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Current Review

RNA Polymerases of Plus-Strand RNA Viruses of Plants

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The replication of (+) strand RNA viruses involves two distinct phases, that is synthesis of a (−) strand RNA copy of the virion RNA and synthesis of progeny virion RNA. RNA-dependent RNA polymerase (RdRp) is the key enzyme involved in the replication of the genetic material of RNA viruses. The term replicase must, in our view, be reserved for enzymes capable of synthesizing both (−) and (+) RNA strands. Because all eucaryotic viral RdRp preparations described to date do not detectably synthesize progeny (+) strands on (−) strands synthesized by the same enzyme, the denotation RdRp will be used here.

In this review, we consider only enzyme preparations that synthesize full-length products complementary to either endogenous or to added homologous or closely related templates. By introducing this restriction, we will neglect the many cases in which an RdRp makes heterogeneous short transcripts on an endogenous template or on any added full-length template. These properties are characteristic of host RdRps that have been reviewed extensively by Hall *et al.* (1982) and Fraenkel-Conrat (1986).

RdRp preparations from virus-infected plants often synthesize both full-length products and products not exceeding a few hundred nucleotides in length on viral templates. The latter activity is generally much greater than it is in uninfected plants. This led to speculations that a viral enzyme could display atypical synthesis *in vitro* or, conversely, that a host enzyme was in some way involved in viral RNA synthesis. However, evidence of host RdRp stimulation, as is observed in whole plants infected with cowpea mosaic virus (CPMV), was not found in infected protoplasts (Van der Meer *et al.* 1984). RdRps from plants infected with CPMV and cucumber mosaic virus (CMV) were purified to near homogeneity and shown to be host proteins of 130K (Dorssers *et al.* 1982) and 100K (Gordon *et al.* 1982), respectively. The CPMV RdRp synthesizing full-length products on endogenous templates was purified and shown immunologically to be free of the 130K protein (Van der Meer *et al.* 1984). The corresponding CMV enzyme was in a much less well-purified state and activity that synthesized small products remained (Jaspars *et al.* 1985). Although it is unlikely that host RdRps are involved in viral RNA synthesis, the remarkable ease with which these enzymes can initiate RNA-dependent RNA synthesis and their stimulation upon viral infection remains intriguing.

The presence of enzymes such as terminal uridyl transferase and host polymerase in the (infected) plant cell masks true viral RdRp activity. Products of viral and host RdRps are usually double-stranded after phenol extraction. Specific nuclease treatment and electrophoretic analysis of RNAs synthesized *in vitro*, in combination with studies of template and substrate specificity, must be used to discriminate between full-length products of the viral RdRp and terminally labeled templates or products (or both) of the host polymerase.

Much of the current research on viral RdRps is directed toward elucidating the protein composition of viral polymerases and studying the interactions of enzyme and template. Rapid progress has been made in the latter area over the last few years. Development of new techniques, for example *in vitro* transcription and site-directed mutagenesis in cloned viral cDNA, have permitted mapping of the interaction of brome mosaic virus (BMV) and alfalfa mosaic virus (AlMV) polymerases with their templates. Our review will focus on the most recent developments that have been published in both areas since the review by Van Kammen (1985).

Viral RdRps are associated with membrane structures and templates in the infected cell. In general, the association of the enzymes with specific membranes has not been accomplished. However, some information on the intracellular sites of viral RNA synthesis has been presented (Garnier *et al.* 1980, 1986; Hatta and Francki 1981; Hills *et al.* 1987; Saito *et al.* 1987; Wellink *et al.* 1988).

Because of these membrane associations, the isolation of RdRps has proven to be extremely difficult. The partially purified viral polymerases described so far can be divided into two groups; namely, template-independent and template-dependent RdRp preparations. The enzyme that has been isolated from plants infected with turnip yellow mosaic virus (TYMV) is highly purified and characterized. It is a special case and is also discussed.

