs (Beachy and Zaitlin, 1975; Ogawa *et al.,* 1983). Additionally, TMV protein synthesis is correlated with double-stranded, but not single-stranded, RNA synthesis (Dawson, 1983). TMV protein synthesis was examined in leaves infected with a *ts* (temperature-sensitive) mutant that, on shift to the restrictive temperature (35°C), stopped genomic single-stranded RNA synthesis, but continued double-stranded RNA synthesis. Syntheses of 126K, 183K, and coat proteins continued uninhibited for at least 16 hours in the absence of detectable single-stranded RNA synthesis, even though other experiments demonstrated that protein synthesis quickly declined after the inhibition of all TMV RNA synthesis.

Also, when the replication machinery was partially disrupted by a heat treatment (40°C), on return of the samples to the permissive temperature, replication resumed only at a minimal rate, and protein synthesis recovered after several hours at the permissive temperature, in parallel with double-stranded RNA synthesis (Dawson, 1983). This recovery occurred several hours before the recovery of single-stranded RNA synthesis. These data suggest that a specific function for mRNA synthesis exists that is different from the function that produces progeny virion RNA. This result was not unexpected for subgenomic mRNA synthesis, but it also appears to be the case for the mRNA for the 126K and 183K proteins.

1. 126K Leader Enhances Translation

The 5' leader of the 126K mRNA is probably involved in regulation of the level of expression of this gene. TMV virion RNA is an exceptionally efficient translation template *in vitro,* and this high efficiency has been related to the leader sequence (Gallie *et al.,* 1987a,b, 1988; Sleat *et al.,* 1987). Addition of the 126K leader to other RNAs greatly stimulates the translation of both eukaryotic and prokaryotic mRNAs

in vitro and *in vivo* (Gallie *et al.*, 1987a,b). The efficient translation of TMV mRNAs in prokaryotic *in vitro* translation systems is exceptional, because most eukaryotic mRNAs are not correctly initiated in bacterial cells (Kozak, 1983).

The 126K leader of TMV, as well as some other viral leaders, contains binding sites (AUU) for a second 80 S ribosome upstream from the start codon (Gallie *et al.*, 1987a; Ahlquist *et al.*, 1979; Filipowicz and Haenni, 1979), thus providing a putative second in-frame translation initiation site (Tyc *et al.*, 1984). The simultaneous binding of two ribosomes (i.e., disome formation) has been suggested to contribute to the stimulation of translation by the leader of the 126K mRNA. However, disome formation by different viral mRNA leaders does not correlate with their efficiency of translation (Gallie *et al.*, 1987a), and deletion of the upstream ribosome binding site does not abolish the enhancement effect of the leader (Gallie *et al.*, 1988).

Although the 126K mRNA has an extraordinarily effective leader for translation, the 126K protein is not produced at extraordinarily high levels. The high efficiency might be necessary to enhance translation of the few molecules of RNA introduced into the cell on inoculation to insure that infection is established. Other factors (e.g., the availability of the mRNA) might reduce the expression of this gene later in the infection.

2. Translation of Tobamovirus Proteins after Heat Shock

TMV protein synthesis is translationally regulated differently than host protein synthesis (Dawson and Boyd, 1987). After a heat shock most host protein synthesis is suppressed, and heat-shock proteins begin to be produced (Key *et al.*, 1981). However, after a heat shock TMV 126K, 183K, and coat protein syntheses continue at their normal rates. This result suggests that TMV mRNAs are recognized differently or that they might function on a different set of ribosomes than the majority of plant mRNAs. The discrimination between host normal mRNAs and heat-shock protein mRNA is due to differences in their leader sequences (McGarry and Lindquist, 1985). However, TMV mRNA leaders do not appear to be similar to the heat-shock protein mRNA leaders.

3. Read-through of the Stop Codon

The 183K protein is produced by read-through of the 126K amber stop codon (Pelham, 1978). The transcription of mRNA for this protein and the efficiency of initiation of translation are thought to be the same as for the 126K protein. Regulation is determined by the frequency of read-through, which occurs about 10% of the time *in vivo*

(Siegel *et al.*, 1978) and provides an effective mechanism to produce the 183K protein in lower amounts.

The read-through in cell-free translation systems can be mediated by the wild-type tyrosine tRNA, amounting to 2–5% read-through. A tyrosine tRNA was isolated from tobacco and other plant species that can enhance suppression of the stop codon up to 30% (Bier *et al.*, 1984). Also, the ratio of production of 126K and 183K proteins *in vitro* is affected by the concentration of message. At lower concentrations of mRNA, read-through occurs more frequently than with higher template concentrations (Joshi *et al.*, 1983). This observation could explain the higher ratio of 183K protein produced early in the infection, when mRNA concentrations are low (Siegel *et al.*, 1978).

Regulation of protein synthesis by read-through is a common phenomenon among numerous RNA virus groups, including tobra-, carmo-, tymo-, and furoviruses from plants and alphaviruses and retroviruses from animals. Regulation appears to depend not only on suppressor tRNAs, but also on the sequence context of the stop codon (Valle and Morch, 1988). However, when the amber stop codon of TMV-L was replaced with an ochre stop codon, the mutant was viable and produced normal amounts of 183K protein, suggesting that the ochre stop codon was suppressed as efficiently as the amber one (Ishikawa *et al.*, 1986). Tobamoviruses provide an ideal system to examine whether the sequence context of the stop codon affects read-through and could at the same time allow the examination of mutants that produce different ratios of 126K to 183K proteins.

