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COMPARISON OF THE REPLICATION OF POSITIVE-STRANDED RNA VIRUSES OF PLANTS AND ANIMALS

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I. INTRODUCTION

Many important animal, bacterial, fungal, and plant viruses have genomes of positive-stranded (messenger-sense) RNA (Murphy *et al.*, 1995). Over the last 12 years it has become clear that some viruses in these different types of hosts have related genes, and sometimes similar arrangements of genes and modes of gene expression, leading to suggestions about evolutionary pathways (Haseloff *et al.*, 1984; Kamer and Argos, 1984; Ahlquist *et al.*, 1985; Goldbach, 1986; Zimmern, 1988; Strauss and Strauss, 1988; Habili and Symons, 1989; Bruenn, 1991; Goldbach *et al.*, 1991; Koonin, 1991a; Dolja and Carrington, 1992; Koonin and Dolja, 1993; Dolja *et al.*, 1994; Goldbach and de Haan, 1994; Ward, 1994). Goldbach and de Haan (1994) outlined four possible evolu-

tionary pathways to account for the observed relationships between viruses of different host types: convergent evolution, transduction (introgression) of (conserved) host genes, common ancestry, interviral recombination. Nucleotide and amino acid sequence similarities provide evidence for common ancestries of genes and, in cases where gene orders and modes of gene expression are similar (e.g., cowpea mosaic virus and poliovirus; Goldbach, 1986), possibly of viruses. However, there is also good evidence that recombination between RNAs of different viruses has played an important role in virus evolution (Lai, 1992, 1995; Simon and Bujarski, 1994). Since mixed infections of positive-stranded RNA viruses and viruses with different genome types, such as single-stranded (ss) DNA, double-stranded (ds) DNA, negative-stranded RNA, or dsRNA, are also possible, acquisition of genes from such disparate viruses is also possible. For example, some animal coronaviruses (positive-stranded RNA) and influenza virus C (negative-stranded RNA) have related hemagglutinin-esterase proteins (Cavanagh and MacNaughton, 1994). Recent studies have also indicated that some positive-stranded RNA viruses have acquired genes from the host, e.g., the HSP70 heat-shock chaperone protein gene analogues found in viruses of the plant closterovirus genus (Dolja *et al.*, 1994; Klaasen *et al.*, 1995); other examples are reviewed by Lai (1995). Recombination between positive-stranded viral RNA and transgenic plant transcripts has been demonstrated (Greene and Allison, 1994). The concept has therefore developed of modular evolution of positive-stranded RNA viruses, with the possibility of genes or groups of genes with related functions, e.g., replication genes or structural genes, having been acquired from different sources.

It is also noteworthy that RNA polymerases that replicate RNA apparently lack proofreading activities, leading to error rates of 10^{-3} to 10^{-5} (Domingo and Holland, 1994). This has led to the concept of the viral RNA "quasi" species (Eigen and Biebriecher, 1988), consisting of a population of closely related sequences, the composition of the population being influenced by positive and negative selection pressures. Such high mutation rates have probably limited the size of (unsegmented) RNA genomes, the largest being the animal coronaviruses (up to ~30 kb) and the plant closteroviruses (up to ~20 kb) and most being <10 kb, although some form of editing remains a possibility for the largest RNA genomes.

Positive-stranded RNA viruses have also evolved (or acquired) genes with functions specific for their hosts. A large number of positive-stranded RNA viruses (over 600 species) are adapted to replication and spread in plant hosts (Goldbach *et al.*, 1994). One important adaptation

is the requirement of plant viruses to spread from cell to cell through
the plasmodesmatal connections. Plant viruses encode specialized move-
ment proteins which modify the plasmodesmata in a variety of ways to
allow the (active) passage of viral nucleic acids, nucleoproteins, or par-
ticles (Waigmann and Zambryski, 1994; Lucas and Gilbertson, 1994).
Since there is evidence that plant proteins and/or mRNAs move through
plasmodesmata during the course of normal plant development (Lucas,
1995), it is possible that plant viruses have become adapted to enable
them to utilize an endogenous transport system for their own move-
ment through the plant. Animal and bacterial viruses also encode spe-
cialized proteins, not needed by plant viruses, for attachment and
entry into cells and for release from cells. Examples include virus at-
tachment proteins which bind to receptors on the surface of cells, and
proteins which direct budding (exocytosis) of enveloped viruses from
the cell surface or which induce cell lysis. Because of the high mutation
rate of RNA viruses (Domingo and Holland, 1994) and the similarities
of present-day viruses, Goldbach and de Haan (1994) considered it
unlikely that common ancestors of plant and animal viruses predated
the evolutionary separation of plants and animals. Insect vectors of
both plant and animal positive-stranded RNA viruses are known, in
some of which the viruses are able to replicate, and these were consid-
ered as the likely bridge between plant and animal viruses. Addition-
ally, the ability of a virus to replicate in two different types of host
[vertebrate and invertebrate, e.g., alphaviruses (Strauss and Strauss,
1994); plant and invertebrate, e.g., marafiviruses (Gamez and Leon,
1988)] is likely to be a significant driving force in evolution. Although
the insect vector hypothesis is persuasive, the possibility of evolution-
ary stasis has also to be considered and the additional hypothesis that
some positive-stranded RNA viruses, which predated the separation of
plants and animals, have provided ancestral gene clusters for current
plant and animal viruses cannot be discounted.

    The replication of the genome can be considered to be the most fun-
damental aspect of the biology of positive-stranded RNA viruses. This
can be envisaged to take place in two main stages: (i) synthesis of a
complementary (negative-stranded) RNA using the genomic positive-
stranded RNA as a template; (ii) synthesis of progeny positive-
stranded RNA using the negative-stranded RNA as a template. At least
one virus-encoded protein, an RNA-dependent RNA polymerase (RdRp),
is required to catalyze RNA synthesis directed by the RNA template
and using the four ribonucleoside triphosphates as substrates. Another
activity is required to unwind the duplex formed between the template
and newly synthesized strands. The poliovirus RdRp ($3D^{pol}$ protein) has

been shown to have unwinding activity (Cho *et al.*, 1993). However, it is
not clear whether the role of this activity in poliovirus RNA replication
is to remove secondary structure in the template RNA, thereby in-
creasing processivity of the enzyme, or to unwind duplexes formed as a
result of RNA synthesis. Many positive-stranded RNA viruses also
encode helicases (or putative helicases), which may function in duplex
unwinding during replication. Other proteins probably have special-
ized roles in the initiation of RNA synthesis. By analogy with the phage
$Q\beta$ RdRp holoenzyme, which contains four host (bacterial) protein
subunits as well as the virus-encoded polymerase subunit (Blumenthal
and Carmichael, 1979; van Duin, 1988), it is likely that RdRp holo-
enzymes of positive-stranded RNA viruses of eukaryotic hosts will also
consist of both virus-encoded and host-encoded protein subunits.
Hence the replication proteins of animal and plant positive-stranded
RNA viruses, which are related in terms of amino acid sequences or
gene order, may nevertheless have adapted to their particular host in
terms of their interactions with host proteins. One aim of this review is
to identify similarities and differences in the replication of positive-
stranded RNA viruses of plants and animals. As the organization and
expression of replication proteins differ considerably between different
groups of viruses within a particular host, a further aim will be to
compare replication strategies of different virus groups. Some positive-
stranded RNA plant viruses also produce subgenomic RNAs which are
used as mRNAs for translation of open reading frames (ORFs) which
are internal in the virus genome. The different strategies which have
evolved for this purpose will be compared. The replication of satellite
RNAs, which by definition generally contain substantial nucleotide
sequence distinct from that of the helper virus genome (reviewed by
Roossinck *et al.*, 1992; Kaper, 1995) will only be discussed when the
results are relevant to replication of the genomic RNA. The subject of
replicase-mediated resistance, which has been recently reviewed (Carr
and Zaitlin, 1993; Baulcombe, 1994; Lomonossoff, 1995; Mueller *et al.*,
1995), will not be considered in this article. RNA replication is
important in RNA recombination in generating hybrid RNA molecules
and defective-interfering (DI) RNAs. This topic is the subject of a
number of excellent recent reviews (Lai, 1992; Bujarski *et al.*, 1994;
Simon and Bujarski, 1994) and will be covered here only insofar as
data on recombination impact on replication mechanisms. The retro-
viruses encapsidate single-stranded, messenger-sense RNA, but repli-
cate via DNA intermediates; the replication of these viruses will not be
considered here, except for brief comparisons of reverse transcriptases
with other types of RNA and DNA polymerases (see Section II,A).

II. VIRUS-ENCODED REPLICATION PROTEINS

A. RNA-Dependent RNA Polymerases

An early debate in the study of plant positive-stranded RNA replication was whether RNA synthesis was catalyzed by host-encoded or virus-encoded RNA polymerases. Infection of plants with a range of positive-stranded RNA viruses from different families or genera leads to increase in activity and amounts of plant RNA polymerases which are able to initiate RNA synthesis utilizing an RNA template (Fraenkel-Conrat, 1986; Schiebel *et al.*, 1993a,b). Similar activities have been reported from animal cells (Volloch, 1986; Volloch *et al.*, 1987). Crude preparations of viral RNA polymerases isolated from infected plants are often contaminated with the host RNA polymerase. The purified enzymes from several plants consist of single polypeptide chains with molecular masses in the range 100–140 kDa, they can utilize ssRNA and ssDNA as templates with no marked sequence specificity, and synthesize relatively short, heterogeneous RNA products (<500 nt). The isolation of replication complexes, containing virus-encoded RdRp but free from the host RdRp, able to synthesize full-length viral RNA, e.g., for cowpea mosaic virus (van der Meer *et al.*, 1984) and cucumber mosaic virus (Hayes and Buck, 1990), argues against a role of the host RdRp in RNA replication, at least for the viruses studied. The biological role of the host RdRp is unknown, although it has been suggested that it may play a role in posttranscriptional suppression of gene expression (Dougherty and Parks, 1995).

It is now clear that all replication-competent positive-stranded RNA viruses which have been sequenced encode an RdRp. For many viruses, putative RdRps have been identified from conserved amino acid sequence motifs in polypeptides whose sequences have been deduced from ORFs in the nucleotide sequences of the viral RNA and the requirement for the ORFs containing these motifs in virus replication has been established by mutation. RNA polymerase activity has been demonstrated biochemically for the specific proteins encoded by these ORFs for only a few viruses, e.g., $Q\beta$ replicase subunit II (Landers *et al.*, 1974), poliovirus 3D protein (van Dyke and Flanegan, 1980; Rothstein *et al.*, 1988; Neufeld *et al.*, 1991), hepatitis C virus NS5B protein (Behrens *et al.*, 1996).

Kamer and Argos (1984) identified several similar motifs between the known poliovirus RdRp (3D[pol] protein) and putative RdRps of several other positive-stranded RNA viruses of animals and plants. The most conserved of these consisted of a central Gly–Asp–Asp (GDD)

triplet flanked by pentapeptides consisting of mainly hydrophobic amino acids, suggesting a β-hairpin structure composed of two hydrogen-bonded antiparallel β-strands connected to a short exposed loop containing the GDD amino acids. Subsequent analyses have extended the range of viruses and identified further conserved motifs (Poch *et al.*, 1989; Habili and Symons, 1989; Bruenn, 1991; Koonin, 1991a; Koonin and Dolja, 1993; Dolja *et al.*, 1994). Koonin (1991a) and Koonin and Dolja (1993) identified eight such motifs, three of which (IV, V, and VI) showed unequivocal conservation, allowing the signature $DX_3[FYWLCA]X_{0-1}DX_n[STM]GX_3TX_3[NE]X_n[GS]DD$ to be proposed as an identifier of RdRps of positive-stranded RNA viruses and some related dsRNA viruses (X indicates an unspecified amino acid residue; alternative amino acids at particular sites are shown in square brackets). The regions of this signature separated by X_n correspond to parts of motifs IV, V, and VI.

The importance of some of these motifs has been confirmed by *in vitro* mutagenesis. Mills *et al.* (1988) showed that many linker insertions in a central region of the phage $Q\beta$ replicase gene, which included motifs I to VIII, were lethal. Furthermore, mutation of the G residue of the GDD box in motif VI to M, P, S, or V reduced the phage $Q\beta$ replication *in vivo* to <1% of wild-type; mutation of the G to A abolished replication (Inokuchi and Hirashima, 1987). Similar mutations in the GDD box of the poliovirus 3D protein considerably reduced its RNA polymerase activity in an *in vitro* assay, although the quantitative effects of the different mutations were different from those in $Q\beta$; mutation of the G to A or S gave 5 to 20% of wild-type activity, while mutation of the G to C, M, P, or V abolished enzyme activity (Jablonski *et al.*, 1991). Furthermore, it was shown that mutation of the Y residue, which flanks the GDD box (YGDD) in the 3D protein of poliovirus and equivalent proteins of some related viruses (Koonin, 1991a), to F had no effect on *in vitro* polymerase activity or virus viability, whereas mutations of Y to S, I, or H considerably reduced or abolished *in vitro* polymerase activity and were lethal. Interestingly, a Y to M mutation had no effect on *in vitro* (primed) polymerase activity, but reduced virus infectivity, giving rise *in vivo* to a second compensatory mutation upstream of the conserved polymerase motifs; this suggested possible interaction of an upstream region of the polymerase with the YGDD sequence of the conserved polymerase motif VI (Jablonski and Morrow, 1993). Sankar and Porter (1992) mutated seven amino acid residues in the encephalomyocarditis 3D protein, which showed a high degree of conservation in motifs IV to VI (D235→E, motif IV; D240→E, motif IV; G294→A, motif V; T298→S, motif V; G332→E, motif VI; D333→E, motif VI; D334→E,

motif VI). All of these mutations abolished, or reduced to a very low level, the *in vitro* RNA polymerase activity of this protein. Mutations to chemically similar residues in regions of these motifs which are conserved only in their hydrophobicity had either no effect on, or a less pronounced reduction in, enzyme activity. Mutations of the GDD box of the potato virus X 166-kDa protein to GED, ADD, or GAD abolished infectivity for plants and reduced RNA replication in protoplasts to undetectable levels (Longstaff *et al.*, 1993). Similarly, a G→R substitution in the GDD box of turnip yellow mosaic virus 66-kDa protein abolished RNA replication (Weiland and Dreher, 1993). Mutations in the central polymerase-like domain of the brome mosaic virus 2a protein, encompassing motif IV and flanking sequences, also gave rise to mutants in which replication became temperature sensitive or was abolished (Kroner *et al.*, 1989; Traynor *et al.*, 1991). A G→E mutation in motif II in the polymerase-like domain of the Sindbis virus nsP4 protein rendered the virus temperature-sensitive for RNA replication (Hahn *et al.*, 1989a).

Argos (1988), Poch *et al.* (1989), Delarue *et al.* (1990), and Heringa and Argos (1994) extended the amino acid sequence comparisons to include RNA-dependent DNA polymerases (reverse transcriptases), DNA-dependent RNA polymerases, and DNA-dependent DNA polymerases, as well as RdRps of negative-stranded and dsRNA viruses. It was found that counterparts to the Koonin (1991a) RdRp sequence motifs IV and VI are present in many of these other types of polymerases (Table I), leading to the suggestion of similar types of protein folds (Delarue *et al.*, 1990; Heringa and Argos, 1994). The three-dimensional structures of the Klenow fragment of *Escherichia coli* DNA polymerase I (Ollis *et al.*, 1985), phage T7 RNA polymerase (Sousa *et al.*, 1993), and human immunodeficiency virus (HIV) type 1 reverse transcriptase (Koehlstaedt *et al.*, 1992) indicate that all these polymerases contain a "hand" structure with a cleft formed between "fingers," "palm," and "thumb" subdomains. Further analysis of the structure of the HIV reverse transcriptase complexed with a template and primer (Jacoba-Molina *et al.*, 1993) indicated that the template and primer are located in the cleft. Sequence motifs equivalent to IV (β-strand) and VI (β-strand-loop-β-strand) were located close together on the floor of the cleft within the palm subdomain, with the three conserved D residues in these motifs (Table I), lying close to the 3'-OH of the primer and the NTP binding site in the polymerase catalytically active site; it was suggested that the function of the three D residues may be to bind the Mg^{2+} ions needed for polymerase activity. Recent studies of the poliovirus RdRp (3D protein) indicate that it also has a "hand" structure (Schultz *et al.*, 1995). Richards *et al.* (1992) located the NTP binding site of the polio-

virus 3D protein to a region spanning motifs II to III, the C-terminal part of the region being 12 residues from the conserved D residue in motif IV. Hayes *et al.* (1994a) and Bates *et al.* (1995) showed that antibodies raised to peptides corresponding to motif VI in the RdRp proteins of cucumber mosaic virus (2a protein) and red clover necrotic mosaic virus (88-kDa protein) inhibited initiation of RNA synthesis by

TABLE I

CONSERVATION OF ASPARTIC ACID (D) RESIDUES IN AMINO ACID SEQUENCE MOTIFS IN RNA-DEPENDENT RNA POLYMERASES AND OTHER TYPES OF NUCLEIC ACID POLYMERASES

Polymerase[a]	Genome[b]	Lineage[c]	Virus or host[d]	Motif IV[e]	Number[f]	Motif VI[e]
RdRp	RNA (+)	1. Picorna	PV	F **D** YTGY.DASLS	80	MIAYG **D.D** VIAS
			CPMV	C **D** YSSF.DGLLS	95	LVTYG **D.D** NLIS
		1. Poty	TEV	A **D** GSQF.DSSLT	85	YYVNG **D.D** LLIA
			RYMV	G **D** GSRF.DSSID	89	FVCNG **D.D** NKFA
		1. Sobemo	SBMV	A **D** ISGF.DWSVQ	78	CIAMG **D.D** SVEG
			PLRV	T **D** CSGF.DWSVA	79	AMAMG **D.D** ALEA
		1. Arteri	IBV	W **D** YPKC.DRAMP	127	LMILS **D.D** GVVC
			EAV	T **D** LESC.DRSTP	100	VYIYS **D.D** VVL.
		1. Astro	HAstV	F **D** WTRY.DGTIP	92	TVVYG **D.D** RLST
		2. Phage	phage Qβ	V **D** LSAASDS.IS	66	VTVYG **D.D** IILP
			phage MS2	I **D** LSSASDS.IS	67	IGIYG **D.D** IICP
		2. Flavi	YFV	D **D** TAGW.DTRIT	117	MAVSG **D.D** CVVR
			TBEV	D **D** TAGW.DTKVT	114	MLVSG **D.D** CVVR
		2. Pesti	HCV	Y **D** TRCF.DSTVT	86	MLVCG **D.D** LVVI
			BVDV	F **D** TKAW.DTQVT	88	IHVCG **D.D** GFLI
		2. Carmo	TBSV	L **D** ASRF.DQHCS	82	LANCG **D.D** CVLI
			RCNMV	L **D** ASRF.DQHCS	83	LANNG **D.D** CVLV
		3. Tymo	TYMV	N **D** YTAF.DQSQH	71	IMVSG **D.D** SLID
			PVX	N **D** YTAF.DQSQD	72	IMVSG **D.D** SLID
		3. Tobamo	TMV	L **D** ISKY.DKSQN	78	GAFCG **D.D** SLLY
			BMV	A **D** LSKF.DKSQG	78	AIFSG **D.D** SLI.
		3. Rubi	SINV	T **D** IASF.DKSQD	80	AAFIG **D.D** NIIH
			BNYVV	I **D** AAAC.DSGQC	76	MAMKG **D.D** GFK.
RdRp	RNA (−)		FLUA	G **D** NTKW.NENQN	124	GLQSS **D.D** FALI
			SENV	T **D** LKKY.CNLWR	94	AMVQG **D.N** QAIA
RdRp	RNA (ds)		BTV	I **D** FGYG.EGRVA	110	EQYVG **D.D** TLFY
			IBDV	I **D** LEKG.EANCT	103	IERSI **D.D** IRGK

TABLE I *(continued)*

Poly-merase[a]	Genome[b]	Lineage[c]	Virus or host[d]	Motif IV[e]	Num-ber[f]	Motif VI[e]
RdDp	RNA (+)		MMTV	I **D** LQDC.FFNI	61	IVHYM **D.D** ILLA
			HIV	C **D** VGDA.YSFV	61	IYQYM **D.D** LYVG
RdDp	DNA		HBV	L **D** VSAA.FYHL	109	AFSYM **D.D** VVLG
			CaMV	F **D** CKSG.FWQV	49	CCVYV **D.D** ILVF
DdDp	DNA		EBV	F **D** FASL.YPSI	158	RIIYG **DTD** SIFV
	DNA		Humα	L **D** FNSL.YPSI	129	EVIYG **DTD** SIMI
	DNA		phage T7	I **D** ASGL.ELRC	168	MAWVH **D.E** IQVG
	DNA		E. coli I	A **D** YSQI.ELRI	169	INQVH **D.E** ELVF

[a] RdRp, RNA-dependent RNA polymerase; RdDp, RNA-dependent DNA polymerase (reverse transcriptase); DdRp, DNA-dependent RNA polymerase; DdDp, DNA-dependent DNA polymerase.

[b] RNA (+), positive-stranded RNA; RNA (ds), double-stranded RNA; RNA (−), negative-stranded RNA.

[c] The numbers and names (given for the positive-stranded RNA viruses only) refer to the RdRp supergroups and lineages from Koonin (1991) and Koonin and Dolja (1993). Two examples of each group are given.

[d] Abbreviations: PV, poliovirus; CPMV, cowpea mosaic virus; TEV, tobacco etch virus; BYMV, barley yellow mosaic virus; SBMV, southern bean mosaic virus; PLRV, potato leafroll virus; IBV, avian infectious bronchitis virus; EAV, equine arteritis virus; HAstV, human astrovirus; YFV, yellow fever virus; BVDV, bovine diarrhea virus; TBSV, tomato bushy stunt virus; RCNMV, red clover necrotic mosaic virus; TYMV, turnip yellow mosaic virus; PVX, potato virus X; TMV, tobacco mosaic virus; BMV, brome mosaic virus; SINV, Sindbis virus; BNYVV, beet necrotic yellow vein virus; FLUA, influenza A virus; SENV, Sendai virus; BTV, Blue tongue virus; IBDV, infectious bursal disease virus; MMTV, mouse mammary tumor virus; HIV, human immunodeficiency virus; HBV, hepatitis B virus; CaMV, cauliflower mosaic virus; EBV, Epstein-Barr virus; Humα, human DNA polymerase α; E. coli I, E. coli DNA polymerase I.

[e] Sequence data and motifs are from Koonin (1991a), Koonin and Dolja (1993), Poch et al. (1989), Delarue et al. (1990), Heringa and Argos (1994). Sequence motifs IV and VI of Koonin (1991a), and Koonin and Dolja (1993) are counterparts of motifs A and C of Poch et al. (1989), Delarue et al. (1990), and Heringa and Argos (1994). The human astrovirus sequences were from Lewis et al. (1994). Aspartic acid (D) residues that are invariant or nearly invariant are shown in bold.

[f] Numbers of amino acid residues between motifs IV and VI.

template-dependent RNA polymerase complexes isolated from plants infected with the respective viruses (shown to contain the 2a and 88-kDa proteins respectively), suggesting that the inhibitory effect of the antibodies may be to interfere with the binding of the template at the catalytically active site of the enzyme. The observation that the antibodies did not inhibit the activity of polymerase complexes con-

taining bound RNA template is consistent with this hypothesis.

On the basis of sequence similarities extending over 300 amino acids, Koonin (1991a) and Koonin and Dolja (1993) classified the RdRps of positive-stranded RNA viruses into three supergroups (1, 2, and 3), with a number of different lineages within each supergroup (Table II). All three supergroups contain RdRps of viruses of animals and plants, and supergroup 2 contains additionally RdRps of the bacterial positive-stranded RNA virus *Leviviridae* family. Although the supergroups and lineages are undoubtably important in establishing likely evolutionary relationships between viral RdRps, it is not yet clear to what extent the sequence differences which define the supergroups reflect any significant differences in the biological properties of the RdRps or their roles in RNA replication. Evolution of RNA replication systems is probably best considered in terms of coevolution of the RNA polymerase proteins with other proteins in the replication complex and with cis-acting sequences required for RNA replication. Isolated polymerase subunits [e.g., Qβ RNA polymerase subunit 2 (Landers *et al.,* 1974); poliovirus 3D protein (van Dyke and Flanegan, 1980; Cho *et al.,* 1993)] are able to catalyze RNA synthesis only on primed templates and synthesis is not template-specific. Initiation of positive-stranded and negative-stranded RNA synthesis on unprimed templates requires interaction of the core polymerase with additional proteins [host proteins in the case of Qβ (Blumenthal and Carmichael, 1979; van Duin, 1988), both host- and virus-encoded proteins in the case of poliovirus (Barton *et al.,* 1995; Xiang *et al.,* 1995a,b; McBride *et al.,* 1996)], some of which will also interact specifically with the viral RNA (which may be terminal or internal structures), conferring template specificity on the replication system. Some replication proteins are clearly multifunctional and may need to interact with different viral or host proteins at various stages of the virus replication cycle. Charged-to-alanine mutagenesis of the poliovirus RdRp (3D protein) yielded multiple temperature-sensitive mutants defective in RNA synthesis, many of which mapped in the N-terminal third of the protein well away from the conserved polymerase motifs (Diamond and Kirkegaard, 1994). Since clustered charged-to-alanine mutagenesis is designed to target residues on the surface of folded proteins, these mutants may be indicative of sites of interaction of the 3D polymerase with other viral or host proteins. Another ts mutant with a M→T mutation in the C-terminal portion of the 3D^{pol} protein was shown to be defective in the initiation of RNA replication, but not in the elongation of nascent chains (Barton *et al.,* 1996). This could also be indicative of a site of interaction with another viral or host protein, or the RNA template. Different modes of expression of the

genes encoding replication proteins, which control the stoichiometry of their synthesis, and the synthesis of subgenomic RNAs by some viruses which requires additional recognitions and controls are clearly also important in considering the evolution of replication systems. Brief details of some additional properties, relevant to replication, of the viruses in the three supergroups are included in Table II, together with virus supergroup designations based on these and other properties of the viruses (Goldbach and de Haan, 1994). The classification of hepatitis E virus in the *Caliciviridae* family (Murphy *et al.*, 1995) may need to be revised because the virus shares many properties with viruses in the alpha-like supergroup.

B. Helicases

Most positive-stranded RNA viruses contain ORFs with the potential to encode proteins with amino acid sequence motifs characteristic of well-defined RNA helicases and in many cases such ORFs have been shown to be essential for RNA replication, e.g., linker insertions in the helicase-like domain of the brome mosaic virus 1a protein were either lethal or rendered the virus temperature-sensitive for replication of all classes of RNA (positive-strand, negative-strand, subgenomic) (Kroner *et al.*, 1990), and some monoclonal antibodies which mapped to the helicase-like domain of the cucumber mosaic virus (CMV) 1a protein partially inhibited a purified CMV replicase complex, shown to contain the 1a protein (Hayes and Buck, 1990; Hayes *et al.*, 1994a). Helicases could function to unwind duplexes formed during RNA replication to allow strands to act as templates for further replication. They could also have an important function in removing secondary structure from RNA templates, e.g., to aid in initiation of negative-strand synthesis on templates with extensive 3' secondary structure, such as those with tRNA-like 3' termini, and to increase the processivity of RNA polymerases through regions of internal secondary structure. In DNA replication, duplex unwinding is generally carried out by helicases and removal of secondary structure from the resultant single-stranded templates by single-stranded DNA-binding proteins (Kornberg and Baker, 1992). However, cellular RNA helicases often have the function of removing secondary structure from ssRNA, e.g., the eukaryotic translational initiation factor, eIF-4A (Merrick, 1992). On the basis of conserved motifs, cellular and viral RNA and DNA helicases (and putative helicases) have been classified into a number of superfamilies (Gorbalenya *et al.*, 1988; Hodgeman, 1988; Habili and Symons, 1989; Gorbalenya and Koonin, 1989; Gorbalenya *et al.*, 1989, 1990; Lain *et al.*, 1989; Koonin,

TABLE II

Properties of Positive-Stranded RNA Viruses

RdRp Super-group[a]	RdRp Lineage[a]	Virus family or genus[b]	Virus examples	Host[c]	No. of genome seg-ments	Mode of expres-sion of RdRp[d]	5' End of RNA	3' End of RNA[e]	Production of sub-genomic RNA	Helicase super family[a]	Helicase lineage[a]	Virus supergroup[f]	Refer-ence[g]
1	Picorna	*Picornaviridae*	Polio, human rhino, hepatitis A, foot and mouth dis-ease, encepha-lomyocarditis	A	1	PP	VPg	poly(A)	No	3	Picorna	Picorna-like	1
1	Picorna	*Sequiviridae*	Parsnip yellow fleck	P	1	PP	VPg?	S		3	?	Picorna-like	2, 3
			Rice tungro spherical	P	1	PP	VPg?	poly(A)		3	Como		3, 4
1	Picorna	*Comoviridae*	Cowpea mosaic, tobacco ringspot	P	2	PP	VPg	poly(A)	No	3	Como	Picorna-like	5, 6
	Picorna	*Caliciviridae*	Feline calici, rabbit hemorrhagic disease	A	1	PP	VPg	poly(A)	Yes	3	Calici	Picorna-like	7
1	Poty	*Potyviridae*	Potato Y, tobacco etch, plum pox	P	1	PP	VPg	poly(A)	No	2	Poty	Picorna-like	8
			Barley yellow mosaic	P	2	PP	VPg	poly(A)	No	2	Poty		8
1	Sobemo	*Sobemovirus*	Southern bean mosaic	P	1	PP	VPg	S	Yes			Sobemo-like	9
			Cocksfoot mottle	P	1	FS, PP?	VPg	S	Yes				10
1	Sobemo	*Luteovirus* subgroup II	Potato leafroll, beet western yellows, barley yellow dwarf (RGV, RMV, RPV)	P	1	FS, PP?	VPg	S	Yes			Sobemo-like	11
1	Sobemo	*Enamovirus*	Pea enation mosaic RNA 1	P		FS, PP?	VPg?	S	Yes				12, 13
1	Sobemo	*Barnaviridae*	Mushroom bacilliform	F	1	FS?	?	S	Probably				14

1	Sobemo	*Nodaviridae*	Black beetle, flock-house, Nodamura	A	2	D	Cap	S	Yes				15
1	Arteri	*Coronaviridae*	Avian infectious bronchitis, mouse hepatitis, Berne	A	1	FS, PP	Cap	poly(A)	Yes	1	Arteri	Corona-like	16, 17, 54
1	Arteri	*Arterivirus*	Equine arteritis, simian hemorrhagic fever	A	1	FS, PP	Cap	poly(A)	Yes	1	Arteri	Corona-like	18, 19
1	Astro	*Astroviridae*	Human astro	A	1	FS, PP?	?	poly(A)	Yes				20, 21
2	Phage	*Leviviridae*	Phage Qβ, R17, MS2	B	1	D	ppp	S	No				22
2	Flavi	*Flaviviridae*	Yellow fever, tick-borne encephalitis	A	1	PP	Cap	S	No	2	Flavi	Flavi-like	23
2	Pesti	*Flaviviridae*	Bovine viral diarrhea, hepatitis C	A	1	PP	?	S	No	2	Flavi	Flavi-like	24, 25
2	Carmo	*Tombusviridae*	Tomato bushy stunt, cucumber necrosis, cymbidium ring-spot, carnation mottle, turnip crinkle	P	1	RT	Cap	S	Yes			Carmo-like	26
2	Carmo	*Machlomovirus*	Maize chlorotic mottle	P	1	RT	Cap	S	Yes			Carmo-like	26–28
2	Carmo	*Necrovirus*	Tobacco necrosis	P	1	RT	pp	S	Yes			Carmo-like	26, 29
2	Carmo	*Dianthovirus*	Red clover necrotic mosaic	P	2	FS	Cap	S	Yes			Carmo-like	26, 30
2	Carmo	*Luteovirus* subgroup I	Barley yellow dwarf (MAV, PAV, SGV)	P	1	FS	?	S	Yes			Carmo-like	11, 26
2	Carmo	*Enamovirus*	Pea enation mosaic RNA 2	P		FS	?	S	Probably				12, 13
2	Carmo	*Umbravirus*	Carrot mottle, groundnut rosette	P	1	?	?	S	Probably				31
2	Carmo	Unclassified	Beet western yellows ST9-associated RNA	P		FS	?	S	Probably				11, 32

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(continued)

TABLE II *(continued)*

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RdRp Super-group[a]	RdRp Lineage[a]	Virus family or genus[b]	Virus examples	Host[c]	No. of genome seg-ments	Mode of expres-sion of RdRp[d]	5' End of RNA	3' End of RNA[e]	Production of sub-genomic RNA	Helicase super family[a]	Helicase lineage[a]	Virus supergroup[f]	Refer-ence[g]
3	Tymo	*Capillovirus*	Apple stem grooving	P	1	D	?	poly(A)	Probably	1	Tymo	Alpha-like	33-35
3	Tymo	*Carlavirus*	Carnation latent, potato M, blue-berry scorch	P	1	PP?	Cap?	poly(A)	Yes	1	Tymo	Alpha-like	36, 55
3	Tymo	*Trichovirus*	Apple chlorotic leafspot, potato T	P	1	D	Cap?	poly(A)	Probably	1	Tymo	Alpha-like	34, 35
3	Tymo	*Tymovirus*	Turnip yellow mosaic, eggplant mosaic	P	1	PP	Cap	tRNA-like	Yes	1	Tymo	Alpha-like	37, 38
3	Tymo	*Potexvirus*	Potato X, cymbidium mosaic, foxtail mosaic	P	1	D	Cap	poly(A)	Yes	1	Tymo	Alpha-like	39
3	Tobamo	*Tobamovirus*	Tobacco mosaic, pepper mild mottle	P	1	RT	Cap	tRNA-like	Yes	1	Tobamo	Alpha-like	40
3	Tobamo	*Tobravirus*	Tobacco rattle, pea early browning, pepper ringspot	P	2	RT	Cap	tRNA-like	Yes	1	Tobamo	Alpha-like	41
3	Tobamo	*Hordeivirus*	Barley stripe mosaic	P	3	D	Cap	tRNA-like	Yes	1	Tobamo	Alpha-like	42
3	Tobamo	*Furovirus*	Soil-borne wheat mosaic	P	2	RT	Cap	tRNA-like	Probably	1	Tobamo	Alpha-like	43
3	Tobamo	*Idaeovirus*	Raspberry bushy dwarf	P	2	D	Cap?	S	Yes	1	Tobamo	Alpha-like	44, 45
3	Tobamo	*Bromoviridae*	Brome mosaic, cucumber mosaic	P	3	D	Cap	tRNA-like	Yes	1	Tobamo	Alpha-like	46, 47
			Alfalfa mosaic, tobacco streak	P	3	D	Cap	S	Yes	1			48

3	Tobamo	*Closterovirus*	Beet yellows, citrus tristeza	P	1	FS, PP	Cap	S	Yes	1	Tobamo	Alpha-like	33, 34
3	Tobamo	*Closterovirus* (Tentative)	Lettuce infectious yellows	P	2	FS, PP	Cap?	S	Yes	1	Tobamo	Alpha-like	49
3	Rubi	*Togaviridae*	Semliki Forest, Sindbis, rubella	A	1	PP	Cap	poly(A)	Yes	1	Rubi	Alpha-like	50, 51
3	Rubi	*Caliciviridae*	Hepatitis E	A	1	PP	Cap	poly(A)	Yes	1	Rubi	Alpha-like?	52
3	Rubi	*Furovirus* (Tentative)	Beet necrotic yellow vein virus	P	4	D	Cap	poly(A)	Yes	1	Rubi	Alpha-like	53

[a] RNA-dependent RNA polymerase supergroups and lineages, and helicase superfamilies are from Koonin and Dolja (1993).