Enzyme preparations independent of added template. A simple approach to the study of a viral RdRp is to leave intact the association of the enzyme with its template and cellular membranes so that most of the activity is sedimentable at about 30,000 × *g*. Elongation of nascent (+) strands initiated *in vivo* is observed with these enzymes, leading to synthesis of full-length products. No stimulation of RdRp activity by exogenous templates occurs (template-independent RNA synthesis). The group of these enzymes comprises: CMV RdRp (Jaspars *et al.* 1985; Nitta *et al.* 1988), tobacco mosaic virus (TMV) RdRp (Watanabe and

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Okada 1986), and velvet tobacco mottle virus RdRp (Rohozinski *et al.* 1986). In the case of plants infected with TMV, such preparations also contain an enzymatic activity that methylates TMV RNA (White 1980).

Template-independent RdRp preparations cannot be used to explain the molecular basis of template recognition and initiation of (−) strand synthesis. However, these preparations may prove to be very useful for characterizing the viral-contributed protein composition of viral RdRps, as was shown in the case of CPMV and TMV. In these studies the enzymes were solubilized from the cellular membranes by treatment with the detergents Triton X-100 and CHAPS, respectively. However, in neither case was the association with the endogenous template disrupted. The CPMV polymerase, although it has not been freed of its template, was purified to a high degree (Dorssers *et al.* 1984). Enzyme activity was clearly associated with a 110K protein, which was immunologically identified as a product of the CPMV component B RNA. Gradient centrifugation separated two enzyme-template complexes, corresponding to the two CPMV genome segments. Recently, Eggen *et al.* (1988) described the synthesis of single-strand progeny RNAs, in addition to double-stranded RNAs, with crude extracts from *Chenopodium amaranticolor* Coste and Reyn. infected with CPMV. Young *et al.* (1987) reported on the copurification of the 126K and 183K proteins encoded by TMV and the solubilized TMV RdRp activity.

Enzyme preparations that respond to added template. A further step in the analysis of plant viral RNA synthesis is to disrupt the binding of the enzyme to both membrane structures and endogenous templates without destroying its activity. Such an enzyme preparation permits the study of the interaction of the RdRp with added templates. Solubilization by nonionic or zwitterionic detergents can easily destroy RdRp activity or change the characteristics of the enzyme drastically (Hall *et al.* 1982). However, using dodecyl-β-D-maltoside (12 M) and Nonidet P-40 (NP-40) proved to be very effective in solubilizing several polymerases from tricornavirus-infected plants.

Template-dependent enzymes were prepared by micrococcal nuclease treatment of the 12 M solubilized enzyme in the case of: BMV RdRp (Miller and Hall 1983), cowpea chlorotic mottle virus (CCMV) RdRp (Miller and Hall 1984), and AlMV RdRp (Houwing and Jaspars 1986). BMV RdRp solubilized by NP-40 treatment was made template dependent by ion exchange chromatography (Maekawa and Furusawa 1984) or by high-speed sedimentation (Quadt *et al.* 1988). The enzymes are capable of synthesizing full-length (−) strand RNAs on added virion RNAs. In the case of BMV RdRp, the enzyme initiated *de novo* at the last 3′ C residue and synthesized a faithful copy of the virion template RNA, as established by labeling and sequencing techniques (Miller *et al.* 1986). In addition, the BMV RdRp was capable of synthesizing, at a low level, full-length complementary strands on the virion RNAs of the closely related CCMV (Quadt *et al.* 1988). Significant template dependency was obtained by solubilization of AlMV RdRp with Lubrol PX without any further treatment (Berna 1986).

In general, the conclusion that a product is synthesized on an exogenous template should be drawn only with care. In very crude preparations, homologous products sometimes appear when heterologous RNAs are added (Saunders and Kaesberg 1985; our unpublished observations with AlMV RdRp), which probably means that the added template is just protecting the endogenous template during incubation.