Read-through of the stop codon and consequent syntheses of both the 126K and 183K proteins are needed for the efficient replication of TMV (Ishikawa *et al.*, 1986). Mutations of TMV-L that prevent production of the 183K protein are lethal. A mutation that changed the amber codon to a tyrosine codon to allow production of the 183K, but not the 126K, protein resulted in a virus population that initially replicated poorly and later began to replicate rapidly. The progeny virus that replicated rapidly contained revertants with an ochre stop codon. This suggests that the mutant was able to replicate minimally with only the 183K protein. However, a mutant with the stop codon deleted entirely and lacking one amino acid in the 183K protein was not infectious. Neither protein alone is sufficient for efficient replication. It will be interesting to determine the effects of altered ratios of these proteins on replication and regulation.

C. Regulation of Internal Genes

Particularly interesting is the regulation of the genes expressed through subgenomic mRNAs, because they are expressed so differ-

ently. The 30K protein is an early gene product produced in minimal amounts, while the coat protein is a late gene product produced at extraordinarily high levels. The 54K protein has not been found in infected tissues, perhaps because of controlled expression during a limited period or because of low amounts of the protein. Although the genes are expressed by similar mechanisms (i.e., subgenomic mRNAs), regulation of the mechanisms appears to occur independently.

Expression of these genes could be controlled at the transcriptional, posttranscriptional, and/or translational levels. Syntheses of the coat protein and 30K mRNAs temporally correlate with production of the corresponding proteins (Ogawa and Sakai, 1984; Watanabe *et al.,* 1984a), suggesting transcriptional regulation. Some of the 30K mRNA is encapsidated by the coat protein, perhaps resulting in posttranslational regulation. Translational regulation is also suggested by the distinctly different leaders of the mRNAs. The coat protein mRNA has a m^7G cap at its 5' terminus and a short (nine-nucleotide) AU-rich leader (Guilley *et al.,* 1979). The leader of the 30K mRNA is not capped (Hunter *et al.,* 1983; Joshi *et al.,* 1983) and is substantially longer (i.e., 75 nucleotides, as found by Lehto *et al.,* 1990c). The leader of the 54K mRNA is even longer, containing approximately 90 nucleotides (Sulzinski *et al.,* 1985).

1. Translational Regulation

Most eukaryotic mRNAs are capped. The cap is a strong determinant of mRNA stability and also strongly enhances the binding of 49 S ribosomal subunits to the 5' end of mRNAs (Shatkin, 1976; Kozak, 1983). The cap-binding protein complex melts the secondary structure of the mRNA leader and facilitates ribosome binding and/or migration to the initiation codon (see Sonenberg, 1987). Removal of the 5' cap impairs ribosomal binding and translation. The addition of cap-binding protein complex to a translation system can relieve translational competition between mRNAs, suggesting that it is a limiting factor in translation initiation (Sarkar *et al.,* 1984). This difference between the coat protein and 30K mRNAs might greatly affect the expression of these genes. The coat protein continues to be produced at relatively high rates several hours after RNA synthesis stops, demonstrating the stability of this mRNA (Dawson, 1983). No information is available concerning the stability of the 30K mRNA.

Start Codon Sequence Context. Another possible type of translational regulation could result from differences in the efficiency of translation initiation, due to differences in the start codon sequence contexts. Kozak's modified scanning model suggests that eukaryotic ribosomes bind to the 5' end of mRNAs and scan in the 3' direction until a start codon within the proper sequence context is found, at

which time translation is initiated (Kozak, 1981, 1983, 1984a,b, 1986a). Usually translation initiates at the first start codon. However, the surrounding sequences are thought to determine whether translation initiates at the first start codon and, if so, with what efficiency.

The most optimal consensus sequence, as defined by the Kozak model, is ACCAUGG, purines at positions −3 and +4 being the most important regulatory signals. This model has been supported by experimental and sequence data from animal, plant, and viral mRNAs. Recently, however, Lutcke *et al.* (1987) suggested that plant ribosomes might not strictly recognize the above consensus sequence as optimal. They suggested that the −3 position was less important in the wheat germ *in vitro* translation system, while the +4 position was more important than that proposed by Kozak. However, some of the most strongly expressed mRNAs in plant systems fit the original consensus.

Comparison of the translation initiation sites of the different tobamoviruses for which sequences are available shows remarkable similarities (Table I).

