Transcribing paramyxovirus RNA polymerase engages the template at its 3’ extremity

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For the non-segmented, negative-stranded RNA viruses, the mechanism controlling transcription or replication is still a matter of debate. To gain information about this mechanism and about the nature of the RNA polymerase involved, the length of an intervening sequence separating the 3’ end of Sendai virus minigenomes and a downstream transcription-initiation signal was increased progressively. It was found that transcription, as measured by green fluorescent protein (GFP) expression, decreased progressively in proportion to the increase in length of the intervening sequence. GFP expression correlated well with the levels of GFP mRNA in the cells, as measured by quantitative primer extension and by RNase protection. Thus, mRNA transcription was inversely proportional to the length of the inserted sequence. These data are evidence that the RNA polymerase initiating transcription at the downstream transcription signal somehow sees the distance separating this signal and the template 3’ extremity. Implication of this observation for the nature of the Sendai virus RNA polymerase and for the mechanism by which it synthesizes mRNAs or replication products is presented.

INTRODUCTION

Viruses of the subfamily Paramyxovirinae are unique among the non-segmented, negative-stranded RNA viruses (NNVs) in several respects. Their genome lengths must be a multiple of six to replicate efficiently (Calain & Roux, 1993). Their genomic and antigenic replication promoters (G/Pr and AG/Pr) are organized similarly with respect to PrE-I and PrE-II. However, whereas G/Pr contains gs1 between PrE-I and PrE-II, AG/Pr contains the complement of the L gene end (ge). The conservation of gs1 between the two essential replication promoter elements, including its precise location relative to the genome 3’ end, implies that this architecture is important in virus replication. Consistent with this notion, the presence of gs1 within either G/Pr or AG/Pr diminishes the accumulation of their respective replication products, presumably by competition for viral RNA-dependent RNA polymerase (vRdRp) (Le Mercier et al., 2003; Vulliémoz et al., 2005). How this competition between gs1 and PrE-I/-II for the two subunits (P and L) of the vRdRp occurs is unclear, but it suggests that this vRdRp is not yet committed to its specialized task. An attractive mechanism that would explain this competition is that it occurs ‘in cis’, e.g. during vRdRp scanning of the genome 3’ end for a new RNA start site, after vRdRp initiation at the genome 3’ end and release of leader RNA (Le Mercier et al., 2003).

There are different views of how NNV RdRp acts as a transcriptase that begins the process of transcription at gs1. For the rhabdovirus VSV, vRdRp is thought to be committed to its mutually exclusive task of replicase or transcriptase before it engages the template. Committed transcriptase interacts directly with and initiates at gs1 (Whelan & Wertz, 2002), whereas replicase does so at the genome/antigenome 3’ end (Fig. 2a). VSV RdRp complexes committed to transcription or replication can be prepared from recombinantly expressed VSV proteins (Qanungo et al., 2004) and, during infection, mRNA expression from gs1 is not affected by UV cross-links.
Fig. 1. (a) Schematic representation of the 3’ ends of the SeV genome and antigenome. PrE-I and PrE-II, promoter elements essential for replication; G/Pr, genomic promoter; AG/Pr, antigenomic promoter; gs1, first gene start; ge, last gene end; RdRp, vRdRp non-committed for transcription or replication; T-RdRp, viral transcriptase; R-RdRp, viral replicase; wt, wild type; black arrow, replication initiation; grey arrow, transcription initiation; grey line with triangle and tail, mRNA; black line with arrow, replication product; grey and black lines preceding mRNA and replication product, nascent RNA (leader or precursor to replication product). (b) Schematic representation of the primary structure of the SeV defective minigenome AGP-GPd12. For more explanation, see Introduction.

Fig. 2. (a) Schematic representation of the 3’ ends of the VSV genome and antigenome. Legend as for Fig. 1(a). wt, Wild type. (b) Schematic representation of the primary structure of the VSV defective interfering RNA DI-LT2 showing replication but no transcription (Semler et al., 1978). For more explanation, see Introduction.
that impede leader RNA synthesis (Whelan & Wertz, 2002). This view is also supported by the properties of the VSV defective interfering (DI) genome DI-LT2. VSV DI-LT2 contains all of the cis-acting sequences of the wild-type VSV genome (Fig. 2a), as well as four intact genes (missing the L gene). It also contains an additional 70 nt at the genome 3′ end derived from AG/Pr, which displaces G/Pr (and gs1) 70 nt from the genome 3′ end (Fig. 2b; Semler et al., 1978; Johnson et al., 1979). This naturally occurring DI-LT2 replicates extremely well during coinfection with wild-type virus, but fails to transcribe mRNAs in vitro and in vivo, presumably because only replicase initiates at AG/Pr (Fig. 2b) and therefore cannot go on to initiate at gs1. Alternatively, VSV transcriptase cannot initiate directly at gs1 because the precise location of gs1 relative to the genome 3′ end is also important for the initiation of transcription from gs1.

