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THE ROYAL SOCIETY

Replication of tobacco mosaic virus RNA

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The replication of tobacco mosaic virus (TMV) RNA involves synthesis of a negative-strand RNA using the genomic positive-strand RNA as a template, followed by the synthesis of positive-strand RNA on the negative-strand RNA templates. Intermediates of replication isolated from infected cells include completely double-stranded RNA (replicative form) and partly double-stranded and partly single-stranded RNA (replicative intermediate), but it is not known whether these structures are double-stranded or largely single-stranded *in vivo*. The synthesis of negative strands ceases before that of positive strands, and positive and negative strands may be synthesized by two different polymerases. The genomic-length negative strand also serves as a template for the synthesis of subgenomic mRNAs for the virus movement and coat proteins. Both the virus-encoded 126-kDa protein, which has amino-acid sequence motifs typical of methyltransferases and helicases, and the 183-kDa protein, which has additional motifs characteristic of RNA-dependent RNA polymerases, are required for efficient TMV RNA replication. Purified TMV RNA polymerase also contains a host protein serologically related to the RNA-binding subunit of the yeast translational initiation factor, eIF3. Study of *Arabidopsis* mutants defective in RNA replication indicates that at least two host proteins are needed for TMV RNA replication. The tomato resistance gene *Tm-1* may also encode a mutant form of a host protein component of the TMV replicase. TMV replicase complexes are located on the endoplasmic reticulum in close association with the cytoskeleton in cytoplasmic bodies called viroplasms, which mature to produce 'X bodies'. Viroplasms are sites of both RNA replication and protein synthesis, and may provide compartments in which the various stages of the virus mutiplication cycle (protein synthesis, RNA replication, virus movement, encapsidation) are localized and coordinated. Membranes may also be important for the configuration of the replicase with respect to initiation of RNA synthesis, and synthesis and release of progeny single-stranded RNA.

Keywords: tobacco mosaic virus; replication; RNA-dependent RNA polymerase; host protein; endoplasmic reticulum

1. MECHANISM OF TOBACCO MOSAIC VIRUS (TMV) RNA REPLICATION

The replication of TMV RNA, like that of other positive-stranded RNA viruses, consists of synthesis of a complementary, negative strand using the positive strand as a template, followed by synthesis of progeny positive strands using the negative strand as a template. The negative strand will also act as a template for the synthesis of subgenomic mRNAs for the movement protein and the coat protein. During the early stages of replication, it is likely that progeny positive strands, as well as acting as translational templates for synthesis of virus-encoded replication proteins, also serve as templates for amplification of the pool of negative strands. In tobacco protoplasts, synthesis of TMV negative strands ceases 6–8 h after inoculation, whereas synthesis of positive strands continues for 16–18 h after inoculation (Ishikawa *et al.* 1991*a*). Later in the replication cycle, after synthesis of negative strands has ceased and much coat protein has been synthesized, most of the progeny positive strands become encapsidated to form virions (Aoki & Takebe 1975; Palukaitis *et al.* 1983).

Virus-specific RNA isolated from TMV-infected plants contains, in addition to genomic- and subgenomic-length positive-strand RNA, genomic-length double-stranded RNA, designated replicative-form (RF) RNA and a heterogeneous family of RNAs, which are partly double-stranded and partly single-stranded, designated replicative-intermediate (RI) RNA (Nillson-Tillgren 1970; Jackson *et al.* 1971). Molecules resembling RF and RI have also been detected following TMV RNA synthesis *in vitro* using polymerase preparations from infected plants or protoplasts (Watanabe & Okada 1986; Young & Zaitlin 1986; Osman & Buck 1996). In RI RNA, the number of positive strands on average exceeds that of the negative strands (Aoki & Takebe 1975) and the structure of an RI molecule has been interpreted as a single negative strand to which are attached several positive strands of different lengths (figure 1). RF and RI RNAs are considered to be intermediates of RNA replication, or derived from intermediates of replication. An RF molecule could arise by initial synthesis of a negative strand on a positive-strand template. The negative strand of the RF could then act as a template for production of progeny positive strands, via RI intermediates. If only one initiation event per negative-strand template took place, an RI structure would have only one single-stranded (positive-strand) tail and an RF would be regenerated after the completion of synthesis of each positive strand. Multiple positive strands in RI structures would arise from repeated initiations of positive-strand synthesis on a negative-strand template,

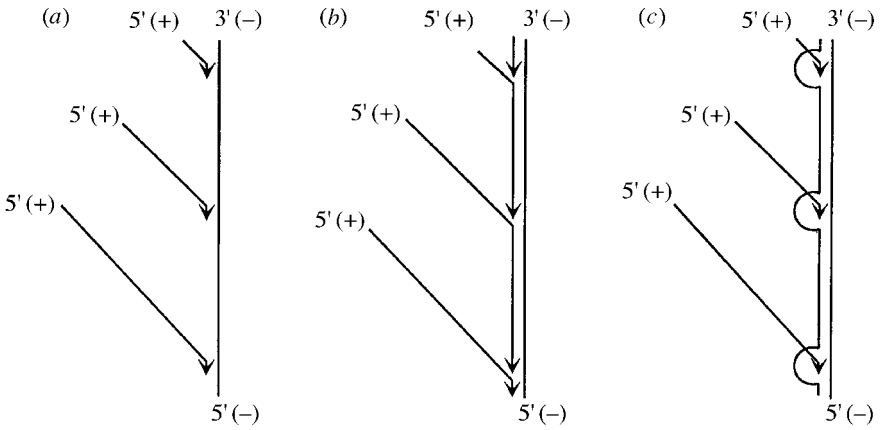

Figure 1. Possible structures of TMV replicative-intermediate (RI) RNA (for details see text).

before synthesis of the preceding progeny positive strand had been completed. Replication of bacteriophage Qβ positive-strand RNA has been shown to occur via predominantly single-stranded RNA intermediates and the purified replicase cannot use double-stranded RNA as a template (reviewed by Blumenthal & Carmichael 1979). However, for eukaryotic positive-stranded RNA viruses, it is uncertain whether completely double-stranded RNA RF is formed *in vivo* or is an artefact resulting from annealing of free positive and negative strands during extraction (reviewed by Buck 1986). Similarly RI RNA could exist *in vivo* in a predominantly single-stranded structure, as for bacteriophage Qβ (figure 1*a*), or in either of two possible double-stranded structures with single-stranded tails, one involving semi-conservative strand displacement (figure 1*b*) and the other involving a conservative mechanism in which the duplex RNA is only transiently unwound at the growing end of the nascent strands (figure 1*c*). During synthesis of TMV RF in *in vitro* systems with labelled NTP substrates, most of the label is found in the positive strand of the RF (Young & Zaitlin 1986; Osman & Buck 1996). This is inconsistent with a conservative mechanism (figure 1*c*), but is consistent with either a semi-conservative strand-displacement mechanism (figure 1*b*) or formation of RF by annealing of free (+) and (−) strands.

Double-stranded RNAs corresponding in size to subgenomic mRNAs have also been isolated from TMV-infected plants (Palukaitis *et al*. 1983). There is no evidence that subgenomic RNAs are self-replicating. TMV subgenomic RNAs are likely to be synthesized by internal initiation on genome-length negative-strand RNA templates, which requires subgenomic promoters containing sequences not found in the subgenomic RNA itself, as shown for brome mosaic virus (Miller *et al*. 1985; Marsh *et al*. 1988; Siegel *et al*. 1997; Adkins *et al*. 1998). Subgenomic double-stranded RNAs (Goelet & Karn 1982) are therefore probably dead-end products formed as a result of synthesis of a negative strand on a subgenomic positive-strand RNA template.

