RNA (2002), *8*:1056–1067. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838202026043

Chemical modification of nucleotide bases and mRNA editing depend on hexamer or nucleoprotein phase in Sendai virus nucleocapsids

FRÉDÉRIC ISENI,[1]* FLORENCE BAUDIN,[2,3]* DOMINIQUE GARCIN,[1]* JEAN-BAPTISTE MARQ,[1] ROB W.H. RUIGROK,[2,3] and DANIEL KOLAKOFSKY[1]

[1]Department of Genetics and Microbiology, University of Geneva School of Medicine, Centre Médicale Universitaire, CH1211 Geneva, Switzerland
[2]European Molecular Biology Laboratory, Grenoble Outstation, BP 181, 38042 Grenoble Cedex 9, France
[3]Laboratoire de Virologie Moléculaire et Structurale EA 2939, Université Joseph Fourier, Faculté de Médecine de Grenoble, 38700 La Tronche, France

ABSTRACT

The minus-strand genome of Sendai virus is an assembly of the nucleocapsid protein (N) and RNA, in which each N subunit is associated with precisely 6 nt. Only genomes that are a multiple of 6 nt long replicate efficiently or are found naturally, and their replication promoters contain sequence elements with hexamer repeats. Paramyxoviruses that are governed by this hexamer rule also edit their P gene mRNA during its synthesis, by G insertions, via a controlled form of viral RNA polymerase "stuttering" (pseudo-templated transcription). This stuttering is directed by a *cis*-acting sequence (3′ UNN UUUUUU CC**C), whose hexamer phase is conserved within each virus group. To determine whether the hexamer phase of a given nucleotide sequence within nucleocapsids affected its sensitivity to chemical modification, and whether hexamer phase of the mRNA editing site was important for the editing process, we prepared a matched set of viruses in which a model editing site was displaced 1 nt at a time relative to the genome ends. The relative abilities of these Sendai viruses to edit their mRNAs in cell culture infections were examined, and the ability of DMS to chemically modify the nucleotides of this *cis*-acting signal within resting viral nucleocapsids was also studied. Cytidines at hexamer phases 1 and 6 were the most accessible to chemical modification, whereas mRNA editing was most extensive when the stutter-site C was in positions 2 to 5. Apparently, the N subunit imprints the nucleotide sequence it is associated with, and affects both the initiation of viral RNA synthesis and mRNA editing. The N-subunit assembly thus appears to superimpose another code upon the genetic code.**

Keywords: chemical probing; hexamer phase; mRNA editing; RNA:protein interactions; Sendai virus

INTRODUCTION

Sendai virus (SeV), a paramyxovirus, is a model nonsegmented negative-strand ([−]) RNA virus. Negative-strand RNA viruses have RNA genomes that are complementary to mRNA. The first step in their replication cycle is the production of mRNA from a helical subviral structure, the nucleocapsid, in which the viral RNA is tightly and stoichiometrically associated with the viral nucleocapsid (N) protein. This complex, together with the attached viral polymerase (composed of the P and L proteins) is the minimum subviral unit that is thought to retain infectivity.

The synthesis of [−] RNA genomes (or plus-strand ([+]) antigenomes) and their assembly with N protein is coupled, and these viral RNAs are only found as nucleocapsids (Gubbay et al., 2001; Lamb & Kolakofsky, 2001). Electron micrographs of negatively stained SeV nucleocapsids have revealed a flexible helical assembly with 13 N subunits per turn and variable pitch, in which each N monomer binds 6 nt (Egelman et al., 1989; Fig. 1). It is possible that biological activity is associated with a specific helical state(s) or that the elongating polymerase modifies the helical parameters. For paramyxoviruses, efficient replication of model minigenomes in transfected cells requires that their total length be a multiple of six, and viruses of this group

Reprint requests to: Daniel Kolakofsky, Department of Genetics and Microbiology, University of Geneva School of Medicine, CMU, 9 Ave de Champel, CH1211 Geneva, Switzerland; e-mail: Daniel.Kolakofsky@Medecine.unige.ch.
*These authors contributed equally to this work.

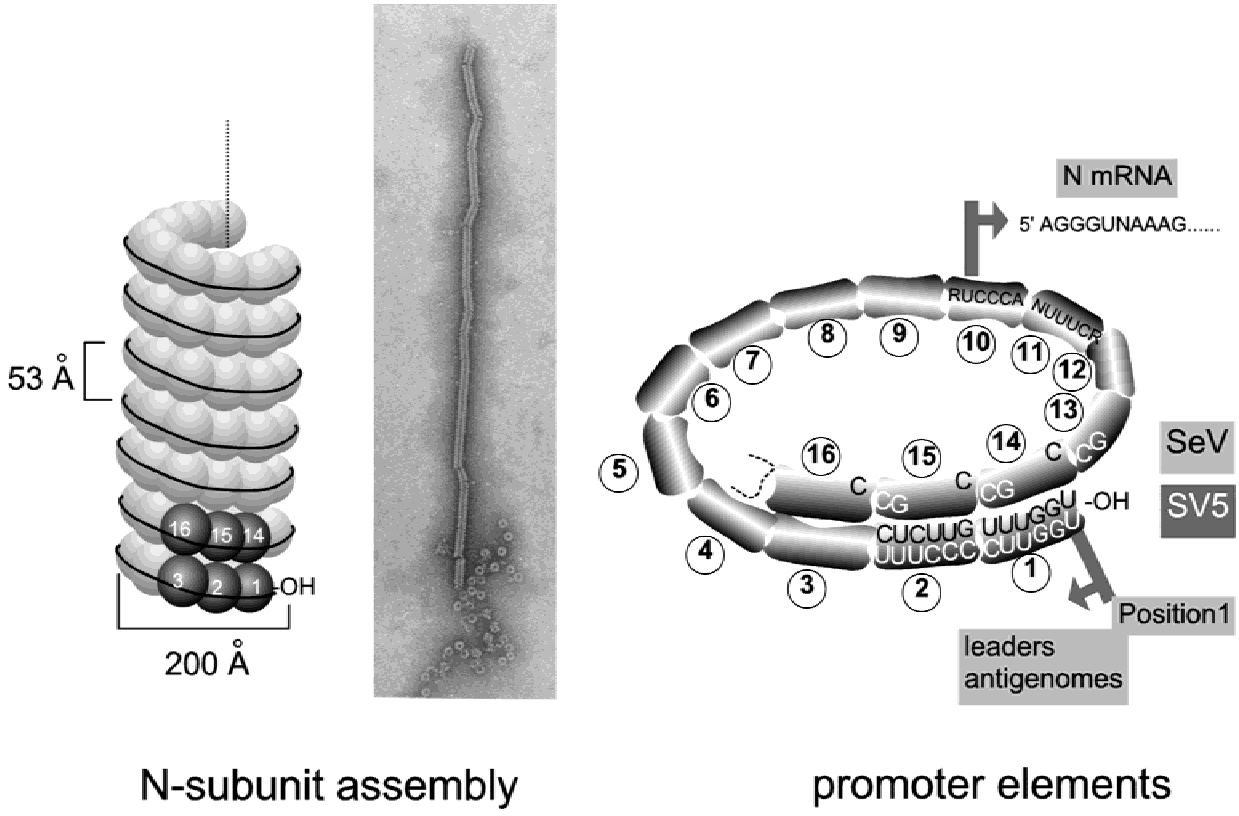

FIGURE 1. SeV nucleocapsid structure and hexamer phasing of the nucleotide sequence. A model of the SeV nucleocapsid as an assembly of N-protein subunits (shaded spheres) in the form of a left-handed helix with 13 subunits per turn (Egelman et al., 1989) is shown on the left, beside an electron micrograph of a negatively stained nucleocapsid. An expanded view of the first 16 subunits (rounded rectangles numbered from the RNA 3′ end) of the nucleocapsid is shown on the right. The invariant elements of the bipartite genomic and antigenomic replication promoters of SeV and SV5 are shown relative to the N subunits; each subunit contains precisely 6 nt. The 5′ starts of the leader and antigenome RNAs (genome position 1), and N mRNAs, (genome position 56) are indicated with arrows. Note that both elements of the replication promoter are found on the same face of the helix, whereas N mRNA synthesis starts on a different face.