[b] Families and genera are from Murphy *et al.* (1995). A genus is only given when not assigned to a family.

[c] A, animal; B,bacterium; F, fungus; P, plant.

[d] PP, polyprotein processing; RT, readthrough; FS, frameshift; D, direct translation.

[e] S, structure other than tRNA-like or poly(A).

[f] Virus supergroups as defined by Goldbach and de Haan (1994).

[g] Data are from Murphy *et al.* (1995) and the following. 1. Wimmer *et al.* (1993). 2. Turnbull-Ross *et al.* (1993). 3. Reavy *et al.* (1993). 4. Shen *et al.* (1993). 5. Peters *et al.* (1995). 6. Mayo and Fritsch (1994). 7. Lambden and Clarke (1995). 8. Riechman, J. L. *et al.* (1992). 9. Othman and Hull (1995). 10. Makinen *et al.* (1995). 11. Miller *et al.* (1995). 12. Demler *et al.* (1993). 13. Demler *et al.* (1994). 14. Revill *et al.* (1994). 15. Ball, L. A. (1995). *J. Virol.* **69**, 720–727. 16. Lai (1990). 17. Snijder and Horzinek (1993). 18. Plagemann and Moenning (1992). 19. Snijder *et al.* (1995). 20. Jiang *et al.* (1993). 21. Willcocks *et al.* (1994). 22. Van Duin (1988). 23. Chambers *et al.* (1990). 24. Collett (1992). 25. Matsuura and Miyamura (1993). 26. Russo *et al.* (1994). 27. Nutter *et al.* (1989). 28. Lommel *et al.* (1991). 29. Meulewater *et al.* (1992). *J. Virol.* **66**, 6419–6428. 30. Giesman-Cookmeyer *et al.* (1995). 31. Gibbs (1995). 32. Passmore *et al.* (1993). 33. Coffin and Coutts (1993). 34. Dolja *et al.* (1994). 35. Candresse (1993). 36. Foster (1992). 37. Kadare *et al.* (1992). 38. Kadare *et al.* (1995). 39. Solovyev *et al.* (1994). 40. Dawson and Lehto (1990). 41. Zerfass and Beier (1992). 42.Donald and Jackson, 1994. 43. Shirako and Wilson (1993). 44. Natsuaki *et al.* (1991). 45. Ziegler *et al.* (1992). 46. Ahlquist (1992). 47. Palukaitis *et al.* (1992). 48. Reusken *et al.* (1995). 49. Klaasen *et al.* (1995). 50. Strauss and Strauss (1994). 51. Frey (1994). 52. Purdy *et al.* (1993). 53. Richards and Tamada (1992). 54. Cavanagh and MacNaughton (1994). 55. Hillman and Lawrence (1995).

1991b; Bork and Koonin, 1993; Koonin and Dolja, 1993), with several
lineages or subfamilies associated with each superfamily. The super-
family and lineage designations for the positive-stranded virus RNA
helicases and putative helicases (Koonin and Dolja, 1993) are given in
Table II. Superfamilies 1 and 2, which have been shown to be distantly
related, were each characterized by seven motifs (which could all be
aligned between the two superfamilies), whereas superfamily 3 has
just three motifs. Two of the motifs, versions of which are present in all
three superfamilies (designated I and II for superfamilies 1 and 2, and
A and B for superfamily 3), are variants of the ATP-binding motifs, first
described by Walker et al. (1982): A, GXXXXGK[TS] and B, $\Phi\Phi\Phi\Phi$D
where X is an unspecified amino acid residue, Φ is a hydrophobic resi-
due, and the residues in square brackets are alternates. Variants of
motif A are present in a vast class of both ATP-binding proteins and
GTP-binding proteins; crystallographic structural studies on several of
these indicate that motif A (I) is in the NTP-binding site located in a
phosphate-binding loop (P-loop) between a β-strand and an α-helix.
The B (II) motif may provide D residues required for chelation of Mg^{2+}
ions which in turn bind to the terminal phosphates and promote NTP
hydrolysis (Saraste et al., 1990; Schulz, 1992). The consensus se-
quences for the motifs for the three viral superfamilies (Koonin and
Dolja, 1993) are shown in Table III.

The (putative) helicases of the positive-stranded RNA viruses in
superfamily 2 constitute a subset of proteins containing variants of the
sequence DEAD (DEAD box proteins), present in motif II as DEXH,
some of which have been demonstrated to have RNA-dependent ATPase
and RNA helicase activity, e.g., eIF-4A (Rozen et al., 1990; Jaramillo et
al., 1990), the human nuclear protein p68 (Hirling et al., 1989), vac-
cinia virus nucleoside triphosphohydrolase (I8R protein) (Schuman,
1992, 1993; Bayliss and Smith, 1996), and human RNA helicase A (Lee
and Hurwitz, 1993). These helicases function in a 3′ to 5′ direction,
requiring a duplex RNA with a 3′ overhang, except for eIF-4A which
has bidirectional activity (but requires also eIF-4B for helicase activ-
ity). Mutagenesis of eIF-4A has shown that the AXXXXGKT motif (see
motif I, Table III) is required for ATP binding, whereas the DEAD
sequence (see motif II, Table III) functions in ATP hydrolysis and coupl-
ing of ATP hydrolysis to helicase activity. Two other motifs, SAT (pres-
ent in motif IV, Table III) and **HRIGRXXR** (the residues shown in bold
are present in motif VI, Table III) were shown to be needed for eIF-4A
RNA helicase activity, the latter sequence functioning specifically in
RNA binding (Pause and Sonenberg, 1992; Pause et al., 1993). The
counterpart of the latter motif in the vaccinia virus DEXH box RNA

TABLE III

Consensus Sequence of Conserved Motifs in Superfamilies of Positive-Stranded RNA Virus Helicase-like Proteins

Super-family[a]	Motif I[b]	N[c]	Motif IA	N	Motif II	N	Motif III	N

1	XXX&XGXPGXGKTXX&XX
	    A A S
		1–9	XXXX&XXXXXXXXXX&	12–52	X&&&DE&X
					      DA
						8–40	&&&&GDXXQ
							AACC
								9–30

2	&&XXXXGSGKTXXX&P
	      A S
		6–14	XRX&UUXPTRXUXXE&
			  K A SK A N
				43–51	&&&&DEXH	16–24	X&XUTATPP
							      S
								39–52

Super-family	Motif A	N			Motif B	N	Motif C	

3	XEP&X&&&XGXXGXGKSXXX
	  D C T
		26–32			XQX&&U&DD
					  E AC E
						30–36	KGXX@XSX&U&XSTNX
							                TS	

Super-family	Motif IV	N	Motif V	N	Motif VI

1	XXXXXXXRX	46–86	XXXXT&XXXQGXT&XXVX&&X
			     SA S AC
				6–14	XXXXX&VAUTRXXX
					        G S

2	X&XUTATPP
	     S
		31–40	&U&XTD&XEXGUX&XXXXXUU
			      N A
				20–28	TXXXXXQRXGRUGR

[a] Superfamilies and data from Koonin and Dolja (1993).

[b] X, unspecified residue; U, bulky aliphatic residue (I, L, V, M); @, aromatic residue (F, Y, W); &, bulky hydrophobic residue (aliphatic or aromatic). Alternate residues are shown below the sequence. Residues conserved in motifs between more than one family are shown in bold. Residues underlined in superfamily 2 are conserved in corresponding motifs in DEAD box proteins (Fuller-Pace, 1994).

[c] N, Number of amino acid residues between the motifs (range).

helicase was required for ATP hyrolysis and RNA unwinding, but not for RNA binding, suggesting that the contribution of conserved helicase motifs to overall protein function may be context-dependent (Gross and Schuman, 1996).

RNA-stimulated ATPase, RNA binding, and RNA helicase activity have also been shown for a number of positive-stranded RNA virus proteins in the DEXH subset of helicase superfamily 2, the CI protein of plum pox virus (PPV) (Table II, Poty helicase lineage) (Lain *et al.*, 1990, 1991; Fernandez *et al.*, 1995), the tamarillo mosaic virus (TaMV) CI protein (poty helicase lineage) (Eagles *et al.*, 1994); the bovine viral diarrhea virus (BVDV) p80 (NS3) protein, and equivalent proteins of yellow fever virus and hepatitis C virus (all flavi helicase lineage) (Suzich *et al.*, 1993; Tamura *et al.*, 1993; Warrener *et al.*, 1993; Warrener and Collett, 1995; unpublished results quoted in Warrener and Collett, 1995). The helicase activities of the PPV CI protein (isolated from infected plants) and the BVDV NS3 protein (expressed in insect cells) were shown to require a dsRNA substrate with a 3' single-stranded overhang and to function in a 3' to 5' direction; dsRNA with a 5' overhang or blunt-ended dsRNA was not a substrate for the helicase. Helicase activity of the TaMV CI protein (isolated from infected plants) also functioned in a 3' to 5' direction (other substrates were not tested). The helicases of all three viruses could be stimulated by all four NTPs, with a slight preference in the case of PPV protein for purine NTPs. Binding of the PPV and TaMV CI proteins to RNA did not require an NTP; deletion mutagenesis of the PPV CI protein (expressed in *E. coli* as a maltose-binding fusion) located the RNA binding site to a region containing motif VI. The RNA-dependent ATPases of West Nile and yellow fever flaviviruses (expressed in *E. coli*) were located to a C-terminal fragment of the NS3 protein containing the seven conserved helicase motifs (Table III) (Warrener *et al.*, 1993; Wengler and Wengler, 1993). It is noteworthy that the basal level of ATPase activity of the viral helicases in the absence of RNA was much higher than that of the cellular RNA helicases in superfamily 2.

In the helicase superfamily 3, RNA (and DNA) helicase activity has been demonstrated for the simian virus 40 (SV40) large T antigen (Scheffner *et al.*, 1989). Like the viral RNA helicases of superfamily 2, the SV40 RNA helicase activity functioned in a 3' to 5' direction; furthermore, mutations of DE to AA in motif B abolished the ATPase activity of this protein (Weiner and Bradley, 1991). No helicase activity has yet been demonstrated for a positive-stranded RNA virus protein in this superfamily, but studies have indicated the importance of the putative helicases of cowpea mosaic virus (58-kDa protein or region of

B polyprotein precursor; como helicase lineage) and poliovirus (2C protein; picorna helicase lineage) in virus replication. Mutation of the conserved K500 residue to T in the motif A sequence GKSRTGK500S in the 58-kDa domain reduced cowpea mosaic virus B RNA replication in cowpea protoplasts to an undetectable level (Peters *et al.*, 1994). A mutation of the conserved D545 to P in the motif B sequence MDD545 was also lethal. Both mutations were shown to act at the protein, rather than RNA, level. However, whereas the K→T mutation in motif A caused a small reduction in ATP binding and an altered distibution of viral proteins, which failed to aggregate into the large cytopathic structures observed in protoplasts infected with wild-type B-RNA, no such effects were observed with the D→P mutation in motif B.

The poliovirus 2C protein and its NTP binding site have been shown to be essential for RNA replication. The 2C protein is present in membranous replication complexes, isolated either from infected cells (Bienz *et al.*, 1990) or from a combined *in vitro* translation–replication system (Barton *et al.*, 1995). Poliovirus RNA replication is inhibited by guanidine hydrochloride at the level of initiation of RNA synthesis (Caliguiri and Tamm, 1973; Barton *et al.*, 1995) and guanidine-resistant (gr) and guanidine-dependent (gd) mutants map to the 2C protein (Wimmer *et al.*, 1993; Tolskaya *et al.*, 1994), implying a role for the 2C protein in the initiation of RNA synthesis. Mutations in the conserved amino acids of the NTP-binding motifs A (129GSPGTGKS136) and B (176DD177), e.g., G129I, K135Q, K135R, S136T, S136A, D176L, and D177L, abolished or greatly reduced RNA synthesis (Mirzayan and Wimmer, 1992; Teterina *et al.*, 1992). The 2C protein, expressed in *E. coli* as a maltose-binding protein fusion (Rodriguez and Carrasco, 1993) or in insect cells not as a fusion (Mirzayan and Wimmer, 1994a), has been shown to have ATPase activity. A K135Q mutation in motif A abolished ATPase activity of the insect-expressed protein. Taken together with the previous observation that this mutation in the virus greatly reduces RNA replication, this result implies that ATP hydrolysis, dependent on this site, is required for RNA replication. The defect in the mutant was probably in ATP binding since the wild-type, but not the mutant, 2C protein bound to ATP-agarose. The guanidine-resistant and guanidine-sensitive mutants mapped to a region overlapping the conserved helicase motifs, although none of the conserved amino acids was altered (Wimmer *et al.*, 1993; Tolskaya *et al.*, 1994). The 2C ATPase activity was not sensitive to concentrations of guanidine which inhibit poliovirus RNA replication *in vivo* (Mirzayan and Wimmer, 1994a) and it was suggested that the region targeted by guanidine, while not being directly involved in ATP binding or hydrolysis, may nevertheless

be important in a coupled downstream event, such as RNA binding and
duplex unwinding (Tolskaya *et al.,* 1994), as described above for some
of the superfamily 2 helicases. However, the 2C ATPase activity was
not stimulated (Mirzayan and Wimmer, 1994a), or modestly stimulated
(twofold) (Rodriguez and Carrasco, 1993), by addition of RNA, al-
though the 2C protein contains a consensus dsRNA-binding sequence
(Paul *et al.,* 1994b). Hence, if the 2C protein is a helicase, it differs from
the superfamily 2 helicases described above, although the possibility
that the (putative) 2C helicase activity requires interaction with an-
other viral or cellular protein for interaction with RNA and unwinding
activity cannot be discounted. It is also noteworthy that the poliovirus
3D^{pol} protein has an unwinding (strand-displacement activity) (Cho *et al.,*
1993), as described in Section I. Although none of these experiments
rules out the hypothesis that the 2C protein is a helicase with a role in
the initiation of RNA replication, the possibility arises that ATP hydrol-
ysis might be coupled to some other function of the 2C protein, such as
virion uncoating (Li and Baltimore, 1988, 1990), encapsidation of viral
RNA, or membrane trafficking (both of which are likely to be important
in RNA replication), as suggested by Mirzayan and Wimmer (1994a).

   RNA helicase activity has not yet been demonstrated for any of the
proteins in superfamily 1. Duplex unwinding in a 3'→5' direction has
been demonstrated for a purified RdRp complex, isolated from plants
infected with alfalfa mosaic virus (de Graaf *et al.,* 1995a). Such RdRp
preparations contain the helicase-like P1 protein (Quadt *et al.,* 1991),
although it was not demonstrated whether the unwinding was due to
the P1 protein, the P2 polymerase, or some other protein component of
the complex. Several members of helicase superfamily 2 have DNA heli-
case activity, e.g., the *E. coli* Rep protein, which has DNA-dependent
ATPase activity, translocates in the 3'→5' direction, and can unwind
RNA/DNA hybrids (Kornberg and Baker, 1992).

   The nsP2 protein of Semliki Forest virus is a multifunctional protein
which has several essential functions in RNA synthesis, as well as a
C-terminal papain-like protease domain (Strauss and Strauss, 1994).
Its N-terminal half contains sequence motifs characteristic of the heli-
case superfamily 1 (rubi lineage, Table II) and a mutant with a C→Y
mutation at residue 304 between motifs IV and V was temperature-
sensitive for subgenomic RNA synthesis (Hahn *et al.,* 1989b). The nsP2
protein when expressed in *E. coli* as a fusion protein with a short
N-terminal addition containing a histidine tag was found to have ATP
and GTP binding activity, ATPase and GTPase activity further stimu-
lated by the presence of ssRNA, and RNA binding capacity. Similar
activities were found with a truncated nsP2 protein containing the

N-terminal 470 amino acid residues, which contained the helicase-like motifs but lacked the C-terminal protease domain. A mutant nsP2 protein in which the conserved lysine residue of motif I was replaced by asparagine exhibited no ATPase or GTPase activity. By analogy with the superfamily 2 and 3 NTPases discussed above, it is likely that this residue functions in NTP binding. It is likely that the NTPase activity of the nsP2 protein is coupled in some way to its function in RNA synthesis, but attempts to demonstrate helicase activity in the *E. coli*–expressed fusion protein were unsuccessful. As discussed for the poliovirus 2C protein, it is possible that interaction with another viral or host protein is needed for helicase activity.

A segment of the rubella virus nonstructural protein containing the helicase-like domain, when expressed as part of a fusion protein in *E. coli*, was shown to have RNA-stimulated NTPase activity (Gros and Wengler, 1996). Although helicase or NTPase activity was not demonstrated for the turnip yellow mosaic virus 140-kDa protein, a K→S substitution in the motif I sequence GFAGCGKT of its helicase-like domain resulted in complete loss of detectable RNA replication (Weiland and Dreher, 1993).

It is noteworthy that all the supergroup 3 RNA polymerases were found in viruses with superfamily 1 helicase-like proteins (Table II). There was also complete correspondence between the polymerase and helicase lineages of these viruses. This probably indicates coevolution and a long-standing association of the polymerase-like and helicase-like genes of these viruses, some of which have animal hosts and others plant hosts, and which constitute the alpha-like (Sindbis-like) virus supergroup (Goldbach and de Haan, 1994). Similarly, the supergroup 1 RNA polymerases of the picorna lineage were found in animal and plant viruses with superfamily 3 helicase-like proteins (Table II), again indicating likely coevolution of these genes. However, plant viruses with supergroup 1 RNA polymerases of the poty lineage and animal viruses with supergroup 2 polymerases of the flavi and pesti lineages both had helicase-like proteins of superfamily 2 (Table II) (Lain *et al.*, 1989). This could indicate creation of new combinations of polymerase and helicase genes in the evolution of these viruses. The coronaviruses, toroviruses, and arteriviruses, which are somewhat divergent from other positive-stranded RNA viruses, contain a unique combination of supergroup 1 polymerases and superfamily 1 helicase-like proteins. As helicase activity has as yet only been demonstrated for positive-stranded RNA viruses of the helicase superfamily 2 and the role of such activities in the replication of the viruses concerned has yet to be confirmed, it is premature to speculate as to what extent the sequences

which define the different helicase superfamilies are reflected in any significant differences in their activities or roles in RNA replication.

It is noteworthy that several families of positive-strand RNA viruses appear to lack the characteristic NTP-binding motifs A (I) and B (II) (Table III). These include some viruses with supergroup I polymerases of the sobemo lineage [the *Sobemovirus* and *Luteovirus* subgroup II genera of plant viruses and the *Nodaviridae* family of animal (mainly insect) viruses] and the animal astroviruses, and some viruses with supergroup II polymerases (the *Tombusviridae* family and *Machlomovirus, Necrovirus, Dianthovirus,* and *Luteovirus* subgroup I genera of plant viruses, and the *Leviviridae* family of ssRNA bacteriophages). All of these viruses, except the astroviruses, have genomes smaller than 6 kb. Typical purine NTP-binding sites could also not be detected in negative-stranded RNA viruses (*Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae*), some dsRNA viruses (*Birnaviridae*), and the retrovirus/pararetrovirus group (*Retroviridae, Hepadnaviridae, Caulimovirus*) (Gorbalenya and Koonin, 1989). There are several possible explanations for this. (i) It is possible that NTP-binding motifs are present, but have diverged too much to be easily recognized by primary sequence comparisons. Such variants in cellular NTP-binding proteins are known (Traut, 1994), e.g., the counterparts of motifs A (I) and B (II) in the ATP binding site of actin are DNGSGLCKA and GIVLDSGDGV, where the residues in bold have been shown from crystallographic structural analysis to make contact with the ligand (Kabsch *et al.*, 1990). Possible variants of motif A (I) (soybean dwarf luteovirus, SDV; southern bean mosaic sobemovirus, SBMV), motif B (II) (SDV), motif IV (maize chlorotic mottle machlomovirus, MCMV; carnation mottle carmovirus, CMoV; cucumber necrosis tombusvirus, CNV; barley yellow dwarf luteovirus, BYDV-PAV; SDV; beet western yellows luteovirus, BWYV; potato leafroll luteovirus, PLRV), and motif VI (MCMV; CMoV; CNV; BYDV-PAV; SDV; BWYV; PLRV; SBMV) have been recognized (Habili and Symons, 1989), but their significance remains to be determined. (ii) The virus polymerase may have unwinding (strand-displacement) activity, as shown for poliovirus RdRp (Cho *et al.*, 1993). (iii) Unwinding might be accomplished by a helix-destabilizing protein which utilizes the energy of stoichiometric binding to single-stranded nucleic acid to drive melting of a duplex in the absence of NTP hydrolysis. The poliovirus $3D^{pol}$ protein has been shown to bind cooperatively to single-stranded RNA under certain conditions (Pata *et al.*, 1995). (iv) The virus might co-opt a cellular helicase to aid in duplex unwinding.

Some viruses with superfamily 1 putative helicases, such as hordei-viruses, potexviruses, and beet necrotic yellow vein virus (BNYVV),

encode a second protein with helicase-like motifs, which is not required for RNA replication. Such proteins have been considered to arise from the replicative helicase-like protein by a gene duplication event, followed by diversification of function (Koonin and Dolja, 1993). The additional helicase-like proteins of hordeiviruses, potexviruses, and BNYVV have been shown to be involved in virus cell-to-cell movement in infected plants (Petty et al., 1990; Beck et al., 1991; Gilmer et al., 1992a).

1. Models for RNA Replication: The Role of Helicases

Different basic models have been proposed for the replication of positive-stranded RNA viruses, which involve intermediates with different structures and which have implications for the involvement of helicases in the replication process. Three models are shown in Fig. 1. In model 1, the RdRp recognizes a promoter at the 3' end of the positive-strand RNA template (a) and starts to synthesize a complementary negative strand. The nascent negative strand only remains base-paired to the positive strand in the region where the polymerase binds to the template and is actively synthesizing RNA. The 5' tail of the nascent negative strand is not base-paired to the template; hence most of the replicative intermediate (b) is in a single-stranded form. Continuation of the reaction leads to the formation of a free negative strand product (c), and releases the positive-strand template. The polymerase then recognizes a promoter at the 3' end of the negative strand and, using the negative strand as a template, starts to synthesize a progeny positive strand, giving a second type of replicative intermediate (d). As before, the nascent strand is only base-paired to the template in the region of the active site of the polymerase where RNA synthesis is taking place, so that this replicative intermediate is also mainly single-stranded. Before the synthesis of the first progeny positive strand has been completed, initiation of synthesis of further positive strands takes place, giving a replicative intermediate consisting of a full-length negative-stranded template, to which are attached several nascent positive strands which again are largely in a single-stranded form (e). The process continues to synthesize and release multiple copies of the progeny positive-stranded RNA (f).

The first stage of model 2 is essentially the same as that of model 1, except that the negative strand formed remains base-paired with the positive-stranded template, giving a replicative intermediate (b), consisting of partially double-stranded and partially single-stranded structure. The reaction continues to give a fully double-stranded RNA (replicative form) (c). In this model, no free negative strand is synthesized. The polymerase then recognizes a promoter at the end of the

MODEL 1
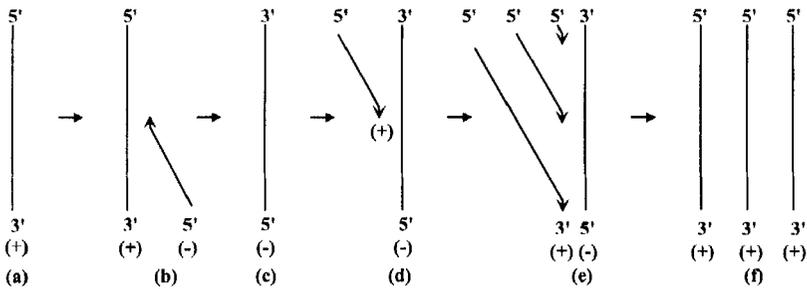
MODEL 2
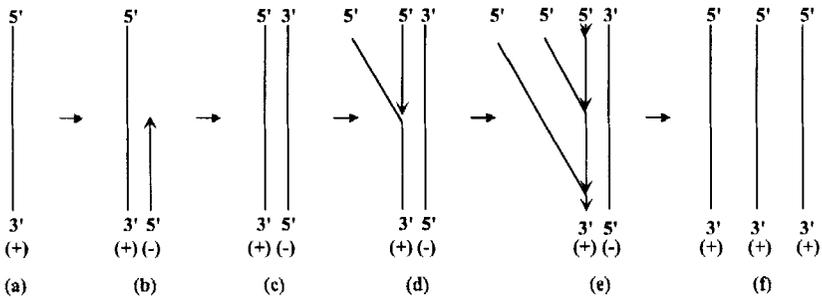
MODEL 3
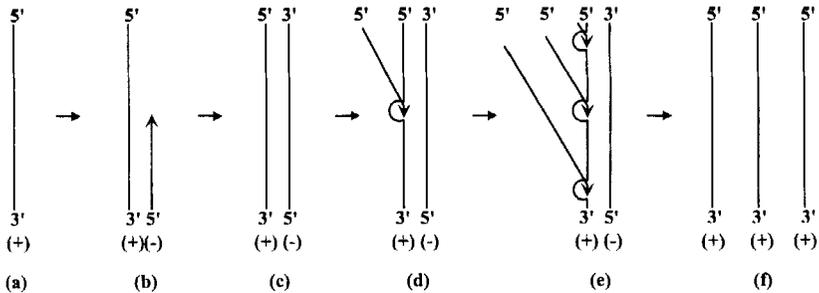
FIG. 1. Models for RNA replication. See text for detail.

replicative form dsRNA containing the 3' end of the negative strand and the 5' end of the positive strand. Synthesis of progeny positive-stranded RNA commences using the negative strand as a template by a strand-displacement mechanism, giving rise to replicative interme-diates consisting of double-stranded RNA with one (d), or following reinitiations several (e), single-stranded 5' tails of the full-length posi-

tive strands. The first full-length positive strand to be released from the replicative intermediate will be the original template strand (a); continued reaction will then result in the synthesis and release of multiple progeny positive strands (f). The formation of the double-stranded replicative form RNA in model 3 is exactly the same as in model 2. However, synthesis of progeny positive-stranded RNA using the negative strand of the dsRNA only displaces the positive strand of the dsRNA transiently in the region where RNA synthesis is taking place. The replicative intermediates formed (d, e) consist of double-stranded RNA with one or several single-stranded tails, but unlike the replicative intermediates in model 2 (d, e) in which the single-stranded tails are the displaced 5′ tails of full-length positive strands, these single-stranded tails belong to the nascent, incomplete progeny positive strands. The synthesis of progeny positive strands from a dsRNA replicative form RNA in model 2 is analogous to the semiconservative transcription of dsRNA by strand displacement characteristic of dsRNA viruses of the *Birnaviridae, Cystoviridae,* and *Partitiviridae* families (Buck, 1979; van Etten *et al.,* 1980; Dobos and Roberts, 1983), whereas that in model 3 is analogous to conservative transcription of dsRNA characteristic of dsRNA viruses of the *Reoviridae* and *Totiviridae* families (Shatkin and Kozak, 1983; Fujimura and Wickner, 1989).

Model 1 has been shown to operate in the replication of Qβ RNA. Qβ RNA replicase holoenzyme catalyzes the complete replication of Qβ RNA; it can utilize either the positive or negative strands as templates and produces free progeny positive and negative strands. Replicative intermediates are largely single-stranded and the replicase cannot utilize Qβ dsRNA as a template (Blumenthal and Carmichael, 1979). There is evidence that the ability of the progeny nascent strands to form stable secondary structures is important in the production of single-stranded RNA progeny. Templates with little secondary structure had a tendency to form extended RNA–RNA duplexes during replication, resulting in reduced synthesis of new RNA strands (Priano *et al.,* 1987; Axelrod *et al.,* 1991). It appears that immediately after the complementary base pairs are formed during Qβ RNA replication, they are rapidly unwound, presumably by one of the proteins in the replication complex (which does not include a recognizable helicase), although the mechanism is unknown.

The nature of the replicative intermediates in the replication of the eukaryotic positive-stranded RNA viruses is less clear. Double-stranded RNAs (usually called replicative form or RF dsRNAs) are usually found in nucleic preparations from cells infected with positive-strand RNA viruses or in the extracted products of *in vitro* RNA synthesis reactions with crude or partially purified polymerase preparations from infected

plants or animal cells (de Graaf and Jaspars, 1994), irrespective of the polymerase or helicase type. Double-stranded RNA could arise *in vivo* as dead-end products formed by annealing of positive and negative strands; even in the case of Qβ, for which model 1 (Fig. 1) is well established, dsRNA accumulates as the concentrations of viral RNAs in the cell increase (Priano *et al.*, 1987). Furthermore, annealing of positive and negative strands can also occur on extraction of nucleic acids from cells by deproteinization. Similar considerations apply to the different types of replicative intermediates. The "closed" intermediates (Fig. 1, model 2, d, e) could be formed by collapse of the "open" intermediates (Fig. 1, model 1, d, e). dsRNAs extracted from *in vitro* RNA synthesis reactions could be formed in the same way, or could result from inadequacies in the *in vitro* systems. Many *in vitro* template-dependent systems synthesize only negative-stranded RNA, indicating a lack of one or more essential components (or the presence of an inhibitor).