2. VIRUS-ENCODED PROTEINS NEEDED FOR TMV RNA REPLICATION

Several lines of evidence indicate that both the TMV-encoded 126-kDa and 183-kDa proteins are required for maximum efficiency of TMV RNA replication. A mutant engineered to produce the 126-kDa protein, but not the 183-kDa protein, did not replicate. A mutant in which the amber UAG stop codon at the end of the open reading frame (ORF) for the 126-kDa protein had been changed to a tyrosine codon (UAU), so that only the 183-kDa protein was produced, replicated in tobacco protoplasts about 20% as efficiently as the wild-type virus. In plants, replication of the mutant was also initially less efficient than that of the wild-type virus. However, at a later stage of infection, the UAU tyrosine codon mutated to an ochre UAA stop codon, allowing synthesis of both the 126-kDa and 183-kDa proteins and more efficient replication (Ishikawa *et al*. 1986).

Both the 126-kDa and 183-kDa proteins were detected in highly purified TMV RNA polymerase preparations by Western blotting, and antibody-linked polymerase assays showed that active TMV RNA polymerase bound to antibodies against the 126-kDa protein (Osman & Buck 1997). Furthermore, antibodies to the N- or C-terminal portions of the 126-kDa protein, or to the read-through portion of the 183-kDa protein, inhibited the initiation of TMV RNA synthesis *in vitro* by a template-dependent, membrane bound RNA polymerase (Osman & Buck 1996).

The read-through portion of the 183-kDa protein contains amino-acid motifs characteristic of RNA-dependent RNA polymerases (RdRps) and hence the 183-kDa protein is likely to provide the catalytic activity for the synthesis of TMV RNA from NTP substrates. Koonin (1991) used eight such motifs to propose evolutionary relationships between RdRps of diverse positive-stranded RNA viruses. Structural analysis of poliovirus RdRp has shown that some of these motifs are clustered round the catalytically active site of the enzyme (Hansen *et al*. 1997) and has enabled comparisons with the structures of DNA-dependent DNA polymerases, DNA-dependent RNA polymerases and RNA-dependent DNA polymerases to be made. Although differences in structure exist, all these polymerases are folded into broadly similar 'hand' structures, consisting of palm, finger and thumb domains, with a deep cleft in which the template, the 3′-end of the nascent strand and NTP substrates are located. Two of the motifs, which are conserved in these four different types of polymerase (Delarue *et al*. 1990), contain aspartic-acid residues which chelate the Mg^{2+} ions essential for polymerase activity. Although the three-dimensional (3D) structure of the TMV RNA polymerase has not yet been determined, the

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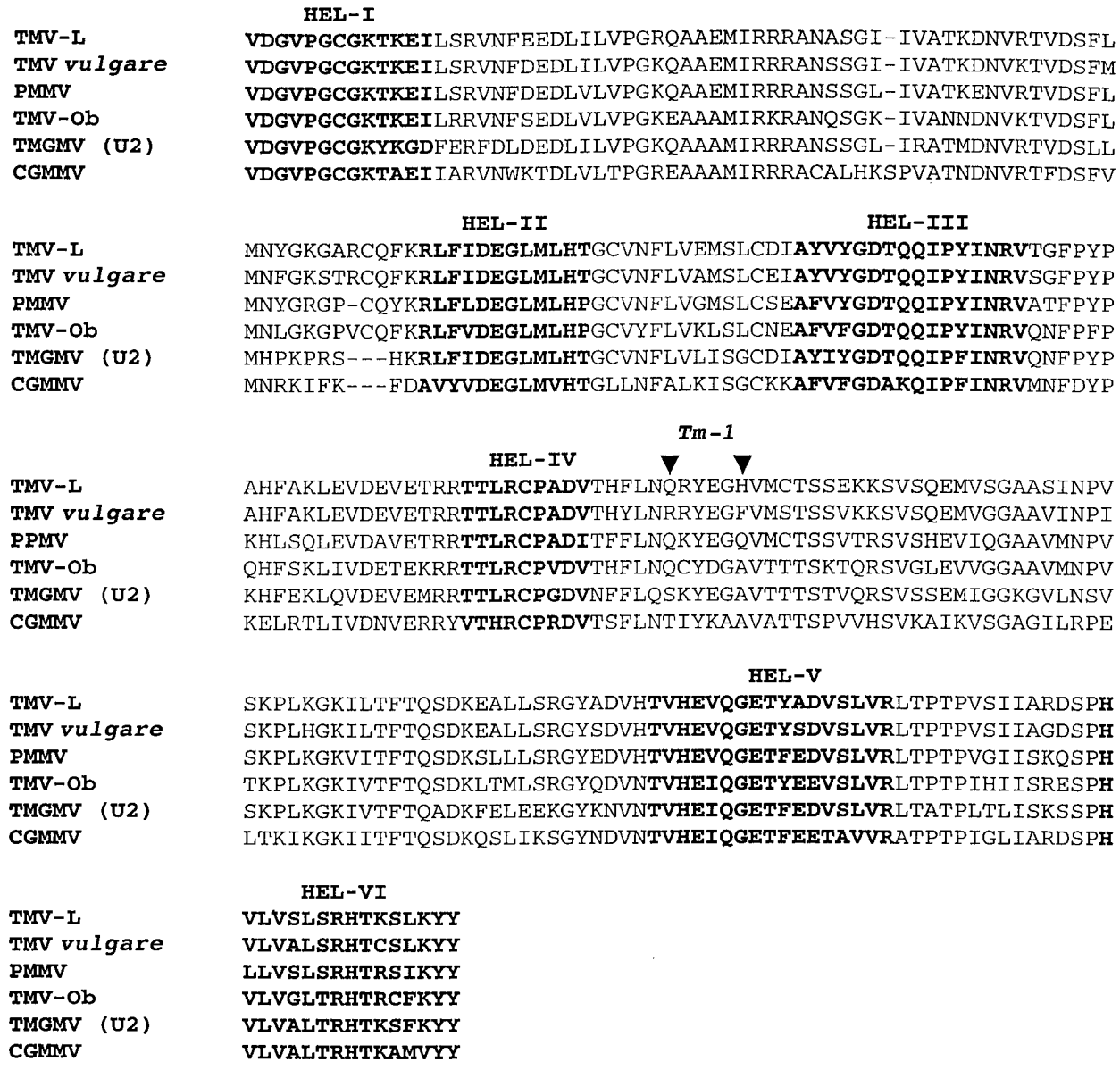

Figure 2. Amino-acid sequences of helicase-like regions encoded by tobamovirus RNAs. Amino-acid sequence motifs HEL-I to HEL-VI conserved in helicase superfamilies 1 and 2 (Koonin & Dolja 1993) are shown in boldface. The positions of mutations in *Tm-1* resistance-breaking strains are indicated by arrowheads. Database accession numbers for the sequence data are: TMV-L (tomato strain), P03587; TMV *vulgare*, P03586; PMMV (pepper mild mottle virus), P29098; TMV-Ob (*N* gene resistance-breaking strain), L11665; TMGMV (tobacco mild green mosaic virus), P18339; CGMMV (cucumber green mottle mosaic virus), P19523. The helicase region of TMV-L extends from amino acids 831 to 1084.

evolutionary relationships demonstrated between the different types of polymerases suggest that it also is likely to be folded into a 'hand' structure.

A subgenomic RNA corresponding to an ORF for a putative 54-kDa protein in the read-through portion of the 183-kDa protein, which contains the RdRp domain, has been detected in RNA isolated from TMV-infected tobacco plants (Sulzinski *et al.* 1985). Translation of encapsidated TMV RNAs gave a 54 kDa-protein which could be immunoprecipitated by antibodies to the 183-kDa protein, but not by antibodies to the 126-kDa protein (Turner *et al.* 1987). However, the 54-kDa protein has not been detected in extracts of TMV-infected or transgenic plants (Carr *et al.* 1992) or in purified TMV RNA polymerase preparations (Osman & Buck 1996, 1997). The putative 54-kDa protein does not appear to be essential for TMV RNA replication, but if the protein is produced *in vivo* and has a short half-life, a regulatory role would be possible.