whose genome has been entirely sequenced do, indeed, have genome lengths that are multiples of six (Calain & Roux, 1993; Kolakofsky et al., 1998). Inefficient replication of non-hexamer-length minigenomes was not due to the lack of encapsidation of the minigenome, but to the inability of viral RNA-dependent RNA polymerase (vRdRP) to initiate at the 3′ end of the mininucleocapsids (Calain & Roux, 1993). It has been suggested that nucleocapsid assembly begins with the first 6 nt at the 5′ end of the nascent chain, and continues by assembling 6 nt at a time until the 3′ end is reached. The efficiency of the 3′ end promoter then presumably depends on the position of the promoter elements relative to the N subunits, and this "phase" is determined by the total number of nucleotides in the genome chain (Calain & Roux, 1993; Kolakofsky et al., 1998; Vulliemoz & Roux, 2001).

The genomic and antigenomic replication promoters of paramyxoviruses are found within the terminal 96 nt of each RNA, and are bipartite in nature (Pelet et al., 1996; Murphy et al., 1998; Tapparel et al., 1998; Hoffman & Banerjee, 2000). There is both an end element comprising approximately the first 30 nt at the 3′ ends (in which the first 12 nt are conserved across each genus), and a downstream element within the 5′ UTR of the N gene ([−] nucleocapsids; Fig. 1) or the 3′ UTR of the L gene ([+] nucleocapsids). For SeV and human parainfluenza virus type 3 (hPIV3), the downstream element is a simple but phased hexameric sequence repeat (3′ $[C^1n^2n^3n^4n^5n^6]_3$ imbedded in what appears to be nonconserved sequences, and that is bound to the 14th, 15th and 16th N-protein subunits (Tapparel et al., 1998; Hoffman & Banerjee, 2000; see Fig. 1). For SV5, $[n^1n^2n^3n^4G^5C^6]_3$ is repeated in subunits 13, 14, and 15 (Murphy & Parks, 1999), such that all these conserved nucleotides are adjacent to the first 12 nt in the helical nucleocapsid with 13 subunits per turn (Fig. 1). This common or contiguous surface of the template may serve as a recognition site for the initiation of RNA synthesis by the polymerase at nt 1. There has long been evidence that the hexamer phase of at least some sequences within the 96-nt-long genomic replication promoter is important for promoter efficiency. Under certain conditions, it is possible to add

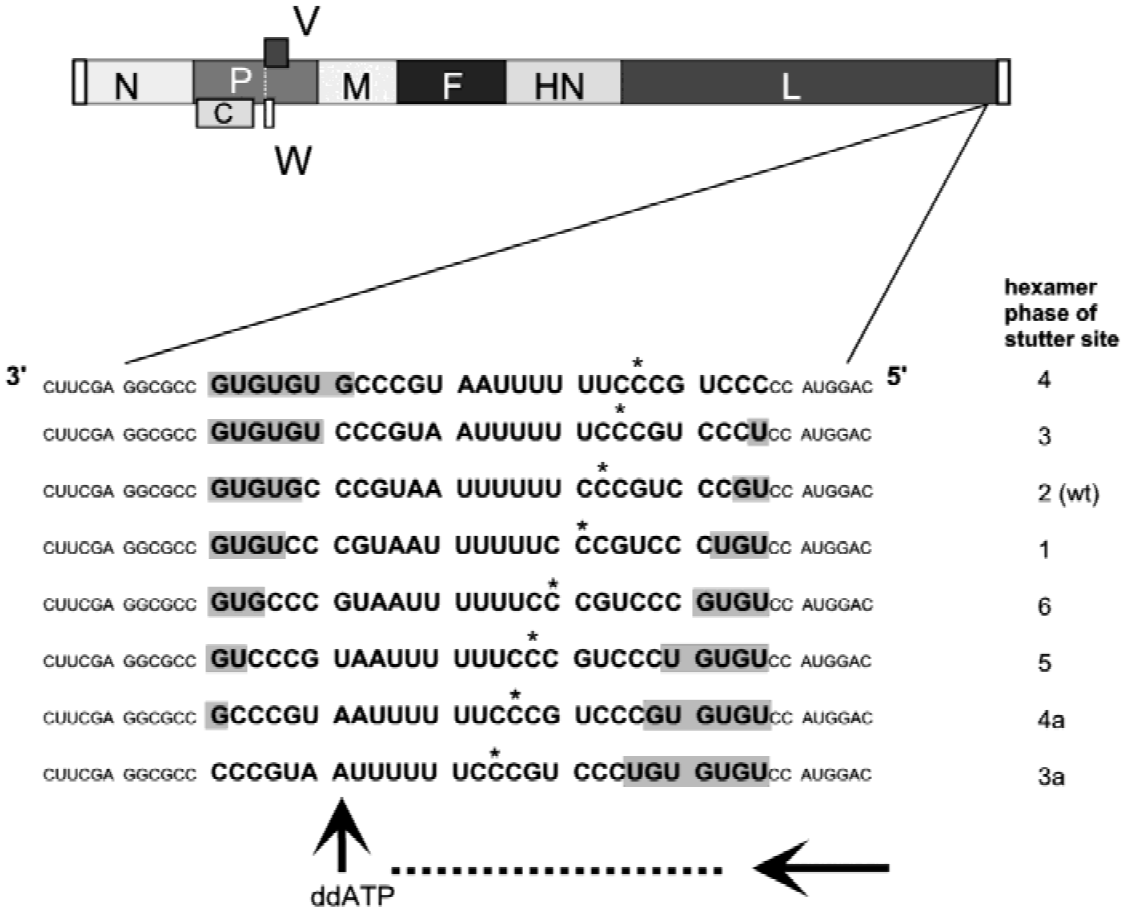

FIGURE 2. SeV genome map and the additional L gene mRNA editing sites. The SeV genome is shown schematically above, with each gene or mRNA transcription unit represented by a box. The single P gene transcription unit is unusual in having several overlapping ORFs, represented by overlapping boxes, two of which are expressed by mRNA editing (V and W). The P gene mRNA editing site is indicated by a dotted line. The various cassettes containing the 3′ UAAUUUUUUCCC hyperediting site in staggered phases that were inserted into the 3′ UTR of the L gene to generate the SeV-L^{edit} series are shown below. The variable-length GU repeats flanking the editing sequence are shaded, as is the unique stutter site, which is also marked with an asterisk and whose hexamer phase is indicated on the right side (the natural P gene stutter-site cytidine is in phase 2). The arrows below the sequences show the primer used for analysis, and the position where ddATP will terminate the primer extension for the experiment shown in Figure 5.