Isolated poliovirus replicative intermediates consist of a full-length negative-strand template to which are attached six to eight nascent positive strands, but conflicting evidence has been presented that such intermediates have either an "open," mainly single-stranded structure (Oberg and Philipson, 1971; Richards *et al.*, 1984; Bienz *et al.*, 1994) or a "closed" structure (Meyer *et al.*, 1978; Nilsen *et al.*, 1981; Troxler *et al.*, 1992). Using electron microscopy and other techniques, Garnier *et al.* (1980) showed that the turnip yellow mosaic virus replicative intermediates formed *in vivo* were mainly in the single-stranded form and considered that the dsRNA isolated from infected leaves was mainly an isolation artifact, although some dsRNA may be formed *in vivo* late in infection. Using an *in vitro* system able to catalyze the complete replication of cucumber mosaic virus RNA, Hayes and Buck (1990) detected free positive and negative strands in the ratio of 7:1, as well as some dsRNA, suggesting that replication occurred according to model 1 and showing that some free negative strands could survive the phenol extraction procedure in the presence of an excess of positive strands. DsRNA was not a template for the replicase complex (Hayes and Buck, 1993), but this could merely indicate that the replicase lacked an essential component required to initiate synthesis on a dsRNA template. Evidence was obtained that flavivirus RNA positive-strand RNA molecules are synthesized from negative-strand templates by a semiconservative mechanism, as in model 2 (Fig. 1) (Chu and Westaway, 1985; Cleaves *et al.*, 1981). Wu and Kaesberg (1991), using a flockhouse virus template-dependent *in vitro* RNA replication system, showed that labelled nucleotides incorporated into dsRNA during a short pulse could be chased into ssRNA using excess unlabelled nucleotides, sug-

gesting "closed" replication intermediates (Fig. 1, model 2). Although free negative-stranded RNA has generally not been isolated from eukaryotic cells infected with positive-stranded RNA viruses, replication of flockhouse virus RNA 2 could be initiated from a negative-stranded template in cells in which replication proteins were provided in trans (Ball, 1994). Furthermore, an isolated cucumber mosaic virus RdRp complex was capable of replicating a satellite RNA when provided with the negative-strand satellite RNA (Wu and Kaper, 1994), an isolated brome mosaic virus RdRp complex could use negative-strand templates to produce subgenomic RNA (Dreher and Hall, 1988), and an isolated alfalfa mosaic virus RdRp could use a negative-strand template to produce full-length and subgenomic RNA (de Graaf *et al.*, 1995a,b). However, attempts to use negative strands for the replication of alphaviruses were not successful (reviewed by Strauss and Strauss, 1994). Finally, some evidence for the formation of double-stranded replication intermediates comes from the observation that transgenic plants expressing a yeast double-stranded RNA-specific ribonuclease showed resistance (albeit incomplete) to tomato mosaic virus, cucumber mosaic virus, and potato virus Y (Watanabe *et al.*, 1995). In model 1, it would be anticipated that the short double-stranded regions where the nascent transcript is bound to the template would be protected by the replicase complex and hence not accessible to the dsRNA ribonuclease.

Model 3 can be eliminated in some cases, e.g., labelled nucleotides incorporated into tobacco mosaic virus RF by an isolated polymerase preparation containing endogenous template were predominantly in the positive strand (Young and Zaitlin, 1986), which is not consistent with model 3. Generally, the available evidence does not allow the "open" and "closed" models (models 1 and 2) to be distinguished unequivocally for any eukaryotic positive-stranded RNA virus and further work is needed in this area. It is possible that different mechanisms operate for different viruses or groups of viruses. This could be related to different mechanisms for the initiation of positive-stranded RNA synthesis and to the requirement for some viruses to synthesize subgenomic RNAs. For example, viruses that initiate with a VPg may employ a different mechanism than those that are capped. Model 2 requires that the double-stranded replicative form RNA is unwound at the end containing the 3' terminus of the negative strand before synthesis can begin. Most RNA helicases, including the cellular and viral helicases discussed above, and most DNA helicases (Kornberg and Baker, 1992) bind to single-stranded regions adjacent to the duplex region to be unwound, and do not act on completely double-stranded structures. Unwinding of a completely double-stranded RF could therefore involve another protein. Conversion of the *E. coli* replication origin to an open

structure requires the sequence-specific DNA-binding protein, the dnaA protein (Bramhill and Kornberg, 1988). Further unwinding of the duplexes as DNA synthesis proceeds at the replication forks utilizes helicases. In the case of positive-stranded RNA replication, a $3' \rightarrow 5'$ helicase bound to the negative template strand of the unwound origin (promoter) could perform the subsequent duplex unwinding. The plum pox and bovine diarrhea virus helicases (Lain *et al.*, 1990; Warrener and Collett, 1995) have the required strand-displacement activity, as does the poliovirus $3D^{pol}$ protein (Cho *et al.*, 1993) and an alfalfa mosaic virus RdRp complex (de Graaf *et al.*, 1995a). Model 2 requires recognition of a double-stranded structure for initiation of positive-strand synthesis (and for subgenomic RNA synthesis for those viruses that utilize subgenomic promoters), whereas model 1 requires recognition of a single-stranded structure. Since secondary structure in the single-stranded RNA appears to be important for recognition of at least some genomic and subgenomic promoters (see Section V,D), model 1 would appear to be favored in this aspect. Model 1 requires an explanation, not required for model 2, as to how the base pairs formed at RNA synthesis are almost immediately unwound by the replicase complex. This could probably be accomplished by a helix-destabilizing protein, or a second molecule of the helicase, suitably positioned in the replicase complex. In this respect, it is noteworthy that purified replication complexes of cucumber mosaic virus (Hayes and Buck, 1990) and brome mosaic virus (Quadt *et al.*, 1993) appear to contain more of the respective 1a protein (which contains the helicase-like domain) than the 2a protein (which contains the polymerase-like domain), and brome mosaic virus RNA virus replication was found to be more sensitive to reductions in the expression of the 1a protein than to reductions in the expression of the 2a protein (Kroner *et al.*, 1990; Traynor and Ahlquist, 1990; Dinant *et al.*, 1993). It should be noted, however, that the 1a protein also has capping functions (see Section II,C).

The different replication models also have some implications for RNA recombination. There is good evidence that, at least in some cases, RNA recombination leading to the formation of viable recombinants or DI RNAs requires RNA replication (reviewed by Lai, 1992; Bujarski *et al.*, 1994; Simon and Bujarski, 1994). The favored mechanism, called "copy choice," requires the polymerase, together with the nascent strand, to switch from one template (the donor) to another template (the recipient), either after first dissociating from the donor template (nonprocessive model) or in a processive fashion without first dissociating. Recombination can occur during negative-strand synthesis, as for poliovirus (Kirkegaard and Baltimore, 1986), brome mosaic

virus (Bujarski *et al.,* 1994), and flockhouse virus (Li and Ball, 1993), or during positive-strand synthesis, as for turnip crinkle virus (Carpenter *et al.,* 1995). In replication model 2 (Fig. 1) in the negative-strand-synthesizing replicative intermediate (b) and the positive-strand-synthesizing replicative intermediate (d, e), the nascent strand remains completely base-paired to its template. This would appear to make it more difficult, in the nonprocessive model, for the polymerase and nascent strand to dissociate from the template or, in the processive model, could create a structure in which the newly synthesized recombinant strand is base-paired to both donor and recipient templates. The problem would be overcome if a second RdRp complex were advancing along the template close behind, carrying out displacement synthesis. If a second polymerase complex "caught up" with a paused complex, the resulting change of structure could facilitate detachment or strand switching, depending on the type of recombination event. Some processive models of recombination require the recipient template strand to be base-paired to the donor template strand in front of the advancing replicase. This would appear to present a problem in replication model 2 for recombination occurring during positive-strand synthesis, unless the recipient template were able to specifically invade the unwound RNA where RNA synthesis is taking place. This problem would not occur with any of the models of RNA replication for recombination taking place during negative-strand synthesis.

In recombination models in which the replicase pauses at a duplex region in the template to allow the helicase to unwind it (or to switch templates), it might be expected that mutations in the helicase protein could affect the site of recombination. Evidence that this occurs has been obtained using mutants of brome mosaic virus with amino acid insertions in the helicase-like domain of the 1a protein (Nagy *et al.,* 1995). It has been suggested that the apparent great propensity of viruses such as the tombus-, carmo-, and luteoviruses to form recombinants and DI RNAs could be due to their missing recognizable NTP-binding and helicase domains (Simon and Bujarski, 1994; Gibbs, 1995). The apparent lack of a virus-encoded helicase could render the RdRps of these viruses less processive and hence more prone to strand switching.

C. Capping and Methylation Enzymes

The presence of a 5′ cap structure is characteristic of most eukaryotic mRNAs, and mRNAs of DNA viruses, retro- and pararetroviruses, negative-strand RNA viruses, some double-stranded RNA viruses, and some positive-stranded RNA viruses. The simplest cap structure (cap 0)

consists of 7MeGpppN, where N is usually A or G. The cap 1 and cap 2 structures are 7MeGpppNmpN' and 7MeGpppNmpN'm, where m represents methylation of the 2'-OH group of the ribose moiety of the nucleoside. Capping of the cellular mRNAs and those of most DNA viruses, retroviruses, and pararetroviruses occurs in the nucleus using cellular capping enzymes; capping of influenza virus mRNAs also occurs in the nucleus and involves endonucleolytic cleavage of capped oligonucleotides from cellular mRNAs for use as primers in transcription of the negative-stranded RNA genome. Capping of mRNAs of some other negative-stranded RNA viruses, such as the rhabdoviruses, and of dsRNA viruses and positive-stranded RNA viruses that possess a cap, and of some DNA viruses, such as the pox viruses, occurs in the cytoplasm, and utilizes virus-encoded enzymes (Mizumoto and Kaziro, 1987; Murphy *et al.*, 1995). The positive-stranded RNA viruses that possess capped genomic RNAs include those in the alpha-like, corona-like, flavi-like, and carmo-like virus supergroups (Table II). The capping reaction of cellular (Mizumoto and Kaziro, 1987), vaccinia virus (Shuman and Moss, 1990), and reovirus (Furiuchi *et al.*, 1976) mRNAs involves the following reactions: (i) pppNpN'..... → ppNpN'..... + p_i (hydrolysis of the 5'-terminal phosphate of the nascent RNA transcript by an RNA triphosphatase); (ii) guanylyltransferase + GTP → guanylyltransferase-GMP + pp_i (interaction of GTP with mRNA:guanylyltransferase to form a covalently bound guanylyltransferase-GMP intermediate); (iii) guanylyltransferase-GMP + ppNpN'..... → GpppNpN'..... + guanylyltransferase (donation of GMP from the guanylyltransferase-GMP intermediate to form a 5'-5' triphosphate linkage); (iv) GpppNpN'..... + AdoMet → 7MeGpppNpN'..... + AdoHcy (methylation of position 7 of the terminal G cap using a specific methyltransferase and *S*-adenosylmethionine [AdoMet] as the methyl donor to generate the cap 0 structure and *S*-adenosylhomocysteine [AdoCys]). For mRNAs that contain a cap 1 or cap 2 structure, further methylation of the 2'-OH groups of the ribose moieties of N and N' is carried out using specific methyltransferases. It has been shown that for reovirus mRNAs, which have a cap 1 structure, capping is coupled to, and occurs at an early stage of, mRNA synthesis (Furiuchi *et al.*, 1976).

Using mutants resistant to methionine starvation (which reduces cellular AdoMet levels) and mycophenolic acid (which reduces cellular GTP levels), evidence was obtained that capping of Semliki Forest virus (SFV) genomic and subgenomic RNAs is linked to RNA synthesis and that these activities reside in the nsP1 protein (Scheidel *et al.*, 1989; Mi *et al.*, 1989; Mi and Stollar, 1990; Scheidel and Stollar, 1991).

The methyltransferase activity of the nsP1 protein of SFV has been expressed in *E. coli* (Mi and Stollar, 1991) and the nsP1 protein of the closely related Sindbis virus in *E. coli* and insect cells (Laakkonen *et al.*, 1994). The Sindbis virus enzymes, as well as an enzyme preparation from Sindbis virus-infected cells, were able to catalyze the transfer of a methyl group from AdoMet to GTP, dGTP and GpppG, but not to 7MeGTP, GpppA, or *in vitro* transcribed RNAs with GpppA or GpppG caps. Subsequently it was shown that the nsP1 protein formed a covalent complex with 7MeGMP, but not with GTP (Ahola and Kääriänen, 1995). It therefore appears that the nsP1 protein has both capping and methylation activities, but that, unlike the cellular and viral capping reactions discussed above, the GTP is first converted to 7MeGTP by the methylase and then the 7MeGTP reacts with the guanylyltransferase to form a guanylyltransferase-7MeGMP complex, which in turn transfers the 7MeGMP to the ppNpN'..... 5' end of the nascent RNA.

Mapping of nsP1 mutants indicated that the methyltransferase domain was located close to its N terminus (Mi *et al.*, 1989; Mi and Stollar, 1990). Sequence comparisons with other positive-stranded RNA viruses has led to the identification of a methyltransferase domain near the N terminus of replication proteins of all members of the alpha-like virus supergroup (Rozanov *et al.*, 1992). Four conserved motifs were identified, with motifs I, II, and IV having an invariant H residue, a DXXR signature and an invariant Y residue respectively. Additional conserved motifs enabled two groups of viruses with the methyltransferase-like domain to be distinguished, the "altovirus" group (*Togaviridae* and *Bromoviridae* families, *Tobamovirus, Tobravirus, Hordeivirus, Furovirus* genera) and the "typovirus" group (*Tymovirus, Potexvirus, Carlavirus, Trichovirus* genera). Mutations which increased the affinity of the SFV nsP1 for AdoMet mapped close to motif II (Mi *et al.*, 1989; Mi and Stollar, 1990), suggesting that this motif may be part of the AdoMet binding site. Furthermore, mutation of any of the conserved H, D, R, and Y residues to A in Sindbis virus abolished virus infectivity and the methyltransferase activity of the *E. coli*–expressed nsP1 protein (Wang *et al.*, 1996).

A guanylyltransferase-like enzyme has been isolated from plants infected with tobacco mosaic virus and identified as the 126-kDa replication protein, which has an N-terminal methyltransferase-like domain and a C-terminal helicase-like domain (Dunigan and Zaitlin, 1990). This transferase formed a covalent complex with GMP in the absence of AdoMet and therefore appears to be different from the Sindbis virus nsP1 protein which only forms a complex with 7MeGMP.

This is surprising since both viruses belong to the alpha-like super-group and may represent some divergence in capping mechanism between the animal alphaviruses and the plant tobamoviruses.

The motifs which characterize the methyltransferase-like domain of the alpha-like virus supergroup could not be found in any of the other virus superfamilies whose members have a 5'-capped RNA (Rozanov *et al.*, 1992). However, the flavivirus NS5 protein and reovirus λ2 protein have been shown to have methyltransferase-like domains related to a class of cellular methyltransferases (Koonin, 1993) and a flavivirus NS3 protein has been shown to have an RNA triphosphatase activity (Wengler and Wengler, 1993). Sequences near the C terminus of this protein had some sequence similarity to a vaccinia virus RNA triphosphatase. Hence it is likely that the flavivirus group also encode capping enzymes. The other groups of capped RNA viruses probably also encode such enzymes, which may be recognized by different, as yet undetermined, motifs.

Transgenic tobacco plants expressing antisense RNA for AdoCys hydrolase, which controls the cellular AdoCys/AdoMet ratio, were shown to be resistant to infection by tobacco mosaic mosaic virus, potato virus X, and cucumber mosaic virus (all with 5'-capped RNAs), but much less resistant to infection by potato virus Y (5' VPg) (Masuta *et al.*, 1995). Resistance was thought to be due partially to undermethylation of the viral cap structure, although induction of host resistance as a result of excess cytokinin levels may also have played a role.

D. Genome-Linked Virus Proteins

Genome-linked virus proteins (VPgs) are virus-encoded proteins that are covalently linked by a phosphodiester linkage to the 5'-terminal nucleotide of the virus genomic RNA. They are found in viruses of the picorna-like and sobemo-like virus supergroups (Table II). Removal of the VPg of some viruses, such as nepoviruses (Hellen and Cooper, 1987) and caliciviruses (Burroughs and Brown, 1978), by protease treatment destroys the infectivity of the RNA. However, this effect is probably due to increased sensitivity of the RNA to exonuclease attack and does not represent a specific requirement for VPg for infectivity. Capped transcripts produced *in vitro* from full-length clones of caliciviruses were infectious (Sosnovtsev and Green, 1995); infectious *in vitro* transcripts have also been produced from comoviruses (Vos *et al.*, 1988), picornaviruses (van der Werf *et al.*, 1986; Sarnow, 1989; Lee *et al.*, 1993), and potyviruses (Dolja *et al.*, 1992) showing that VPg is not needed for infectivity.

The poliovirus and cowpea mosaic virus VPgs consist of 22 and 28 amino acid residues, repectively, and are linked to the 5' end of the virus RNAs by tyrosine and serine phosphodiester linkages, respectively (Wimmer *et al.*, 1993; Jaegle *et al.*, 1987). The VPg of tobacco etch potyvirus is larger, with a mass of 21 kDa (Riechmann *et al.*, 1992). The poliovirus Vg is removed from the genomic RNA by a host enzyme soon after infection and the 5' ends of viral RNA isolated from polysomes mainly consist of pUpU..... (Wimmer *et al.*, 1993). Mutational and other analyses have shown that the poliovirus VPg (and/or its precursor 3AB) are essential for RNA replication (Reuer *et al.*, 1990; Xiang *et al.*, 1995a). Viral VPgs are probably involved in the initiation of both negative-strand and positive-strand synthesis; VPg is found covalently linked to the 5' ends of newly synthesized negative and positive strands of poliovirus (Wimmer *et al.*, 1993) and cowpea mosaic virus (Lomonossoff *et al.*, 1985). VPg of poliovirus has been shown to be present in infected cells both as free VPg and as VPg-pUpU (Crawford and Baltimore, 1983). Furthermore, VPg-pU can be synthesized in membrane-bound replication complexes isolated from infected cells and elongated to longer VPg-RNA molecules (Takeda *et al.*, 1986; Toyoda *et al.*, 1987). Such a molecule (or a precursor 3AB-pU (see Section II,E,4) could act as a primer for RNA synthesis, although other models have been suggested (see Section III).

E. Expression of, and Interactions between, Replication Proteins

1. Alpha-like Virus Supergroup

The polymerase, helicase, and methyltransferase functions of viruses in the alphavirus-like virus supergroup can be expressed in several different ways. For some viruses, the methyltransferase-like, helicase-like, and polymerase-like domains are within the same protein (in that order), which is translated from the 5' ORF. An example is the 166-kDa protein encoded by ORF 1 of the genomic RNA of potato virus X (Huisman *et al.*, 1988; Longstaff *et al.*, 1993). The other downstream genes of the virus, which are required for cell-to-cell movement and encapsidation, are translated from subgenomic RNAs.

For some viruses, which have divided genomes, the methyltransferase and helicase-like domains and the polymerase-like domain are in two separate proteins, translated from two separate RNAs. Viruses in the *Bromoviridae* family, such as brome mosaic virus (BMV) (Ahlquist, 1992), cucumber mosaic virus, and alfalfa mosaic virus, fit into this category. BMV has three genomic RNAs, of which only RNAs 1 and 2

are essential for RNA replication. The BMV 1a protein, encoded by RNA 1, contains the methyltranferase-like domain near its N terminus and the helicase-like domain near its C terminus, and the 2a protein, encoded by RNA 2, contains the polymerase-like domain. RNA 3 encodes a cell-to-cell movement protein and the coat protein, the latter being translated from a subgenomic RNA (RNA 4).

For some other viruses, the methyltransferase-like and helicase-like domains are present on a protein which is translated from the 5' ORF and the polymerase domain, which is located downstream, is translated by readthrough (suppression) of the stop codon to give a fusion protein, e.g., the 126-kDa protein of tobacco mosaic virus (TMV) contains the methyltransferase-like and helicase-like domains, and the 183-kDa protein, translated by readthrough of an amber stop codon (UAG), additionally contains the polymerase-like domain (Dawson and Lehto, 1990). As a result, the methyltransferase-like and helicase-like domains are present in excess over the polymerase-like domain, being present both in the more abundant 126-kDa protein and in the 183-kDa protein. Mutational analysis has shown that both the 126-kDa protein and the 183-kDa protein are required for efficient RNA replication (Ishikawa *et al.*, 1986, 1991b). This could imply, as suggested for BMV and cucumber mosaic virus (CMV) (see Section II,B,1), that two or more copies of the helicase domain are required for different unwinding functions of RNA replication, or that the helicase functions in the 126-kDa protein and the methyltransferase functions in the 183-kDa protein (or vice versa). The other two downstream ORFs, which encode the cell-to-cell movement protein and the capsid protein, are not required for RNA replication; they are translated from subgenomic RNAs.

The BMV 1a and 2a proteins copurify with an RdRp complex isolated from infected plants (Quadt and Jaspars, 1990; Quadt *et al.*, 1993). Furthermore, the 1a and 2a proteins, produced by translation *in vitro* of RNA 1 and RNA2, form a complex; a complex was also formed when purified 2a protein expressed in insect cells was mixed with purified helicase-like domain expressed in *E. coli*, showing that no other proteins were needed to mediate the 1a–2a interaction. Certain mutations in the 1a protein which blocked virus RNA replication or made replication temperature-sensitive also prevented or made temperature-sensitive, respectively, the interaction between the 1a and 2a proteins, suggesting that this interaction is required for BMV RNA replication (Kao *et al.*, 1992). The region of interaction has been mapped to the N-terminal 115 amino acids of the 2a protein and the helicase-like domain of more than 50 kDa of the 1a protein (Kao and Ahlquist, 1992; O'Reilly *et al.*, 1995). Mutational analysis has shown that the N-

terminal region of the 2a protein, which lies upstream of the conserved polymerase-like domain, is essential for virus RNA replication (Traynor *et al.*, 1991). Requirement of the large size of the helicase-like domain of the 1a protein for the interaction suggested involvement of a higher-order structure and it was shown that this domain, and analogous domains of three other viruses in the *Bromoviridae* family [alfalfa mosaic virus, cowpea chlorotic mottle virus (CCMV), CMV], folded into protease-resistant structures; mutations which rendered the region protease-sensitive were defective in RNA replication (O'Reilly *et al.*, 1995). The BMV 1a protein is homologous to the TMV 126-kDa protein and the BMV 1a–2a protein complex is homologous in structure to the TMV 183-kDa protein. The homologous proteins may serve similar functions in the replication complexes of the two viruses. Using different combinations of the 1a and 2a proteins of BMV and CMV expressed transiently in plant protoplasts to support replication of BMV or CCMV RNA 3 templates, Dinant *et al.* (1993) found that the combination of CCMV 1a and BMV 2a did not support detectable synthesis of negative-strand, positive-strand, or subgenomic RNA, whereas the combination of BMV 1a and CCMV 2a was preferentially defective in positive-strand and subgenomic RNA accumulation, and negative-strand RNA was only slightly affected. The latter combination suggested that partial incompatibility of 1a–2a can distinguish some aspect of negative-stranded and positive-stranded RNA synthesis (including synthesis of subgenomic RNA), possibly the capping function of the 1a protein, since only positive-stranded RNAs are capped. Isolation of replication complexes from yeast expressing the 1a and 2a proteins has shown that the replication complexes, which probably contain host and viral proteins (see Section III), can only form in the presence of an RNA template (Quadt *et al.*, 1995).

A different strategy is used by animal viruses of the *Togaviridae* family (Strauss and Strauss, 1994; Frey, 1994), which are the only enveloped viruses in the alpha-like virus supergroup. The viruses of the *Alphavirus* genus are the best studied. The nonstructural proteins are encoded at the 5′ end of the genome and are translated as a polyprotein which is cleaved by virus-encoded proteinase activity. The structural proteins (the capsid and envelope proteins) are encoded at the 3′ end of the genome and are translated from a subgenomic RNA (26S RNA) as a polyprotein, which is cleaved by virus and host proteinases. Only the nonstructural proteins are required for RNA replication. Translation of Sindbis virus RNA gives a polyprotein P123, containing the sequences of nsP1, nsP2, and nsP3. Readthrough of an opal termination codon (UGA), which is followed by an essential (C), results in

the production of P1234. The readthrough rate in translation *in vitro* was found to be 10–20% at 30°C and <5% at 40°C. Since nsP1 is associated with methyltransferase and guanylyltransferase (see Section II,C), nsP2 has a helicase-like domain (see Section II,B) and nsP4 has a polymerase-like domain (see Section II,A), the relationship between P123 and P1234 is similar to that of the TMV 126-kDa and 183-kDa proteins. However, unlike the TMV proteins, there is a domain for a papain-like cysteine proteinase near the C terminus of nsP2. The specificity of nsP2 is modulated by the surrounding sequences, so that the pattern of cleavage of the polyproteins changes through the replication cycle (reviewed by Strauss and Strauss, 1994). In P1234, the proteinase can cleave in cis between nsP3 and nsP4 to give P123 and nsP4. The cleavage site is six amino acid residues downstream of the opal terminal codon, but as the C-terminal region of nsP3 is variable and deletions in it are well tolerated (Lastarza *et al.*, 1994b), P123 formed by primary translation and P123 formed by cleavage of P1234 are considered equivalent. P123 cannot be cleaved in cis, but once sufficient concentrations have built up, it is cleaved in trans to produce nsP1 and P23. The P23 proteinase can cleave the P2/P3 site in polyproteins (including itself) very efficiently, and nsP2 and nsP3 are produced. The P3/P4 site can only be cleaved by nsP2 polyproteins containing nsP3 sequences. Late in infection it is likely that that the P2/P3 site is cleaved (in trans) in the nascent polypeptide; hence the P3/P4 site is not cleaved and the main products are nsP1, nsP2, and P34, but the proteins produced late in infection are not thought to be involved in replication.

   Mutations which prevented the cleavage to form nsP4 were lethal (reviewed by Strauss and Strauss, 1994), and the nsP4 protein is believed to be active in elongation of negative and positive RNA strands. However, mutations which inactivated the nsP2 proteinase and resulted in uncleaved P123 led to increased accumulation of negative-stranded RNA and decreased accumulation of positive-stranded genomic and subgenomic RNAs. Another mutation which greatly increased the rate of cleavage of P123 was lethal. These results, together with further mutational analysis, led to the proposal that P123 + nsP4 function in negative-stranded RNA synthesis (Shirako and Strauss, 1994; Lemm *et al.*, 1994). However, it is likely that nsP1 + P23 + nsP4 can also function in negative-stranded RNA synthesis, since temperature-sensitive mutations with lesions in nsP1 and nsP3 complement, whereas those in nsP2 and nsP3 do not rescue defects in negative-stranded RNA synthesis (Sawicki and Sawicki, 1994; Lastarza *et al.*, 1994a; Wang *et al.*, 1994). Complete cleavage of P123 to form nsP1, nsP2, and nsP3 not only results in inactivation of negative-strand RNA synthesis, but also

switches on positive-stranded genomic and subgenomic RNA synthesis (Shirako and Strauss, 1994; Lemm *et al.*, 1994).

Although polyprotein processing is common in the expression of viral genes (Dougherty and Semler, 1993), it is relatively uncommon in the alpha-like virus superfamily. In the case of the alphaviruses, and probably also rubella virus (Frey, 1994), it may have evolved as a method of inducing conformational changes in replication complexes to switch from negative-strand to positive-strand synthesis. After cleavage, all four proteins remain in association because they are found in isolated replication complexes (Barton *et al.*, 1991), although their relative juxtapositions may alter. All the nonstructural alphavirus proteins are multifunctional and have functions in both negative-strand and positive-strand synthesis, which have been distinguished by mutational analysis (reviewed in Strauss and Strauss, 1994). The nsP1 protein functions as a guanylyltransferase and methyltransferase in the capping of positive-stranded RNA (see Section II,C), it is required for negative-strand synthesis and in polyproteins it modulates the proteinase activity of the nsP2 protein by inhibiting cleavage of the site between nsP2 and nsP3. The nsP2 protein has an N-terminal helicase domain (see Section II,B) and a C-terminal proteinase domain; it is specifically required for the synthesis of subgenomic RNA and plays a role in the conversion of negative-strand-synthesizing to positive-strand-synthesizing enzymes (Dé *et al.*, 1996). The nsP3 protein has separate functions in negative-stranded RNA and subgenomic RNA synthesis (Lastarza *et al.*, 1994a; Wang *et al.*, 1994), some of which may be host-specific (Lastarza *et al.*, 1994b). Its presence in polyproteins modulates the activity of the nsP2 proteinase (see above). The nsP4 protein is likely to be the RNA polymerase that functions in negative-stranded, positive-stranded, and subgenomic RNA synthesis. Presumably all these proteins must also contain domains that keep them associated after proteolytic cleavage. The N-terminal region of the nsP4 protein is thought to be important for this purpose. Another possibility is that complete cleavage of the polyprotein could enable an additional copy of an individual subunit, such as the nsP2 protein which contains the helicase-like domain, to be recruited for a specific function in the synthesis of positive-stranded RNA, which was not needed for negative-stranded RNA synthesis.

Turnip yellow mosaic virus is an example of a plant alpha-like virus in which cleavage of a polyprotein is essential for virus RNA replication. Its genome contains three ORFs. ORF 1 encodes a 206-kDa protein which contains methyltransferase-like, helicase-like, and polymerase-like domains. ORF 2 encodes a cell-to-cell movement protein and ORF 3

encodes the coat protein, which is translated from a subgenomic RNA; neither of these proteins is required for RNA replication. The 206-kDa primary *in vitro* translation product is cleaved in cis by a papain-like cysteine proteinase, located between the methyltransferase-like and helicase-like domains, to give a protein of 66 kDa from the C-terminal end (which contains the polymerase-like domain) and a protein of 140 kDa from the N-terminal end, which contains the other three domains (Bransom *et al.,* 1991; Bransom and Dreher, 1994; Kadaré *et al.,* 1995). The proteinase was active and cleaved the same site when expressed in *E. coli* (Kadaré *et al.,* 1995) Although this cleavage is needed for virus RNA replication (Weiland and Dreher, 1993), its precise function has not been determined. Purified preparations of a RdRp complex isolated from infected plants were reported to contain a virus-encoded protein of 115 kDa (Mouches *et al.,* 1984; Candresse *et al.,* 1986), but the relationship of this protein to the 140-kDa and 66-kDa proteins is not clear.

The genome of beet yellows closterovirus contains a long ORF (1a) which could encode a protein of 295 kDa, which has a papain-like cysteine proteinase domain, a methyltransferase-like domain, and a helicase-like domain. The next ORF (1b), which has the capacity to encode a 53-kDa protein with a polymerase-like domain, overlaps the end of ORF 1a and is probably translated by a +1 frameshift (Agranovsky *et al.,* 1994). The leader proteinase is cleaved autocatalytically after translation *in vitro.* There is also a potential aspartic proteinase between the methyltranferase-like and helicase domains, but the significance of this for proteolytic processing or RNA replication is not known.

It is noteworthy that the order of the homologous methyltransferase-like, helicase-like, and polymerase-like domains is conserved in all the viruses in the alpha-like supergroup. It may be speculated that this could reflect some basic common structural feature in the organization of the replicase complex, particularly as the three domains can apparently function in one protein in some viruses, e.g., potato virus X. However, a single replication protein is not sufficient for tobacco mosaic virus replication. A tobacco mosaic virus mutant, engineered to remove the stop codon at the end of the ORF for the 126-kDa protein so that only the 183-kDa protein was produced, replicated poorly and the stop codon was reintroduced by reversion (Ishikawa *et al.,* 1986). The stop codon near the end of nsP3 in Sindbis virus, however, is not essential. Some alphaviruses, such as Semliki Forest virus, do not have the stop codon, so that the only primary translation product is P1234. It has been suggested that the extra amount of P123 produced when the stop codon is present provides extra proteinase to accelerate the switch from negative-strand to positive-strand synthesis (Strauss and Strauss,

1994). The phenotype of Sindbis virus mutants in which the stop codon had been replaced by a sense codon (Li and Rice, 1989) were consistent with this hypothesis. It is noteworthy that there is no apparent counterpart of the alphavirus nsP3 protein in the plant alpha-like viruses. This protein has functions distinct from its effect on nsP2 proteinase specificity, perhaps in protein folding or protein–protein or protein–RNA interactions, which must be circumvented or achieved in different ways in the plant virus replication complexes. Switches in functions to achieve synthesis of negative-stranded, positive-stranded, or subgenomic RNA may perhaps be achieved by a variety of methods, including proteolytic cleavage, protein–protein interactions, protein–RNA interactions, modification of proteins, for instance by phosphorylation, or changes in the stoichiometry of the different components in replication complexes. Further studies will be required to determine whether or not the considerable diversity in expression of replicase functions within the alpha-like supergroup merely reflects variations on a common theme.

A subset of the plant alpha-like viruses, carla-, furo-, hordei-, and tobraviruses, contain 3′-proximal genes for small cysteine-rich proteins, some of which have been shown to have RNA-binding activity and display some sequence similarity to other nucleic-acid-binding proteins (Koonin et al., 1991). One of the functions of these proteins is to regulate the synthesis of the capsid protein, which in these viruses is encoded by 5′-proximal genes. Although these proteins are not absolute requirements for RNA replication, they have been shown to affect replication in some instances. Beet necrotic yellow vein virus has four RNA components. RNA 1 can replicate alone in protoplasts and contains a long ORF containing methyltransferase-like, helicase-like, and polymerase-like functions (Richards and Tamada, 1992). RNA 2 has several ORFs, including the coat protein gene at the 5′ end and a gene for a small cysteine-rich protein (P14) at the 3′ end. Null mutations in P14 not only reduced coat protein accumulation, but also had a specific inhibitory effect on RNA 2 accumulation (Hehn et al., 1995). The defect in RNA 2 accumulation could not be complemented in trans, which is surprising since it is expressed as a single protein via a subgenomic RNA, and the defect in coat protein accumulation could be complemented in trans; it was suggested that translation of P14 and replication of RNA 2 were tightly coupled.