The TMV 126-kDa protein contains two domains. An N-terminal domain with amino-acid motifs and predicted secondary structure typical of S-adenosylmethionine binding proteins, methyltransferases and guanylyltransferases (Koonin & Dolja 1993; O'Reilly *et al.* 1998) is probably required for synthesis of the 5′ m^7GpppG cap structure. The TMV 126-kDa protein has been shown to have guanylyltransferase activity (Dunigan & Zaitlin 1990). 5′-cap structures increase stability of mRNAs by protecting them from degradation by 5′ exonucleases and are required for cap-dependent translation. There is no direct evidence that the synthesis of the TMV 5′ cap plays a role in RNA replication, but *in vitro* mutagenesis of

other positive-strand RNA viruses suggests that the capping domain is important for replication (Wang *et al*. 1996; Ahola *et al*. 1997; Khromykh *et al*. 1998; O'Reilly *et al*. 1998). It is noteworthy that the coat protein subgenomic RNA, like the genomic RNA, is capped (Keith & Fraenkel-Conrat 1975; Zimmern 1975; Guilley *et al*. 1979), whereas indirect evidence (analogue inhibitor studies) suggests that the movement protein subgenomic RNA may not be capped (Hunter *et al*. 1983; Joshi *et al*. 1983).

The C-terminal domain of the TMV 126-kDa protein is helicase-like. Although helicase activity has not yet been demonstrated for the 126-kDa protein of any tobamovirus, these proteins contain six amino-acid motifs (figure 2), which are highly conserved in known helicases, such as the translational initiation factor eIF-4A (Koonin & Dolja 1993). Motifs I and II are variants of motifs found in a wide variety of ATP- and GTP-binding proteins and are involved in ATP binding, chelation of Mg^{2+} ions and helicase-coupled ATP hydrolysis. ATP binding has been demonstrated for the TMV 126-kDa protein (Evans *et al*. 1985). Motif VI is involved in RNA binding (reviewed by Buck 1996). Helicase activity is likely to be essential for TMV RNA replication to unwind the duplex RNA formed during RNA synthesis, whether replication occurs via mainly single-stranded RNA intermediates, in which case duplex formation occurs transitorily at the replication fork (figure 1*a*), or whether it requires unwinding of completely double-stranded RF molecules (figure 1*b*). Helicase activity would also be needed to remove secondary structure in single-stranded RNA templates to allow replication to take place. A single amino-acid change (S643F) upstream of the conserved helicase motifs was sufficient to reduce TMV RNA replication and produce a symptomless phenotype (Lewandowski & Dawson 1993), but whether this mutation affects helicase function is not known.

Both the 126-kDa and 183-kDa proteins potentially have capping and helicase activity. The requirement of both proteins for maximum replication suggests that the two different helicase functions may lie in different proteins. It is noteworthy that replication of the RNA of potato virus X (PVX), an evolutionarily related virus in the alpha-like virus supergroup, requires only one virus-encoded protein, P1 (equivalent to the TMV 183-kDa protein), which contains both helicase-like and polymerase-like domains (Longstaff *et al*. 1993). However, it is possible that the PVX P1 protein functions as a dimer, as do reverse transcriptases (Jacobo-Molina *et al*. 1993) and *Escherichia coli* DNA polymerase III holoenzyme (Yuzhakov *et al*. 1996). O'Reilly *et al*. (1998) have observed an intermolecular interaction between two protein 1a molecules of brome mosaic virus (BMV), another alpha-like virus, involving the N-terminal capping domain. It was proposed that the BMV replicase contains two copies of the 1a protein (the counterpart of the TMV 126-kDa protein) together with the 2a protein (the counterpart of the polymerase domain of the TMV 183-kDa protein). The TMV replicase could therefore have a similar structural organization to the BMV replicase, in which the 126-kDa and 183-kDa proteins are bound together via their N-terminal capping domains.

In tobacco protoplasts, TMV mutants with the coat protein or movement-protein genes deleted replicate as well as the wild-type virus, indicating that neither of these genes is absolutely required for TMV RNA replication (Meshi *et al*. 1987; Takamatsu *et al*. 1987). However it is likely that in infected plants, viral RNA replication is closely coordinated with cell-to-cell movement and RNA encapsidation. The recent demonstration that the TMV movement protein is found associated with replication bodies (Heinlein *et al*. 1998) *in vivo* does not necessarily imply a direct role for the movement protein in virus replication, but suggests that viral RNA synthesis, viral protein synthesis and the assembly of movement complexes take place in close proximity in replication bodies in a highly coordinated and interactive fashion. The TMV movement protein binds to single-stranded RNA in a sequence-non-specific fashion (Citovsky *et al*. 1990, 1992), so it is possible that interaction of the movement protein with the replicase complex could confer some specificity on the movement process. Association between RNA replication and movement is also suggested by the finding that some mutations in the capping domain (O'Reilly *et al*. 1998) of the 126-kDa and 183-kDa proteins of the masked strain of TMV affect phloem-dependent virus accumulation (Derrick *et al*. 1997), and by the observation that viral RNA trafficking is inhibited in replicase-mediated resistant transgenic tobacco plants (Nguyen *et al*. 1996).

3. HOST PROTEINS INVOLVED IN TMV RNA REPLICATION

The notion that replication of eukaryotic positive-stranded RNA viruses may require host, as well as viral, proteins originally stemmed from the discovery that bacteriophage Qβ RNA polymerase holoenzyme contains four host proteins, as well as the phage-encoded core polymerase subunit (table 1). Two of the functions of these host proteins, which are all ribosome-associated or translation factors, are (i) to bind at an internal site in the viral RNA to prevent initiation of protein synthesis, which would proceed on the template in the opposite direction to RNA synthesis, and (ii) to bring the RNA polymerase close to the 3′-end of the RNA to enable initiation of RNA synthesis to take place (Blumenthal & Carmichael 1979; Miranda *et al*. 1997). It is likely that host proteins are also important for replication of both positive-stranded and negative-stranded RNA viruses in eukaryotes. In general, isolated catalytic subunits, e.g. the poliovirus 3D polymerase subunit (Neufeld *et al*. 1991) or the hepatitis C NS5B polymerase subunit (Lohman *et al*. 1997), can elongate chains on a variety of templates *in vitro*, but cannot initiate RNA synthesis, a function which requires additional host and/or viral proteins. In contrast, isolated polymerase holoenzymes, which contain both virus-encoded and host-encoded proteins, such as those of BMV (Quadt *et al*. 1993), cucumber mosaic virus (Hayes & Buck 1990) and TMV (Osman & Buck 1996), are able to initiate RNA synthesis *in vitro* in the absence of a primer and show template specificity. Lai (1998) has suggested that RNA-dependent RNA polymerases may be similar to DNA-dependent RNA polymerases in that the core polymerases lack template-specificity, the ability to bind to and initiate RNA synthesis at specific promoters being provided by ancillary proteins (transcription

Table 1. *Host proteins required for replication of bacteriophage Qβ RNA*

host protein	function in phage RNA replication	cellular function
elongation factor EF-Ts	not known, but probably functions as a complex with EF-Tu	forms a complex with EF-Tu.GDP, resulting in the release of GDP. GTP then binds to EF-Tu and releases EF-Ts
elongation factor EF-Tu	EF-Tu.GTP complex binds to sequences near the 3′-end of negative-strand RNA templates	EF-Tu.GTP complex binds to amino-acyl-tRNA and delivers it to the A site on the ribosome. GTP is then hydrolysed
ribosomal protein S1	binds to internal sites (S and M) in Qβ RNA. Binding to S site blocks ribosome binding and translation of coat protein cistron. Binding to M site is essential for replication. Essential for synthesis of negative strands on positive-strand template, but not for synthesis of positive strands on negative-strand template	binding of 30S ribosomal subunits to mRNA. Possible helix-destabilizing function in translation
host factor HF-1	binds to an internal site and the 3′-end of Qβ RNA. Brings the phage-encoded polymerase subunit close to the 3′-end of Qβ RNA. Essential for synthesis of negative strands on positive-strand template, but not for synthesis of positive strands on negative-strand template	loosely bound to ribosomes. RNA-binding protein. Required for translation of ρ^S

factors). Many different host proteins have been invoked to be involved in eukaryotic virus RNA replication on the basis of specific binding to 5′ or 3′ untranslated regions of (+) or (−) RNA, requirement for *in vitro* transcription, binding to catalytic polymerase subunits or copurification with RdRps (table 2). The most compelling evidence for a role of host proteins comes from the use of host mutants deficient in the ability to support virus RNA replication. Ishikawa *et al.* (1997) showed that yeast mutations in multiple complementation groups inhibit BMV RNA replication, implying the need for several host proteins for the replication of this virus in yeast.

