INITIATION AND REGULATION OF PARAMYXOVIRUS TRANSCRIPTION AND REPLICATION

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Abstract

The paramyxovirus family has a genome consisting of a single strand of negative sense RNA. This genome acts as a template for two distinct processes: transcription to generate subgenomic, capped and polyadenylated mRNAs, and genome replication. These viruses only encode one polymerase. Thus, an intriguing question is, how does the viral polymerase initiate and become committed to either transcription or replication? By answering this we can begin to understand how these two processes are regulated. In this review article, we present recent findings from studies on the paramyxovirus, respiratory syncytial virus, which show how its polymerase is able to initiate transcription and replication from a single promoter. We discuss how these findings apply to other paramyxoviruses. Then, we examine how *trans*-acting proteins and promoter secondary structure might serve to regulate transcription and replication during different phases of the paramyxovirus replication cycle.

INTRODUCTION

The family *Paramyxoviridae* is large and diverse. It encompasses viruses that infect reptilian, avian and mammalian hosts, and includes a number of human pathogens, such as respiratory syncytial virus (RSV), mumps (MuV), measles (MeV), parainfluenza viruses (PIV 1 to 5), and the newly emerged Nipah and Hendra viruses. The family is divided into two subfamilies, the *Pneumovirinae and Paramyxovirinae*, which contain two and seven genera, respectively (1). The paramyxoviruses have a single stranded, negative sense RNA genome, and so are members of the non-segmented, negative sense (NNS) RNA virus order. The paramyxoviruses also share a similar (although not identical) cohort of genes as each other. During their replication cycle, the viral genome is transcribed to produce subgenomic, capped and polyadenylated mRNAs and replicated to produce encapsidated antigenome and genome RNAs (2). Despite the fact that approximately two-thirds of the paramyxovirus

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genome encodes proteins involved in performing and regulating gene expression and genome replication, paramyxoviruses only encode one polymerase. This raises questions that have puzzled researchers for more than three decades, namely how does the polymerase become committed to either mRNA transcription or genome replication, and how can these processes be differentially regulated? In this review, we attempt to address these questions. The review is divided into three parts. Part 1 presents an overview of paramyxovirus transcription and replication and discusses previously proposed models. In part 2, we describe relatively new findings regarding RSV transcription and replication, propose a revised model that fits these data, and discuss if this revised model can be applied across the paramyxovirus family. With models to describe possible mechanisms of transcription and replication initiation, it is possible to consider how these processes might be regulated during infection and in part 3 we describe information available regarding regulation of transcription and replication, highlighting similarities and differences across the family.

PART 1: PARAMYXOVIRUS TRANSCRIPTION AND REPLICATION

Overview of paramyxovirus transcription and replication

The general strategy of paramyxovirus transcription and replication is similar to that of other NNS RNA viruses (2, 3) and much of what we know has been as a result of studies on another virus in the order, vesicular stomatitis virus (VSV), a member of the family *Rhabdoviridae*. However, there is a considerable body of research on paramyxoviruses, as reviewed previously (2), and this is described below.

The core polymerase is a complex of two proteins, the large polymerase subunit L, which contains the enzymatic domains involved in RNA synthesis, capping and cap methylation, and the phosphoprotein, P, which is an essential cofactor (4) (5) (6) (7). The polymerase executes transcription and replication by recognizing and responding to a number of essential *cis*-acting elements within the virus genome (Figure 1). At the beginning and end of each gene are short (~10–13 nt) conserved signals termed *gene start* (*gs*) and *gene end* (*ge*) signals, respectively, and between each gene is a short, non-transcribed intergenic region. At the 3′ end of the genome, before the first gene, is a *leader* (*le*) promoter region, which is ~40–55 nt long, and at the 5′ end is a trailer region which is variable in length, depending on the virus (2). An important feature of the genome template is that it is associated along its length with an interlinking polymer of nucleoprotein (N) to form a helical nucleocapsid, such that *cis*-acting RNA elements are buried within the N-RNA structure (8–10). Most likely for this reason, when the polymerase transcribes the genome to produce mRNAs, it cannot access individual genes independently. Instead, it first engages the template at or near the 3′ end of the genome, within the *le* promoter. The polymerase then moves along the genome, presumably with the N subunits of the nucleocapsid being displaced and replaced as the polymerase passes by. As the polymerase proceeds, it responds to the *gs* and *ge* signals it encounters to generate the subgenomic mRNAs: at a *gs* signal, the polymerase initiates mRNA synthesis (opposite the first nucleotide of the *gs*) and at the *ge* signal, it releases the RNA (2). The polymerase can then scan the intergenic region to locate the next *gs* signal and begin mRNA synthesis of the next gene (11). This allows the polymerase to generate subgenomic RNAs. The mRNAs are also modified to contain a 5′

methyl cap and 3′ poly A tail. Work with VSV and the paramyxovirus, Sendai virus (SeV) indicates that the complement of the *gs* signal, which lies at the 5′ end of the mRNA, contains a signal that directs the capping reaction and methylation of the cap (12) (13, 14). The *ge* signal contains a poly U tract, and it is thought that stuttering of the polymerase on this U-stretch leads to polyadenylation of the mRNA (3). Similarly to cellular capping, there is evidence that addition of the cap is important to allow the transcribing polymerase to transition into an elongation mode: in the case of RSV, if capping is inhibited, the polymerase aborts RNA synthesis approximately 45–50 nt after initiating at the *gs* signal (15).