2. Carmo-like Virus Supergroup

Viruses in the Tombusviridae family (Carmovirus and Tombusvirus genera) and the Dianthovirus, Luteovirus (subgroup I), and Necrovirus genera have a 5′-proximal ORF which encodes a protein of 22–33 kDa

(Russo *et al.*, 1994). Readthrough of a stop codon at the end of this ORF (carmoviruses, necroviruses, and tombusviruses), or a ribosomal frameshift prior to the stop codon (dianthoviruses and subgroup I luteoviruses), gives rise to a fusion protein (82–99 kDa). The C-terminal region of the fusion protein contains a polymerase-like domain. Mutants of cymbidium ringspot virus, red clover necrotic mosaic virus, tomato bushy stunt virus, and turnip crinkle virus which produce only the smaller protein, or only the fusion protein, failed to replicate (Hacker *et al.*, 1992; Wei *et al.*, 1992; Dalmay *et al.*, 1993a; Gieseman-Cookmeyer *et al.*, 1995; White *et al.*, 1995), indicating that both proteins are required for RNA replication. For cymbidium ringspot virus and barley yellow dwarf virus-PAV, it was shown that the 5′-encoded protein and the readthrough or frameshift protein were the only virus-encoded proteins needed for RNA replication (Kollar and Burgyan, 1994; Mohan *et al.*, 1995). It was also shown that when these two proteins were expressed in transgenic plants from chromosomal insertions of the genes, replication of DI RNAs was supported. Furthermore, nonviable mutants of turnip crinkle virus with mutations in either the gene for the 28-kDa protein or the 88-kDa readthrough protein could complement each other (White *et al.*, 1995). These two experiments show that the two replication proteins act in trans. A purified RdRp complex isolated from plants infected with red clover necrotic mosaic virus was shown to contain both the 27-kDa protein and the 88-kDa frameshift fusion protein (Bates *et al.*, 1995). The function of the smaller replication protein, which is produced in a large excess over the fusion protein, is not known. It could be a helix-destabilizing protein which substitutes for the apparent absence of a helicase-like domain in these viruses. Evidence also suggests that it is involved in membrane localization of the replicase complex (see Section IV). RNAs of viruses in the carmo-like supergroup are capped and presumable capping enzymes are encoded by one or both of the essential ORFs, although no sequence similarity to capping enzymes of the alpha-like viruses has been detected. The other proteins encoded by these viruses, which are not needed for replication, are translated from subgenomic RNAs or additionally in the case of the dianthoviruses, from an additional RNA segment.

Carmo-like viruses have not been found in animals. The polymerase-like domains of carmo-like viruses fall into supergroup 2, like those of the animal flaviviruses, pestiviruses and hepatitis C virus. The *Flaviviridae* are clearly very different from the carmo-like viruses. They produce several structural and nonstructural proteins by proteinase cleavage of a polyprotein. They possess helicase-like domains of superfamily 2, with similarity to those of the potyviruses (see Section II,B),

but are clearly also different from the picorna-like viruses, e.g., they possess a 5' cap. Very little is known about proteins involved in *Flaviviridae* replication, beyond that discussed in Section II,A–C, the requirement for certain proteolytic cleavages (e.g., Chambers *et al.*, 1993; Nestorowicz *et al.*, 1994), and the inhibition of *in vitro* conversion of RF to RI by an isolated dengue virus RNA polymerase fraction using antibodies to the NS3 (helicase) and NS5 (polymerase) regions (Bartholomeusz and Wright, 1993). However, replication of viruses in the Flavi-like supergroup is likely to have significant differences from that of the other virus supergroups.

3. Sobemo-like Virus Supergroup

These include the sobemoviruses and the subgroup II luteoviruses. Viruses of these two genera are similar at the 5' end of the genome, which contains three overlapping ORFs, 1, 2, and 3, but differ at the 3' ends. The 3' ends of subgroup II luteoviruses more closely resemble those of subgroup I luteoviruses and it has been suggested that the subgroup II luteoviruses were derived by recombination between a sobemovirus and a subgroup I luteovirus (Miller *et al.*, 1995). For beet western yellows luteovirus, it has been shown that only ORFs 2 and 3 are required for RNA replication (Reutenauer *et al.*, 1993). For both viruses in both genera, ORF 3, which contains a polymerase-like domain, is translated by ribosomal frameshifting from near the end of ORF 2. ORF 2 contains motifs suggestive of a VPg and characteristic of a serine proteinase (Miller *et al.*, 1995; Makinen *et al.*, 1995). Genes downsteam of ORF 3 are translated from subgenomic RNAs. The sobemo-like virus supergroup contains no animal viruses, although the gene order VPg-proteinase-polymerase is similar to that in the picorna-like supergroup and the human astroviruses have a similar arrangement of a proteinase motif fused to a polymerase by a probable frameshift. Viruses in the *Nodaviridae* family have a polymerase-like domain which has been classified in the supergroup 1 sobemo-like lineage (Table II). However these viruses have a capped 5' terminus and a simpler bipartite genome structure. Only one protein (the polymerase), which is expressed as a 5'-proximal ORF of RNA 1, is required for RNA replication (Ball, 1995). Their replication is likely to be significantly different from that of the sobemoviruses.

4. Picorna-like Virus Supergroup

Viruses in this family have VPg at their 5' ends and poly(A) at their 3' ends, their gene products are expressed by proteinase cleavage of a polyprotein translated from the genomic RNA, and a gene order of

helicase-VPg-proteinase-polymerase is conserved. The positions of the structural proteins differ, being translated at the 5' end (*Picornaviridae*) or 3' end (*Potyviridae*) of a polyprotein encoding the replication genes, on a separate RNA (*Comoviridae*), or from a subgenomic RNA (*Caliciviridae*).

By far the best-studied viruses in this supergroup are the picornaviruses (Wimmer *et al.*, 1993; Mirzayan and Wimmer, 1994b). The genome map of poliovirus may be divided into three regions, P1, P2, and P3. P1 encodes the virus capsid proteins and P2 and P3 encode the nonstructural proteins. The order of the genome is (N terminus to C terminus) 1A–1B–1C–1D–2A–2B–2C–3A–3B(=VPg)–3C–3D. Translation starts at an initiation site several hundred nucleotides from the 5' end and continues until a stop codon is reached in the 3'-terminal region. Theoretically, a polyprotein of 2209 amino acids could be produced, but this is partially cleaved while still nascent by proteinases present within the polyprotein, which probably act in cis. The first cleavage is mediated by 2A, a chymotrypsin-like cysteine proteinase, which cleaves a YG linkage between the P1 and P2 regions. Once the 3C region, which encodes another chymotrypsin-like proteinase specific for QG linkages, has been translated, further cleavages can occur. Many intermediates are formed, some of which have functions distinct from the completely cleaved products. Most subsequent cleavages are mediated by 2A^{pro}, 3CD^{pro}, and, to a lesser extent, 3C^{pro}. Many of the partly or completely cleaved products have roles in RNA replication and interact at different stages of replication. The product 2A^{pro} is a multifunctional protein which has functions in replication which are distinct from its functions as a proteinase (cleavage of poliovirus polyprotein, inhibition of host protein synthesis) and as an enhancer of poliovirus translation (Yu *et al.*, 1995; Lu *et al.*, 1995). Protein 2B (or its precursor 2BC) may be required in cis during assembly of the replication complex, because RNA-negative 2B mutants are noncomplementable (Johnson and Sarnow, 1991; van Kuppeveld *et al.*, 1995). The NTPase and putative helicase function of the 2C protein was discussed in Section II,B. The 3AB protein is the precursor of VPg (3B). 3AB has also been shown to stimulate the activity of the polymerase (3D^{pol}) in catalyzing RNA synthesis on various primed templates by up to 100-fold, possibly because of its ability to bind both the template-primer and 3D^{pol} (Lama *et al.*, 1994; Paul *et al.*, 1994a; Plotch and Palant, 1995). 3AB also forms a complex with 3CD^{pro} (which has no polymerase activity), resulting in accelerated autoprocessing of 3CD^{pro} to form 3C^{pro} and the active polymerase 3D^{pol} (Molla *et al.*, 1994). The 3AB–CD^{pro} complex was found to form a complex with a cloverleaf structure at the

5′ terminus of poliovirus RNA and formation of the complex was essential for RNA replication (Harris *et al.*, 1994; Xiang *et al.*, 1995a,b). 3AB or 3CD^{pro} alone did not bind to the cloverleaf. The 3AB–CD^{pro} complex, or 3AB alone, formed a complex with the 3′-terminal sequence of poliovirus. These observations led Harris *et al.* (1994) to propose a model for poliovirus RNA replication. Initiation of negative-strand synthesis using VPg-pU as a primer occurs following formation of a 3AB–3CD^{pro} complex at the 3′ end of poliovirus RNA, cleavage of 3CD^{pro}, and formation of [3AB–D]^{superpol}. After negative-strand synthesis to form a double-stranded duplex, the end of the duplex is unwound, possibly by the putative 2C helicase, allowing formation of the 3AB–3CD^{pro}-cloverleaf complex and initiation of positive-strand synthesis by [3AB–3D]^{superpol} on the free 3′ end of the negative-stranded RNA. The multiplicity of functions of precursors and fully cleaved proteins, combined with the formation of functional complexes and their membrane locations (see Section IV), may lead to explanations of why some mutations, for instance in 3A or 3D^{pol}, are complementable and others are not. Evidence has been obtained that poliovirus translation and replication are coupled, probably in the early stages of replication (Novak and Kirkegaard, 1994); such coupling could be required for the assembly of new replication complexes. Complete replication of poliovirus has been achieved *in vitro* using a combined translation and replication system (Molla *et al.*, 1991).

The plant comoviruses encode their replication proteins on one RNA segment (RNA 1 or B), which can replicate in protoplasts alone, and their capsid and cell-to-cell movement proteins on a second segment (RNA 2 or M), which requires RNA B for its replication (Eggen and van Kammen, 1988). The gene order of RNA B is (N terminus to C terminus) 32K–58K–VPg–24K–87K. Both RNAs are translated as polyproteins, which are cleaved in cis and trans by a 24K proteinase which resembles the poliovirus 3C^{pro}. There appears to be no direct equivalent to the poliovirus 2A^{pro}, but there is a 32K protein at the N terminus of the B polyprotein that acts as a regulator for processing of the B polyprotein and as a cofactor in cleavages in the M polyprotein. The 58K protein contains the helicase-like domain. The 87K protein contains a polymerase-like domain like that in poliovirus 3D^{pol}, but the active form, detected in isolated replication complexes, appears to be the 110K precursor (24K–87K) (Dorssers *et al.*, 1984). This represents a significant difference from poliovirus, because 3CD^{pro} has no polymerase activity. VPg is probably involved in initiation of RNA synthesis (see Section II,D), but its mode of processing may be different from that in poliovirus. In the latest model, it is suggested that initiation may involve cleavage of the 112K polymerase precursor (VPg–24K–87K)

(Peters *et al.*, 1995), although in a crude RNA polymerase preparation, the 60K precursor (58K–VPg) was the most prominent protein detected by anti-VPg serum (Eggen *et al.*, 1988).

Various insertional and frameshift mutants of RNA B, one of which did not contain any of the B-encoded genes, could not be replicated in trans by wild-type RNA B, suggesting that translation and replication may be coupled (van Bokhoven *et al.*, 1993). M RNA must be replicated in trans, but mutational analysis of RNA M indicated that the N-terminal region of the M-encoded 58K protein must be translated to allow replication of RNA M to proceed in the presence of B replication proteins, again indicating a link between translation and replication (van Bokhoven *et al.*, 1993).

The gene order of a typical potyvirus, tobacco etch virus (TEV), is (N terminus to C terminus) P1–HCPro–P3–CI–6K–N1a–N1b–coat protein. The polyprotein translation product is cleaved by three proteinases (Dougherty and Semler, 1993). The P1 proteinase autocatalytically cleaves itself from the rest of the polyprotein. The HC-proteinase (HC-Pro) also autocatalytically cleaves itself at its C terminus in a cotranslational event. The remainder of the cleavages are carried out by the NIa proteinase, which has similarities with the picornavirus and comovirus proteinases. Like the picornavirus proteins, potyvirus proteins are multifunctional and several function in RNA replication (Riechmann *et al.*, 1992). The NIb protein contains the RNA polymerase-like domain. Mutants in the NIb region could be complemented by wild-type NIb protein expressed in transgenic plants with efficiencies varying from 1% to 100% (Li and Carrington, 1995). VPg constitutes the N-terminal region of the NIa proteinase and both NIa and the cleaved VPg have been found linked to the 5′ end of the viral RNA. A large proportion of the NIa and NIb proteins is found in the nucleus. A predominantly nuclear location was also found for the alphavirus nsP2 protein, but this is irrelevant for replication, since a proportion of the protein is localized to the cytoplasmic membranes where replication tales place (see Section IV). Localization of a proportion of the NIa and NIb proteins to the membranes where TEV RNA replication takes place may be mediated by the adjacent 6K protein, which has been shown to be essential for RNA replication (Restrepo-Hartwig and Carrington, 1994). 6K–VPg and 6K–NIa polyproteins were detected in extracts from infected plants. The 6K protein was shown to have a membrane location and may function in a similar way to the poliovirus 3A protein as a membrane anchor (see Section IV). The NIa and NIb proteins have been shown to interact in yeast cells (Hong *et al.*, 1995). The CI helicase was described in Section II,B. The CI, NIb, and NIb–

NIa proteins have been detected in polymerase preparations from infected plants (Martin *et al.*, 1995). HC-Pro also has an essential role in RNA replication, which requires its cleavage activity (Kasschau and Carrington, 1995). The P1 protein has RNA-binding activities (Soumounou and Laliberte, 1994) and has a stimulatory effect on RNA replication (Verchot and Carrington, 1995).

5. Corona-like Virus Supergoup

The coronavirus, torovirus, and arterivirus replication proteins are encoded in gene 1, which consists of two overlapping ORFs, 1a and 1b. ORF 1a is expressed as a polyprotein by direct translation of the viral RNA, while ORF 1b is expressed by ribosomal frameshifting to give a 1a–1b fusion protein. The latter ranges in size from 345 kDa (arteriviruses) up to 800 kDa (coronaviruses) (Snijder and Horzinek, 1993). Polymerase-like and helicase-like domains are found in that order in the frameshift part of the polyprotein. Clearly, this order is different from that in the other virus supergroups and the location of the helicase-like domain in the frameshift portion is also unusual. The gene 1 polyprotein is broken down into functional products by virus-encoded proteinases, some of which are papain-like, another resembles the poliovirus 3C proteinase, and another has similarities to both types (Kim *et al.*, 1995; Snijder *et al.*, 1995). It has been shown for mouse hepatitis virus that RNA replication is inhibited by a cysteine proteinase inhibitor (Kim *et al.*, 1995). Genes downstream of gene 1 are expressed via subgenomic RNAs which are synthesized by a mechanism different from that of other positive-stranded RNA viruses (see Section V,D,3).

F. Effect of Capsid Proteins on RNA Replication

Capsid proteins (CPs) play an important regulatory role in the replication cycle of positive-stranded RNA phages (Witherell *et al.*, 1991). However, it has been found for many eukaryotic positive-stranded RNA viruses that CPs are not essential for virus RNA replication. CP genes have been replaced by other genes in a number of expression vectors derived from animal and plant positive-stranded RNA plant viruses, such as alphaviruses, bromoviruses, and tombusviruses (reviewed by Mushegian and Shepherd, 1995; Schlesinger, 1995). In the absence of the capsid protein, levels of accumulation of positive-stranded RNA are sometimes reduced and this has been ascribed to degradation of the RNA in the absence of the protective capsid, e.g., in beet western yellows virus (Reutenauer *et al.*, 1993), cowpea mosaic virus (van Bokhoven *et al.*, 1993), and cucumber mosaic virus (Boccard and Baulcombe, 1993).

In some other cases, there appears to be little or no effect, e.g., brome mosaic virus (French and Ahlquist, 1987; Marsh *et al.*, 1991a), tomato bushy stunt virus (Scholthof *et al.*, 1993), turnip crinkle virus (Hacker *et al.*, 1992). For poliovirus, a large deletion in the capsid-encoding region led to an increase in RNA accumulation (Collis *et al.*, 1992), although there is evidence that poliovirus replication and encapsidation are linked (Pfister *et al.*, 1995).

Plant viruses in the *Alfamovirus* and *Ilarvirus* genera of the *Bromoviridae* family appear to be exceptional in their requirement for CP in both early and late functions. Alfalfa mosaic virus (AlMV) has a tripartite genome, similar to that of brome mosaic virus. RNAs 1 and 2 encode the replication proteins P1 and P2 (equivalent to 1a and 2a), whereas RNA 3 encodes the movement protein and the CP, the latter being translated from a subgenomic RNA. Unlike brome mosaic virus, plants cannot be infected by a mixture of RNA 1, RNA2, and RNA 3; infection requires additionally a small amount of CP or its subgenomic RNA, a process called genome activation (reviewed by Jaspars, 1985). The 3' terminal 145 nt of the three RNAs are homologous and can be folded into a structure consisting of a series of stem–loops, separated by AUGC motifs, which contains a high affinity CP-binding site (site 1); an additional similar upstream binding site (site 2) is found in RNA 3 (Reusken *et al.*, 1994; Houser-Scott *et al.*, 1994). Mutations in two of the AUGC motifs in binding site 1 of RNA 3 reduced or abolished binding. The RNA-binding site is located near the N terminus of the CP and one or more lysine residues in this region are needed for gene activation (Baer *et al.*, 1994; Yusibov and Loesch-Fries, 1995). When RNA 3 was used to inoculate transgenic plants expressing P1 and P2 from chromosomal expression cassettes (P12 plants), replication of RNA 3 was achieved in the absence of CP in the inoculum (Neeleman *et al.*, 1993). It was suggested that the role of the CP in gene activation may be to stabilize the RNAs in the inoculum to allow translation of the replicase genes and the formation of a replicase complex to occur.

P12 plants could be infected by RNA 3 with deletions in the CP gene; this had little effect on negative-strand synthesis, but reduced the accumulation of positive strands by 100-fold, indicating that the CP also had an additional role in asymmetric positive-strand accumulation (van der Vossen *et al.*, 1994). This was unlikely to be due to stabilization of the RNA by encapsidation because, using infections with chimeric AlMV–tobacco streak ilarvirus (TSV) constructs, it was found that the CP of TSV could encapsidate AlMV RNA, but not stimulate positive-strand synthesis (Reusken *et al.*, 1995). Further analysis indicated that some AlMV CP mutations affected the early (gene activa-

tion) and late (asymmetric positive-strand accumulation) functions in a quantitatively different way and that AUGC motif mutations that abolished CP binding reduced positive-strand but not negative-strand accumulation (van der Vossen *et al.*, 1994). Quadt *et al.* (1991) detected CP in a purified RdRp preparation from AlMV-infected plants, in addition to P1 and P2. De Graaf *et al.* (1995b) showed that synthesis of full-length and subgenomic positive-stranded RNA on a negative-strand AlMV RNA 3 template by an RdRp preparation isolated from P12 plants was strongly stimulated by addition of CP and suggested that this stimulation could explain both gene activation and asymmetric positive-strand accumulation.

The coronaviruses probably also use the nucleocapsid N protein in the control of genomic and subgenomic RNA synthesis (see Section V,D,3).

Binding of a potyvirus CP to the NIb (polymerase) protein was observed in yeast (Hong *et al.*, 1995), but the significance is not known.

G. Replication Complexes and Virus Movement in Plants

As outlined in the Introduction (Section I), plant viruses have evolved specialized proteins to enable them to move from cell to cell in the plant. Generally it has been found that replication in single cells (protoplasts) is not affected when the movement protein gene is deleted. However, two observations suggest that there may be a link between replication and virus movement in the plant. (1) Mutations in the C-terminal region of the 2a brome mosaic virus replication protein had no effect on replication in single cells, but were deleterious for virus spread in the plant (Traynor *et al.*, 1991). (2) Transgenic plants expressing a truncated cucumber mosaic virus 2a protein were resistant to infection by the virus. The major effect was on inhibition of RNA replication, but to a lesser extent virus spread in the plant was also affected (Carr *et al.*, 1994). It is therefore possible that there may be some interactions between replication complexes and the virus-encoded or host proteins that are involved in virus spread through the plant.

III. Host Proteins in RNA Replication

The notion that replication of eukaryotic positive-stranded viral RNA may require host proteins stems from the requirement of host proteins for ssRNA phage replication. Phage Qβ RNA polymerase complex contains, in addition to the phage-encoded polymerase subunit, 30S ribosomal protein S1, and protein synthesis elongation factors EF-

Tu and EF-Ts (Blumenthal and Carmichael, 1979). Another ribosome-associated protein, termed host factor I (HF-I), is required for RNA synthesis on genomic RNA, but not negative-strand RNA, templates.

Various approaches have been used to attempt to obtain evidence for a role of host proteins in eukaryotic viral RNA replication. The first involves purification of solubilized RdRp and searching for host proteins that copurify with the RdRp in multiple purification steps. Co-purification of host proteins with RdRps has been found for several viruses, such as brome mosaic virus (BMV) (Quadt and Jaspars, 1990), cowpea mosaic virus (Dorrsers et al., 1984), cucumber mosaic virus (Hayes and Buck, 1990), red clover necrotic mosaic virus (Bates et al., 1995), Sindbis and Semliki Forest viruses (Barton et al., 1991), and turnip yellow mosaic virus (Mouches et al., 1984). All such studies require further evidence that such copurification is not fortuitous. One of the host proteins that copurified with the BMV RdRp was identified as the barley analogue of the p41 subunit of the wheat germ eukaryotic translation initiation factor eIF-3, or a closely related protein (Quadt et al., 1993). The BMV RdRp-associated host protein and the p41 subunit of wheatgerm eIF-3 were found to bind with high affinity and specificity to the BMV 2a polymerase-like protein. Addition of wheatgerm eIF-3 or its p41 subunit to the BMV RdRp gave a threefold stimulation of negative-strand synthesis. Biochemical functions of eIF-3 include stabilization of Met-tRNA$_i$^{Met} (ternary complex) binding to the 40S ribosomal subunit, mRNA binding to the ribosome, and dissociation of 80S ribosomes into 60S and 40S subunits, and it is thought to play a key role in assembly of the initiation complex (Merrick, 1992; Hannig, 1995). The specific function in protein synthesis of the p41 subunit of wheatgerm eIF-3, which is composed of 10 subunits, is not known.

Another approach has been to search for proteins that bind specifically to terminal sequences of viral RNAs. Specific binding of host proteins to viral terminal sequences has been reported for diverse viruses: alpha-like supergroup, BMV [3' (+), 3' (−), and 5' (+); barley] (Duggal et al., 1994; Duggal and Hall, 1995), cucumber mosaic virus, tobacco mosaic virus [3' (−); tobacco, spinach] (Hayes et al., 1994b), Sindbis virus [3' (−); chicken, mosquito] (Pardigon and Strauss, 1992, 1996; Pardigon et al., 1993), rubella, virus [3' (−); 3' (+); 5' (+); simian] (Nakhasi et al., 1990, 1991, 1994); flavi-like supergroup, West Nile virus [3' (+); hamster] (Blackwell and Brinton, 1995); picorna-like supergroup, hepatitis A [3' (+), together with internal sites] (Nuesch et al., 1993; Kusov et al., 1996), poliovirus [5' (+); human] (Najita and Sarnow, 1990), rhinovirus [3' (+); human] (Todd et al., 1995), corona-like supergroup, mouse hepatitis virus [3' (−); 5' (+); human] (Furuya

and Lai, 1993), [3' (+); murine] (Yu and Leibowitz, 1995), [intergenic (+); murine] (Zhang and Lai, 1995); carmo-like supergroup, red clover necrotic mosaic virus [3' (–); tobacco, spinach] (Hayes *et al.,* 1994b). In all these cases, it will be important to distinguish between binding required for translational regulation which can require both 5' (+) and 3 (+) sequences (Gallie, 1991; Ehrenfeld and Gebhard, 1994; Gallie and Kobayashi, 1994; Schmid and Wimmer, 1994; Standart and Jackson, 1994), binding required for replication, and irrelevant binding because the protein recognizes a structure in a viral RNA which is fortuitously related to structures recognized by the protein in its normal function.

Two proteins that bound to the 5' (+) terminus of rubella virus RNA were shown to be Ro/SS-A-related antigens and it was suggested that they may have a role in translational control of rubella virus RNA (Pogue *et al.,* 1993). A 60-kDa protein that interacted with a rubella virus 3' (–) sequence (Nakhasi *et al.,* 1991) was also shown to interact with a 3' (+) stem–loop structure in the genomic RNA, important for the initiation of negative-strand synthesis (Nakhasi *et al.* 1994). This protein has been identified as the simian homologue of human calreticulin (Singh *et al.,* 1994) and the rubella virus RNA-binding activity has been located to the N-terminal region of the protein (Atreya *et al.,* 1995). Calreticulin is a major calcium storage protein found in the lumen of the endoplasmic reticulum in animals and plants, but it may have other functions, such as in the regulation of gene expression (Dedhar, 1994). Ro/SS-A protein, La/SS-B protein, and calreticulin may be present in RNA–protein complexes; autoantibodies to them are found in cases of systemic lupus erythematosus and Sjögren's syndrome (Zhu and Newkirk, 1994).

One of the proteins that bound to the Sindbis virus 3' (–) terminal sequence has been shown to be the mosquito homologue of the La autoantigen (Pardigon and Strauss, 1996). The La (SS-B) protein is an abundant cellular protein which belongs to the RNP class of RNA binding proteins; it is found in both the nucleus and the cytoplasm. It binds to the 3'-oligouridine stretch found on all newly synthesized RNA polymerase III transcripts and is required for correct transcript termination and release, and also facilitates reinitiation (Maraia *et al.,* 1994). It can unwind both RNA/DNA hybrids and dsRNA (Xiao *et al.,* 1994). It may also be involved in the internal initiation of translation of poliovirus RNA and in poliovirus-infected cells is largely redistributed to the cytoplasm (Meerovitch *et al.,* 1993). It has also been found to bind to several other viral RNAs (van Verooj *et al.,* 1993).

A complex of a cellular protein and the poliovirus 3CD^{pro} protein was found to bind to the cloverleaf structure at the 5' end of poliovirus RNA

(Andino *et al.*, 1990). The cloverleaf structure has been shown to be essential for virus RNA replication (Andino *et al.*, 1993) and this domain functions independently from the internal ribosomal entry site (IRES) required for translation (Rohll *et al.*, 1994). The cellular protein was identified as an N-terminal fragment of EF-1α (Harris *et al.*, 1994). The significance of this complex is not clear in view of the finding that a 3AB–3CD[pro] complex also binds to the clovercleaf (see Section II,E,4), although it has been found that mouse cells are temperature-sensitive for initiation of poliovirus positive-strand synthesis, implicating a host factor in this process (Shiroki *et al.*, 1993). A putative host factor, a 67-kDa phosphoprotein which possesses autophosphorylation activity and can phosphorylate the α-submit of eIF-2 *in vitro*, has been shown to be able to function in the initiation of poliovirus negative-strand RNA synthesis *in vitro* (Morrow *et al.*, 1985). It has also been suggested that a terminal uridylyltransferase may act as a host factor in the initiation of poliovirus negative-strand synthesis by adding uridine residues to the 3' poly(A) end of virion RNA, which could anneal back to the poly(A) to form a hairpin primer for the polymerase (Andrews and Baltimore, 1986). dsRNA molecules joined at one end in a hairpin structure have been isolated from poliovirus-infected cells (Young *et al.*, 1985). A model was proposed in which VPg cleaved the hairpin and became covalently attached to the 5' UMP of the negative strand in a self-catalyzed transesterification reaction (Tobin *et al.*, 1989). Other models were suggested by Lubinski *et al.* (1986). Good evidence for involvement of a host protein in poliovirus replication comes from the use of a combined *in vitro* translation/replication system. Pre-initiation complexes isolated after inhibiting the initiation of RNA synthesis with guanidine-HCl were shown to require the addition of soluble cellular factors for initiation of RNA synthesis (Barton *et al.*, 1995).

Recently it has been shown that the human protein Sam68 binds strongly to the poliovirus 3D[pol] (McBride *et al.*, 1996). In uninfected cells, Sam68 was found to be located mainly in the nucleus. It is known to associate with Src during mitosis (Taylor and Shalloway, 1994; Fumagalli *et al.*, 1994), it has SH-2 and SH-3 binding domains, and it binds to ssRNA and dsRNA (Taylor *et al.*, 1994, 1995). In poliovirus-infected cells, Sam68 was relocated to the cytoplasm where it was bound to the 3D[pol] protein in membrane-bound replication complexes together with other virus proteins, including 2BC and 2C (McBride *et al.*, 1996).

A host protein may be involved in the resistance to tobacco mosaic virus conferred by the Tm-1 gene in tomato. Expression of this gene causes inhibition of RNA replication (Watanabe *et al.*, 1987) and mutations in resistance-breaking strains of the virus map close to the helicase-

like motif IV in the 126-kDa protein (Meshi *et al.,* 1988). A possible explanation is that the resistance gene could be an allele of a gene encoding an essential host component of the replicase complex. The product of the resistance gene would be a mutant of this host protein, which may be unable to form a functional replicase. The resistance-breaking strains would contain compensating mutations to allow production of an active replicase. Host-specific alterations in brome mosaic virus RNA accumulation, dependent on RNA 1, have also been reported (de Jong and Ahlquist, 1995). A recessive mutation *tom1* in *Arabidopsis thaliana* reduced replication of tobacco mosaic virus RNA to low levels, suggesting that the product of the wild-type gene was required for replication (Ishikawa *et al.,* 1993). Studies of host-range mutants (Kowal and Stollar, 1981) and analysis of the effects of some cis-acting seqences of alphaviruses (Kuhn *et al.,* 1992) also suggest a role of host factors in replication.

Other evidence for roles of host factors come from studies of inhibitors of host transcription, e.g., there are steps in the replication of cowpea mosaic virus (de Varennes *et al.,* 1985), Sindbis virus (Baric *et al.,* 1983a), and tobacco mosaic virus (Dawson, 1978) that are sensitive to actinomycin D. Another study suggested that a host protein may negatively control production of a tobacco mosaic virus subgenomic RNA (Blum *et al.,* 1989). A new approach that should uncover host genes required for the replication of brome mosaic virus has been opened up by the demonstration that this virus can replicate in yeast cells (Janda and Ahlquist, 1993). This opens up the powerful yeast genetic system for the creation of host mutants defective in virus RNA replication, and for the isolation of the genes involved. The ability of brome mosaic virus to replicate in yeast clearly depends on the ability of yeast proteins to substitute for plant proteins in the replication of the virus RNA and implies a degree of conservation. However, several animal and plant genes have been cloned by complementation in yeast, and yeast proteins can often substitute for mammalian proteins, e.g., yeast eIF-3 can substitute for the mammalian factor in a heterologous reconstituted *in vitro* assay system (Naranda *et al.,* 1994). The method may therefore be applicable to a range of animal and plant viruses.