All of the known 126K start codon contexts are identical and would be considered strong by both the Kozak and Lutcke models. The leader for the 126K/183K mRNA has already been shown to strongly enhance translation and must be near-optimal *in vivo*, supporting both models. All of the coat protein start codons have an A at the −3 position and, in contrast with both models, U1, L, OM, and O have a U at the +4 position. The coat protein is produced at such extraordinarily high levels that this start codon context also must be near-optimal. At the 30K start codon the U1, L, OM, and CGMMV strains have similar contexts with −3 U and +4 G, which is defined by the Kozak model as a "weak" start codon context. The TMV-O 30K start codon context is defined as "strong" and that of the Cc strain is intermediate. The

TABLE I

COMPARISON OF THE TRANSLATION INITIATION SITES OF DIFFERENT TOBAMOVIRUS GENES[a]

Strain	126K Protein	54K Protein	30K Protein	Coat protein
U1	ACAAUGG	GAUAUGC	UAGAUGG	AAUAUGU
L	ACAAUGG	GACAUGU	UUGAUGG	AAUAUGU
OM	ACAAUGG		UAGAUGG	AAUAUGU
CGMMV			UAGAUGG	ACGAUGG
Cc			GUGAUGU	UUGAUGAUGG
O			ACAAUGG	AAUAUGU

[a]Underlined residues indicate the start codons.

theoretical nonoptimal start codon context might be one factor responsible for down-regulation of the expression of this gene and the production of 30K protein at minimal levels.

We examined the start codon context of the 30K gene by changing the weak start codon context (UAGAUGG) to stronger contexts by site-directed mutagenesis and examination of the expression of the 30K gene (Lehto and Dawson, 1990a). We chose to examine the mutants *in planta,* because similar experiments have resulted in different translation efficiencies *in vitro* than those observed *in vivo* (Roner *et al.,* 1989). Two mutants were produced:

wild type	GUUUAUAGAUGGCUCUAG
KK1	GUUUAUAGACUCGAGAUGGCUCUAG
KK2	GUUUAUAGACGAUGGCUCUAG

Mutant *KK1* had a −3 G, which, according to the Kozak model, is a strong start codon context; mutant *KK2* had an even stronger context. Complicating this experiment was that the sequences in front of the 30K start codon could not be modified without also altering the 183K gene, which overlaps the 30K ORF by 14 nucleotides, as well as the subgenomic promoter/leader sequences that control the 30K gene. Mutant *KK1,* which contained an insertion of seven nucleotides, possessed an altered 183K ORF, leading to changes in four amino acids at the carboxy terminus. Mutant *KK2* was designed to contain the strongest possible start codon context and minimal modifications to the 183K protein and promoter/leader sequence. The mutation in *KK2* modified the 183K protein by the insertion of only one amino acid at position 5 from the carboxy terminus; this amino acid was identical to the adjacent amino acid. Three nucleotides were added to the leader of *KK2.* Neither of the 183K protein alterations appeared to have an effect on replication of these mutants in terms of the amounts of progeny RNA and viral proteins produced.

Although start codon contexts often affect translation efficiency in eukaryotic systems severalfold, alteration of the start codon contexts of the TMV-U1 30K gene to more "optimal" contexts, as defined by Kozak's ribosome scanning model, did not enhance the expression of the 30K gene. Mutant *KK2,* with what should have been an optimal start codon context, produced amounts of the 30K protein approximately equal to those of the wild-type virus. Mutant *KK1,* with G at the −3 position, produced only about 30% as much 30K protein as the wild-type virus. This result suggests that the consensus sequence recognized by most eukaryotic ribosomes is not a major factor in the regulation of TMV 30K protein synthesis.

The virus strains with a -3 U in the 30K start codon sequence have additional in-frame (potential) start codons within stronger contexts. The common strains, U1 and OM, have additional start codons at positions 20, 43, and 97, each of which has a -3 A and a $+4$ G, and TMV-L has similar start codons at positions 34, 42, and 96. CGMMV has one start codon at position 100. A possibility is that the 5' start codons are weaker, so that ribosomes occasionally progress to the internal start codons before translation initiates, producing shorter proteins. In fact, *in vitro* translation of the 30K protein mRNA results in multiple proteins with common carboxy termini (Hunter *et al.*, 1983; Joshi *et al.*, 1983). It is not known whether these truncated proteins are active *in vivo;* in fact, they have not been found *in vivo.* The predominant protein produced *in vivo* appears to be the protein initiated at the first start codon. However, it cannot be excluded that a few of the amino-terminal truncated proteins are produced to provide a specific function.

A mutant was produced to examine whether the putative internally initiated carboxy-coterminal 30K proteins could mediate cell-to-cell movement. The 5' AUG of this ORF was changed to ACG to prevent initiation at the first start codon. However, this mutant was not able to infect intact plants, suggesting that the intact 30K protein is needed for viability in the plant (Lehto and Dawson, 1990a).

2. Effect of Subgenomic RNA Promoter/Leader Sequences on Gene Expression

The kinetics of the syntheses of 30K and coat protein mRNAs parallel those of their respective proteins (Ogawa and Sakai, 1984; Watanabe *et al.*, 1984a), which indicates that the regulation of these two genes occurs at least partially at the level of transcription. Bromoviruses replicase preparations are able to initiate the synthesis of subgenomic RNA 4 from a specific internal promoter sequence on the full-length minus-sense RNA (Miller *et al.*, 1985). The replicase recognizes a specific sequence on this RNA to initiate synthesis of the subgenomic RNA. The bromovirus promoter is contained within the 60–70 nucleotides upstream from and including the translation initiation site (Marsh *et al.*, 1987; Marsh *et al.*, 1988; French and Ahlquist, 1988). In contrast to bromoviruses, tobamoviruses should have three subgenomic RNA promoters, one each for the 54K, 30K, and coat protein mRNAs. The lack of similarities in these regions suggests that these promoters might be regulated differently. One possibility is that the genes are expressed at a level determined by an efficiency of transcrip-

tion initiation characteristic of the specific promoter (i.e., that the coat protein promoter might be stronger than the 30K and 54K promoters).