To replicate the genome, the polymerase also initiates RNA synthesis at the *le* promoter. In this case, it must initiate precisely opposite the first nucleotide of the template. During replication, the polymerase does not respond to the gene junction signals, but instead elongates the nascent RNA along the complete length of the genome to produce a positive sense antigenome. The 3′ end of the antigenome contains the complement of the trailer, referred to here as *tr* promoter. The *tr* promoter in turn signals the polymerase to initiate and perform genome RNA synthesis. The antigenome and genome RNAs are not capped, but instead are encapsidated with N protein, which is delivered to the elongating RNA in a complex with P (N_0P, where N_0 is a monomer of N) (5). It is thought that concurrent encapsidation causes the polymerase to enter a super-processive mode, allowing it to disregard the *ge* signals and extend to the end of the template (16) (17) (18). Encapsidation initiation appears to be dependent on *cis*-acting elements in the *le* and *tr* promoters (18). These elements could function in the context of the promoter within the template strand, to recruit a specific pool of polymerase that is capable of delivering N protein onto the RNA that it is synthesizing. Alternatively, they could function at the 5′ end of the nascent RNA product to signal an initial nucleation event that begins polymerization of N protein onto the growing RNA chain.

What emerges from this description of transcription and replication is that the *le* and *tr* promoters and the *gs* signals are all multifunctional entities, which are not only important for directing initiation of RNA synthesis, but also directing modification of the RNA products. These modifications enable the polymerase to elongate the RNA and also serve to protect the RNA from nucleases. This multifunctional nature of the *cis*-acting signals complicates analysis of the initial events in RNA synthesis, particularly in cell-based assays in which abortive (i.e. prematurely released), unmodified RNAs might be unstable, and this is the reason why understanding mechanisms underlying transcription and replication initiation and regulation has proven difficult.

NNS RNA virus transcription and replication initiation models

A complexity in understanding mechanisms by which the polymerase is coordinated between transcription and RNA replication is that it is difficult to conclusively define where transcription begins on the viral genome. The mRNA for the first protein-coding gene is initiated at the first *gs* signal (at ~ nt 40–55), but how the polymerase accesses this signal has been the focus of debate (19–21). Three models have been proposed to explain how this

could happen, based largely on studies with paramyxoviruses (mainly SeV, PIV-3 and RSV) and VSV.

Model 1: According to this model, transcription and replication are both initiated in exactly the same way, opposite the first nucleotide of the *le* promoter (16, 22). In its simplest version, this model postulates that a single pool of polymerase can initiate both processes. The polymerase begins transcription by first synthesizing an RNA transcript complementary to the *le* region (le+). At or near the end of the *le,* this RNA is released and the polymerase is able to scan the template to locate the first *gs* signal and reinitiate RNA synthesis. It is then committed to mRNA transcription. Replication would occur when N protein accumulates to a sufficiently high concentration to initiate encapsidation very quickly after initiation of le+ synthesis. If the le+ RNA becomes encapsidated before being released, this stabilizes the polymerase-template-nascent RNA complex and commits the polymerase to replication. As described in detail in later sections, this model is the most consistent with what is known regarding paramyxoviruses.

Model 2: The second model proposes that there are two pools of polymerase, a transcriptase and a replicase that consists of L-P in complex with different proteins (20, 21, 23). According to this model, the replicase initiates at the $3'$ end of the genome, whereas the transcriptase initiates directly at the first *gs* signal and then proceeds to synthesize capped and polyadenylated mRNAs. This model is consistent with results from a number of studies with the rhabdovirus VSV (24) (25) (23) (26).

Model 3: Finally, a universal model has been proposed which attempts to tie together the two models described above (19). This model postulates that N_0P is required for RNA synthesis initiation from position 1 of the template, to aid initiation from the $3'$ end of the linear genome. According to this model, when the virus first enters the cell, when N_0P would not be present, the polymerase cannot initiate at the $3'$ end, and instead relies on cellular factors to help the polymerase access the first *gs* signal to begin transcription. However, once N_0P has been synthesized, the polymerase instead switches to initiating at the $3'$ end. When N_0P is present at a low level, the polymerase synthesizes a le+ RNA, but because this is not encapsidated, the polymerase aborts RNA synthesis and reinitiates mRNA synthesis at the *gs* signal. At high levels of N_0P, the le+ transcript becomes encapsidated and the polymerase is committed to encapsidation. This model is supported by the finding that in an *in vitro* SeV experiment, low concentrations of N_0P enhanced transcription, whereas high concentrations caused a shift from transcription to replication. It is also based on results from VSV UV mapping experiments, which showed that the polymerase initiated transcription at the $3'$ end of the *le* if the nucleocapsid template was derived from a virus particle, but at the *gs* signal in infected cells (26). However, arguments have been made against this model (20, 21) and more recent studies with VSV and RSV have shown that the polymerase can initiate opposite nucleotide 1 of the template independently of N_0P (6, 27).

One problem with trying to develop a universal transcription/replication model to explain all the data available for VSV and the paramyxoviruses is that there are differences between the viruses and they might not utilize a common mechanism. One difference that is of particular

significance is that the organization of transcription signals in the *le* promoter is different in the paramyxoviruses than in VSV, with the paramyxovirus transcription signals being relatively circumscribed (as described below) and the VSV transcription signals existing throughout almost all of *le* (25, 28) (Figure 2). This distinction could reflect the presence of a 3′ terminal transcription start site on the paramyxovirus promoter versus an internal start site on the VSV promoter.

PART 2: A REVISED MODEL FOR PARAMYXOVIRUS TRANSCRIPTION AND REPLICATION INITIATION

If the data regarding VSV are put aside, model 1, described above, is highly consistent with almost all available data regarding the paramyxoviruses. However, based on our recent studies with RSV we propose a slightly revised version of this model, which is extremely simple and is supported with experiments performed *in vitro*, using a minigenome system and analyzing RNA from RSV infected cells. This model is also consistent with what is known about mechanisms of RNA synthesis initiation by RNA dependent RNA polymerases of other RNA viruses.