IV. The Role of Membranes in RNA Replication

The genome of animal and plant positive-stranded RNA viruses from a number of different virus supergroups is replicated *in vivo* in membrane-bound complexes. Isolated membrane complexes have been

found to be capable of *in vitro* synthesis of replicative intermediates, elongation and release of genomic-length RNA, and in some cases, initiation of RNA synthesis on endogenous templates which remain bound to the replication complex. Examples include alpha-like virus supergroup, Sindbis virus (Barton *et al.*, 1991), alfalfa mosaic virus (de Graaf *et al.*, 1993), cucumber mosaic virus (Jaspars *et al.*, 1985), foxtail mosaic virus (Rouleau *et al.*, 1993), tobacco mosaic virus (Young and Zaitlin, 1986); picorna-like virus supergroup, poliovirus (Takeda *et al.*, 1986; Bienz *et al.*, 1990); cowpea mosaic virus (Eggen *et al.*, 1988); plum pox virus (Martin *et al.*, 1995); flavi-like virus supergroup, West Nile virus (Grun and Brinton, 1988), Kunjin virus (Chu and Westaway, 1992), dengue virus (Bartholomeusz and Wright, 1993); carmo-like virus supergroup, red clover necrotic mosaic virus (Bates *et al.*, 1995), turnip crinkle virus (Song and Simon, 1994); sobemo-like virus supergroup, velvet tobacco mottle virus (Rohozinski *et al.*, 1986); corona-like virus supergroup, mouse hepatitis virus (Brayton *et al.*, 1982, 1984). In the case of flockhouse virus, it was possible to remove the bound RNA from the membrane-bound complex by nuclease digestion to produce a template-dependent RNA polymerase. Such preparations synthesized only negative-strand RNA (isolated as dsRNA) with a genomic RNA template, but on addition of certain neutral or negatively charged phosphoglycerolipids (PGLs) both negative and positive strands were produced, giving complete replication of the genomic RNA (Wu and Kaesberg, 1991; Wu *et al.*, 1992). It was suggested that initiation of positive-strand synthesis may result from a direct GPL-replicase interaction, analogous to activation of the *E. coli* replication initiator protein dnaA by diphosphatidylglycerol (Sekimizu and Kornberg, 1988), or a change in membrane configuration to mimic similar changes that may occur during replication *in vivo*. Membranes also appear to be important for the replication of poliovirus RNA in a combined translation and replication system (Molla *et al.*, 1991, 1992, 1993; Barton *et al.*, 1995). For some other viruses it has been possible to produce template-dependent RNA polymerases after detergent solubilization and removal of the endogenous RNA (reviewed by de Graaf and Jaspars, 1994). Generally, only the complementary strand has been synthesized by such preparations, although a solubilized polymerase able to catalyze the complete replication of cucumber mosaic virus (CMV) RNA has been described (Hayes and Buck, 1990). *In vitro* replication of CMV RNA in this system was, however, very inefficient with only a small fraction of the template being copied and the ratio of positive to negative strands much lower than that *in vivo*.

Infection of cells with many positive-stranded RNA viruses results in the formation of multiple vesicles or invaginations in the membranes of various organelles. It has been suggested that continuous synthesis of lipid is required for the replication of some viruses. Infection of cells by poliovirus (Guinea and Carrasco, 1990; Maynell *et al.*, 1992) or Semliki Forest virus (Perez *et al.*, 1991) led to increased lipid synthesis; conversely, replication of both these viruses was inhibited by cerulenin, an inhibitor of lipid biosynthesis. Cerulenin, which has several activities apart from inhibition of lipid biosythesis (Odd and Wu, 1993), also inhibited poliovirus replication in an *in vitro* translation/replication system (Molla *et al.*, 1993). It remains uncertain whether increased lipid synthesis in cells infected by these viruses is a requirement for, or a consequence of, virus replication.

Infection of cells with poliovirus results in the formation of numerous vesicles, derived largely from the rough endoplasmic reticulum, which have been compared to the intermediate or transport vesicles involved in cellular protein sorting and secretion (Bienz *et al.*, 1987), although it has recently been shown that the virus-induced membranous structures are bounded by double lipid bilayers (Schlegel *et al.*, 1996). Lysosomes, trans-Golgi, and the trans-Golgi network also contribute to the virus-induced membranous structures (Schlegel *et al.*, 1996). Brefeldin A, an inhibitor of the cellular secretory pathway, inhibits poliovirus replication, possibly by preventing the formation of these vesicles (Maynell *et al.*, 1992). Expression of the 2C or 2BC proteins separately in cells using vaccinia virus vectors induced the formation of similar vesicles, but did not cause an increase in lipid synthesis (Cho *et al.*, 1994; Aldabe and Carrasco, 1995). 2BC has also been reported to induce vesicle formation in yeast (Barco and Carrasco, 1995). The replication complex was located on the surface of the vesicles; proteins 2B, 2C, and 2BC were located exclusively with the complex and may be responsible for its organization, whereas 3D and its precursors were also found in the peripheral cytoplasm (Bienz *et al.*, 1990). Isolated replication complexes were shown to consist of a central replication complex consisting of small, densely packed vesicles, surrounded by larger vesicles in a rosette-like arrangement, which could be disrupted by guanidine, suggesting a role of the 2C protein in the organization of the rosette (Bienz *et al.*, 1992, 1994; Troxler *et al.*, 1992). The larger vesicles may aid in the release of newly synthesized RNA and in its subsequent encapsidation (Pfister *et al.*, 1995). Expression of proteins 2B and 3A separately blocked cellular secretion in the absence of virus infection; evidence indicated that in virus-infected

cells, 3A may function to block the fusion of the virus-induced vesicles with the Golgi membranes (Doedens and Kirkegaard, 1995). The 3A and 2B proteins may attach to membranes via hydrophobic domains (Datta and Dasgupta, 1994; van Kuppeveld *et al.*, 1995); the 2C protein may attach via the hydrophobic side of an amphipathic helix (Paul *et al.*, 1994b). It has been suggested that the 3A protein forms a membrane anchor for VPg (in the form of 3AB) in the initiation of RNA synthesis. Mutations in the hydrophobic domain of 3A affected initiation of RNA synthesis, *in vitro* uridylylation of VPg, and *in vivo* positive-stranded RNA synthesis (Giachetti and Semler, 1991) (see also Section II,E,4).

Large arrays of membranous vesicles, analogous to those seen in poliovirus-infected cells and containing viral RNA and nonstructural proteins, have been detected in cells infected with cowpea mosaic virus, a plant picorna-like virus (Wellinck *et al.*, 1988). Similar structures were observed when the RNA B-encoded 200K or 60K (58K-VPg) protein was expressed in insect cells, and the 60K protein was shown to be associated with the vesicles (van Bokhoven *et al.*, 1992). The cowpea mosaic virus RNA B-encoded 58K protein is the equivalent of the poliovirus 2C protein (see Section II,E,4) and hence may induce the vesicles in a similar way to the 2C protein. Cowpea mosaic virus has no proteins with significant sequence homology to the poliovirus 2B and 3A proteins, but it is possible that the 58K protein might have domains which serve the same functions.

The cytopathic structures formed by viruses in another plant picorna-like virus genus, the potyviruses, are somewhat different and can include formation of pinwheels by the cytoplasmic inclusion (CI) protein (which has the helicase-like domain) and invaginations in the nuclear membrane (Lesemann, 1988). The tobacco etch potyvirus 6-kDa protein expressed in transgenic plants apparently causes the formation of, and localizes to, membranous proliferations associated with the periphery of the nucleus (Restrepo-Hartwig and Carrington, 1994). These may be the sites of virus replication in infected cells. The possible role of the 6-kDa protein (probably equivalent to the poliovirus 3A protein) as a membrane anchor for VPg and its association with other replication proteins was discussed in Section II,E,4. Overall, it appears that there are likely to be similarities and differences between potyvirus and picornavirus replication.

There also appear to be diversities in the membrane localization of replication complexes in the alpha-like supergroup. RNA synthesis of alphaviruses, such as Sindbis and Semliki Forest virus, takes place on membranous structures called type I cytopathic vacuoles (CPVIs), on the surface of which are located the virus replication proteins nsP1,

nsP2, nsP3, and nsP4 (Froshauer *et al.*, 1988; Peränen and Kääriäinen, 1991). CPVIs are modified endosomes and lysosomes, which have characteristic invaginations (spherules), which may represent the attachment sites for the replication complexes. Spherules are also found at the plasma membrane. When nsP1 was expressed alone in cells synchronously, it was located first on the cytoplasmic side of the plasma membrane and then moved to endosomes and later lysosomes (Peränen *et al.*, 1995). Evidence indicated that nsP1 could be acylated, possibly at the plasma membrane, and that it may be attached to membranes by a fatty acid residue; it may act as an anchor for the other replication proteins. A large proportion of the nsP2 protein is normally targeted to the nucleus, but this is irrelevant for replication, because redirection to the cytoplasm by removal of the nuclear localization signal did not affect virus replication (Rikkonen *et al.*, 1994).

Replication complexes of tobacco mosaic virus, a plant alpha-like virus, are associated with cytoplasmic inclusions, called viroplasms, which enlarge during the course of infection to form "X bodies." They are composed of aggregates of tubules, possibly derived from the endoplasmic reticulum, which may be twisted round each other to form ropes, embedded in a ribosome-rich matrix (Esau and Crinshaw, 1967; 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 are therefore the likely sites of RNA replication. In contrast, cytoplasmic invaginations of the chloroplast outer membrane are the site of RNA synthesis of turnip yellow mosaic virus RNA (reviewed in Garnier *et al.*, 1986); the chloroplast outer membrane is probably also the site of alfalfa mosaic virus RNA replication (de Graaf *et al.*, 1993). Replication of cucumber mosaic virus may be associated with invaginations in the vacuolar membrane (tonoplast) (Hatta and Francki, 1981). In the case of brome mosaic virus, the 1a and 2a replication proteins were initially localized to punctate structures in the cytoplasm, which aggregated into well-defined structures adjacent to the nucleus, containing nascent viral RNA (Restrapo-Hartwig and Ahlquist, 1995). The mechanism of targeting of replication complexes to different regions of the cell is unknown, but it is interesting that replication complexes of brome mosaic virus in yeast were membrane-bound (Quadt *et al.*, 1995). Binding of brome mosaic virus or alfalfa mosaic virus replication proteins to membranes in yeast (Quadt *et al.*, 1995) or plants (de Graaf *et al.*, 1995b) did not require the presence of viral RNA.

In the carmo-like virus supergroup, the formation of multivesicular bodies (MVBs) derived from peroxisome membranes is characteristic of several tombusviruses (artichoke mottled crinkle, cymbidium ringspot,

eggplant mottle crinkle, tomato bushy stunt). In some cases, dsRNA has been located to the MBVs indicating that they are the likely site of replication (Russo *et al.*, 1983; Lupo *et al.*, 1994). However, with another tombusvirus, carnation Italian ringspot virus, MBVs were found to be derived by peripheral vesiculation of mitochondria (Russo *et al.*, 1995). The replication proteins of tombusviruses (see Section II,E,2) are known to be membrane-bound (Lupo *et al.*, 1994; Scholthof *et al.*, 1995). Analysis of hybrids between cymbidium ringspot and carnation Italian ringspot viruses indicated that the subcellular origin of the MBVs was determined by the N-terminal region of the preread-through protein encoded by the 5'-proximal ORF (Russo *et al.*, 1995; M. Russo, personal communication).

V. CIS-ACTING NUCLEOTIDE SEQUENCES REQUIRED FOR RNA REPLICATION

Essential cis-acting sequences will include promoters for negative-strand and positive-strand RNA synthesis. It is also possible that additional sequences might be required for the assembly of replication complexes. Assembly of brome mosaic virus replication complexes in yeast requires the presence of viral RNA (Quadt *et al.*, 1995). However, once complexes have been assembled *in vivo* in yeast or plants, after isolation and removal of the RNA template, they remain competent to initiate negative-strand synthesis on added positive-strand templates (Miller and Hall, 1983; Quadt *et al.*, 1995; Sun and Kao, 1996). It is therefore possible that assembled replication complexes, having copied one template, could be recycled for use on another template. Hence the cis-acting sequences required for replicase assembly and for promoter recognition and initiation of negative-strand synthesis may not be identical, although they are likely to have elements in common. Replication complexes assembled on positive-strand templates could be recycled and modified to recognize negative-strand templates, and evidence suggests that this is the case for the alphaviruses (see Section II,E,1). However, the ability of flockhouse virus to initiate RNA synthesis on negative-strand templates (Ball, 1994) suggests that, for this virus, a functional RNA polymerase can be assembled on a negative-strand template, although whether this occurs during a normal virus infection remains to be shown. Since accumulation of virus RNA *in vivo* depends on expression of replication proteins and encapsidation, as well as the RNA replication process *per se*, methods for identifying cis-acting sequences specifically required for RNA replication have

relied on DI (and in special cases satellite) RNAs, *in vitro* transcription systems, and in the case of viruses with segmented genomes, genomic RNA segments not required for replication.

A. 3'-Terminal Sequences of Positive Strands

1. tRNA-like Sequences in RNAs of Some Plant Alpha-like Viruses

Sequences that can be folded into tRNA-like structures have been found at the 3'-termini of several genera of plant viruses in the alpha-like virus supergroup (reviewed by Florentz and Gierge, 1995). Such termini are substrates for specific aminoacyl-tRNA synthetases and can be aminoacylated with valine (tymoviruses, sunnhemp mosaic tobamovirus), tyrosine (bromoviruses, cucumoviruses), histidine (most tobamoviruses), although aminoacylation is much less efficient than with the cognate canonical tRNA. They can also be adenylated by tRNA nucleotidyltransferases and, after aminoacylation, can bind elongation factors, such as EF1-α. Some RNAs are also substrates for RNase P.

The structural requirements in the tRNA-like sequence of brome mosaic virus (BMV) for RNA replication, aminoacylation and adenylation have been examined in detail (reviewed by David *et al.*, 1992; Duggal *et al.*, 1994). A 134 nt 3'-terminal fragment containing the tRNA-like structure could act as a template for negative-strand synthesis by an isolated RdRp *in vitro*, but large deletions which removed several stem–loops and pseudoknots in the region from nt 135 to 280 upstream of the 3' terminus reduced accumulation of RNA 3 *in vivo* to undetectable levels, indicating that a larger 3' region was needed for efficient replication *in vivo* (Lahser *et al.*, 1993). Furthermore, although the 3'-terminal 200 nt of RNAs 1, 2, and 3 are very similar in sequence, reciprocal exchanges led to aberrant replication, again suggesting a requirement for compatibility with upstream sequences (Duggal *et al.*, 1992). In the case of tobacco mosaic virus (TMV), removal of a single pseudoknot structure upstream of the tRNA-like structure reduced RNA replication (Takamatsu *et al.*, 1990). The terminal A residue of the BMV template is not copied or required for infectivity; it is added on by a posttranscriptional mechanism, possibly by the RdRp as with Qβ RNA (Blumenthal and Carmichael, 1979) or by tRNA nucleotidyltransferase (see below). Initiation of RNA synthesis therefore takes place internally on the template (initiating with a 5'-G) and it is noteworthy that the infectivity of RNA transcripts, produced from cDNA clones, is generally tolerant to 3' extensions of moderate length (Boyer and Haenni, 1994). Mutations in the

terminal C residues of the –CCA terminus of RNA 3 that greatly reduced *in vitro* RNA synthesis were rapidly repaired *in vivo*, probably by nuclease degradation and resynthesis by tRNA nucleotidyltransferase, an enzyme known to be able to repair tRNA CCA ends (Rao *et al.*, 1989), consistent with the suggestion of a telomere-like function for the 3′ ends of genomic RNA molecules (Weiner and Maizels, 1987). Regions of the tRNA-like structure that are important for replication, aminoacylation, and adenylation overlapped, but were distinguishable. Some aminoacylation-defective mutants of RNA 3 were not greatly debilitated in RNA replication, indicating that charging with tyrosine is not essential for replication of this RNA (Dreher *et al.*, 1989). Further experiments indicated a possible correlation between aminoacylation and replication for RNA 1 (Duggal *et al.*, 1994) and RNA 2 (Rao and Hall, 1991). *In vitro* transcription studies with turnip yellow mosaic virus (TYMV) RdRp indicated that 3′ tRNA-like structure contained the promoter for negative-strand synthesis (Morch *et al.*, 1987; Gargouri-Bouzid *et al.*, 1991). Moreover, TYMV RNAs with anticodon loop substitutions that resulted in decreased valylation failed to replicate efficiently (Tsai and Dreher, 1991). In some cases, second-site suppressor mutations that restored both valylation and replication appeared (Tsai and Dreher, 1992). Nevertheless, a requirement of aminoacylation for efficient replication cannot be established unequivocally by such correlations, because sequences needed for aminoacylation may also be required for template recognition by the replicase. The 3′ termini of tobraviruses and furoviruses can be folded into tRNA-like structures that cannot be aminoacylated.

Reciprocal exchanges of 3′ tRNA-like termini have been carried out to determine the template specificity of replicase complexes. It was found that TMV RNA containing a 3′ terminal region of BMV RNA 3 was amplified by TMV replicase, although much less efficiently than the wild-type 3′ end, indicating that the TMV replicase can recognize some feature of the BMV RNA 3 3′-terminal structure (Ishikawa *et al.*, 1991a). However, BMV RNA 3 containing a 3′ terminal region of TMV RNA was not amplifiable by BMV replicase provided by RNA1 and 2, or by TMV RNA, or a mixture of all three. Similarly, replacement of the 3′-terminal tRNA-like structure of TYMV with that of BMV or TMV gave only low viral accumulation in protoplasts and no systemic symptoms on plants (Skuzeski *et al.*, 1996). In contrast, BMV RNA 3 containing a 3′ terminal region of cucumber mosaic virus (CMV) RNA 3 could be amplified by BMV RNA 1 and RNA 2 to give both the hybrid RNA 3 and a subgenomic RNA (Rao and Grantham, 1994), probably reflecting the greater structural similarity of the 3′ end of BMV and

CMV RNAs than BMV and TMV RNAs. BMV RNA 2 containing a 3'-terminal region of CMV RNA 2 could not be amplified by the BMV RNAs, perhaps reflecting the need for interactions with upstream sequences, since only the terminal 186 nt were exchanged. Reciprocal 3' exchanges between the 3' ends of the RNAs 3 of BMV and a closely related bromovirus, cowpea chlorotic mottle virus (CCMV), indicated that the 3' sequences were not the sole or the major determinants of template specificity (Pacha and Ahlquist, 1991). The 3' sequences of these two viruses are, however, much more closely related to each other than the other examples discussed above. Overall, it may be concluded that 3' terminal sequences are important determinants of template specificity, with replicase complexes being able to recognize some common structural elements in related viruses.

2. Other 3' Structural Elements in Alpha-like Viruses

Whereas the bromoviruses and cucumoviruses have 3' tRNA-like structures, viruses in the other two genera of the *Bromoviridae* family, the alfamoviruses and ilarviruses, have 3' structures consisting of a series of stem–loop structures (see Section II,F). In an *in vitro* assay with a template-dependent RNA polymerase isolated from plants infected with alfalfa mosaic virus, it was shown that 3' deletions of up to 133 nt in RNA 3 did not affect its ability to act as a template for negative-strand synthesis (van der Kuyl *et al.*, 1990). However, a 3' deletion of 163 nt completely abolished its template activity. In an *in vivo* assay in which deletion mutants of RNA 3 were used to infect transgenic plants expressing the P1 and P2 replication proteins, it was found that deletions of 11 to 133 nt from the 3' end of the RNA-reduced replication to about 1% of that of the full-length RNA 3, whereas a 3' deletion of 200-nt-reduced replication to undetectable levels (van der Kuyl *et al.*, 1991). It is noteworthy that nucleotides 11 to 127 and nucleotides 133 to 208 of RNA 3 contain independent coat protein (CP) binding sites (Reusken *et al.*, 1994; see Section II,F), the replicase complex in virus-infected plants contains the CP (Quadt *et al.*, 1991) and replicase in P1P2 plants can assemble *in vivo* in the absence of CP. It is possible that the region from nucleotides 11 to 127 is important for the assembly of the replication complex lacking CP and as the promoter for negative-strand synthesis. It is known that CP inhibits the activity of isolated replication complexes in synthesizing negative strands on positive-strand templates, probably by competing for the replicase binding site (Houwing and Jaspars, 1986; Quadt *et al.*, 1991).

Some alpha-like viruses have 3' poly(A) tails (Table II) and in some cases it has been shown that there is a poly(U) sequence at the 5' end

of the negative strand, as in potato virus X (Dolja *et al.,* 1987). Deletion
of the poly(A) tail from beet necrotic yellow vein virus (BNYVV) RNA 3
caused a great reduction in ability to replicate (in the presence of RNAs
1 and 2) and the poly(A) tail was restored in the progeny, together with
a short upstream U-rich sequence not originally present (Jupin *et al.,*
1990a). Sequence evidence suggested that the poly(A) sequence was
not restored by recombination with RNA 1 or 2. Addition of poly(A) also
occurred during infection with 3' poly(A)-deficient transcripts of white
clover mosaic virus (Guilford *et al.,* 1991). Further deletion analysis
with BNYVV RNA 3 showed that a 67-nt sequence upstream of the
poly(A), which can be folded into a double-hairpin secondary structure
that is conserved in all four of the virus RNAs, was essential for repli-
cation and that a further 50-nt upstream contributed to efficient repli-
cation (Jupin *et al.,* 1990b; Richards and Tamada, 1992).

Viruses in the animal *Alphavirus* and *Rubivirus* genera of the
Togaviridae family also have 3' poly(A) tails. In the alphaviruses, there
is a highly conserved 19-nt U-rich sequence upstream of the poly(A)
that is required for RNA replication (reviewed by Strauss and Strauss,
1994). All naturally occurring Sindbis and Semliki Forest virus DI
RNAs were found to contain a minimum of 50 nt from the 3' end of the
genome, including the conserved 19-nt sequence. Within the 3' un-
translated region of alphaviruses, there are variable numbers of 40–60
nt repeat sequences; these appear to have a host-specific effect on RNA
replication. The 19 nt alphavirus conserved sequence is not found at
the 3' end of rubella virus RNA, but there is a sequence 58 nt upstream
of the poly(A) that can be folded into a stable stem–loop structure, with
a GC-rich stem and a loop composed only of U residues (reviewed by
Frey, 1994). There is evidence that this structure may be required for
RNA replication and it has been implicated in the the binding of
calreticulin (Nakhasi *et al.,* 1994; see Section III).

3. 3'-Terminal Structures in the Picorna-like Viruses

The 3' termini of all the viruses in the picorna-like virus supergoup
are polyadenylated and several lines of evidence suggest that the
poly(A) tract is present, not just to protect the RNA from 3' exonuclease
degradation, but also to provide an essential cis-acting sequence for
RNA replication. The poly(A) tail has been shown to be required for the
infectivity of poliovirus (Spector and Baltimore, 1974; Sarnow, 1989)
and cowpea mosaic virus (Eggen *et al.,* 1989a). The poly(A) sequence is
transcribed from a poly(U) sequence at the 5' end of the negative
strand. However, in poliovirus, the poly(A) tract has been reported to
be longer than the poly(U) tract (Larsen *et al.,* 1980). The additional A

residues may be added on by slippage or by terminal adenylyl transfer-
ase activity (Neufeld *et al.*, 1994). Addition of poly(A) tracts occurs during
infection with 3′ poly(A)-deficient transcripts of cowpea mosaic virus
(Eggen *et al.*, 1989b) and plum pox virus (Riechmann *et al.*, 1990).

Sequences upstream of the 3′ poly(A) are also important for replica-
tion, and may form secondary structures that include part of the
poly(A) tail. The 3′ 151 nt of cowpea mosaic virus RNA M contains all
the 3′-terminal cis-acting elements required for RNA replication (Rohll
et al., 1993). The 3′ 65 nt upstream of the poly(A) in both RNAs B and
M have a high degree of sequence similarity and can be folded to create
a stem–loop, containing four A residues of the poly(A), linked to a Y-
shaped structure (Eggen *et al.*, 1989a; Rohll *et al.*, 1993). Mutagenesis
showed the importance of both these features for virus RNA replica-
tion. Another upstream putative stem–loop structure within the 3′-
terminal 151 nt of RNA M was also needed for replication (Rohll *et al.*,
1993), although the sequences of RNAs M and B have little sequence
similarity in this region. Nevertheless, replacement of the 3′-terminal
210 nt of RNA M by the 3′ 500 nt of RNA B had only a small effect on
replication (van Bokhoven *et al.*, 1993).

The 3′ end of poliovirus RNA, upstream of the poly(A) tract, can be
folded into a tRNA-like structure (Pilipenko *et al.*, 1992), although this
is less similar to cellular tRNAs than those discussed in Section V,A,1.
Further analysis indicated that a pseudoknot, formed between the 3′
untranslated region and sequences upstream of the translational ter-
minator, was important for replication and one of the stem–loop
structures involved base-pairing with five A residues of the poly(A) tail
(Jacobson *et al.*, 1993). Moreover, an 8-nt insertion which would affect
the 5′-proximal of the 3′ stem–loop structures conferred a ts replication
phenotype (Sarnow *et al.*, 1986). Further analysis has confirmed the
importance for replication of secondary structure in the 3′ untrans-
lated regions of poliovirus and other picornaviruses (Rohll *et al.*, 1995).
Mutagenesis of the single 3′-terminal stem–loop formed by a human
rhinovirus untranslated region indicated the importance of the loop
sequence, the stability of the stem, and its proximity to the poly(A)
tract (Rohll *et al.*, 1995). Binding of picornavirus replication complexes
to the 3′-terminal sequences may involve both viral and host proteins.
Cui *et al.* (1993) reported that the encephalomyocarditis virus (EMC)
3D^{pol} protein bound specifically to the 3′ noncoding region of EMC
RNA. Further analysis showed that binding was dependent on cova-
lent attachment of the 3′ noncoding region and the poly(A), a U-rich
sequence upstream of the poly(A) and part of the poly(A) sequence; a
stem–loop structure with a pseudoknot linking the poly(A) to a U-rich

loop was proposed (Cui and Porter, 1995). Evidence also suggested that binding of poliovirus 3CD^{pro} protein to the poliovirus 3' pseudoknot-poly(A) structure in the presence of the 3AB protein occurred via contact points in 3D^{pol} (Harris *et al.*, 1994). Host proteins, inducible by virus infection, which bound to the 3' regions of rhinovirus and poliovirus RNAs, have also been reported (Todd *et al.*, 1995).

4. *3'-Terminal Structures in the Carmo-like Viruses*

RNAs of all viruses in the carmo-like virus supergroup, *together* with several satellite and DI RNAs, have –CCC 3' termini with no poly(A) tail (Russo *et al.*, 1994). Clones of cymbidium ringspot virus (CyRSV) RNA with the terminal CCC deleted, so that the 3' terminus was –G, were infectious for plants with a delay of 2 to 3 days in appearance of symptoms. Analysis of the progeny RNA showed that the 3' end had been repaired by the addition of one or more C residues (Dalmay *et al.*, 1993b). Similarly, –GGGG termini were repaired to –GCCC, although –GGCC termini were stable. Artificially added 3' poly(A) tails were removed *in vivo*. Removal of four nucleotides from the 3' end led to complete loss of infectivity. A repair mechanism also operated with a CyRSV satellite RNA which had heterogeneous 3' termini (–C, –CC, –CCC, –CCCA). Up to eight residues could be removed from the longest 3' terminus without complete loss of infectivity and the molecules were repaired to give termini mostly identical to the wild-type (Dalmay and Rubini, 1995). The satellite is therefore even more tolerant than the genomic RNA to 3'-terminal deletions. Short repeat units were characteristic of the 3' termini of both the genomic (**GCA GCA AU GCA GC CC**) and satellite (**ACA ACA AC CCA**). Turnip crinkle satellite RNA D molecules with 3'-terminal truncations also had their 3' termini restored *in vivo* to the motif $(C_{1-2})UG(C_{1-3})$, giving ends similar or identical to the wild-type satellite (CCUGCCC) (Carpenter and Simon, 1996). This also probably occurred by a repair mechanism, rather than by recombination with the genomic RNA. Whether all these repairs are carried out by the viral polymerase or a cellular enzyme is not known. However, as with the tRNA-like termini (see Section V,A,1), analogies to telomerase, an enzyme that contains an RNA template, and to the short repeated sequences in the telomeres of cellular chromosomes (Blackburn, 1993) have been made (Dalmay and Rubino, 1995; Carpenter and Simon, 1996).

A CyRSV DI RNA retained a block of 102 nt from the 3' terminus of the genomic RNA, of which 77 nt were required for replication (Havelda *et al.*, 1995). Similar 3' regions are retained in DI RNAs of other tombusviruses, such as cucumber necrosis virus and tomato bushy stunt

virus (Knorr *et al.*, 1991; Finnen and Rochon, 1993; White and Morris, 1994; Chang *et al.*, 1995). The 77-nt CyRSV DI RNA 3′ domain could be folded into a structure composed of three hairpins and two non-base-paired regions. Mutational analysis showed that replication competence depended on maintaining the structure of the stems (Havelda and Burgyan, 1995). The ability to form 3′ stem–loop structures is conserved in all the carmo-like viruses. Using an *in vitro* transcription system, it has been shown that the promoter for negative-strand synthesis of a turnip crinkle virus (TCV) satellite RNA (sat-RNA C) is contained within the 3′-terminal 29 nt of the positive strand (Song and Simon, 1995). Structural probing revealed the presence of hairpin structure within this region. Mutagenesis showed that the primary sequence or size of the loop was not important for replication. However mutations that altered the structural integrity of the lower part of the stem strongly reduced template activity *in vitro*. The 3′ 37 nt of sat-RNA C could be joined to an inactive template and the resultant hybrid was competent for transcription *in vitro* by the TCV RdRp. The 3′-terminal sequences of TCV genomic RNA and sat-RNA C are 90% identical and the 3′ genomic RNA could be folded into a similar hairpin structure. However, upstream motifs in the 3′ untranslated region of TCV RNA, close to the end of the coat protein ORF, are also important for RNA replication *in vivo* (Carpenter *et al.*, 1995). It is noteworthy that the TCV RdRp could utilize negative strands of sat-RNA C as templates (Song and Simon 1994). Template activity depended on 5′-proximal sequences, but was insensitive to 3′ deletions, suggesting that the RdRp recognized a 5′ sequence and then scanned the RNA for a 3′ terminus to initiate positive-strand synthesis. It is possible that the RdRp might recognize a stem–loop structure at the 5′ end of the negative strand.

5. 3′-Terminal Structures in Coronavirus RNAs

Analysis of mouse hepatitis virus (MHV) DI RNAs has shown that a 3′-terminal sequence of 436 nt is needed for RNA replication (Kim *et al.*, 1993). This sequence can be folded into a structure containing several hairpins which may correspond to binding sites for host proteins (Yu and Leibowitz, 1995). However, the specific requirements of negative-strand synthesis were investigated using artificial DI RNAs carrying 5′ deletions that prevented complete replication (Lin *et al.*, 1994). It was found that the cis-acting signal for negative-strand synthesis lay in the 55 nt from the 3′ end plus poly(A) tail of the MHV genome, which included the 3′-proximal hairpin structure. No further upstream sequences were required, but DI RNAs which transcribed subgenomic

RNAs synthesized less negative-stranded RNA. It is noteworthy that the poly(U) tract at the 5' terminus of bovine coronavirus negative strands (8–20 nt) is shorter than the poly(A) tract at the 3' end of the positive strands (100–130 nt) (Hofmann and Brian, 1991). This is similar to the situation discussed for poliovirus (see Section V,A,3).

6. 3'-Cis-Acting Sequences of Novaviridae RNAs

The cis-acting sequences required for the replication of flockhouse virus (FHV) RNA 2 have been studied using an *in vivo* system in which the templates were transcribed intracellularly from DNA plasmids containing FHV cDNA flanked by a T7 promoter and a ribozyme, using T7 RNA polymerase expressed from a vaccinia virus recombinant; replication proteins were provided by FHV RNA 1. These studies revealed that 50–60 nt at the 3' end of FHV RNA 2 were required for replication (Ball and Li, 1993; Ball, 1994).

B. 5'-Terminal Sequences of Positive Strands and 3'-Terminal Sequences of Negative Strands

The 5'-terminal regions of positive strands and 3'-terminal regions of negative strands are considered together, because mutations which affect one will necessarily also affect the other, and complementary secondary structures can sometimes be formed for both termini.

The ability of the 5'-terminal 90 nt of poliovirus RNA to fold into a cloverleaf structure, its ability to bind 3CD[pro] in the presence of 3AB or an N-terminal fragment of EF-1α, its requirement for RNA replication, and a model for its role in initiating positive-strand synthesis were discussed in Sections II,E,4 and III. Both the 5'-positive and 3'-negative termini can form cloverleaf structures, but mutagenesis showed that only the positive-strand cloverleaf is functional (Andino *et al.*, 1990). The 5' 44 nt of RNAs B and M of cowpea mosaic virus, a plant picorna-like virus, have a high degree of sequence similarity and are interchangeable (van Bokhoven *et al.*, 1993), but whether they are structurally and functionally analogous to the poliovirus 5' cloverleaf structure is not known.

