Sendai virus genome synthesis and assembly are coupled: a possible mechanism to promote viral RNA polymerase processivity

Oliver Gubbay, Joseph Curran and Daniel Kolakofsky

Dept of Genetics and Microbiology, University of Geneva School of Medicine, CMLU, 9 Ave de Champel, CH1211 Geneva, Switzerland

A cell-free system for studying Sendai virus RNA synthesis was reconstituted from N protein:RNA templates and transfected cell extracts in which the viral N, P and L proteins were expressed. Both transcription (mRNA synthesis) and replication (genome and antigenome synthesis) took place concurrently in these reactions. Viral RNA polymerases engaged in replication (replicases) were found to elongate their chains at a constant speed along the genome (1.7 nt/s), in a highly processive manner. In contrast, viral RNA polymerases engaged in transcription (transcriptases), although capable of synthesizing RNA at a comparable speed to replicases, were poorly processive. In this system, therefore, transcriptases require special reaction conditions to promote processivity that are not required by replicases. In addition, during replication, incomplete nascent genome chains were shown to be assembled with N protein, providing direct evidence that the synthesis and assembly of genomes are concurrent events. The strong processivity of replicases, independent of the reaction conditions, may thus be due to the coupling of genome synthesis and assembly. A model is proposed to explain how pausing of viral polymerase on the template is restricted when assembly and synthesis of the nascent chain are coupled.

Introduction

Non-segmented negative-strand RNA virus (mononegavirus) genomes contain five to ten tandemly linked genes separated by conserved gene junctions. These junctions contain cis-acting sequences that specify the initiation and polyadenylation/termination of the individual mRNAs. A defining feature of mononegavirus replication is that the (-) genome is used first as a template for mRNA synthesis (transcription), and then as a template for the synthesis of full-length (+) antigenomes (the intermediates of genome replication). Both types of RNA synthesis are thought to be carried out by the same, or similar, viral RNA polymerase (vRNAP). For Sendai virus (SeV), this vRNAP consists of a homotetramer of the P protein bound to a single L protein (Curran, 1998; Tarboureich et al., 2000). Another feature of mononegaviruses is that genome (and antigenome) templates are not found as free RNA, but as helical N protein:RNA nucleocapsids (reviewed by Lamb & Kolakofsky, 1996).

During transcription, vRNAP (or transcriptase) initiates at the genome 3' end, with synthesis of the short leader region. The transcriptase is thought to terminate the leader RNA and reinitiate at the start of the first gene, to synthesize the N mRNA. This transcriptase then continues across the genome, in turn polyadenylating and terminating each mRNA, reinitiating and capping the mRNA of the following gene. Polyadenylation is achieved by the transcriptase reiteratively copying a short run of template uridylates (vRNAP ‘stuttering’) before releasing the mRNA with a tail of adenylates. The transcriptase then crosses a short intergenic region, without synthesis, and reinitiates at the beginning of the adjacent downstream gene. During replication, vRNAP (or replicase) initiates synthesis at the 3' end of the genome and antigenome. However, unlike the transcriptase, the replicase ignores all the junctions (and editing signals) to produce an exact complement of the 15·384 kb genome. The fact that full-length antigenomes and genomes, in contrast to the mRNAs, are found only as assembled nucleocapsids has led to the notion that genome/antigenome synthesis and encapsidation are coupled (see below).
Much of our knowledge of mononegavirus RNA synthesis has come from studying vesicular stomatitis virus (VSV), a model rhabdovirus with a particularly robust virion transcriptase activity. Iverson & Rose (1981) studied the kinetics of VSV transcription in vitro by hybridizing short DNA fragments corresponding to specific genome sequences to the VSV virion reaction products. They found that VSV RNAP synthesized mRNA at an average rate of 3-7 nt/s within genes and at 1-4 nt/s over regions that span gene junctions. Assuming that VSV RNAP synthesizes mRNA at a constant rate, the extra time spent in crossing the junctions was presumably due to vRNAP stuttering to form the poly(A) tail and reinitiation of the next mRNA. Even for VSV, however, there is as yet little information on how vRNAP proceeds across the template during genome replication and how this differs from the process of transcription.