6 nt at either nt 47 or nt 67 within this promoter without deleterious effect. However, simultaneously altering the phase of the sequence strongly reduces promoter efficiency (Pelet et al., 1996). More recently, by engineering genomes with functional 96-nt-long promoters that are not at the RNA extremities, it has been possible to show that these internal replication promoters do, in fact, retain significant activity, but only when present in the bona fide hexamer phase (Vulliemoz & Roux, 2001). Finally, viruses within the *Paramyxovirinae* share another property unique to this virus subfamily; they edit their P gene mRNA by adding one to six guanylates

FIGURE 3. DMS modification of the genome RNA within SeV nucleocapsids. Autoradiograms of 12% sequencing gels of the cDNA fragments produced after reverse transcription of DMS-modified rSeV virion RNA. Lanes Ct are incubation controls of unmodified RNAs. Lanes 1 and 2 represent incubation of viral RNA with 0.1 and 0.3 μL of DMS. Lanes A, C, G, and U represent the sequence of the viral RNA determined using the same primer. The hexamer phase of the editing site is indicated above each gel. The reactivities of the cytidines are shown on the left of each panel by arrowheads as follows; black: hyperreactive; grey: reactive; white: nonreactive. A horizontal line indicates that the reactivity could not be determined due to a band in Ct lane. The star indicates a band compression that is partially resolved in the phase 3 gel. The genome positions covered by this analysis (numbered from the genome 3′ end) are shown on the right of the gels of the 4 and 6 viruses, along with the insertions (numbers in italics). The positions and lengths of the GU repeats that frame the editing sites are also shown on the right side of each gel.

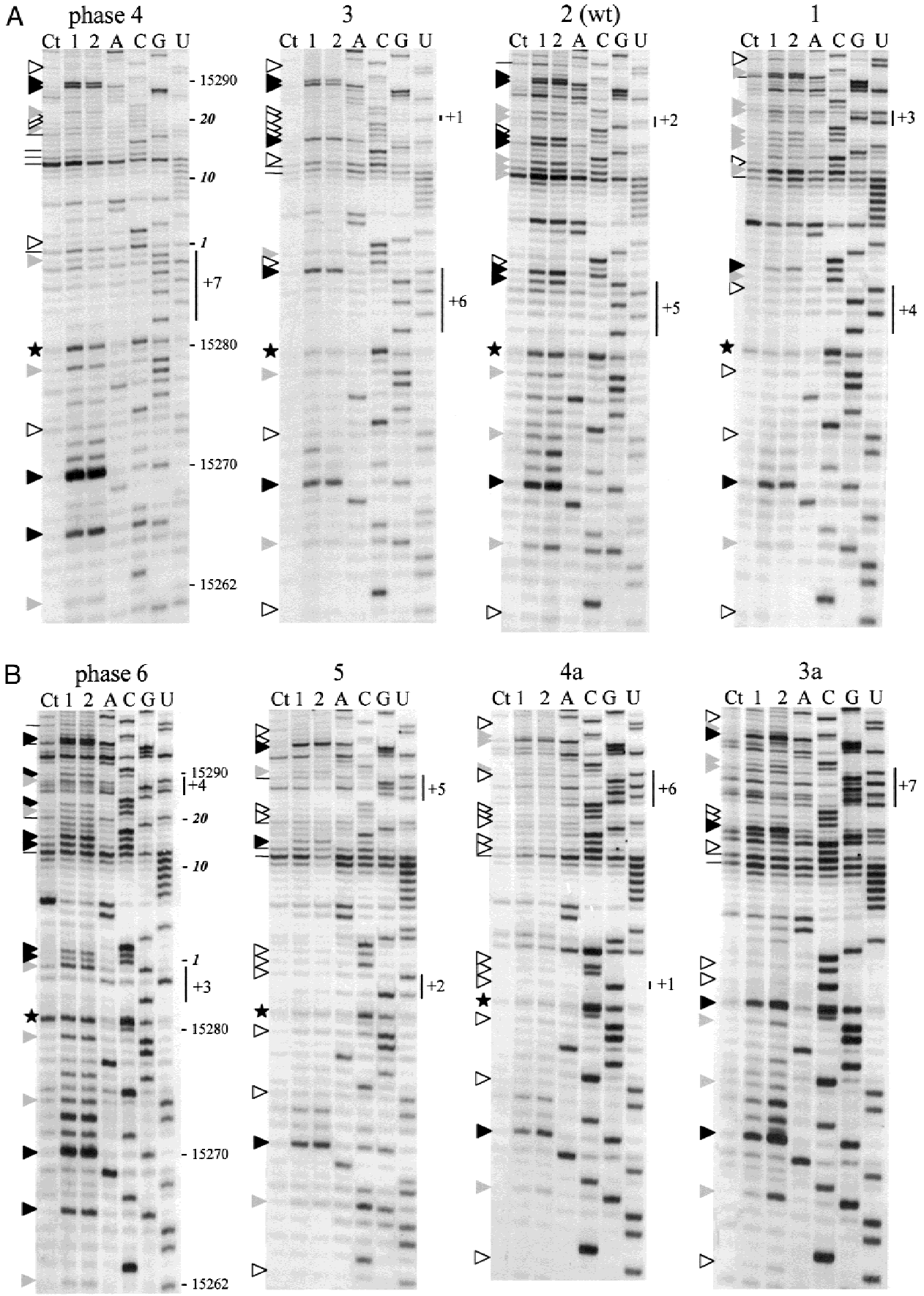
A
phase 4
3
2 (wt)
1
Ct 1 2 A C G U
15290
20
10
1
15280
15270
15262
+7
+1
+2
+3
+6
+5
+4
B
phase 6
5
4a
3a
15290
+4
20
10
1
+3
15280
15270
15262
+5
+2
+6
+1
+7

FIGURE 3.

within short runs of Gs (three to seven long) via pseudotemplated transcription (Jacques & Kolakofsky, 1991). The G insertions are genetically controlled by an upstream sequence, whose hexamer (or N subunit) phase is conserved within each genus of the *Paramyxovirinae* (Kolakofsky et al., 1998).

The phase of the downstream element of the replication promoter (3′ $[C^1n^2n^3n^4n^5n^6]_3$; Fig. 1) is known to be essential for replication efficiency. The present work explores whether there is a correlation between the chemical reactivities of cytidines within SeV *cis*-acting regulatory sequences and biological activity. We would clearly like to know whether the chemical reactivity of the conserved cytidines in the above replication promoter element might be affected by its phase. However, a change in phase here leads to loss of replication, and these viruses cannot be propagated. We therefore studied the effect of phase on C reactivity within a model mRNA editing cassette as an alternative. A matched set of eight recombinant SeV (rSeV) was prepared in which the editing signal is displaced 1 nt at a time relative to the genome ends, such that it is found in all possible hexamer phases with two repetitions. These rSeV were then used to examine ectopic mRNA editing during cell culture infections, and the ability of DMS to chemically modify the cytidines of this editing cassette within viral nucleocapsids. Hexamer phase was found to affect the distribution of G insertions during mRNA editing, and this suggests that the N subunits remain intimately associated with the template RNA even during transcription. Hexamer phase also strongly influences C reactivity to DMS, and this suggests a physical basis for the effect of hexamer phase on the replication promoter and mRNA editing.

