MECHANISM OF GENOME TRANSCRIPTION IN SEGMENTED dsRNA VIRUSES

Jeffrey A. Lawton,[1] Mary K. Estes,[2]
and B. V. Venkataram Prasad[1]

Verna and Maars McLean Department of Biochemistry and Molecular Biology[1]
and Department of Molecular Virology and Microbiology[2],
Baylor College of Medicine
Houston, Texas 77030

1. INTRODUCTION

A. Unity and Diversity among Segmented dsRNA Viruses

Viruses with double-stranded RNA (dsRNA) genomes are widespread in nature and infect hosts ranging from bacteria and fungi to species throughout the plant and animal kingdoms (reviewed in Murphy *et al.*, 1995). Although these viruses vary considerably in their genetic and structural complexity, they share several basic similarities. Besides having genomes consisting of dsRNA, which is often arranged in multiple segments, these viruses also appear to exhibit certain common architectural similarities, and all of them, at the start of their replication cycles, produce mRNA transcripts within intact particles using an endogenous transcription apparatus, which is an integral part of the viral architecture.

185

Viruses having dsRNA genomes are currently classified into five major families (Murphy *et al.*, 1995). This classification scheme is summarized in Table 4.1. All of these viruses have an icosahedral capsid architecture, and, with the exception of those in the family *Totiviridae,* all have genomes consisting of multiple segments. None of the dsRNA viruses infecting eukaryotes have a lipid membrane; however, the cystoviruses, which infect prokaryotes, contain a lipid envelope in the mature particle. The classification scheme for dsRNA viruses is likely to continue to expand as currently unclassified dsRNA viruses are better characterized.

The most well-studied family of segmented dsRNA viruses is the *Reoviridae.* Although the viruses in the family *Reoviridae* infect a wide variety of hosts throughout the plant and animal kingdoms and cause illnesses ranging from subclinical to life-threatening, they are classified together primarily because of the following major distinguishing structural characteristics (reviewed in Fields, 1996). Members of the *Reoviridae* are large, complex particles, often having a diameter greater than 700 Å. Capsids have an icosahedral architecture and are composed of multiple proteins arranged generally in concentric layers. None of the mature viruses in this family contain a lipid envelope. Genomes consist of 10 to 12 segments of double-stranded RNA (dsRNA) packed within the core of the virion, and each segment generally encodes only one protein. Like other dsRNA viruses, members of the *Reoviridae* also have an endogenous transcription apparatus that operates within the viral interior. Viruses in this family are currently classified into nine genera (Table 4.1).

Several prototypical members of the family *Reoviridae,* namely mammalian orthoreovirus, bluetongue virus, rotavirus, and cypovirus, have figured prominently in studies aimed at understanding how dsRNA viruses undergo replication. However, related studies on several other types of dsRNA viruses have also provided substantial insights. These viruses include cystovirus φ6 of the plant pathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola* and L-A virus of *Saccharomyces cerevisiae.* Unlike members of the *Reoviridae,* which have between 10 and 12 monocistronic genome segments, φ6 has three polycistronic segments, and L-A virus contains only a single polycistronic segment. Because both φ6 and L-A virions share striking similarities with members of the *Reoviridae,* and because of the greater extent to which the genetics and molecular biology of these systems may be manipulated, these two viral systems have also made significant contributions to the understanding of the way in which dsRNA viruses progress through the important stages of their life cycles.

TABLE 4.1
CLASSIFICATION OF MAJOR FAMILIES AND GENERA OF DSRNA VIRUSES[a]

Genus	Primary Host	Prototype virus	Genome segments
Reoviridae			
Orthoreovirus	Mammals	Reovirus 3	10
Orbivirus	Mammals	Bluetongue virus 1	10
Rotavirus	Mammals	Simian rotavirus SA11	11
Coltivirus	Mammals	Colorado tick fever virus	12
Aquareovirus	Fish	Golden shiner virus	11
Cypovirus	Insects	Bombyx mori cypovirus 1	10
Fijivirus	Plants	Fiji disease virus	10
Phytoreovirus	Plants	Wound tumor virus	12
Oryzavirus	Plants	Rice ragged stunt virus	10
Cystoviridae			
Cystovirus	Bacteria	Pseudomonas phage φ6	3
Birnaviridae			
Aquabirnavirus	Fish	Infectious pancreatic necrosis virus	2
Avibirnavirus	Birds	Infectious bursal disease virus	2
Entomobirnavirus	Insects	Drosophila X virus	2
Totiviridae			
Totivirus	Fungi	Saccharomyces cerevisiae L-A virus	1
Giardiavirus	Protozoa	Giardia lamblia virus	1
Leishmaniavirus	Protozoa	Leishmania RNA virus 1–1	1
Partitiviridae			
Partitivirus	Fungi	Gaeumannomyces graminis virus 019/6A	2–4
Chrysovirus	Fungi	Penicillium chrysogenum virus	3
Alphacryptovirus	Plants	White clover cryptic virus 1	2
Betacryptovirus	Plants	White clover cryptic virus 2	2

[a] The complete classification scheme for dsRNA viruses also includes the family *Hypoviridae*, a group of viral genetic elements infecting several species of plant pathogenic fungi (reviewed in Wickner, 1996). However, these viral genetic elements are not encapsidated, and they exhibit more genetic similarities with the plant potyviruses than with other families of dsRNA viruses.

B. Genome Transcription in Segmented dsRNA Viruses

After cell entry, a virus must present its genetic information to host cell ribosomes in a translatable form so that the virally encoded proteins needed for genome replication and the assembly of progeny virions may be synthesized. As host cells do not have any biochemical

machinery capable of transcribing mRNA from a dsRNA template, viruses with segmented dsRNA genomes produce mRNA transcripts within structurally intact particles using a virally encoded endogenous transcriptase. Biochemical and structural studies carried out on many types of segmented dsRNA viruses suggest that, despite differences in viral architecture, the basic events surrounding the process of endogenous genome transcription are notably similar. The results obtained from studying transcription in one viral system are often applicable to the entire family as a whole, and together these studies have helped to provide a better picture of the way in which segmented dsRNA viruses accomplish this important step in their life cycles.

Rotaviruses, which are prototypical members of the *Reoviridae*, represent a useful experimental system in which to investigate the mechanism of endogenous genome transcription in segmented dsRNA viruses from a structural perspective and to study the structural and biochemical constraints on the efficiency of mRNA production within intact particles. The review presented here is written from the perspective of rotavirus as a model system; however, relevant observations from other dsRNA viruses are included as well to enchance the understanding of genome transcription in dsRNA viruses as a whole.

II. Organization of Transcription Apparatus

A. Overview of Virus Architecture

One interesting observation about the life cycle of viruses having segmented dsRNA genomes is that transcription only occurs within the context of an intact viral particle. From this observation, it follows that, not only are the transcription enzymes in the core required for efficient mRNA production, but the integrity of the capsid is necessary as well. For this reason, a basic presentation of virus architecture is helpful for understanding how the transcription apparatus is organized and how this apparatus functions under transcribing conditions to generate mRNA transcripts.

1. Rotavirus

Physical characterization of the rotaviruses has been made possible through the combined application of biochemical and structural techniques. Studies using purified virions and baculovirus-expressed recombinant virus-like particles in conjunction with electron cryomicroscopy have defined the organization of the capsid layers and localized each

protein within the virion (reviewed in Prasad and Estes, 1997). Much of what is known about the structural organization of rotavirus has come from studies using simian and rhesus rotavirus strains.

Mature, infectious rotaviruses have a multilayered capsid structure as depicted in Fig. 4.1 (see also color insert) (Prasad *et al.,* 1988; Yeager *et al.,* 1990; Prasad *et al.,* 1996). The innermost capsid layer is formed by 120 molecules of a 102 kDa protein VP2 arranged as 60 dimers on a T=1 icosahedral lattice (Lawton *et al.,* 1997b). Such an organization with two molecules in the icosahedral asymmetric unit has also been referred to as a 'T=2' structure (Grimes *et al.,* 1998). This protein shell is believed to define the basic icosahedral architecture of the virus, as VP2 is the only rotavirus protein capable of self-

FIG 4.1. Three-dimensional structures of the mature infectious rotavirus particle and the transcriptionally competent subviral particle. (Left) The mature rotavirus particle consists of three concentric capsid layers. In this illustration, produced from a three-dimensional reconstruction of rotavirus at 24 Å resolution, the inner capsid protein VP2 is shown in green, the intermediate capsid protein VP6 in blue, and the outer capsid protein VP7 in yellow. The VP4 spike proteins are shown in orange. The transcription enzymes VP1 and VP3, along with the eleven genomic dsRNA segments, are housed in the interior within the VP2 capsid layer. (Center) Rotavirus enters the cell by penetration of the plasma membrane, and as a consequence of cell entry, the outer capsid proteins VP7 and VP4 are removed. (Right) Once inside the cell, the resulting subviral particle becomes transcriptionally active, releasing capped mRNA transcripts into the cytoplasm. See also color insert.

assembling into stable virus-like particles (VLPs) (Labbé et al., 1991; Crawford et al., 1994). The VP2 layer serves as a scaffold for the assembly of the intermediate layer, which consists of 780 copies of a 45 kDa protein (VP6) arranged as 260 trimers on a T=13(levo) icosahedral lattice. The addition of VP6 protein to VLPs formed from VP2 imparts greater morphological homogeneity and long-term stability to the particles (Zeng et al., 1996), suggesting that VP6 may play a major role in providing structural integrity to the rotavirus capsid. The icosahedral organization of the VP6 capsid layer defines a set of channels leading to the viral interior; these channels play an important role in the transcription process, facilitating the import of RNA precursor molecules and the export of mRNA transcripts (Prasad et al., 1988). The outermost capsid layer in the mature particle consists of 780 copies of a 37 kDa glycoprotein VP7 assembled in a 1:1 stoichiometry onto the top of each VP6 molecule. The outer capsid layer also contains 60 dimers of VP4, which form spikes extending 120 Å outward from the viral surface (Prasad et al., 1990; Shaw et al., 1993; Yeager et al., 1994).

The atomic resolution structure of the VP6 capsid protein of rotavirus was recently determined using X-ray crystallographic techniques (Mathieu et al., 2000). The VP6 structure is similar to the structure of the homologous VP7 protein in the orbivirus bluetongue virus (Grimes et al., 1995). The rotavirus VP6 protein folds into two distinct domains of roughly equal size. The upper domain, which interacts with the outermost capsid protein VP7 in the mature particle, folds into an eight-stranded β-barrel structure, a motif often seen in viral capsid proteins. The upper domains of the individual monomers interact closely with one another to stabilize the trimeric structure of VP6. At the base of the trimer, the contacts between monomers are sparse with a large cavity along the 3-fold axis of the trimer. The lower domain of VP6, composed of several α helices, interacts with the underlying VP2 capsid layer as well as with neighboring trimers. Combination of the X-ray crystallographic structure of VP6 with the structure of the native virus determined using electron cryomicroscopy (Prasad et al., 1996) suggests that the VP6 molecule may function like a tripod in which the domains forming the base of the trimers are capable of slight adjustments to adapt to various quasi-equivalent locations on the T=13 icosahedral surface lattice of the viral capsid. This feature also allows the VP6 to compensate for the symmetry mismatch between the VP6 (T=13) and VP2 (T=1) layers of the virion.

Although mature, infectious rotavirus particles consists of three capsid layers, genome transcription occurs only in particles that have shed the outer capsid layer composed of VP4 and VP7 (Fig. 4.1). The

transcriptionally competent rotavirion thus contains only the inner VP2 and intermediate VP6 capsid layers. This structural form of rotavirus has been described elsewhere as a double-layered particle (Prasad *et al.*, 1996), inner capsid particle (Taylor *et al.*, 1996), single-shelled particle (Prasad and Chiu, 1994), and subviral particle (López *et al.*, 1994). Because the configuration of the capsid in the transcriptionally competent virion differs from one type of dsRNA virus to another, the term *transcriptionally competent particle* (TCP) is used in this review to describe the structural form of the virus that normally functions during the viral life cycle to transcribe the genome.

2. Other Viruses with dsRNA Genomes

Electron cryomicroscopy has also been used extensively to study other common viruses having dsRNA genomes, including bluetongue virus (Prasad *et al.*, 1992; Hewat *et al.*, 1992; Grimes *et al.*, 1997) and Broadhaven virus (Schoehn *et al.*, 1997) of the genus orbivirus, mammalian orthoreovirus (Dryden *et al.*, 1993; Dryden *et al.*, 1998), insect cypovirus (Hill *et al.*, 1999; Zhang *et al.*, 1999), aquareovirus (Shaw *et al.*, 1996), rice dwarf virus of the genus phytoreovirus (Lu *et al.*, 1998), cystovirus φ6 (Butcher *et al.*, 1997), infectious bursal disease virus of the family *Birnaviridae* (Böttcher *et al.*, 1997), and L-A virus of the family *Totiviridae* (Cheng *et al.*, 1994; Castón *et al.*, 1997). These studies have served to highlight the common structural themes arising across multiple genera of dsRNA viruses and also to highlight important architectural differences, which, among dsRNA viruses with segmented genomes, are typically seen within the vicinity of the icosahedral 5-fold axes.

The atomic structure of the intact bluetongue virus TCP has been determined at 3.5 Å resolution (Grimes *et al.*, 1998; Gouet *et al.*, 1999), providing unprecedented insight into the basic architecture of the transcriptionally competent particle. Like the rotavirus TCP, the bluetongue virus TCP consists of two capsid layers enclosing the viral genome and the endogenous transcription enzymes. The outer capsid layer in the bluetongue virus TCP is composed of 780 copies of a protein VP7 arranged as trimers on a T=13 *(levo)* icosahedral lattice, and the inner capsid layer is composed of 120 copies of a protein VP3 arranged as dimers on a T=1 icosahedral lattice. The VP3 protein folds into a thin, relatively flat structure having the shape of a narrow triangular wedge. Each protein is composed of three distinct domains, each domain having a unique fold unlike that of any other reported protein structure. The VP3 capsid layer is most flexible in the region surrounding the icosahedral vertices. Some of the internal features of

the virion corresponding to the dsRNA genome and the enzymes involved in transcription are also partially visible in the viral interior.

The three-dimensional structure of orthoreovirus has also been extensively studied (reviewed in Nibert, 1998). Like rotavirus, mature orthoreovirus particles may be described as having three concentric capsid layers, although the radial boundaries between the capsid regions in orthoreovirus are not as readily apparent because of a greater degree of interdigitation among capsid proteins belonging to different layers. The innermost capsid layer consists of 120 molecules of λ1 arranged as dimers on a T=1 icosahedral lattice, much like the VP2 capsid layer of rotavirus. Unlike rotavirus, however, the inner capsid layer also contains 150 copies of a protein σ2, of which 120 associate in a 1:1 stoichiometry with λ1, and 30 are positioned at the icosahedral 2-fold axes (Reinisch et al., 2000). The intermediate capsid layer, roughly equivalent to the VP6 layer of rotavirus, is formed by 600 copies of μ1, which are organized as trimers on an incomplete T=13 (levo) icosahedral lattice. The icosahedral distribution of μ1 trimers is described as "incomplete" because the protein is excluded from the regions immediately surrounding the icosahedral vertices, where instead of μ1, a turret-shaped pentamer of λ2 extends from the viral core through the intermediate capsid layer. The outermost capsid layer, similar to the VP7 layer of rotavirus, is composed of 600 copies of σ3 assembled in a 1:1 stoichiometry onto the underlying μ1 protein. The mature orthoreovirus particle also contains trimers of a long flexible spike protein σ1, anchored at each of the icosahedral vertices and extending up to 400 Å away from the periphery of the particle.

Unlike rotavirus and orbivirus, in which the transcriptionally competent form of the virus consists of two complete capsid layers, the capsid structure in the transcriptionally competent orthoreovirion consists only of the inner λ1/σ2 capsid layer and the λ2 turrets at the icosahedral vertices (Dryden et al., 1993). The atomic structure of the orthoreovirus TCP has been determined recently (Reinisch et al., 2000) and, when compared with the atomic structure of bluetongue virus, will provide additional insight into the manner in which mRNA production occurs in dsRNA viruses. The TCP of aquareovirus is likely to have a similar structure with that of orthoreovirus (Nason et al., 2000). The capsid configuration of the mature cypovirus, which has only a single capsid layer and is transcriptionally competent, also shows many remarkable similarities with the TCP of orthoreovirus, including turret-like structures at the icosahedral vertices (Zhang et al., 1999; Hill et al., 1999).

B. Products of Transcription Reaction

At the start of the rotavirus life cycle, mature triple-layered particles attach to specific receptors on the cell surface and then enter the cell either by direct penetration of the plasma membrane or by receptor mediated endocytosis (reviewed in Estes, 1996). As a consequence of cell entry, the outermost capsid proteins VP4 and VP7 present in the mature particle are removed, and the resulting double-layered TCPs become transcriptionally active, releasing capped mRNA transcripts into the cytoplasm (Fig. 4.1). The production of viral mRNA *in vivo* begins shortly after cell entry and gradually increases as the life cycle progresses (Stacy-Phipps and Patton, 1987).