In contrast to the VSV virion transcription reaction, that of SeV is much less active and requires high concentrations of polyanions and other special conditions for optimal activity (Vidal & Kolakofsky, 1989). A previous study to measure transcriptional read-through of the leader (le) junction by SeV vRNAP in vitro virion reactions demonstrated that the extent of read-through varies between different SeV strains (5–10% for strain H and 20–40% for strain Z). In SeVZ virion reactions, vRNAP that had initiated at position 1 (the genome 3′ end) and read through the le/N junction (position 55/56) did not proceed further than 250–300 nt from the genome 3′ end. In contrast, >90% of vRNAP that had (re)initiated at position 56 synthesized the complete N mRNA (terminating at position 1737). These experiments suggest that vRNAP initiates RNA synthesis on the template as a relatively non-processive enzyme, but becomes processive (independent of concurrent assembly) upon (re)initiation at position 56 (and possibly also capping of the mRNA). The reinitiated transcriptase also responded to junction signals at very high frequency (close to 100%). Alternatively, vRNAP that has initiated at position 1 may also become processive during genome synthesis, as a replica. Unlike transcriptases, however, replicates are expected to exhibit a sufficiently high degree of processivity that allows them to ignore junction (and editing) signals. The precise difference between vRNAPs engaged in transcription and replication is unknown; these two forms of vRNAPs have been defined so far essentially by the RNA products they generate.

Besides high concentrations of polyanions, the SeV virion reaction can be stimulated by high concentrations of cytoplasmic extracts (Curran et al., 1992). In either case, however, the limited activity of these reactions, and the fact that SeV cores contain about 50 vRNAPs scattered across each nucleocapsid (Portner et al., 1988), makes them unsuitable for detailed kinetic analysis. This paper reports a reconstituted SeV in vitro reaction, in which the progress of vRNAP along the templates during transcription, as well as replication, can be followed.

Methods


- **In-vitro transcription and replication.** RNA synthesis was performed essentially as described by Curran et al. (1994) and Curran (1998). Briefly, N-RNA (non-defective) templates were isolated from SeV-infected (strain Z) egg allantoic fluid by banding twice on 20–40% CsCl linear gradients. Templates were resuspended at a concentration of about 250 µg/ml in TE (10 mM Tris–HCl, pH 7.4, 1 mM EDTA) containing 1 mM DTT and 10% glycerol and stored at −70 °C. A549 cells (in 9 cm diameter Petri dishes) were infected with a recombinant vaccinia virus expressing T7 polymerase (vTF7-3; Fuerst et al., 1986) and transfected with 2.5 µg pGEM-N, 2.5 µg pGEM-P[Δ7] and 1 µg pGEM-L. At 24 h p.i., medium was removed and the cells were incubated for 1 min with 1 ml ice-cold buffer A (150 mM sucrose, 30 mM HEPES–NaOH, pH 7.4, 5 mM MgCl2, 7 mM KCl, 4.5 mM magnesium acetate) containing 250 µg/ml lysochelin. The cells were then scraped into 300 µl energy mix (100 mM HEPES–NaOH, pH 7.4, 150 mM NH4Cl, 45 mM magnesium acetate, 1 mM DTT, 1 mM ATP/CTP/UTP, 10 µM GTP, 40 U/ml creatine phosphokinase, 1 mM creatine phosphate) and cell membranes were disrupted by pipetting up and down 20 times. Nuclei and cell debris were removed by centrifugation at 12000 g for 5 min at 4 °C.

RNA synthesis was carried out in 150 µl reactions containing 5–10 µl of the N-RNA template, 20 µg/ml actinomycin D, 100 µl of a vTF7-3-infected and transfected A549 cell extract and either 30 µCi [α-32P]GTP or 1 µM GTP at 30 °C for 3 h. Nucleocapsid and non-nucleocapsid RNAs were separated by centrifugation on 20%, 40%, 57% CsCl step gradients and analysed on agarose–formaldehyde gels. During a kinetic analysis, aliquots were removed at the times indicated in the figure legends and the reaction was stopped by addition of 20 mM EDTA.

- **RNase protection assay.** Riboprobes, corresponding to sequences at the ends of the M, N and P genes, were prepared as SP6 run-off transcripts from pGEM-M (linearized with Ndel; 227 nt), pGEM-N (linearized with MscI; 253 nt) and pGEM-P (linearized at an MluI site that was introduced at position 1436 on the P gene; 307 nt), respectively. The 142 nt riboprobe corresponding to sequences of the leader and first 87 nt of the N gene was described by Vidal & Kolakofsky (1989). Riboprobes were purified from 6% acrylamide–urea gels and co-purified with CsCl-purified RNA from SeV in vitro reactions and RNase protection analysis was then performed essentially as outlined by Vidal & Kolakofsky (1989). After treatment with RNase A, RNA duplexes were recovered by precipitation with an inactivation buffer (Ambion), according to the manufacturer’s instructions. Protected fragments were resolved on a 6% acrylamide–urea gel, visualized by autoradiography and quantified by using a phosphorimager.