RESULTS

Hexamer phase and cytosine reactivity in situ

Cassettes containing a modified P gene mRNA editing site were inserted at the 5′ end of the [−] genome RNA (that encodes the 3′ UTR of the L gene; Fig. 2), where they are likely to be well tolerated. In this manner, the common sequence 3′ CCCGUAAUUUUUUC**C**CGU CCC, containing a model editing site (underlined) flanked by C-rich sequences, was progressively displaced through all six phases (with two repetitions). The sequences of these cassettes and their hexamer phases in the various rSeV are shown in Figure 2 (listed by the hexamer phase or N monomer position of the stutter-site cytidine, phase 2 is the wild-type or P gene phase). All eight rSeV had 6*n* genome lengths and grew similarly to SeV-wt in both chicken eggs and cell culture (data not shown).

Dimethyl sulfate (DMS) reacts with the Watson–Crick (W-C) positions of adenines (N1-A) and cytidines (N3-C) in single-stranded RNA. The extent of reactivity at W-C positions of these nucleotide bases can be estimated by primer extension analysis because methylation prevents reverse transcriptase from copying the modified bases, leading to the presence of a band 1 nt downstream of the modified nucleotide (Ehresmann et al., 1987). DMS reactions can be carried out with purified virions because DMS can easily cross lipid membranes. For influenza virus and vesicular stomatitis virus (VSV), DMS reacts in the same manner with the RNA inside purified virions as with the RNA in purified nucleocapsids (Klumpp et al., 1997; Iseni et al., 2000). Purified virus preparations of all eight rSeV were reacted with DMS. Total RNA was extracted and the region containing the L editing site was examined for base reactivity. The primer extension analysis is shown in Figure 3A, B. The numbers above the gels refer to the recombinant viruses presented in Figure 2. The most striking feature from these eight gels is that the adenines show only a very weak reactivity when compared to the cytidines. This is contrary to what is usually found, that is, adenines usually react more strongly than cytidines (Blackburn, 1996).

The reactivity of the cytidines is indicated on the left of the gels by arrows; white for nonreactive, gray for reactive, and black for hyperreactive. When the reactivity of a base could not be determined because of a band in the untreated control, this is indicated by a line. These reactivities are also represented in Figure 4A where the nonreactive bases are blue (white arrows in 3A,B), the reactive bases are yellow (gray arrows), and the hyperreactive bases are red (black arrows). Cytidines whose reactivity could not be determined are not colored. The same colors are used in the histogram in Figure 4B, where the percentages of reactive Cs relative to their hexamer phases are noted. The yellow/red-striped columns indicate the percentage of reactive Cs (reactive (yellow) plus hyperreactive (red) Cs of Fig. 4A) and the red columns the percentage of hyperreactive bases only. It is clear that all Cs at positions 1 and 6 are reactive, that the Cs at positions 2 and 4 are least reactive, and that positions 3 and 5 are intermediate. The hyperreactive bases are mainly found in positions 1 and 6 and some at position 5 but none at positions 2, 3, and 4. Because the same nucleotide sequence is displaced across the hexamer positions in the eight mutant viruses, this result is not due to the sequence context. Clearly, the position of the cytidine on/within the N monomer influences its reactivity towards DMS.

Base reactivities of the sequence outside the GU repeats were also determined in this analysis. These bases, in contrast to those within the GU repeats, are not shifted through the six phases. Because there are only 12 Cs outside of the insertion (Fig. 4A), we have added more examples from a separate experiment that examined the end of the HN gene (not shown). The

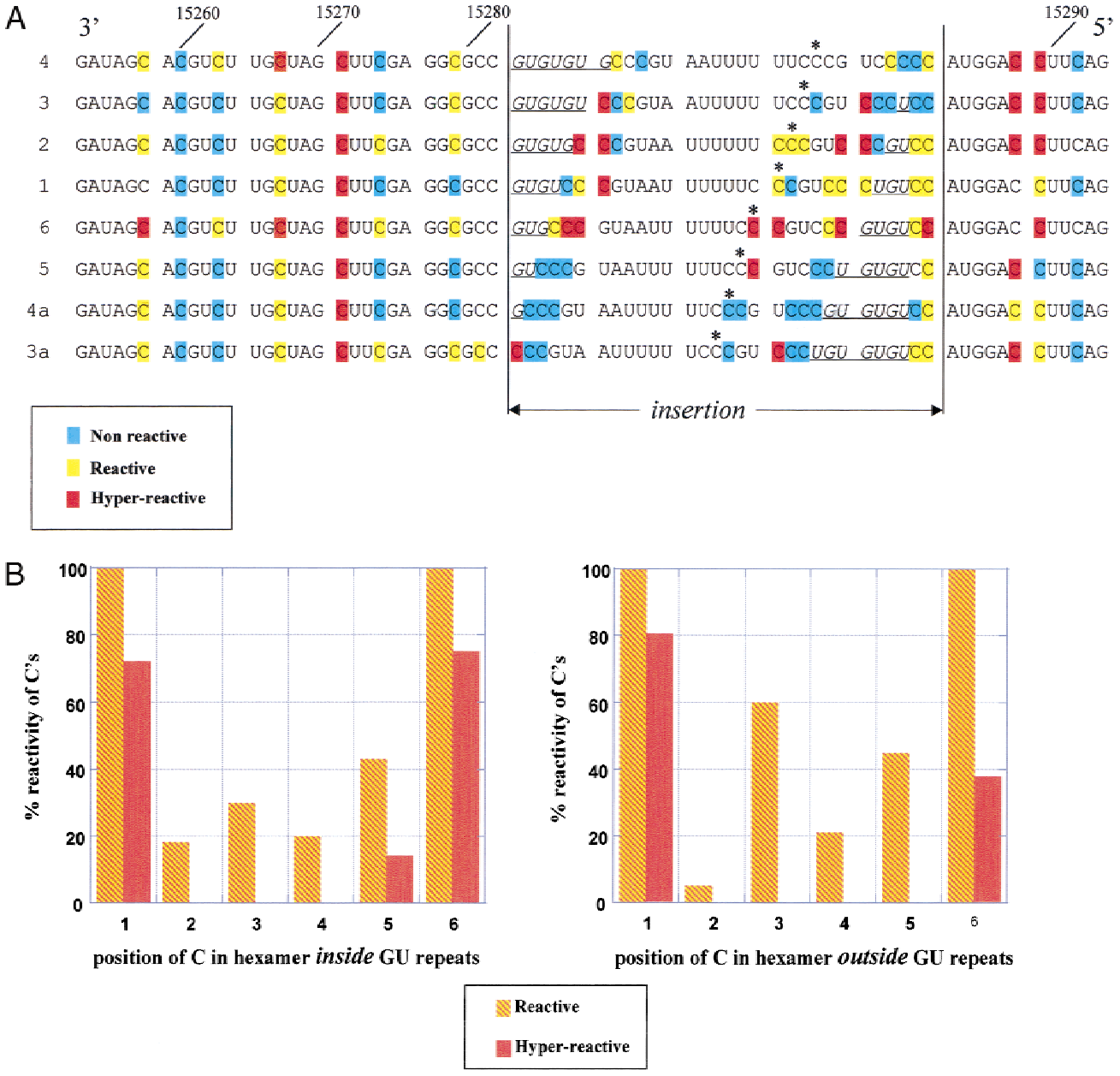

FIGURE 4. Reactivity of cytidines by hexamer position. **A:** Reactivities of cytidines as determined in Figure 3 are placed on the sequences of the eight viruses. Red are hyperreactive Cs, yellow are reactive Cs, and blue are nonreactive Cs. If the reactivity could not be determined, the respective C is not colored. **B:** Left histogram: the percentage of reactive Cs inside the GU repeats as a function of their position on the nucleoprotein. The red/yellow striped (orange) bars indicate the percentage of all reactive Cs (yellow plus red Cs in **A**) and the red bars indicate the percentage of hyperreactive Cs only. Right histogram: percentage of reactive Cs outside of the GU repeats. Because in the region that we studied, there are only 12 Cs outside of the insertion (**A**), we have added more examples from a separate experiment that examined the end of the HN gene (not shown). Therefore, this right-hand histogram represents a compilation of the reactivities of 17 Cs at position 1 (17 C-1), 20 C-2, 10 C-3, 14 C-4, 20 C-5, and 18 C-6.

