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# Elucidation of the genome organization of tobacco mosaic virus

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Proteins unique to tobacco mosaic virus (TMV)-infected plants were detected in the 1970s by electrophoretic analyses of extracts of virus-infected tissues, comparing their proteins to those generated in extracts of uninfected tissues. The genome organization of TMV was deduced principally from studies involving *in vitro* translation of proteins from the genomic and subgenomic messenger RNAs. The ultimate analysis of the TMV genome came in 1982 when P. Goelet and colleagues sequenced the entire genome. Studies leading to the elucidation of the TMV genome organization are described below.

**Keywords:** genome organization; subgenomic mRNAs; replicase; movement protein; coat protein; *in vitro* translation

## 1. PROTEINS ASSOCIATED WITH TMV INFECTION

Until 1970, the only protein 'found to be unequivocally associated with infection' had been the viral coat protein (Zaitlin & Hariharsubramanian 1970). My colleague V. Hariharasubramanian (now known as V. Hari) had the good idea of examining mixed extracts of uninfected and TMV-infected tobacco tissues, labelled with $^{14}$C-leucine and $^{3}$H-leucine, respectively. The tissues were infiltrated beforehand with actinomycin D to inhibit *de novo* host mRNA and protein synthesis. The mixed extracts were run on polyacrylamide gels in tubes; the data obtained were hard won, as each gel had to be cut into about 100 1-mm slices, which were then solubilized and counted. (As an aside, the one-page paper describing how to solubilize the gel slices generated many more reprint requests [>1200] than any other paper I have published!) Plotting the ratio of the $^{3}$H to $^{14}$C incorporations revealed that there were at least four virus-stimulated proteins and one inhibited protein in virus-infected tissue (figure 1; Zaitlin & Harihasubramanian 1970, 1972). Subsequent studies described below have shown that TMV RNA encodes at least four proteins; namely, the coat protein (CP), the 126-kDa and 183-kDa proteins of the replicase, and the 30-kDa movement protein. In contrast, Singer (1971), using a somewhat similar double-labelling technique, concluded that the high-molecular weight proteins were 'an aggregate containing coat protein', which is now known not to be the case.

## 2. WHERE IS THE CP GENE IN TMV RNA?

The first gene whose location on the genome was sought was that of the CP. Kado & Knight (1968) concluded that the CP gene was located in that half of the viral RNA nearest the 5′-end. Their conclusions were based on experiments in which the partially stripped, RNase-treated TMV was mixed with another strain of TMV. (Treating TMV virions with certain detergents or alkali removes protein subunits sequentially and selectively from one end of the molecule.) They examined how the degree of stripping using sodium dodecyl sulphate (SDS) affected the capacity to produce TMV CP in the mixed inoculum. It is now known that the CP is at the 3′-end of the viral genome. Possible explanations for the erroneous conclusion of Kado & Knight are given by Beachy *et al*. (1976). Basically, as is now known, the population of rods from a TMV infection consists of full-length, encapsidated subgenomic RNAs and broken fragments (Beachy & Zaitlin 1977); thus, without sorting these rods, stripping would yield a mixed population of molecules, leading to the erroneous conclusion. Additionally, uncertainty as to which end was stripped with SDS, the reagent used by Kado & Knight (1968), contributed to the confusion. It is now known that SDS strips from the 3′-end (Wilson *et al*. 1976).

In contrast, Richards *et al*. (1975) correctly identified the 3′-region of the genome as the location of the CP gene, which was still the only viral-encoded protein known at that time. They were able to show that viral CP bound to a specific fragment of T1 RNase-digested TMV RNA which was within the CP cistron. Using this knowledge, they found similar CP binding to the RNA of TMV virions which had been partially stripped of their CP, leaving the 3′-terminal portion of the RNA exposed.