Organization of the RSV *le* promoter

The RSV *le* region is 44 nt in length and is followed by a 10 nt *gs* signal for the first gene (29). Mutation analysis of the RSV *le* region, using a minigenome system, has shown it can be divided into three segments. The 3′ terminal ~12 nt are required for both transcription and RNA replication, nucleotides 13 to ~36 are not required for transcription, but are required for replication, and a U-rich region at the end of *le*, nucleotides ~37–44, increases transcription efficiency, but is not essential for transcription; this region has no effect on replication (18). The first 13 nt were shown to be sufficient to signal RNA synthesis initiation, indicating this region contains the core promoter (30, 31). This core promoter is capable of recruiting both transcription- and replication-competent polymerase, suggesting that it either recruits a single pool of polymerase that subsequently becomes differentiated, or has the capability to recruit both transcriptase and replicase forms of polymerase (30). Nucleotides located at positions 3, 5, 8, 9, 10 and 11 of the *le* region are of particular significance: if any of these nucleotides was mutated, both transcription and RNA replication were completely, or almost completely abrogated (32). Thus, the 3′ end of the RSV *le* region contains a core promoter necessary for signaling transcription and replication initiation (Figure 3).

Transcription initiation in RSV

A hint as to how transcription is initiated came serendipitously from minigenome studies examining how the RSV polymerase is able to initiate antigenome synthesis opposite nt 1 of the *le* (described in more detail below). In these studies, it was noticed that some RNA appeared to be initiated at position 3 of the *le* promoter, in addition to the expected initiation site at position 1 (33). Primer extension analysis of RNA isolated from RSV infected cells confirmed this finding, clearly showing that RNA is initiated from both the position 1 and 3 sites in the *le* promoter. Indeed in infected cells, RNA initiated from position 3 was significantly more abundant than RNA initiated from position 1, suggesting that initiation

from position 3 is the dominant initiation event (31). Inspection of the *le* core promoter sequence showed that it bears very strong resemblance to one of the RSV *gs* sequences, with the essential nucleotides from positions 3–11 of *le* aligning perfectly (Figure 3). These findings suggest that nt 3–11 of the *le* promoter element function similarly to a *gs* signal and position the polymerase to initiate opposite position 3. Further analysis of the RNAs generated from the position 1 and 3 initiation sites indicated that the RNA initiated from position 1 was elongated relatively efficiently, consistent with it being a replication product, as expected. In contrast, RNA initiated from position 3 was short and heterogeneous in length, with the majority of transcripts varying from approximately 20–25 nucleotides long (31). Why these transcripts were released after such a short distance, whereas mRNA transcripts are elongated, is not completely clear.

These findings suggest a very simple and logical model for transcription initiation, illustrated in Figure 4A. According to this model, the polymerase interacts with nucleotides 3–11 of the *le* region. This core promoter positions the polymerase so that its active site is opposite nucleotide 3, similarly to the way in which a *gs* signal positions the polymerase to reinitiate mRNA synthesis at a gene junction. The polymerase initiates RNA synthesis at position 3, but is unable to enter a stable elongation mode and releases the RNA after approximately 25 nucleotides. Synthesis of this abortive RNA allows the polymerase to break contacts with the promoter. Having released the abortive RNA, the polymerase can scan the template (as it does at the gene junctions) and locate the *gs* signal of the first gene, aided by the U-rich region at the end of *le*. Here it can reinitiate RNA synthesis, extend the RNA beyond 25 nucleotides, and cap the 5′ end. This commits the polymerase to transcription.

Replication initiation in RSV

While the results described above provide a compelling explanation for how RSV transcription is initiated, they raise the question: if the *gs*-like element in the *le* core promoter positions the polymerase to initiate opposite nucleotide 3 of the genome, how does the polymerase initiate replication from position 1? In considering this, it is helpful to appreciate that replication of a linear viral genome presents a challenge to a polymerase. The reason being that RNA synthesis initiation is a complex enzymatic process, in which the polymerase and template must stabilize and position the two incoming NTPs sufficiently well to allow formation of the first phosphodiester bond. This is even more challenging at the end of a linear template because there is limited RNA template for the polymerase to associate with. For this reason, viruses with linear genomes have evolved complex mechanisms of replication initiation that help provide stability to the initiating complex (34). An initiation processes that has been particularly well characterized is that utilized by phi6 bacteriophage. In this case, the polymerase enters internally on the template and then ratchets in a 5′ to 3′ direction to reposition the active site opposite nucleotide 1 (35). Based on this model and what is known regarding other RNA viruses, a logical mechanism for RSV replication initiation would be internal entry at the promoter element at nt 3–11 followed by events to enable initiation at position 1. Evidence as to how this might happen during RSV replication came from studies probing the role of the first nucleotide in determining the replication start site (36). It was found that if the first nucleotide of the *tr*

promoter was mutated, the replication product was restored to wild type sequence in a single round of replication. These findings indicate that when the polymerase initiates RNA replication, it is able to select the initiating ATP independently of the template. Similar results were obtained for the RSV *le* promoter, and in this case there was evidence that the second nucleotide, a cytidine, was also selected in a template independent manner (33). Studies with rotavirus and Dengue virus, have shown that their viral polymerases can self generate a dinucleotide primer (37, 38). Taken together, these findings suggest a replication initiation model in which the polymerase becomes loaded with the first two nucleotides of the replication product, ATP and CTP, independently of the template and self-generates a primer. It could then bind the core promoter element, ratchet backwards by one nucleotide to position the 5′ AC primer opposite nucleotides 1 and 2, and then use the primer to initiate RNA synthesis. This would give the appearance that replication is initiated from position 1, although the first templated nucleotide insertion would be opposite position 3 (Figure 4B).

There are also data to suggest a mechanism for how the RNA initiated at position 1 can become encapsidated. Studies performed *in vitro*, using purified, recombinant RSV L-P complexes, have shown that they initiate RNA synthesis at both the position 1 and 3 sites of the promoter (39). Thus, in the case of RSV, N protein is not required for replication initiation at position 1, indicating that in infected cells N protein becomes recruited after initiation of RNA synthesis. This suggests that encapsidation begins by N binding to a signal at the 5′ end of the nascent RNA. In a minigenome experiment, in which a position 3C-to-U substitution was introduced into the *le* promoter to create a promoter sequence: 3′ UGUGCUUUU (the 3C-to-U substitution is underlined), a high level of full-length encapsidated replication product was generated from position 3 (33). This suggests that 5′ AC at the end of the RNA correlates with encapsidation and replication elongation. Based on these findings, we propose that when the polymerase initiates antigenome synthesis at position 1, the nascent RNA that is synthesized contains *cis*-acting elements that signal initiation of encapsidation, with 5′ AC playing a key role.