The precise sequences of the subgenomic RNA promoters of TMV have not been characterized, but the coat protein subgenomic RNA promoter appears to be within approximately 100 nucleotides upstream from the coat protein ORF. The insertion of 250 nucleotides from upstream from the coat protein ORF in front of the chloramphenicol acetyltransferase (CAT) ORF resulted in the production of a new subgenomic RNA (Dawson et al., 1989). In other experiments, when the 30K gene was deleted, leaving only 96 nucleotides of the putative coat protein subgenomic RNA promoter region, the coat protein was still produced, although in reduced amounts (Meshi et al., 1987). No work examining the 30K and 54K promoter regions has been described.

We examined the regulation of gene expression by the coat protein subgenomic RNA promoter/leader by inserting this sequence in front of the 30K ORF and determining its effect on 30K protein synthesis (Lehto et al., 1990c). If the coat protein subgenomic RNA promoter, which controls the highest expressed gene, causes a much higher level of transcription, much higher production of 30K protein should have occurred. Studies of the bromovirus subgenomic RNA promoter show that sequences that contain the subgenomic RNA promoter also contain the leader for the mRNA. A complication is that it probably is not possible to use the coat protein subgenomic RNA promoter to produce an mRNA with the wild-type 30K leader, because insertion of a subgenomic RNA promoter in front of an ORF also changes the leader of the mRNA. Since the minimal unit of the coat protein promoter was not known, we made two mutants, each containing different amounts of the promoter region, anticipating that the insertion of the minimal active unit would perturb the virus less. Mutant *KK7* contained 49 nucleotides of the sequence 5' from the coat protein ORF plus 16 non-TMV nucleotides inserted upstream from its 30K ORF. Mutant *KK6* contained 253 nucleotides from the coat protein promoter region plus the same 16 non-TMV nucleotides (Fig. 3).

Mutant *KK6*, with the large insertion (Fig. 3), produced a new 30K protein mRNA with a shorter leader, with similarities to the coat protein mRNA leader. The *KK6* 30K protein mRNA leader was 24 nucleotides, compared to a 75-nucleotide leader of the wild-type 30K mRNA and nine nucleotides of the wild-type coat protein mRNA leader. The *KK6* 30K protein mRNA leader contained the sequences of the coat protein mRNA, as would be expected if promoted by the coat protein promoter. Additionally, it contained nine extra nucleotides
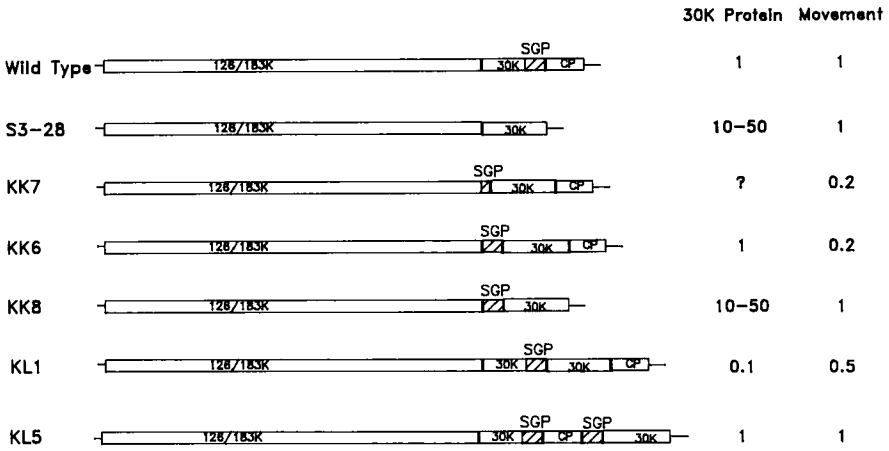
FIG. 3.   TMV mutants and relative amounts of movement and amounts of 30K protein produced. Hatched areas show the coat protein (CP) subgenomic RNA promoter region (SGP). The amount of 30K protein was estimated by densitometric measurements of Western immunoblots and compared to that produced by wild-type TMV, arbitrarily set a 1.0. The relative amounts of movement were estimated by the diameter of local lesions produced in Xanthi nc, with wild-type TMV equal to 1.0.

from the XhoI site created to make the construct and six extra nucleotides added because the subgenomic RNA was initiated six nucleotides upstream from the normal coat protein mRNA initiation site.

Another difference between the wild-type 30K and coat protein mRNAs is that the latter is capped, while the former is not. What determines whether a virus RNA becomes capped is not known. In fact, we do not know how cytoplasmic viruses such as TMV are capped, since host enzymes involved in this process are thought to be limited to the nucleus. Possible controls of capping of the TMV RNAs are the subgenomic RNA promoter and leader. However, the new 30K protein mRNA of KK6 that was initiated by the coat protein subgenomic RNA promoter and contained a hybrid coat protein mRNA leader was not capped (Lehto et al., 1990c). Yet, since both the promoter and the leader were modified by being repositioned in front of the 30K ORF, this does not exclude that they control capping in the native position within the genome.