## 3. SUBGENOMIC MESSENGER RNAS

The notion that the 5′-most open reading frame of a polycistronic eukaryotic mRNA is almost always the only open reading frame to be translated (Kozak 1989) was a critical antecedent to the concept of subgenomic mRNAs, and studies on TMV were important in supporting both these concepts. In parallel studies on the alphavirus Sindbis, a 26S RNA had been observed, which was contained within the 49S genomic RNA, but it was not considered to be a subgenomic RNA at that time (Simmons & Strauss 1972). Wengler *et al*. (1974) translated

 

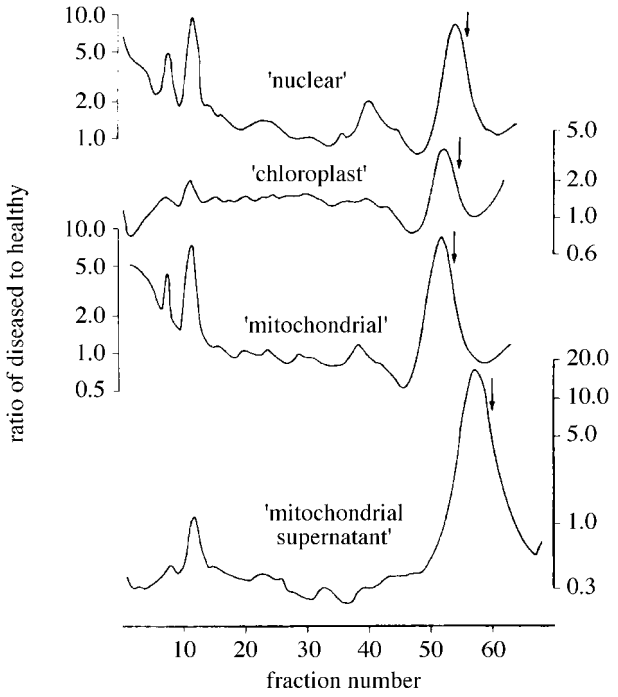


Figure 1. The ratio of the incorporation of $^{3}$H-leucine into subcellular fractions of TMV-infected tobacco leaves to $^{14}$C-leucine into comparable healthy tissue fractions. Note the virus-specific incorporation into several slow-migrating components in fractions 5–11, and the prominent CP at about fraction 55. The arrows represent the position of the bromophenol blue dye marker in the gels. From Zaitlin & Hariharasubramanian (1972).