Together, these proposed mechanisms for transcription and replication initiation integrate to form a cohesive model. The binding site for the RSV polymerase is located at nt 3–11 of the *le* region and RSV L-P complex alone, with no other viral proteins, is able to interact with this signal to initiate RNA synthesis. Most frequently, the polymerase initiates directly at position 3. Because the RNA initiated at this site lacks a complete encapsidation signal, the polymerase is unable to enter an efficient elongation mode and generates abortive transcripts. Having released the le+ transcript, the polymerase can scan forward and locate the *gs* signal at position 45 to reinitiate RNA synthesis and become committed to transcription. Less frequently, the polymerase is able to become loaded with ATP and CTP and self-generate a primer. When this happens the polymerase can generate an RNA that is apparently initiated at position 1, and engage in RNA replication.

Can the RSV transcription and replication initiation model be applied to other paramyxoviruses?

As described above, paramyxoviruses are divided into the paramyxovirinae and pneumovirinae. These two sub-families clearly share many similarities in transcription and

replication mechanisms, but they differ significantly in the organization of their promoters. Therefore, is it reasonable to think that the model proposed for RSV, a pneumovirus, will apply to other paramyxoviruses?

There are three differences in the organization of the pneumovirus and paramyxovirus promoters. First, whereas the RSV promoters are contained entirely within the *le* or *tr* regions the promoters of the paramyxovirinae are bipartite. One promoter element (promoter element I) lies within approximately the first 12 nt of the template (40–42) and is sensitive to mutation at almost all positions (40), similarly to the core promoter of RSV. The other (promoter element II) lies further downstream, between nucleotides 79 and 96 of the respiro- and morbilliviruses and between 77 and 94 of the rubulaviruses, and is a more simple motif repeated three times, either $(CNNNNN)_3$ or $(NNNNGC)_3$ (40–46). Second, the paramyxovirinae promoter elements need to be positioned in the appropriate phase relative to N protein and aligned adjacent to each other on the helical nucleocapsid to be functional (43, 44, 47)(see Figure 8 in reference 43 for an illustration of the promoter alignment on the nucleocapsid helix). In contrast N phasing has no effect on the efficiency of the RSV promoters (48). Third, whereas the RSV promoter must lie near the 3′ end of the template to be functional, this is not the case in the paramyxovirinae (30, 47, 49). Together, these data suggest that the structure that the polymerase recognizes to form its initial contacts with the promoter is distinct between the pneumo- and paramyxovirinae, with the pneumovirus polymerase relying, at least in part, on the unique structure that would be present at the 3′ end of the nucleocapsid, and the polymerase of the paramyxovirinae recognizing promoter bases by virtue of their positioning within the N-RNA helix. However, having bound the promoter in the nucleocapsid, it would be expected that the N-RNA structure would become relaxed at the initiation site to allow the RNA into the polymerase active site. Thus, in both the pneumo- and paramyxovirinae the next step would be expected to involve a direct polymerase-RNA interaction.

Aside from this difference in initial promoter recognition, other aspects of transcription initiation are conserved between pneumo and paramyxovirinae. Aside from the necessity for a core promoter, the *le* sequence prior to the first *gs* signal is not essential for transcription (50–52). In the paramyxovirinae, the first *gs* signal lies on the opposite face of the helical nucleocapsid from the promoter elements, suggesting that it is not seen in conjunction with them (53), and like the RSV *gs* signal, it can function if it is placed at varying distances relative to the promoter, although its natural position is optimal (51, 52, 54, 55). Finally, it has been known for a long time that the SeV and MeV polymerases synthesize a heterogeneous population of abortive transcripts from a site at, or near, the 3′ end of *le* (16, 56, 57). These data are very consistent with what has been found for RSV, and with a model that the polymerase initiates transcription from the 3′ end of the promoter.

The question that remains is: does the polymerase of the paramyxovirinae use a *gs*-like sequence at the 3′ end of the *le* promoter for initiation, and are transcription and replication initiated from two different start sites? Figure 5 shows the 3′ terminal promoter elements (promoter element I) and *gs* sequences for a representative virus species of each paramyxovirus genus. The most striking observation is that while the promoter sequences do not obviously align, all of them begin with 3′ UG, meaning that the replication product is

initiated 5′ AC. This suggests that the mechanism that RSV uses to select initiating NTPs to begin replication is probably conserved throughout the family. On the other hand, identity between the promoter and *gs* signals is not that obvious in the paramyxovirus subfamily. However, as noted above, these signals are multifunctional and differences between them might be important for determining the fate of the RNA products (e.g. whether they will be capped, encapsidated, or aborted). Indeed, we only noticed the identity between the RSV promoter and L *gs* signal because the 3′ terminal *le* nucleotides required specifically for transcription (rather than replication) had been identified, but this information is not available for the paramyxovirinae. Thus, the promoters and *gs* signals of the paramyxovirinae might contain a conserved polymerase binding and initiation signal, even if not readily apparent. With this in mind, a closer inspection of the sequence alignments suggests that there might be a *gs*-like signal in the paramyxovirus promoters. By color coding the pyrimidine and purine nucleotides, it is apparent that the promoters and *gs* signals both contain a motif consisting of a pyrimidine stretch, followed by 1–3 nt of variable sequence that typically contains a purine, followed by a second pyrimidine stretch. This motif typically aligns slightly internally on the promoter, although the optimal alignment varies between viruses. This observation, coupled with the fact that apparently all paramyxoviruses begin replication with 5′ AC suggests that the members of the paramyxovirus family share a common mechanism of replication initiation, in which the polymerase binds to a pyrimidine-rich motif, slightly internally on the template, and perhaps self-generates a primer to initiate opposite position 1.