Purified rotavirus TCPs also readily synthesize mRNA *in vitro* when provided with the necessary precursors and proper conditions (Cohen, 1977; Mason *et al.*, 1980; Spencer and Arias, 1981; Flores *et al.*, 1982). Much of what is known about the characteristics of the endogenous transcription machinery and the behavior of actively transcribing particles has come from such *in vitro* studies. Similar studies have been carried out on orthoreoviruses (Shatkin and Sipe, 1968; Skehel and Joklik, 1969; Banerjee and Shatkin, 1970; Bartlett *et al.*, 1974), cypoviruses (Shimotohno and Miura, 1973; Yazaki and Miura, 1980), orbiviruses (Huismans and Verwoerd, 1973; Van Dijk and Huismans, 1980; Van Dijk and Huismans, 1988), and cystovirus φ6 (Rimon and Haselkorn, 1978; Partridge *et al.*, 1979; Gottlieb *et al.*, 1990; Ojala and Bamford, 1995), to name a few.

1. Mature mRNA Transcripts

Rotavirus transcripts have a standard eukaryotic cap sequence $^m7GpppG^m$ at the 5' end (Imai *et al.*, 1983; McCrae and McCorquodale, 1983) but lack a poly(A) tail at the 3' end. A similar methylated cap structure is also observed in orthoreovirus (Furuichi *et al.*, 1975), cypovirus (Furuichi and Miura, 1975), and orbivirus (Mertens and Sanger, 1985). All of the segments contain one functional open reading frame (ORF), and some segments contain alternative translation initiation sites that are also used. Both of the untranslated regions upstream and downstream of the ORF in each segment contain conserved sequences that are believed to function as important signals during genome assortment, replication, and packaging (Patton *et al.*, 1997). Transcription in rotaviruses, as in the other members of the *Reoviridae,* is conservative; all transcripts are synthesized *de novo* and represent precise end-to-end copies of the plus strands from the eleven genome segments (McCrae and McCorquodale, 1983). Interestingly, in

cystovirus φ6, transcription is semiconservative; as mRNA synthesis occurs, the plus strand from the dsRNA genome is displaced and translocated out of the particle to become the mRNA transcript (Emori et al., 1980; Usala et al., 1980; Van Etten et al., 1980).

In addition to directing the synthesis of viral proteins, the mRNA transcripts also serve as templates for minus strand synthesis during genome replication later in the viral life cycle (reviewed in Estes, 1996). In this way, genome transcription and genome replication may be thought of as complementary processes and in fact appear to use much of the same enzymatic machinery. This relationship between transcription and replication is being understood in greater detail from studies conducted using cystovirus φ6 (Gottlieb et al., 1990; Van Dijk et al., 1995; Qiao et al., 1997; Mindich, 1999), where a complete viral reconstitution system is available to allow recovery of infectious virions from mRNA transcripts or cDNA clones of the viral genome (Olkkonen et al., 1990). Although no reconstitution system is yet available for any of the reoviruses, studies conducted using baculovirus-expressed recombinant rotavirus-like particles containing the viral RNA polymerase coexpressed with the inner capsid protein have begun to clarify the mechanism by which the dsRNA genome is synthesized from the mRNA templates in rotavirus (Chen et al., 1994; Patton et al., 1996; Zeng et al., 1996).

2. Oligonucleotides

In addition to full-length mRNA transcripts, several members of the Reoviridae also produce significant quantities of short oligonucleotides, which correspond in sequence to the extreme 5' end of the mRNA transcripts and likely represent prematurely terminated transcription products. This phenomenon was first observed in the context of mammalian orthoreoviruses. Biochemical analysis of mature virions revealed that a substantial proportion of the nucleic acid present in the particle consisted of single-stranded oligonucleotides ranging in length from 3 to 20 bases (Bellamy et al., 1972). These short oligonucleotides were believed to be the result of reiterative transcription initiation events that occurred in vivo within maturing particles during the assembly of the outer capsid. Completion of the outer capsid layer was believed to have halted transcript elongation, and the aborted transcripts then remained trapped within the viral core. Under transcription conditions in vitro, mature orthoreovirus particles are observed to produce short oligonucleotides corresponding in sequence to the extreme 5' end of the genome segments, and some of these transcripts are properly capped (Yamakawa et al., 1982). Likewise,

orthoreovirus TCPs also produce a significant molar excess of short oligonucleotides in addition to full-length transcripts under transcribing conditions *in vitro* (Yamakawa *et al.,* 1981; Zarbl *et al.,* 1980). Short oligonucleotide transcription products have also been observed in cypovirus TCPs (Furuichi, 1981) and in both mature and transcriptionally competent rotavirus particles (Lawton *et al.,* 1999).

C. Components of Endogenous Transcription Apparatus

One of the more interesting observations about the life cycle of dsRNA viruses is that genome transcription occurs only within structurally intact TCPs. In rotavirus, the transcriptionally competent form of the virus has a double-layered capsid consisting of the structural proteins VP2 and VP6 surrounding the dsRNA genome segments and the enzymatic machinery in the core. Not only is the RNA-dependent RNA polymerase unable to synthesize RNA efficiently unless associated with the inner capsid protein VP2 (Patton *et al.,* 1997), but the intermediate VP6 capsid layer must also be present (Bican *et al.,* 1982; Kohli *et al.,* 1993; Sandino *et al.,* 1986) and the outermost VP7 capsid layer absent (Cohen *et al.,* 1979; Chen and Ramig, 1993; Lawton *et al.,* 1999). These observations have led to the proposal that the functional endogenous transcription apparatus contains three components: (1) the enzymes required for RNA synthesis and capping, (2) a scaffold consisting of the intact VP2 capsid layer functionally enhanced by the presence of VP6 on the exterior surface, and (3) the viral nucleic acid (Prasad *et al.,* 1996). The identities of the proteins involved in the function of the transcription apparatus in several of the major prototype reoviruses are summarized in Table 4.2.

TABLE 4.2
FUNCTIONS OF PROTEINS COMPRISING TRANSCRIPTIONALLY COMPETENT PARTICLE IN
SEVERAL MEMBERS OF REOVIRIDAE

Component	Rotavirus	Orthoreovirus	Bluetongue virus
RNA-dependent RNA polymerase	VP1	λ3	VP1
Guanylyltransferase (cap formation)	VP3	λ2	VP4
Methyltransferase (cap formation)	VP3	λ2	VP4
Helicase	?	λ1, μ2	VP6
Inner capsid (scaffold)	VP2	λ1, σ2	VP3
Outer capsid	VP6	μ1	VP7

1. RNA-Dependent RNA Polymerase

Because no cellular proteins are capable of transcribing or replicating a dsRNA template, all dsRNA viruses encode an RNA-dependent RNA polymerase as an essential component of the endogenous transcription apparatus. In rotavirus, several lines of evidence suggest that RNA synthesis is performed by VP1, a minor structural protein present in the viral core. The VP1 amino acid sequence contains the four major motifs commonly observed in RNA-dependent polymerases (Cohen et al., 1989; Poch et al., 1989; Bruenn, 1991; Suzuki et al., 1992), and some temperature-sensitive mutations mapping to the gene encoding VP1 render the virus unable to polymerize RNA (Chen et al., 1990). VP1 is specifically targeted by the nucleotide analog 8-azido-ATP (adenotn phosphate), which can ordinarily function as a substrate for RNA polymerization but becomes covalently cross-linked to VP1 upon ultraviolet irradiation, suggesting that VP1 is the protein that interacts with nucleotide substrates during RNA elongation (Valenzuela et al., 1991). The polymerase activity of VP1 in rotavirus has also been inferred from the observation that protein complexes formed from the coexpression of VP1 and the inner capsid protein VP2 are capable of using single-stranded mRNA as a template to generate dsRNA (Zeng et al., 1996; Patton et al., 1997). Although the polymerase activity of VP1 alone has yet to be demonstrated, the protein does bind specifically to the 3' end of viral transcripts in vitro and presumably in vivo, as would be expected of the viral polymerase (Patton, 1996). Structural and biochemical studies indicate that there are likely 12 copies of VP1 per virion (Liu et al., 1988; Prasad et al., 1996), probably existing as a heterodimeric complex with VP3, the other enzymatic component of the transcription apparatus (Prasad et al., 1996). RNA polymerase activity has been directly demonstrated in the homologous proteins VP1 of bluetongue virus (Urukawa et al., 1989), λ3 of mammalian orthoreovirus (Starnes and Joklik, 1993), and the polymerase enzyme of cypovirus (Dai et al., 1982).

2. Enzymes Involved in Cap Formation

The eukaryotic translation machinery requires the presence of a cap sequence at the 5' end of a mRNA transcript for efficient translation. For this reason, all dsRNA viruses infecting eukaryotic hosts contain the necessary enzymatic activities to synthesize a eukaryotic cap sequence on the end of each mRNA transcript before release. Rotavirus mRNA transcripts contain the cap sequence m^7GpppG^m at the 5' end (Imai et al., 1983; McCrae and McCorquodale, 1983). To produce such a

cap structure, four distinct enzyme activities are required to act on the penultimate pppG present in each transcript: (1) a nucleotide phosphohydrolase activity to remove the terminal orthophosphate to generate ppG; (2) a guanylytransferase activity to attach a guanine nucleotide to the 5' end to generate GpppG; (3) a methyltransferase activity to attach a methyl group to the N^7 position of the G cap to generate m^7GpppG; and (4) a second methyltransferase activity to generate m^7GpppG^m (Furuichi *et al.*, 1976). The guanylyltransferase requires GTP (Pizarro *et al.*, 1991a), and the two methyltransferase reactions use the amino acid derivative *S*-adenosylmethionine (SAM) as the methyl donor (Spencer and Garcia, 1984). In rotaviruses, the minor structural protein VP3, which is present in the viral core, possesses guanylyltransferase activity, suggesting that it is the enzyme responsible for catalyzing the second of these four steps in the capping reaction (Pizarro *et al.*, 1991a; Liu *et al.*, 1992; Patton and Chen, 1999). As expected, VP3 preferentially binds to single-stranded, uncapped RNA as a preferred substrate (Patton and Chen, 1999). VP3 is likely incorporated into the viral core as a heterodimer with VP1 (Prasad *et al.*, 1996). Not surprisingly, the capping reactions also appear to be quite similar in orthoreoviruses (Furuichi *et al.*, 1976), orbiviruses (Mertens and Sanger, 1985), and cypoviruses (Furuichi and Miura, 1975).

Indirect evidence from other viral systems suggests that the rotavirus VP3 protein may also be responsible for the phosphohydrolase and the two methyltransferase activities as well. In the orbivirus bluetongue virus, the four enzymatic activities required for cap formation all reside within the core structural protein VP4, homologous to rotavirus VP3 (Martinez-Costas *et al.*, 1998; Ramadevi *et al.*, 1998; Ramadevi and Roy, 1998). Furthermore, in orthoreovirus, the guanylyltransferase enzyme λ2 has been shown to contain a binding site for SAM (Koonin, 1993; Luongo *et al.*, 1998). This enzyme is also the target of the photoaffinity reagent 8-azido-*S*-adenosylmethionine (Seliger *et al.*, 1987), suggesting that both guanylyltransferase and methyltransferase activities needed for cap formation reside within a single protein in orthoreovirus as well. A nucleoside triphosphate phosphohydrolase activity potentially involved in cap formation has also been described in cypovirus, but the enzyme responsible for this activity has not been identified (Shimotohno and Miura, 1977). Whether the phosphohydrolase, guanylyltransferase, and methyltransferase enzyme activities all reside within a single protein in rotavirus remains to be determined. Nevertheless, it is likely that all of the cap formation activities are colocalized within the same protein in most, if not all, viruses in the family *Reoviridae*.

3. RNA Helicase

Because the nucleic acid template used during genome transcription is double-stranded, a helicase activity is likely required during transcription to unwind the RNA duplex ahead of the RNA polymerase. In the orbivirus bluetongue virus, the minor core protein VP6 uses ATP hydrolysis to unwind the dsRNA helix (Stäuber *et al.*, 1997). In orthoreovirus, helicase and nucleoside triphosphate phosphohydrolase activities have been detected in the λ1 protein, which, interestingly, is architecturally analogous to the VP2 capsid protein in rotaviruses (Bisaillon *et al.*, 1997; Noble and Nibert, 1997a), with the minor core protein μ2 also playing a possible role (Noble and Nibert, 1997b).

Although no protein with helicase activity has been identified in rotaviruses, VP3 exhibits several of the properties expected of an ATP-dependent helicase. While nucleotidyl transfer obviously requires a form of ATP which can be hydrolyzed to adenosine monophosphate (AMP) as directed by the template nucleotide sequence, elongation in rotavirus also has an absolute requirement for a form of ATP which can be hydrolyzed to adenosine diphosphate (ADP) (Spencer and Garcia, 1984). VP3, rather than the polymerase VP1, is believed to be the enzyme responsible for this (Pizarro *et al.*, 1991b). During the rotavirus life cycle, a helicase should be necessary only during transcription, as replication makes use of a single-stranded template to generate double-stranded RNA. As expected, the inhibition of ATPase activity in VP3 affects only transcription and has virtually no effect on the efficiency of reverse-strand synthesis during genome replication (Pizarro *et al.*, 1991b). A similar requirement for ATP hydrolysis during transcription but not replication has been observed in orthoreoviruses (Rankin *et al.*, 1989), although the protein responsible for this has not been directly identified.

4. Scaffolding

Numerous biochemical studies attest to the notion that genome transcription in segmented dsRNA viruses is a highly organized process involving the careful coordination of events in the viral core, and for this process to occur efficiently, the structural integrity of the particle is absolutely required. These observations suggest the existence of a scaffold on which the enzymatic and nucleic acid components of the transcription apparatus are assembled and organized during the transcription process.

In rotavirus, the inner capsid protein VP2 is proposed to have a dual role in defining the overall architecture of the virion, not only defining the icosahedral architecture of the capsid layers, but also acting as a scaffold for the proper assembly of the protein and nucleic acid compo-

nents within the viral core (Prasad *et al.*, 1996; Lawton *et al.*, 1997b). The inner capsid layer is formed from 60 dimers of quasiequivalent VP2 molecules organized on a T=1 icosahedral lattice (Fig. 4.2, see also color
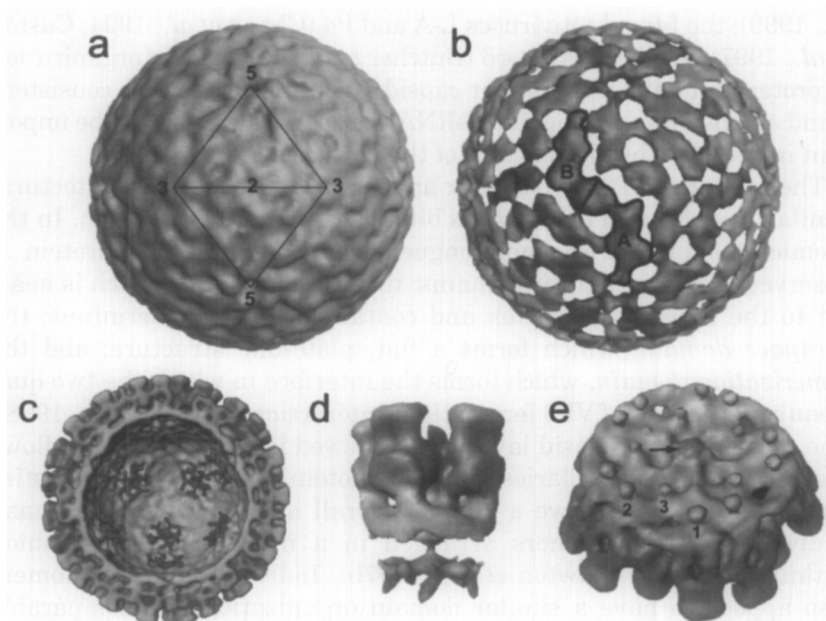

FIG 4.2. Role of VP2 as a scaffold for the internal components of the endogenous transcription apparatus. (a) When represented using a normal contour threshold (~0.4 σ), the VP2 capsid layer appears as a thin, relatively featureless closed shell, and the volume accounts for the expected 120 molecules of VP2. The boundaries between individual VP2 monomers are difficult to distinguish. Several of the icosahedral symmetry axes are indicated. (b) When represented at an elevated contour threshold (~2.3 σ), the surface appears to separate into 60 antiparallel strips of mass density, with each strip extending from one icosahedral vertex (5-fold axis) to a point near an adjacent vertex. One of these strips is outlined. Each strip appears to have an approximate center of symmetry and, as such, may be a dimer of quasiequivalent VP2 molecules. These two types of VP2 are designated as A (red) and B (purple). (c) The transcription enzymes VP1 and VP3 form a complex that is anchored to the inner surface of the VP2 capsid layer. In this cross-sectional illustration produced from a three-dimensional reconstruction of 1/3/2/6-VLP (Prasad *et al.*, 1996) and shown oriented along the icosahedral 3-fold axis, 9 of 12 complexes are shown, each anchored at an icosahedral vertex. (d) The VP1/3 complex appears as a flower-shaped structure (*orange*) attached to a small inward protrusion of VP2 at the 5-fold axis. (e) The inner surface of the VP2 capsid layer in the TCP serves as a scaffold for the enzymatic and nucleic acid components of the transcription apparatus. In this illustration, the capsid region surrounding the icosahedral vertex has been computationally isolated from the three-dimensional structure of the TCP to reveal the locations of three distinct classes of VP2-dsRNA contacts (numbered as 1, 2, and 3) and the anchor point for the VP1/3 enzyme complex (arrow). Illustrations in (a), (b), and (e) adapted from Lawton *et al.* (1997b). Used by permission. See also color insert.

insert). This sort of inner capsid architecture is also seen in other seg-
mented dsRNA viruses including the orbiviruses bluetongue (Grimes *et
al.*, 1998) and Broadhaven viruses (Schoehn *et al.*, 1997), orthoreovirus
(Dryden *et al.*, 1998), the phytoreovirus rice dwarf virus (Lu *et al.*, 1998),
aquareovirus (Shaw *et al.*, 1996), cypovirus (Hill *et al.*, 1999; Zhang *et
al.*, 1999), the fungal totiviruses L-A and P4 (Cheng *et al.*, 1994; Castón
et al., 1997), and cystovirus φ6 (Butcher *et al.*, 1997). This organization
of protein subunits in the inner capsid layer appears to be a consistent
trend among many segmented dsRNA viruses and indeed may be impor-
tant in defining the architecture of the viral core.