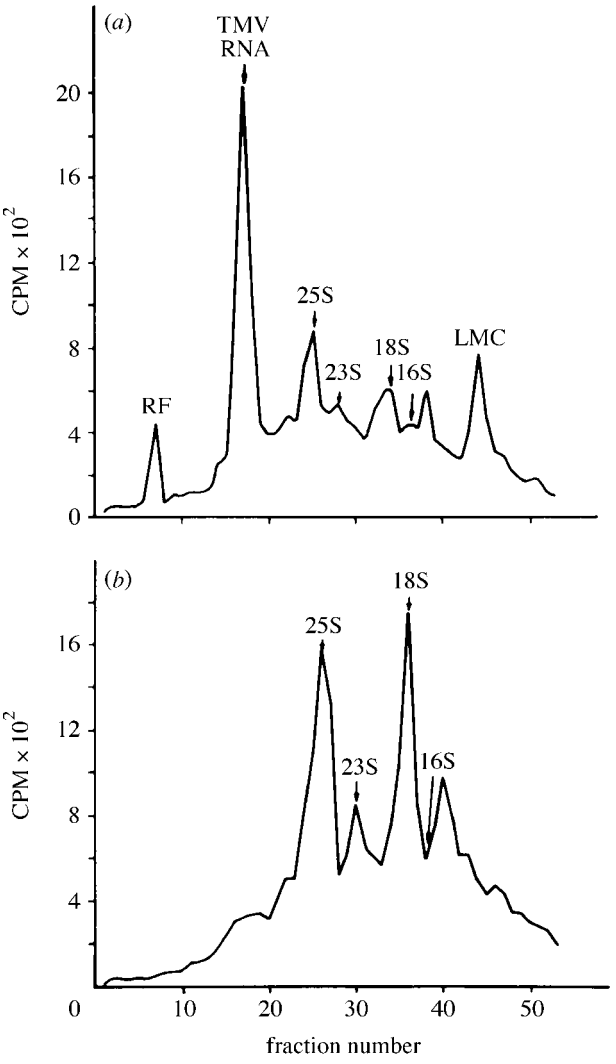


Figure 2. Incorporation of $^{3}$H-uridine into RNA of cells from (*a*) TMV-infected or (*b*) healthy leaf tissues. Note the virus-specific incorporation into the replicative form (RF), TMV RNA and the CP mRNA, the 'LMC'. From Jackson *et al.* (1972).

the 26S RNA of the related alphavirus Semliki Forest, and concluded that it served as a mRNA.

Our own studies (at the University of Arizona) in which it was shown that there was a possible separate messenger for TMV CP were also published in 1972 (Jackson *et al.* 1972). These studies used leaf cells obtained from virus-infected tobacco leaves by digestion of the middle lamella of the cells with pectinase enzymes. The cells were fed $^{3}$H-uridine and analysed for incorporation of $^{3}$H into single- and double-stranded RNA. Label accumulated in a RNase-resistant species interpreted to be the 'replicative form', in TMV RNA, and in a fast-running, single-stranded, viral-related RNA, termed the 'LMC' or 'low molecular weight component'.

Later we postulated that the LMC was the CP mRNA (Siegel *et al.* 1973). In 1976, Hunter *et al.* translated TMV RNA extracted from virions, in reticulocyte lysates *in vitro* and in injected *Xenopus laevis* oocytes; only large protein products were generated, but no CP. Moreover, they determined that these large proteins (later shown to be the replicase proteins) did not contain internal CP sequences. However, when total RNA was extracted from TMV-infected leaves and fractionated on sucrose gradients, *in vitro* translation of the slower-sedimenting fractions (especially I4 as shown in figure 3) in a wheat germ cell-free system produced CP, thus demonstrating that the LMC was indeed the CP mRNA (Hunter *et al.* 1976). Siegel *et al.* (1976) also reached the same conclusion about the LMC. Both groups noted that there were other viral RNAs which might also be messenger RNAs for other viral-encoded proteins.

Many of the studies in which the genome organization was ascertained involved use of the so-called cowpea strain of TMV ($C_c$). This tobamovirus, which is very distinct in nucleotide sequence from the common strain of TMV (also variously called *vulgare* or U1) is now known as sunnhemp mosaic tobamovirus. Sunnhemp mosaic virus has the same genome organization as the common strain, but unlike the common strain, its recognition site for initiation of encapsidation by CP is in the CP gene (Fukada *et al.* 1980) and thus the CP mRNA is encapsidated separately in easy-to-isolate short rods. My laboratory (Bruening *et al.* 1976; Beachy & Zaitlin 1977), and that of Paul Whitfeld in Canberra (Higgins *et al.* 1976) were working on this problem independently. I was editor of *Virology* at that time, and was surprised to receive a letter from Whitfeld, with whom I had worked earlier, telling me about his intention to submit his study for publication in *Virology*. Thus, it was easy for me to arrange to have the two studies published together in the same issue. Both groups had isolated RNA from the full-length and the short rods, and ascertained that only RNA from the short rods could translate CP. Bruening *et al.* (1976)

