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Reference


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Minireview

Viral RNA polymerase scanning and the gymnastics of Sendai virus RNA synthesis

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Abstract

mRNA synthesis from nonsegmented negative-strand RNA virus (NNV) genomes is unique in that the genome RNA is embedded in an N protein assembly (the nucleocapsid) and the viral RNA polymerase does not dissociate from the template after release of each mRNA, but rather scans the genome RNA for the next gene-start site. A revised model for NNV RNA synthesis is presented, in which RNA polymerase scanning plays a prominent role. Polymerase scanning of the template is known to occur as the viral transcriptase negotiates gene junctions without falling off the template.

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This minireview summarizes current understanding of Sendai virus (SeV) RNA synthesis, drawing on work from other nonsegmented negative-strand RNA viruses (NNV), and observations of bacterial RNA polymerase and RNA pol II. We first describe NNV genome organization and our current understanding of its expression, then what is known about the structure of the viral proteins that carry out RNA synthesis, and then what is known about cellular RNA synthesis that is likely to apply to NNV RNA synthesis as well. New experiments on VSV and SeV RNA synthesis are discussed, and a general model for NNV RNA synthesis that integrates the new results is presented, in which RNA polymerase scanning of the template plays a prominent role.

NNV genome organization and expression

Sendai virus, a Respirovirus of the Paramyxovirinae subfamily of the Paramyxoviridae, is a model NNV. The other subfamily, the Pneumoviridae, contains respiratory syncytial virus (RSV) and human meta-pneumovirus. Other families of the order Mononegavirales that carry out their RNA synthesis in the cytoplasm include the Rhabdoviridae (with vesicular stomatitis virus (VSV) and rabies virus), and the Filoviridae (Ebola and Marburg viruses). NNV RNA synthesis requires the action of three viral proteins, the nucleocapsid protein N, which assembles the genome and antigenome RNAs into helical nucleocapsids (N:RNAs), and the P and L proteins that form the core viral RNA-dependent RNA polymerase (vRdRp). The genomic RNA of NNV functions firstly as a template for mRNA synthesis, and then as a template for a full-length complementary copy (the [+] antigenome), which like the genome is found only as N:RNA nucleocapsids (Fig. 1). For the Paramyxovirinae, each N subunit of the N:RNA is associated with precisely six nucleotides and this stoichiometry is functionally important (Calain and Roux, 1993; Egelman et al., 1989). This “rule of six”, however, does not apply to the other NNV. During infection, ostensibly the same vRdRp carries out both types of RNA synthesis on ostensibly the same N:RNA templates. The first is termed transcription, in which vRdRp responds to cis-acting signals and “stutters” (see below) to polyadenylate and terminate each mRNA. As a consequence, vRdRp restarts at successive mRNA start sites to synthesize each mRNA in turn. During RNA replication, in contrast, RNA synthesis and assembly with N occur concomitantly, and vRdRp ignores all polyadenylation/termina-
tion signals to form a full-length antigenome nucleocapsid (Lamb and Kolakofsky, 2001). Paramyxov-, Rhabdo-, and Filoviridae genomes are 11–18 kb long and contain 5–10 genes in tandem, separated by conserved junctional sequences that act as mRNA start and polyA/stop sites (Fig. 1). The first mRNA (usually N) starts around 50 nt from the genome 3' end, and the 50 nt upstream are called the leader region (le, Fig. 1). The last (L) mRNA ends a similar but variable distance from the genome 5' end, and is followed by the trailer region (tr, Fig. 1). Exactly how NNV RdRp switches between its dual function as transcriptase and replicase remains unclear. However, a self-regulatory model of viral RNA synthesis was proposed after the discovery that short le and tr RNAs were the first products transcribed from viral genomes and antigenomes, respectively (Leppert et al., 1979). More importantly, unlike the synthesis of genomes and antigenomes (and similar to the synthesis of mRNAs), le and tr RNA synthesis not only occurred, but was enhanced in the absence of on-going protein synthesis (Blumberg et al., 1981). As on-going protein synthesis is required for genome synthesis at any time of infection, the component limiting for replication was assumed to be unassembled N protein (N°), as each mature genome is associated with many N subunits (each 15,384-nt long SeV genome has 2564 N subunits). This assumption was later elegantly shown to be the case for VSV (Arnheiter et al., 1985). Moreover, as the site for the initiation of nucleocapsid assembly on VSV le RNA mapped to the first 14 nucleotides at its 5' end (Blumberg et al., 1983), nucleocapsid assembly was proposed to initiate on nascent le and tr RNAs before vRdRp had released these chains at or near the le/tr-gene junctions. The sequences responsible for measles virus nucleocapsid assembly are also contained within the le region (Castaneda and Wong, 1990). Antigenome RNA synthesis and assembly would then become coupled, and this vRdRp becomes a "replicase" that synthesizes complementary N:RNAs. In the absence of this coupling, vRdRp would release the le chain near the N mRNA start site, and be free to initiate N mRNA synthesis. Upon N mRNA initiation (including mRNA 5'-end modification), this vRdRp becomes a "transcriptase" that responds to transcriptional signals, that is, that stutter to form a mature polyA tail and releases the mRNA, and thus synthesizes each mRNA in turn (Iverson and Rose, 1981; McGeoch, 1979; Rose, 1980; Schubert et al., 1980). A self-regulatory model was proposed, in which the relative levels of transcription and replication are controlled by the availability of N° (Kolakofsky, 1982). In the absence of sufficient levels of N° to permit its assembly on the nascent le chain, vRdRp only transcribes the genome, to generate more mRNA that generates more N°. When N° levels rise, some of the vRdRp are devoted to genome replication,
which depletes the pool of N° and keeps its steady-state levels low.