In striking contrast to VSV DI-LT2, a corresponding Sendai virus (SeV) DI, produced artificially, in which gs1 is displaced 90 nt from its natural position in the genome, expresses mRNAs from gs1 [Vulliémoz et al., 2005; Fig. 1b (AGP-6-GPd12)]. Thus, either SeV RdRp, which initiates at the genome 3′ end, can go on to initiate at gs1, meaning that this VrdRp is not a committed replicase, or the SeV RdRp, which initiates at gs1, is a committed transcriptase, which does so, in apparent contrast to VSV, independently of its precise location relative to the genome 3′ end. Such a transcriptase, then, should not be affected by the distance separating gs1 and the genome 3′ end. We therefore investigated the effect of progressive further displacement of gs1 from the genome 3′ end.

**METHODS**

**Virus and cells.** BSR-T7/5 cells, a baby hamster kidney cell line constitutively expressing a T7 RNA polymerase, a gift from K.-K. Conzelmann (Buchholz et al., 1999), were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) in a 5% CO2 atmosphere. The AGP-55 recombinant Sendai virus (rSeV-AGP-55) was constructed and rescued as described previously (Calain & Roux, 1995). To score replication, the 32P-labelled riboprobe contained promoter- and GFP-specific sequences, which allow detection of the helper virus genome. The blotted membranes were quantified by using a Phospholmager (ImageQuant version 5.0; Molecular Dynamics).

**RNase protection.** The negative-sense probe described in Fig. 3(a), RNaseP probe, was synthesized from the appropriate plasmid by the T7 RNA polymerase using [α-32P]ATP by T4 polynucleotide kinase (Promega) and annealed with 20 μg (1x) or 60 μg (3x) infected-cell CsCl gradient-pelleted RNAs. Reverse transcription was carried out at 42°C according to the supplier’s protocol by using M-MLV reverse transcriptase (Promega). The reverse transcription product was analysed by electrophoresis on a 6% polyacrylamide gel. The gel was fixed by acetic acid/ethanol, dried and quantified by using a Phospholmager (ImageQuant version 5.0; Molecular Dynamics).

**Northern blot analysis.** Northern blots were performed as described previously (Calain & Roux, 1995). To score replication, the 32P-labelled riboprobe contained promoter- and GFP-specific sequences, which allow detection of the helper virus genome. The blotted membranes were quantified by using a Phospholmager (ImageQuant version 5.0; Molecular Dynamics).

**Analysis of GFP expression.** Infected/transfected cells were collected in PBS. Flow cytometry was performed on a Becton-Dickinson FACSCan2. R1 and M1 were adjusted on BSR-T7/5 cells infected with rSeV-AGP-55 only and transfected with the plasmid harbouring AGP-GPd12. Data analysis was performed with Becton Dickinson software.

**GFP relative expression.** The method has been described previously in detail (Vulliémoz et al., 2005). In brief, GFP gene transcription, by measures of mean GFP fluorescence, was standardized to the amount of the template available for GFP mRNA synthesis. Replication itself was corrected for the fraction of cells carrying the minigenomes, taken as the percentage of cells positively gated in the flow-cytometry analysis. In the end, GFP fluorescence was expressed as the mean fluorescence divided by the corrected replication value. To be able to integrate the data of more than one experiment, the results were expressed as a percentage relative to one template taken as the reference for the series; this reference is indicated in the figure legends [see Figs 3(f), 4(b) and 5(c)], as is the number of independent experiments (three or four) performed and the average of the mean.
RESULTS