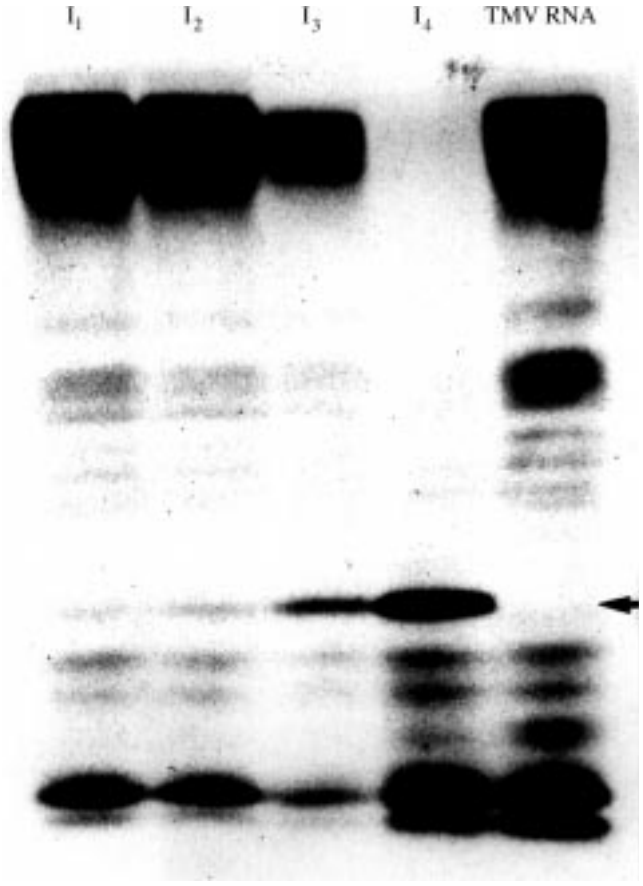


Figure 3. *In vitro* translation of different size classes of RNA extracted from TMV-infected leaves, ranging from the largest class (I1) to the smallest (I4). The CP translation product (indicated by the arrow) is seen predominantly in the smallest fractions, and is not found at all in the translation products of TMV RNA extracted from virions (right lane). Photo provided by Tony Hunter for data used in Hunter *et al.* (1976).

also demonstrated that the *in vitro*-produced CP was functional, in that it could be assembled with TMV RNA to reconstitute infectious virions. Higgins *et al.* (1976) found that RNA isolated from the longer rods could be translated, generating a large spectrum of proteins of various sizes, but not CP. In addition, Bruening *et al.* (1976) isolated RNAs from sucrose gradient-generated fractions containing viral rods of various lengths and determined that one rod of intermediate length contained RNA (a subgenomic, termed I2 RNA) which encoded a 30-kDa protein, later shown to be the movement protein.

In subsequent studies, Beachy *et al.* (1976) and Beachy & Zaitlin (1977) explored the properties of the subgenomic RNAs and found that there was yet another subgenomic RNA, the I1 RNA, which was the mRNA for the mysterious 54-kDa protein (discussed below). Later we revisited the subgenomic RNA question (Palukaitis *et al.* 1983) because of confusion in several studies concerning the number of such RNAs, and double-stranded RNAs, found in TMV-infected tissue. Without going into details, we concluded that there were artefacts created in the hybridization experiments, whereby fragments of viral RNA stack up and run together with ribosomal RNAs in polyacrylamide gels, giving an artefactual hybridization with a viral RNA probe. We concluded that there were four subgenomic polyribosome-associated RNAs and four corresponding double-stranded RNAs. Our 1985 map of the genome (Sulzinski *et al.* 1985) is given as figure 4.

When we discovered that TMV encoded a 30-kDa protein, I asked my graduate student Alain Asselin to see if it was a constituent of the virion. Alain devised a protocol to get highly purified virions, disrupted them with glacial acetic acid and then analysed the proteins by polyacrylamide gel electrophoresis. He did not find a 30-kDa protein in the virions but did show that virions had about one copy per virion of another protein, which he termed H-protein because we considered it to be of host origin (Asselin & Zaitlin 1978). Many years later, following the efforts of a number of people in independent studies, we discovered that H-protein was a fusion protein consisting of CP linked by an isopeptide bond to the host-encoded protein, ubiquitin, a first for virology (Dunigan *et al.* 1988). Its role in the TMV infection–replication process, if it has one, was never uncovered. In more recent (unpublished) studies we concluded that virions were ubiquitinated after assembly and that the free CP pool was not ubiquitinated.