The rotavirus VP2 capsid layer appears to share many architectural
similarities with the homologous bluetongue virus VP3 protein. In the
atomic structure of the bluetongue virus TCP, the VP3 protein is
observed to fold into three domains: the *apical domain*, which is near-
est to the icosahedral vertex and contains the amino terminus; the
carapace domain, which forms a flat, plate-like structure; and the
dimerization domain, which forms the interface in which the two qua-
siequivalent types of VP3 form a tight interaction (Grimes *et al.*, 1998).
The rotavirus VP2 capsid layer, when viewed in a manner that allows
the approximate boundaries between protein monomers to be distin-
guished, appears to have a similar overall architecture, with quasi-
equivalent VP2 monomers arranged in a nearly identical fashion
within the capsid (Lawton *et al.*, 1997b). Individual VP2 monomers
also appear to have a similar domain organization and comparable
overall dimensions, suggesting that the rotavirus VP2 polypeptide
probably folds into a structure closely resembling that of the blue-
tongue virus VP3 protein.

The asymmetric unit of the rotavirus VP2 capsid layer consists of
two quasiequivalent VP2 monomers, designated as type A and type B
(Fig. 4.2b). From the three-dimensional structure, the interaction
between these two molecules appears to be the strongest at the dimer-
ization domain interface, where the two molecules interact in an end-
to-end arrangement, and comparatively weaker along the sides,
suggesting that the naturally occurring assembly subunit may consist
of a pair of VP2 molecules interacting end-to-end via the dimerization
domain. Further evidence for this notion comes from electron micro-
scopic examination of various forms of purified recombinant VP2 (Zeng
et al., 1994). At low protein concentrations, the recombinant VP2 pro-
tein self-assembles into empty icosahedral capsids closely resembling
the VP2 capsid layer observed in the native virus. However, at high
protein concentrations, the capsids undergo spontaneous conversion
into long helical structures (see Zeng *et al.*, 1994). The width of these

helical structures and the morphology of the subunits suggest that the helices likely consist of dimers of VP2 arranged in a braided configuration. Interestingly, at intermediate concentrations, helical structures can be seen extending away from clusters of partially disassembled particles, and the helical structures formed at high concentration are known to revert back to icosahedral capsids when the protein concentration is reduced. It is possible that the helical structure is favored when both VP2 molecules in the dimer exist in an identical conformation, whereas the icosahedral capsid is favored when the two molecules are constrained to adopt slightly different, quasiequivalent conformations.

The inner surface of VP2 forms the structural platform on which the transcription enzymes are assembled in the viral core. In the three-dimensional structure, the two enzymes VP1 and VP3 are visualized as a heterodimeric complex anchored to a small inward protrusion of VP2 at each of the twelve icosahedral 5-fold axes (Prasad et al., 1996) (Fig. 4.2c, 4.2d). The region within the VP2 amino acid sequence that forms the binding site for the VP1/VP3 enzyme complex may be near the amino terminus (Zeng et al., 1998; Lawton et al., 1997b). The binding site appears to be formed at the point where the five symmetrically related VP2 molecules surrounding the icosahedral vertex (type A monomers) interact with one another (Lawton et al., 1997b). The interaction between the VP2 capsid and the VP1/VP3 enzyme complexes is probably critical for the incorporation of these enzymes into the core during particle morphogenesis (Zeng et al., 1996), and the interaction with VP2 is apparently also required for the enzymatic activity of VP1 (Patton et al., 1997). A similar arrangement of transcription enzymes at the icosahedral vertices is also seen in orthoreovirus (Dryden et al., 1998), orbiviruses (Schoehn et al., 1997; Grimes et al., 1998), cypovirus (Hill et al., 1999; Zhang et al., 1999), and cystovirus φ6 (Butcher et al., 1997).

In orthoreovirus, the λ2 enzyme involved in cap formation is not housed entirely within the viral core as a complex with the polymerase (Dryden et al., 1993; Dryden et al., 1998), as is the case for rotavirus and orbivirus. Rather, the capping enzymes exists as pentamers, forming large turret-like projections anchored within the inner capsid layer and extending outward into the outer capsid region at each icosahedral vertex. Such pentameric turret-like projections are also seen in cypovirus (Hill et al., 1999; Zhang et al., 1999) and aquareovirus (Shaw et al., 1996), although the identity of this feature as the capping enzyme remains to be confirmed in these other viral systems. The presence or absence of a turret-like projection at the icosahedral vertex

represents an important architectural distinction among members of the *Reoviridae,* with some members more closely resembling orthoreovirus and others more closely resembling rotavirus in regard to the position of the capping enzyme (Hill *et al.,* 1999).

The inner capsid protein scaffold serves not only to anchor the transcription enzymes but also to provide organization to the genome within the core. In rotavirus, the inner surface of VP2 forms numerous contacts with the dsRNA genome, especially within the vicinity of the icosahedral vertices (Lawton *et al.,* 1997b) (Fig. 4.2e). Whereas the transcription enzymes appear to interact with only one of the two types of quasiequivalent VP2 molecules, both types of VP2 appear to be involved in forming contacts with the genome. The RNA-binding region of VP2 has been localized to the amino terminus (Labbé *et al.,* 1994), although the affinity for dsRNA is considerably weaker than for ssRNA (Boyle and Holmes, 1986; Patton *et al.,* 1997). The large number of protein-nucleic acid contacts seen in the viral core are believed to serve two purposes. First, because of the stiffness of dsRNA, these interactions are likely necessary to induce the bending and folding required for proper genome packaging (Kapahnke *et al.,* 1986). Also, the extensive order imposed on the dsRNA genome through its numerous contacts with VP2 and the anchored VP1/VP3 complex may be critical for coordinating the efficient movement of the dsRNA template through the active site of the transcription enzyme complex (Prasad *et al.,* 1996). Extensive protein–dsRNA contacts are also seen in other segmented dsRNA viruses studied using structural techniques, including bluetongue virus (Gouet *et al.,* 1999), cypovirus (Zhang *et al.,* 1999), and cystovirus φ6 (Bamford *et al.,* 1993).

5. Organization of Viral Genome

The genomes of dsRNA viruses differ considerably in organization and complexity. One of the simplest is the totivirus L-A virus, which has a single genome segment encoding the major capsid protein and, through ribosomal frameshifting, the polymerase enzyme (reviewed in Wickner, 1996). Birnaviruses contain two genome segments, one that encodes an autoproteolytically cleaved polyprotein consisting of several structural proteins, and the other that encodes the RNA polymerase (Kibenge *et al.,* 1988). Cystovirus φ6 virions package three polycistronic segments (reviewed in Mindich, 1999). The reoviruses are the most complex dsRNA viruses, having between 10 and 12 unique segments, all of which are generally monocistronic.

The 11 SA11 rotavirus genome segments are 667 to 3302 nucleotides (nt) long, with a total length of 18,525 nt for the entire genome

(reviewed in Estes, 1996). Each genome segment is likely associated with its own polymerase enzyme in the viral core. This idea is a logical extension of the observation that the core of the virion contains 12 polymerase enzyme complexes and 11 genome segments (Prasad *et al.*, 1996) and is also supported by the observation that no dsRNA virus containing more than 12 segments has ever been isolated (Payne and Mertens, 1983). Kinetic studies of transcription in cypovirus clearly suggest that each genome segment is transcribed by an independently functioning transcription enzyme complex, as transcripts corresponding to all 10 segments are generated simultaneously, are synthesized at the same rate, and accumulate in molar quantities that are inversely proportional to their length (Smith and Furuichi, 1982). Likewise, orthoreovirus TCPs also appear to be capable of simultaneous transcription of all 10 genome segments in molar quantities that are inversely proportional to segment length (Skehel and Joklik, 1969; Banerjee and Shatkin, 1970; Gillies *et al.*, 1971; Bartlett *et al.*, 1974). The association of one transcription enzyme complex with one genome segment may arise initially during particle assembly and then remain throughout the lifetime of the virion. Viral mRNA is packaged and replicated to become dsRNA during particle assembly, and it is conceivable that the polymerase responsible for replicating and packaging a particular genome segment may also be the polymerase responsible for transcribing that same segment during a subsequent round of infection.

It is not known how the individual segments of dsRNA are organized within the particle, although several structural studies have provided helpful insights. In the three-dimensional structure of rotavirus at 19 Å resolution, structurally discernible portions of the genome appear as concentric shells having decreasing order toward the center of the viral core (Prasad *et al.*, 1996) (Fig. 4.3a, see also color insert). In the outermost shell, which accounts for approximately 25% of the genome, individual helices of dsRNA are clearly visible as tubes of mass positioned along the inner surface of the VP2 capsid and encircling the transcription enzyme complexes (Fig. 4.3b). Low angle X-ray scattering studies of mammalian orthoreovirus particles paint a similar picture, indicating that the dsRNA genome is tightly packed as parallel helices in a semicrystalline array (Harvey *et al.*, 1981). Regardless of the manner in which the nucleic acid is organized within the rotavirus core, the genome appears to fill nearly all of the space not occupied by protein, although biophysical studies of particles having rearranged genomes suggest that the viral interior is nonetheless able to accommodate up to 10% more dsRNA than is found in the standard rotavirus
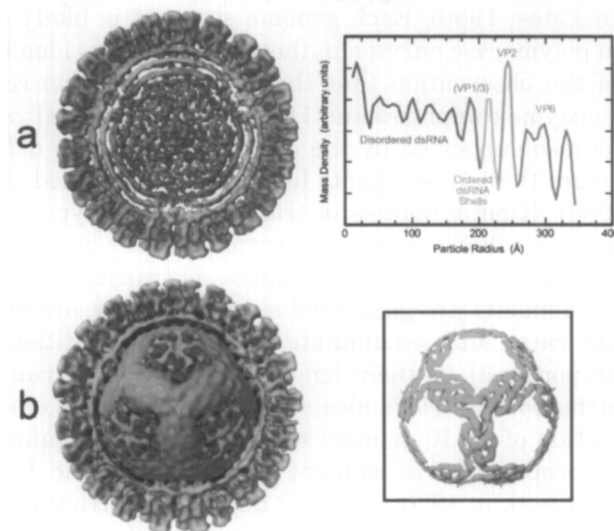
FIG 4.3. Internal organization of the rotavirus core. The enzymatic and nucleic acid components of the transcription apparatus are housed within the core of the virion. (a) A cross section of the viral core, produced from a three-dimensional reconstruction of the rotavirus TCP at 19 Å resolution, illustrates how structurally discernible portions of the dsRNA genome appear as concentric shells (yellow and orange). These shells have the greatest degree of order near the inner surface of the viral capsid and progressively less order toward the viral center (maroon). (Inset) An averaged radial density profile, computed from the three-dimensional reconstruction of the TCP, further illustrates the concentric organization of the two capsid layers and the genomic dsRNA. The color scheme matches that used in the three-dimensional structures. The VP6 capsid layer extends between the radii 260 and 350 Å, VP2 between 230 and 260 Å, and two shells of icosahedrally ordered dsRNA between ~160 and 230 Å. The structurally discernible portions of the VP1/3 transcription enzyme complexes are embedded within these two ordered dsRNA layers (Prasad *et al.*, 1996). (b) the VP6 (blue) and VP2 (green) capsid layers have been computationally removed, revealing the organization of the viral interior. A significant portion (approximately 25%) of the genomic dsRNA forms a well-organized layer (yellow) immediately inside the capsid. The surface of this RNA shell has been made slightly transparent to reveal the outlines of the RNA double helices, which have a higher density than protein. The dsRNA makes extensive contact with the inner surface of the VP2 capsid layer near the icosahedral vertices. (Inset) When displayed using a higher density threshold appropriate for nucleic acid, the dsRNA shell immediately inside the VP2 capsid layer is resolved into parallel tubes of mass, which likely represent dsRNA helices. Illustrations adapted from Prasad *et al.* (1996). Used by permission. See also color insert.

genome without a corresponding change in the volume of the core (Hundley *et al.*, 1987).

Based on the observation that, in rotavirus, 11 segments of dsRNA are packaged within a particle containing 12 transcription enzyme complexes, each segment may be arranged in association with its own

polymerase at an icosahedral vertex (Prasad *et al.*, 1996). Figure 4.4 illustrates the manner in which the genome segments organized in this way may be spatially distributed within the viral core. In this model, each genome segment occupies a defined region of the viral interior in the vicinity of the polymerase with which it is associated. The packaging of the linear genome segments within the viral core requires the nucleic acid to assume a collapsed configuration. Linear duplex DNA is known to collapse into a pear-shaped toroidal confor- mation under conditions of high ionic strength on the introduction of homonemic supercoils (Eickbush and Moudrianakis, 1978), and it is conceivable that the same may be true of the linear dsRNA segments in rotavirus. Aligning the 11 collapsed genome segments with one another in a manner such that the pointed ends of the pear-shaped lobes are all oriented toward the center of the virus allows the entire genome to be packaged with maximal efficiently inside the icosahedral core, as suggested from theoretical modeling studies of DNA incorpo- ration within the icosahedral head of the bacteriophage lambda (Witkiewicz and Schweiger, 1985).

The precise manner in which the genome segments are organized to facilitate repeated cycles of transcription remains one of the more puz- zling questions about the architecture of dsRNA viruses. The linear genome segments must be packaged in a manner that allows for reini- tiation at the 3' end of the minus strand once the 5' end has been reached at the end of one transcription cycle. The mechanism of reini-
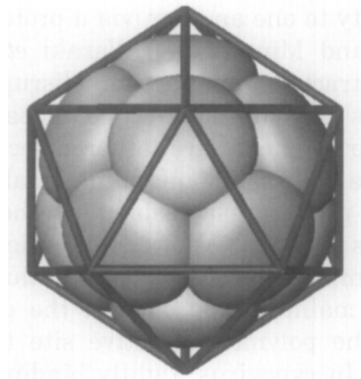

FIG 4.4. Model for the arrangement of genome segments within the rotavirus core. The packaging of the rotavirus dsRNA genome within the icosahedral core may involve the collapse of the 11 segments into pear-shaped toroidal lobes packed with the pointed ends oriented toward the center (Witkiewicz and Schweiger, 1985). Such a configuration could result from the introduction of homonemic supercoiling (Eickbush and Moudri- anakis, 1978). The 11 dsRNA genome segments may be organized such that each seg- ment is associated with its own polymerase complex at one of the icosahedral vertices.

tiation would be simple to envision if the ends of the linear genome segments were somehow tethered together throughout most of the transcription cycle, effectively "circularizing" the genome segment (Banerjee and Shatkin, 1970; Yazaki *et al.*, 1986). On reaching the 5' end of the minus strand, the 3' end would then be positioned close to the polymerase active site for easy reinitiation. Although the genome segments are clearly not covalently linked into closed circular configurations, they could conceivably be tethered through limited nucleic acid base-pairing in *cis* or via a protein interacting simultaneously with both ends. The genomes of some dsDNA viruses are known to circularize via nucleic acid base-pairing, as occurs, for example, in the bacteriophage lambda (reviewed in Campbell, 1996). Furthermore, the dsRNA replicative intermediate forms of several picornaviruses are known to be capable of forming circular molecules (Romanova and Agol, 1979; Robberson *et al.*, 1982). In all of these viral systems, circular forms of the genome arise through base pair formation involving single-stranded regions at one or both ends of the linear molecule. The genome segments of rotavirus, however, like those of other reoviruses, have blunt ends and limited sequence complementarity between opposite ends (Imai *et al.*, 1983), making it unlikely that the genome segments could circularize simply via the formation of nucleic acid base pairs between the ends of the molecules.

There is good evidence from electron microscopy studies of cypovirus that the ends of the segments are tethered within the viral core as "open-ended circles," (i.e., both ends of the linear genome segments are held in close proximity to one another, via a protein component of the viral core) (Yazaki and Miura, 1980; Yazaki *et al.*, 1986). Indeed, genome segments extracted from partially disrupted cypovirus TCPs under mild conditions take on a supercoiled appearance, implying that the ends of the linear segments are tethered securely enough within the core to maintain a certain amount of torsional strain in the double helix. The structural protein responsible for tethering the ends of the genome segments has not been positively identified; however, the transcription enzyme complex itself is a logical candidate, as such an arrangement would maintain the ends of the genome segments in close proximity to the polymerase active site throughout repeated transcription cycles. In cypovirus, tightly binding protein complexes can be copurified along with the genome segments under certain conditions, and these complexes possess RNA polymerase and methyltransferase activities, strongly suggesting that they are the enzymatic components of the endogenous transcription apparatus (Dai *et al.*, 1982; Yazaki *et al.*, 1986).

Given the possibility that the ends of the linear genome segments may be tethered to the transcription enzyme complexes throughout most of the transcription cycle, it would be interesting to know how the endogenous transcription apparatus handles the accumulation of topological strain in the dsRNA template during transcript elongation. Topological strain arises as the nucleic acid template moves through the anchored transcription enzyme complex. As the helical axis is forced to rotate, the polymerase generates positive supercoils ahead of the transcription bubble and leaves negative supercoils in its wake (Liu and Wang, 1987). If the template nucleic acid were to be in the form of a closed circle, the positive and negative supercoils would cancel each other, and the topological strain introduced during transcription would be only transient. In reoviruses, however, where the genome segments may be tethered to the transcription enzyme complex in an "open circle" configuration, the ends would presumably have to be bound in a manner that allows some freedom for them to rotate during transcript elongation to allow dissipation of topological strain. Atomic resolution structural studies of the transcription enzymes may shed light on the manner in which the ends of the genome segments are tethered within the viral core.