This simple self-regulatory model, proposed >20 years ago, is generally accepted in broad outline. However, the model has been difficult to pin down unambiguously and important details are missing. There have been some advances. For example, by reconstituting SeV genome replication in a synchronous fashion in vitro, it has been possible to show that the replicase traverses the N:RNA at a constant speed from beginning to end (albeit at 2 nt/s) even when mRNA synthesis is poorly processive. More importantly, genome RNA synthesis and assembly were found to occur concurrently (Gubbay et al., 2001). Similarly, although we know little of how the coupling of genome synthesis and assembly constrains vRdRp to ignore template stutter signals and act as a replicase, the assembly process itself has been better described. VSV, rabies virus, and SeV N° expressed by themselves tend to aggregate nonspecifically, often with RNA (Horikami et al., 1992; Masters and Banerjee, 1988; Peluso and Moyer, 1988; Schoehn et al., 2001); the N° that assembles on nascent genome/antigenome RNA is chaperoned by an oligomeric P protein (probably P₄–N° for SeV (unpublished), and P₂–N° or a dimer thereof for rabies virus (Mavrikas et al., 2003)). There is biochemical evidence that P tetramers interact with each other in vitro (Tarbouriech et al., 2000a), and the addition of supplemental P₄ to P₄–L/N:RNA transcription reactions strongly enhances SeV mRNA synthesis (Curran, 1996). This enhancement requires the binding of P₄ to the template. Thus, both P₄ and P₄–L appear to be present in the transcription elongation complex (TEC). The replication elongation complex, by analogy, would contain P₄–N° along with P₄–L. The P₄ of the P₄–N° assembly complex may similarly interact with that of the polymerizing P₄–L during replication, and the ability of vRdRp to stutter in response to polyA signals may be modulated in this way (see below).