Marsh and Hall (1987) discovered sequences in the 5' untranslated regions of brome mosaic virus (BMV) RNAs that resemble consensus sequences for the internal control regions (ICR1 and ICR2) of tRNA promoters. In the mature tRNA, ICR1 (box A), and ICR2 (box B) correspond to the D-loop and T-loop respectively (Geiduschek and Kassavetis, 1992). ICR-like sequences have also been found in other bromoviruses, cucumoviruses, tobamoviruses, tobraviruses, tymoviruses, and tobacco

necrosis satellite virus (Marsh *et al.*, 1989). Pogue and Hall (1992) proposed that the 5'-terminal region of BMV RNA 2 could be folded into a stem–loop structure with the ICR2-like motif in the loop and the ICR1-like motif comprising part of the stem. Similar structures were predicted for the 5' termini of BMV RNAs 1 and 3, RNAs of cucumber mosaic virus (CMV), and cowpea chlorotic mottle virus (CCMV) (Pogue and Hall, 1992) and alfalfa mosaic virus (van der Vossen *et al.*, 1993). Mutational analysis established the importance of the proposed structure and the ICR-like motifs in the replication of BMV RNA 2 (Pogue and Hall, 1992; Pogue *et al.*, 1990, 1992). In particular, it was shown that the structure was important in the 5'-terminal region of the positive strand, but not in the 3'-terminal region of the negative strand. ICR2-like motifs were located within 27-nt repeats in the 5' untranslated sequence of alfalfa mosaic virus RNA 3, which were shown to be important for the replication of this RNA (van der Vossen *et al.*, 1993). These results led to a model in which a host factor, possibly a transcription factor associated with RNA polymerase III, binds to the ICR-like region at the 5' terminus of the positive strand of the double-stranded replicative form dsRNA and plays a role in the formation of an initiation complex for positive-stranded RNA synthesis (Pogue and Hall, 1992; Pogue *et al.*, 1994). Support for the model and involvement of ICR-like motifs comes from the finding that binding of some host proteins to the 5'-terminal sequence of the positive strand and the 3'-terminal sequence of the negative strand did not occur in RNA 2 mutants with substitutions in the ICR2-like motif and known to be debilitated in replication (Duggal and Hall, 1995).

It is unlikely that the ICR-like motifs are the only 5' elements involved in positive-strand RNA replication in these plant alpha-like viruses, since longer 5' regions (~90 nt) are required for efficient replication of RNA 3 of BMV (French and Ahlquist, 1987), CCMV (Pacha *et al.*, 1990), and CMV (Boccard and Baulcombe, 1993), and ICR-like motifs are not readily discernible in the 5' region of CMV RNA 3. The 5' untranslated sequence of tobacco mosaic virus lacks G residues and contains multiple CAA repeats. The sequence is highly conserved and the 5'-terminal 31 nt are almost identical in different strains. Deletion analysis of the 5'-terminal region of the L strain showed that large deletions (nucleotides 9–47 or 25–71) abolished replication, but of approximately 10-nt deletions across the whole region, only a deletion of nucleotides 2–8 abolished replication (Takamatsu *et al.*, 1991). The progeny of *in planta* replication of some TMV subgenomic replicons included a molecule with only 23 nt at the 5' terminus (Raffo and Dawson, 1991). There is no evidence for the involvement of ICR-like

motifs in this region. The 312-nt 5' untranslated region of beet necrotic yellow vein virus RNA 3 can be folded into a structure containing several stem–loop structures, which involves long-range base-pairing between different cis-active elements. Chemical and enzymatic probing, and mutational analysis, has provided evidence for the structure and its involvement in the promotion of positive-strand synthesis (Gilmer et al., 1992b, 1993).

The structure of the 3' end of the negative strand may also be important for replication. Double-stranded RNAs isolated from cells infected by CMV and a satellite (Collmer and Kaper, 1985), potato virus X (Dolja et al., 1987), and Semliki Forest and Sindbis viruses (Wengler et al., 1979, 1982) contained an unpaired G residue at the 3' terminus of the negative strand. Wu and Kaper (1994) showed that the negative strand of a CMV satellite would only act as a template for an isolated CMV RdRp if it contained this additional nontemplated G. Whether this also applies to replication of the genomic RNAs is not known. It is noteworthy, however, that long 5'-terminal extensions generally render RNA transcripts produced from cDNA clones noninfectious (Boyer and Haenni, 1994). Since a 5'-terminal positive-strand extension would be copied to produce a 3'-terminal negative-strand extension, it may be the unpaired base (rather than the length or sequence of the extension) that is important. This could be required for unwinding an RF structure by helicases that require a 3' extension (see Section II,B) and possible recognition by the replicase after unwinding.

In the animal alphaviruses, there is a conserved stem–loop structure at the 5' terminus which is important for replication and this has been proposed to be functional as its complement at the 3' end of the negative strand (reviewed by Strauss and Strauss, 1994). This sequence is found in some Sindbis virus DI RNAs. However, in some Sindbis virus DI RNAs, the 5' terminus consists of nt 10 to 75 of a cellular tRNA^{Asp} or a sequence derived from the 5' end of the subgenomic RNA, suggesting that a structure at the 5' terminus, rather than a linear sequence, is important for replication. The 5' terminus of rubella virus RNA can also be folded into a stem–loop structure, with the potential to form a pseudoknot (Frey, 1994).

Analysis of 5'-terminal structures of viruses in other supergroups also indicates the ability to fold into secondary structures and a requirement for RNA replication. Examples include: carmo-like virus supergroup, cucumber necrosis virus (Finnen and Rochon, 1993; Chang et al., 1995), cymbidium ringspot virus (Havelda et al., 1995), tomato bushy stunt virus (Knorr et al., 1991; White and Morris, 1994; Chang et al., 1995); corona-like virus supergroup, mouse hepatitis

virus virus (Kim *et al.*, 1993). The smallest 5′ cis-acting element required for RNA replication appears to be that of RNA 2 of flockhouse virus, which consisted of between 3 and 14 nt, and probably less than 6 nt (Ball and Li, 1993; Ball, 1994).

C. Internal Sequences

Internal cis-acting elements, in either intercistronic or coding regions, which are required for efficient RNA replication have been identified for a number of virus RNAs. In some cases, such sequences may be required to maintain an optimal RNA structure for binding of the replicase complex to promoters at the termini of the positive- or negative-stranded RNAs, or to promote processivity of the replicase during RNA synthesis. In other cases, it is possible that the replicase could bind to internal sequences for a particular purpose, e.g., translational repression, or for an obligatory step in the assembly or modification of RNA complexes. Phage Qβ replicase, lacking the additional host factor, HF1 (see Section III), binds strongly to internal sites on the Qβ RNA positive strand, but weakly if at all to the 3′ terminus. The purpose of this internal binding, which requires the ribosomal S1 protein component of the replicase, is probably to prevent binding of ribosomes upstream of the coat protein cistron, because the replicase is unable to dislodge ribosomes travelling along the RNA in the opposite direction (van Duin, 1988). Addition of HF1, which binds to both an internal and the 3′-terminal region of the RNA, serves to bring the 3′-terminal region in contact with the replicase to allow initiation of negative-strand synthesis (Barrera *et al.*, 1993). Qβ replicase does not bind internally on the RNA negative strand and does not require HF1 for initiation of positive-strand synthesis (van Duin, 1988; Barrera *et al.*, 1993).

A sequence of about 150 nt in the 5′ region of the 244-nt intercistronic region of brome mosaic virus (BMV) RNA 3, which separates the movement protein (3a) and coat protein ORFs, is required in cis for efficient replication of this RNA (French and Ahlquist, 1987). Removal of this region decreased RNA 3 replication to less than 1% of wild-type levels. This decrease was not due to effects on movement protein or coat protein gene expression, because frameshift mutations which abolished synthesis of these proteins had little effect on replication. This intercistronic region contains ICR-like motifs; removal of the motif which best fit the tRNA gene ICR2 consensus sequence reduced RNA replication to 15% of wild-type levels (Pogue *et al.*, 1992), indicating that this sequence contributes to the function of the intercistronic region in RNA 3 replication. In yeast, synthesis of BMV negative

strand RNA 3 required intercistronic sequences, as well as the 3'-terminal sequence (Quadt *et al.*, 1995). *In vitro* synthesis of BMV negative-strand RNA 3 by an isolated RdRp extract did not require the intercistronic sequences; this could indicate that these sequences are required specifically for the assembly of the replication complex or that current RdRp extracts lack essential components and contain only a basal activity. Sequences of the intercistronic regions of RNA 3 of cucumber mosaic virus, which contain an ICR2-like motif (Boccard and Baulcombe, 1993), and alfalfa mosaic virus (van der Vossen *et al.*, 1995), have also been shown to be important for RNA replication. In contrast, replication of cowpea chlorotic mottle virus RNA 3 did not require its intercistronic region and was less susceptible than BMV RNA 3 to large deletions in the RNA (Pacha *et al.*, 1990).

It has also been suggested that some of the BMV 2a protein coding region may be required in cis for replication of RNA 2, since RNA 2 mutants lacking the C-terminal region of the 2a protein could be replicated in trans in the presence of wild-type RNA 2, but mutants with larger deletions, encompassing the central and N-terminal region of 2a, could not (Pogue *et al.*, 1990; Marsh *et al.*, 1991b). In contrast, there do not appear to be any essential cis-acting replication elements in the coding regions of tobacco mosaic virus RNA. Mutants with deletions across the 126-kDa or 183-kDa replication protein ORFs could be replicated in protoplasts in trans by the wild-type proteins; deletion of the C-terminal part of the 183-kDa ORF increased replication (Ogawa *et al.*, 1992). TMV-derived replicons with most of the 126-kDa/183-kDa ORFs removed also replicated well and spread in plants in the presence of a helper virus (Raffo and Dawson, 1991). Furthermore, TMV mutants lacking the movement protein and coat protein ORFs replicated in protoplasts as well as wild-type; a decline in the accumulation of the mutant positive strand relative to that of the wild-type was only observed late in the infection and was ascribed to degradation of the RNA in the absence of the coat protein (Ishikawa *et al.*, 1991b).

The animal alphaviruses have a conserved 51-nt sequence near the beginning of the P123/4 ORF, which can be folded into a structure with two stem–loops. Of 21 silent mutations in this region, 19 resulted in a decrease of virus growth of two to four orders of magnitude (Niesters and Strauss, 1990). Some of the deleterious mutations could potentially disrupt the secondary structure, whereas others would not be expected to. The 51-nt sequence was also found to be necessary for efficient accumulation of DI RNAs, although deletion of the element did not reduce DI RNA replication as much as expected when compared to the effect of mutations in this region in the genomic RNA (Levis *et*

al., 1986; Schlesinger et al., 1987). Internal regions of the DI RNAs derive from other parts of the alphavirus genome and are presumably selected for efficiency of replication and packaging of the RNA (reviewed by Strauss and Strauss, 1994).

A 3'-proximal element of the capsid-coding region (P1) of human rhinovirus 14 RNA was shown to be required for efficient RNA replication (McKnight and Lemon, 1996). Although the P1 region of another picornavirus, poliovirus, was not required for replication (see Section II,F), McKnight and Lemon (1996) suggested that an element within the P2–P3 region of the poliovirus genome, which must undergo translation in cis for RNA replication to proceed (Novak and Kirkegaard, 1994), may be analogous to the human rhinovirus P1 replication element.

Sequences derived from the coding region of polymerase genes have been found to be essential cis-acting elements for the replication of a number of DI RNAs derived from viruses in the carmo-like virus supergroup, such as cucumber necrosis and tomato bushy stunt viruses (Chang et al., 1995) and cymbidium ringspot virus (Havelda et al., 1995). In the corona-like virus supergroup, a 135-nt internal sequence was required for DI RNA replication of one strain of mouse hepatitis virus, but not for another (Kim et al., 1993; Lin and Lai, 1993; van der Most et al., 1994). Although genomic sequences, retained by DI RNAs and required for DI RNA replication, provide useful information, cis-acting elements involved in genomic and DI RNA replication are not necessarily identical, since the selection pressures operating on the two types of RNA, as well as their structures, are different.

D. Sequences Required for Subgenomic RNA Synthesis

1. Alpha-like Virus Supergroup

Subgenomic RNA synthesis in this supergroup occurs by internal initiation on negative-strand templates, first shown in vitro by Miller et al. (1985) for brome mosaic virus (BMV) using an isolated RdRp and in vivo by Gargouri et al. (1989) for turnip yellow mosaic virus. Following convention, sequences of subgenomic promoters will be discussed on the positive strand, although it is the negative strand that acts as the template. The BMV subgenomic promoter, for synthesis of the coat protein subgenomic mRNA, has been defined in vitro (Marsh et al., 1988) and in vivo (French and Ahlquist, 1988). It is contained within the 250-nt intergenic region between the movement protein and coat protein genes, and extends between 74 and 95 nt upstream and 16 nt downstream of the transcriptional initiation site. It consists of three

functional domains. The first of these is the core promoter, which includes the initiation site, 20 nt upstream and about 15 nt downstream; this is sufficient for a low basal level of transcription and determines correct initiation. Immediately upstream of the core promoter is a poly(A) tract, which acts as an activator and possible spacer; similar poly(A) tracts are present in equivalent locations in other bromoviruses. Upstream of the poly(A) is a further enhancer region which contains imperfect direct repeats of sequences in the core promoter. The subgenomic promoter is distinct from, but may overlap at its 5' extremity, the cis-acting intergenic sequence required for efficient replication of RNA 3 (see Section V,C); mutations in the ICR2-like (box B) motif of the latter reduced RNA 3 synthesis without affecting subgenomic RNA synthesis (Smirnyagina *et al.*, 1994).

The subgenomic promoter of cucumber mosaic virus (CMV) is also located in the 286-nt intergenic region in RNA3 and is contained within a sequence extending about 70 nt upstream and 20 nt downstream of the transcriptional initiation site (Boccard and Baulcombe, 1993). Unlike the BMV subgenomic promoter, this sequence contains an ICR2-like motif. The intergenic region in alfalfa mosaic virus RNA 3 only extends 13 nt upstream of the transcriptional initiation site for the coat protein subgenomic mRNA and the subgenomic promoter extends into the C-terminal end of the movement protein ORF (van der Kuyl *et al.*, 1990, 1991; van der Vossen *et al.*, 1995). The basal AlMV subgenomic promoter was located from −26 to +1, where +1 is the transcriptional start site. The basal level of transcription was increased more than tenfold by extending the upstream sequence to −136 and the downstream sequence to +12. The upstream sequence enhancer sequence was mapped to −136/−94; the downstream element is a U-rich sequence with high homology to the 5'-terminal sequences of AlMV RNAs 1 and 2 (van der Vossen *et al.*, 1995).

The transcriptional initiation site for the 26S subgenomic RNA of the animal alphaviruses lies in the last codon of the P123/4 polyprotein. The basal promoter lies in a "junction" sequence from −19 to +5, which is highly conserved in different alphaviruses (Levis *et al.*, 1990). Full promoter activity (about fivefold greater than the basal promoter) is contained within the −98 to +14 sequence (Raju and Huang, 1991). Sequence comparisons between the animal alphavirus and plant alpha-like virus subgenomic promoters identified a number of conserved motifs (Marsh *et al.*, 1988; French and Ahlquist, 1988; van der Vossen *et al.*, 1995). Mutation of some of these in the AlMV subgenomic promoter led to a decrease in activity (van der Vossen *et al.*, 1995). The poly(A) tract found in bromovirus subgenomic promoters

was not found in the animal alphavirus subgenomic promoters or those of the other plant alpha-like viruses. Mutants of BMV RNA 3 lacking the poly(A) tract synthesize little coat protein, but second-site mutations in the intergenic region can suppress the transcriptional defect (Smirnyagina et al., 1994). One of these mutations was a duplication of the sequence UAUUAUUA immediately 5' to the deleted poly(A); this sequence had previously been shown to be an important enhancer element in the wild-type subgenomic promoter (Marsh et al., 1988). Hence the BMV subgenomic promoter, like those of other alpha-like viruses, can function in the absence of a poly(A) tract.

Positional effects on the activities of subgenomic promoters have also been noted. For BMV (French and Ahlquist, 1988) and CMV (Boccard and Baulcombe, 1993), the promoter closest to the 3' end of the positive strand was the most active, whereas with AlMV (van der Vossen et al., 1995) and Sindbis virus (Raju and Huang, 1991), the promoter closest to the 5' end of the positive strand was the most active. With tobacco mosaic virus (TMV), the level of expression of genes closest to the 3' end of the positive strand was the highest, but this has been shown to be due to translational control, there being little difference in the levels of subgenomic RNAs produced with promoters at different positions (Culver et al., 1993). Wild-type TMV RNA has two subgenomic promoters, one for the movement protein which is expressed early and another for the coat protein which is expressed late. Expression of the movement protein under the control of the coat protein subgenomic promoter led to late expression, suggesting that the promoter sequence (rather than its position) may control the timing of expression (Lehto et al., 1990).

The promoter for subgenomic RNA synthesis on beet necrotic yellow vein virus RNA 3 differs from those described above in that most of the promoter was located downstream (between + 100 and + 208) of the transcriptional initiation site and only extended to −16 upstream (Balmori et al., 1993).

2. Carmo-like and Sobemo-like Virus Supergroups

Although there are fewer studies, it is probable that subgenomic RNAs of viruses in these supergoups are also synthesized by internal initiation on a negative-strand template. Cucumber necrosis virus (tombusvirus) produces two 3' coterminal subgenomic RNAs, one of 2.1 kb, which directs the synthesis of the coat protein and one of 0.9 kb which directs the synthesis of two proteins, p21 and p20. The core subgenomic promoter for the 0.9 kb RNA was located to a region extending from between −10 and −20 to +6 (Johnston and Rochon, 1995).

The sequence from −11 to +3 was highly conserved in the equivalent regions of different tombusviruses. However, there was very limited similarity between this region, the 5′-terminal sequence of the genomic RNA, and the sequence surrounding the 5′ end of the 2.1 kb subgenomic RNA. This suggests that the two subgenomic promoters may be controlled independently, possibly in a temporal fashion as suggested above for the two tobacco mosaic virus subgenomic promoters (see Section V,D,1).

For several other viruses, there is considerable sequence similarity between the 5′ termini of the genomic and subgenomic RNAs, suggesting that these sequences may be part of the promoters for genomic and subgenomic RNA synthesis. Thirteen of the first fourteen nucleotides of the 5′ termini of the red clover necrotic mosaic virus (dianthovirus) genomic RNA 1 and of the positive strand of the coat protein subgenomic dsRNA are identical (Zavriev *et al.*, 1996). It was hypothesized that the subgenomic promoter consisted of a stem–loop structure composed of nucleotides −53 to +27. Sequence similarity in the 5′-terminal sequences of genomic and subgenomic RNAs has also been found for several luteoviruses (reviewed in Miller *et al.*, 1995). There is also similarity between the 5′-terminal sequences of two subgenomic RNAs of tobacco necrosis virus (necrovirus) (Meulewater *et al.*, 1992); this did not extend upstream of the transcriptional initiation site, suggesting that the two promoters may be controlled independently, consistent with temporal differences in their expression observed *in vivo*. In contrast, the 5′-terminal sequences of two carnation mottle virus (carmovirus) subgenomic RNAs had little similarity, but sequences upstream in the genomic RNA were highly conserved (Carrington and Morris, 1986). In the case of maize chlorotic mottle virus (machlomovirus), a sequence element partially homologous to the 5′-terminal genomic RNA sequence was found upstream of the start of the subgenomic RNA start site (Lommel *et al.*, 1991).

3. Coronaviruses

Coronaviruses synthesize a nested set of five to seven 3′-coterminal subgenomic mRNAs for translation of the internal ORFs (reviewed by Lai, 1990). The mechanism of subgenomic RNA synthesis is very different from that of the animal and plant alpha-like viruses, and the plant carmo-like and sobemo-like viruses, discussed above. Every subgenomic RNA has an identical 5′-terminal leader sequence which varies in length (60–90 nt), depending on the coronavirus. The leader sequence is only found at the 5′ end of the genomic RNA, which implies that subgenomic mRNAs are formed by fusion of two noncontiguous

elements. Ultraviolet irradiation inactivation experiments established that the leader and body of the mRNAs were not joined together by a splicing mechanism (Stern and Sefton, 1982). Upstream of each gene are conserved sequence elements, referred to as IS elements. There is sequence homology between sequence elements (present in varying numbers of copies) in the 3′ region of the leader sequence and the IS elements. For mouse hepatitis virus (MHV), every IS contains the sequence (AAUCUAAAC) or a closely related sequence. These IS elements are believed to function as promoters for subgenomic mRNA synthesis (van der Most et al., 1994). For MHV, the smallest mRNAs are generally synthesized in larger amounts that the larger ones, although this is not the case for all coronaviruses (Hiscox et al., 1995).

Several models have been proposed to account for the preceding observations in the synthesis of coronavirus mRNAs (Lai, 1990). One group of models proposes that the virus genomic RNA acts as a template for the synthesis of a full-length negative strand, which in turn acts as a template for the synthesis of both progeny-genomic RNA and the subgenomic RNAs. In the leader-primed hypothesis, the 5′ leader is first synthesized and this then acts as a primer for initiation of subgenomic RNA synthesis at one of the IS elements, either on the same RNA (by looping out of the intervening RNA), or by detachment of the polymerase with its leader and reattachment to another RNA template. The discovery of full-length negative strands and the isolation of replicative intermediates containing nascent subgenomic RNAs with the leader attached was consistent with this model (Baric et al., 1983b). Evidence that the polymerase and leader can detach from one template and attach to another comes from observations that the leader sequences undergo rapid exchanges between two RNA molecules, for example between mRNAs of two MHV strains or between an MHV RNA and a DI RNA (Makino et al., 1986; Makino and Lai, 1989). Also an exogenously added leader can be incorporated into mRNAs in an in vitro transcription system (Baker and Lai, 1990) or in vivo from a helper virus when supplied as a negative strand containing an IS sequence (Hiscox et al., 1995). Reattachment to new templates could be aided by sequence homology between the 3′ leader region and IS elements. It may also involve host proteins which bind to the IS regions (Zhang and Lai, 1995). Mutations in IS regions which reduced subgenomic RNA synthesis also reduced host protein binding. Further studies showed that the leader sequence can act both in trans and in cis (Zhang et al., 1994) and that leader switching is facilitated by a 9-nt sequence (UUUAUAAAC) located immediately downstream of the leader sequence, possibly at the end of a stem–loop structure (Zhiang

and Lai, 1996). The leader-primed model has some features in common with the model proposed by Carpenter *et al.* (1995) for recombination in turnip-crinkle-virus-associated RNAs, although the former is clearly a more directed process.

The discovery of subgenomic-length negative strands and sub-genomic replicative intermediates (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990; Schaad and Baric, 1994) opened up additional possibilities for the synthesis of coronavirus subgenomic RNAs. The suggestion that subgenomic RNAs might be formed by the leader-primed mechanism and then replicate independently seems unlikely in view of the inability of transfected positive-stranded subgenomic RNAs to be replicated in the presence of helper virus (Brian *et al.*, 1994). Sawicki and Sawicki (1990, 1995) have suggested a model in which discontinuous synthesis of subgenomic RNAs occurs during negative-strand synthesis. The polymerase is proposed to pause at an IS element in the positive-strand template and then either continue synthesis or detach with the nascent strand and reattach on the same or different RNA molecules to copy the leader sequence. It was suggested that having copied an IS element, the polymerase might retract in a way analogous to some DNA-dependent RNA polymerases (Kassavetis and Geiduschek, 1993) and suggested for some types of recombination between brome mosaic virus RNAs (Bujarski *et al.*, 1994), and remain associated with the template. The IS sequences at the 3' end of the nascent strand could then align with complementary sequences on the positive-strand template in the 3' region of the leader sequence. The newly synthesized subgenomic negative strand could then act as a preferred template for synthesis of positive-strand subgenomic RNA by the same polymerase complex. It has been shown that MHV downstream IS elements have a negative impact on transcription from upstream IS elements, whereas upstream IS elements have little effect on transcription from downstream IS elements (van Marle *et al.*, 1995), consistent with the greater synthesis of the shorter mRNAs. This is nicely explained by the model of Sawicki and Sawicki (1995) because the polymerase would have a choice of detachment or continued synthesis at each IS element encountered and therefore fewer polymerase molecules would reach the IS elements upstream in the positive-strand template. The various models have not yet been unequivocally resolved because other work has suggested that the subgenomic negative strands, apparently present in the cell in double-stranded RNA form, may be a dead-end product (Lin *et al.*, 1994). Double-stranded RNA subgenomic RNAs of the alpha-like viruses are generally considered to be dead-end products, because they lack the upstream promoter sequences. However, further

studies are needed to resolve this issue for coronavirus subgenomic RNAs.

An additional control mechanism must be invoked to account for the predominance of full-length genomic RNA molecules late in infection. It has been suggested by Lai (1990) that the nucleocapsid (N) protein might perform this function, in a manner analogous to the role of the vesicular stomatitis virus nucleocapsid protein in controlling the switch from mRNA synthesis to production of genome-length positive strands, which can then be used as templates for the synthesis of progeny (negative strand) virus RNA molecules (Blumberg *et al.*, 1981). The coronavirus N protein is known to contain an RNA-binding domain and to form high affinity complexes with the 3′ end of the leader sequence (Baric *et al.*, 1988; Nelson and Stohlman, 1993).

VI. CONTROL OF ASYMMETRIC POSITIVE- AND NEGATIVE-STRAND SYNTHESIS

During the replication of positive-stranded RNA viruses, a large excess of positive over negative strands is produced. Estimates range from 10:1 (flaviviruses; Chambers *et al.*, 1990), 50–100:1 (coronaviruses; Lai, 1990), 100:1 (brome mosaic virus, French and Ahlquist, 1987), 1000:1 (alfalfa mosaic virus; Nassuth and Bol, 1983). This could be due to down-regulation of negative-strand synthesis and/or up-regulation of positive-strand synthesis. In the alpha-like virus superfamily, it has been shown for Sindbis virus and other alphaviruses (Strauss and Strauss, 1994), alfalfa mosaic virus (van der Vossen *et al.*, 1994), and tobacco mosaic virus (Ishikawa *et al.*, 1991b) that negative-strand synthesis is switched off or greatly reduced a few hours after infection, whereas positive-strand synthesis continues throughout the replication cycle. There are many examples of mutations in virus replication proteins which affect positive- and negative-strand synthesis differently, e.g., in alphaviruses (reviewed by Strauss and Strauss, 1994), bromoviruses (reviewed by Ahlquist, 1992, Duggal *et al.*, 1994), and tobamoviruses (reviewed by Dawson and Lehto, 1990). Hence replication complexes which synthesize negative and positive strands (and subgenomic RNAs) may be different or one may be modified to form the other. It is noteworthy that the phage Qβ replicase complexes for synthesis of positive and negative strands differ. The enzyme for negative-strand synthesis contains the host factor (HF1 protein), whereas that for positive-strand synthesis does not. When HF1 protein is in excess, equal amounts of positive and negative strands are produced. When HF1 is in limiting amount, as in the cell, negative-strand synthesis is

limited and positive-strand synthesis predominates (Blumenthal and Carmichael, 1979).

In the animal alphaviruses, the initial unstable replication complex that synthesizes the negative strand is converted by a process that includes proteolytic cleavage into a stable complex that synthesizes the positive strands (Strauss and Strauss, 1994; Sawicki and Sawicki, 1994; see Section II,E,1). It appears that replication complexes are only formed on the positive-strand templates and the number of replication complexes formed depends on the number of negative strands synthesized. Once negative-strand synthesis has ceased, positive-strand synthesis carries on using the stable replication complex already formed, leading to the observed asymmetry in positive- and negative-strand accumulation.

The model of Pogue and Hall (1992) for the replication of brome mosaic virus also suggests that the replicase complex, having assembled on a positive-strand template and synthesized a negative strand, is then modified to use the negative strand as a template. This does not account for the strand asymmetry, because infection of protoplasts with RNA 1 and 2, which encode the 1a and 2a replication proteins, produced a 1:1 ratio of positive to negative strands. However infection with RNAs 1, 2 and 3 produced a 100:1 ratio of positive to negative strands (Marsh et al., 1991a). RNA 3 therefore controls the strand asymmetry. Mutational analysis showed that the coat protein, which is synthesized from a subgenomic RNA derived from RNA 3, while contributing to the strand asymmetry, was not a major determinant of it. A deletion of the subgenomic core promoter and the first 5' 43 nt of the subgenomic RNA synthesis reduced the strand asymmetry to 1.8:1. It has been suggested that sequences in the intergenic region of RNA 3 may form a platform on which a positive-strand-synthesizing replicase could be assembled (Duggal et al., 1994). It is noteworthy that deletion of the core subgenomic promoter in RNA 3 resulted in production of an RdRp in yeast with increased ability to synthesize negative strands in vitro (Quadt et al., 1995), perhaps again indicating differences between replicase complexes able to synthesize positive and negative strands. Differences in replicase complexes for positive- and negative-strand synthesis have also been invoked for alfalfa mosaic virus, but for this virus the coat protein was shown to be the main activator of positive-strand synthesis (see Sections II,E,1 and II,F). Nevertheless, the shutoff of negative-strand RNA 3 synthesis in P12 transgenic plants appeared to be independent of the coat protein (van der Vossen et al., 1994), a further indication that negative-strand and positive-strand synthesis for this virus may be regulated separately.

Early shut-off of negative-strand synthesis does not occur for all positive-stranded RNA viruses. Negative-stranded RNA synthesis of the flavivirus dengue virus continued throughout the entire replication cycle (Cleaves *et al.,* 1981). Although negative strand-synthesis of coronaviruses peaked at 5–6 hours after infection, some synthesis continued until late in infection (Sawicki and Sawicki, 1986). Viruses in the picorna-like virus supergroup pose a different problem because both positive and negative strands have VPg 5′ termini (see Section II,D). Hence one polyprotein molecule has to be synthesized for every RNA molecule synthesized (unless VPg released from RNAs destined to be translated can be recycled, which seems unlikely for poliovirus if the primer is 3ABpU; see Sections II,D and II,E,4). However poliovirus RNA synthesis is not completely linked to translation throughout the whole replication cycle, because synthesis of both positive and negative strands can continue for some time after addition of inhibitors of protein synthesis (Novak and Kirkegaard, 1994).

VII. CONCLUDING REMARKS

It is clear that there are some similarities in RNA replication for all eukaryotic positive-stranded RNA viruses, i.e., the mechanism of polymerization of the nucleotides is probably similar for all. It is noteworthy that all appear to utilize host membranes as a site of replication. Membranes appear to function not just as a way of compartmentalizing virus RNA replication, but also appear to have a central role in the organization and functioning of the replication complex and further studies in this area are needed. Within virus supergroups, similarities are evident between animal and plant viruses, e.g., in the nature and arrangements of replication genes and in sequence similarities of functional domains. However, it is also clear that there has been considerable divergence, even within supergroups. For example, the animal alphaviruses have evolved to encode proteinases which play a central controlling function in the replication cycle, whereas this is not common in the plant alpha-like viruses and even when it occurs, as in the tymoviruses, the strategies that have evolved appear to be significantly different. Some of the divergence could be host-dependent and the increasing interest in the role of host proteins in replication should be fruitful in revealing how different systems have evolved. Even within the plant alpha-like viruses, there are significant differences in regulation mechanisms. An outstanding question which needs to be solved is, for those viruses which appear to show some form of cis-

preferential replication, such as poliovirus (Wimmer *et al.,* 1993; Novak and Kirkegaard, 1994) and turnip yellow mosaic virus (Weiland and Dreher, 1993), whether or not the replication complex assembles on the RNA from which it has been translated. Other possibilities have been discussed by Wimmer *et al.* (1993) and Novak and Kirkegaard (1994). Finally, there are virus supergroups which appear to have no close relatives between animals and plants, such as the animal coronavirus-like supergroup and the plant carmo-like supergroup. Nevertheless, our knowledge of positive-stranded RNA virus replication is still in its infancy and future research may reveal unsuspected similarities. More comprehensive comparisons must await further knowledge of the assembly and structures of replication complexes and how they are modified to utilize and initiate RNA synthesis at different promoters on positive- and negative-strand RNA templates.

Acknowledgments

References

Agranovsky, A. A., Koonin, E. V., Boyko, V. P., Maiss, E., Frotschi, R., Lunini, N. A., and Atabekov, J. G. (1994). *Virology* **198**, 311–324.

Ahlquist. P. (1992). *Curr. Opin. Genet. Devel.* **2**, 71–76.

Ahlquist, P., Strauss, E. G., Rice, C. M., Strauss, J. M., Haseloff, J., and Zimmern, D. (1985). *J. Virol.* **53**, 536–542.

Ahola, T., and Kääriänen, L. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 507–511.

Aldabe, R., and Carrasco, L. (1995). *Biochem. Biophys. Res. Comm.* **206**, 64–76.

Andino, R., Rieckhof, G. E., and Baltimore, D. (1990). *Cell* **63**, 369–380.

Andino, R., Rieckhof, G. E., Achacoso, P. L., and Baltimore, D. (1993). *EMBO J.* **12**, 3587–3598.

Andrews, N. C., and Baltimore, D. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 221–225.

Argos, P. (1988). *Nucleic Acids Res.* **16**, 9909–9916.

Atreya, C. D., Singh, N. K., and Nakhasi, H. L. (1995). *J. Virol.* **69**, 3848–3851.

Axelrod, V. D., Brown, E., Priano, C., and Mills, D. R. (1991). *Virology* **184**, 595–608.

Baer, M. L., Houser, F., Loesch-Fries, L. S., and Gehrke, L. (1994). *EMBO J.* **13**, 727–735.

Baker, S. C., and Lai, M. M. C. (1990). *EMBO J.* **9**, 4173–4179.

Ball, L. A. (1994). *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12443–121447.

Ball, L. A. (1995). *J. Virol.* **69**, 720–727.