D. Conditions Required for Efficient mRNA Production

The conditions required for optimum mRNA production in several viral systems have been worked out mainly from *in vitro* studies of rotavirus (Spencer and Arias, 1981), bluetongue virus (Van Dijk and Huismans, 1980), orthoreovirus (Shatkin and Sipe, 1968; Skehel and Joklik, 1969; Banerjee and Shatkin, 1970), cypovirus (Shimotohno and Miura, 1973), and cystovirus φ6 (Rimon and Haselkorn, 1978; Partridge *et al.*, 1979), to name a few. Although the precise concentrations of the mRNA precursors and the exact conditions needed for optimum synthesis rates vary from one virus to another, the general requirements are similar. These studies have also shed additional light on the common mechanisms of mRNA production in segmented dsRNA viruses.

1. mRNA Precursors

All viral *in vitro* transcription systems in the family *Reoviridae* uniformly require the four nucleoside triphosphates (ATP, GTP, CTP, UTP) for the synthesis of RNA, and SAM is required for methylation of the cap at the 5' end of the transcripts. In rotavirus, some nucleotide analogs are able to substitute for the NTPs (Pizarro *et al.*, 1991b;

Valenzuela *et al.*, 1991), but a form of ATP capable of being hydrolyzed to ADP must be included (Spencer and Garcia, 1984), presumably for helicase function. SAM is not explicitly required for RNA synthesis *in vitro*, but its presence enhances the rate of transcription in rotavirus (Spencer and Garcia, 1984), orbivirus (Van Dijk and Huismans, 1980), and cypovirus (Furuichi, 1981; Mertens and Payne, 1983), although apparently not in orthoreovirus (Shatkin, 1974). SAM may increase the rate of RNA synthesis by introducing a conformational change in the transcription enzyme complex, increasing the frequency of initiation (Wertheimer *et al.*, 1980). As is generally the case with polymerases, Mg^{2+} is also required to facilitate nucleotide binding in the enzyme active site. When Mn^{2+} is supplied instead of Mg^{2+}, the transcription enzymes retain some enzymatic activity (Cohen, 1977; Spencer and Arias, 1981; Skehel and Joklik, 1969; Yamakawa *et al.*, 1982; Shimotohno and Miura, 1973; Van Dijk and Huismans, 1980) but lose the ability to discriminate properly between various NTPs (Gorziglia and Esparza, 1981; Pizarro *et al.*, 1991a; Pelletier *et al.*, 1996).

2. Temperature

Like many other enzymatic processes, transcription in segmented dsRNA viruses is critically dependent on the temperature of the environment. Biochemical studies investigating the relationship between temperature and the efficiency of mRNA production in calf rotavirus found that transcription is generally inhibited below 20°C or above 60°C and that mRNA production is most efficient around 50°C (Cohen, 1977). A more comprehensive biochemical study conducted on mammalian orthoreovirus observed a similar phenomenon (Kapuler, 1970). The production of mRNA in orthoreovirus TCPs could not be detected below ~30°C and, after reaching a maximum around 50°C, declined sharply above ~55°C. Furthermore, the effect of temperature on the efficiency of transcription was completely reversible. Particles preincubated at the optimum temperature and then shifted to a lower temperature were not able to transcribe any more efficiently than particles that had been maintained at the lower temperature throughout the duration of the experiment. This observation suggests that temperature does not induce a one-time "transcriptional activation" of some sort but rather represents an environmental factor continuously required for transcriptional efficiency. Temperature may thus be acting to hold the components of the endogenous transcription apparatus in a transcriptionally active conformation (Kapuler, 1970).

Like orthoreovirus, the rate of mRNA synthesis in rotavirus is affected immediately on changes in temperature, (Fig. 4.5). Three important observations follow from this result. First, the effect of temperature on the efficiency of genome transcription in rotavirus appears to be completely reversible, as has been previously observed for mammalian orthoreovirus (Kapuler, 1970). Second, changes in temperature appear to have an immediate and dramatic effect on the efficiency of the transcription process. This effect could be observed within the first minute after increasing or decreasing the temperature of the transcription reaction. Third, the rate of transcription at a particular temperature appears to be the same regardless of whether the virus had previously transcribed at a different temperature. A period of rapid transcription at 50°C did not somehow predispose the virus to be able to transcribe more efficiently than expected at 30°C. Likewise, a period of slow transcription at 30°C did not somehow cause the virus to transcribe less efficiently than expected at 50°C.

Whatever the effect of temperature on the structure of the particle, temperature changes likely bring about immediate conformational changes in the particle. Above and below the allowed temperature
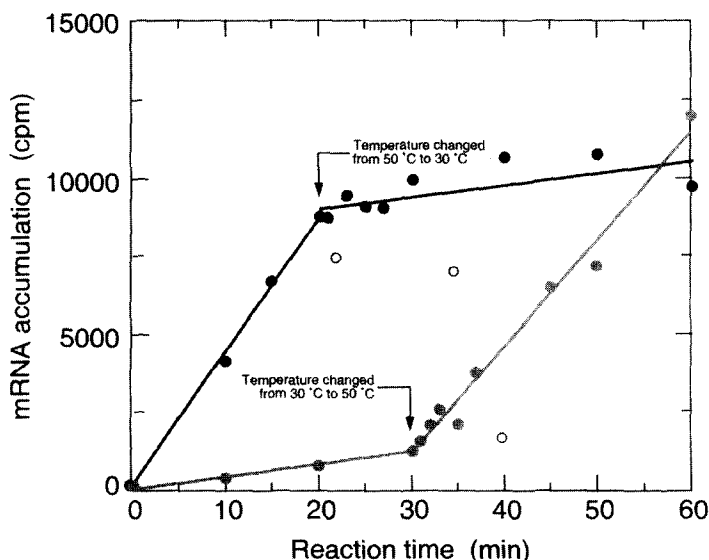

FIG 4.5. Effect of temperature changes on the rate of transcription in rotavirus. One reaction was incubated first at 30 °C and then switched to 50 °C (lower), whereas the other reaction was incubated first at 50 °C and then switched to 30 °C (upper). The accumulation of mRNA during each reaction segment was fitted to a straight line using linear regression, omitting the points indicated with a hollow circle.

range, such structural changes may render the particle transcription-
ally incompetent. These changes may be quite subtle, perhaps only
affecting the conformation of critical amino acids within VP1 or VP3,
rendering the proteins enzymatically inactive, or the structural
changes may occur on a larger scale, affecting the movement of the
dsRNA template or preventing the efficient release of nascent mRNA
transcripts through the channels in the capsid.

3. pH

Like temperature, proper pH is also an important factor required for
the efficiency of the transcription process in rotavirus. Biochemical
studies conducted with calf and human rotaviruses have shown that
transcription occurs most efficiently between pH 8.0 and pH 8.5 and is
essentially inhibited below pH 6.0 and above pH 10.0 (Cohen, 1977;
Spencer and Arias, 1981). Similar results have been obtained in stud-
ies of orthoreovirus (Shatkin and Sipe, 1968) and cypovirus (Shimo-
tohno and Miura, 1973). The transcriptional behavior of simian
rotavirus SA11 as a function of pH is shown in Fig. 4.6. Transcription
appears to be most efficient around pH 8.5. Below pH 5.3 and above
pH 10.4, transcription is almost completely inhibited. These results
are consistent with those previously observed for calf and human
rotaviruses. Within a moderate pH range (5 to 10), the effect of pH on
transcriptional efficiency is completely reversible (Fig. 4.7). These
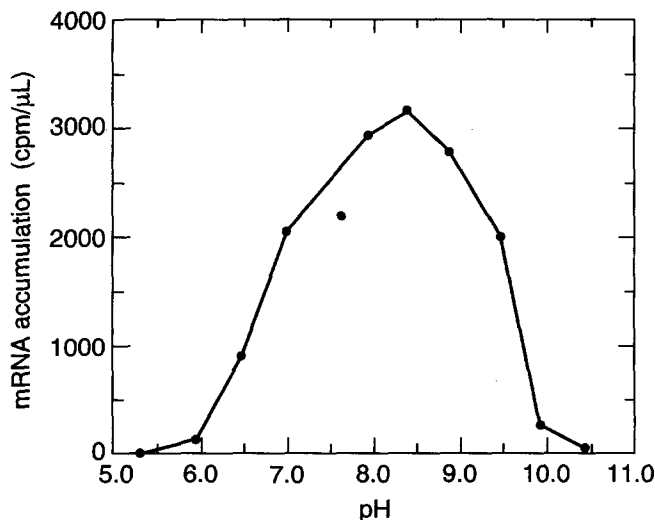

FIG 4.6. Effect of pH on the rate of transcription in rotavirus. Each data point depicts
the accumulation of mRNA after 30 minutes of *in vitro* transcription at the pH indicated.
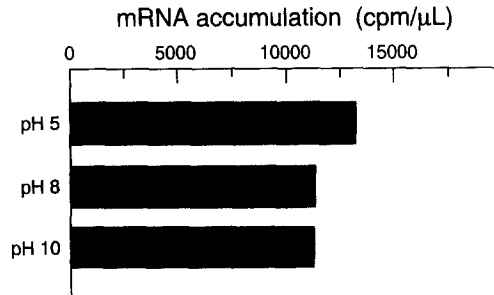
mRNA accumulation (cpm/μL)

FIG 4.7. Recovery of transcriptional activity in rotavirus after exposure to low and high pH conditions. Rotavirus TCPs were incubated in the indicated buffers for 4 hours and then transferred to a standard *in vitro* transcription reaction at pH 8.0. mRNA accumulation represents the yield of mRNA after 30 minutes of incubation at pH 8.0.

results indicate that exposure to such pH conditions does not cause irreversible damage to the endogenous transcription apparatus.

Although exposure to moderate pH conditions does not cause irreversible structural changes, the integrity of the genome is known to be disrupted above pH 11 because of the introduction of singe-strand breaks in the dsRNA segments due to base-catalyzed hydrolysis of the phosphate backbone (Brown and Todd, 1952). After exposure to pH 11 for ~30 minutes, the genome is degraded into fragments 200 to 500 base pairs long (Yeager *et al.*, 1994). Damage of this sort to the nucleic acid would be expected to cause irreversible transcriptional inhibition.

Because pH is known to have an effect on the structure and biological activity of many proteins, the observed effect of pH on transcriptional efficiency is not surprising. Although the structural integrity of the rotavirus TCP is not compromised over a moderate pH range (between 5 and 10), pH may nevertheless affect the structure of the protein or the nucleic acid components of the endogenous transcription apparatus in a way that renders the particle unable to transcribe when the pH is too low or too high. Alternatively, improper pH may disrupt interactions that are important for the function of the endogenous transcription apparatus, perhaps preventing the polymerase from binding correctly to the dsRNA template or preventing the transcription precursors from binding to the active site of the polymerase. Changes in pH may also affect the protonation state of key amino acids in the transcription enzymes, thereby interfering with the binding of substrates, or changes in the protonation state of the mRNA precursors may affect their suitability as substrates for mRNA synthesis.

III. Mechanism of mRNA Production

Numerous structural and biochemical studies attest to the notion that genome transcription in segmented dsRNA viruses is a highly organized process involving the careful coordination of events in the viral core. The endogenous transcription enzymes, along with the genomic dsRNA segments and the capsid layers, function together as a complex macromolecular machine to produce mRNA transcripts. The key to understanding the mechanism of genome transcription in these viruses is to delineate the way in which the components of this machine work together to facilitate the production of mRNA.

A. Transcriptional Events in Viral Cores

The process of viral mRNA synthesis may be considered to involve three distinct events: (1) *initiation,* in which the polymerase complex and the nucleotides at the 3′ end of the minus strand interact in a manner that allows nucleotidyl transfer and capping to occur; (2) *elongation,* in which nucleotidyl transfer progresses as the polymerase complex moves along the helical axis relative to the dsRNA template; and (3) *translocation,* in which the growing mRNA transcript is transported across the intact capsid through specific channels to exit the particle. Initiation and elongation primarily involve enzymatic components of the transcription apparatus and occur in the viral core, whereas translocation primarily involves the capsid region of the virus.

1. Transcript Initiation

During transcription initiation, the end of double-stranded nucleic acid template must be partially unwound, presumably by the action of the viral helicase, to allow the active site of the polymerase access to the nucleotide base-pairing face of the minus strand. This is then followed by limited nucleotidyl transfer and capping of the initiated transcript. Although few details are known about the way in which transcript initiation and elongation occur in rotavirus, biochemical studies in orthoreoviruses and cypoviruses have provided important insights that may have relevance for all members of the *Reoviridae.* In both of these viral systems, the progression from transcript initiation to sustained elongation seems to be the overall rate-limiting step during genome transcription. Under transcribing conditions, particles generate not only full-length transcripts but also an enormous molar excess of short oligonucleotides corresponding in sequence to the extreme 5′ end of the plus strands (Bellamy *et al.,* 1972; Zarbl, *et al.,*

1980; Yamakawa *et al.,* 1981; Furuichi, 1981). Whereas some of these oligonucleotides are properly capped, others contain an incomplete cap structure, and most are uncapped. These oligonucleotides are believed to be aborted transcripts that arose when the transcription apparatus either failed to complete the initiation phase correctly or to progress properly into the elongation phase.

2. Transcript Elongation

To progress into the elongation stage, the growing transcript must be properly positioned to exit the core, the helicase must be properly engaged, and the nucleic acid template must have the necessary freedom to move relative to the polymerase. Given that structural studies of these viruses have indicated the polymerase complex to be an integral part of the capsid while the genome is relatively less constrained, elongation clearly must involve the nucleic acid template moving through the active site of an anchored polymerase rather than the polymerase moving along the nucleic acid (Prasad *et al.,* 1996; Dryden *et al.,* 1998), as has been suggested from earlier studies of genome transcription (Yazaki and Miura, 1980; Shatkin and Kozak, 1983). The notion of a "moving template" is consistent with the emerging paradigm for eukaryotic nuclear gene transcription, in which the RNA transcription complex is fixed to the nuclear matrix and the DNA template slides through the enzyme active site (Iborra *et al.,* 1996).

The rate of polymerization has been measured to be ~6 nucleotides per second in cypoviruses (Smith and Furuichi, 1982) and as high as 60 nucleotides per second in orthoreoviruses, depending on the conditions (Banerjee and Shatkin, 1970). It is not clear to what extent the rate of synthesis is specifically regulated with regard to individual segments; for cypoviruses, all segments are transcribed at the same rate *in vitro* (Smith and Furuichi, 1982), but for rotaviruses and orbiviruses, some studies suggest that elongation may occur more quickly for some segments than for others (Huismans and Verwoerd, 1973; Bernstein and Hruska, 1981; Van Dijk and Huismans, 1988). The transcription apparatus nonetheless is clearly able to complete multiple initiation–elongation cycles, as has been observed for orthoreoviruses (Skehel and Joklik, 1969), cypoviruses (Smith and Furuichi, 1982), and rotaviruses (Cohen, 1977).

B. Pathway of mRNA Release

One of the most striking observations about transcription in viruses with segmented dsRNA genomes is that efficient mRNA production

only occurs within the context of the fully intact particle. The structural integrity of the capsid serves several important functions. First, the enzymatic and nucleic acid components of the endogenous transcription apparatus are held in their proper arrangement throughout repeated cycles of initiation and elongation. Additionally, by sequestering the dsRNA genome within the intact capsid, the virion is able to minimize the likelihood that the cellular interferon response system will become active, as viral dsRNA is a potent inducer of the interferon response and subsequent antiviral activities (reviewed in Samuel, 1998). However, this arrangement presents a considerable engineering problem for the transcription apparatus, as the newly synthesized mRNA transcripts must be efficiently translocated across the intact capsid with minimal disruption of the particle.

Much of what is known about the way in which transcription occurs within segmented dsRNA viruses has come from biochemical and electron microscopy studies of actively transcribing virions. Early observation of actively transcribing orthoreovirus particles suggested that all 10 genome segments can be transcribed simultaneously and that the transcripts are released through turret-like projections at the icosahedral vertices (Bartlett *et al.*, 1974; Gillies *et al.*, 1971). Later biochemical studies conducted on actively transcribing cypoviruses confirmed that genome transcription involves the simultaneous synthesis of a complete set of mRNA transcripts from all of the dsRNA segments (Smith and Furuichi, 1982), and observation of actively transcribing cypovirus particles suggested that, like orthoreoviruses, mRNA transcripts likely exit through turret-like projections at the icosahedral vertices (Yazaki and Miura, 1980).

Although these early studies of transcriptionally active particles provided valuable insights into the transcriptional behavior of segmented dsRNA viruses, several limitations precluded a more complete understanding of the mechanism of mRNA translocation through the intact capsid. First, nascent mRNA was visualized using the Kleinschmidt specimen preparation technique (Freifelder and Kleinschmidt, 1965) in conjunction with conventional electron microscopy. Such specimen preparation techniques, although useful for visualizing the overall morphology of biological macromolecules, may make a high-resolution analysis of the structure difficult, as the use of stains, fixatives, and desiccation during specimen preparation can introduce structural artifacts (Cohen *et al.*, 1984). In this way, the conditions under which specimens were prepared and images collected precluded a three-dimensional visualization of the pathway of mRNA translocation through the capsid. Second, although observa-

Rb binding
106-114
Origin
DNA binding
131-259

Zinc finger
302-320

ATP binding
418-528

PO₄

J domain
1-82

NLS

ATPase
418-616

PO₄

1 ——————————————————————————— 708

82   246   259   708   Topo I binding

strong site   weak site

195 ——————— 313   DNA pol binding

351 ———▬▬— 450   529 ——————— 651   p53 binding/p300?