The Sendai virus RNA polymerase cofactor

P₄, the polymerase cofactor

NNV L proteins (approximately 2200 aa) are thought to contain all the catalytic sites of vRdRp (NMP polymerization, 5′ end guanylylation and methylation), but L does not bind to the N:RNA by itself. L in virions and infected cells is stably complexed with the more abundant P protein, which in SeV (and probably all Paramyxovirinae) is a parallel coiled-coil tetramer (Fig. 2) (Curran et al., 1995a; Tarbouriech et al., 2000a; Tarbouriech et al., 2000b). L is unstable in the absence of P, and coexpression of P and L is required for active P₄–L complex formation (Horikami et al., 1992). Paramyxovirus P genes are complex, and are divided in half by editing sites; specialized vRdRp stutter sites that induce vRdRp to add pseudo-templated guanylylates to the mRNA (Lamb and Kolakofsky, 2001). The net result of these nucleotide insertions in the mRNA is that different carboxyl segments (from overlapping ORFs) are fused to a common P amino-terminal segment (called PNT). X-ray and NMR studies have determined the structure of most of the longest of these carboxyl modules (called PCT), that of the SeV P protein (Blanchard et al., 2003; Marion et al., 2001; Tarbouriech et al., 2000b). Although never expressed independently in nature, PCT by itself is sufficient for all aspects of transcription in vitro (Curran, 1996), and has been dubbed the “polymerase cofactor”. The overall size and shape of PCT and its subdomains have been studied by small angle X-ray and neutron scattering (Blanchard et al., 2003; Tarbouriech et al., 2000a). PCT appears to be a rod ca. 160 Å long with a diameter of 36 Å (Fig. 2). The polymerase cofactor module contains a long α-helix that forms a stable coiled-coil tetramer of approximately 100 Å long. The coiled coil is buttressed at its NH₂-terminus by short α-helical segments, and is followed by a flexible linker of ca 30 aa, and then the X domain that contains the major site(s) for stable N:RNA binding via N-tail (see below). A 95-aa X protein is also expressed during some infections (Curran and Kolakofsky, 1988), and it is the structure of this monomer that was determined by NMR. The structure of the measles virus X domain has also been determined, by crystallography (Johansson et al., 2003). Both X domains are structurally well conserved, and their most prominent feature is a triple α-helical bundle at the C-terminus that is predicted to be present in the X domains of all the Paramyxovirinae (Curran et al., 1995a). The three helices show an asymmetric charge distribution and this bundle contains grooves between the helices to which N-tail may bind either through hydrophobic interactions or via its conserved, highly negatively charged peptide, forming a four-helix bundle (see below). As expected from the different methods used, the X-ray study stresses the atomic details of the crystallized protein, whereas NMR spectroscopy stresses the highly dynamic nature of this protein domain in solution. This latter property is likely to be important for P protein function in vRdRp (Blanchard et al., 2003).

The region where L is bound to P, mapped broadly by deletion analysis (Curran et al., 1994; Smallwood et al., 1994) (residues 412–445, Fig. 2) and more finely by alanine scanning mutagenesis (Bowman et al., 1999) (relevant side chains shown in Fig. 2), lies in the carboxyl half of the coiled coil. Unlike classic coiled coils that have hydrophobic interfaces of uniform diameter, the internal channel here is of variable size and widest at the center where water molecules are also found. Because RNA synthesis occurs one nucleotide at a time whereas displacement of P on the template occurs in steps of N subunits (6 nt at a time, Calain and Roux, 1993), P₄ is proposed to cartwheel across the N:RNA as L transcribes the template RNA (Curran, 1998). This would require some mobility at the P–L interface,
which may be provided by the plasticity of the non-canonical coiled coil due to its increased radius, and the exceptional mobility of the interacting side chains at the P–L interface, as indicated by their temperature factors (Tarbouriech et al., 2000b). The coiled coil of P is proposed to act as a molecular axle that allows P₄ rotation while L slides along the genomic RNA.

Genome replication, in contrast to transcription, requires the participation of at least part of the 317-aa amino-terminal segment of SeV P encoded upstream of the editing site (PNT). This segment of P contains a site near its amino-terminus (residues 33–41) that forms a complex with N⁺. This site is thought to chaperone N⁺ during the nascent chain assembly step of genome replication, and to ensure specificity of assembly (Curran et al., 1995b). Both the N⁺ chaperone domain and the surrounding sequence that has properties of an acidic activation domain are required for genome replication, but the remainder of the amino-terminal segment (residues 78–320) can be deleted without destroying its activity (at least for mini-genome synthesis) (Curran et al., 1994) (Fig. 2). The unexpected non-lethality of this massive deletion may be related to the finding that this segment in measles virus (and probably all the Paramyxovirinae) is intrinsically unstructured (Karlin et al., 2002). This segment of SeV P is similarly predicted to be unstructured, and the four N⁺ chaperone domains of P₄ appear to be connected to the rigid coiled coil of the polymerase cofactor module by a long
flexible linker (which is nonessential for activity under some conditions). This arrangement might prove useful in providing the flexibility required to position N subunits onto the growing end of the nascent nucleocapsid, as the product RNA emerges from the surface of vRdRp for assembly (bottom cartoon, Fig. 3).