Insertion of the coat protein subgenomic RNA promoter/leader sequence into mutant KK6 allowed efficient replication of the virus, but expression of the 30K protein was not greatly increased to a level similar to that of the coat protein. Instead, the time course of production of the 30K protein was altered. Instead of being produced earlier

than the 126K protein, the 30K protein of *KK6* was produced later than the 126K protein. In mechanically inoculated tobacco leaves the wild-type TMV 30K protein accumulated to a maximal level (2–3 days after infection) earlier than the 126K protein (3–5 days). In contrast, the maximal accumulation of *KK6* 30K protein occurred 4–7 days later (6–10 days) than that of the 126K protein (4–5 days). Thus, insertion of the coat protein subgenomic promoter/leader sequence greatly delayed production of the 30K protein.

Mutant *KK7*, with the 49-nucleotide coat protein subgenomic RNA promoter/leader region insert (Fig. 3), initially replicated slowly, but later began replicating like wild-type TMV. Progeny virus that replicated well had the insert precisely deleted, resulting in wild-type virus. A comparison of mutant *KK6*, which was stably maintained as progeny virus, to *KK7*, which quickly lost the inserted sequences, suggests that the 49 inserted nucleotides did not contain a functional subgenomic RNA promoter. If the insert failed to promote a subgenomic mRNA, it would be expected to lengthen the leader of the mRNA induced by the native 30K protein subgenomic mRNA promoter. Apparently, this mutant replicated in this manner until the inserted sequences were deleted.

Insertion of the promoter/leader sequences into mutant *KK6* allowed efficient replication of the virus, but greatly altered the time of accumulation of the 30K protein. We do not know whether the delay of 30K protein synthesis by mutant *KK6* is due primarily to the inserted promoter with delayed transcription of the mRNA or the modified leader, resulting in translational regulation, or both. Determining whether insertion of the 30K promoter/leader sequence in front of the coat protein ORF will cause the coat protein to become an early product is important. It is possible that the sequences upstream from each of the internal (i.e., 54K, 30K, and coat protein) ORFs determine their times of expression during infection.

3. Control of 30K Protein Subgenomic RNA Synthesis

The D1 and/or D2 domain of the 126K or 183K protein (Fig. 2) might be involved in the production of the 30K protein mRNA. An attenuated strain of TMV-L was isolated that is produced in reduced amounts in tomato plants and induces only mild symptoms. However, this virus replicates like wild-type virus in protoplasts (Nishiguchi *et al.*, 1982). Sequencing demonstrated that the attenuated strain differed from TMV-L by 10 nucleotides within the 126K gene, three of which result in amino acid substitutions, one in the D1 domain and two in the D2 domain (Nishiguchi *et al.*, 1985). During replication in protoplasts, the synthesis of the 30K protein and its mRNA are specifically reduced in

isolate $L_{11}A$, which has all three amino acid alterations, but the reduction is less in isolate L_{11}, which has only the D1 alteration (Watanabe et al., 1987). Syntheses of genomic RNA, coat protein mRNA, and 126K, 183K, and coat proteins were not reduced. This finding suggests that alterations in these domains of the 126K protein can be involved in the production of 30K protein, but not coat protein, mRNA. If so, the specific subgenomic RNA promoters are recognized independently.

4. Effect of Actinomycin D on 30K Protein Synthesis

A recent observation is that actinomycin D appears to alter the regulation of the 30K gene. It selectively enhances the synthesis of this protein up to 100-fold, while stimulating the syntheses of other viral proteins no more than twofold (Blum et al., 1989). Actinomycin D treatment of TMV-infected protoplasts not only greatly increases production of the 30K protein, but also causes the protein to be produced for longer periods. Instead of synthesis peaking at 8–10 hours and then declining, in actinomycin D-treated protoplasts, the maximal enhanced rate of synthesis continued 16–24 hours after infection, the latest time at which samples were taken.

5. Effect of Position Relative to the 3′ Terminus on Gene Expression

Positioning of the 30K gene nearer to the 3′ terminus by the deletion of portions of the coat protein gene proportionally increases the amount of the 30K protein produced (Lehto et al., 1990b). Mutants with the coat protein gene completely deleted produce approximately 10–50 times as much 30K protein as the wild-type virus. This occurred with mutants with the native 30K protein subgenomic RNA promoter in front of the 30K ORF (mutant S3-28, wild-type TMV with the entire coat protein gene deleted) and a mutant with the coat protein subgenomic RNA promoter/leader sequences in front of the 30K ORF (KK8, mutant KK6 with the coat protein gene removed) (Fig. 3). This increase in 30K protein synthesis is similar to that observed in actinomycin D-treated protoplasts (Blum et al., 1989).

Initially, we thought that the coat protein might be a negative regulator that repressed 30K protein synthesis. However, mutants with the start codons altered so that no coat protein was produced, but with the rest of the coat protein ORF left intact, produced only wild-type amounts of the 30K protein. When mutants with different sizes of deletions in their coat protein genes were examined, the increased production of the 30K protein was always proportional to the number of nucleotides removed, demonstrating that the position of the 30K gene determines its level of expression. The level of expression was

affected little by which promoter/leader sequence controlled the gene. This suggests that one reason the coat protein is produced in greater amounts than the 30K protein is because of their relative positions to the 3' terminus. The same logic suggests that the 54K protein, if it exists, would be produced in amounts proportionally less than the 30K protein. This argument is consistent with the observation by French and Ahlquist (1988), who showed that the level of production of sub-genomic RNA from bromovirus RNA 3 was progressively greater when the promoter was inserted into different positions nearer the 3' terminus.