259 ——————————————————— 627   SS DNA binding

131 ——————————————————————— 522   Nonspecific DNA binding

ori-binding   region 2

131 ——————————————————————— 616   Helicase

164 ———— 249   RPA binding

1 ——————————— 250   Transcriptional activation

1 ———————— 147   Chromosome rearrangements

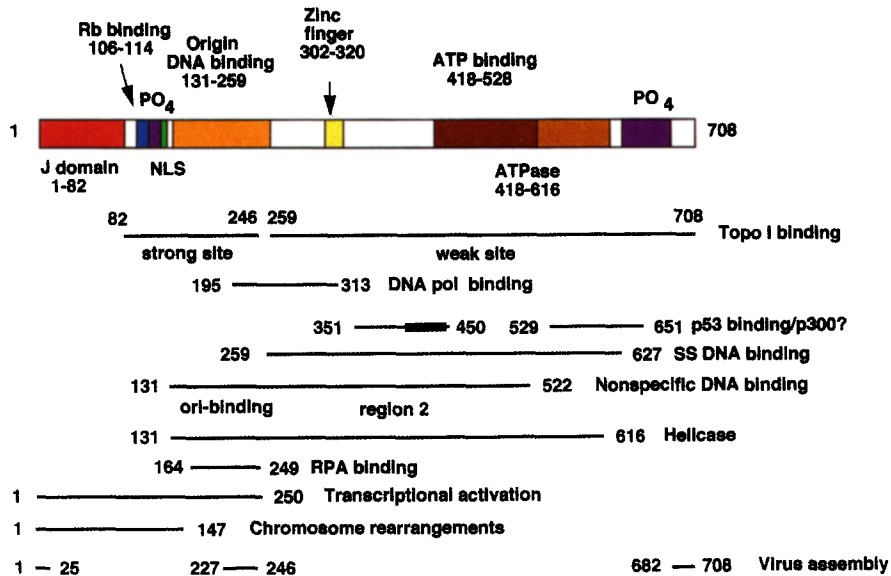1 — 25   227 —— 246   682 — 708   Virus assembly

FIG 2.1. Map of structural and functional domains in SV40 T antigen. The major domains or structurally important regions are shown in color. Map positions of other activities are shown as black lines. See text for discussion and references. The heavy black line in the p53 binding domain corresponds to a region that appears to be the most important. NLS, nuclear localization signal. [For text discussion, see pp.77 in article by Simmons, this volume.]

DNA pol alpha/primase

RPA

RPA

RPA

topo I   T

RNA

5'   3'

3'   5'
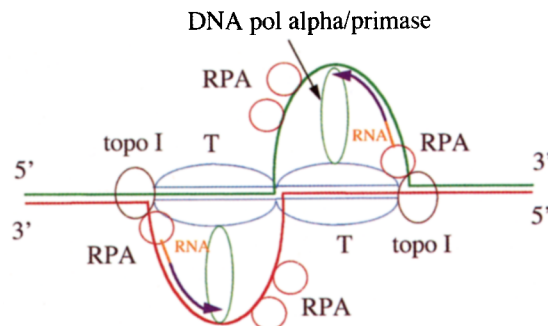
RPA

RNA

T   topo I

RPA

FIG 2.5. Model of DNA replication initiation at the SV40 origin. Initiation of replication is presumed to require T antigen, topo I, RPA, and DNA pol α/primase. RPA fulfills at least two functions: binding to single-stranded DNA and interaction with the initiation complex to promote primer synthesis. T antigen interacts with topo I, RPA, and DNA pol α. RPA and DNA pol also interact (not shown for clarity). RNA primers are shown in orange, newly synthesized DNA in purple. [For text discussion, see pp. 92 in article by Simmons, this volume.]
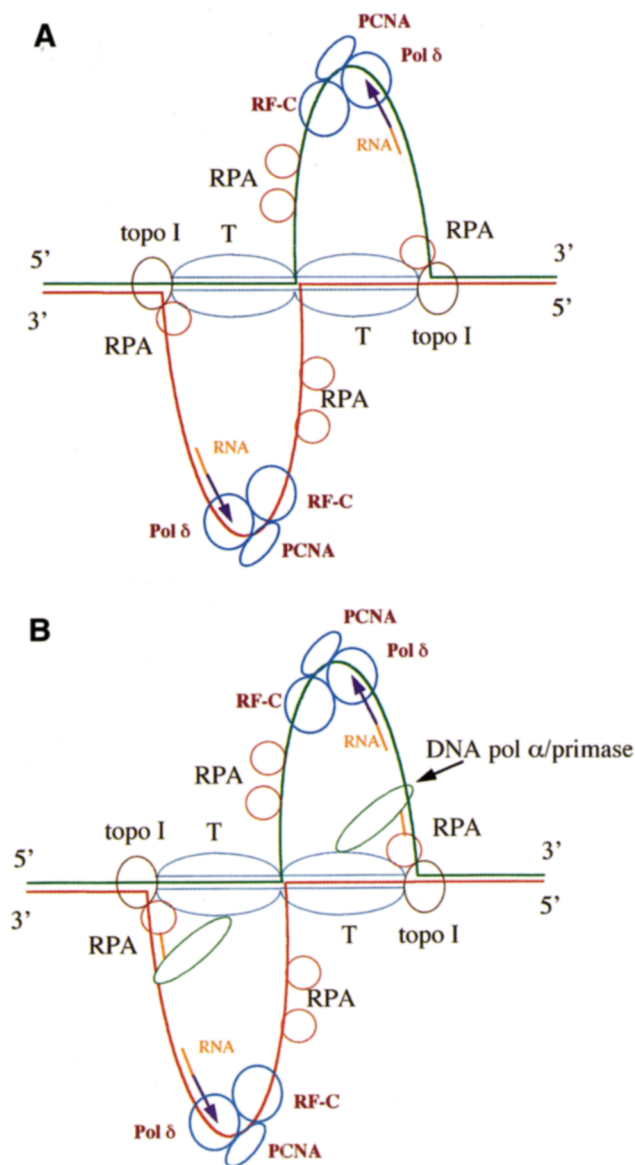
FIG 2.6. Further steps in DNA synthesis on SV40 DNA. (A) DNA pol α/primase is replaced by RF-C, PCNA and DNA pol δ. This enzyme processively extends DNA previously layed down by DNA pol α. These newly synthesized chains are presumably leading strands. (B) Lagging strand synthesis is initiated when another molecule of DNA pol α/primase attaches to each T antigen hexamer, synthesizes an RNA primer that is extended a short distance by pol α. RF-C, PCNA and pol δ complete the synthesis of the lagging strand until it reaches the previous primer. Gap synthesis (not shown) is needed to ligate new chains together. [For text discussion, see pp. 94 in article by Simmons, this volume.]
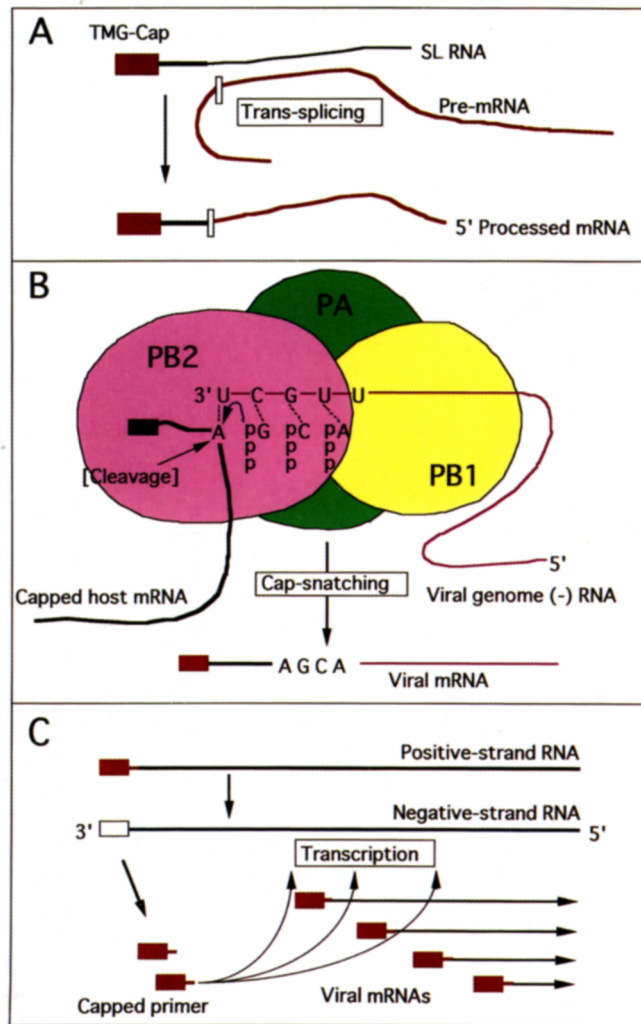
FIG 3.6. Schematic representation of the mechanism for adding preformed 5′ cap sequences to cellular and viral mRNAs. (A) *Trans*-splicing in nematodes; (B) cap-snatching in influenza virus; (C) possible capped leader priming in coronavirus transcription. The horizontal boxes with red or black indicate capped oligonucleotides. [For text discussion, see pp. 163 in article by Furuichi and Shatkin, this volume.]
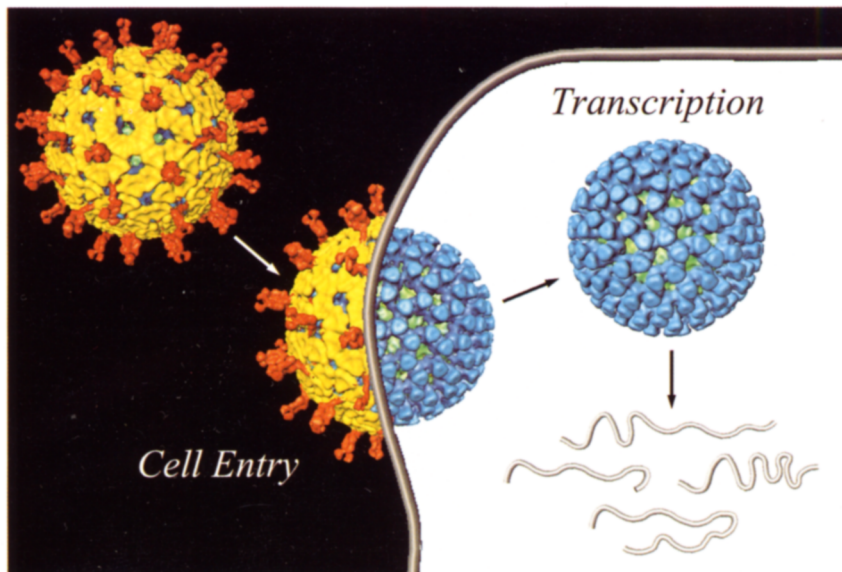
FIG 4.1. Three-dimensional structures of the mature infectious rotavirus particle and the transcriptionally competent subviral particle. (Left) The mature rotavirus particle consists of three concentric capsid layers. In this illustration, produced from a three-dimensional reconstruction of rotavirus at 24 Å resolution, the inner capsid protein VP2 is shown in green, the intermediate capsid protein VP6 in blue, and the outer capsid protein VP7 in yellow. The VP4 spike proteins are shown in orange. The transcription enzymes VP1 and VP3, along with the eleven genomic dsRNA segments, are housed in the interior within the VP2 capsid layer. (Center) Rotavirus enters the cell by penetration of the plasma membrane, and as a consequence of cell entry, the outer capsid proteins VP7 and VP4 are removed. (Right) Once inside the cell, the resulting subviral particle becomes transcriptionally active, releasing capped mRNA transcripts into the cytoplasm. [For text discussion, see pp. 189 in article by Lawton *et al.,* this volume.]
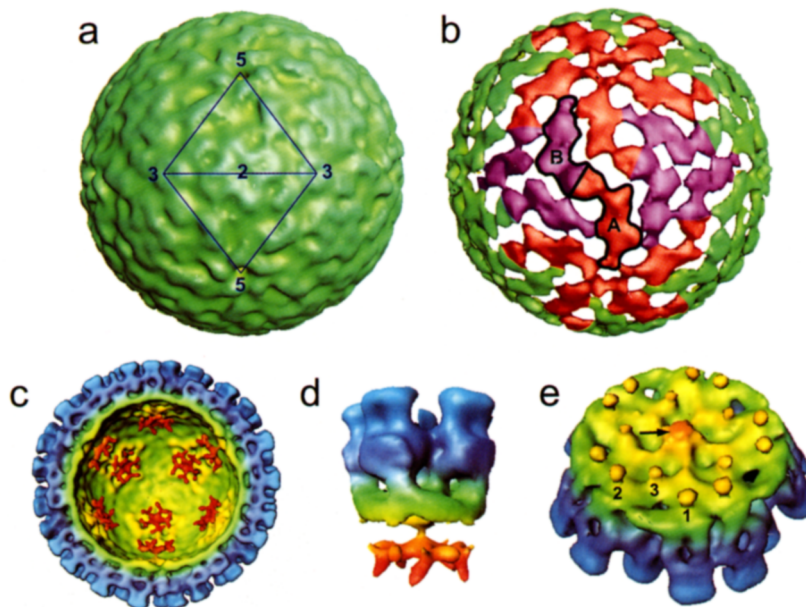
FIG 4.2. Role of VP2 as a scaffold for the internal components of the endogenous transcription apparatus. (a) When represented using a normal contour threshold (~0.4 σ), the VP2 capsid layer appears as a thin, relatively featureless closed shell, and the volume accounts for the expected 120 molecules of VP2. The boundaries between individual VP2 monomers are difficult to distinguish. Several of the icosahedral symmetry axes are indicated. (b) When represented at an elevated contour threshold (~2.3 σ), the surface appears to separate into 60 antiparallel strips of mass density, with each strip extending from one icosahedral vertex (5-fold axis) to a point near an adjacent vertex. One of these strips is outlined. Each strip appears to have an approximate center of symmetry and, as such, may be a dimer of quasiequivalent VP2 molecules. These two types of VP2 are designated as A (red) and B (purple). (c) The transcription enzymes VP1 and VP3 form a complex that is anchored to the inner surface of the VP2 capsid layer. In this cross-sectional illustration produced from a three-dimensional reconstruction of 1/3/2/6-VLP (Prasad *et al.,* 1996) and shown oriented along the icosahedral 3-fold axis, 9 of 12 complexes are shown, each anchored at an icosahedral vertex. (d) The VP1/3 complex appears as a flower-shaped structure (*orange*) attached to a small inward protrusion of VP2 at the 5-fold axis. (e) The inner surface of the VP2 capsid layer in the TCP serves as a scaffold for the enzymatic and nucleic acid components of the transcription apparatus. In this illustration, the capsid region surrounding the icosahedral vertex has been computationally isolated from the three-dimensional structure of the TCP to reveal the locations of three distinct classes of VP2-dsRNA contacts (numbered as 1, 2, and 3) and the anchor point for the VP1/3 enzyme complex (arrow). Illustrations in (a), (b), and (e) adapted from Lawton *et al.* (1997b). Used by permission. [For text discussion, see pp. 199 in article by Lawton *et al.,* this volume.]
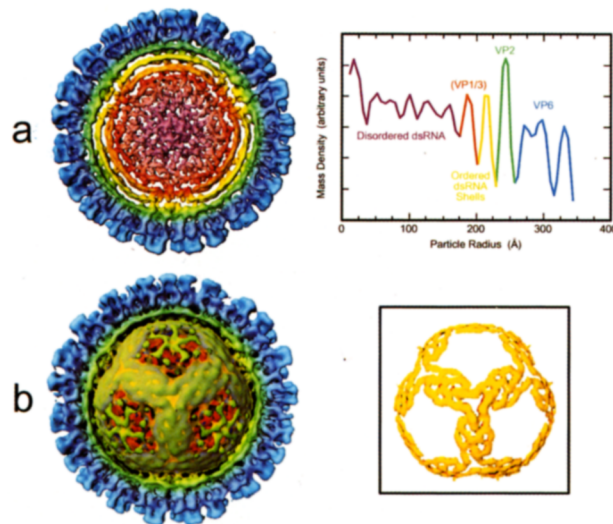
Fig 4.3. Internal organization of the rotavirus core. The enzymatic and nucleic acid components of the transcription apparatus are housed within the core of the virion. (a) A cross section of the viral core, produced from a three-dimensional reconstruction of the rotavirus TCP at 19 Å resolution, illustrates how structurally discernible portions of the dsRNA genome appear as concentric shells (yellow and orange). These shells have the greatest degree of order near the inner surface of the viral capsid and progressively less order toward the viral center (maroon). (Inset) An averaged radial density profile, computed from the three-dimensional reconstruction of the TCP, further illustrates the concentric organization of the two capsid layers and the genomic dsRNA. The color scheme matches that used in the three-dimensional structures. The VP6 capsid layer extends between the radii 260 and 350 Å, VP2 between 230 and 260 Å, and two shells of icosahedrally ordered dsRNA between ~160 and 230 Å. The structurally discernible portions of the VP1/3 transcription enzyme complexes are embedded within these two ordered dsRNA layers (Prasad *et al.*, 1996). (b) the VP6 (blue) and VP2 (green) capsid layers have been computationally removed, revealing the organization of the viral interior. A significant portion (approximately 25%) of the genomic dsRNA forms a well-organized layer (yellow) immediately inside the capsid. The surface of this RNA shell has been made slightly transparent to reveal the outlines of the RNA double helices, which have a higher density than protein. The dsRNA makes extensive contact with the inner surface of the VP2 capsid layer near the icosahedral vertices. (Inset) When displayed using a higher density threshold appropriate for nucleic acid, the dsRNA shell immediately inside the VP2 capsid layer is resolved into parallel tubes of mass, which likely represent dsRNA helices. Illustrations adapted from Prasad *et al.* (1996). Used by permission. [For text discussion, see pp. 203 in article by Lawton *et al.,* this volume.]
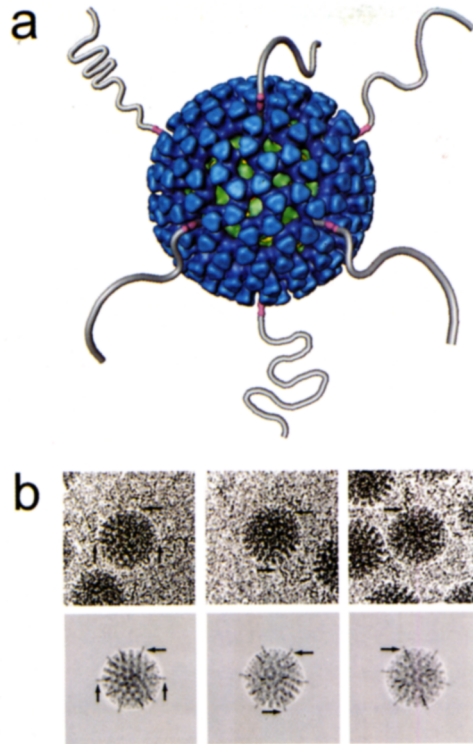
FIG 4.8. Release of newly synthesized mRNA during transcription. (a) During transcription in rotavirus, capped mRNA transcripts corresponding to the 11 dsRNA genome segments are synthesized in the viral core and then translocated 140 Å through a system of channels penetrating the two capsid layers at the icosahedral vertices. In the three-dimensional structure of the actively transcribing TCP at 25 Å resolution, the structurally discernible portions of exiting mRNA transcripts appear as small hourglass-shaped plugs (pink) suspended above the channels at the icosahedral vertices. Hypothetical mRNA transcripts (gray) are shown passing through the capsid. (b) A comparison of several transcribing particle images with simulated images (mathematical projections) of the three-dimensional structure indicate that the particle-associated mRNA strands seen in the cryomicrograph images are aligned with the icosahedral vertices (arrows). To mark the locations of the icosahedral vertices in the simulated images, the mRNA plugs (pink) in the three-dimensional structure were replaced with high-density spikes which, in projection, appear dark. Illustrations adapted from Lawton *et al.* (1997a). Used by permission. [For text discussion, see pp. 215 in article by Lawton *et al.*, this volume.]
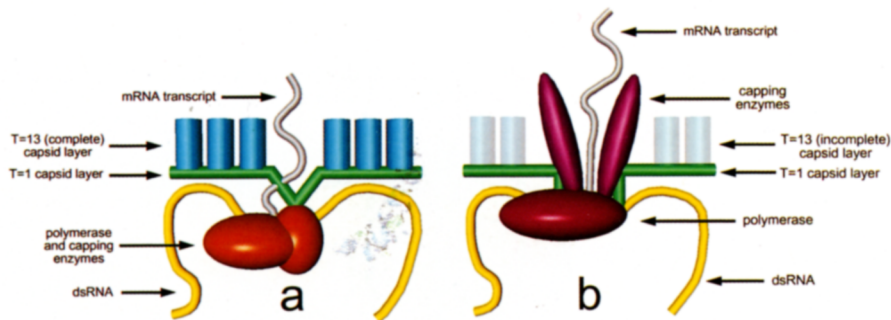
FIG 4.9. Model for the organization of the endogenous transcription apparatus. (a) In members of the *Reoviridae* resembling rotavirus and orbivirus, the transcription and capping enzymes are housed entirely within the viral core, and nascent mRNA transcripts are released through channels penetrating the two capsid layers at the icosahedral vertices. (b) In members of the *Reoviridae* resembling orthoreovirus, the polymerase enzyme is housed within the core, but the capping enzymes are incorporated as pentameric turret-like projections extending through the inner capsid layer, and nascent mRNA transcripts are released through this turret structure. This structural difference at the icosahedral vertex represents an important architectural distinction among members of the *Reoviridae* (Hill *et al.,* 1999). In the turreted viruses having multiple capsid layers, the major capsid protein defining the incomplete T=13 capsid layer (faded blue) is absent in the transcriptionally competent form of the virus. [For text discussion, see pp. 217 in article by Lawton *et al.,* this volume.]
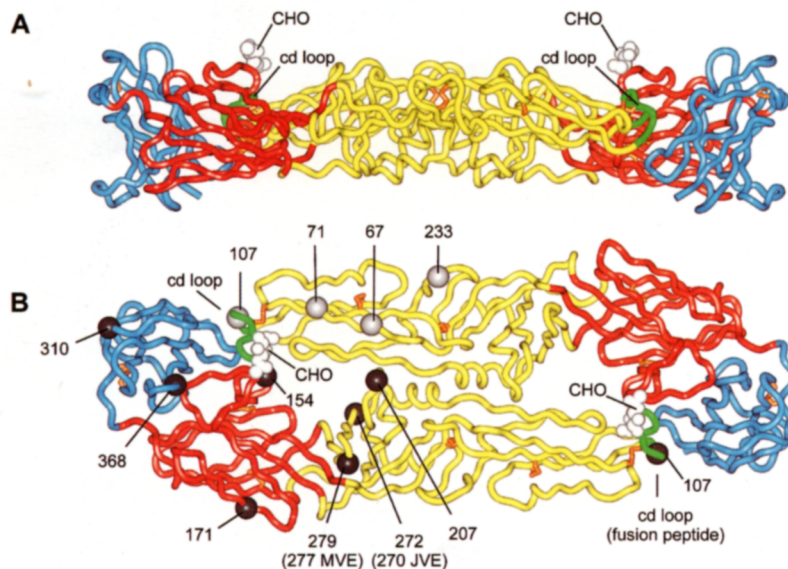