Although the sequence alignments in Figure 5 suggest that there may be a *gs*-like sequence within the promoters of the paramyxovirinae, it is difficult to conclude that their polymerase initiates transcription internally, in a similar fashion to RSV. A method to identify initiation sites experimentally is to perform primer extension analysis on RNA from virus-infected cells. However, to detect RNA initiated from position 3 of the RSV *le* promoter, we needed to use a primer that hybridized very close to the 5′ end of the RNA to detect the short abortive transcripts. We could find no evidence in the literature of a similar analysis for the paramyxovirinae. Sequence alignment also does not give a clear indication of an internal initiation site, particularly given that RNA polymerases typically initiate synthesis with a purine residue and so initiation opposite position 2 or 3 of the promoter would be unlikely. In the absence of any data indicating the presence of an internal initiation site for transcription, it is probably prudent to assume that the paramyxovirinae differ from the pneumovirinae in terms of the exact transcription initiation site, and instead initiate both transcription and replication in the same way, opposite the first nucleotide of the *le*. As described below, the model that the polymerase of the paramyxovirinae initiates transcription opposite position 1 is supported by data regarding transcription and replication regulation, which appears to be slightly different in RSV versus SeV and measles virus, as described below.

PART 3: REGULATION OF PARAMYXOVIRUS POLYMERASE ACTIVITY

Kinetics of paramyxovirus RNA synthesis

Although paramyxoviruses are relatively simple viruses, it would be expected that they would have evolved mechanisms to regulate RNA synthesis to maximize use of available templates and avoid production of non-functional and potentially detrimental RNAs (e.g. unencapsidated negative sense RNA could hybridize to mRNAs). Consistent with this idea, studies in which RSV, measles and SeV RNAs were examined over a single cycle of infection have provided evidence for temporal control of RNA synthesis (58–60). In the case of measles virus, where quantitative analysis of each RNA species was performed, it was found that mRNA accumulated in an exponential fashion from 2–24 hours post infection, at which point it reached a plateau. In contrast, genome and antigenome levels remained at a low level until 12 hours post infection, then increased exponentially at an equivalent rate until 24 hours post infection; thereafter, the rate of replicative RNA accumulation decreased, but the rate of genome accumulation exceeded that of antigenome (59). These findings are essentially similar to those with RSV (58). The experiments with SeV measured antigenome and genome RNAs, specifically. In this case, it was found that in the initial stages of infection, the ratio of antigenome to genome RNA increased significantly compared to input virus, reflecting very high use of the *le* promoter and synthesis of positive sense antigenome RNA, but at later stages, the ratio reversed due to very active production of genome RNA from the *tr* promoter (60). These studies indicate that there are at least two points in which paramyxovirus gene expression and genome replication are regulated: first, between transcription and replication, and second, between positive and negative sense RNA synthesis from the *le* and *tr* promoters, respectively. The mechanisms by which these two transitions might be regulated are described below. In addition, other regulatory mechanisms might come into play to temper RNA synthesis, to avoid over-stimulation of the innate immune response, and/or to ready nucleocapsids for packaging (61–67).

Transition from mRNA to antigenome synthesis

The factor responsible for "switching on" replication during paramyxovirus infection is the viral N protein, which is required to encapsidate the replication product as the RNA is being synthesized (5, 68). Although the N protein is required to enable replication of all paramyxoviruses, the extent to which it regulates a switch between transcription and replication appears to differ depending on the virus. In experiments using the RSV minigenome system it was shown that while increasing the level of N (or N and P) resulted in an increase in antigenome synthesis, there was no apparent inhibition of transcription, even at very high levels of N protein (69). The reason why RSV transcription is not affected by N protein concentration is now clear: the dominant initiation event from the *le* promoter is from position 3, not position 1 (31). There is no evidence that any RNA initiated at position 3 can be elongated into a replication product. This means that transcription would always be the dominant initiation event, regardless of how much N protein is available for encapsidation. However, a corollary of the RSV transcription/replication model is that if the polymerase initiates at position 1, but N levels are low, then this polymerase could abort RNA synthesis after ~25 nt and engage in transcription. This would mean that N protein levels would control a switch between transcription and replication, but only for the

relatively small proportion of polymerase that happens to initiate at position 1, not polymerase that initiates at position 3. Thus, in the case of RSV, rather than thinking of transcription switching to replication, it is more appropriate to think in terms of transcription being a constitutive event, and replication being switched on once N has accumulated, with just a small cost to transcription.

If transcription and replication were both initiated from position 1 of the *le* promoter by a single pool of polymerase, then these would be expected to be interchangeable events. This indeed seems to be the case for at least two of the paramyxovirinae. In experiments performed *in vitro* with SeV and *in cellulo* with measles virus, increasing the level of N protein led to an increase in replication and a discernable decrease in transcription (19, 59). This difference in the effect of N protein on transcription between RSV, MeV and SeV provides evidence that different paramyxoviruses might initiate transcription using different initiation sites.

Transition from positive to negative sense RNA synthesis

As described above, at a late stage of infection there is a transition from synthesis of positive to negative sense RNA (58–60). This transition ensures accumulation of a high proportion of genome sense nucleocapsids for packaging into virus particles (70). One plausible mechanism by which this transition could occur is by action of a *trans*-acting protein that is either absent, or at only a low level in virus particles, but which accumulates over the course of infection. There does not appear to be a common protein to control this transition in the paramyxoviruses and consideration of this provides further evidence of how divergent the paramyxoviruses are.

The protein most likely responsible for regulating a shift from positive to negative sense RNA synthesis in the pneumovirinae is the M2-2 protein. M2-2 is expressed from an alternative and downstream open reading frame in the M2 gene (71, 72). If M2-2 is over-expressed, it inhibits all RNA synthesis (72–74). However, examination of the kinetics of RNA accumulation in a recombinant RSV containing a deletion of M2-2 suggests the protein specifically inhibits positive sense RNA synthesis (58). How M2-2 functions to accomplish this is not known, but it binds to the L protein (74), and in this and other respects, shares similarities with a protein encoded by some of the paramyxovirinae, the C protein.