Although 30K protein synthesis was markedly increased by removing the coat protein gene and positioning the 30K gene nearer the 3' terminus, the resulting level of synthesis of the 30K protein was still substantially less than wild-type levels of coat protein synthesis. Two contributing factors might be that the 30K gene is larger than the coat protein gene and its 5' end is still positioned farther from the 3' terminus in the mutant than that of the coat protein gene in the wild-type genome. However, even another 10-fold increase in the 30K protein would still be substantially less than the amount of coat protein produced. This suggests that other differences between these genes affect their expression. For example, the 5' cap of the coat protein mRNA probably gives it more longevity than the mRNA of the 30K gene (Dawson, 1983).

6. Effects of Coat Protein Gene Deletions on Coat Protein Synthesis

Factors other than distance from the 3' terminus, subgenomic promoter, and subgenomic mRNA leader are involved in the regulation of the internal genes of TMV. We examined a series of mutants with deletions in the coat protein gene and determined the amount of altered coat protein they produced (Dawson et al., 1988). The mutants varied widely in their production of altered coat proteins, from amounts equivalent to the wild-type level of coat proteins to levels too low to detect. There was no correlation between the amount of coat protein produced and the number of nucleotides deleted. Often, mutants differing by less than 10 nucleotides in length differed in coat protein production by several orders of magnitude. In general, mutants that maintained the coat protein ORF through the deletion and produced the normal carboxy terminus produced more coat protein. Pulse-labeling of proteins in mutant infected cells demonstrated that the observed differences in the accumulation of proteins were due to reduced synthesis of the truncated proteins, rather than degradation of proteins without normal carboxy termini (unpublished observations).

7. Effects of Coat Protein on Gene Expression

Initially, we suspected that coat protein might be a regulatory molecule, perhaps binding to specific areas of the RNA to positively or negatively affect replication. We constructed the series of coat protein deletion mutants (Dawson *et al.*, 1988) to examine this phenomenon, but we have found no evidence of altered regulation of these mutants. Mutants with the coat protein start codon changed to ACG produce no coat protein, but produce normal amounts of 126K, 183K, and 30K proteins (J. N. Culver and W. O. Dawson, unpublished observations). This also suggests that the encapsidation of mRNAs does not reduce gene expression by removing either 126K/183K or 30K mRNAs, which could support the argument that there are separate functions for the production of mRNAs and virion RNAs (Dawson, 1983).

D. Effects of Genome Organization on Gene Expression

We examined the effects of different genomic organizations on virus replication, genome stability, and the level of gene expression by constructing different chimeric mutants of TMV. A hybrid, *CAT-CP*, with a gene cartridge consisting of the CAT ORF fused behind the coat protein subgenomic RNA promoter inserted into the TMV genome between the 30K and coat protein ORFs replicated efficiently and produced additional subgenomic RNA and CAT activity (Dawson *et al.*, 1989). However, this hybrid was not stably maintained. Large amounts of the hybrid virus were produced in inoculated leaves, but progeny virus with the inserted sequences deleted predominated in systemically infected tissues. Virus hybrids with two coat protein genes were even less stable (Beck and Dawson, 1990). In fact, they were too transient to detect in their original form: Progeny virus contained only one coat protein gene. These results indicated that there is strong selection against propagating viruses with unnecessary sequences in their genomes. In contrast, however, mutant *KK6* (Fig. 3), which contained the 269-nucleotide insertion, was propagated stably for months (Lehto *et al.*, 1990c). Thus, the selection pressures appear to operate differentially on differently altered genomes.

At present we have little information concerning why the stability of genome organizations differed so greatly during propagation. Viral protein synthesis is precisely regulated, both temporally and quantitatively. Apparently, the gene products are needed in different amounts and at different times for optimal virus replication. Genomic organization, along with specific regulatory sequences, must provide the regulation of individual genes. However, another factor could be the efficiency of replication of the genomic RNAs. There appears to be

selection based on how well the genomic RNA molecule is replicated, indicating that the genomic organization must provide for both efficient replication and effective gene expression. The optimal genome organization might balance gene expression against efficient replication of the RNA.

We created a series of TMV hybrids with two 30K ORFs to examine how well they would replicate and what effect genome position has on gene expression (Lehto and Dawson, 1990b). We have seen that inserted or altered sequences can be deleted or rearranged quickly and that selection is a strong force determining the constitution of the progeny population. A major question, then, is what determines whether a virus can compete with its altered progeny. Can modified viruses be propagated and maintained as the major component of a progeny population?

A mutant with a second 30K ORF fused to the coat protein ORF, *CP30K,* produced more fusion protein than wild-type 30K protein but substantially less than wild-type coat protein (Fig. 3). Simple fusion of an ORF to the amino-terminal two-thirds of the coat protein ORF did not provide the same level of expression as that of the native coat protein. Expression of the fusion protein gene might have been partially decreased by its position relative to 3' end, since the insertion of 30K ORF moved the coat protein subgenomic RNA promoter approximately 700 nucleotides farther from the 3' terminus than that of the wild-type virus. This insertion also decreased the amount of native 30K protein produced by this mutant. This reduction in 30K protein apparently was due to positioning the native gene farther from the 3' terminus.