An early step in the multiplication of TMV is sensitive to actinomycin D, showing that host transcription and possible synthesis of a host protein are required (Dawson & Schlegel 1976; Dawson 1978). However, as inoculations were carried out with virus particles in these experiments, it is not known if the actinomycin D-sensitive step was involved in uncoating of the particles or an early step in viral RNA replication. Uncoating of particles is known to be bidirectional with 5′→3′ uncoating assisted by ribosomes in a cotranslational disassembly process and the later 3′→5′ uncoating probably assisted by the 126-kDa or 183-kDa replication proteins (Wu & Shaw 1996, 1997). It is possible that either of these stages could require host RNA and protein synthesis.

Arabidopsis mutants have been obtained in which accumulation of TMV RNA was reduced to low levels in both plants and protoplasts compared to the wild-type (Ishikawa *et al.* 1993; Ohshima *et al.* 1998). Two distinct mutants had unlinked, single, recessive mutations, designated *tom-1* and *tom-2* respectively, indicating that at least two different host proteins are needed for replication of TMV in *Arabidopsis*. Neither of these mutations affected the replication of cucumber mosaic virus, turnip crinkle virus or turnip yellow mosaic virus. Tentative evidence was also obtained for another host gene required for replication of both TMV and cucumber mosaic virus, but not for replication of turnip crinkle virus.

A highly purified TMV RNA polymerase, isolated from infected tomato plants by differential and density gradient centrifugation, solubilization and anion exchange chromatography on columns of DEAE-Biogel and High Q, was found to contain, in addition to the virus-encoded 126-kDa and 183-kDa proteins, three host proteins of 56, 54 and 50 kDa (Osman & Buck 1997). The 56-kDa protein was found to cross-react with antibodies to translation initiation factor eIF3 from yeast and from wheat germ. Yeast eIF3 contains subunits of 115, 90, 55, 39, 33, 29, 21 and 16 kDa (Asano *et al.* 1997), whereas wheat-germ eIF3 contains subunits of 116, 107, 87, 83, 56, 45, 41, 36, 34 and 26 kDa (Heufler *et al.* 1988). Sequences of some of the yeast subunits have been obtained, but no sequence data on the wheat eIF3 subunits are available and the correspondence between yeast and wheatgerm subunits is not known. Further work showed that the 56-kDa protein in the TMV RNA polymerase preparation cross-reacted specifically with antibodies to the yeast 55-kDa (GCD10) subunit. These antibodies also reacted specifically with a 56-kDa protein in wheatgerm extract, suggesting that the yeast eIF3 GCD10 protein and the 56-kDa subunit of wheat eIF3 are related (Osman & Buck 1997).

A number of lines of evidence indicate that the 56-kDa GCD10-related host protein in the purified TMV RNA polymerase is a genuine component of the polymerase holoenzyme: (i) the protein was not detected in an equivalent fraction prepared from uninfected tomato leaves; (ii) the 183-, 126- and 56-kDa proteins were co-selected by immunoaffinity chromatography using antibodies to either the 126-kDa protein or to the yeast GCD10 protein, showing that these three proteins are part of a complex; (iii) antibody-linked polymerase assays showed that the active TMV polymerase bound

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Table 2. *Eukaryotic cellular proteins invoked to be involved in viral RNA-dependent RNA synthesis*[a]

protein	virus	evidence
actin	human parainfluenza 3	required for *in vitro* transcription
calreticulin	rubella	binds to 3′-NTR (+)
EF-1α	polio	fragment binds to 5′-NTR (+)
	turnip yellow mosaic	binds to 3′-NTR (+)
	vesicular stomatitis	associated with RdRp
	West Nile	binds to 3′-NTR (+)
eIF-3 p45 subunit	brome mosaic	associated with RdRp
GPDH	hepatitis A	binds to 5′-NTR (+)
heat shock protein	canine distemper	stimulates polymerase activity *in vitro*
HnRNP A1	mouse hepatitis	binds to 5′ (-) and 3′ (+) leaders; IG (−)
la (lupus autoantigen)	Sindbis	binds to 3′-NTR (−)
MAB1, MAB2, MAB3	brome mosaic	yeast mutations
nucleolin	polio	binds to 3′-NTR (+)
poly-(rC) binding proteins	polio	binds to 5′-NTR (+)
Sam68	polio	binds to $3D^{pol;}$ coimmunoprecipation
tubulin	respiratory syncytial, Sendai, vesicular stomatitis	required for *in vitro* transcription; antibody inhibition

[a] Data from Lai (1998) and references cited therein; Waggoner & Sarnow 1998.

to antibodies to the GCD10 protein as well as to antibodies to the 126-kDa protein; (iv) antibodies to the GCD10 protein inhibited the synthesis of single-stranded and double-stranded TMV RNA by a membrane-bound, template-dependent RNA polymerase *in vitro* (Osman & Buck 1997).

The cellular functions of eIF3 in the initiation of protein synthesis include (i) binding to 40S ribosomal subunits and dissociating 80S ribosomes into 40S and 60S subunits; (ii) prevention of dissociation of the Met $tRNA_i$.eIF2.GTP ternary complex; (iii) mRNA binding to 40S ribosomal subunits partly by binding the eIF4G subunit of the cap-binding complex, eIF4F and partly by binding directly to mRNA and 18S rRNA (attributed to the GCD10 subunit which has been shown to have RNA-binding activity) (Garcia-Barrio *et al.* 1995; Hennig 1995). If the tomato GCD10-related protein also has RNA-binding activity, its function in the TMV replicase could be related to this activity. Antibodies to the GCD10 protein inhibited the replicase activity of a template-dependent TMV RNA polymerase *in vitro*, but had no effect on the activity of a polymerase preparation containing endogenous template RNA (Osman & Buck 1997). This suggests that the GCD10-related protein may have a role in initiation of RNA synthesis, possibly by binding to the viral RNA. Although the RNA-binding activity of the yeast GCD10 protein is not sequence-specific, it is possible that specificity could be conferred by interaction with the 126-kDa or 183-kDa proteins.

A host protein related to the 41-kDa subunit of wheatgerm eIF-3 was found to copurify with the BMV RdRp isolated from infected barley plants (Quadt *et al.* 1993). Furthermore, addition of wheatgerm eIF3 or its 41-kDa subunit to BMV RdRp increased synthesis of negative-strand RNA by a factor of three. The BMV RdRp did not cross-react with antibodies to the wheatgerm eIF3 56-kDa subunit and therefore its host protein component described above is not related to the yeast GCD10 protein. The TMV and BMV RNA polymerases contain host proteins that are related to different eIF3 subunits and probably have different functions in virus RNA synthesis.