It must be noted that the viral RdRp preparations are relatively crude; therefore, little is known about the composition of proteins contributing to RdRp activity. Comparison of peptide maps showed that the 110K protein specifically present in BMV RdRp preparations corresponded with the *in vitro* translation product of BMV RNA 1 (Bujarski *et al.* 1982). Incubation with antibodies directed against C-terminal peptides of BMV nonstructural proteins gave almost complete inhibition of (−) strand synthesis in the case of P1 and had no effect in the case of P2, although both proteins were detected on immunoblots (Quadt *et al.* 1988). This is the first experimental evidence of a nonstructural protein of a tricornavirus being present in the RdRp complex.

By *in vitro* transcription of cloned BMV cDNAs, it is possible to generate well-defined modified RNAs. By using mutant RNAs as templates for the BMV RdRp, it was shown that the overall 3′ terminal tRNA-like structure is indispensable for the (−) strand promoter activity *in vitro* (Dreher *et al.* 1984; Bujarski *et al.* 1985; Miller *et al.* 1986; Dreher and Hall 1988a). The structural and sequence elements in the tRNA-like structure important for promoter function were mapped in detail by Dreher and Hall (1988a). Distortion of the tRNA-like structure led to a sharp decrease of (−) strand RNA synthesis. Interestingly, the removal of one of the arms of this structure greatly enhanced (−) strand promoter activity. These data indicate that recognition of the template and initiation are not due solely to the presence of specifically recognized sequence tracts. The 3′ terminus CCA_{OH} is required for initiation by the RdRp, as shown by mutational analysis (Dreher *et al.* 1984). Further evidence was presented by Miller *et al.* (1986). It was shown that removal of the terminal A-1 and the penultimate C-2 residue had a relatively slight detrimental effect on (−) strand RNA synthesis, whereas removal of a third base, the C-3 residue, almost completely abolished template activity.

The 3′ terminal tRNA-like structure is present in all virion RNAs of bromoviruses, cucumoviruses, hordeiviruses, tobamoviruses, and tymoviruses and can be adenylated and aminoacylated (reviewed by Haenni *et al.* 1982). The role of adenylation and aminoacylation in viral RNA synthesis *in vivo* is not yet known. A correlation between aminoacylation and RNA synthesis has been proposed by Hall (1979). Mutant BMV RNAs with 3′ extensions show almost no aminoacylation *in vitro*, whereas the (−) strand promoter activity is partially retained (Miller *et al.* 1986). This indicates that aminoacylation is not mandatory for *in vitro* RNA synthesis. Mutant BMV RNAs (Dreher and Hall 1988b) might prove to be suitable tools in elucidating the role of tRNA-like functions in viral replication *in vivo*.

BMV (−) strands are not significantly recognized by the BMV RdRp. However, the enzyme is capable of synthesizing the subgenomic messenger RNA 4 by internal initiation on (−) strand RNA 3 (Miller *et al.* 1985). By using mutant (−) strand transcripts, the responsible promoter and enhancer regions have been delineated *in vitro* (Marsh *et al.* 1988). Constructs with multiple inserts

of promoter or enhancer sequences in RNA 3 and even in RNA 1 yield the expected subgenomic RNAs *in vivo* (French and Ahlquist 1988). This clearly indicates that as far as the synthesis of the subgenomic messenger is concerned, the *in vitro* function of the BMV RdRp is similar to the function *in vivo*.

The AlMV RdRp has been purified to a level similar to that achieved for the BMV enzyme. The template specificity of the AlMV RdRp is less than that of the BMV enzyme. Nevertheless, it has been used for the analysis of regions on AlMV RNAs essential for promoter function (Langereis 1987; A. C. Van der Kuyl and J. F. Bol, personal communication). In contrast to the BMV case, large portions of the 3′ homologous region in AlMV RNA 4 can be deleted. At least 100 and possibly 133 nucleotides can be removed from the 3′ terminus without a significant effect on the (−) strand synthesis *in vitro*. The 5′ boundary of the subgenomic promoter on AlMV RNA 3 was mapped.