The L protein polymerase

There is no detailed structure for any NNV L protein (ca. 2200 aa) as yet. However, NNV L proteins contain conserved sequence motifs characteristic of other RNA polymerases (Poch et al., 1989), and mutational analysis has

![Image of SeV RdRp elongation complex](image_url)
confirmed their importance for polymerase function. Given the conservation in overall structure of all RNA polymerases determined to date (Bressanelli et al., 1999; Cramer et al., 2001; Gnatt et al., 2001; Hansen et al., 1997; Korzheva et al., 2000; Tao et al., 2002; Zhang et al., 1999), it is reasonable that the basic architecture of NNV L proteins will be similar. The catalytic domain of nucleic acid polymerases is organized around a central cleft in an arrangement reminiscent of a right hand (Ollis et al., 1985). The remainder of the cleft accommodates the nascent chain–template chain hybrid (the hybrid) that maintains polymerase register during RNA synthesis (8–9 bp long for cellular DdRp (Nudler et al., 1997), and postulated to be 7 bp long for SeV RdRp (Hausmann et al., 1999a). One of the most striking features of recent bacterial and yeast DdRp structures in different phases of RNA synthesis is the network of contacts that interconnect side chains in the active site cleft with the hybrid and the nascent RNA exit channel. The central feature of this interaction network is a moveable “clamp” that contacts all the above. Comparison of these structures reveals several changes related to transcription, including the movement of this massive clamp by >30 Å between the proposed (open) initiation state and the transcribing complex (closed state), in which it largely surrounds the hybrid (Cramer et al., 2000; Cramer et al., 2001; Gnatt et al., 2001; Landick, 2001). Five polypeptide segments, termed switches, connect the clamp to the stationary parts of DdRp. The switches are disordered when the clamp is open, but they fold cooperatively upon interacting with the hybrid (and the bridge helix) when the clamp closes in the TEC (Cramer et al., 2001). The bridge helix spans the active site cleft and positions the nascent RNA 3′ end in the active site. This induced fit interaction is thought important in maintaining catalysis and TEC processivity, especially in negotiating pause sites. Polymerase pausing, the prelude to arrest and termination for cellular DdRp (Landick, 1999), is triggered by a conformational change in the interaction network that partially opens the active site cleft and misaligns the hybrid with the key active site residues (Landick, 2001). This inhibits nucleotide addition, either directly, or by allowing backtracking of the TEC. Polymerase backtracking is also the proposed prelude to NNV RdRp pseudo-templated transcription (Hausmann et al., 1999b). Cellular RNAP are thought to bind to their templates in a more open-handed conformation, and the initiating complex converts to a more closed, stable TEC that “grips” the template after formation of the hybrid and the entry of the nascent RNA 5′end into the RNA exit channel (promoter clearance).

**Cellular and NNV RNA synthesis, and polymerase processivity**

Synthesis of mature mRNAs is not guaranteed when TEC leaves a promoter in either bacteria or eucaryotes, as TECs (lacking elongation factors) are prone to extensive pausing and arrest during elongation. Eucaryotic elongation factors such as TFIIF, elongin, and ELL all suppress polymerase pausing via interaction with RNAPII (TFIIF is thought to act by preventing the displacement of the nascent RNA 3′end from the active site) (Price, 2000; Shilatifard et al., 2003). There is much evidence that cellular factors stimulate NNV RNA synthesis (reviewed in Gubbay et al., 2001). In a similar vein, bacteriophage λ Q-protein is stably incorporated into *E. coli* DdRp shortly after initiation, and fundamentally alters its elongation properties (Roberts et al., 1998). Q-modified DdRp display diminished pausing and are resistant to all downstream termination sites, much like NNV RNA synthesis that is coupled to concurrent assembly of the nascent chain. Mutations that uncouple this phage property suggest that Q-induced anti-termination is due to the stabilization of DdRp interactions with the DNA–RNA hybrid that optimize the alignment of nucleic acids in the catalytic site (Santangelo et al., 2003).