Results

An SeV in vitro system suitable for kinetic analysis can be reconstituted with virion N-RNA cores (from which the resident P and L proteins have been removed) and cytoplasmic extracts of transfected cells expressing the P and L proteins (Curran et al., 1992). Conditions can then be created in which N-RNA templates are in excess such that all the vRNAP should, at least in principle, engage their templates at the start
of the reaction. Furthermore, when the transfected cell extracts contain the N protein as well as the P and L proteins, predominantly mRNAs and genomes are synthesized in the same reaction (Horikami et al., 1992); SeV virions (like their infected cells) contain relatively large amounts of antigenomes relative to genomes (10–20% of the combined total; Kolakofsky & Bruschi, 1975). The antigenomes act as templates for genome synthesis, whereas the majority of the genomes present in the viral cores act as templates mostly for mRNA synthesis, but also for some antigenome synthesis (see below). This in-vitro reaction thus mimics, in part, vRNA synthesis in vivo.

The radiolabelled RNA products of an in-vitro reaction were separated by CsCl density gradients into banded and pelleted material on a formaldehyde–agarose gel. The banded material contains encapsidated RNA and thus represents the nucleocapsid products of replication. Pelleted material, however, contains unencapsidated RNA and largely represents the products of transcription (see below). The replication (nucleocapsid-assembled) products formed defined bands that migrated progressively more slowly with time of reaction, until the full-length genome of approximately 15·384 kb was completed (Fig. 1A). The relative intensities of bands, when normalized for their G content (by reference to RNA size markers), were roughly the same (data not shown). This reaction is thus highly processive for a significant fraction of the replicases, in that most of these replicases completed their chains. The recovery of genome chains of intermediate length as nucleocapsid-assembled material finally provides direct evidence that genome synthesis and assembly are concurrent events. From these data, we calculated that genome synthesis occurred at approximately 1·2–2·2 nt/s throughout the time-course of the reaction. Thus, many of the vRNAPs engaged in genome replication began synthesis at the beginning of the reaction, as expected, and continued in a relatively synchronous fashion across the template at an average rate of 1·7 nt/s. In particular, processivity of vRNAPs during replication is emphasized by the fact that replicases showed no evidence of slowing down towards the end of the reaction.

In contrast to the encapsidated replicative products, unencapsidated RNAs migrated as a smear, 0·5–2·0 kb in length, as well as two defined bands approximately 2 kb in length, corresponding to the sizes of N and P mRNAs (Fig. 1B). The preponderance of sub-mRNA-length fragments did not appear to be due simply to RNase degradation, as the smear did not migrate faster with time. However, to examine further whether the smear was due to random degradation, the relative abundance of sequences representing the 5′ and 3′ ends of the N mRNA present in the total unencapsidated RNA products was determined by RNase protection with specific (unlabelled) riboprobes (Fig. 2). Unencapsidated RNAs were largely synthesized by vRNAPs that terminated at the end of the leader and reinitiated at the beginning of the N gene. However, 5% of the unencapsidated RNAs resulted from read-through of le/N gene sequences (as documented previously; Vidal & Kolakofsky, 1989). By comparing the 5′ and 3′ ends of the N mRNA, the 5′ end was shown to be 3- to 4-fold more abundant than the 3′ end (irrespective of whether replication and transcription were taking place concurrently). However, for the products of replication, the two ends of the N gene were almost equally abundant. Thus, many of the transcriptases that started the N mRNA apparently failed to finish the complete mRNA.

In addition, we also examined whether the 3′ ends of the second (P) and third (M) mRNAs could be detected in the transcription products. Whereas the 3′ end of the P mRNA
could be detected easily, surprisingly, the M mRNA could not be detected (Fig. 3). In contrast, the 3’ ends of all three mRNAs were detected at roughly equal levels when unlabelled CsCl fractions of SeV-infected cells were analysed with radiolabelled riboprobes (as documented previously; Homann et al., 1990).

The apparent absence of M mRNA in these in-vitro reactions is also inferred by the presence of only two bands, corresponding to the sizes of mature N and P mRNAs, when SeV radiolabelled transcription products were analysed by agarose gel electrophoresis (Fig. 1B). Thus, in contrast to the viral replicases, the

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**Fig. 2.** Relative abundance of 5’ and 3’ N gene sequences. Two reconstituted radiolabelled SeV reactions were carried out, in duplicate, as described in Fig. 1, except that one reaction contained transfected cell extract in which only the P and L proteins were expressed (–N). As negative controls, parallel reactions were carried out in the presence of 10 mM EDTA (+EDTA). After 180 min at 30 °C, the products of replication (CsCl banded) and transcription (CsCl pelleted) were annealed to excess (non-radiolabelled) riboprobe. Riboprobe-complementary sequences along the genome are depicted above (dashed line represents gene boundary). RNAs were digested with RNase and the protected fragments were separated on a 6% sequencing gel. The bands indicated were quantified by using a phosphorimager. The ratio of the 5’ and 3’ fragments for replication (142 and 253 nt) and transcription (87 and 253 nt), respectively, were normalized for G content and are shown below.
transcriptases in this in-vitro reaction apparently completed the M mRNA rarely (if ever), and mostly synthesized incomplete N and P mRNAs of various lengths.