right-hand histogram in Figure 4B represents a compilation of the reactivities of 17 Cs at position 1 (17 C-1), 20 C-2, 10 C-3, 14 C-4, 20 C-5, and 18 C-6. As before, the adenines showed only a very faint reactivity towards DMS. The reactivities of the Cs outside the GU repeats behave in essentially the same manner with regard to hexamer phase as those within the GU repeats (Fig. 4B, left) although position 3 was slightly more reactive. All hyperreactive bases were found again only at positions 1 and 6. Overall, N-subunit position and not the immediate sequence context seems to exert the major effect on cytosine reactivity.

The ribose-phosphate backbone of the genome RNA within influenza virus and VSV nucleocapsids is protected against chemical attack, whereas the nucleotide bases are exposed to the solvent (Baudin et al., 1994;

Klumpp et al., 1997; Iseni et al., 2000). If we assume that the ribose-phosphate backbone of the SeV nucleocapsid lies in a groove or channel of the N-subunit assembly, this might explain why the cytidines at the outside positions of the N monomer seem to be more exposed towards chemical modification than those at the middle positions. However, this simplistic view does not explain why the As are virtually nonreactive, especially as adenines are bigger than cytidines and their W-C positions can extend further from the backbone. Figure 3 also shows that some Cs are particularly reactive towards DMS. C15271 (N monomer position 1), in the constant region, for example, is particularly hyperreactive in all the viruses tested (Fig. 3, and much data not shown). The two uridines 5′ to this residue are also reactive (Fig. 3A,B) although uridines are normally not reactive towards DMS [DMS-reactive uridines were also observed in influenza virus and VSV RNA (Klumpp et al., 1997; Iseni et al., 2000)]. Why this particular region of the genome is so reactive to DMS has not been investigated.

The effect of N subunit phase on mRNA editing

Editing of the SeV P mRNA occurs within the [−] 3′ UUUUUU C<u>C</u>C "slippery" sequence. G insertions in the mRNA are due to pseudo-templated transcription by RdRP (or "stuttering") at a single template position during mRNA synthesis (underlined above) (Jacques & Kolakofsky, 1991; Hausmann et al., 1999a, 1999b). The number of Gs inserted is thought to reflect the number of RdRP stutter cycles during elongation (Vidal et al., 1990; Pelet et al., 1991). Stuttering requires realignment of the nascent mRNA 3′ end that is hybridized to the template, and recopying of the underlined template C before RdRP returns to strictly templated mRNA synthesis (for a schematic representation of a single cycle of pseudo-templated transcription, based on previous work, see Fig. 5A). The number of Gs inserted is determined genetically for each paramyxovirus, and this property maps just upstream of the 3′ U_6C_3 slippery sequence (Hausmann et al., 1999a, 1999b). Natural SeV mRNA editing (3′ U**UG** U_6C_3) is relatively restrained, as only 30% of the mRNAs are modified and essentially only a single G insertion occurs. For the related human and bovine strains of PIV3 (3′ U**AA** U_6C_3), however, about 70% of the mRNA is edited and insertions of one to six Gs occur at roughly equal frequency (Pelet et al., 1991; Galinski et al., 1992). When the SeV P gene is engineered to contain a 3′ U**AA** UUUUUU C<u>C</u>C editing site, the P mRNA editing profile resembles that of PIV3-like (Hausmann et al., 1999b). We have placed this PIV3 hyperediting site at the 5′ end of the [−] genome RNA that codes for the 3′ UTR of the L gene (Fig. 2) and varied its hexamer phase in the eight mutant viruses mentioned above. Cells were infected with these viruses and CsCl-pelleted RNA (devoid of genomes and antigenomes as nucleocapsids band in the CsCl gradient) was isolated. The L mRNA 3′ UTR was then amplified by RT/PCR. The presence of insertions within the editing site was examined by poisoned primer extension as schematically indicated in Figure 2. To control for spurious bands not associated with the editing process, reactions were also carried out on DNA that was PCR amplified directly from the plasmids used to generate each rSeV, and run alongside each mRNA lane.

The results of this analysis are shown in Figure 5B. Even though this biological process is clearly influenced by the hexamer phase, editing was not abolished at any position. The hexamer phase of the editing sequence (that operates during RdRP elongation) is thus apparently less important for activity than that of the replication promoter elements (that operate during RdRP initiation; see introduction). The effect of phase on the pattern of G insertions is subtle. Editing is most extensive when the stutter-site C (our point of reference for the entire *cis*-acting sequence) is at hexamer position 2 (the wild-type position) or positions 3 and 4, and least extensive at position 1. When the stutter-site C is in the phases 2, 3, and 4 (Fig. 5B, lanes 11–16), the editing sequence directs a pattern of insertions that closely resembles PIV3 mRNA editing, that is, ~70% of the mRNA is edited and insertions of one to six or more Gs occur at roughly equal frequency (Pelet et al., 1991; Galinski et al., 1992). When the stutter-site C is at position 1, the editing pattern more closely resembles that of the wild-type SeV P gene, where mRNAs with only one to three inserted Gs are found, in strongly decreasing abundance. The traces in the bottom panel of Figure 5B are from an independent analysis that is similar to that in the top panel, and where the differences in the range of G insertions between phases 1 and 3 is even more striking. Phases 3 and 4 are repeated in this series of viruses. The editing profiles of phase 3a and 4b viruses are similar to the profiles of phase 3 and 4 viruses in that the number of Gs inserted is equally extensive. However, the insertion of one to six or more Gs occurs in a more decreasing fashion for the 3a and 4a viruses (Fig. 5B). The phases of the editing cassettes relative to the N subunits were displaced by altering the lengths of a GU repeat on either side of this sequence. The different GU repeats upstream of the editing site may influence the editing profiles as upstream sequences can affect editing frequency (Hausmann et al., 1999a), and this may account for the somewhat different editing patterns of duplicate phases 3 and 4. However, it is also clear that the number of stutter cycles that RdRP carries out before resuming strictly templated synthesis (in response to the *cis*-acting sequence during mRNA synthesis) varies in a cyclical manner when the editing sequence is displaced across successive hexamer phases.