Ball, L. A., and Li, Y. (1993). *J. Virol.* **67**, 3544–3551.

Balmori, E., Gilmer, D., Richards, K., Guilley, H., and Jonard, G. (1993). *Biochimie* **75**, 517–521.

Barco, A., and Carrasco, L. (1995). *EMBO J.* **14**, 3349–3364.

Baric, R. S., Carlin, L. J., and Johnston, R. E. (1983a). *J. Virol.* **48**, 200–205.

Baric, R. S., Stohlman, S. A., and Lai, M. M. C. (1983b). *J. Virol.* **48**, 633–640.

Baric, R. S., Nelson, G. W., Fleming, J. O., Deans, R. J., Keck, J. G., Casteel, N., and Stohlman, S. A. (1988). *J. Virol.* **62**, 4280–4287.

Barrera, I., Schuppli, D., Sogo, J. M., and Weber, H. (1993). *J. Mol. Biol.* **232**, 512–521.

Bartholomeusz, A. I., and Wright, P. J. (1993). *Arch. Virol.* **128**, 111–121.

Barton, D. J., Sawicki, S. G., and Sawicki, D. L. (1991). *J. Virol.* **65**, 1496–1506.

Barton, D. J., Black, P. E., and Flanegan, J. B. (1995). *J. Virol.* **69**, 5516–5527.

Barton, D. J., Morasco, B. J., Eisner-Smerage, L., Collins, P. S., Diamond, S. E., Hewlett, J. J., Merchant, M. A., O'Donnell, B. J., and Flanegan, J. B. (1996). *Virology* **217**, 459–469.

Bates, H. J., Farjah, M., Osman, T. A. M., and Buck, K. W. (1995). *J. Gen. Virol.* **76**, 1483–1491.

Baulcombe, D. (1994). *Trends Microbiol.* **2**, 60–62.

Bayliss, C. D., and Smith, G. L. (1996). *J. Virol.* **70**, 794–800.

Beck, D. L., Guiford, P. J., Voot, D. M., Andersen, M. T., and Forster, R. L. (1991). *Virology* **183**, 695–702.

Behrens, S.-E., Tomei, L., and De Francesco, R. (1996). *EMBO J.* **15**, 12–22.

Bienz, K., Egger, D., and Pasamontes, L. (1987). *Virology* **160**, 220–226.

Bienz, K., Egger, D., Troxler, M., and Pasamontes, L. (1990). *J. Virol.* **64**, 1156–1163.

Bienz, K., Egger, D., Pfister, T., and Troxler, M. (1992). *J. Virol.* **66**, 2740–2747.

Bienz, K., Egger, D., and Pfister, T. (1994). *Arch. Virol.* **S9**, 147–157.

Blackburn. E. (1993). *In* "The RNA World" (R. F. Gesteland and J. F. Atkins, eds.), pp. 557–576, Cold Spring Harbor Laboratory Press, Plainview, NY.

Blackwell, J. L., and Brinton, M. A. (1995). *J. Virol.* **69**, 5650–5658.

Blum, H., Gross, H. J., and Beier, H. (1989). *Virology* **169**, 51–61.

Blumberg, B. M., Leppert, M., and Kolakofsky, D. (1981). *Cell* **23**, 837–845.

Blumenthal, T., and Carmichael, G. G. (1979). *Annu. Rev. Biochem.* **48**, 525–548.

Boccard, F., and Baulcombe, D. (1993). *Virology* **193**, 563–578.

Bork, P., and Koonin, E. V. (1993). *Nucleic Acids Res.* **21**, 751–752.

Boyer, J. C., and Haenni, A.-L. (1994). *Virology* **198**, 415–426.

Bramhill, D., and Kornberg, A. (1988). *Cell* **52**, 743–748.

Bransom, K. L., and Dreher, T. W. (1994). *Virology* **184**, 351–358.

Bransom, K. L., Weiland, J. J., and Dreher, T. W. (1991). *Virology* **184**, 351–358.

Brayton, P. R., Stohlman, S. A., and Lai, M. M. C. (1982). *J. Virol.* **42**, 847–853.

Brayton, P. R., Lai, M. M. C., Patton, C. D., and Stohlman, S. A. (1984). *Virology* **133**, 197–201.

Brian, D. A., Chang, R-Y., Hofmann, M. A., and Sethna, P. B. (1994). *Arch. Virol.* **S9**, 173–180.

Bruenn, J. A. (1991). *Nucleic Acids Res.* **19**, 217–226.

Brun, J. B., and Brinton, M. A. (1988). *J. Gen. Virol.* **69**, 3121–3127.

Buck, K. W. (1979). *In* "Viruses and Plasmids in Fungi" (P. A. Lemke, ed.), pp. 94–151. Marcel Dekker, New York.

Bujarski, J. J., Nagy, P. D., and Flasinski, S. (1994). *Adv. Virus Res.* **43**, 275–302.

Burroughs, J. N., and Brown, F. (1978). *J. Gen. Virol.* **41**, 443–446.

Caliguiri, L. A., and Tamm, I. (1973). *In* "Selective inhibitors of Viral Functions" (W. Carter, ed.), pp. 257–294. CRC Press, Boca Raton.

Candresse, T. (1993). *In* "Encyclopaedia of Virology" (R. G. Webster and A. Granoff, eds.), vol. 1, pp. 242–248. Academic Press, London.

Candresse, T., Mouches, C., and Bové, J. M. (1986). *Virology* **152**, 322–330.

Carpenter, C. D., and Simon, A. E. (1996). *J. Virol.* **70**, 478–486

Carpenter, C. D., Oh, J.-W., Zhang, C., and Simon, A. E. (1995). *J. Mol. Biol.* **245**, 608–622.

Carr, J. P., and Zaitlin, M. (1993). *Semin. Virol.* **4**, 339–347.

Carr, J. P., Galon, A., Palukaitis, P., and Zaitlin, M. (1994). *Virology* **199**, 439–447.

Carrington, J. C., and Morris, T. J. (1986). *Virology* **150**, 196–206.

Cavanagh, D., and MacNaughton, M. R. (1994). *In* "Principles and Practice of Clinical Virology" (A. J. Zuckerman, J. R. Pattison, and J. E. Banatvala, eds.), 3rd ed., pp. 325–335. John Wiley and Sons, New York.

Chambers, T. J., Hahn, C. S., Galler, R., and Rice, C. M. (1990). *Annu. Rev. Microbiol.* **44**, 649–688.

Chambers, T. J., Nestorowicz, A., Amberg, S. E., and Rice, C. M. (1993). *J. Virol.* **67**, 6797–6807.

Chang, Y. C., Borja, M., Scholtof, H. B., Jackson, A. O., and Morris, T. J. (1995). *Virology* **210**, 41–53.

Cho, M. W., Richards, O. C., Dmitrieva, T. M., Agol, V., and Ehrenfeld, E. (1993). *J. Virol.* **67**, 3010–3018.

Cho, M. W., Teterina, N., Egger, D., Bienz, K., and Ehrenfeld, E. (1994). *Virology* **202**, 129–145.

Chu, P. W. G., and Westaway, E. G. (1985). *Virology* **140**, 68–79.

Chu, P. W. G., and Westaway, E. G. (1992). *Arch. Virol.* **125**, 177–191.

Cleaves, G. R., Ryan, T. E., and Schlesinger, R. W. (1981). *Virology* **111**, 73–83.

Coffin, R. S., and Coutts, R. H. A. (1993). *J. Gen. Virol.* **74**, 1475–1483.

Collett, M. S. (1992). *Comp. Immunol. Microbiol. Infect. Dis.* **15**, 145–154.

Collis, P. S., O'Donnell, B. J., Barton, D. J., Rogers, J. A., and Flanegan, J. B. (1992). *J. Virol.* **66**, 6480–6488.

Collmer, C. W., and Kaper, J. M. (1994). *Virology* **145**, 249–259.

Crawford, N. M., and Baltimore, D. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7452–7455.

Cui, T., and Porter, A. (1995). *Nucleic Acids Res.* **23**, 377–382.

Cui, T., Sankar, S., and Porter, A. (1993). *J. Biol. Chem.* **268**, 26093–26098.

Culver, J. N., Lehto, K., Close, S. M., Hilf, M. E., and Dawson, W. O. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2055–2059.

Dalmay, T., Rubino, L., Burgyan, J., Kollar, A., and Russo, M. (1993a). *Virology* **194**, 697–704.

Dalmay, T., Russo, M., and Burgyan, J. (1993b). *Virology* **192**, 551–555.

Dalmay, T., and Rubino, L. (1995). *Virology* **206**, 1092–1098.

Datta, U., and Dasgupta, A. (1994). *J. Virol.* **68**, 4468–4477.

David, C., Gargouri-Bouzid, R., and Haenni, A-L. (1992). *Prog. Nucleic Acid Res. Mol. Biol.* **42**, 157–227.

Dawson, W. D. O. (1978). *Intervirology* **9**, 304–309.

Dawson, W. D. O., and Lehto, K. M. (1990). *Adv. Virus Res.* **38**, 307–342.

Dedhar, S. P. (1994). *Trends Biol. Sci.* **19**, 269–271.

Dé, I., Sawicki, S. G., and Sawicki, D. L. (1996). *J. Virol.* **70**, 2706–2719.

de Graaf, M., and Jaspars, E. M. J. (1994). Annu. Rev. Phytopathol. 32, 311–335.

de Graaf, M., Coscoy, L., and Jaspars, E. M. J. (1993). *Virology* **194**, 878–881.

de Graaf, M., Houwing, C. J., Lukacs, N., and Jaspars, E. M. J. (1995a). *FEBS Lett.* **371**, 219–222.

de Graaf, M., Man In 'T Veld, M. R., and Jaspars, E. M. J. (1995b). *Virology* **208**, 583–589.

de Jong, W., and Ahlquist, P. (1995). *J. Virol.* **69**, 1485–1492.

de Varennes, A., Davies, J. W., Shaw, J. G., and Maule, A. J. (1985). *J. Gen. Virol.* **66**, 817–825.

Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990). *Protein Eng.* **3**, 461–467.

Demler, S. A., Rucker, D. G., and de Zoeten, G. A. (1993). *J. Gen. Virol.* **74**, 1–14.

Demler, S. A., Borkhsenious, O. N., Rucker, D. G., and de Zoeten, G. A. (1994). *J. Gen. Virol.* **75**, 997–1007.

Diamond, S. E., and Kirkegaard, K. (1994). *J. Virol.* **68**, 863–876.

Dinant, S., Janda, M., Kroner, P. A., and Ahlquist, P. (1993). *J. Virol.* **67**, 7181–7189.

Dobos, P., and Roberts, T. E. (1983). *Can. J. Microbiol.* **29**, 377–384.

Doedens, J. R., and Kirkegaard, K. (1995). *EMBO J.* **14**, 894–907.

Dolja, V. V., and Carrington, J. C. (1992). *Semin. Virol.* **3**, 315–326.

Dolja, V. V., Grama, D. P., Morozov, S. Y., and Atabekov, J. G. (1987). *FEBS Lett.* **214**, 308–312.

Dolja, V. V., McBride, H. J., and Carrington, J. C. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10208–10212.

Dolja, V. V., Karasev, A. V., and Koonin, E. V. (1994). *Annu. Rev. Phytopathol.* **32**, 261–285.

Domingo, E., and Holland, J. J. (1994). *In* "The Evolutionary Biology of Viruses" (S. S. Morse, ed.), pp. 161–184. Raven Press, New York.

Donald, R. G. K., and Jackson, A. O. (1994). *In* "Encyclopaedia of Virology" (R. G. Webster and A. Granoff, eds.), Vol. 2, pp. 661–664. Academic Press, New York.

Dorssers, L., Van der Krol, S., Van der Meer, J., Van Kammen, A., and Zabel, P. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1951–1955.

Dougherty, W. G., and Semler, B. L. (1993). *Microbiol. Reviews* **57**, 781–822.

Dougherty, W. G., and Parks, T. D. (1995). *Curr. Opin. Cell Biol.* **7**, 399–405.

Dreher, T. W., and Hall, T. C. (1988). *J. Mol. Biol.* **201**, 31–40.

Dreher, T. W., Rao, A. L. N., and Hall, T. C. (1989). *J. Mol. Biol.* **206**, 425–438.

Duggal, R., and Hall, T. C. (1995). *Virology* **214**, 638–641.

Duggal, R., Rao, A. L. N., and Hall, T. C. (1992). *Virology* **187**, 261–270.

Duggal, R., Lahser, F. C., and Hall, T. C. (1994). *Annu. Rev. Phytopathol.* **32**, 287–309.

Dunigan, D. D., and Zaitlin, M. (1990). *J. Biol. Chem.* **265**, 7779–7786

Eagles, R. M., Balmori-Melian, E., Beck, D. L., and Gardner, R. C. (1994). *Eur. J. Biochem.* **224**, 677–684.

Eigen, M., and Briebricher, C. (1988). *In* "RNA Genetics" (E. Domingo, J. J. Holland, and P. Ahlquist, eds.), Vol. 3, pp. 211–245. CRC Press, Boca Raton.

Eggen, R., and Van Kammen, A. (1988). *In* "RNA Genetics" (P. Ahlquist, J. J. Holland, and E. Domingo, eds.), Vol. 1, pp. 49–69. CRC Press, Boca Raton.

Eggen, R., Kaan, A., Goldbach, R., and Van Kammen, A. (1988). *J. Gen. Virol.* **69**, 2711–2720.

Eggen, R., Verver, J., Wellink, J., Pleij, K., Van Kammen, A., and Goldbach, R. (1989a). *Virology* **173**, 456–464.

Eggen, R., Verver, J., Wellink, J., De Jong, A., Golbach, R., and Van Kammen, A. (1989b). *Virology* **173**, 447–455.

Ehrenfeld, E., and Gebhard, J. G. (1994). *Arch. Virol.* **S9**, 269–277.

Esau, K., and Cronshaw, J. (1967). *J. Cell Biol.* **33**, 665–678.

Fernandez, A., Lain, S., and Garcia, J. A. (1995). *Nucleic Acids Res.* **23**, 1327–1332.

Finnen, R. L., and Rochon, D. M. (1993). *J. Gen. Virol.* **74**, 1715–1720.

Florentz, C., and Gierge, R. (1995). *In* "tRNA: Structure, Biosynthesis, and Function" (D. Soll and U. RajBhandary, eds.), pp. 141–163. American Society for Microbiology, Washington, DC.

Foster, G. D. (1992). *Res. Virol.* **143**, 103–112.

Fraenkel-Conrat, H. (1986). *Crit. Rev. Plant Sci.* **4**, 213–226.

French, R., and Ahlquist, P. (1987). *J. Virol.* **61**, 1457–1465.

French, R., and Ahlquist, P. (1988). *J. Virol.* **62**, 2411–2420.

Frey, T. K. (1994). *Adv. Virus Res.* **44**, 69–160.

Froshauer, S., Kartenbeck, J., and Helenius, A. (1988). *J. Cell Biol.* **107**, 2075–2086.

Fujimura, T., and Wickner, R. B. (1989). *J. Biol. Chem.* **264**, 10872–10877.

Fuller-Pace, F. V. (1994). *Trends Cell Biol.* **4**, 271–274.

Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtneidge, S. A. (1994). *Nature* **368**, 867–871.

Furiuchi, Y., Muthakrishnan, S., Tomaz, J., and Shatkin, A. J. (1976). *J. Biol. Chem.* **251**, 5043–5053.

Furuya, T., and Lai, M. M. C. (1993). *J. Virol.* **67**, 7215–7222.

Gallie, D. R. (1991). *Genes Dev.* **5**, 2108–2116.

Gallie, D. R., and Kobayashi, M. (1994). *Gene* **142**, 159–165.

Gamez, R., and Leon P. (1988). *In* "The Plant Viruses" (R. Koenig, ed.), Vol. 3, pp. 213–233. Plenum Press, New York.

Gargouri, R., Joshi, R. L., Astier-Manifacier, S., and Haenni, A-L. (1989). *Virology* **171**, 386–393.

Gargouri-Bouzid, R., David, C., and Haenni, A. L. (1991). *FEBS Lett.* **294**, 56–58.

Garnier, M., Mamoun, R., and Bové, J. M. (1980). *Virology* **104**, 357–374.

Garnier, M., Candresse, T., and Bové, J. M. (1986). *Virology* **151**, 100–109.

Geiduschek, E. P., and Kassavetis, G. A. (1992). *In* "Transcriptional Regulation" (S. L. McKnight and K. R. Yamamoto, eds.), Vol. 1, pp. 247-280. Cold Spring Harbor Laboratory Press, Plainview, NY.

Giachetti, C., and Semler, B. L. (1991). *J. Virol.* **65**, 2647–2654.

Gibbs, M. J. (1995). *In* "Molecular Basis of Viral Evolution" (A. J. Gibbs, C. H. Calisher, and F. Garcia-Arenal, eds.), pp. 351–368. Cambridge University Press, Cambridge.

Gieseman-Cookmeyer, D., Kim, K-H., and Lommel, S. A. (1995). *In* "Pathogenesis and Host Specificity in Plant Diseases" (R. P. Singh, U. P. Singh, and K. Kohmoto, eds.), Vol. III, pp. 157–176. Elsevier, Oxford.

Gilmer, D., Bouzoubaa, S., Guilley, H., Richards, K., and Jonard, G. (1992a). *Virology* **189**, 40–47.

Gilmer, D., Bouzoubaa, S., Richards, K., Jonard, G., and Guilley, H. (1992b). *Virology* **190**, 55–67.

Gilmer, D., Allmang, C., Ehresmann C., Guilley, H., Richards, K., Jonard, G., and Ehresmann, B. (1993). *Nucleic Acids Res.* **21**, 1389–1395.

Goldbach, R. W. (1986). *Annu. Rev. Phytopathol.* **24**, 289–310.

Goldbach, R., and de Haan, P. (1994). *In* "The Evolutionary Biology of Viruses" (S. S. Morse, ed.), pp. 105–120. Raven Press, New York.

Goldbach, R., Le Gall, O., and Wellink, J. (1991). *Semin. Virol.* **2**, 19–25.

Goldbach, R., Wellink, J., Verver, J., Van Kammen., A., Kasteel, D., and Van Lent, J. (1994). *Arch. Virol.* **S9**, 87–97.

Gorbalenya, A. E., and Koonin, E. V. (1989). *Nucleic Acids Res.* **17**, 8413–8440.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1988). *FEBS Lett.* **239**, 16–24.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinow, V. M. (1989). *Nucleic Acids Res.* **17**, 4713–4730.

Gorbalenya, A. E., Koonin, E. V., and Wolf, Y. I. (1990). *FEBS Lett.* **243**, 103–113.

Greene, A. E., and Allison, R. F. (1994). *Science* **263**, 1423–1425.

Gros, C., and Wengler, G. (1996). *Virology* **217**, 367–372.

Gros, C. H., and Shuman, S. (1996). *J. Virol.* **70**, 1706–1713.

Grun, J. B., and Brinton, M. A. (1988). *J. Gen. Virol.* **69**, 3121–3127.

Guilford, P. J., Beck, D. L., and Forster, R. L. S. (1991). *Virology* **182**, 61–67.

Guinea, R., and Carrasco, L. (1990). *EMBO J.* **9**, 2011–2016.

Habili, N., and Symons, R. H. (1989). *Nucleic Acids Res.* **17**, 9543–9555.

Hacker, D. L., Petty, I. T. D., Wei, N., and Morris, T. J. (1992). *Virology* **186**, 1–8.

Hahn, Y. S., Grakoui, C. M., Rice, C. M., Strauss, E. G., and Strauss, J. H. (1989a). *J. Virol.* **63**, 1194–1202.

Hahn, Y. S., Strauss, E. G., and Strauss, J. H. (1989b). *J. Virol.* **63**, 3142–3150.

Hannig, E. M. (1995). *Bioessays* **17**, 915–919.

Harris, K. S., Xiang, W., Alexander, L., Lane, W. S., Paul, A. V., and Wimmer, E. (1994). *J. Biol. Chem.* **269**, 27004–27014.

Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R., and Kaesberg, P. (1984). *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4358–4362.

Hatta, T., and Francki, R. I. B. (1981). *J. Gen. Virol.* **53**, 343–346.

Havelda, Z., and Burgyan, J. (1995). *Virology* **214**, 269–272.

Havelda, Z., Dalmay, T., and Burgyan, J. (1995). *J. Gen. Virol.* **76**, 2311–2316.

Hayes, R. J., and Buck, K. W. (1990). *Cell* **63**, 363–368.

Hayes, R. J., and Buck, K. W. (1993). *In* "Molecular Virology: A Practical Approach" (A. J. Davison and R. M. Elliott, eds.), pp. 1–34. IRL Press, Oxford.

Hayes, R. J., Pereira, V. C. A., McQuillin, A., and Buck, K. W. (1994a). *J. Gen. Virol.* **75**, 3177–3184.

Hayes, R. J., Pereira, V. C. A., and Buck, K. W. (1994b). *FEBS Lett.* **352**, 331–334.

Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K., and Jonard, G. (1995). *Virology* **210**, 73–81.

Hellen, C. U. T., and Cooper, J . I. (1987). *J. Gen. Virol.* **68**, 2913–2917.

Heringa, J., and Argos, P. (1994). *In* "The Evolutionary Biology of Viruses" (S. S. Morse, ed.), pp. 87–103. Raven Press, New York.

Hillman, B. I., and Lawrence, D. M. (1995). *In* "Pathogenesis and Host Specificity in Plant Diseases" (R. P. Singh, U. P. Singh and K. Kohmoto, eds.), Vol. III, pp. 35–50. Elsevier, Oxford.

Hills, G. J., Plaskitt, K. A., Young, N. D., Dunigan, D. D., Watts, J. W., Wilson, T. M. A., and Zaitlin, M. (1987). *Virology* **161**, 488–496.

Hirling, H., Scheffner, M., Restle, T., and Stahl, H. (1989). *Nature* **339**, 562–564.

Hiscox, J. A., Mawditt, K. L., Cavanagh, D., and Britton, P. (1995). *J. Virol.* **69**, 6219–6227.

Hodgeman, T. C. (1988). *Nature* **333**, 22–33; 579 (Erratum).

Hofmann, M. A., and Brian, D. A. (1991). *J. Virol.* **65**, 6331–6333.

Hong, Y., Levay, K., Murphy, J. F., Klein, P. G., Shaw, J. G., and Hunt, A. G. (1995). *Virology* **214**, 159–166.

Houser-Scott, F., Baer, M. L., Liem, K. F., Cai, J.-M., and Gehrke, L. (1994). *J. Virol.* **68**, 2194–2205.

Houwing, C. J., and Jaspars, E. M. J. (1986). *FEBS Lett.* **209**, 284–288.

Huisman, M. J., Linthorst, H. J. M., Bol, J. F., and Cornellison, B. J. C. (1988). *J. Gen. Virol.* **69**, 1789–1798.

Inokuchi, Y., and Hirashima, A. (1987). *J. Virol.* **61**, 3946–3949.

Ishikawa, M., Meshi, T., Motoyoshi, F., Takamatsu, N., and Okada, Y. (1986). *Nucleic Acids Res.* **14**, 8291–8305.

Ishikawa, M., Kroner, P., Ahlquist, P., and Meshi, T. (1991a). *J. Virol.* **65**, 3451–3459.

Ishikawa, M., Meshi, T., Ohno, T., and Okada, Y. (1991b). *J. Virol.* **65**, 861–868.

Ishikawa, M., Naito, S., and Ohno, T. (1993). *J. Virol.* **67**, 5328–5338.

Jablonski, S. A., and Morrow, C. D. (1993). *J. Virol.* **65**, 373–381.

Jablonski, S. A., Luo, M., and Morrow, C. D. (1991). *J. Virol.* **65**, 4565–4572.

Jacoba-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hiza, A., Hughes, S. H., and Arnold, E. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6320–6324.

Jacobson, S. J., Konigs, D. A. M., and Sarnow, P. (1993). *J. Virol.* **67**, 2961–2971.

Jaegle, M., Wellink, J., and Goldbach, R. (1987). *J. Gen. Virol.* **68**, 627–632.

Janda, M., and Ahlquist, P. (1993). *Cell* **72**, 961–970.

Jaramillo, M., Browning, K., Dever, T. E., Blum, S., Trachsel, H., Merrick, W. C., Ravel, J. M., and Sonenberg, N. (1990). *Biochim. Biophys. Acta* **1050**, 134–139.

Jaspars, E. M. J. (1985). *In* "Molecular Plant Virology" (J. W. Davies, ed.), pp. 155–211. CRC Press, Boca Raton.

Jaspars, E. M. J., Gill, D. S., and Symons, R. H. (1985). *Virology* **144**, 410–425.

Jiang, B. M., Monroe, S. S., Koonin, E. V., Stine, S. E., and Glass, R. I. (1993). *Proc Natl. Acad. Sci. U.S.A.* **90**, 10539–10543.

Johnson, K. L., and Sarnow, P. (1991). *J. Virol.* **65**, 4341–4349.

Johnston, J. C., and Rochon, D. M. (1995). *Virology* **214**, 100–109.

Jupin, I., Bouzoubaa, S., Richards, K., Jonard, G., and Guilley, H. (1990a). *Virology* **178**, 281–284.

Jupin, I., Richards, K., Jonard, G., Guilley, H., and Pleij, C. W. A. (1990b). *Virology* **178**, 273–280.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990). *Nature* **347**, 37–44.

Kadaré, G., Drugeon, G. Savithri, H. S., and Haenni, A-L. (1992). *J. Gen. Virol.* **73**, 493–498.

Kadaré. G., Rozanov, M., and Haenni, A-L. (1995). *J. Gen. Virol.* **76**, 2853–2857.

Kamer, G., and Argos, P. (1984). *Nucleic Acids Res.* **12**, 7269–7282.

Kaper, J. M. (1995). *In* "Pathogenesis and Host Specificity in Plant Diseases" (R. P. Singh, U. P. Singh, and K. Kohmoto, eds.), Vol. III, pp. 373–392. Elsevier, Oxford.

Kao, C. C., and Ahlquist, P. (1992). *J. Virol.* **66**, 7293–7302.

Kao, C. C., Quadt, R., Hershberger, R. P., and Ahlquist, P. (1992). *J. Virol.* **66**, 6322–6329.

Kassavetis, G. A., and Geiduschek, E. P. (1993). *Science* **259**, 944–945.

Kasschau, K. D., and Carrington, J. C. (1995). *Virology* **209**, 268–273.

Kim, J. C., Spence, R. A., Currier, P. A., Lu, X., and Denison, M. R. (1995). *Virology* **206**, 1–8.

Kim, Y. N., Jeong, Y. S., and Makino, S. (1993). *Virology* **197**, 53–63.

Kirkegaard, K., and Baltimore, D. (1986). *Cell* **47**, 433–443.

Klaasen, V. A., Boeshore, M. L., Koonin, E. V., Tian, T., and Falk, B. W. (1995). *Virology* **208**, 99–110.

Knorr, D. A., Mullin, R. H., Hearne, P. Q., and Morris, T. J. (1991). *Virology* **181**, 193–202.

Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, T. A. (1992). *Science* **256**, 1783–1790.

Kollar, A., and Burgyan, A. (1994). *Virology* **201**, 169–172.

Koonin, E. V. (1991a). *J. Gen. Virol.* **72**, 2197–2206.

Koonin, E. V. (1991b). *Nature* **352**, 290.

Koonin, E. V. (1993). *J. Gen. Virol.* **74**, 733–740

Koonin, E. V., and Dolja, V. V. (1993). *Crit. Rev. Biochem. Mol. Biol.* **28**, 375–430.

Koonin, E. V., Boyko, V. P., and Dolja, V. V. (1991). *Virology* **181**, 395–398.

Kornberg, A., and Baker, T. A. (1992). "DNA Replication," 2nd Ed. W. H. Freeman and Co., New York.

Kowal, K. J., and Stollar, V. (1981). *Virology* 114, 140–148.

Kroner, P., Richards, D., Traynor, P., and Ahlquist, P. (1989). *J. Virol.* 63, 5302–5309.

Kroner, P. A., Young, B. M., and Ahlquist, P. A. (1990). *J. Virol.* 64, 6110–6120.

Kuhn, R. J., Griffin, D. E., Zhang, H., Niiesters, H. G. M., and Strauss, J. H. (1992). *J. Virol.* 66, 7121–7127.

Kusov, Y., Weitz, M., Dollenmeier, G., Gauss-Müller, V., and Siegl, G. (1996). *J. Virol.* 70, 1890–1897.

Laakkonen, P., Hyvonen, M., Peranen, J., and Kääriäinen, L. (1994). *J. Virol.* 68, 7418–7425.

Lahser, F. C., Marsh, L. E., and Hall, T. C. (1993). *J. Virol.* 67, 3295–3303.

Lai, M. M. C. (1990). *Annu. Rev. Microbiol.* 44, 303–333.

Lai, M. M. C. (1992). *Microbiol. Rev.* 56, 61–79.

Lai, M. M. C. (1995). *In* "Molecular Basis of Viral Evolution" (A. J. Gibbs, C. H. Calisher, and F. Garcia-Arenal, eds.), pp. 119–132. Cambridge University Press, Cambridge.

Lain, S., Riechmann, J. L., Martin, M.T., and Garcia, J. A. (1989). *Gene* 82, 357–362.

Lain, S., Riechmann, J. L., and Garcia, J. A. (1990). *Nucleic Acids Res.* 18, 7003–7006.

Lain, S., Martin, M. T., Riechmann, J. L., and Garcia, J. A. (1991). *J. Virol.* 65, 1–6.

Lama, J., Paul, A. V., Harris, K. S., and Wimmer, E. (1994). *J. Biol. Chem.* 269, 66–70.

Lambden, P. R., and Clarke, I. N. (1995). *Trends Microbiol.* 3, 261–265.

Landers, T. A., Blumenthal, T., and Weber, K. (1974). *J. Biol. Chem.* 249, 5801–5808.

Larsen, G. R., Dorner, A. J., Harris, T. J. R., and Wimmer, E. (1980). *Nucleic Acids Res.* 8, 1217–1229.

Lastarza, M. W., Lemm, J. A., and Rice, C. M. (1994a). *J. Virol.* 68, 5781–5791.

Lastarza, M. W., Grakoui, A., and Rice, C. M. (1994b). *Virology* 202, 224–232.

Lee, C-G., and Hurwitz, J. (1993). *J. Biol. Chem.* 268, 16822–16830.

Lee, W. M., Monroe, S. S., and Rueckert, R. R. (1993). *J. Virol.* 67, 2110–2122.

Lehto, K., Grantham, G. L., and Dawson, W. O. (1990). *Virology* 174, 145–157.

Lemm, J. A., Rumenapf, T., Strauss, E. G., and Strauss, J. H. (1994). *EMBO J.* 13, 2925–2934.

Lesemann, D. E. (1988). *In* "The Plant Viruses. IV. The Filamentous Plant Viruses," pp. 179–235. Plenum Press, New York.

Levis, R., Weiss, B. G., Tsiang, M., Huang, H., and Schlesinger, S. (1986). *Cell* 44, 137–145.

Levis, R., Schlesinger, S., and Huang, H.V. (1990). *J. Virol.* 64, 1726–1733.

Lewis, T. L., Greenberg, H. B., Herrman, J. E., Smith, L. S., and Matsui, S. M. (1994). *J. Virol.* 68, 77–83.

Li, G., and Rice, C. M. (1989). *J. Virol.* 67, 5062–5067.

Li, J. P., and Baltimore, D. (1988). *J. Virol.* 62, 4016–4021.

Li, J. P., and Baltimore, D. (1990). *J. Virol.* 64, 1102–1107.

Li, X. H., and Carrington, J. C. (1995). *Proc. Natl. Acad. Sci. U.S.A.* 92, 457–461.

Li, Y., and Ball, L. A. (1993). *J. Virol.* 67, 3854–3960.

Lin, Y-J., and Lai, M. M. C. (1993). *J. Virol.* 67, 6110–6118.

Lin, Y-J., Liao, C-L., and Lai, M. M. C. (1994). *J. Virol.* 68, 8131–8140.

Lommel, S. A., Kendall, T. L., Xiong, Z., and Nutter, R. C. (1991). *Virology* 181, 382–385.

Lomonossoff, G. P. (1995). *Annu. Rev. Phytopathol.* 33, 323–343.

Lomonossoff, G. P., Shanks, M., and Evans, D. (1985). *Virology* 144, 351–362.

Longstaff, M., Brigneti, G., Boccard, F., Chapman, S., and Baulcombe, D. (1993). *EMBO J.* 12, 379–386.

Lu, H-L., Li, X., Cucanati, A., and Wimmer, E. (1995). *J. Virol.* **69**, 7445–7452.

Lubinski, J. M., Kaplan, G., Racaniello, V. R., and Dasgupta, A. (1986). *J. Virol.* **58**, 459–467.

Lucas, W. J. (1995). *Curr. Opin. Cell Biol.* **7**, 673–680.