NNV transcriptases respond to cis-acting “stutter” signals that cause TEC to copy the same template base(s) repetitively (pseudo-templated transcription). Other transcriptase properties, like termination and initiation of the next mRNA, depend on the extensive vRdRp stuttering that forms the mRNA polyA tail (Barr and Wertz, 2001). The repetitive copying of template nucleotides requires cycles of realignment of the two strands of the hybrid (Hausmann et al., 1999a; Vidal et al., 1990), and this realignment implies conformational changes in the active site cleft–hybrid interactions that would permit this realignment (loosening of induced fit beyond that necessary to permit simple polymerase translocation after each nucleotide addition). Replicases, in contrast, do not (or very rarely, Bilsel and Nichol, 1990; Castaneda and Wong, 1990; Hausmann et al., 1996) respond to stutter signals. One way in which the coupling of nascent chain synthesis and assembly could commit vRdRp to replication is by simply locking this induced fit in place, preventing pseudo-templated transcription of any kind and maintaining near-absolute vRdRp processivity. The coupling of NNV nascent chain synthesis and assembly could act in a manner similar to the interaction of eucaryotic elongation factors with RNAP II, or bacteriophage λ Q-protein and *E. coli* DdRp.

SeV RdRp that has initiated at the genome 3′end (and read through the le/N junction in the absence of nascent chain assembly) will respond to a gene-end signal placed 80 nt downstream of the le/N junction as efficiently as the transcriptase at natural gene junctions (Le Mercier et al., 2002). Although SeV RdRp that initiates le chains retains this property of a transcriptase, it is relatively non-processive (le/N chains >300 nt long are not detected; Vidal and Kolakofsky, 1989). A similar situation is found for VSV polR mutants, which read through the le/N junction at high frequency (Perrault et al., 1983). In contrast, a bona fide SeV or VSV transcriptase that has initiated the N mRNA produces matures mRNAs at a frequency of >90%. SeV
and VSV RdRp become processive independent of nascent chain assembly upon initiation (and capping) of the N mRNA, and they do so while retaining their ability to respond to stutter signals. How this processivity is acquired is unknown, but work with VSV has indicated that processivity is acquired only after the modification of the nascent mRNA 5’ end when it is approximately 50 nt long (Stillman and Whitt, 1999). Similarly, VSV RdRp does not respond to a polyA/stop site within the first 50 nucleotides of the gene-start site (Whelan et al., 2000). mRNA 5’ end modification has been proposed to be a quality-control checkpoint for VSV mRNA synthesis, similar to that described for nuclear mRNA synthesis (Lis, 1998). This checkpoint could act as well to ensure that other interactions have been formed, which may be required for the transcriptase to negotiate gene junctions (see below).

NNV RdRp scanning

Intrinsic or factor-independent termination sites in E. coli act by forming 2’ structures in the DdRp RNA exit channel that pry open the active site cleft, such that the TEC becomes unstable (Landick, 1999b). Most RNA polymerases are thought to dissociate from their templates upon nascent chain release and loss of the hybrid that tethers it to its template. However, a hallmark of NNV mRNA synthesis is that vRdRp does not dissociate from its template upon release of the mature mRNA. Rather, this transcriptase scans the template (in either direction) for a nearby (re)start site. RdRp scanning has long been suspected for the Pneumovirus RSV, whose M-2 and L genes overlap by 68 nt (Collins et al., 1987) and then shown to occur experimentally (Fears and Collins, 1999). Scanning was also unexpectedly found for the rhabdovirus VS(NJ)V (Stillman and Whitt, 1998). Scanning at gene junctions appears to be a general property of NNV, as the overlapping arrangement of gene ends is found in filoviruses (Sanchez et al., 1993) and some rhabdoviruses (Teninges et al., 1993). RdRp scanning neatly explains why the intergenic regions of many NNV are not conserved (and can vary from 1 to 52 nt in length); this would have little consequence when scanning is efficient. Scanning also explains how vRdRp is released from the template after completing the last mRNA; vRdRp would simply run off the template 5’ end. Finally, scanning may also explain the unusual situation for the rubulavirus SV41, where M gene transcripts are produced exclusively as M–F dicistronic read-throughs, yet abundant monocistronic F mRNA is produced (Tsurudome et al., 1991). In this case, however, scanning would have to occur over much larger distances (i.e., 1400 nt from the start of the M gene).