The mechanism of the genesis of the subgenomic mRNAs of TMV has never actually been elucidated. Several postulates were put forth by Siegel *et al.* (1976) for the CP mRNA (the LMC RNA), i.e. that it was 'split from a strand of viral RNA or it may be an incomplete transcript of a complementary RNA strand'. Probably neither is correct. Miller *et al.* (1985) demonstrated very elegantly with brome mosaic virus that the subgenomic RNA4, the CP mRNA, was derived from RNA3 by internal initiation on the (−) strand of RNA3. Thus, we can analogize that the TMV subgenomic RNAs are generated in a similar manner, i.e. by internal initiations on the full-length genomic RNA (−) strand.

## 4. REPLICASE

High molecular weight proteins were found to be associated with TMV infections in the early 1970s, as described above. Pelham (1978) discovered that the 126-kDa and 183-kDa proteins (he termed them 110K and 160K) were co-initiated but that there was a leaky UAG termination codon at the 3′-end of the 126-kDa open reading frame that led to occasional translation of the 183-kDa protein. Beier *et al.* (1984) subsequently demonstrated that plants contain suppressor Tyr-tRNAs that carry out this function. Attempts to purify the replicase enzyme, yielded an imperfect enzyme which would not respond to exogenous template RNA, but which did synthesize TMV RNAs; it contained the two high molecular weight proteins (Young *et al.* 1987). The recent preparation of a fully functional TMV replicase showed that it was comprised of the 126-kDa and the 183-kDa proteins, along with at least one protein of host origin (Osman & Buck 1997).

## 5. MOVEMENT PROTEIN

The notion that TMV RNA encoded a protein involved in the capacity to move the genome from cell to cell derives from the work of Nishiguchi *et al.* (1978). They

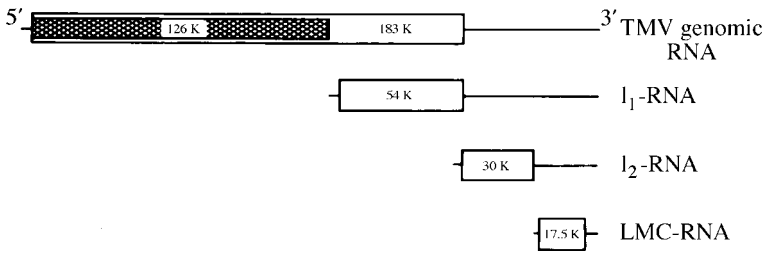


Figure 4. 'Genetic' map of the genome of tobacco mosaic virus given in Sulzinski *et al*. (1985). The narrow lines represent RNAs and the boxes the open reading frames for the virus-encoded proteins.

isolated a temperature-sensitive mutant of the tomato strain of TMV (Ls-1) which, at restrictive temperatures, replicates and assembles normally in epidermal cells of inoculated leaves and in leaf protoplasts inoculated *in vitro*, but does not move from cell to cell in inoculated leaves. Later, Leonard & Zaitlin (1982) demonstrated by *in vitro* translation and peptide mapping from a subgenomic TMV RNA population, that the 30-kDa protein of the Ls-1 isolate was slightly modified, suggesting that it was the 'movement protein'. It was subsequently shown by Ohno *et al*. (1983) that the Ls-1 30-kDa protein had a single amino acid change from that of the parent strain L: a serine was substituted for a proline. Ultimate proof that the 30-kDa protein was the movement protein was provided by Deom *et al*. (1987) who demonstrated that plants transformed with the 30-kDa protein complemented the temperature-sensitive defect in Ls-1.