Increasing the distance between the replication and transcription signals

Our starting construct has been described previously (Vulliémoz et al., 2005). It contains, at the DI genome 3' end, 96 nt of AG/Pr, 6 nt of spacer and 84 nt of G/Pr in which the first 12 nt have been deleted (Fig. 3a, construct 1). This construct generates minigenomes that replicate via the external AG/Pr and express GFP from gs1 of the internal G/Pr. AG/Pr was chosen as the genome 3'-end replication promoter because of the absence of gs1 and because no transcripts other than the trailer RNA and antigenomes are known to be initiated from AG/Pr. The deletion of the first 12 nt of the internal G/Pr ensures that it cannot function as a replication promoter (Vulliémoz et al., 2005). Further minigenome constructs were generated containing intervening sequences of increasing size (240, 474 and 720 nt) between the tandem promoters (Fig. 3a, constructs 2, 3 and 4). gs1 was thus moved progressively downstream, from position 146 to positions 384, 618 and 864 relative to the minigenome 3' end. It is noteworthy that, in all of the minigenomes, the phase context of gs1 is identical and
corresponds to that found on the natural genome (second position of the hexamer). The minigenomes of these constructs were first rescued from plasmids by using helper SeV and were found to replicate to similar levels (Fig. 3b, c). This is expected, as all of these minigenomes have identical replication promoters. In contrast, measurement of GFP fluorescence by flow cytometry indicated that GFP expression decreased steadily with increasing distance between the replication and transcription signals (Fig. 3f, FLUO bars).

GFP mRNA levels were also measured directly by primer extension and RNase protection. For primer extension (Fig. 3d), we used a [32P]5′-phosphorylated primer of negative polarity that extends 228 nt to the 5′ end of the GFP mRNA (see Fig. 3a, bottom line). Lanes 1 and 3 of Fig. 3d show duplicate samples that contained different amounts (3× and 1×) of CsCl gradient-pelleted RNA to demonstrate the quantitative nature of the analysis. For RNase protection (Fig. 3e), we used a negative-polarity probe of 142 nt, of which 62 and 112 nt are complementary, respectively, to the GFP message and to the RNA transcript of the constructs depicted in (a). A riboprobe of positive polarity was used to evaluate the template available for transcription. (c) The bands obtained in (b) were quantified and the replication mean values (three experiments) were expressed relative to construct 1 and plotted with SD from the mean. (d) Primer-extension analysis (see Methods). Lane numbers represent minigenome numbers as in (a); No indicates helper virus infection only; 3× and 1× indicate relative fraction of total RNA used. Two independent experiments were carried out. (e) RNase-protection assay (see Methods) carried out using 10 μg (1×) or 50 μg (5×) total RNA. Lane numbers as in (d). (f) GFP transcription levels estimated by GFP mean fluorescence (FLUO, see Methods), primer extension (PE) (mean of two separate experiments (mean and SD)) and RNase protection (RNaseP) are plotted as values relative to those obtained for AGP-GPd12. Black bars, PE; dark-shaded bars, FLUO; light-shaded bars, RNaseP.

Rescue of the minigenomes in the absence of helper virus

The use of a helper virus to rescue minigenomes in transmissible form leads to competition between the helper virus and minigenome promoters for available vRdRp during intracellular replication. To examine whether the nature of the rescue system affected our results, we repeated the experiment in BSR-T7/5 cells in which vRdRp (P and L) and N protein, as well as the minigenomes, were produced 'constitutively' from plasmids via T7 RNA polymerase, in the absence of helper virus (Fig. 4). Under these conditions, once more, GFP expression levels as measured by flow cytometry showed a steady decrease proportional to the increasing distance between the replication and transcription start sites (Fig. 4c). Thus, SeV RdRp that initiates at gS1 is not independent of its precise location relative to the genome 3′ end. It is rather affected by the distance between the genome 3′ end and gS1, as it progressively initiates at gS1 less frequently as the distance increases.