A number of viruses within the paramyxovirinae have the capability to express C protein from an alternative open reading frame within the P gene (2, 75). The SeV C protein has been studied extensively. It is a multifunctional protein, which plays a role in allowing the virus to evade the host immune response and inhibit apoptosis. However, it also plays an important role in modulating viral RNA synthesis. If the C protein is over-expressed it inhibits all RNA synthesis (76, 77). However, at normal expression levels, the protein functions in a promoter specific manner, inhibiting RNA synthesis from the *le* promoter, but not from the *tr* promoter (49, 76–78). Indeed, in recent work using recombinant viruses lacking C protein, it was shown that the *le* promoter is a stronger promoter than the *tr*, contrary to what had been thought previously (60), and that C protein is the key factor that ensures that genome RNA is more abundant in cells than antigenome (60, 70). C protein

binds to the L subunit of the polymerase and the strength of this interaction correlates with the ability of C protein to exert its inhibitory effect (79, 80). Thus, C protein acts directly to modify the polymerase complex and affect its behavior at the *le* promoter.

The ferla, avula and rubulavirus genera of the paramyxovirinae do not express a C protein (75) and it is not clear if they have a functional homolog. However, one possible candidate is their V protein. The V proteins are multi-functional proteins, expressed from the same gene that expresses P, by almost all paramyxovirinae. The V proteins can inhibit RNA replication by binding N protein (64), but the V proteins of some viruses have also been shown to bind to L (65, 81). In the case of PIV-2, a rubula virus, L-V interaction was responsible for inhibiting RNA synthesis in a minigenome system (65). Thus, it is possible that V might bind L to fulfill a similar function as C protein in some of the paramyxovirinae.

Finally, another factor that could play a role in regulating the polymerase between different RNA synthesis activities is P protein phosphorylation. The P proteins are heavily phosphorylated (as indicated by their name), but in most cases in which the effect of phosphorylation status has been examined, there has been either inconsistent results between *in vitro* assays and recombinant virus, or no detectable effect (82–87). However, in experiments with the rubulaviruses, PIV5 and mumps virus, regulatory effects of phosphorylation have been clearly demonstrated (88–90). In addition, sumoylation been shown to impact RNA synthesis (91). Given these findings, it would be interesting to determine to what extent phosphorylation and other post-translational modifications can play a role in temporal regulation.

Can RNA secondary structure in the promoters play a regulatory role?

As described above, it is well established that the genome and antigenome RNAs of the paramyxoviruses are encapsidated along their length with N protein at all times of infection. Thus, these RNAs are not expected to form secondary structures, and *cis*-acting signals are comprised entirely of primary sequence. However, recent work with RSV indicates that the *tr* promoter might not be completely encapsidated at all times, suggesting the possibility that promoter RNA secondary structure could play a regulatory role. It was found that antigenome sense RNA isolated from RSV infected cells is heterogeneous, containing one to three nucleotide additions at the 3′ end (6). Nucleotide addition was shown to occur because the *tr* RNA could adopt a secondary structure that allowed limited templated nucleotide addition. A similar phenomenon has been described for Borna disease virus, another NNS RNA virus (92). In the case of Borna disease virus, the 3′ nucleotide addition enables the virus to generate replicative RNA lacking a 5′ triphosphate, allowing it to escape immune surveillance (93), but this is not the case in RSV and so the significance of the nucleotide addition is not clear. However, it was found that in the context of a naked RNA template, the three-nucleotide addition significantly inhibited RSV *tr* promoter activity (6). Thus, one possible explanation for this finding is that the capacity for forming a secondary structure and adding nucleotides onto the *tr* promoter serves as a mechanism for sensing available N protein. According to this hypothesis, if N protein levels are low as the polymerase completes antigenome synthesis, encapsidation of the RNA lags behind and there is the opportunity for the RNA to form a secondary structure and additional nucleotides to be

added onto the 3′ end of the *tr* promoter. In this case, the newly synthesized antigenome RNA cannot act as a template to produce genome RNA, and polymerase remains available to reinitiate RNA synthesis on available genome templates, resulting in increased N protein expression. On the other hand, if N protein is abundant, the newly synthesized antigenome RNA is encapsidated as it is synthesized and does not have the opportunity to form a secondary structure to modify the 3′ end of the *tr* promoter. In this case, the polymerase can efficiently initiate genome RNA synthesis from the *tr* promoter. While considerable work is required to test this hypothesis, the idea that limited RNA secondary structure might play a role in regulating transcription and replication is intriguing.

The possibility that promoter secondary structure might play a role in its regulation has also been suggested for SeV (60). This idea emerged from studies to define the sequence within the *le* promoter that is responsible for inhibition by C protein. Swaps between *le* and *tr* promoter sequences failed to identify a specific primary sequence that could be responsible (60, 77). However, there was a correlation between lack of predicted promoter secondary structure and C protein inhibition (60). It is difficult to reconcile the idea that RNA secondary structure plays a role in SeV given that the bipartite promoter appears to function by being positioned on the same face of a helical nucleocapsid. Furthermore, mutation analysis of the PIV-3 promoters using the minigenome system failed to identify a role for RNA secondary structure (94). However, promoter secondary structures might form as the N-RNA template is relaxed during polymerase binding and such secondary structure effects might not be detectable in a minigenome assay due to the excess of N protein in this system. Therefore, while the data to support the idea are limited, it is interesting to speculate that perhaps the genome and antigenome 3′ termini are not entirely encapsidated at all times during infection, and that under certain circumstances, secondary structures may play a role in governing promoter activity.