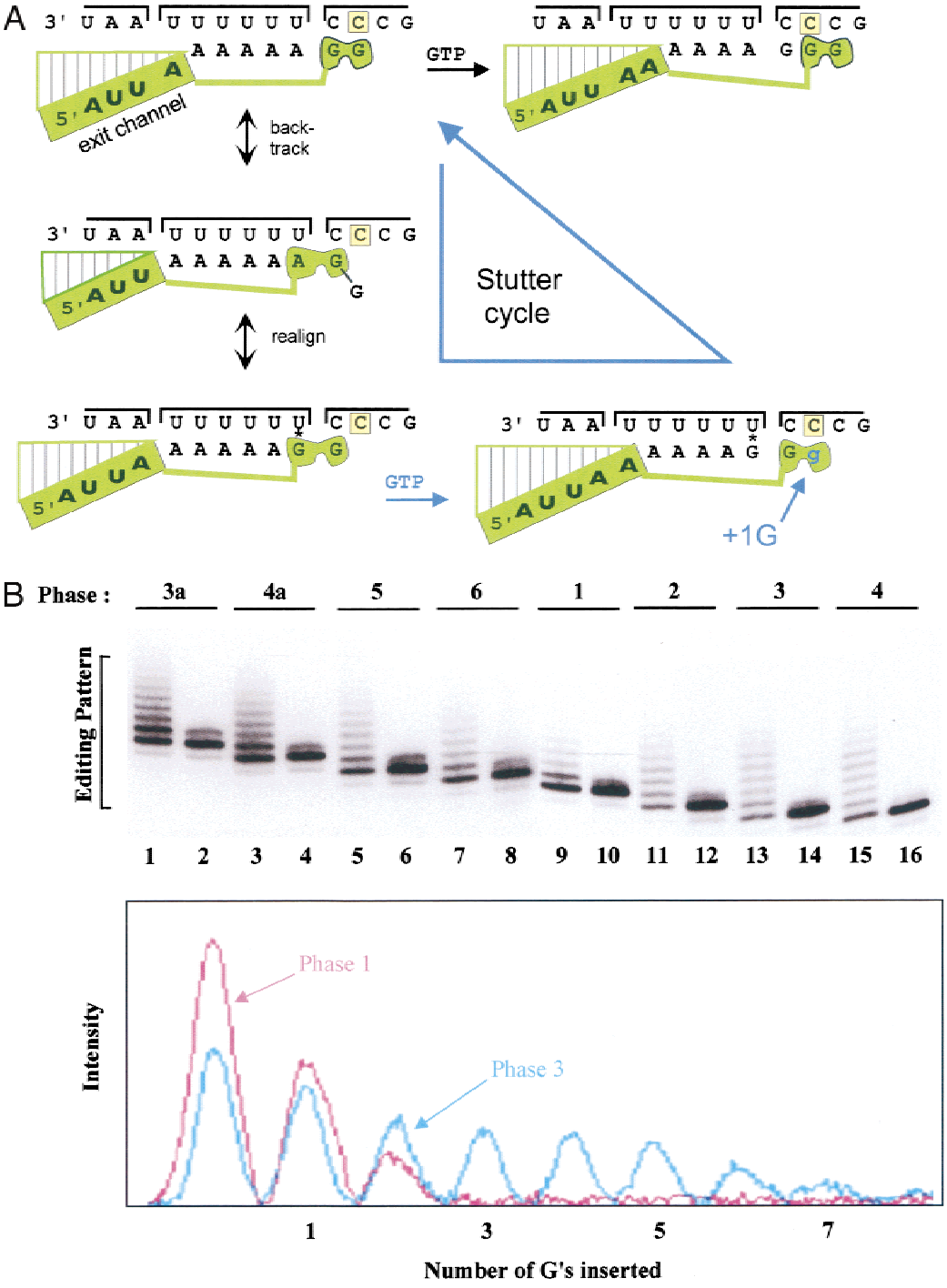

FIGURE 5. Hexamer phase and L mRNA editing. **A:** A schematic view of the SeV transcription elongation complex at the editing site and the proposed cycle of pseudo-templated G insertion (after Hausmann et al., 1999a, 1999b). The editing site of the wild-type [−] genome is shown on top; its hexamer phase is indicated by overhead brackets and spacing. The unique C stutter site is boxed. The nascent mRNA chain is shown below, and the 7-bp mRNA/template hybrid is indicated by the close apposition of the sequences. The bipartite active site and mRNA exit channel of RdRP are shown schematically. The wedge or rudder that fixes the limits of the mRNA/template hybrid (Landick, 2001) is represented by a striped triangle. The stutter cycle begins at the top left, just after the strictly templated G is incorporated. At this point, RdRP can either continue strictly templated transcription (top right), or backtrack along the template, realign the 7-bp mRNA/template hybrid, and pseudo-transcribe the stutter site C, creating a 1-G insertion. This stutter cycle can be repeated before strictly templated transcription resumes, and leads to multiple G insertions in the mRNA. **B:** HeLa cells were infected with 20 pfu/cell of the various SeV-L^{edit} stocks. CsCl pellet RNA was isolated from each infected culture at 24 h postinfection. The L mRNA 3′ UTR carrying the editing site was amplified by RT/PCR, and subjected to poisoned primer extension analysis (Materials and Methods; Fig. 2). As negative controls, the same analysis was carried out with the DNA plasmids used to generate the various SeV-L^{edit}, and these controls (even-numbered lanes) were run alongside the infected cell RNA analysis (odd-numbered lanes). The various viruses are listed above according to the hexamer phase of the stutter-site cytidine, as in Figure 2. The upper panel shows a film of the sequencing gel of a complete experiment. The lower panel shows tracings from the phase 1 and phase 3 virus infections of a separate analysis, to indicate the reproducibility of the range of G insertions (see text).

DISCUSSION

For influenza virus and VSV, two [−] RNA viruses of other families, the nucleoprotein in resting nucleocapsids (not engaged in RNA synthesis) binds to the ribose-phosphate backbone of the genome RNA and the nucleotide bases are exposed to the solvent (Baudin et al., 1994; Klumpp et al., 1997; Iseni et al., 2000). Apart from the nucleotides at the conserved 3′ and 5′ ends of influenza virus RNA that are protected from chemical modification when the polymerase is present, all nucleotides are more or less equally reactive at their W-C positions to the various chemical probes that were tested (Klumpp et al., 1997). For VSV, variability in nucleotide reactivity was observed, but no regularity in this variation was detected (Iseni et al., 2000). Experiments on isolated SeV nucleocapsids and intact virions showed that the bases were relatively nonreactive to chemical modification at their W-C positions. Using dimethyl sulfate to probe N1-A and N3-C, we observed a very reduced reactivity of the adenines and a strongly variable reactivity of the cytidines. There is thus a gradient of interaction of N subunits and genome RNAs of resting influenza, rhabdo- and paramyxovirus nucleocapsids, culminating in the relative nonreactivity of all adenines in SeV nucleocapsids. This contrasts strongly with influenza virus and VSV nucleocapsids, where adenines are generally more reactive than cytidines, consistent with their relative reactivities as free bases (Blackburn, 1996). It is unclear how the SeV N/RNA interactions in resting nucleocapsids can specifically prevent DMS reaction with adenines. Cytidines within resting SeV nucleocapsids are most reactive at hexamer positions 1 and 6. Remarkably, the downstream replication promoter element for SeV consists of 3 Cs at hexamer position 1, and for SV5 there are 3 Cs at position 6 plus 3 Gs at position 5 (Fig. 1). It may be coincidental that the conserved nucleotides of this *cis*-acting element are, in all cases, in the most reactive positions in resting nucleocapsids. However, if cytidine reactivity with DMS in resting nucleocapsids also reflects the accessibility of RdRP to the *cis*-acting promoter sequences of the template, this may be more than coincidence. It is presumably this resting structure that RdRP engages to initiate viral RNA synthesis at the 3′ end of the template.