NNV RdRP scanning is presumably related to the unusual nature of NNV templates that are composed mostly of protein; the SeV template is 97% N protein by weight. Moreover, N is thought to be as much a part of the TEC as P₄–L itself (Perrault et al., 1983; Vidal and Kolakofsky, 1989). SeV and VSV P are found tightly bound to the assembled N subunits of the template (Nass) via its hypervariable, protease-sensitive carboxyl tail (N-tail) (Iseni et al., 1998; Ryan et al., 1990). The N proteins of the Paramyxovirinae are divided into a well-conserved, protease-resistant core (N-core, the amino-terminal 80% of the polypeptide) and the carboxyl tail. N-core by itself can self-assemble and bind RNA, and N-core is sufficient to drive mini-genome replication (a measure of nascent chain assembly). However, templates composed of N-core are apparently inactive for RNA synthesis (Curran et al., 1995a). N-tail is thus required for template function, at least during replication. “Tail” is an apt description of the carboxyl domain of Nass, as this domain was recently shown to be intrinsically disordered (Longhi et al., 2003). Many polypeptides have now been found to contain little or no ordered structure under physiological conditions, and a majority also undergo some degree of folding in the presence of their physiological partner(s). Bacterially expressed measles virus N-tail was found to be intrinsically disordered by biochemical and biophysical criteria, and to undergo a structural transition (a significant increase in α-helical content of the complex) upon binding to PCT (Longhi et al., 2003).

These results explain the protease hypersensitivity of N-tail, and they also help explain how vRdRp might scan the N:RNA for a new start site so efficiently after releasing their transcripts. The functional importance of disorder is thought to reside in the advantages of flexible as compared to rigid structures. In particular, induced folding can result in complexes that are highly specific but of low affinity. For acidic activation domains of transcription factors, affinity is attained via multiple activation domains, often through oligomerization (Melcher, 2000). For SeV, as long as two X domains of P₄ are in continual contact with N-tails, this interaction will be stable. We note that the net change in Gibbs free energy of P₄ cartwheeling on successive N-tails itself would be zero, and if X domain–N-tail and P₄–L interactions are broken and remade simultaneously, the activation energy barriers to P₄ cartwheeling on the template might be minimal. This is exactly what is needed if the X domains of P₄–L are to continually make and break contact with successive Nass subunits within the TEC as the L active site cleft slides along the genome RNA in search of a new start site. Rotation of the coiled coil with the P binding site of L is presumably coordinated with X domain/ N-tail cartwheeling, and the flexible linker domain of PCT may be involved in this. During RNA synthesis, vRdRp translocation is coupled to nucleotide addition (pseudotemplated transcription is of course an exception here) and is unidirectional. During vRdRp scanning, the absence of the hybrid permits vRdRp to traverse the template RNA in both directions. Whether vRdRp scanning requires the input of much chemical energy is unclear. It should be
noted in this context that, like P, N-tail is the site of extensive phosphorylation and de-phosphorylation (Roux and Kolakofsky, 1974; Vidal et al., 1988). The free energy of phosphate hydrolysis can be harnessed to drive vRdRp scanning.

**Recent experiments with VSV**

To date, there is no way of discriminating between transcriptases and replicases until these vRdRp have been committed to their specialized tasks. The question of when this commitment occurs thus remains open. Le RNA is the first product of VSV RNA synthesis in vitro (Colonno and Banerjee, 1976), and whether this short promoter-proximal transcript is synthesized by a transcriptase, a replicase, or a generic vRdRp is at the center of a debate of how vRdRp switches between transcription and replication. There has long been the feeling that these vRdRp may be committed to their specialized tasks before they engage the template (Barr et al., 2002), and evidence has recently been presented that in infected cells, VSV RdRp may initiate N mRNA synthesis directly, without first having initiated le RNA synthesis (Whelan and Wertz, 2002). These authors propose that VSV RdRp can recognize an overlapping set of cis-acting promoter sequences differently, such that its active site is positioned to initiate at the genome 3′end for replication, or at the nearby N mRNA start site (ca. 50 nt downstream) for transcription. How this alternate vRdRp-positioning mechanism would respond to N⁺ availability (Armentier et al., 1985) is not specified.