In order to clarify the relative rates at which transcriptases synthesize complete mRNAs, we employed a similar RNase protection analysis using unlabelled riboprobes that complement sequences at the ends of the N and P genes (Fig. 4). In addition, the same riboprobes, hybridized to nucleocapsid-assembled products, will determine the extent of antigenome synthesis.

The accumulation of the nucleocapsid-assembled N and P gene sequences was comparable during antigenome synthesis (Fig. 4A), indicating that replicases traversed these two genes at the same speed. Linear regression analysis indicated that the fastest replicases traversed the N and P genes at rates of 1·9 and 1·8 nt/s, respectively. The levelling off of RNA synthesis with time (N and P gene sequences stopped accumulating at around 90 min) presumably reflects the time taken for the slowest replicases to pass beyond the two genes and continue downstream, in the relative absence of continued initiation of replication. Thus, the total products of replication, mostly genomes (Fig. 1A) and antigenomes (Fig. 4A), were made at similar overall rates (1·7 and 1·9 nt/s, respectively).

The kinetics of mRNA synthesis, determined by analysing unencapsidated RNAs in these reactions, differed from those of antigenome synthesis in several respects (Fig. 4B). Firstly, the unencapsidated N and P gene sequences accumulated linearly throughout the entire course of the reaction, without any tendency to level off. Secondly, the N and P mRNAs accumulated at clearly different rates; the P gene sequences accumulated about half as fast as the N gene sequences (as indicated by the relative gradients of each graph). Lastly, the fastest transcriptases (analysed by linear regression analysis) completed the N mRNA at an average of 3·0 nt/s, whereas they appeared to have completed the P mRNA at an average of only 2·1 nt/s. Assuming that vRNAP synthesizes mRNA at a constant rate (of 3·0 nt/s), the extra time spent synthesizing the complete P gene (calculated to be 8·5 min) may be due to the combined effects of the N/P intergenic and P editing sites. However, even though the fastest transcriptases thus traversed the N and P genes of the genome template at least as rapidly as the replicases, this reaction appeared to be very poorly processive. For example, most of the transcriptases that initiated the N mRNA apparently did not finish this chain (Fig. 1B) and only about half the transcriptases that finished the N mRNA continued to finish the P mRNA. Furthermore, very few (if any) of the transcriptases then continued to finish the M mRNA (Fig. 3). We suspect that most of these transcriptases have paused heterogeneously along the template and eventually released their chains. vRNAPs without nascent chains would then, presumably, be free to disengage from the template and re-initiate mRNA synthesis at the beginning of the leader or adjacent start sites.

Discussion

Paramyxovirus mRNA synthesis in vitro

By comparing directly the products of transcription with the products of replication from the same reaction, we have demonstrated a difference of vRNAP processivity between the two modes of SeV RNA synthesis. The lack of processivity during mRNA synthesis in these reconstituted N:RNA + vRNAP reactions was unexpected. Moreover, in contrast to the products of replication, the heterogeneous length of transcription products varied between different reactions; at times, either predominantly incomplete mRNAs or full-length N and P mRNAs accumulated (data not shown). We do not understand these variations in transcriptase processivity; they do not appear to be related to the overall level of RNA synthesis (data not shown). These variations may be related to some aspect of the preparation of the transfected cell extract that alters the array of cellular factors so critical for paramyxovirus RNA synthesis in vitro. Some paramyxovirus reactions are stimulated by tubulin, but not actin (Moyer et al.,
Fig. 4. Kinetic analysis of transcription and replication in reconstituted vRNAP reactions by RNase protection. The reconstituted in-vitro reaction was carried out as described for Fig. 1. The radiolabelled encapsidated (A) and unencapsidated (B) product were annealed to an excess of non-radiolabelled riboprobes corresponding to the 3′ regions of the N and P genes. Riboprobe-complementary sequences along the genome are depicted above (dashed lines represent gene boundaries). The protected fragments were separated on a 6% sequencing gel and quantified by using a phosphorimager. In the right-hand lanes of each gel (marked as ‹), 5-fold more cold riboprobe was added to the sample from the last time-point. The intensities of each band were normalized for G content and are plotted on the right. Rates of synthesis were determined by linear regression.
independent of the cellular and viral factors required for RNAP processivity during mRNA synthesis.