We have also shown that the hexamer phase of a mRNA editing site affects the pattern of RdRP stuttering during mRNA editing. These differences due to hexamer phase, although subtle, are nevertheless likely to be important for viral replication. The P protein (unedited mRNA, +3Gs, etc.) is an essential RdRP subunit whereas the V (+1G, +4Gs, etc.) and W proteins (+2Gs, +5Gs, etc.) are inhibitors of viral RNA synthesis. V and W also act to counteract the innate immune response of the host, whereas P is inactive in this respect (Lamb & Kolakofsky, 2001). Even modest differences in the relative proportions of these mRNAs can thus have important consequences on how these viruses replicate in nature in the presence of powerful host innate defenses. It is presumably these differences that account, at least in part, for the conservation of the hexamer phase of the P gene editing site. Pneumoviruses and rhabdoviruses, the two virus groups most closely related to the *Paramyxovirinae*, also form their mRNA poly(A) tails in the cytoplasm by RdRP stuttering. However, pneumoviruses (Samal & Collins, 1996) and rhabdoviruses (Pattnaik et al., 1995) do not edit their mRNAs, nor do they appear to be governed by a hexamer (or any integer) rule. RdRP stuttering in the middle of the P gene ORF that leads to a limited number of G insertions needs to be relatively precise, as compared to the stuttering that adds ~250 adenosines at the ends of each gene transcript. It has thus been suggested that hexamer phase and mRNA editing coevolved in the *Paramyxovirinae* to provide the additional information required to control the RdRP stuttering involved in mRNA editing (Kolakofsky et al., 1998).

The finding that the hexamer phase of a mRNA editing site affects this process, albeit subtly, indicates that the template nucleoprotein influences RdRP during elongation. This could mean that the nucleoprotein remains associated with the genome RNA even during SeV mRNA synthesis. Biochemically, it is impossible to remove the nucleoprotein from the genomic RNA of rabies virus, VSV, or SeV in resting nucleocapsids without denaturation of the protein, and the genomic RNA within N:RNA is resistant to RNase A digestion under all salt conditions (Lynch & Kolakofsky, 1978; Iseni et al., 2000, and references therein). The close association of N protein and genomic RNA in rhabdovirus and paramyxovirus nucleocapsids has raised the question of how this RNA is made available for the base pairing that has to take place during RNA-directed RNA synthesis. This base pairing includes not only that involved in nucleotide addition, but also the hybrid between the 3′ end of the nascent chain and the template that is essential to maintain polymerase register, as shown for *Escherichia coli* RNA polymerase (Nudler et al., 1997). This hybrid has been postulated to be 7 bp in length from studies of SeV mRNA "editing" (Hausmann et al., 1999a, 1999b). One obvious explanation is that the N subunits are sufficiently displaced from the template RNA during RNA synthesis so that the hybrid can be accommodated in the putative active site channel of RdRP. This is thought to occur in all other R/DdRPs for which high-resolution structures are available (Landick, 2001).

Given that the N-subunit phase affects SeV RdRP during mRNA editing, there are two very different views of how this might occur. If SeV N remains associated with the template RNA even during RNA synthesis, the catalytic L subunit of SeV RdRP then presumably does not bind the ribose-phosphate backbone of the tem-

plate. This would be in contrast to positive-strand RNA virus RdRP that surrounds the template/nascent chain hybrid during elongation, similar to cellular DdRP (Bressanelli et al., 1999; Zhang et al., 1999; Korzheva et al., 2000; Gnatt et al., 2001; Landick, 2001). Consistent with this, the catalytic L subunit of SeV RdRP does not bind to N:RNA directly, but via the P protein (Horikami et al., 1992; Curran et al., 1994), in contrast to other RdRPs. In this view, successive N subunits would be as integral a component of the transcription elongation complex as the L and P proteins themselves. The N subunits could then directly imprint another code upon the genetic code. Alternatively, if N is transiently displaced from the template RNA so that the L protein can encircle the hybrid, N would presumably remain associated with another surface of L, so as to indirectly affect mRNA editing.

MATERIALS AND METHODS

Preparation of rSeV

Five picomoles of oligonucleotides carrying the editing site (3′-UAAUUUUUUCCC-5′) in the relative hexamer phase 4, 3, 2, 1, 6, 5, 4a, 3a, as follows:

Phase 4:
5′-CTCCCCGCGGCACACACGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCCCATGGGG-5′
Phase 3:
5′-CTCCCCGCGGCACACAGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCTCCATGGGG
Phase 2:
5′-CTCCCCGCGGCACACGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCGTCCATGGGG
Phase 1:
5′-CTCCCCGCGGCACAGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCTGTCCATGGGG
Phase 6:
5′-CTCCCCGCGGCACGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCGTGTCCATGGGG
Phase 5:
5′-CTCCCCGCGGCAGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCTGTGTCCATGGGG
Phase 4a:
5′-CTCCCCGCGGCGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCGTGTGTCCATGGGG
Phase 3a:
5′-CTCCCCGCGGGGGCATTAAAAAAGG-3′
3′-CCCGTAATTTTTTCCCGTCCCTGTGTGTCCATGGGG

were resuspended in TE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) and boiled for 2 min. The primers were annealed by slow return to room temperature in 0.3 M NaCl. The annealed primers were precipitated and filled in using the Klenow enzyme. The dsDNAs were then digested by *Sac*II and *Kpn*I (underlined in the primer sequences) and inserted in the same sites in the 3′ UTR of the L gene (between nt 15277 and 15281) in the pFL5 infectious clone (Fig. 2). Virus recovery was carried out as previously described (Schnell et al., 1994; Garcin et al., 1995), except that vaccinia virus coinfection was eliminated. BSR T7 cells were transfected with pFL5 and IRES-containing pTM1 plasmids encoding the N, P, and L proteins for 48 h at 37 °C. Acetyltrypsin (1.2 μg/mL, in serum-free medium) was then added to the cells for 24 h to activate the fusion protein. The supernatant was then inoculated into 8-day-old embryonated chicken eggs, and allantoic fluid was recovered after 3 days at 33 °C. After a second passage in eggs, the allantoic fluid was titered and stored at −70 °C, or centrifuged on a 25% glycerol cushion in TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA). The virus pellet was resuspended in 50 mM sodium cacodylate, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 5% glycerol, at a concentration of 10 mg/mL (by Bradford assay; Bio-Rad).

Chemical probing and detection of the modified positions

The RNA modification procedure and the chemistry of methylation by DMS have been described previously (Ehresmann et al., 1987; Baudin et al., 1994; Klumpp et al., 1997; Iseni et al., 2000) and was adapted for use with intact virus particles. For methylation, 0, 0.1, and 0.3 μL DMS (representing conditions of control, 1, and 2, respectively) were added to 250 μg (25 μL) of purified virus in 50 mM sodium cacodylate buffer, pH 7.5, 20 mM magnesium acetate, 0.3 M KCl, 5 mM DTT. The virus was prewarmed for 10 min at 37 °C and then incubated for 3 min with DMS at 37 °C. The virus suspension was then centrifuged for 3 min at 150,000 × *g* in an Airfuge (Beckman). The pellet was directly phenol/chloroform extracted and the modified template was precipitated with ethanol in the presence of 0.3 M sodium acetate, pH 6.8.