The notion that vRdRp can initiate N mRNA synthesis independently of prior le RNA synthesis is based on experiments in which UV cross-links are introduced in the le region in a variable manner within viable VSV, as barriers to vRdRp movement during elongation. The effect of these barriers on downstream mRNA synthesis was then examined. When this experiment was carried out in vitro, mRNA synthesis was found to strictly depend on prior le RNA synthesis. These results confirmed earlier work that found that vRdRp initiates exclusively at the genome 3′end in vitro, using reconstituted VSV reactions (Emerson and Wertz, 2002). More importantly, these results show that VSV RdRp cannot scan the template and reinitiate at the N start site upon release of the le chain, at least in vitro. This vRdRp must fall off the N:RNA upon release of the le chain. However, when the same experiment is carried out in infected cells in which N⁺ accumulation is prevented by cycloheximide, exactly the opposite result is found; mRNA synthesis is now independent of the number of UV cross-links in the le region, hence, the conclusion that direct initiation at N must have occurred in vivo (but not in vitro). This conclusion, however, begs the question of why vRdRp cannot initiate at N directly in vitro if it can do so in vivo. Moreover, these experiments do not rule out other possibilities, which are in fact suggested by considering the question of when vRdRp becomes scan-competent during NNV RNA synthesis.

**The problem of determining when vRdRp is committed to its specialized tasks**

When gene-start sites are introduced into the SeV promoter for [−] genome synthesis, this diminishes the use of this 3′end promoter for replication. Similarly, when gene-start sites are removed from the promoter for anti-genomes synthesis in SeV mini-genomes, these mini-genomes compete more effectively with their helper genomes for the limiting supply of replication substrates provided by the latter. These experiments suggest that mRNA start sites and 3′end replication promoters compete for a common pool of vRdRp (Le Mercier et al., 2003). These experiments, as such, do not rule out that committed transcriptases and replicases are present before they engage the template, and that initiation at a gene-start site impedes access of a potential replicase to the genome 3′end, or otherwise impedes replication. However, in mini-genomes in which the gene-start site is displaced further downstream from the genome 3′end (from positions 56 to 68), the gene-start site is equally effective in reducing replication (Le Mercier et al., 2003). The precise position of the gene-start site within the genomic promoter is thus not critical for its ability to reduce 3′end promoter efficiency, and these results disfavor interference models. However, it will be necessary to examine the properties of mini-genomes in which the gene-start site is displaced even further downstream from the genome 3′end, to be more certain that mRNA start sites and 3′end replication promoters compete for a common pool of vRdRp, or generic vRdRp.

Evidence has also been presented that supports the notion that committed transcriptases and replicases are present before they engage the template, but this evidence remains equally open to alternative interpretations. For example, (1) mutant VSV P proteins exist that are defective in transcription and not in replication (Pattnaik et al., 1997). However, as (i) we do not know in what way these P proteins cannot carry out transcription and (ii) at least some of these mutant P proteins are not transcription defective in insect cell extracts (Gupta et al., 2003), further conclusions are difficult to draw.

(2) Insect cell extracts containing coexpressed N, P, and L (when combined with purified N:RNA templates) are more efficient in replication than mixed extracts in which N + L and P + L are coexpressed. Moreover, a tripartite complex containing N, P, and L can be immunoprecipitated from the former cell extracts (Gupta et al., 2003). This has been taken as evidence that there are distinct replicases (containing N as well as P and L) and transcriptases (that do not contain N) in the absence of the template. However, at this stage, the active complex in the triply expressed extract remains uncharacterized, and this extract may contain other complexes with
various combinations of N, P, and L that interact to initiate replication.

(3) VSV le RNA and mRNA synthesis in vitro have different optimal requirements for ATP (Helfman and Perrault, 1988). However, until we know how ATP hydrolysis participates in the initiation of le and mRNA synthesis, again, further interpretation is difficult.

(4) A spontaneous mutation in the VSV N protein (polR) was found that (i) strongly increases vRdRp read-through of the le/N junction, producing incomplete le/N transcripts of various lengths, and (ii) these mutants synthesizes more N mRNA than le RNA in vitro, in contrast to the wild-type reaction (Chuang and Perrault, 1997). This is the best evidence so far that vRdRp can directly initiate the N mRNA synthesis. However, other evidence (cited above) indicates that (wild type) VSV RdRp cannot directly initiate at the N mRNA in vitro, at least when the N\textsuperscript{nos} of the template is mutated. These mutants synthesizes mature polyadenylated mRNAs and negotiations gene junctions efficiently, and thus synthesizes each mRNA in turn.