Removing PrE-I and PrE-II

The cis-acting PrE-I and PrE-II sequences of G/Pr and AG/Pr that are important for replication are spread over 96 nt of the genome and antigenome 3′ ends (see Fig. 1a). However, in tandem promoter constructs such as AGP-GPd12, the PrE-I and PrE-II domains, essential for replication, can be removed completely from the internal G/Pr, leaving only 30 nt (49–78) containing gS1 in its bona fide hexamer phase, yet transcription continues (Vulliémoz et al., 2005). To determine whether PrE-I/II of G/Pr are nevertheless important for how Sendai virus RdRp that initiates at gS1 is affected by the distance between the genome 3′ end and gS1, we progressively increased the distance between the genome 3′ end and this minimal gS1 constructs 7–9, Fig. 5c(ii), as when it initiates from the efficient gS1 of G/Pd12 [Fig. 5c(i)]. Note the difference in scale of the two graphs of Fig. 5c, reflecting a general decreased GFP expression due to the PrE-I/II deletions. This was expected, as sequences upstream of nt 49
in G/Pr also contribute to gs1 expression, as described previously (Vulliémoz et al., 2005). Thus, in the end, we were unable to find any evidence that PrE-I/II of G/Pr are important for how the SeV RdRp that initiates at gs1 is affected by the intervening distance between gs1 and the genome 3' end. Our data support the conclusion that SeV RdRp does not initiate directly at gs1, but rather first interacts with the genome 3' end and then reaches gs1 in a manner that senses the distance separating gs1 from the genome 3' end.

**DISCUSSION**

Two models can be envisioned of how SeV RdRp, which initiates at gs1, is affected by the distance between the genome 3' end and gs1. First, SeV RdRp that initiates at gs1 could, for example, be a dimer (Smallwood et al., 2002) with one subunit bound to the genome 3' end and the other to gs1. In this view, initiation at gs1 by the downstream vRdRp depends on the upstream vRdRp interaction with the genome 3' end. Increasing the intervening distance, then,
will decrease the likelihood of interaction of gs1 and the genome 3′ end (via the two vRdRp subunits) that is required for the downstream vRdRp to initiate at gs1. Second, all SeV RdRps, both those that go on to make antigenomes and those that go on to make mRNAs, initially initiate RNA synthesis at the genome 3′ end. The latter initiate at gs1 after having presumably released the nascent transcript and having scanned the template linearly for gs1. NNV RdRps are unique in that they apparently do not disengage from the template upon release of the mature mRNA, but scan the template linearly for a new start site, even when this gs is upstream of the gene-end site (Stillman & Whitt, 1998; Fearns & Collins, 1999). If similar events occur upon release of the nascent RNA (trailer RNA in the present case?), the increasing distance between gs1 and the genome 3′ end will lower the number of scanning RdRps that reach gs1.

Both models account for the reduction of gs1 efficiency with its increasing displacement from the genome 3′ end, by invoking a required interaction of vRdRp with the genome 3′ end for subsequent vRdRp initiation at gs1. However, in the first case, this interaction serves to position a second vRdRp on the template that initiates at gs1, without prior RNA synthesis (de novo initiation). In the second case, this interaction serves to initiate RNA synthesis, thereby stably attaching vRdRp to the N–RNA template, and it is this stable attachment that permits subsequent vRdRp scanning of the N–RNA for gs1 after nascent RNA release (vRdRp reinitiation; Kolakofsky et al., 2004). There are two reasons why we favour the second model.

First, the interaction of the genome 3′ end and gs1 via SeV RdRp, independent of RNA synthesis and independent of the precise location of gs1 downstream, i.e. vRdRp searching in space for gs1 by looping out the intervening sequence, would require a remarkable flexibility in the N–RNA template. This flexibility is inconsistent with this structure, as revealed by electron-microscopy image reconstruction of the SeV and measles virus nucleocapsids (Egelman et al., 1989; Bhella et al., 2004; Schoehn et al., 2004) and the general inaccessibility of the RNA bases of this N–RNA assembly to the solvent (Iseri et al., 2002). Second, we were unable to provide evidence that the PrE-I/II sequences, which are essential for the initiation of genome replication, are also important in determining how the vRdRp initiates at gs1. In case of a de novo initiation at gs1, the PrE-I/II sequence would be expected to affect this initiation. This negative result is more consistent with a model in which vRdRp scans the template linearly for gs1, where the essential task of attaching vRdRp stably to the template (so that linear scanning for gs1 can occur) takes place, in our case during trailer RNA synthesis. Reinitiation, rather than de novo initiation at gs1, can presumably take place on a more limited cis-acting sequence (nt 49–78, containing gs1 in its bona fide hexamer phase). In the end, in the same way that the lack of VSV DI-LT2 transcription supports the existence of VSV RdRp committed to replication or transcription before it engages the template, the transcription of SeV (AGP-6-GPd12) and its derivatives supports the existence of an uncommitted pool of SeV RdRp that engages the template at the genome 3′ end and becomes committed to transcription after release of the nascent RNA.

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REFERENCES