FIG 5.4. Ribbon diagram of the TBE virus E protein dimer (Rey *et al.,* 1995). (A) Side view (B) Top view. Domain I is colored red, Domain II yellow, and Domain III blue. The cd loop (putative fusion peptide) is shown in green and the six disulfide bridges in orange. The black spheres on the lower ribbon in B show the positions of mutations affecting fusion characteristics. The gray spheres on the upper ribbon show the positions of mutations defining epitopes of TBE virus MAbs that inhibit fusion (67 and 71: MAb A3; 233: MAb A4). Mutations affecting fusion are discussed in section VII H and inhibition of fusion by MAbs in section VII I. CHO: First residue of the carbohydrate attached to Asn 154. [For text discussion, see pp. 242 in article by Heinz and Allison, this volume.]

tions of actively transcribing orthoreoviruses and cypoviruses provided a consistent picture that nascent mRNA was translocated across the capsid through special turret-like projections at the icosahedral vertices, it was not apparent that other members of the *Reoviridae,* such as rotavirus or orbivirus, would also release mRNA transcripts in the same manner, as these particles do not have any special structures penetrating the capsid region at the icosahedral vertices.

Three-dimensional structural techniques were used in conjunction with electron cryomicroscopy to visualize the pathway of mRNA translocation in rotavirus (Lawton *et al.,* 1997a). In electron cryomicrograph images of actively transcribing particles, fibrous material corresponding to newly synthesized mRNA transcripts could be seen in the periphery of many of the virions. Likewise, in the three-dimensional structure, a narrow plug of mass, approximately 55 Å long, representing the structurally discernible portion of exiting mRNA could be seen in each of the channels penetrating the VP6 capsid layer at the icosahedral vertices (Fig. 4.8a, see also color insert). Comparison of back projections of the reconstruction with selected particle images confirmed that the fibers seen in the images were indeed aligned in projection with the channels at the icosahedral vertices (Fig. 4.8b). The pathway through the underlying VP2 capsid layer was not clear in these studies but may involve a set of small channels that are slightly offset from the icosahedral 5-fold axis and are located beneath the channels in the VP6 layer.

Although observation of the actively transcribing rotavirus particle by electron cryomicroscopy has not provided a clear definition of the pathway of mRNA translocation through the inner capsid layer, atomic resolution structural studies of the bluetongue virus TCP have suggested a possible route (Grimes *et al.,* 1998). The inner capsid protein VP3, structurally analogous to the VP2 protein of rotavirus, forms a shell having very few openings into the underlying viral core. However, at each of the icosahedral vertices, a small pore measuring 9 Å in diameter penetrates the inner capsid layer immediately above the transcription enzyme complex. Although this pore is likely too narrow at 9 Å to allow mRNA to pass through unhindered, this region of the structure is relatively flexible, and small conformational changes accompanying transcriptional activation may allow the channel to open considerably wider to facilitate the passage of nascent mRNA transcripts. High resolution structural studies of actively transcribing TCPs may serve to clarify the pathway of mRNA translocation across the inner capsid layer.
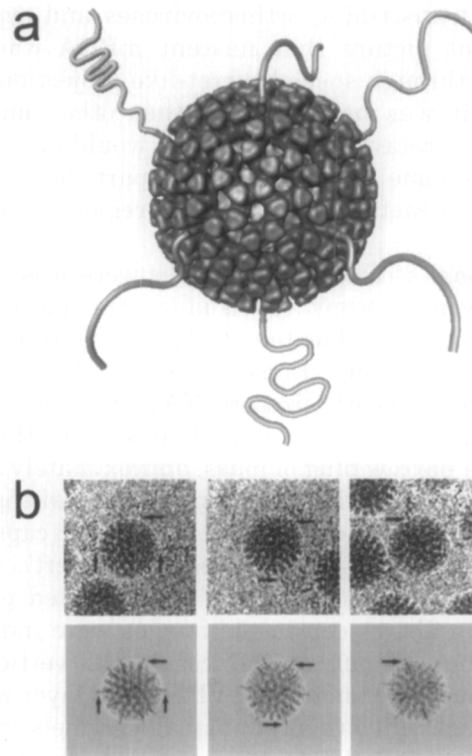
FIG 4.8. Release of newly synthesized mRNA during transcription. (a) During transcription in rotavirus, capped mRNA transcripts corresponding to the 11 dsRNA genome segments are synthesized in the viral core and then translocated 140 Å through a system of channels penetrating the two capsid layers at the icosahedral vertices. In the three-dimensional structure of the actively transcribing TCP at 25 Å resolution, the structurally discernible portions of exiting mRNA transcripts appear as small hourglass-shaped plugs (pink) suspended above the channels at the icosahedral vertices. Hypothetical mRNA transcripts (gray) are shown passing through the capsid. (b) A comparison of several transcribing particle images with simulated images (mathematical projections) of the three-dimensional structure indicate that the particle-associated mRNA strands seen in the cryomicrograph images are aligned with the icosahedral vertices (arrows). To mark the locations of the icosahedral vertices in the simulated images, the mRNA plugs (pink) in the three-dimensional structure were replaced with high-density spikes which, in projection, appear dark. Illustrations adapted from Lawton *et al.* (1997a). Used by permission. See also color insert.

Actively transcribing orthoreovirus TCPs have also been studied using electron cryomicroscopy (Yeager *et al.,* 1996). In agreement with earlier studies using conventional electron microscopy, exiting mRNA transcripts appeared to be passing through the turret-like projections

at the icosahedral vertices. These observations indicated that, despite significant differences between rotavirus and orthoreovirus in the architecture of the capsid region surrounding the icosahedral vertices, the overall strategy for mRNA release may be quite similar in all of the reoviruses, involving translocation through channels at the vertices.

From an engineering perspective, the most efficient way to generate mRNA within the confines of the intact particle would be to release the newly synthesized transcripts through channels that lie in closest proximity to the polymerase complexes. In rotavirus, the VP1 and VP3 enzymes are positioned along the inner surface of the capsid at each icosahedral vertex, and this location is directly beneath the system of channels through which mRNA transcripts are observed to come out during transcription. Although the precise location of the enzyme active site is not known, it is conceivable that the polymerase is positioned to allow mRNA to move directly out of the core during synthesis (Fig. 4.9a, see also color insert). It is conceivable that the proper threading of a newly initiated transcript into the exit channel system may be the critical event required for the successful transition from initiation to sustained elongation (Lawton *et al.*, 1999).
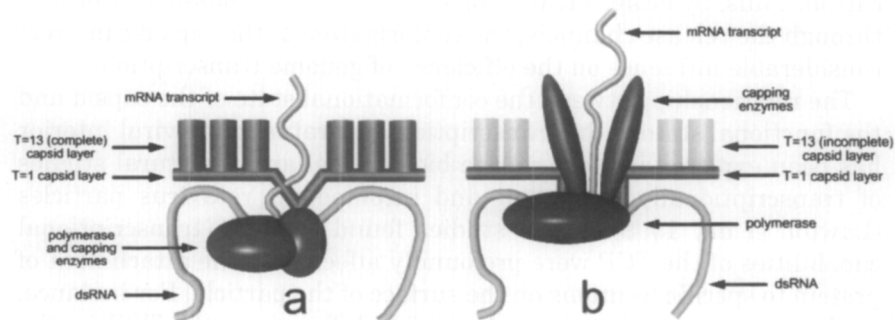

FIG 4.9. Model for the organization of the endogenous transcription apparatus. (a) In members of the *Reoviridae* resembling rotavirus and orbivirus, the transcription and capping enzymes are housed entirely within the viral core, and nascent mRNA transcripts are released through channels penetrating the two capsid layers at the icosahedral vertices. (b) In members of the *Reoviridae* resembling orthoreovirus, the polymerase enzyme is housed within the core, but the capping enzymes are incorporated as pentameric turret-like projections extending through the inner capsid layer, and nascent mRNA transcripts are released through this turret structure. This structural difference at the icosahedral vertex represents an important architectural distinction among members of the *Reoviridae* (Hill *et al.*, 1999). In the turreted viruses having multiple capsid layers, the major capsid protein defining the incomplete T=13 capsid layer (faded blue) is absent in the transcriptionally competent form of the virus. See also color insert.

A similar juxtaposition of transcription enzymes and mRNA release channels is seen in orthoreoviruses (Dryden et al., 1998). The projections through which newly synthesized mRNA transcripts are released are formed from pentamers of λ2, the multifunctional enzyme responsible for capping. The polymerase λ3 is anchored at the inner surface of the capsid immediately beneath the capping enzyme complex. During transcription, newly synthesized mRNA comes off the polymerase and passes through the hollow region in the center of the λ2 pentamer (Yeager et al., 1996). In this way, the arrangement of the transcription machinery facilitates easy release of nascent transcripts after their synthesis (Fig. 4.9b).

C. Effect of Capsid Conformation on Efficiency of mRNA Translocation

Because of the requirement for particle integrity during transcription in segmented dsRNA viruses, mRNA production may be thought of as a coordinated process during which the nucleotidyl transfer reaction in the core of the virion must be synchronized with the translocation of the newly synthesized mRNA through specific channels in the capsid. Thus, by either facilitating or hindering the passage of mRNA through the release channels, the conformation of the capsid can exert considerable influence on the efficiency of genome transcription.

The relationship between the conformational state of the capsid and the functional state of the transcription apparatus in the viral interior has been explored in comparative biochemical and structural studies of transcriptionally competent and incompetent rotavirus particles (Lawton et al., 1999). These studies found that the transcriptional capabilities of the TCP were profoundly affected by the attachment of protein to specific locations on the surface of the particle. For instance, in the mature rotavirus particle, which differs from the TCP by the presence of an additional capsid layer on the VP6 surface, transcript elongation was arrested after only 5–7 nt had been polymerized, and the aborted transcripts remained trapped within the viral interior. The VP2 and VP6 capsid layers in these two particle forms appeared to be similar in structure, although minor differences in the conformation of the trimers making up the VP6 capsid layer were observed within the vicinity of the VP2–VP6 capsid interface. Although the relationship between the subtle structural changes seen in the capsid and the inhibition of transcription in the core was not readily apparent in these studies owing to limitations in resolution, nearly identical structural and biochemical results were observed in a transcriptionally

incompetent virus–Fab complex in which anti-VP6 Fabs were bound to the VP6 surface in a manner similar to that of VP7 in the mature particle. Higher resolution structural studies of transcriptionally incompetent particles will better characterize the mechanisms by which subtle conformational changes in the capsid architecture may prevent the proper function of the transcription apparatus in the viral interior.

Biochemical studies of mature orthoreovirions and transcriptionally active orthoreovirus cores have provided evidence of a similar phenomenon. Mature orthoreovirions incubated with the precursors needed for RNA synthesis cannot produce full-length mRNA transcripts but are nevertheless observed to generate large quantities of short oligonucleotides corresponding in sequence to the 5' ends of the mature transcripts (Bellamy *et al.*, 1972; Yamakawa *et al.*, 1982). Interestingly, although these oligonucleotides are generated in both mature virions and TCPs, the fact that they are generated in the complete absence of full-length transcripts in the mature particles demonstrates that the polymerase enzyme is fully capable of transcript initiation even in the presence of the outer capsid layer and suggests that the inhibition of genome transcription in the mature particle occurs at the stage of transcript elongation and/or translocation through the capsid (Yamakawa *et al.*, 1982). In support of these observations, comparative structural studies of mature and transcriptionally competent orthoreovirus particles have provided evidence of significant conformational changes occurring in the inner capsid regions near the icosahedral vertices on removal of the outer capsid proteins (Dryden *et al.*, 1993). It is quite likely that these conformational changes are involved in transforming the capsid to a state that facilitates, rather than hinders, the translocation of mRNA during transcription.