It has been suggested that the product of the *Tm-1* resistance gene, which confers resistance to TMV in tomatoes, may be an altered form of a host protein involved in virus RNA replication (Meshi *et al.* 1988). The *Tm-1* gene inhibits the multiplication of many tobamoviruses, but is not effective against viruses in other genera, such as cucumber mosaic virus (Pelham 1966, 1972; Dawson *et al.* 1979; Watanabe *et al.* 1987*a*). The *Tm-1* gene is dominant and dose-dependent, inhibition of virus multiplication being greater in *Tm-1*/*Tm-1* tomato plants (homozygous for the *Tm-1* gene) than in *Tm-1*/+ plants (heterozygous for the *Tm-1* gene). Resistance is also expressed in protoplasts and suspension-culture cells, and is equally effective when either virus particles or viral RNA is used as inoculum or when a mutant lacking most of the movement-protein and coat protein genes is used (Yamafuji *et al.* 1991). Synthesis of all virus-specific genomic and subgenomic RNAs and virus-encoded proteins (126-kDa and 183-kDa replication proteins, the 30-kDa movement protein and the coat protein) is almost completely blocked (*Tm-1*/*Tm-1*) or greatly reduced (*Tm-1*/+). These results suggest that the *Tm-1* gene operates after virus uncoating at an early stage of RNA replication. The *Tm-1* locus has been mapped to the short arm of chromosome 2, close to a ribosomal RNA gene (Ohmori *et al.* 1996).

The *Tm-1* gene is not durable and was overcome by resistance-breaking strains of the virus within a year of its introduction into commercial tomato cultivars (Pelham *et al.* 1970). Ltal is a spontaneous mutant derived from a tomato strain of TMV, TMV-L. Unlike TMV-L, Ltal spread systemically and caused mosaic symptoms in *Tm-1*/*Tm-1* tomato plants (Watanabe *et al.* 1987*a*). The nucleotide sequence of Ltal RNA showed that it differed from that of TMV-L RNA at five positions (Meshi *et al.* 1988). Two of the mutations (in the ORFs for the 30-kDa movement protein and coat protein) were silent (i.e. did not change the amino

TMV strain/ mutant	amino acid position 979 984	tomato R	tomato S	tobacco
L	THFLN**Q**RYEG**H**VMCTSSEKKS	--	++	++
Lta1	*****E****Y**********	++	++	++
T2	**********Y**********	+	++	++
T3	*****E***************	++	++	++
TMV-1	**Y**E****F**S***V***	++	++	++
TLAsp	*****D***************	++	++	++
TLLys	*****K***************	++	++	++
TLAsn	*****N***************	+	++	++
TLArg	*****R***************	+	++	++
TLHys	*****H***************	--	++	++
TLIle	*****I***************	--	--	++

Figure 3. Effect of amino-acid changes in the TMV 126-kDa/183-kDa proteins on TMV replication in tomato and tobacco plants. R, tomato with *Tm-1*/*Tm-1* genotype; S, tomato with +/+ genotype. *, sequence identity to that of the TMV-L 126-kDa/183-kDa proteins. Amounts of virus multiplication are indicated as: (++), similar to L and Lta1 multiplication in susceptible hosts; (+), reduced accumulation; (−−), no detectable accumulation. Data from Meshi *et al.* 1988; Hamamoto *et al.* 1997*a*,*b*; B. J. Hirst, A. A. Brunt and K. W. Buck, unpublished results.

acid encoded) and a third, insertion of a U residue in the 5′-untranslated region, was found in other *Tm-1*-sensitive isolates. The other two mutations, a C→G transversion at nucleotide 3006 and a C→U transition at nucleotide 3021, resulted in amino-acid changes in the common region of the 126-kDa and 183-kDa proteins, glutamine (Q) to glutamic acid (E) at position 979 and histidine (H) to tyrosine (Y) at position 984 (figure 3).

In vitro mutagenesis of a full-length cDNA clone of TMV-L RNA was used to investigate the minimum amino-acid change(s) needed for *Tm-1* resistance breaking (Meshi *et al.* 1988). When inoculated on to *Tm-1*/*Tm-1* tomato plants, mutant T1, which differed from TMV-L RNA only at positions 979 (E) and 984 (Y) of the 126-kDa/183-kDa sequence, induced mosaic symptoms and amounts of progeny virus similar to those induced by Lta1. Furthermore, these two changes were stable in the 126-kDa/183-kDa sequence of the progeny virus and no further sequence changes were found. Another mutant, T3, which differed from TMV-L RNA only at position 979 (E) also replicated well in *Tm-1*/*Tm-1* tomato plants and the introduced mutation was stable, but additional amino-acid changes were found in the 126-kDa/183-kDa sequence of the progeny virus, which included H984Y in two of the progeny isolates but also H984E and other changes in other progeny isolates. A mutant T2, which differed from TMV-L at only position 984 (Y) of the 126-kDa/183-kDa sequence, was able to infect *Tm-1*/*Tm-1* tomato plants, but produced only weak mosaic symptoms with variable and lower amounts of progeny virus. Again the introduced mutation was stable, but additional amino-acid changes were found in the 126-kDa/183-kDa sequence of the progeny virus, which included Q979E in one of the progeny (Meshi *et al.* 1988). An English *Tm-1* resistance-breaking strain of TMV (TMV-1), isolated from a natural infection of a tomato crop, was also found to have an E residue at position 979 in the 126-kDa/183-kDa sequence, but had other amino acids in this region which differed from TMV-L, but which were the same as those of TMV *vulgare* including an F residue at position 984 (figure 3) (B. J. Hirst, A. A. Brunt and K. W. Buck, unpublished results). It therefore appears that position 979 is the most important in determining Tm-1 resistance breaking, but that other amino acids in this region also contribute. It was originally suggested that the amino-acid change Q979E caused resistance breaking by decreasing the local net charge of the of the 126-kDa/183-kDa proteins (Meshi *et al.* 1988). This has now been disproved by showing that mutations of Q979 to several other amino acids, including lysine (K), cause resistance breaking (figure 3) and the mutations were stable in the progeny virus isolated from *Tm-1*/*Tm-1* tomato plants (Hamamoto *et al.* 1997*a*). Interestingly, a mutant with a Q979I mutation was unable to replicate in either (+/+) or (*Tm-1*/*Tm-1*) tomato plants, although it replicated normally in tobacco (Hamamoto *et al.* 1997*b*). This is further evidence to support the hypothesis that compatible interactions between the 126-kDa and/or 183-kDa proteins and host factors are needed for productive replication of TMV RNA.

The mutations that occur in *Tm-1* resistance-breaking strains are located within the helicase-like region of the 126-kDa/183-kDa proteins close to motif IV (figure 2). If the Tm-1 protein is an altered form of a normal host component of the TMV replicase holoenzyme, it is therefore likely that this host component binds to the helicase-like region of the 126-kDa or 183-kDa protein. The Tm-1 protein might not be able to bind to the helicase-like region of non-resistance-breaking strains of TMV or might bind to this region, but be unable to function in the assembly of a functional replicase. TMV resistance-breaking strains are able to replicate as efficiently as non-resistance-breaking strains in tobacco and tomato (+/+) plants. This would imply that the helicase-like domains of the 126-kDa and/or 183-kDa proteins of resistance-breaking strains would need to be able to interact, not only with the Tm-1 protein, but also with its counterparts in tobacco and tomato (+/+ plants).

Two other hypotheses to explain *Tm-1* resistance need to be considered. The first is that the Tm-1 protein is not a host component of the TMV replicase, but interacts with the replicase to inactivate it or degrade it (Meshi *et al.* 1988). There is evidence that the *Tm-1* gene does not cause degradation of the 126-kDa or 183-kDa proteins (Yamafuji *et al.* 1991), but inactivation of the replicase by binding of the Tm-1 protein to the helicase-like domain cannot be excluded. In this hypothesis, the Tm-1 protein would bind to the helicase-like domain of non-resistance-breaking strains, but not to that of resistance-breaking strains.