Lucas, W. J., and Gilbertson, R. L. (1994). *Annu. Rev. Phytopathol.* **32**, 387–411.

Lupo, R., Rubino, L., and Russo, M. (1994). *Arch. Virol.* **138**, 135–142.

Makinen, K., Tamm, T., Naess, V., Truve, E., Puurand, U., Munthe, T., and Saarma. M. (1995). *J. Gen. Virol.* **76**, 2817–2825.

Makino, S., and Lai, M. M. C. (1989). *J. Virol.* **63**, 5285–5292.

Makino, S., Stohlman, S. A., and Lai, M. M. C. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4204–4208.

Maraia, R. J., Kenan, D. J., and Keene, J. D. (1994). *Mol. Cell Biol.* **14**, 2147–2158.

Marsh, L. E., and Hall, T. C. (1987). *Cold Spring Harbor Symp. Quant. Biol.* **52**, 331–341.

Marsh, L. E., Dreher, T. W., and Hall, T. C. (1988). *Nucleic Acids Res.* **16**, 981–995.

Marsh, L. E., Pogue, G. P., and Hall, T. C. (1989). *Virology* **172**, 415–427.

Marsh, L. E., Huntley, C. C., Pogue, G. P., Connell, J. P., and Hall, T. C. (1991a). *Virology* **182**, 76–83.

Marsh, L. E., Pogue, G. P., Szybiak, U., Connell, J. P., and Hall, T. C. (1991b). *J. Gen. Virol.* **72**, 2367–2374.

Martin, M. T., Cervera, M. T., and Garcia, J. A. (1995). *Virus Res.* **37**, 127–137.

Masuta, C., Tanaka, H., Dehara, K., Kuwata, S., Koiwai, A., and Noma, M. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6117–6121.

Matsuura, Y., and Miyamura, T. (1993). *Semin. Virol.* **4**, 297–304.

Maynell, L. A., Kirkegaard, K., and Klymkowsky, M. W. (1992). *J. Virol.* **66**, 1985–1994.

Mayo, M. A., and Fritsch, C. (1994). *FEBS Lett.* **354**, 129–130.

McBride, A. E., Schlegel, A., and Kirkegaard, K. (1996). *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2296–2301.

McKnight, K. L., and Lemon, S. M. (1996). *J. Virol.* **70**, 1941–1952.

Meerovitch, K., Svitkin, Y. V., Lee, H. S., Lejbkowicz, F., Kenan, D. J., Chan, E. K. L., Agol, V. I., Keene, J. D., and Sonenberg, N. (1993). *J. Virol.* **67**, 3798–3807.

Merrick, W. C. (1992). *Microbiol. Rev.* **56**, 291–315.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., and Okada, Y. (1988). *EMBO J.* **7**, 1575–1581.

Meyer, J., Lundquist, R. E., and Maizel, J. V. (1978). *Virology* **85**, 445–455.

Meulewater, F., Cornelissen, M., and Van Emmelo, J. (1992). *J. Virol.* **66**, 6419–6428.

Mi, S., and Stollar, V. (1990). *Virology* **178**, 429–434.

Mi, S., and Stollar, V. (1991). *Virology* **184**, 423–427.

Mi, S., Durbin, R., Huang, H. V., Rice, C. M., and Stollar, V. (1989). *Virology* **170**, 385–391.

Miller, W. A., and Hall, T. C. (1983). *Virology* **125**, 236–241.

Miller, W. A., Dreher, T., and Hall, T. C. (1985). *Nature* **313**, 68–70.

Miller, W. A., Dinesh-Kumar, S. P., and Paul, C. P. (1995). *Crit. Rev. Plant Sci.* **14**, 179–211.

Mills, D. R., Priano, C., DiMauro, P., and Binderow, B. D. (1988). *J. Mol. Biol.* **205**, 751–764.

Mirzayan, C., and Wimmer, E. (1992). *Virology* **189**, 547–555.

Mirzayan, C., and Wimmer, E. (1994a). *Virology* **199**, 176–187.

Mirzayan, C., and Wimmer, E. (1994b). *In* "Encyclopedia of Virology" (R. G. Webster and A. Granoff, eds.), Vol. 3, pp. 1119–1123. Academic Press, London.

Mizumoto, K., and Kaziro, Y. (1987). *Prog. Nucleic Acids Res. Mol. Biol.* **34**, 1–28.

Mohan, B. R., Dinesh-Kumar, S. P., and Miller, W. A. (1995). *Virology* **212**, 186–195.

Molla, A., Paul, A. V., and Wimmer, E. (1991). *Science* **254**, 1647–1651.

Molla, A., Paul, A. V., and Wimmer, E. (1992). *Dev. Biol. Stand.* **78**, 39–56.

Molla, A., Paul, A. V., and Wimmer, E. (1993). *J. Virol.* **67**, 5932–5938.

Molla, A., Harris, K. S., Paul, A. V., Shin, S. H., Mugavero, J., and Wimmer, E. (1994). *J. Biol. Chem.* **269**, 27015–27020.

Morch, M. D., Joshi, R. L., Denial, T. M., and Haenni, A. L. (1987). *Nucleic Acids Res.* **15**, 4123–4130.

Morrow, C. D., Gibbons, G. F., and Dasgupta, A. (1985). *Cell* **40**, 913–921.

Mouches, C., Candresse, T., and Bove, J. M. (1984). *Virology* **134**, 78–91.

Mueller, E., Gilbert, J., Davenport, G., Brigneti, G., and Baulcombe, D. C. (1995). *Plant J.* **7**, 1001–1013.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., and Summers, M. D., eds. (1995). "Virus Taxonomy. Classification and Nomenclature of Viruses. Sixth Report of the International Committee on Taxonomy of Viruses," *Arch. Virol.* **Suppl. 10**, 1–586. Springer Verlag, Vienna.

Mushegian, A. R., and Shepherd, R. J. (1995). *Microbiol. Rev.* **59**, 548–578.

Nagy, P. D., Dzianott, A., Ahlquist, P., and Bujarski, J. J. (1995). *J. Virol.* **69**, 2547–2556.

Najita, L., and Sarnow, P. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5846–5850.

Nakhasi, H. L., Rouault, T. A., Haile, D., Liu, T.-Y., and Klausner, R. D. (1990). *New Biol.* **2**, 255–264.

Nakhasi, H. L., Cao, X.-Q., Rouault, T. A., and Liu, T-Y. (1991). *J. Virol.* **65**, 5961–5967.

Nakhasi, H. L., Singh, N. K., Pogue, G. P., Cao, X-Q., Rouault, T. A. (1994). *Arch. Virol.* **S9**, 255–267.

Naranda, T., MacMillan, S. E., and Hershey, J. W. B. (1994). *J. Biol. Chem.* **269**, 32286–32292.

Nassuth, A., and Bol, J. F. (1983). *Virology* **124**, 75–85.

Nassuth, A., Ablas, F., and Bol, J. F. (1981). *Virology* **24**, 75–85.

Natsuaki, T., Mayo, M. A., Jolly, C. A., and Murant, A. F. (1991). *J. Gen. Virol.* **72**, 2183–2189.

Neeleman, L., Van der Vossen, E. A. G., and Bol, J. F. (1993). *Virology* **196**, 883–887.

Nelson, G. W., and Stohlman, S. A. (1993). *J. Gen. Virol.* **74**, 1975–1979.

Nestorowicz, A., Chambers, T. J., and Rice, C. M. (1994). *Virology* **199**, 114–123.

Neufeld, K. L., Richards, K. L., and Ehrenfeld, E. (1991). *Virus Res.* **19**, 173–188.

Neufeld, K. L., Galarza, J. M., Richards, D. F., Summers, D. F., and Ehrenfeld, E. (1994). *J. Virol.* **68**, 5811–5818.

Niesters, H. G. M., and Strauss, J. H. (1990). *J. Virol.* **64**, 1639–1647.

Nilsen, T. W., Wood, D. L., and Baglioni, C. (1981). *Virology* **109**, 82–93.

Novak, J. E., and Kirkegaard, K. (1994). *Genes Develop.* **8**, 1726–1737.

Nuesch, J. P. F., Weitz, M., and Siegl, G. (1993). *Arch. Virol.* **128**, 65–79.

Nutter, R. C., Sheets, K., Panganiban, L. C., and Lommel, S. A. (1989). *Nucleic Acids Res.* **17**, 3163–3177.

Oberg, B. F., and Philipson, L. (1971). *J. Mol. Biol.* **58**, 725–737.

Odd, T., and Wu, H. C. (1993). *J. Biol. Chem.* **268**, 12596–12602.

Ogawa, T., Watanabe, Y., and Okada, Y. (1992). *Virology* **191**, 454–458.

Ollis, D. L., Brick, P., Hamlin, R., Xung, N. G., and Steitz, T. A. (1985). *Nature* **313**, 762–766.

O'Reilly, E. K., Tang, N., Ahlquist, P., and Kao, C. C. (1995). *Virology* **214**, 59–71.

Othman, Y., and Hull, R. (1995). *Virology* **206**, 287–297.

Pacha, R. F., and Ahlquist, P. (1991). *J. Virol.* **65**, 3693–3703.

Pacha, R. F., Allison, R. F., and Ahlquist, P. (1990). *Virology* **174**, 436–443.

Palukaitis, P., Roosinck, M., Dietzgen, R. G., and Francki, R. I. B. (1992). *Adv. Virus Res.* **41**, 281–348.

Pardigon, N., and Strauss, J. H. (1992). *J. Virol.* **66**, 1007–1015.

Pardigon, N., and Strauss, J. H. (1996). *J. Virol.* **70**, 1173–1181.

Pardigon, N., Lenches, E., and Strauss, J. H. (1993). *J. Virol.* **67**, 5003–5011.

Passmore, B. K., Sanger, M., Chin, L.-S., Falk, B. W., and Bruening, G. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10168–10172.

Pata, J. D., Schultz, S. C., and Kirkegaard, K. (1995). *RNA* **1**, 466–477.

Paul, A. V., Cao, X., Harris, K. S., Lama, J., and Wimmer, E. (1994a). *J. Biol. Chem.* **269**, 29173–29181.

Paul, A. V., Molla, A., and Wimmer, E. (1994b). *Virology* **199**, 188–199.

Pause, A., and Sonenberg, N. (1992). *EMBO J.* **11**, 2643–2654.

Pause, A., Méthot, N., and Sonenberg, N. (1993). *Mol. Cell Biol.* **13**, 6789–6798.

Peränen, J., and Kääriäinen, L. (1991). *J. Virol.* **64**, 1888–1896.

Peränen, J., Laakkonen, P., Hyvonen, M., and Kääriäinen, L. (1995). *Virology* **208**, 610–620.

Perez, L., Guinea, R., and Carrasco, L. (1991). *Virology* **183**, 74–82.

Peters, S. A., Verver, J., Nollen, E. A. A., van Lent, J. W. M., Wellink, J., and van Kammen, A. (1994). *J. Gen. Virol.* **75**, 3167–3176.

Peters, S. A., Mesnard, J-M., Kooter, I. M., Verver, J., Wellink, J., and Van Kammen, A. (1995). *J. Gen. Virol.* **76**, 1807–1813.

Petty, I. T. D., French, R., Jones, R. W., and Jackson, A. O. (1990). *EMBO J.* **9**, 3453–3457.

Pfister, T., Egger, D., and Bienz, K. (1995). *J. Gen. Virol.* **76**, 63–71.

Pilipenko, E. V., Maslova, S. V., Sinyakov, A. N., and Agol, V. I. (1992). *Nucleic Acids Res.* **20**, 1739–1745.

Plagemann, P. G. W., and Moenning, V. (1992). *Adv. Virus Res.* **41**, 99–192.

Plotch, S. J., and Palant, O. (1995). *J. Virol.* **69**, 7169–7179.

Poch, O., Sauvaget, I., Delarue, M., and Tordo, N. (1989). *EMBO J.* **8**, 3867–3874.

Pogue, G. P., and Hall, T. C. (1992). *J. Virol.* **66**, 674–684.

Pogue, G. P., Marsh, L. E., and Hall, T. C. (1990). *Virology* **178**, 152–160.

Pogue, G. P., Marsh, L. E., Connell, J. P., and Hall, T. C. (1992). *Virology* **188**, 742–753.

Pogue G. P., Cao, X.-Q, Singh, N. K., and Nakhasi, H. L. (1993). *J. Virol.* **67**, 7106–7117.

Pogue, G. P., Huntley, C. C., and Hall, T. C. (1994). *Arch. Virol.* **S9**, 181–194.

Priano, C., Kramer, F. R., and Mills, D. R. (1987). *Cold Spring Harbor Symp. Quant. Biol.* **33**, 373–398.

Purdey, M. A., Tam, A. W., Huang, C.-C., Yarbough, P. O., and Reyes, G. R. (1993). *Semin. Virol.* **4**, 319–326.

Quadt, R., and Jaspars, E. M. J. (1990). *Virology* **178**, 189–194.

Quadt, R., Rosdorff, H. J. M., Hunt, T. W., and Jaspars, E. M. J. (1991). *Virology* **182**, 309–315.

Quadt, R., Kao, C. C., Browning, K. S., Hershberger, R. P., and Ahlquist, P. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1498–1502.

Quadt, R., Ishikawa, M., Janda, M., and Ahlquist, P. (1995). *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4892–4896.

Raffo, A. J., and Dawson, W. O. (1991). *Virology* **184**, 277–289.

Raju, R., and Huang, H. V. (1991). *J. Virol.* **65**, 2501–2510.

Rao, A. L. N., Dreher, T. W., Marsh, L. E., and Hall, T. C. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5335–5339.

Rao, A. L. N., and Hall, T. C. (1991). *Virology* **180**, 16–22.

Rao, A. L. N., and Grantham, G. L. (1994). *Virology* **204**, 478–481.

Reavy, B., Mayo, M. A., Turnbull-Ross, A. D., and Murant, A. F. (1993). *Arch. Virol.* **131**, 441–446.

Restrapo-Hartwig, M. A., and Ahlquist, P. (1995). Abstr. Am. Soc. Virol. Meeting, Wisconsin, p. 136.

Restrapo-Hartwig, M. A., and Carrington, J. C. (1994). *J. Virol.* **68**, 2388–2397.

Reuer, Q., Kuhn, R. J., and Wimmer, E. (1990). *J. Virol.* **64**, 2967–2975.

Reusken, C. B. E. M., Neeleman, L., and Bol, J. F. (1994). *Nucleic Acids Res.* **22**, 1346–1353.

Reusken, C. B. E. M., Neeleman, L., and Bol, J. F. (1995). *J. Virol.* **69**, 4552–4555.

Reutenauer, A., Ziegler-Graff, V., Lot, H., Scheidecker, D., Guilley, H., Richards, K., and Jonard, G. (1993). *Virology* **195**, 692–699.

Revill, P. A., Davidson, A. D., and Wright, P. J. (1994). *Virology* **202**, 904–911.

Richards, K. E., and Tamada, T. (1992). *Annu. Rev. Phytopathol.* **30**, 291–313.

Richards, O. C., Martin, S. C., Jense, H. G., and Ehrenfeld, E. (1984). *J. Mol. Biol.* **173**, 325–340.

Richards, O. C., Yu, P., Neufeld, K. L., and Ehrenfeld, E. (1992). *J. Biol. Chem.* **267**, 17141–17146.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1990). *Virology* **177**, 710–716.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1992). *J. Gen. Virol.* **73**, 1–16.

Rikkonen, M., Peränen, J., and Kääriäinen, L. (1994). *Arch. Virol.* **S9**, 369–377.

Rodriguez, P. L., and Carrasco, L. (1993). *J. Biol. Chem.* **268**, 8105–8110.

Rohll, J. B., Holness, C. L., Lomonossoff, G. P., and Maule, A. J. (1993). *Virology* **193**, 672–679.

Rohll, J. B., Percy, N., Ley, R., Evans, D. J., Almond, J. W., and Barclay, W. S. (1994). *J. Virol.* **68**, 4384–4391.

Rohll, J. B., Moon, D. H., Evans, D. J., and Almond, J. W. (1995). *J. Virol.* **69**, 7835–7844.

Rohozinski, J., Francki, R. I. B., and Chu, P. W. G. (1986). *Virology* **155,** 27–38.

Roossinck, M. J., Sleat, D., and Palukaitis, P. (1992). *Microbiol. Rev.* **56**, 265–279.

Rothstein, M. A., Richards, O. C., Amin, C., and Ehrenfeld, E. (1988). *Virology* **164**, 301–308.

Rouleau, M., Bancroft, J. B., and Mackie, G. A. (1993). *Virology* **197**, 695–703.

Rozanov, M. N., Koonin, E. V., and Gorbalenya, A. E. (1992). *J. Gen. Virol.* **73**, 2129–2134.

Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W, C., and Sonenberg, N. (1990). *Mol. Cell. Biol.* **10**, 1134–1144.

Russo, M., Di Franco, S., and Martelli, G. P. (1983). *J. Ultrastruct. Res.* **82**, 52–63.

Russo, M., Burgyan, J., and Martelli, G. P. (1994). *Adv. Virus Res.* **44**, 381–428.

Russo, M., Burgyan, J., and Rubino, L. (1995). Abstr. Fourth Internat. Symp. Positive Strand RNA Viruses, Utrecht, P6-58.

Saito, T., Hosokawa, D., Meshi, T., and Okada, Y. (1987). *Virology* **160**, 477–481.

Sankar, S., and Porter, A. G. (1992). *J. Biol. Chem.* **267**, 10168–10176.

Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990). *Trends Biochem. Sci.* **15**, 430–434.

Sarnow, P. (1989). *J. Virol.* **63**, 467–470.

Sarnow, P., Bernstein, H. D., and Baltimore, D. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 571–575.

Sawicki, S. G., and Sawicki, D. L. (1986). *J. Virol.* **57**, 328–334.

Sawicki, S. G., and Sawicki, D. L. (1990). *J. Virol.* **64**, 1050–1056.

Sawicki, D. L., and Sawicki, S. G. (1994). *Arch. Virol.* **S9**, 393–405.

Sawicki, S. G., and Sawicki, D. L. (1995). *In* "Corona- and Related Viruses" (P. J. Talbot and G. A. Levy, eds.), pp. 499–506. Plenum Press, New York.

Schaad, M. C., and Baric, R. S. (1994). *J. Virol.* **68**, 8169–8179.

Scheffner, M., Knippers, R., and Stahl, H. (1989). *Cell* **57**, 955–963.

Sheidel, L. M., Durbin, R. K., and Stollar, V. (1989). *Virology* **173**, 408–414.

Scheidel, L. M., and Stollar, V. (1991). *Virology* **181**, 490–499.

Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sänger, H. L. (1993a). *J. Biol. Chem.* **263**, 11851–11857.

Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sänger, H. L. (1993b). *J. Biol. Chem.* **263**, 11858–11867.

Schlegel, A., Giddings, T. H., Ladinsky, M. S., and Kirkegaard, K. (1996). *J. Virol.*, in press.

Schlesinger, S. (1995). *Mol. Biotech.* **3**, 155–165.

Schlesinger, S., Levis, R., Weiss, B. G., Tsiang, M., and Huang, H. (1987). *UCLA Symp. Mol. Cell Biol. New Ser.* **54**, 241–250.

Schmid, M., and Wimmer, E. (1994). *Arch. Virol.* **S9**, 279–289.

Scholthof, H. B., Morris, T. J., and Jackson, A. O. (1993). *Mol. Plant-Microbe Interact.* **6**, 309–322.

Scholthof, K.-B. G., Scholthof, H. B., and Jackson, A. O. (1995). *Virology* **208**, 365–369.

Schulz, G. E. (1992). *Curr. Opin. Struct. Biol.* **2**, 61–67.

Schultz, S. C., Hansen, J. L., and Long, A. (1995). Abstr. Fourth Internat. Symp. Positive Strand RNA Viruses, Utrecht, p. 13.

Shuman, S. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11798–11802.

Shuman, S. (1993). *J. Biol. Chem.* **268**, 11798–11802.

Sekimizu, K., and Kornberg, A. (1988). *J. Biol. Chem.* **263**, 7131–7135.

Sethna, P. B., Hung, S.-L., and Brian, D. A. (1989). *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5626–5630.

Shatkin, A. J., and Kozak, M. (1983). In "The Reoviridae" (W. K. Joklik, ed.), pp. 79–106. Plenum Press, New York.

Shen, P., Kaniewska, M. B., Smith, C., and Beachy, R. N. (1993). *Virology* **193**, 621–630.

Shuman, S., and Moss, B. (1990). *Methods Enzymol.* **181**, 170–180.

Shirako, Y., and Wilson, T. M. A. (1993). *Virology* **195**, 16–32.

Shirako, Y., and Strauss, J. H. (1994). *J. Virol.* **68**, 1874–1885.

Shiroki, K., Kato, H., Koike, S., Odaka, T., and Nomoto, A. (1993). *J. Virol.* **67**, 3989–3996.

Shuman, S. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10935–10939.

Simon, A. E., and Bujarski, J. J. (1994). *Annu. Rev. Phytopathol.* **32**, 337–362.

Singh, N. K., Atreya, C. A., and Nakhasi, H. L. (1994). *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12770–12774.

Skuzeski, J. M., Bozarth, C. S., and Dreher, T. W. (1996). *J. Virol.* **70**, 2107–2115.

Smirnyagina, E., Hsu, Y.-H., Chua, N., and Ahlquist, P. (1994). *Virology* **198**, 427–436.

Snijder, E. J., and Horzinek, M. C. (1993). *J. Gen. Virol.* **74**, 2305–2316.

Snijder, E. J., Wassenaar, A. L. M., Spaan, W. J. M., and Gorbalenya, A. E. (1995). *J. Biol. Chem.* **270**, 16671–16676.

Solovyev, A. G., Novikov, V. K., Merits, A., Savenkov, E. I., Zelenina, D. A., Tyulkina, L. G., and Morozov, S. Y. (1994). *J. Gen. Virol.* **75**, 259–267.

Song, C., and Simon, A. E. (1994). *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8792–8796.

Song, C., and Simon, A. E. (1995). *J. Virol.* **69**, 4020–4028.

Sosnovtsev, S., and Green, K. Y. (1995). *Virology* **210**, 383–390.

Soumounou, Y., and Laliberte, J-F. (1994). *J. Gen. Virol.* **75**, 2567–2573.

Sousa, R., Chung, Y. J., Rose, J. P., and Wang, B. C. (1993). *Nature* **364**, 593–599.

Spector, D. H., and Baltimore, D. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2983–2987.

Standart, N., and Jackson, R. J. (1994). *Biochimie* **76**, 867–879.

Stern, D. F., and Sefton, B. M. (1982). *J. Virol.* **42**, 755–759.

Strauss, J. H., and Strauss, E. G. (1988). *Annu. Rev. Microbiol.* **42**, 657–683.

Strauss, J. H., and Strauss, E. G. (1994). *Microbiol. Rev.* **58**, 491–562.

Sun, J., and Kao, C. C. (1996). *Virology*, in press.

Suzich, J. A., Tamura, J. K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C. M., Feinstone, S. M., and Collett, M. S. (1993). *J. Virol.* **67**, 6153–6158.

Takamatsu, N., Watanabe, Y., Meshi, T., and Okada, Y. (1990). *J. Virol.* **64**, 3686–3693.

Takamatsu, N., Watanabe, Y., Iwasaka, T., Shiba, T., Meshi, T., and Okada, Y. (1991). *J. Virol.* **65**, 1619–1622.

Takeda, N., Kuhn, R. J., Yang, C. F., Takegami, T., and Wimmer, E. (1986). *J. Virol.* **60**, 43–53.

Tamura, J. K., Warrener, P., and Collett (1993). *Virology* **193**, 1–10.

Taylor, S. J., and Shalloway, D. (1994). *Nature* **368**, 867–871.

Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995). *J. Biol. Chem.* **270**, 10120–10124.

Teterina, N. L., Kean, K. M., Gorbalenya, A. E., Agol, V. L., and Girard, M. (1992). *J. Gen. Virol.* **73**, 1977–1986.

Tobin, G. J., Young, D. C., and Flanegan, J. B. (1989). *Cell* **59**, 511–519.

Todd, S., Nguyen, J. H. C., and Semler, B. L. (1995). *J. Virol.* **69**, 3605–3614.

Tolskaya, E. A., Romanova, L. I., Kolesnikova, M. S., Gmyl, A. P., Gorbalenya, A. E., and Agol, V. I. (1994). *J. Mol. Biol.* **236**, 1310–1323.

Toyoda, H., Yang, C. F., Takeda, N., Nomoto, A., and Wimmer, E. (1987). *J. Virol.* **61**, 2816–2822.

Traut, T. W. (1994). *Eur. J. Biochem.* **222**, 9–19.

Traynor, P., and Ahlquist, P. (1990). *J. Virol.* **64**, 69–77.

Traynor, P., Young, B. M., and Ahlquist, P. (1991). *J. Virol.* **65**, 2807–2815.

Troxler, M., Egger, D., Pfister, T., and Bienz, K. (1992). *Virology* **191**, 687–697.

Tsai, C-H, and Dreher, T. W. (1991). *J. Virol.* **65**, 3060–3067.

Tsai, C-H, and Dreher, T. W. (1992). *J. Virol.* **66**, 5190–5199.

Turnbull-Ross, A. D., Mayo, M. A., Reavy, B., and Murant, A. F. (1993). *J. Gen. Virol.* **74**, 555–561.

van Bokhoven, H., Van Lent, J. W. M., Custers, R., Vlak, J. M., Wellinck, J., and Van Kammen, A. (1992). *J. Gen. Virol.* **73**, 2775–2784.

van Bokhoven, H., Le Gall, O., Kasteel, D., Verver, J., Wellinck, J., and Van Kammen, A. (1993). *Virology* **195**, 377–386.

van der Kuyl, A. C., Langereis, K., Houwing, C. J., Jaspars, E. M. J., and Bol, J. F. (1990). *Virology* **176**, 346–354.

van der Kuyl, A. C., Neeleman, L., and Bol, J. F. (1991). *Virology* **183**, 687–694.

van der Meer, J., Dorssers, L., Van Kammen, A., and Zabel, P. (1984). *Virology* **132**, 413–425.

van der Most, R. G., De Groot, R. J., and Spaan, W. J. M. (1994). *J. Virol.* **68**, 3656–3666.

van der Vossen, E. A. G., Neeleman, L., and Bol, J. F. (1993). *Nucleic Acids Res.* **21**, 1361–1367.

van der Vossen, E. A. G., Neeleman, L., and Bol, J. F. (1994). *Virology* **202**, 891–903.

van der Vossen, E. A. G., Notenboom, T., and Bol, J. F. (1995). *Virology* **212**, 663–672.

van der Werf, S., Bradley, J., Wimmer, E., Studier, F. W., and Dunn, J. J. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2330–2334.

van Duin, J. (1988). *In* "The Bacteriophages" (R. Calandar, ed.), pp. 117–167. Plenum Press, New York.

van Dyke, T. A., and Flanegan, J. B. (1980). *J. Virol.* **35**, 733–740.

van Etten, J. L., Burbank, D. E., Cuppels, D. A., Lane, L. C., and Vidaver, A. K. (1980). *J. Virol.* **33**, 769–773.

van Kuppeveld, F. J. M., Galama, J. M. D., Zoll, J., and Melchers, W. J. G. (1995). *J. Virol.* **69**, 7782–7790.

van Marle, G., Luytjes, W., Van der Most, R. G., Van Der Straaten, T., and Spaan, W. J. M. (1995). *J. Virol.* **69**, 7851–7856.

van Verooj, W. J., Slobbe, R. L., and Pruijn G. J. M. (1993). *Mol. Biol. Rep.* **18**, 113–119.

Verchot, J., and Carrington, J. C. (1995). *J. Virol.* **69**, 3668–3674.

Volloch, V. (1986). *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1208–1212.

Volloch, V., Schweitzer, B., and Rits, S. (1987). *J. Cell Biol.* **105**, 137–143.

Vos, P., Jaegle, M., Wellink, J., Van Kammen, A., and Goldbach, R. (1988). *Virology* **165**, 33–41.

Waigmann, E., and Zambryski, P. (1994). *Curr. Biol.* **4**, 713–716.

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). *EMBO J.* **1**, 945–951.

Wang, H.-L., O'Rear, J., and Stollar, V. (1996). *Virology* **217**, 527–531.

Wang, Y.-F., Sawicki, S. G., and Sawicki, D. L. (1994). *J. Virol.* **68**, 6466–6475.

Ward, C. W. (1994). *Res. Virol.* **144**, 419–453.

Warrener, P., and Collett, M. C. (1995). *J. Virol.* **69**, 1720–1726.

Warrener, P., Tamura, J. K., and Collett, M. C. (1993). *J. Virol.* **67**, 989–996.

Watanabe, Y., Kishibayashi, N., Motoyoshi, F., and Okada, Y. (1987). *Virology* **161**, 527–532.

Watanabe, Y., Ogawa, T., Takahashi, H., Ishida, I., Takeuchi, Y., Yamamoto, M., and Okada, Y. (1995). *FEBS Lett.* **372**, 165–168.

Wei, N., Hacker, D. L., and Morris, T. J. (1992). *Virology* **190**, 346–355.

Weiland, J. J., and Dreher, T. W. (1993). *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6095–6099.

Weiner, B. M., and Bradley, M. K. (1991). *J. Virol.* **65**, 4973–4984.

Weiner, A. M., and Maizels, N. (1987). *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7383–7387.

Wellink, J., Van Lent, J., and Goldbach, R. (1988). *J. Gen. Virol.* **69**, 751–755.

Wengler, G., and Wengler, G. (1993). *Virology* **197**, 265–273.

Wengler, G., Wengler, G., and Gross, H. J. (1979). *Nature* **282**, 754–756.

Wengler, G., Boege, U., Wengler, G., Bischoff, H., and Wahn, K. (1982). *Virology* **118**, 401–410.

White, K. A., and Morris, T. J. (1994). *J. Virol.* **68**, 14–24.

White, K. A., Skuzeski, J. M., Li, W., Wei, N., and Morris, T. J. (1995). *Virology* **211**, 525–534.

Willcocks, M. M., Brown, T. D. K., Madeley, C. R., and Carter, M. J. (1994). *J. Gen. Virol.* **75**, 1785–1788.

Wimmer, E., Hellen, C. U. T., and Cao, X. (1993). *Annu. Rev. Genet.* **27**, 353–436.

Witherell, G. W., Gott, J. M., and Uhlenbeck, O. C. (1991). *Prog. Nucleic Acid Res. Mol. Biol.* **40**, 185–220.

Wu, G., and Kaper, J. M. (1994). *J. Mol. Biol.* **238**, 655–657.

Wu, S.-X., and Kaesberg, P. (1991). *Virology* **183**, 392–396.

Wu, S.-X., Ahlquist, P., and Kaesberg, P. (1992). *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11136–11140.

Young, N. D., and Zaitlin, M. (1986). *Plant Mol. Biol.* **6**, 455–465.

Young, D. C., Tuschall, D. M., and Flanegan, J. B. (1985). *J. Virol.* **54**, 256–264.

Yu, W., and Leibowitz, J. L. (1995). *J. Virol.* **69**, 2016–2023.

Yu, S. F., Benton, P., Bovee, M., Sessions, L., and Lloyd, R. E. (1995). *J. Virol.* **69**, 247–252.

Yusibov, V. M., and Loesch-Fries, L. S. (1995). *Virology* **208**, 405–407.

Xiang, W., Cuconati, A., Paul, A. V., Cao, X., and Wimmer, E. (1995a). *RNA* **1**, 892–904.

Xiang, W., Harris, K. S., Alexander, L., and Wimmer, E. (1995b). *J. Virol.* **69**, 3658–3667.

Xiao, Q., Sharp, T. S., Jeffrey, I. W., James, M. C., Pruijn, G. J. M., van Venrooij, W. J., and Clemens, M. J. (1994). *Nucleic Acids Res.* **22**, 2512–2518.

Zavriev, S. K., Hickey, C. M., and Lommel, S. A. (1996). *Virology* **216**, 407–410.

Zerfass, K., and Beier, H. (1992). *EMBO J.* **11**, 4167–4173.

Zhang, X., and Lai, M. M. C. (1995). *J. Virol.* **69**, 1637–1644.

Zhang, X., and Lai, M. M. C. (1996). *J. Virol.* **70**, 705–711.

Zhang, X., Liao, C.-L., and Lai, M. M. C. (1994). *J. Virol.* **68**, 4738–4746.

Zhu, J., and Newkirk, M. M. (1994). *Clin. Invest. Med.* **17**, 196–205.

Ziegler, A., Natsuaki, T., Mayo, M. A., Jolly, C. A., and Murant, A. F. (1992). *J. Gen. Virol.* **73**, 3213–3218.

Zimmern, D. (1988). *In* "RNA Genetics" (J. Holland, E. Domingo, and P. Ahlquist, eds.), Vol. 2, pp. 211–240. CRC Press, Boca Raton.