Efficient genome transcription in viruses having built-in transcriptional machinery is likely dependent on the continuous exit of newly synthesized mRNA, especially as the endogenous transcriptase complexes can perform multiple cycles of initiation followed by elongation and translocation. If mRNA translocation through the capsid is somehow disrupted, transcript elongation at the enzyme active site in the core is probably affected immediately, as the failure to translocate nascent mRNA away from the site of synthesis would likely hinder the movement of the dsRNA template through the polymerase, given the dense packing of the genome observed in the core of the virion (Harvey *et al.*, 1981; Prasad *et al.*, 1996; Zhang *et al.*, 1999). During genome transcription in segmented dsRNA viruses, the processes of transcript elongation in the core and translocation through the capsid need to be

coordinated, and with the disruption of mRNA elongation comes premature termination of transcription, as has been described for some DNA-dependent RNA polymerases (Spencer and Groudine, 1990).

IV. SUMMARY

Genome transcription is a critical stage in the life cycle of a virus, as this is the process by which the viral genetic information is presented to the host cell protein synthesis machinery for the production of the viral proteins needed for genome replication and progeny virion assembly. Viruses with dsRNA genomes face a particular challenge in that host cells do not produce proteins which can transcribe from a dsRNA template. Therefore, dsRNA viruses contain all of the necessary enzymatic machinery to synthesize complete mRNA transcripts within the core without the need for disassembly. Indeed one of the more striking observations about genome transcription in dsRNA viruses is that this process occurs efficiently only when the transcriptionally competent particle is fully intact. This observation suggests that all of the components of the TCP, including the viral genome, the transcription enzymes, and the viral capsid, function together to produce and release mRNA transcripts and that each component has a specific and critical role to play in promoting the efficiency of this process.

This review has examined the process of genome transcription in dsRNA viruses from the perspective of rotavirus as a model system. However, despite numerous architectural and organizational differences among the families of dsRNA viruses, numerous studies suggest that the basic mechanism of mRNA production may be similar in most, if not all, viruses having dsRNA genomes. Important functional similarities include (1) the presence of a capsid-bound RNA-dependent RNA polymerase, which produces single-stranded mRNA transcripts from the dsRNA genome and regenerates the dsRNA genome from single-stranded RNA templates; (2) in viruses infecting eukaryotic hosts, the presence of all the enzymatic activities needed to generate the 5' cap required by the eukaryotic translation machinery; (3) the high degree of structural order present in the packaged genome, suggesting the requirement for organization in the viral core; (4) the role of the innermost capsid protein as a scaffold on which the core components of the transcription apparatus are assembled; and (5) the release of nascent mRNA transcripts through channels at the icosahedral vertices.

The process of genome transcription in dsRNA viruses will become better understood as structural studies progress to higher resolution and as more viruses become amenable to study using site-directed mutagenesis coupled with viral reconstitution to generate recombinant particles having precise functional and structural changes. Future studies will dissect important intermolecular interactions required for efficient mRNA synthesis and will shed further light on the reasons for which the viral core must be structurally intact in order for transcription to occur efficiently. Structural studies of the capping enzymes at atomic resolution will reveal how multiple enzyme activities reside within a single polypeptide and how they act in concert to synthesize the 5' cap on the end of each mature transcript. Perhaps most interestingly, high resolution structural studies of actively transcribing virions will provide insight into the conformational changes that occur within the core during mRNA synthesis. Together, these studies will clarify the function of this complex macromolecular machine and will also shed additional light on the basic principles of virus architecture and assembly, as well as provide avenues for the design of antiviral therapies.

REFERENCES

Bamford, J. K. H., Bamford, D. H., Li, T., and Thomas, G. J. Jr. (1993). Structural studies of the enveloped dsRNA bacteriophage φ6 of *Pseudomonas syringae* by Raman spectroscopy. II. Nucleocapsid structure and thermostability of the virion, nucleocapsid, and polymerase complex. *J. Mol. Biol.* **230,** 473–482.

Banerjee, A. K., and Shatkin, A. J. (1970). Transcription *in vitro* by reovirus-associated ribonucleic acid- dependent polymerase. *J. Virol.* **6,** 1–11.

Bartlett, N. M., Gillies, S. C., Bullivant, S., and Bellamy, A. R. (1974). Electron microscopy study of reovirus reaction cores. *J. Virol.* **14,** 315–326.

Bellamy, A. R., Nichols, J. L., and Joklik, W. K. (1972). Nucleotide sequences of reovirus oligonucleotides: Evidence for abortive RNA synthesis during virus maturation. *Nature New Biol.* **238,** 49–51.

Bernstein, J. M., and Hruska, J. F. (1981). Characterization of RNA polymerase products of Nebraska calf diarrhea virus and SA11 rotavirus. *J. Virol.* **37,** 1071–1074.

Bican, P., Cohen, J., Charpilienne, A., and Scherrer, R. (1982). Purification and characterization of bovine rotavirus cores. *J. Virol.* **43,** 1113–1117.

Bisaillon, M., Bergeron, J., and Lemay, G. (1997). Characterization of the nucleoside triphosphate phosphohydrolase and helicase activities of the reovirus λ1 protein. *J. Biol. Chem.* **272,** 18298–18303.

Böttcher, B., Kiselev, N. A., Stel'mashchuk, V. Y., Perevozchikova, N. A., Borisov, A. V., and Crowther, R. A. (1997). Three-dimensional structure of infectious bursal disease virus determined by electron cryomicroscopy. *J. Virol.* **71,** 325–330.

Boyle, J. F., and Holmes, K. V. (1986). RNA-binding proteins of bovine rotavirus. *J. Virol.* **58,** 561–568.

Brown, D. M., and Todd, A. R. (1952). Nucleotides. Part X. Some observations on the structure and chemical behavior of the nucleic acids. *J. Chem. Soc.* Part 1, 52–58.

Bruenn, J. A. (1991). Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. *Nucleic Acids Res.* **19,** 217–226.

Butcher, S. J., Dokland, T., Ojala, P. M., Bamford, D. H., and Fuller, S. D. (1997). Intermediates in the assembly pathway of the double-stranded RNA virus φ6. *EMBO J.* **16,** 4477–4487.

Campbell, A. M. (1996). Bacteriophages. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.), pp. 587–608. Raven Press, New York.

Castón, J. R., Trus, B. L., Booy, F. P., Wickner, R. B., Wall, J. S., and Steven, A. C. (1997). Structure of L-A virus: A specialized compartment for the transcription and replication of double-stranded RNA. *J. Cell Biol.* **138,** 975–985.

Chen, D., Gombold, J. L., and Ramig, R. F. (1990). Intracellular RNA synthesis directed by temperature sensitive mutants of simian rotavirus SA11. *Virology* **178,** 143–151.

Chen, D., and Ramig, R. F. (1993). Rescue of infectivity by sequential *in vitro* transcapsidation of rotavirus core particles with inner capsid and outer capsid proteins. *Virology* **194,** 743–751.

Chen, D., Zeng, C. Q., Wentz, M. J., Gorziglia, M., Estes, M. K., and Ramig, R. F. (1994). Template-dependent, *in vitro* replication of rotavirus RNA. *J. Virol.* **68,** 7030–7039.

Chen, D., Luongo, C. L., Nibert, M. L., and Patton, J. T. (1999). Rotavirus open cores catalyze 5'-capping and methylation of enogenous RNA: evidence that VP3 is a methyl transferase. *Virology.* **265,** 120–130.

Cheng, R. H., Caston, J. R., Wang, G. J., Gu, F., Smith, T. J., Baker, T. S., Bozarth, R. F., Trus, B. L., Cheng, N., Wickner, R. B., and Steven, A. C. (1994). Fungal virus capsids, cytoplasmic compartments for the replication of double-stranded RNA, formed as icosahedral shells of asymmetric Gag dimers. *J. Mol. Biol.* **244,** 255–258.

Cohen, J. (1977). Ribonucleic acid polymerase activity associated with purified calf rotavirus. *J. Gen. Virol.* **36,** 395–402.

Cohen, J., LaPorte, J., Charpilienne, A., and Scherrer, R. (1979). Activation of rotavirus RNA polymerase by calcium chelation. *Arch. Virol.* **60,** 177–186.

Cohen, H. A., Jeng, T. W., Grant, R. A., and Chiu, W. (1984). Specimen preparation methods for electron crystallography of soluble proteins. *Ultramicroscopy* **13,** 19–25.

Cohen, J., Charpilienne, A., Chilmonczyk, S., and Estes, M. K. (1989). Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. *Virology* **171,** 131–140.

Crawford, S. E., Labbé, M., Cohen, J., Burroughs, M. H., Zhou, Y.-J., and Estes, M. K. (1994). Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J. Virol.* **68,** 5945–5952.

Dai, R. M., Wu, A. Z., Shen, X. R., Li, Q., and Sun, Y. K. (1982). Isolation of genomeenzyme complex from cytoplasmic polyhedrosis virus of silkworm *Bombyx mori. Scientia Sinica (Series B)* **25,** 29–35.

Dryden, K. A., Wang, G., Yeager, M., Nibert, M. L., Coombs, K. M., Furlong, D. B., Fields, B. N., and Baker, T. S. (1993). Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: Analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* **122,** 1023–1041.

Dryden, K. A., Farsetta, D. L., Wang, G., Keegan, J. M., Fields, B. N., Baker, T. S., and Nibert, M. L. (1998). Internal structures containing transcriptase-related proteins in top component particles of mammalian orthoreovirus. *Virology* **245,** 33–46.

Eickbush, T. H., and Moudrianakis, E. N. (1978). The compaction of DNA helices into either continuous supercoils or folded-fiber rods and toroids. *Cell* **13,** 295–306.

Emori, Y., Iba, H., and Okada, Y. (1980). Semi-conservative transcription of double-stranded RNA catalyzed by bacteriophage φ6 RNA polymerase. *J. Biochem.* **88,** 1569–1575.

Estes, M. K. (1996). Rotaviruses and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.), pp. 1625–1655. Raven Press, New York.

Fields, B. N. (1996). Reoviridae. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath and B. Roizman, eds.), pp. 1553–1555. Raven Press, New York.

Flores, J., Myslinski, J., Kalica, A. R., Greenberg, H. B., Wyatt, R. G., Kapikian, A. Z., and Chanock, R. M. (1982). *In vitro* transcription of two human rotaviruses. *J. Virol.* **43,** 1032–1037.

Freifelder, D., and Kleinschmidt, A. K. (1965). Single-strand breaks in duplex DNA of coliphage T7 as demonstrated by electron cryomicroscopy. *J. Mol. Biol.* **14,** 271–278.

Furuichi, Y. (1981). Allosteric stimulatory effect of S-adenosylmethionine on the RNA polymerase in cytoplasmic polyhedrosis virus. A model for the positive control of eukaryotic transcription. *J. Biol. Chem.* **256,** 483–493.

Furuichi, Y., and Miura, K. (1975). A blocked structure at the 5'-terminus of mRNA of cytoplasmic polyhedrosis virus. *Nature* **253,** 374–375.

Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975). Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m-7G (5')ppp(5')G-MpCp. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 362–366.

Furuichi, Y., Muthukrishnan, S., Tomasz, J., and Shatkin, A. J. (1976). Mechanism of formation of reovirus mRNA 5'-terminal blocked and methylated sequence, m7GpppGmpC. *J. Biol. Chem.* **251,** 5043–5053.

Gillies, S., Bullivant, S., and Bellamy, A. R. (1971). Viral RNA polymerases: Electron microscopy of reovirus reaction cores. *Science* **174,** 694–696.

Gorziglia, M., and Esparza, J. (1981). Poly(A) polymerase activity in human rotavirus. *J. Gen. Virol.* **53,** 357–362.

Gottlieb, P., Strassman, J., Qiao, X., Frucht, A., and Mindich, L. (1990). *In vitro* replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage φ6: Studies with procapsids assembled from plasmid-encoded proteins. *J. Bacteriol.* **172,** 5774–5782.

Gouet, P., Diprose, D. I., Grimes, J. M., Malby, R., Burroughs, J. N., Zientara, S., Stuart, D. I., and Mertens, P. C. C. (1999). The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell.* **97,** 481–490.

Grimes, J. M., Basak, A. K., Roy, P., and Stuart, D. I. (1995). The crystal structure of bluetongue virus VP7. *Nature* **373,** 167–170.

Grimes, J. M., Jakana, J., Chosh, M., Basak, A. K., Roy, P., Chiu, W., Stuart, D. I., and Prasad, B. V. V. (1997). An atomic model of the outer layer of the bluetongue virus core

derived from X-ray crystallography and electron cryomicroscopy. *Structure* **5,** 885–893.

Grimes, J. M., Burroughs, J. N., Gouet, P., Diprose, J. M., Malby, R., Zientara, S., Mertens, P. P., and Stuart, D. I. (1998). The atomic structure of the bluetongue virus core. *Nature* **395,** 470–478.

Harvey, J. D., Bellamy, A. R., Earnshaw, W. C., and Schutt, C. (1981). Biophysical studies of reovirus type 3. IV. Low-angle x-ray diffraction studies. *Virology* **112,** 240–249.

Hewat, E. A., Booth, T. F., and Roy, P. (1992). Structure of bluetongue virus particles by cryo-electron microscopy. *J. Struct. Biol.* **109,** 61–69.

Hill, C. L., Booth, T. F., Prasad, B. V. V., Grimes, J. M., Mertens, P. P. C., Sutton, G. C., and Stuart, D. I. (1999). The structure of a cypovirus and the functional organisation of dsRNA viruses. *Nature Struct Biol.* **6,** 565–568.

Huismans, H., and Verwoerd, D. W. (1973). Control of transcription during the expression of the bluetongue virus genome. *Virology* **52,** 81–88.

Hundley, F., McIntyre, M., Clark, B., Beards, G., Wood, D., Chrystie, I., and Desselberger, U. (1987). Heterogeneity of genome rearrangements in rotaviruses isolated from a chronically infected, immunodeficient child. *J. Virol.* **61,** 3365–3372.

Iborra, F. J., Pombo, A., McManus, J., Jackson, D. A., and Cook, P. R. (1996). The topology of transcription by immobilized polymerases. *Exp. Cell Res.* **229,** 167–173.

Imai, M., Akatani, K., Ikegami, N., and Furuichi, Y. (1983). Capped and conserved terminal structures in human rotavirus genome double-stranded RNA segments. *J. Virol.* **47,** 125–136.

Kapahnke, R., Rappold, W., Desselberger, U., and Riesner, D. (1986). The stiffness of dsRNA: Hydrodynamic studies on fluorescence-labeled RNA segments of bovine rotavirus. *Nucleic Acids Res.* **14,** 3215–3228.

Kapuler, A. M. (1970). An extraordinary temperature dependence of the reovirus transcriptase. *Biochemistry* **9,** 4453–4457.

Kibenge, F. S. B., Dhillon, A. S., and Russell, R. G. (1988). Biochemistry and immunology of infectious bursal disease virus. *J. Gen. Virol.* **69,** 1757–1775.

Kohli, E., Pothier, P., Tosser, G., Cohen, J., Sandino, A. M., and Spencer, E. (1993). *In vitro* reconstitution of rotavirus transcriptional activity using viral cores and recombinant baculovirus expressed VP6. *Arch. Virol.* **133,** 451–458.

Koonin, E. V. (1993). Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and λ2 protein of reovirus. *J. Gen. Virol.* **74,** 733–740.

Labbé, M., Charpilienne, A., Crawford, S. E., Estes, M. K., and Cohen, J. (1991). Expression of rotavirus VP2 produces empty corelike particles. *J. Virol.* **65,** 2946–2952.

Labbé, M., Baudoux, P., Charpilienne, A., Poncet, D., and Cohen, J. (1994). Identification of the nucleic acid binding domain of the rotavirus VP2 protein. *J. Gen. Virol.* **75,** 3423–3430.

Lawton, J. A., Estes, M. K., and Prasad, B. V. V. (1997a). Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat. Struct. Biol.* **4,** 118–121.

Lawton, J. A., Zeng, C. Q., Mukherjee, S. K., Cohen, J., Estes, M. K., and Prasad, B. V. V. (1997b). Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: Implications for the architecture of the VP2 capsid layer. *J. Virol.* **71,** 7353–7360.

Lawton, J. A., Estes, M. K., and Prasad, B. V. V. (1999). Comparative structural analysis of transcriptionally-competent and incompetent rotavirus-antibody complexes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 5428–5433.

Liu, L. F., and Wang, J. C. (1987). Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7024–7027.

Liu, M., Offit, P. A., and Estes, M. K. (1988). Identification of the simian rotavirus SA-11 genome segment 3 product. *Virology* **163,** 26–32.

Liu, M., Mattion, N. M., and Estes, M. K. (1992). Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. *Virology* **188,** 77–84.

López, S., Espinosa, R., Greenberg, H. B., and Arias, C. F. (1994). Mapping the subgroup epitopes of rotavirus protein VP6. *Virology* **204,** 153–162.

Lu, G., Zhou, Z. H., Baker, M. L., Jakana, J., Cai, D., Wei, X., Chen, S., Gu, X., and Chiu, W. (1998). Structure of double-shelled rice dwarf virus. *J. Virol.* **72,** 8541–8549.