Template modification studies provided detailed information on the interaction of RdRps with their templates. Because these studies were performed with crude enzyme preparations, it is not known whether host factors, different subunits of the enzyme, or even different subunits are involved in the interactions. Further purification of the enzymes is needed to broaden our understanding of the molecular basis of viral RNA synthesis.

With the AlMV polymerase, it should be possible to test the hypothesis that ilarviruses need coat protein (CP) for infectivity, because CP is necessary for (−) strand initiation (reviewed by Jaspars 1985). However, the addition of small amounts of CP inhibited rather than stimulated the (−) strand synthesis. It is difficult to remove all endogenous CP from the crude enzyme preparation. The inhibition of RNA synthesis by CP *in vitro* is interesting, because it may explain why RNA synthesis in the infected cell is mainly (+) strand synthesis. According to this hypothesis, after early CP-dependent (−) strand synthesis CP accumulates in the cells and blocks further (−) strand synthesis by occupying the 3′ terminal regions of the (+) strands (Nassuth and Bol 1983). In protoplasts inoculated with only B and M virions, which contain RNAs 1 and 2, respectively, viral RNA synthesis takes place in the absence of CP synthesis. Much more (−) strand synthesis occurs in these cells than in cells infected by the complete set of virions. However, crude RdRp preparations from cells inoculated with B and M virions synthesized mainly (+) strand RNA, and the preparations were shown to contain parental CP. Whether this parental CP was in contact with the RdRp in the intact cell is unknown. This would mean that the CP rather than the P3 protein (which is certainly absent) is the regulating factor determining the (+) and (−) balance of RNA synthesis (Houwing and Jaspars 1987). With BMV RdRp, inhibition of (−) strand synthesis by CP also was observed, but at much higher concentrations of CP (Horikoshi *et al.* 1987).

TYMV polymerase. TYMV RdRp is the best characterized plant viral enzyme in terms of protein composition (Mouchès *et al.* 1984; Bové and Bové 1985; Candresse *et al.* 1986). The enzyme was solubilized from membranes by Lubrol W treatment and obtained in a template-dependent and homogenous state by an extensive purification procedure. The complex was shown to be composed of a 115K viral-encoded subunit and a 45K host-encoded subunit. Antibodies directed against the viral RdRp preparation were shown to bind to *in vitro* translation products of TYMV RNA. Furthermore, it was observed that the 115K protein was present only in enzyme preparations from plants infected with TYMV. Moreover, the incubation of the viral RdRp with antibodies directed against the viral 115K protein reduced RdRp activity *in vitro* by 40% (Candresse *et al.* 1986).

Garnier *et al.* (1980, 1986) showed that both viral RNA synthesis occurred and the location of the 115K protein was at invaginations of the chloroplast envelope. The function of the RdRp subunits is not known. It is clear, however, that the 45K protein is not the host enzyme stimulated by TYMV infection, which was shown to be functionally different from the viral RdRp. That is, the two enzymes behaved differently on sedimentation and chromatography. The viral RdRp is inhibited by the addition of small RNAs containing the 3′ terminal sequence of TYMV RNA (Morch *et al.* 1987). As mentioned previously, a correlation between aminoacylation and RNA synthesis has been proposed by Hall (1979). However, no stimulation of RdRp activity by the addition of elongation factor 1α (EF-1α) was observed (Joshi *et al.* 1986). This indicates that aminoacylation is not mandatory for *in vitro* RNA synthesis. Unfortunately, it has not been shown whether the homogeneous RdRp preparation is still capable of synthesizing full-length RNA strands. When this is demonstrated, the enzyme preparation will be extremely valuable in elucidating the biochemical basis of viral RNA synthesis.