A third hypothesis is that non-resistance-breaking TMV strains induce a host response in *Tm-1* tomato plants, which would result in a non-virus-specific inhibition of virus replication. Mutations in a strain of TMV (TMV-Ob) which determine the ability to overcome *N*-mediated resistance also map to (other parts of) the helicase-like region of the 126-kDa/183-kDa proteins (Padgett *et al.* 1997) and that both *N*-mediated and *Tm-1*-mediated resistance

are temperature sensitive. There are differences between *N*-gene- and *Tm-1*-gene-mediated resistance, in that the *N* gene induces a hypersensitive response in resistant plants, whereas the *Tm-1* gene does not, and the *Tm-1* gene is active in protoplasts, whereas the *N* gene is not. However, the potato *Rx* gene, which confers resistance to potato virus X and is similar to the *Tm-1* gene in giving an essentially null phenotype in resistant plants and being active also in protoplasts, apparently induces non-specific inhibition of virus replication, since co-inoculation of *Rx* protoplasts with potato virus X and cucumber mosaic virus results in the inhibition of replication of both viruses (Kohm *et al*. 1993). Cucumber mosaic virus can replicate in potato protoplasts lacking the *Rx* gene either alone or when co-inoculated together with potato virus X, and can replicate in *Rx* protoplasts in the absence of PVX. Evidence which argues against the induction of a non-specific host response by the *Tm-1* gene is as follows: (i) *Tm-1* resistance is not affected by actinomycin D and therefore host transcription is not needed for expression of resistance (Watanabe *et al*. 1987*a*); (ii) resistance in protoplasts is shown at an early stage after inoculation (Watanabe *et al*. 1987*a*); (iii) co-inoculation of *Tm-1*/*Tm-1* protoplasts with equal amounts of RNA of TMV-L and a resistance-breaking strain did not prevent replication of the resistance-breaking strain and did not allow replication of TMV-L (Yamafugi *et al*. 1991). Further work is needed to distinguish the three hypotheses unequivocally.

It has recently been found that protein synthesis elongation factor EF1α colocalizes with the TMV replication complex in tobacco leaves (Ding *et al*. 1998). EF1α is the eukaryotic counterpart of the prokaryotic translation elongation factor Tu, an essential component of Qβ replicase (q.v.). TMV replication bodies are found in close association with components of the cellular cytoskeleton, including actin and tubulin (Ding *et al*. 1998; Heinlein *et al*. 1998). EF1α has also been found to associate with a cytoskeletal network surrounding protein bodies in maize endosperm cells (Clore *et al*. 1996). The suggestion that EF1 may be important for virus replication was first made by Litvak *et al*. (1973), who showed that aminoacylated, but not uncharged, TMV RNA and turnip yellow mosaic virus RNA, bound unfractionated EF1αβγ. EF1 has been shown to bind to aminoacylated RNA of BMV (Bastin & Hall 1976). However, EF1 was found to be absent from a template-dependent turnip yellow mosaic virus RNA polymerase, and addition of EF1 did not stimulate polymerase activity (Joshi *et al*. 1986). No evidence has been found that EF1 is required for BMV replication (Duggal *et al*. 1994). More recently EF1α has been found to bind to the 3′ stem-loop region of West Nile virus RNA (Blackwell & Brinton 1997). Evidence has also been obtained that EF1 is associated with, and may be required for the activity of, the RNA polymerase of vesicular stomatitis virus (Das *et al*. 1998). The finding that EF1α colocalizes with the TMV replicase (Ding *et al*. 1998) does not prove that it is required for replication. Beachy & Zaitlin (1975) demonstrated that protein synthesis occurs in isolated replication complexes and suggested that protein synthesis can initiate on the 5′-ends of nascent RNA strands in replicative intermediates (figure 1) before synthesis of a positive strand has been completed, as also demonstrated for Semliki Forest virus (Friedman 1968) and bacteriophage R17 (Robertson 1975). Hence the high concentration of EF1α in replication complexes may merely reflect associated protein synthesis, although a role in replication cannot be discounted.

4. *CIS*-ACTING SEQUENCES REQUIRED FOR TMV RNA REPLICATION

The importance of the 3′-terminal region of TMV RNA has been shown by the demonstration that a mutant lacking the 3′-terminal 346 nucleotides is not infectious (Dawson *et al*. 1986). The 3′-untranslated region of TMV *vulgare* (approximately 200 nucleotides) can be folded into three major structural domains, connected by a central core. A 3′ structural domain mimics a tRNA acceptor branch, another is analogous to a tRNA anticodon branch and the third upstream domain contains three pseudoknots, each of which contains two double-helical segments (Van Belkum *et al*. 1985; Felden *et al*. 1996). Mutational analysis of the upstream pseudoknot domain (UPD) indicated that five out of the six double-helical domains were not essential for replication. However, deletion of double-helical segment I just upstream of the tRNA-like domains, or point mutations which destabilized the helix, resulted in loss of ability to replicate. Compensatory mutations which restored the double-helical structure also restored virus replication to near wild-type levels, indicating that the secondary structure, but not the primary structure, of double helical segment I is essential for replication (Takamatsu *et al*. 1990). The tRNA-like domain and UPD play roles in increasing RNA stability and in the regulation of translation, as well as acting as recognition sites for the virus RdRp. The TMV RNA 5′ cap structure and 3′ UPD interact to regulate translation (Leathers *et al*. 1993; Gallie & Kobayashi 1994). A 102-kDa RNA-binding protein that binds specifically to the poly(CAA) region within the 5′-untranslated leader and to the 3′ UPD, and is widely conserved in plant species, has been isolated from wheat, and evidence obtained that this binding is important for efficient translation (Tanguay & Gallie 1996).

The 3′-terminal tRNA-like structures of the common strains of TMV (*vulgare*, U1, OM) and other tobamoviruses, such as the tomato strain TMV-L and cucumber green mottle mosaic virus (CGMMV), are similar and all can be aminoacylated by the host histidyl-tRNA synthetase. The 3′-terminal region of the cowpea strain of TMV (TMV-Cc; also called sunnhemp mosaic virus) can be folded into a tRNA-like structure, which differs considerably from those of the other tobamoviruses, but which is similar to that of turnip yellow mosaic virus (TYMV), and like the latter, can be aminoacylated with valine. Chimaeras in which the 3′ non-coding region of TMV-L was replaced by those of TMV-OM, CGMMV or TMV-Cc were all able to replicate in tobacco protoplasts and plants, although the replication of the latter two chimeras was much reduced compared to that of TMV-L (Ishikawa *et al*. 1988). This suggests that recognition of the 3′ structure by the replicase has some flexibility. This notion is supported by the observation that a chimaera in which the 3′ non-translated region of TMV-L RNA had been replaced by the 3′ non-translated region of BMV RNA, which can be folded into a tRNA-like structure which

accepts tyrosine, could also replicate in tobacco protoplasts, though at very much lower efficiency than that of TMV-L (Ishikawa *et al*. 1991*b*). It is possible that the TMV replicase can recognize common structural features in these otherwise-divergent tRNA-like structures and that these structures may be modified by interaction with the replicase. Polymerases of other viruses apparently have more stringent requirements, since BMV RNA 3 derivatives carrying the 3′ non-coding region of TMV-L were not amplified in protoplasts when co-inoculated with BMV RNAs 1 and 2 (Ishikawa *et al*. 1991*b*). Similarly TYMV hybrids in which the 3′ tRNA-like structure had been replaced by that of TMV-U1 or TMV-Cc did not replicate to detectable levels in turnip protoplasts, although limited replication was observed in protoplasts, but not plants, if the hybrids included the TMV-U1 UPD (Skuzeski *et al*. 1996).

Whether aminoacylation of the TMV tRNA-like structures is required for replication is not known. Extensive experiments with BMV led to the conclusion that tyrosylation was not a requirement for replication (Duggal *et al*. 1994). TYMV RNA is known to be aminoacylated with valine *in vivo* and mutagenesis of its 3′ tRNA-like sequence indicated a good correlation between extent of aminoacylation *in vitro* and ability to replicate *in vivo*, although the nature of the amino acid appeared to be flexible and a mutant which could be aminoacylated with methionine, but not valine, was able to replicate (Dreher *et al*. 1996). Recently, however, TYMV hybrids have been obtained containing the TMV tRNA-like region and UPD, modified to introduce TYMV sequences into the anticodon loop and amino-acid acceptor arm. These gave rise to stable infections in plants. Some of the progeny virus isolates were highly infectious, but could not be detectably aminoacylated *in vitro* (Goodwin *et al*. 1997). It appears that aminoacylation is not an absolute requirement for replication of this virus.