Luongo, C. L., Contreras, C. M., Farsetta, D. L., and Nibert, M. L. (1998). Binding site for S-adenosyl-L-methionine in a central region of mammalian reovirus λ2 protein. Evidence for activities in mRNA cap methylation. *J. Biol. Chem.* **273,** 23773–23780.

Martinez-Costas, J., Sutton, G., Ramadevi, N., and Roy, P. (1998). Guanylyltransferase and RNA 5'-triphosphatase activities of the purified expressed VP4 protein of bluetongue virus. *J. Mol. Biol.* **280,** 859–866.

Mason, B. B., Graham, D. Y., and Estes, M. K. (1980). *In vitro* transcription and translation of simian rotavirus SA11 gene products. *J. Virol.* **33,** 1111–1121.

Mathieu, M., Petitpas, I., Prasad, B. V. V., Kohli, E., Pothier, P., Cohen, J., and Rey, F. A. (2000). Atomic resolution structure of a major capsid protein of rotavirus and its implications in the virion structure. Submitted for publication.

McCrae, M. A., and McCorquodale, J. G. (1983). Molecular biology of rotaviruses. V. Terminal structure of viral RNA species. *Virology* **126,** 204–212.

Mertens, P. P., and Payne, C. C. (1983). The effects of S-adenosyl methionine (AdoMet) and its analogues on the control of transcription and translation *in vitro* of the mRNA products of two cytoplasmic polyhedrosis viruses. *Virology* **131,** 18–29.

Mertens, P. P. C. and Sanger, D. V. (1985). Analysis of the terminal sequences of the genome segments of four orbiviruses. *In* "Bluetongue and related orbiviruses" (T. L. Barger and M. M. Yochim, eds.), pp. 371–387. Alan R. Liss, New York.

Mindich, L. (1999). Precise packaging of the three genomic segments of the double-stranded-RNA bacteriophage φ6. *Microbiol. Mol. Biol. Rev.* **63,** 149–160.

Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martalli, G. P., Mayo, M. A., and Summers, M. D. (1995). Virus taxonomy—the classification and nomenclature of viruses. *In* "Sixth Report of the International Committee on Taxonomy of Viruses" Springer-Verlag, Vienna.

Nason, E. L., Samal, S. K., and Prasad, B. V. V. (2000). Trypsin-induced structural transformation in aquareovirus. *J. Virol.* **74,** 6546–6555.

Nibert, M. L. (1998). Structure of mammalian orthoreovirus particles. *In* "Reoviruses I: Structure, Proteins, and Genetics. (K. L. Tyler and M. B. A. Oldstone, eds.), pp. 1–30. Springer-Verlag, New York.

Noble, S. and Nibert, M. L. (1997a). Characterization of an ATPase activity in reovirus cores and its genetic association with core-shell protein λ1. *J. Virol.* **71,** 1282–1291.

Noble, S. and Nibert, M. L. (1997b). Core protein μ2 is a second determinant of nucleoside triphosphatase activities by reovirus cores. *J. Virol.* **71,** 7728–7735.

Ojala, P. M. and Bamford, D. H. (1995). *In vitro* transcription of the double-stranded RNA bacteriophage φ6 is influenced by purine NTPs and calcium. *Virology* **207,** 400–408.

Olkkonen, V. M., Gottlieb, P., Strassman, J., Qiao, X. Y., Bamford, D. H., and Mindich, L. (1990). *In vitro* assembly of infectious nucleocapsids of bacteriphage φ6: Formation of a recombinant double-stranded RNA virus. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 9173–9177.

Partridge, J. E., Van Etten, J. L., Burbank, D. E., and Vidaver, A. K. (1979). RNA poly-merase activity associated with bacteriophage φ6 nucleocapsid. *J. Gen. Virol.* **43,** 299–307.

Patton, J. T. (1996). Rotavirus VP1 alone specifically binds to the 3′ end of viral mRNA, but the interaction is not sufficient to initiate minus-strand synthesis. *J. Virol.* **70,** 7940–7947.

Patton, J. T. and Chen, D. (1999). RNA-binding and capping activities of proteins in rotavirus open cores. *J. Virol.* **73,** 1382–1391.

Patton, J. T., Wentz, M., Xiaobo, J., and Ramig, R. F. (1996). *cis*-Acting signals that pro-mote genome replication in rotavirus mRNA. *J. Virol.* **70,** 3961–3971.

Patton, J. T., Jones, M. T., Kalbach, A. N., He, Y.-W., and Xiaobo, J. (1997). Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. *J. Virol.* **71,** 9618–9626.

Payne, C. C. and Mertens, P. C. C. (1983). Cytoplasmic polyhedrosis viruses. *In* "The Reoviridae" (W. G. Joklik, ed.), pp. 425–504. Plenum Press, New York.

Pelletier, H., Sawaya, M. R., Wolfle, W., Wilson, S. H., and Kraut, J. (1996). A structural basis for metal ion mutagenicity and nucleotide selectivity in human DNA poly-merase β. *Biochemistry* **35,** 12762–12777.

Pizarro, J. L., Sandino, A. M., Pizarro, J. M., Fernández, J., and Spencer, E. (1991a). Characterization of rotavirus guanylyltransferase activity associated with polypep-tide VP3. *J. Gen. Virol.* **72,** 325–332.

Pizarro, J. M., Pizarro, J. L., Fernández, J., Sandino, A. M., and Spencer, E. (1991b). Effect of nucleotide analogues on rotavirus transcription and replication. *Virology* **184,** 768–772.

Poch, O., Sauvaget, I., Delarue, M., and Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J.* **8,** 3867–3874.

Prasad, B. V. V. and Chiu, W. (1994). Structure of rotavirus. *In* "Rotaviruses" (R. F. Ramig, ed.), pp. 9–29. Springer-Verlag, New York.

Prasad, B. V. V. and Estes, M. K. (1997). Molecular basis of rotavirus replication: Struc-ture-function correlations. *In* "Structural biology of viruses" (W. Chiu, R. Burnett, and R. Garcia, eds.), pp. 239–268. Oxford University Press, New York.

Prasad, B. V. V., Wang, G. J., Clerx, J. P., and Chiu, W. (1988). Three-dimensional struc-ture of rotavirus. *J. Mol. Biol.* **199,** 269–275.

Prasad, B. V. V., Burns, J. W., Marietta, E., Estes, M. K., and Chiu, W. (1990). Localiza-tion of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. *Nature* **343,** 476–479.

Prasad, B. V. V., Yamaguchi, S., and Roy, P. (1992). Three-dimensional structure of sin-gle-shelled BTV. *J. Virol.* **66,** 2135–2142.

Prasad, B. V. V., Rothnagel, R., Zeng, C. Q., Jakana, J., Lawton, J. A., Chiu, W., and Estes, M. K. (1996). Visualization of ordered genomic RNA and localization of tran-scriptional complexes in rotavirus. *Nature* **382,** 471–473.

Qiao, X., Qiao, J., and Mindich, L. (1997). Stoichiometric packaging of the three genomic segments of double-stranded RNA bacteriophage φ6. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 4074–4079.

Ramadevi, N., Burroughs, N. J., Mertens, P. P., Jones, I. M., and Roy, P. (1998). Capping and methylation of mRNA by purified recombinant VP4 protein of bluetongue virus. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 13537–13542.

Ramadevi, N. and Roy, P. (1998). Bluetongue virus core protein VP4 has nucleoside triphosphate phosphohydrolase activity. *J. Gen. Virol.* **79,** 2475–2480.

Rankin, J. T. J., Eppes, S. B., Antczak, J. B., and Joklik, W. K. (1989). Studies on the mechanism of the antiviral activity of ribavirin against reovirus. *Virology* **168**, 147–158.

Reinisch, K. M., Nibert, M. L., and Harrison, S. C. (2000). Structure of the reovirus core at 3.6Å. *Nature* **404**, 960–967.

Rimon, A. and Haselkorn, R. (1978). Transcription and replication of bacteriophage φ6 RNA. *Virology* **89**, 206–217.

Robberson, D. L., Thornton, G. B., Marshall, M. V., and Arlinghaus, R. B. (1982). Novel circular forms of mengovirus-specific double-stranded RNA detected by electron microscopy. *Virology* **116**, 454–467.

Romanova, L. I. and Agol, V. I. (1979). Interconversion of linear and circular forms of double-stranded RNA of encephalomyocarditis virus. *Virology* **93**, 574–577.

Samuel, C. E. (1998). Reoviruses and the interferon system. *In* "Reoviruses II: Cytopathogenicity and Pathogenesis" (K. L. Tyler and M. B. A. Oldstone, eds.), Vol. 233, pp. 125–145. Springer-Verlag, New York.

Sandino, A. M., Jashes, M., Faundez, G., and Spencer, E. (1986). Role of the inner protein capsid on *in vitro* human rotavirus transcription. *J. Virol.* **60**, 797–802.

Schoehn, G., Moss, S. R., Nuttall, P. A., and Hewat, E. A. (1997). Structure of Broadhaven virus by cryoelectron microscopy: Correlation of structural and antigenic properties of Broadhaven virus and bluetongue virus outer capsid proteins. *Virology* **235**, 191–200.

Seliger, L. S., Zheng, K., and Shatkin, A. J. (1987). Complete nucleotide sequence of reovirus L2 gene and deduced amino acid sequence of viral mRNA guanylyltransferase. *J. Biol. Chem.* **262**, 16289–16293.

Shatkin, A. J. (1974). Methylated messenger RNA synthesis *in vitro* by purified reovirus. *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3204–3207.

Shatkin, A. J. and Sipe, J. D. (1968). RNA polymerase activity in purified reoviruses. *Proc. Natl. Acad. Sci. U.S.A.* **61**, 1462–1469.

Shatkin, A. J. and Kozak, M. (1983). Reovirus transcription and translation. *In* "The Reoviridae" (W. G. Joklik, ed.), pp. 425–504. Plenum Press, New York.

Shaw, A. L., Rothnagel, R., Chen, D., Ramig, R. F., Chiu, W., and Prasad, B. V. V. (1993). Three-dimensional visualization of the rotavirus hemagglutinin structure. *Cell* **74**, 693–701.

Shaw, A. L., Samal, S. K., Subramanian, K., and Prasad, B. V. V. (1996). The structure of aquareovirus shows how the different geometries of the two layers of the capsid are reconciled to provide symmetrical interactions and stabilization. *Structure* **4**, 957–967.

Shimotohno, K. and Miura, K. (1973). Single-stranded RNA synthesis *in vitro* by the RNA polymerase associated with cytoplasmic polyhedrosis virus containing double-stranded RNA. *J. Biochem. (Tokyo)* **74**, 117–125.

Shimotohno, K. and Miura, K. (1977). Nucleoside triphosphate phosphohydrolase associated with cytoplasmic polyhedrosis virus. *J. Biochem. (Tokyo)* **81**, 371–379.

Skehel, J. J. and Joklik, W. K. (1969). Studies on the *in vitro* transcription of reovirus RNA catalyzed by reovirus cores. *Virology* **39**, 822–831.

Smith, R. E. and Furuichi, Y. (1982). The double-stranded RNA genome segments of cytoplasmic polyhedrosis virus are independently transcribed. *J. Virol.* **41**, 326–329.

Spencer, E. and Arias, M. L. (1981). *In vitro* transcription catalyzed by heat-treated human rotavirus. *J. Virol.* **40**, 1–10.

Spencer, E. and Garcia, B. I. (1984). Effect of S-adenosylmethionine on human rotavirus RNA synthesis. *J. Virol.* **52**, 188–197.

Spencer, C. A. and Groudine, M. (1990). Transcription elongation and eukaryotic gene regulation. *Oncogene* **5**, 777–785.

Stacy-Phipps, S. and Patton, J. T. (1987). Synthesis of plus- and minus-strand RNA in rotavirus-infected cells. *J. Virol.* **61,** 3479–3484.

Starnes, M. C. and Joklik, W. K. (1993). Reovirus protein λ3 is a poly(C)-dependent poly(G) polymerase. *Virology* **193,** 356–366.

Stäuber, N., Martinez-Costas, J., Sutton, G., Monastyrskaya, K., and Roy, P. (1997). Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function an a helicase activity that catalyze the unwinding of double-stranded RNA substrates. *J. Virol.* **71,** 7220–7226.

Suzuki, N., Tanimura, M., Watanabe, Y., Kusano, T., Kitagawa, Y., Suda, N., Kudo, H., Uyeda, I., and Shikata, E. (1992). Molecular analysis of rice dwarf phytoreovirus segment S1: Interviral homology of the putative RNA-dependent RNA polymerase between plant- and animal-infecting reoviruses. *Virology* **190,** 240–247.

Taylor, J. A., O'Brien, J. A., and Yeager, M. (1996). The cytoplasmic tail of NSP4, the endoplasmic-reticulum located non-structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. *EMBO J.* **15,** 4469–4476.

Urukawa, T., Ritter, D. G., and Roy, P. (1989). Expression of largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: Association of VP1 protein with RNA polymerase activity. *Nucleic Acids Res.* **17,** 7395–7401.

Usala, S. J., Brownstein, B. H., and Haselkorn, R. (1980). Displacement of parental RNA strands during *in vitro* transcription by bacteriophage φ6 nucleocapsid. *Cell* **19,** 855–862.

Valenzuela, S., Pizarro, J., Sandino, A. M., Vasquez, M., Fernandez, J., Hernandez, O., Patton, J., and Spencer, E. (1991). Photoaffinity labeling of rotavirus VP1 with 8-azido-ATP: Identification of the viral RNA polymerase. *J. Virol.* **65,** 3964–3967.

Van Dijk, A. A. and Huismans, H. (1980). The *in vitro* activation and further characterization of the bluetongue virus-associated transcriptase. *Virology* **104,** 347–356.

Van Dijk, A. A. and Huismans, H. (1988). *In vitro* transcription and translation of bluetongue virus mRNA. *J. Gen. Virol.* **69,** 573–581.

Van Dijk, A. A., Frilander, M., and Bamford, D. H. (1995). Differentiation between minus- and plus-strand synthesis: Polymerase activity of dsRNA bacteriophage φ6 in an *in vitro* packaging and replication system. *Virology* **211,** 320–323.

Van Etten, J. L., Burbank, D. E., Cuppels, D. A., Lane, L. C., and Vidaver, A. K. (1980). Semiconservative synthesis of single-stranded RNA by bacteriophage φ6 RNA polymerase. *J. Virol.* **33,** 769–773.

Wertheimer, A. M., Chen, S.-Y., Borchardt, R. T., and Furuichi, Y. (1980). *S*-adenosylmethionine and its analogs: Structural features correlated with synthesis and methylation of mRNAs of cytoplasmic polyhedrosis virus. *J. Biol. Chem.* **255,** 5924–5930.

Wickner, R. B. (1996). Viruses of yeasts, fungi, and parasitic microorganisms. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds.), pp. 557–585. Raven Press, New York.

Witkiewicz, H. and Schweiger, M. (1985). A model of lambda DNA arrangement in the viral particle. *J. Theor. Biol.* **116,** 587–605.

Yamakawa, M., Furuichi, Y., Nakashima, K., LaFiandra, A. J., and Shatkin, A. J. (1981). Excess synthesis of viral mRNA 5-terminal oligonucleotides by reovirus transcriptase. *J. Biol. Chem.* **256,** 6507–6514.

Yamakawa, M., Furuichi, Y., and Shatkin, A. J. (1982). Reovirus transcriptase and capping enzymes are active in intact virions. *Virology* **118,** 157–168.

Yazaki, K. and Miura, K. (1980). Relation of the structure of cytoplasmic polyhedrosis virus and the synthesis of its messenger RNA. *Virology* **105,** 467–479.

Yazaki, K., Mizuno, A., Sano, T., Fujii, H., and Miura, K. (1986). A new method for extracting circular and supercoiled genome segments from cytoplasmic polyhedrosis virus. *J. Virol. Methods* **14,** 275–283.

Yeager, M., Dryden, K. A., Olson, N. H., and Baker, T. S. (1990). Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* **110,** 2133–2144.

Yeager, M., Berriman, J. A., Baker, T. S., and Bellamy, A. R. (1994). Three-dimensional structure of the rotavirus haemagglutinin VP4 by cryo-electron microscopy and difference map analysis. *EMBO J.* **13,** 1011–1018.

Yeager, M., Weiner, S., and Coombs, K. M. (1996). Transcriptionally active reovirus core particles visualized by electron cryo-microscopy and image reconstruction. *Biophys. J.* **70,** A116.

Zarbl, H., Hastings, K. E., and Millward, S. (1980). Reovirus core particles synthesize capped oligonucleotides as a result of abortive transcription. *Arch. Biochem. Biophys.* **202,** 348–360.

Zeng, C. Q.-Y., Labbé, M., Cohen, J., Prasad, B. V. V., Chen, D., Ramig, R. F., and Estes, M. K. (1994). Characterization of rotavirus VP2 particles. *Virology* **201,** 55–65.

Zeng, C. Q.-Y., Wentz, M. J., Cohen, J., Estes, M. K., and Ramig, R. F. (1996). Characterization and replicase activity of double-layered and single-layered rotavirus-like particles expressed from baculovirus recombinants. *J. Virol.* **70,** 2736–2742.

Zeng, C. Q.-Y., Estes, M. K., Charpilienne, A., and Cohen, J. (1998). The N terminus of rotavirus VP2 is necessary for encapsidation of VP1 and VP3. *J. Virol.* **72,** 201–208.

Zhang, H., Zhang, J., Yu, X., Lu, X., Zhang, Q., Jakana, J., Chen, D. H., Zhang, X., and Zhou, Z. H. (1999). Visualization of protein-RNA interactions in cytoplasmic polyhedrosis virus. *J. Virol.* **73,** 1624–1629.