**The effect of nascent chain assembly on RNAP processivity during replication**

In these *in-vitro* reactions, genome, antigenome and mRNA synthesis all take place simultaneously using the same pool of P and L proteins (as transfected cell extract). Nevertheless, vRNAPs engaged in replication are highly processive, whereas those engaged in transcription are not. The main difference between these two modes of vRNA synthesis is that RNA synthesis and assembly are coupled during replication. As a consequence of this coupling, replicases are envisaged to interact with P–N assembly complexes involved in nascent chain assembly (Curran, 1998; Horikami et al., 1992). We cannot rule out that the presence of N (and P) protein may lead to a conformational change in vRNAP that itself promotes processivity (Mooney & Landick, 1999). However, we argue that the coupling of assembly and synthesis promotes processivity simply by preventing vRNAP pausing, independent of possible changes in vRNAP conformation (see below).

Bacterial and eukaryotic DNA-dependent RNAPs synthesize RNA at 30–100 nt/s *in vitro*, which is nevertheless insufficient to account for the rates observed *in vivo* (Reines et al., 1996; Uptain et al., 1997; Shilatifard, 1998). In addition to their slower overall rate *in vitro*, transcription elongation by these cellular RNAPs is disrupted by frequent pausing. Pausing is thought to occur at specific sites where NTP addition is disfavoured, and RNAP has a propensity to backtrack on the template and nascent RNA, removing the nascent RNA 3’ end from the RNAP active site (Reeder & Hawley, 1996; Guajardo & Sousa, 1997; Komissarova & Kashlev, 1997a, b; Nudler et al., 1997). During the pause, RNAP is envisaged as oscillating between the backtracked inactive state and the active (forward) state. SeV vRNAP backtracking is thought to be essential for the vRNAP stuttering that forms the mRNA poly(A) tail (as well as the G additions for P mRNA editing); this is envisaged to be achieved by nascent RNA–template hybrid realignment within the elongating vRNAP (Hausmann et al., 1999a, b).

Although we have provided direct evidence that genome synthesis and assembly take place concurrently, how closely the assembly of the nascent genome/antigenome chain follows its synthesis is unclear. It is possible that assembly occurs as soon as the nascent RNA emerges from the vRNAP exit channel (Mooney & Landick, 1999) and is available for interaction with the P–N assembly complex, since replicases traverse their templates relatively slowly (1.8 nt/s). In this case, the nascent chain assembled with N subunits would hinder vRNAP backtracking sterically, as backtracking requires the most recently exposed RNA chain to be drawn back within the exit channel (Reeder & Hawley, 1996; Komissarova & Kashlev, 1997a, b; Mooney & Landick, 1999); this model is summarized in Fig. 5. The close coupling of genome synthesis and assembly would thus favour vRNAP read-through of the gene junctions and other transcriptional pause sites and promote vRNAP processivity throughout genome synthesis.

Additional evidence to suggest that genome synthesis and assembly are tightly coupled is provided by the fact that a stretch of unprotected genome would be available for the formation of mRNA/antigenome dsRNA and thus potentiate the cellular innate antiviral response. For measles virus, the hyperbiased U-to-C (or A-to-I) mutations confined to the M gene in some subacute sclerosing panencephalitis (SSPE) cases are thought to have resulted from the annealing of the M mRNA to the genome during this long-term persistent
infection and mutation of multiple adenosines by a dsRNA-dependent deaminase (Cattaneo et al., 1988).

Following on from the model described above, the uncoupling of assembly from genome synthesis would permit vRNAP backtracking at poly(A)/termination sites and produce truncated genomes. A rare polyadenylated transcript representing the leader and N gene sequences (as read-through) is found in measles virus-infected cells, but only as a nucleocapsid, whereas the N mRNA is found only in a non-assembled form (Castaneda & Wong, 1990). This rare measles virus fusion transcript was presumably made by vRNAP that began the synthesis of an antigenome nucleocapsid but nevertheless polyadenylated and terminated the chain at the second junction. However, both these examples of uncoupling are rare events. Some mechanism must therefore exist to ensure that genome synthesis and assembly are tightly coupled, especially as replicase approach poly(A) signals. For example, replicase progression along the template could be driven by its interaction with the P–N assembly complex once genome synthesis and assembly have become coupled. The idea that the interaction between elongating replicases and P–N assembly complexes would not only promote but also maintain vRNAP processivity is therefore a corollary of the above model.

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