Sequences in the 5′ untranslated region of TMV RNA have also been shown to be important for replication (Takamatsu *et al*. 1991). Deletion analysis of the 5′-terminal region of TMV-L RNA showed that large deletions (nucleotides 947 or 2571) reduced replication to undetectable levels, whereas of several approximately 10-nucleotide deletions across the whole of the 71-nucleotide untranslated region, only a deletion of nucleotides 2–8 abolished replication. The TMV 5′-untranslated region is a translational enhancer sequence (Gallie *et al*. 1987), but *in vitro* translation experiments indicated that the effects of 5′ deletions on replication were not primarily due to effects on translation (Takamatsu *et al*. 1991). The absence of effects of most small deletions, but inhibition by large deletions, suggests that there may be more than one binding site for a replication protein in the 5′ region; deletion of one binding site may have no effect, but deletion of all binding sites could abolish replication. However, the progeny of replication of some TMV subgenomic replicons in plants included a molecule with only 23 nucleotides at the 5′-terminus (Raffo & Dawson 1991).

The dispensability of genes for the 30-kDa movement protein and the coat protein for replication of TMV RNA in tobacco protoplasts was referred to earlier. TMV derivatives with most of the coding regions for the 126-kDa/183-kDa proteins removed also replicated well in protoplasts, and spread in plants, in the presence of a wild-type TMV helper virus (Raffo & Dawson 1991). Similar observations were reached by Ogawa *et al*. (1991, 1992), who also noted that removal of the 3′-terminal one-third of the read-through portion of the coding region for the 183-kDa protein increased the accumulation of this subgenomic replicon, suggesting that this sequence may have a regulatory role in RNA replication.

Tobamoviruses are also able to support the replication of a satellite virus, satellite tobacco mosaic virus (STMV). The 1059-nucleotide STMV RNA contains two open reading frames, one for a 6.6-kDa protein of unknown function and the other for its 17.5-kDa coat protein. Neither of these proteins is required for replication of STMV (Routh *et al*. 1995, 1997), which relies completely on replication proteins of its tobamovirus helper. The natural helper for STMV is tobacco mild green mosaic virus (TMGMV), although other tobamoviruses, including TMV-U1, can act as less efficient helpers. The 3′-end of STMV can be folded into a tRNA-like structure and UPD (Gultyaev *et al*. 1994), which presumably acts as a binding site for the replicase to initiate negative-strand RNA synthesis. However, this structure resembles that of the 3′-end of TMV-U1 RNA more closely than the 3′-end of the RNA of its natural helper, TMGMV (Gultyaev *et al*. 1994). There is little sequence similarity between the 5′-terminal regions of STMV and those of its helpers. Nevertheless, the STMV 5′ sequence, not the 3′ sequence, determines the helper specificity (Yassi & Dodds 1998).

Although TMV can act as a helper for replication *in trans* of various subgenomic replicons and STMV, evidence has been presented for *cis*-preferential synthesis of negative strands from TMV genomic RNA templates (Lewandowski & Dawson 1998). The observation that a *Tm-1*-resistance-breaking strain of TMV did not allow the replication in *trans* of a sensitive strain when co-inoculated onto *Tm-1*-resistant tomato plants (Yamafugi *et al*. 1991) is consistent with a model in which translation of the RNA is closely coupled with, but not exclusively required for, negative-strand RNA synthesis. Coupling of translation and replication has also been observed for poliovirus (Novak & Kirkegaard 1994).

Relatively little is known about *cis*-acting sequences required for the synthesis of TMV subgenomic RNAs. By analogy with BMV (Duggal *et al*. 1994; Siegel *et al*. 1997), it is likely that sequences upstream and downstream of the transcriptional start sites for the TMV subgenomic RNAs contribute to the activity of subgenomic promoters. Goelet *et al*. (1982) proposed that A-U-rich sequences near the 5′-ends of the movement-protein and coat protein genes, termed Butler boxes, could be signal sequences for subgenomic RNA synthesis. The subgenomic promoter for the movement-protein gene probably lies largely within the C-terminal region of the gene for the 183-kDa protein and this region must therefore be bifunctional. Mutations upstream of the TMV movement-protein gene reduce the accumulation of the movement protein and its mRNA and attenuate virulence of the virus (Nishiguchi *et al*. 1985; Watanabe *et al*. 1987*b*). Some of these mutations may have affected the subgenomic promoter of the movement-protein gene. Similarly the subgenomic promoter for the coat protein gene is probably largely within a bifunctional region of

the movement-protein gene. Experiments using mutants with deletions in the movement-protein gene (Meshi *et al.* 1987) and using hybrid-virus constructs (Dawson *et al.* 1989) suggest that the coat protein subgenomic promoter is within 100 nucleotides upstream of the coat protein coding region. The sequences on the negative-strand template for initiation of synthesis of subgenomic mRNAs for the movement protein and coat protein are identical (3′ CAAA 5′) and similar to that for initiation of synthesis of the genomic RNA (3′ CAUA 5′). They follow a +1 pyrimidine and +2 adenylate rule found at initiation sites for synthesis of genomic and subgenomic RNAs for most members of the alphavirus-like superfamily of positive-stranded RNA viruses (Adkins *et al.* 1998).

5. REGULATION OF TMV RNA SYNTHESIS

There are several factors that are likely to influence the synthesis of TMV genomic and subgenomic RNA species. TMV genomic positive-strand RNA can act as a template for protein synthesis, as well as a template for negative-strand synthesis. Since ribosomes travel down the RNA in a 5′→3′ direction, whereas the polymerase copies the template in the opposite (3′→5′) direction, the two processes cannot occur on the same RNA molecule. The first event to occur at the start of the virus multiplication cycle is cotranslational disassembly of the virions in a 5′→3′ direction, which will uncoat the coding regions of the 126-kDa and 183-kDa proteins and concurrently result in their synthesis. These proteins may then assist in the coreplicational disassembly of the remainder of the particle in a 3′→5′ direction (Wu & Shaw 1996, 1997) and could result in the synthesis of negative-strand RNA corresponding to the 3′-end of the template (tRNA-like structure, coding regions of the coat protein and movement protein). However, ribosomes will still be travelling down the RNA in the opposite (5′→3′) direction, continuing to synthesize more copies of the 126-kDa and 183-kDa proteins. A similar scenario would result if the inoculum were RNA rather than virions and for synthesis of negative strands on progeny positive-strand templates, which occurs during the early stages of the replication cycle (Ishikawa *et al.* 1991*a*). Although the problem could be solved by compartmentalization of translation and RNA synthesis, the location of replicative intermediates on polyribosomes (Beachy & Zaitlin 1985) suggests a close association between replication and translation. It is therefore likely that a mechanism exists to remove ribosomes from the genomic RNA, or to prevent their attachment, to allow synthesis of the negative strand to proceed to the 5′-end of the template. It is unlikely that the advancing polymerase itself could remove ribosomes in front of it; indeed it has been found that the poliovirus RNA-dependent RNA polymerase is unable to synthesize RNA on viral RNA templates undergoing translation. Evidence has been obtained that the poliovirus 3CD protein, the precursor of the 3D polymerase, controls the switch from translation to replication by binding to a cloverleaf structure at the 5′-end of the genomic RNA and preventing further initiation of translation (Gamarnik & Andino 1998). For phage Qβ, the switch is achieved by the S1 host component of the replicase, which binds to ribosome-binding sites on Qβ RNA and prevents further binding of ribosomes (Blumenthal & Carmichael 1979). How the switch from translation to replication is achieved for TMV RNA is not known, but it is possible that the 126-kDa or 183-kDa protein, a host protein or a complex of both could bind to the 5′-end of TMV RNA and prevent the further attachment of ribosomes. Once the already-bound ribosomes had been released from the RNA after translation, synthesis of negative-strand RNA could then proceed towards the 5′-end of the positive-strand template.