Another hybrid, *KL1,* has two 30K ORFs in tandem, the 5' ORF driven by the native 30K protein subgenomic RNA promoter/leader sequences and the 3' ORF driven by the coat protein subgenomic RNA promoter/leader sequences (Fig. 3). At the time this mutant was built, we expected that with two 30K genes, one driven by its native promoter and the other controlled by the coat protein subgenomic RNA promoter, more 30K protein would be produced. However, this mutant produced greatly reduced amounts of the 30K protein, at least one-tenth of that produced by wild-type TMV. This result might be due in part to decreased expression of the native 30K gene, because of its being positioned farther from the 3' terminus by the size of the insertion (i.e., 800 nucleotides). However, the position of the inserted 30K ORF relative to the 3' terminus was identical to that of *KK6,* which produced normal amounts of the 30K protein, but later in the infection (Lehto *et al.,* 1990c). *KL1* produced only barely detectable amounts of the 30K protein at any time. This suggests that factors other than

increased distance from the 3' terminus caused the decreased production of the 30K protein by this mutant.

Mutant *KL5* has the 30K ORF fused behind the coat protein subgenomic RNA promoter/leader region and inserted between the coat protein ORF and the 3'-nontranslated region (Fig. 3). The second 30K gene was in the position of the coat protein gene relative to the 3' terminus. *KL5* produces increased amounts of the 30K protein compared to the other mutant with two 30K ORFs, but this occurred at the expense of reduced abilities for replication and stable propagation. This mutant replicated poorly and did not move out of inoculated leaves, and most progeny virus had altered sequences. The hybrids that we have examined with insertions between the coat protein ORF and the 3'-nontranslated region—*KL5* and *CP-CAT* (Dawson *et al.,* 1989)—replicated poorly, and virus with the inserted sequences removed predominated the progeny population. However, mutant *KL5* reaffirmed that genes positioned nearer the 3' terminus could be expressed at higher rates.

III. Effects of Alteration of Production of 30K Protein on Phenotype

The alteration of gene expression also allows examination of the effects of protein levels on gene function. For example, a wide range of levels of 30K protein production appears to be sufficient for normal cell-to-cell movement within the plant. Mutant *KK1* produced only about 30% as much 30K protein as wild-type TMV, but there was no detectable decrease in the mutant's ability to move (Lehto and Dawson, 1990a). Mutants with greater decreases in the 30K protein resulted in a decreased ability to move in inoculated, but not into systemically infected, leaves. Mutant *KL1* (Fig. 3) produced barely detectable amounts of the 30K protein, which apparently resulted in a reduction in the size of local lesions to approximately one-half the diameter of those produced by wild-type TMV (Lehto and Dawson, 1990b). However, *KL1* systemically infected upper leaves as well as wild-type TMV. Thus, even this greatly reduced amount of 30K protein was sufficient for long-distance systemic movement. A surprising result was that, with this greatly reduced amount of 30K protein and its reduced ability to spread in inoculated leaves, *KL1* was able to compete effectively during the course of infection of tobacco plants with the wild-type virus that arose by deletion of the inserted sequences. The wild-type virus only gradually overtook the *KL1* population.

Increases in the production of the 30K protein did not increase the ability of mutants to move from cell to cell. The coat protein deletion mutants (Fig. 3), which had up to 50 times more 30K protein than did wild-type TMV, moved in inoculated leaves identically to wild-type virus (Lehto *et al.*, 1990b). Apparently, an amount of 30K protein above a threshold is sufficient for normal movement, and increased amounts have no effect.

Delayed production of the 30K protein by mutant *KK6* (Fig. 3) greatly affected cell-to-cell as well as long-distance movement (Lehto *et al.*, 1990c). The final accumulation of the 30K protein of *KK6* was equal to that of wild-type TMV, but mutant *KK6* local lesions in inoculated leaves were much smaller than wild-type lesions and *KK6* moved more slowly and to only limited areas in upper leaves. The movement defect of *KK6* was due to to delayed production of the 30K protein, rather than to reduced levels, which suggests that the 30K protein is needed during the early hours of the replication cycle to properly mediate cell-to-cell movement.

IV. Conclusions and Speculation

Tobamoviruses appear to use several strategies to control gene expression:

1. Different subgenomic RNA promoter/leader sequences control timing of expression of genes.
2. Genes expressed via subgenomic mRNAs are expressed in decreasing amounts with increasing distances from the 3' terminus.
3. TMV mRNAs appear to be translationally regulated differently from host mRNAs.
4. Capped mRNAs probably are translated at higher levels than noncapped mRNAs.

However, there certainly is much regulation that we do not understand.