Indirect data on the composition of viral RdRp. Because the viral RdRps prefer their cognate RNAs as template, one would expect that the enzymes or at least one of their subunits would be virus-encoded. Extensive and scattered data suggest indirectly that viral proteins are involved in viral RNA-dependent RNA synthesis. In this review, we will simply enumerate genetic analyses of multipartite viruses and homology data for the aforementioned viruses.

In some multipartite viruses it is observed that a certain genome part or a certain combination of genome parts can replicate independently in protoplasts. This means that the viral information for RNA replication, including that for a RdRp complex, is present in these genome parts; namely, in component B RNA of CPMV (Goldbach *et al.* 1980), and in RNAs 1 and 2 of AlMV (Nassuth *et al.* 1981), BMV (Kiberstis *et al.* 1981), and CMV (Nitta *et al.* 1988). Furthermore, it was observed that mutations in RNAs 1 and 2 of AlMV have remarkably similar effects on viral RNA synthesis (Huisman *et al.* 1985) and that in constructing BMV-CCMV hybrids, RNAs 1 and 2 cannot be exchanged independently (Allison *et al.* 1988).

The nucleotide sequences of many plant viruses have been determined; data on the conservation of viral proteins have become available. Because RNA genomes have a high mutation rate (Van Vloten-Doting *et al.* 1985), the conservation is very likely to be of biological significance. It was shown that the nonstructural proteins of poliovirus and CPMV have a similar genetic organization and large homologous regions. Extensive homologies are also found for the 183K TMV nonstructural protein, the 206K TYMV polyprotein (Morch *et al.* 1988), and for the RNA 1- and RNA 2-encoded proteins of AlMV, BMV, and CMV (reviewed by Goldbach *et al.* 1987). In these homologous

regions distinct motifs can be recognized.

The segment Gly-Asp-Asp (GDD), flanked by hydrophobic residues, is conserved, as far as it is known, in positive RNA viruses (Kamer and Argos 1984; Goldbach 1987). The GDD motif is present in the polioviral 3D protein and the Qβ replicase, which are capable of elongating preinitiated viral RNAs (Rothstein *et al.* 1988; Blumenthal 1980). Furthermore, mutational analysis of the GDD motif in Qβ replicase showed the significance of this motif in viral RNA synthesis *in vivo* (Inokuchi and Hirashima 1987). It is, therefore, generally assumed that the viral proteins containing the GDD motif are RdRps or catalytic subunits thereof. This conserved segment is present in the RNA 2 products of AlMV, BMV, and CMV. Furthermore, this motif is present in the 183K TMV protein, the 206K TYMV polyprotein (Morch *et al.* 1988), and the 110K CPMV protein, which was shown experimentally to be involved in CPMV RNA synthesis (Dorssers *et al.* 1984).

Gorbalenya *et al.* (1985, 1988) reported on the conservation of several motifs in the 126K TMV protein and the RNA 1-encoded proteins of AlMV, BMV, and CMV. Such motifs have also been found in the 206K polyprotein of TYMV (Morch *et al.* 1988). These motifs are characteristic of the NTP-binding site of ATP- and GTP-utilizing enzymes. The predicted NTP-binding capacity of the 126K TMV protein was experimentally proven by Evans *et al.* (1985). Furthermore, this protein copurifies with the TMV RdRp activity (Young *et al.* 1987). The conserved regions are also found in bacterial helicases. A helicase activity of an RdRp subunit could increase the accessibility of the template for the enzyme.

Hodgman (1988) identified six conserved segments in a set of 21 proteins related by common involvement in nucleic acid replication or recombination. This set includes the RNA 1-encoded nonstructural proteins of AlMV, BMV, and CMV and the 126K TMV-encoded nonstructural protein. The presence of the P1 protein in the RdRp complex of BMV was shown experimentally (Quadt *et al.* 1988).

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