## 6. THE ELUSIVE 54-KDA PROTEIN

There is one open reading frame in the TMV genome in which the function of its protein product (if any) remains an unresolved mystery. This is the 54-kDa open reading frame in the read-through portion at the 3′-terminus of the 126-kDa replicase gene, thus coincident with the 3′-portion of the 183-kDa gene. There is an AUG start codon for the 54-kDa open reading frame at residues 3495–3497 and an internal sequence encoding the Gly-Asp-Asp (GDD) motif, characteristic of RNA replicases (polymerases). However, in many (unpublished) frustrating studies over a number of years, I and a number of associates have been unable to find the protein in infected leaf tissues or isolated protoplasts. Moreover, recent studies of the purified replicase enzyme from the tomato strain (L) of tobacco mosaic virus contained no such protein (Osman & Buck 1997).

In spite of these observations, I feel that the 54-kDa gene product must have some real function in the TMV replication cycle. My reasoning stems from the observation that there is a subgenomic mRNA (the $I_1$-RNA) from which a 54-kDa protein can be generated by *in vitro* translation (Sulzinski *et al*. 1985), and further, that this RNA is associated with polyribosomes in infected tobacco leaves, implying the translation of a 54-kDa protein in infected tissues (Sulzinski *et al*. 1985).

Happily, some very valuable results did come from the studies in which we sought a role for the 54-kDa protein. The cloned gene was transformed into tobacco plants with the ultimate aim to see if the transgene could complement TMV mutants with a defective 54-kDa sequence. Complementation studies could never be done, however, because we found that the transformed plants were highly resistant to infection by TMV (Golemboski *et al*. 1990). This led to the very useful virus-resistance phenomenon now known as 'replicase-mediated resistance', recently reviewed by Palukaitis & Zaitlin (1997).

## 7. STILL ANOTHER PROTEIN?

Morozov and his colleagues (1993) examined the sequences of several tobamoviruses and found an open reading frame (ORF-X), which began near the 3′-end of the movement protein gene and extended into the CP gene. They cloned this sequence and translated a transcript *in vitro*, yielding a protein of about 4-kDa. Interestingly, this protein associated with a complex which was derived from the *in vitro* translation system, which they later (Fedorkin *et al*. 1995) identified as the elongation factor EF-1-α. They did not, to my knowledge, seek this protein in diseased tissues, nor did they seek a subgenomic mRNA, so its role (if any) in the TMV replication cycle is unresolved.

## 8. THE ULTIMATE CONFIRMATION OF THE GENOME ORGANIZATION

When the RNA of the *vulgare* isolate of TMV was sequenced in its entirety (Goelet *et al*. 1982), the exact locations of the translational starts of the proteins and the sites of the initiation of the subgenomic RNAs were determined with precision. The RNA, 6395 nucleotides long, is capped by $m^7G^{5'}ppp^{5'}Gp$ (Zimmern 1975), has a 5′ untranslated region of 68 nucleotides (part of the Ω fragment), followed in order by the 126-/183-kDa replicase genes, the 30-kDa movement protein gene, the 17.5-kDa CP gene and an untranslated region of 204 nucleotides at the 3′-end of the molecule. We now know the precise sites for the initiation of the subgenomic RNAs, for the region of encapsidation, and of the initiation and termination of translation of the proteins. The 126-/183-kDa proteins initiate at nucleotide 69, terminate at UAG residues 3417–3419, or after read-through to produce the 183-kDa protein which terminates by a UAA at residues 4917–4919. The 54-kDa gene initiates at residue 3495. The 30 kDa gene (in a different reading frame) begins at residue 4903, overlapping the 54-kDa and 183-kDa genes by 14 nucleotides. The CP gene begins at residue 5712 and ends at residue 6191.

The subgenomic mRNA for the elusive 54-kDa protein (I1-RNA) initiates at nucleotide 3405; that of the 30-kDa movement protein ($I_2$-RNA) at 4838, and that of the CP (LMC RNA) at 5703. The site of initiation of CP assembly on the RNA is within the 30-kDa gene of the common strain (Zimmern 1977), whereas for the cowpea strain (sunnhemp mosaic virus) it is in the CP gene, thereby explaining how the CP mRNA comes to be encapsidated to form a short rod in that tobamovirus, but not in most others.

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