FUTURE DIRECTIONS

In our view, the main gaps in knowledge in paramyxovirus transcription and replication research fall in two main areas. First, it will be valuable to have an understanding of how the RNA synthesis machinery functions, at a molecular level. While there are structures for many paramyxovirus proteins, there is relatively little structural information available regarding the L protein. An exciting development in NNS RNA virology in recent years includes establishment of enzymatic assays using recombinant SeV, VSV, and RSV polymerase (6, 12, 27, 95–97). This breakthrough has opened up the opportunity to perform detailed mechanistic and structural studies of the polymerase complex (4, 98). The second main area to pursue involves developing a better appreciation of temporal and perhaps spatial regulation of polymerase and nucleocapsid function. There is relatively little known about what happens to the paramyxovirus nucleocapsid and polymerase once they have been delivered into a cell, or how cellular proteins might alter their structures and functions. The ability to generate recombinant paramyxoviruses, coupled with developments in high-throughput "omics" approaches and high-resolution microscopy, has the potential to provide enormous insight into the interplay between the virus and the host cell. Finally, as we think this review conveys, a true understanding of paramyxovirus transcription and replication

mechanisms will come from continued research on a variety of viruses so that we can appreciate the similarities and differences between them.

Acknowledgments

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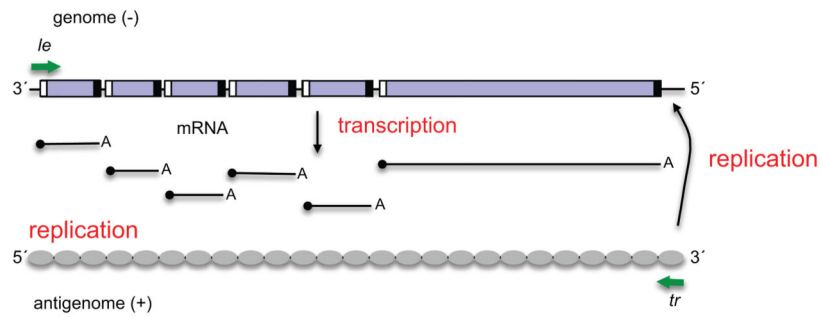
Figure 1.

Schematic diagram illustrating a representative paramyxovirus genome and transcription and RNA replication products. The genes are represented by purple boxes, and *gs* and *ge* signals are illustrated with white and black boxes, respectively. The *le* and *tr* promoters at the 3′ ends of the genome and antigenome, respectively, are indicated with green arrows. The genome acts as a template for mRNA and antigenome synthesis, and the antigenome as a template for genome RNA synthesis. The mRNA caps are indicated with black circles. The antigenome is shown covered with grey ovals, representing N protein, to indicate that it is encapsidated. The genome is also encapsidated, but this is not shown so that the *cis*-acting signals can be clearly seen.
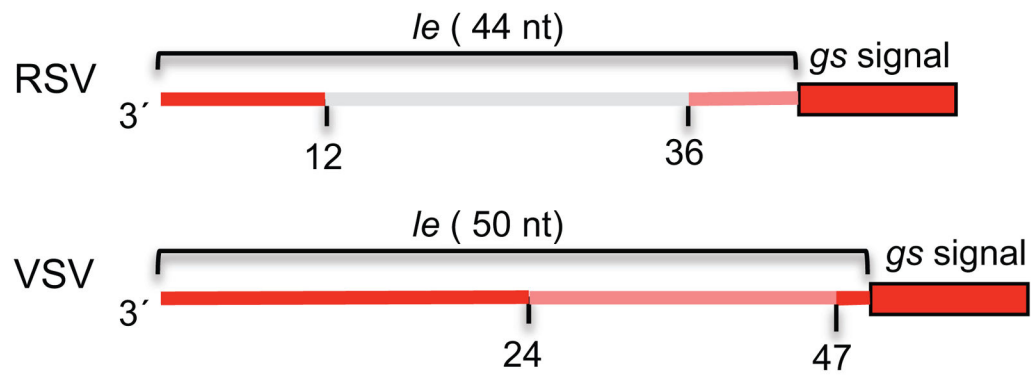
Figure 2.
Schematic diagrams of the *le* and *gs* regions of RSV (a paramyxovirus) and VSV (a rhabdovirus) showing the positions of the transcription-specific signals in the respective viruses. Regions in which mutations reduced transcription to less than 15% of wt levels are shown in red. Regions in which mutations reduced transcription to 15–40% of wt levels are shown in orange. Substitution of the region shown in gray had no effect on transcription. The numbers underneath indicate nucleotide positions. It should be noted that not all signals have been mapped precisely.
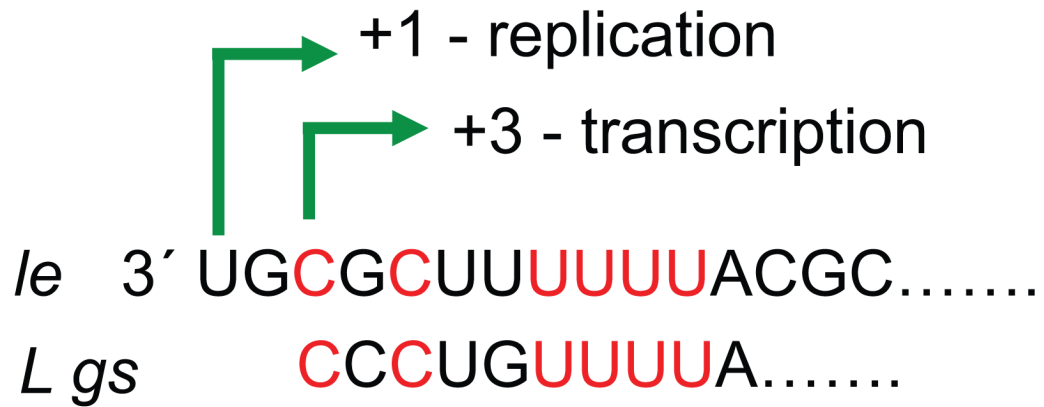
+1 - replication

+3 - transcription

le   3′ UGCGCUUUUUUACGC.......