Genome organization also affects gene expression, but it appears to be equally important for the efficiency of replication and the ability of the genomic structure to be stably propagated. What advantages do different genome organizations have? Different virus groups have evolved different gene arrangements. Examples of similar viruses that express genes via subgenomic mRNAs that have different genome organizations are shown in Fig. 4. We have little understanding of why the particular genomic structure of tobamoviruses arose. Within the tobamoviruses we have established that different types of reg-
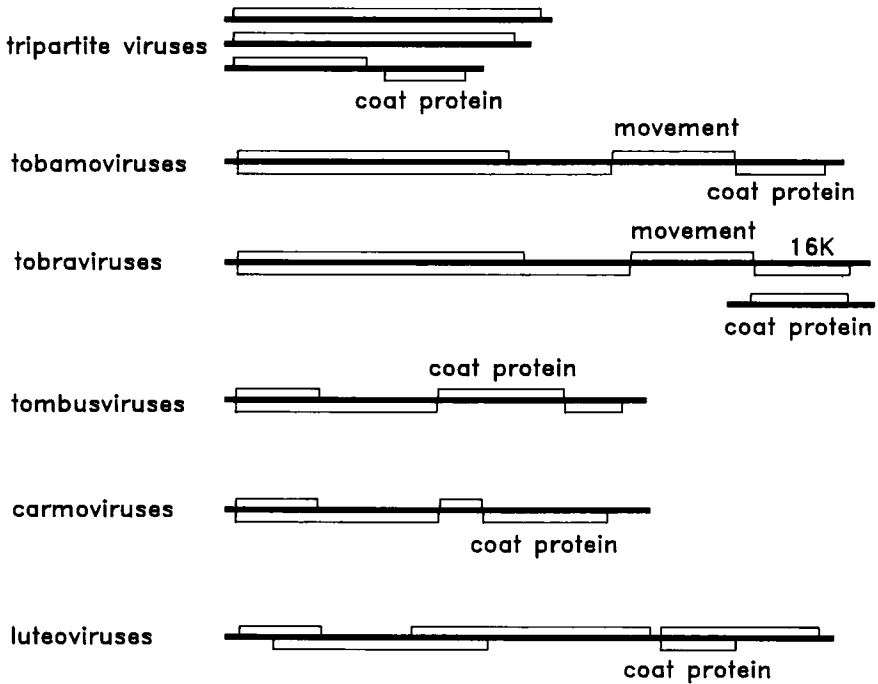
FIG. 4.   Generalized genomes of plant virus groups. Open reading frames are shown as open boxes (Ahlquist *et al.*, 1984; Goelet *et al.*, 1982; Hamilton *et al.*, 1987; Rochon and Tremaine, 1989; Carrington *et al.*, 1989; van der Wilk *et al.*, 1989).

ulation exist, but our understanding of regulatory mechanisms is meager. However, we have observed enough to make it tempting to speculate on the relationships of RNA virus genomic organizations to the regulation of gene expression.

Genes that need to be expressed first to produce enzymes required for later production of subgenomic mRNAs and late gene products are expected to be at the 5′ terminus. We assume that genes expressed through subgenomic RNAs would require the prior production of replicase and could not be expressed initially. However, within the Sindbis supergroup that includes tobamoviruses, tripartite viruses, and alphaviruses, even the replicase domains are expressed differently. With alphaviruses the D1, D2, and D3 domains (Fig. 2) are expressed as a polyprotein that cleaves into proteins containing these domains singularly. The D1 and D2 domains of tripartite viruses and tobamoviruses are contained within a single protein. Tripartite viruses

express the D3 domain as a single protein, but tobamoviruses express this domain in a protein that contains all three domains, or, if the 54K protein is produced, expresses it both ways. With tobamoviruses the initial expression of the 126K and 183K genes allows production of all three domains without the requirement of RNA synthesis. Does the 183K protein function, which differs from the 126K protein function, require the D1 and D2 domains, or is this simply a method to produce the D3 domain without requiring RNA synthesis (Fig. 2)?

Also, tripartite viruses express their putative movement protein from a genomic RNA instead of from a subgenomic mRNA, as do tobamoviruses. Does this mean that this protein must be produced initially? It will be interesting to determine whether a TMV hybrid with the D3 and 30K domains on separate genomic RNAs in the 5' position will replicate.

Viruses that express genes via subgenomic mRNAs generally produce structural proteins in greater amounts than nonstructural proteins. In most of these viruses, the structural protein gene(s) is positioned at the 3' terminus of the genome (Fig. 4). Tobamovirus genes expressed via subgenomic mRNAs appear to be expressed in increasing amounts when positioned nearer the 3' terminus. This has similarities to rhabdovirus transcriptional regulation, in which amounts of transcript are progressively decreased as genes are positioned farther from the genomic 3' end (see Banerjee, 1987). If this observation can be extended to other virus groups (e.g., tobraviruses), the 16K gene which is positioned at the 3' terminus (Fig. 4) should be expressed in greater amounts than the movement protein, as recently demonstrated (Angenent *et al.*, 1989). Tombusviruses (Fig. 4) do not have their coat protein genes positioned at the 3' terminus, and whether their 3' genes are expressed at higher levels than their coat protein genes remains to be seen. Tombusviruses and carmoviruses (Fig. 4) appear to be similar, except for their gene order (Carrington *et al.*, 1989; Rochon and Tremaine, 1989). Do they differ in levels of expression of the coat protein? What advantages do each of these genome organizations provide? We have only begun to understand the regulation of RNA viruses and how it relates to the evolution of genome organizations.

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