4. The bottom cartoon shows the replication elongation complex, which can be formed from either of the first two TECs by the coupling of RNA synthesis and assembly. The coupling is proposed to prevent loosening of the L active site cleft–hybrid interaction that would be required to permit realignment of the two chains of the hybrid during pseudo-templated transcription. This vRdRp would be extremely processive, as it does not respond to stutter signals (or does so very rarely). There would be no need for P–N-tail interactions to tether vRdRp to its template during replication. Nevertheless, as N-tail is required for template function (see text), it presumably is involved in other tasks as well, for example, coordinating the proposed transient displacement of N\textsuperscript{nos} from the template RNA.

A general model for the control of NNV RNA synthesis

In the end, there is as yet no compelling evidence for or against the existence of committed transcriptases and replicases before they engage the template. It is nevertheless useful to consider a general model of NNV RNA synthesis based on the premise of a generic vRdRp that initiates the genome 3'end. This model can accommodate all the available information, including the results of Whelan and Wertz. Four forms of vRdRp are proposed, two forms that are poorly processive, one incompetent and the other competent to scan the template for a new start site, a transcriptase, and a replicase, and these are shown in cartoon form in Fig. 3.

1. The top cartoon shows the TEC shortly after initiating at the genome 3'end. This vRdRp is poorly processive because it readily responds to pause sites that open the active site cleft of L, and this promotes release of the nascent RNA, and is scanning incompetent as P–N-tail interactions have not yet been formed. This vRdRp falls off the template upon nascent RNA release (and joins the polR mutants have wild-type growth phenotype (Perrault et al., 1983) as cited as evidence that VSV RdRp can directly initiate the N mRNA in vitro (at least in polR mutants), as this can explain the absence of an obvious growth defect in VSV polR. However, efficient vRdRp scanning for the N start site can equally explain the absence of an obvious growth defect.

2. If P–N-tail interactions are formed before the nascent le chain is released (second cartoon), this probably does not affect vRdRp processivity, but it prevents the loss of P\textsubscript{4}–L from the N:RNA upon RNA release, and thus permits vRdRp scanning for a nearby start site. If this situation predominates for VSV in vivo, N mRNA synthesis will be independent of precise le RNA synthesis. RdRp scanning should not be impeded by UV cross-links; RNA synthesis stops at cross-links because the polymerase cannot incorporate an NTP opposite the modified base. The coupling is proposed to prevent loosening of the L active site cleft–hybrid interaction that would be required to permit realignment of the two chains of the hybrid during pseudo-templated transcription. This vRdRp would be extremely processive, as it does not respond to stutter signals (or does so very rarely). There would be no need for P–N-tail interactions to tether vRdRp to its template during replication. Nevertheless, as N-tail is required for template function (see text), it presumably is involved in other tasks as well, for example, coordinating the proposed transient displacement of N\textsuperscript{nos} from the template RNA.

Two P tetramers are proposed to cooperate for efficient SeV RNA synthesis; the P\textsubscript{4}–L polymerase itself, along with an upstream P\textsubscript{4} for the transcriptase (not shown), and a P\textsubscript{4}–N\textsuperscript{−} complex for the replicase. Only the carboxyl half of the leading P\textsubscript{4} is shown in the first three cartoons (and only two of the four chains are shown for clarity), and the amino-terminal half of the trailing P\textsubscript{4} (in complex with N\textsuperscript{−} in the act of assembling the nascent chain) is shown as well in the replication complex. This already-crowded representation is likely to be oversimplified, as there is new evidence that L proteins can directly interact with each other, and that this interaction helps RNA synthesis (Smallwood et al., 2002). Although the cartoon in Fig. 3 implies otherwise, it is not impossible that the trailing P\textsubscript{4} for replication is resolved with both N\textsuperscript{−} and L, as these binding sites are at opposite ends of P\textsubscript{4}. Two L proteins could interact during RNA synthesis, the trailing (presumably non-polymerizing) L could help stabilize the leading L as both P tetramers rotate on/in their respective L proteins and the entire replication elongation complex (P\textsubscript{4}–N\textsuperscript{−}–L plus P\textsubscript{4}–L) rolls along the template.

The above model can form the basis for new experiments to examine NNV RNA synthesis. We can now look forward to studies that examine vRdRp scanning that is unique to NNV, and which may shed light on whether this process also occurs at the genome 3'end.
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