L gs   CCCUGUUUUA.......

Figure 3.
Diagram showing the alignment of the RSV *le* and L *gs* sequences. The figure shows the 3′ terminal 15 nt of the *le* and the first 10 nt of the L gene. The nucleotides shown in red in the *le* region were shown to be essential for both transcription and replication. These nucleotides align with the L *gs* signal. The green arrows show the experimentally determined initiation sites at positions 1 and 3 of *le*, which we propose are the replication and transcription initiation sites, respectively.
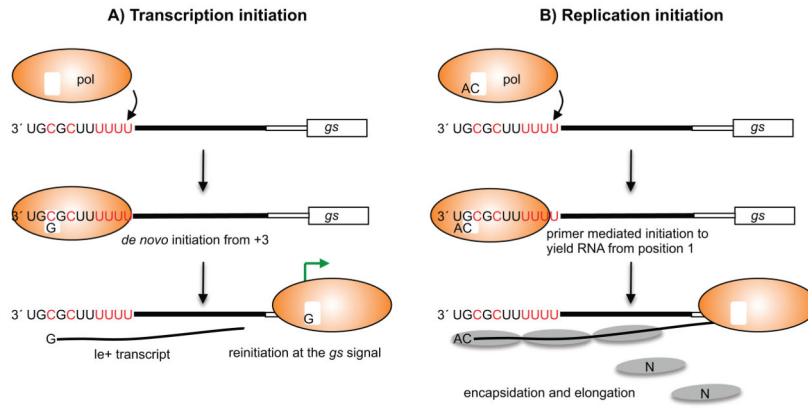
A) Transcription initiation

B) Replication initiation

Figure 4.
Diagrams showing the models for initiation of transcription (A) and replication (B) in RSV infection. The figure shows the relationship between the polymerase, NTPs, the *le* promoter region and *gs* signal in each case. The *le* region is shown as three sections, reflecting the distribution of *cis*-acting signals, as determined by mapping analysis. The first eleven nucleotides of the *le* region are written, with the core promoter element shown in red type. The black line in the middle of *le* indicates sequence required specifically for replication, and the white box at the end of *le* indicates a U-rich sequence that enhances transcription. The *gs* signal is shown as a large white box. In the replication initiation model (B), N protein binding to the nascent le+ RNA allows encapsidation. N is likely delivered to the RNA as a soluble N$_0$P complex, but the P protein is not shown for simplicity.

Sendai virus
(Respirovirus)

```
le  UGGUUUGUUCUC
tr  UGGUCUGUUCUC
N   UCCCAGUUUC
P   UCCCACUUUC
M   UCCCACUUUC
F   UCCCUAUUUC
HN  UCCCACUUUC
L   UCCCACUUAC
```

Atlantic salmon paramyxovirus
(Aquaparamyxovirus)

```
le  UGGUUUGUUCUUC
tr  UGGUUUGUUCUUC
N   UCCUAGUUUC
P   UCCUAGUUUC
M   UCCUAGUUUC
F   UCCUAGUUUC
HN  UCCUAGUUUC
L   UCCUAGUUUC
```

Measles virus
(Morbillivirus)

```
le  UGGUUUGUUUC
tr  UGGUCUGUUUC
N   UCCUAAGUUC
P   UCCUUGGUCC
M   UCCUCGUUUC
F   UCCCGGUUCC
H   UCCCACGUUC
L   UCCCAGGUUC
```

Hendra virus
(Henipavirus)

```
le  UGGCUUGUUCCC
tr  UGGCUUGUUCCC
N   UCCUUGGUUC
P   UCCUAGGUUC
M   UCCUCUGUCC
F   UCCUCGGUUC
G   UCCUGGGUUC
L   UCCUGGGUUC
```

Newcastle disease virus
(Avulavirus)

```
le  UGGUUUGUCUCU
tr  UGGUUUGUCUCU
N   UGCCCAUCUU
P   UGCCCAUCUU
M   UGCCCAUCUU
F   UGCCCAUCUU
HN  UGCCCAUCUU
L   UGCCCAUCCU
```

Fer de Lance virus
(Ferlavirus)

```
le  UGGGUUGUUCCCCUUUAU
tr  UGGGUUGUUCCCCUUUAU
N   UCCUAGUUUC
P   UCCUAGUUUC
M   UCCUUGUUUC
F   UCCUCGUUUC
HN  UCCUAGUUUC
L   UCCUAGUUUC
```

Mumps
(Rubulavirus)

```
le  UGGUUCCCCUUUU
tr  UGGUUCCCCUCUU
N   UUCGGGCCU
P   UCCGGGCCU
M   UUCGUGCUU
F   UUCGGAUCU
SH  UUCUUACUU
HN  UUCGGUCUU
L   UCCGGUCUU
```

Human metapneumovirus
(Metapneumovirus)

```
le  UGCGCUUUUUUUG
tr  UGCCGUUUUUUUG
N   CCCUGUUCA
P   CCCUGUUCA
M   CCCUGUUCA
F   CCCUGUUUA
M2  CCCUGUUUA
SH  CCCUAUUCA
G   CCCUGUUCA
L   CUCUGUUUA
```

Human RSV
(Pneumovirus)

```
le   UGCGCUUUUUU
tr   UGCUCUUUUUU
NS1  CCCCGUUUA
NS2  CCCCGUUUA
N    CCCCGUUUA
P    CCCCGUUUA
M    CCCCGUUUA
SH   CCCCGUUUA
G    CCCCGUUUA
F    CCCCGUUUA
M2   CCCCGUUUA
L    CCCUGUUUUA
```

Figure 5.
Promoter and *gs* sequences for one virus species from each of the paramyxovirus genera
(indicated in parentheses). The *gs* signals for each of the viral genes are shown. The
promoter sequences shown were defined as those nucleotides that were identical or almost
identical between the *le* and *tr* promoters; the minimal core promoters might be more
constrained than what is shown. Pyrimidine and purine residues are shown in orange and
green type, respectively.