After the first negative strands have been synthesized, synthesis of positive strands can commence. It is likely that different RNA polymerase holoenzymes are responsible for the synthesis of negative strands and positive strands. Several observations are consistent with this possibility: (i) TMV RNA synthesis, like that of other positive-strand RNA viruses, is asymmetric, the ratio of positive to negative strands synthesized during infection being about 100:1 (Kielland-Brandt 1974); (ii) a temperature-sensitive TMV mutant has been described which is specifically deficient in the synthesis of progeny single-stranded positive-sense RNA at the restrictive temperature (Dawson & White 1979); (iii) the template sequences for initiation of genomic positive-strand synthesis (3′ CAUA 5′) and negative-strand synthesis (3′ ACCC 5′) are different; (iv) in protoplasts, synthesis of negative strands ceases about 8 h after inoculation, whereas synthesis of positive strands continues for up to 18 h. This is not simply due to removal of positive-strand templates by encapsidation, because a similar cessation of negative-strand synthesis occurs with a coat protein deletion mutant (Ishikawa *et al.* 1991*a*). A similar shut-off of negative-strand RNA synthesis occurs with Sindbis virus, another member of the alphavirus-like supergroup. Evidence suggests that there are distinct Sindbis virus RNA polymerase complexes which synthesize negative strands and positive strands, respectively, and that proteolytic processing regulates the composition and template preference of these complexes (Lemm *et al.* 1994; Shirako & Strauss 1994). The polymerase complex for Sindbis virus negative-strand RNA synthesis is considered to contain the uncleaved polyprotein P123 (which has methyltransferase, helicase and protease domains) plus nsP4 (the RNA polymerase subunit), or alternatively nsP1 plus P23 plus nsP4. The recent establishment of a template-dependent RNA polymerase system, containing P123 (modified to be protease deficient) plus nsP4, able to initiate negative-strand synthesis on a positive-strand RNA template (Lemm *et al.* 1998), is consistent with this view. Complete cleavage of P123 to form nsP1, nsP2 and nsP3 not only switches off negative-strand RNA synthesis, but also switches on positive-stranded genomic and subgenomic RNA synthesis (Lemm *et al.* 1994; Shirako & Strauss 1994). Phage Qβ also uses different polymerases to synthesize positive and negative strands. Host factor HF1 is needed for negative-strand synthesis, but not for positive-strand synthesis. Equal amounts of positive and negative strands are produced when the HF1 protein is in excess, but when the amount of HF1 is limiting, as in the cell, negative-strand synthesis is limited and positive strands are produced in excess (Blumenthal & Carmichael 1979). In the case of TMV what controls conversion of a polymerase able to synthesize negative

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strands into one able to synthesize positive strands is not known, but could involve a change of polymerase conformation, e.g. as result of post-translational modification such as phosphorylation, and/or addition or loss of viral or host proteins in the polymerase complex. A template-dependent RNA polymerase able to catalyse the complete synthesis of TMV RNA has been described (Osman & Buck 1996). This system contained polymerase activities able to copy both positive-strand and negative-strand RNA templates. However, the ratio of positive to negative strands synthesized starting with a genomic RNA template was much less than that found *in vivo*, and additional factors may be required to achieve the *in vivo* ratio.

Full-length negative strands also have to act as templates for the synthesis of the subgenomic mRNAs for the movement protein and coat protein by internal initiation from subgenomic promoters. The movement protein is expressed transiently, early in infection and in relatively small amounts, whereas the coat protein is expressed in large amounts mainly late in the replication cycle (Watanabe *et al.* 1984; Lehto *et al.* 1990*a*). It is likely that the subgenomic promoter sequence, rather than its position in the RNA, determines the temporal control of subgenomic RNA synthesis, since expression of the movement protein under the control of the coat protein promoter led to late expression (Lehto *et al.* 1990*b*). Similarly, there was little difference in the amounts of subgenomic mRNAs produced with constructs in which promoters were placed at different positions in the RNA. The high level of coat protein produced appears to be controlled mainly at the translational level (Culver *et al.* 1993). Whether the same polymerase synthesizes genomic-length and subgenomic RNAs is not known. However, expression of the movement protein in tobacco protoplasts was greatly and selectively enhanced by addition of actinomycin D 1 h after inoculation (Blum *et al.* 1989). It was suggested that actinomycin D, which inhibits host transcription, selectively inhibits the synthesis of a host factor which limits expression of the movement protein.

6. THE ROLE OF MEMBRANES IN THE SYNTHESIS OF TMV RNA

TMV replication complexes are associated with cytoplasmic inclusions or viroplasms, which enlarge during the course of infection to form 'X bodies'. They are composed of aggregates of tubules, embedded in a ribosome-rich matrix (Saito *et al.* 1987; Hills *et al.* 1987). The viroplasms contain the 126-kDa and/or 183-kDa replication proteins, which are associated with the tubules and therefore are the likely sites of RNA replication. The presence of ribosomes in the viroplasms is consistent with the observation that isolated replicative intermediate RNA is found on polyribosomes, and with the close association of TMV replication and protein synthesis (Beachy & Zaitlin 1975). TMV RNA polymerase preparations are membrane-bound (Watanabe & Okada 1986; Young *et al.* 1987; Osman & Buck 1996) and TMV negative strands isolated as double-stranded RNA from infected tobacco leaves were found to cofractionate with membranes (Ralph *et al.* 1971; Nillson-Tillgren *et al.* 1974). Esau & Cronshaw (1967*a*,*b*) noted that viroplasms contained endoplasmic reticulum. Endoplasmic reticulum has been shown to be the site of replication of BMV (Restrepo-Hartwig & Ahlquist 1996) and tobacco etch virus (Schaad *et al.* 1997), and poliovirus replication complexes are located on membranous vesicles derived from the rough endoplasmic reticulum (Bienz *et al.* 1994). Recently, Heinlein *et al.* (1998) have obtained evidence that the TMV replicase is associated with the endoplasmic reticulum, particularly the cortical endoplasmic reticulum, and reported unpublished experiments of C. Reichel and R. N. Beachy, which confirmed that both the replicase and movement protein are associated with endoplasmic reticulum isolated from infected tissues.

There are several possible reasons why the replicase complexes of TMV and those of other positive-stranded RNA viruses are bound to membranes. Compartmentalization will increase the local concentrations of virus-encoded proteins and the efficiency of processes such as virus translation, replication, movement and replication, which must all be coordinated in a highly regulated fashion. Membranes may also play an important role in the configuration of the replication complex. Osman & Buck (1996) observed that a template-dependent RNA polymerase could be obtained only from the membrane-bound enzyme; no template-dependent activity could be obtained after solubilization of the polymerase. A similar observation was made with a template-dependent RNA polymerase of Sindbis virus (Lemm *et al.* 1998). Hence, membranes appear to be required for initiation of RNA synthesis with these viruses. The membrane configuration may also affect the production of progeny single-stranded RNA. Osman & Buck (1997) found that solubilization of membrane-bound TMV RNA polymerase containing endogenous viral RNA template resulted in synthesis of greatly increased amounts of single-stranded, positive-sense RNA and a decrease in the amount of double-stranded RNA. Synthesis of single-stranded RNA by a template-dependent flock house virus RNA polymerase was dependent on added phosphoglycerolipids (Wu *et al.* 1992). Even solubilized polymerases probably contain fragments of cellular membranes in mixed micelles with the detergent used for solubilization and these could have effects on RNA polymerase reactions *in vitro*. The nature of the linkage of the TMV replication complex to the endoplasmic reticulum is unknown. However, of a wide range of detergents tested for the solubilization of the TMV RNA polymerase, only sodium taurodeoxycholate and to a lesser extent sodium deoxycholate were effective (Osman & Buck 1997). This suggests that the replicase may be located in cholesterol-rich regions of the membrane.

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