The precipitated RNA was washed with 70% ethanol, vacuum-dried, and redissolved in double distilled water. Cuts in the RNA or modified positions were detected by the primer extension method using reverse transcriptase. An oligodeoxyribonucleotide complementary to nucleotides 15235–15253 of [−] genome RNA (5′-GCTCGTAATAATTAGTCCC-3′) was labeled at its 5′ end with [γ^{32}P]ATP and was used as a primer for reverse transcription. The RNA template was then hydrolysed by addition of 3 μL 3 M KOH and incubation for 3 min at 95 °C, followed by 1 h at 37 °C. The cDNA fragments were then precipitated and separated by PAGE on 12% acrylamide/0.5% bis(acrylamide)/8 M urea slab gels at 1,500 V for 2 h. Dideoxy sequencing reactions were carried out in parallel using the naked unmodified template (Sanger et al., 1977). Incubation controls were run in parallel to detect nicks in the unmodified RNA and reverse transcriptase pauses due to RNA 2° structures.

Virus infection and purification of total RNA

HeLa cell cultures at 60% confluence in 10 cm Petri dishes were infected with the various rSeV stocks at a m.o.i. of 20 for 1 h in 1 mL of serum-free DMEM, followed by incubation at 33 °C in medium containing 5% FCS. Two days postinfection, the cells were lysed in 0.5% NP-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA. After a brief spin to remove

cell debris, the supernatant was loaded on a 20–40% CsCl and centrifuged overnight in a SW55 rotor at 38,000 rpm. The RNA pellet (free of encapsidated genome and antigenome RNA) was resuspended in a buffer containing 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and recovered by ethanol precipitation. The RNA pellet was then resuspended in 10 μL of water.

Poisoned primer extension and sequencing reactions

Half of the total pellet RNA (5 μL) was used for reverse transcription with the oligonucleotide 5′ CTT ACT ATT GTC ATA TGG ATA AG 3′ (downstream of the L editing site) and 200 U of MMLV-reverse transcriptase (Gibco-BRL) for 1 h at 42 °C. A 1/10 sample was then amplified by PCR with the above primer and the oligonucleotide 5′ GAT GGA TCA CTG GGT GAT ATC G located upstream of the L editing site. A similar PCR reaction was performed directly on the plasmids encoding the eight rSeV genomes. After purification on a 2% agarose gel, the PCR products were annealed to 5′ ^{32}P-ATA AGT CCA AGA CTT CCA GGT ACC 3′, complementary to the sequence immediately downstream of the editing site (Fig. 2). Poisoned primer extension was performed in 10 μL at 37 ° for 10 min with 1 U of T7 DNA polymerase (Pharmacia), in the presence of 40 μM dGTP, dTTP, dCTP, and 4 μM dideoxy-ATP. Then, 200 μM dNTP was added and the mix incubated a further 5 min. The reaction was stopped by adding a solution of 95% formamide, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The extension products were boiled for 1 min and electrophoresed on a 12.5% polyacrylamide sequencing gel. The gel was dried and exposed to X-OMAT film (Kodak).

ACKNOWLEDGMENTS

F. Iseni is supported by a long-term European Molecular Biology Organisation fellowship, and a grant from the Roche Research Foundation. The early contributions of Jonathan Freeman and Joseph Curran (Geneva) and Estelle Garino (Grenoble) are gratefully acknowledged. This work was also supported by a grant from the Swiss National Science Fund.

Received January 10, 2002; returned for revision January 29, 2002; revised manuscript received May 7, 2002

REFERENCES

Baudin F, Bach C, Cusack S, Ruigrok RWH. 1994. Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panandle RNA and exposes the bases to the solvent. *EMBO J 13*:3158–3165.

Blackburn GM. 1996. Covalent interactions of nucleic acids with small molecules. In: *Nucleic acids in chemistry and biology*, 2nd ed. Oxford: Oxford University Press. p 291.

Bressanelli S, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, De Francesco R, Rey FA. 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci USA 96*:13034–13039.

Calain P, Roux L. 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J Virol 67*:4822–4830.

Curran J, Pelet T, Kolakofsky D. 1994. An acidic-like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. *Virology 202*:875–884.

Egelman EH, Wu S-S, Amrein M, Portner A, Murti G. 1989. The Sendai virus nucleocapsid exists in at least four different helical states. *J Virol 63*:2233–2243.

Ehresmann C, Baudin F, Mougel M, Romby P, Ebel JP, Ehresmann B. 1987. Probing the structure of RNAs in solution. *Nucleic Acids Res 15*:9109–9128.

Galinski MS, Troy RM, Banerjee AK. 1992. RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. *Virology 186*:543–550.

Garcin D, Pelet T, Calain P, Roux L, Curran J, Kolakofsky D. 1995. A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: Generation of a novel copy-back nondefective interfering virus. *EMBO J 14*:6087–6094.

Gnatt AL, Cramer P, Fu J, Bushnell DA, Kornberg RD. 2001. Structural basis of transcription: An RNA polymerase II elongation complex at 3.3 A resolution. *Science 292*:1876–1882.

Gubbay O, Curran J, Kolakofsky D. 2001. Sendai virus genome synthesis and assembly are coupled: A possible mechanism to promote viral RNA polymerase processivity. *J Gen Virol 82*: 2895–2903.

Hausmann S, Garcin D, Delenda C, Kolakofsky D. 1999a. The versatility of paramyxovirus RNA polymerase stuttering. *J Virol 73*:5568–5576.

Hausmann S, Garcin D, Morel AS, Kolakofsky D. 1999b. Two nucleotides immediately upstream of the essential A6G3 slippery sequence modulate the pattern of G insertions during Sendai virus mRNA editing. *J Virol 73*:343–351.

Hoffman MA, Banerjee AK. 2000. Precise mapping of the replication and transcription promoters of human parainfluenza virus type 3. *Virology 269*:201–211.

Horikami SM, Curran J, Kolakofsky D, Moyer SA. 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *J Virology 66*: 4901–4908.

Iseni F, Baudin F, Blondel D, Ruigrok RW. 2000. Structure of the RNA inside the vesicular stomatitis virus nucleocapsid. *RNA 6*: 270–281.

Jacques JP, Kolakofsky D. 1991. Pseudo-templated transcription in prokaryotic and eukaryotic organisms. *Genes & Dev 5*: 707–713.

Klumpp K, Ruigrok RWH, Baudin F. 1997. Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. *EMBO J 16*:1248–1257.

Kolakofsky D, Pelet T, Garcin D, Hausmann S, Curran J, Roux L. 1998. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: The rule of six revisited. *J Virol 72*: 891–899.

Korzheva N, Mustaev A, Kozlov M, Malhotra A, Nikiforov V, Goldfarb A, Darst SA. 2000. A structural model of transcription elongation. *Science 289*:619–625.

Lamb RA, Kolakofsky D. 2001. Paramyxoviridae: The viruses and their replication. In: Knipe DM, Howley PM, eds. *Fields virology*. Philadelphia: Lippincott, Williams & Wilkins. pp 1305–1340.

Landick R. 2001. RNA polymerase clamps down. *Cell 105*:567–570.

Lynch S, Kolakofsky D. 1978. Ends of the RNA within Sendai virus defective interfering nucleocapsids are not free. *J Virol 28*: 584–589.

Murphy SK, Ito Y, Parks GD. 1998. A functional antigenomic promoter for the Paramyxovirus Simian virus 5 requires proper spacing between an essential internal segment and the 3′ terminus. *J Virol 72*:10–19.

Murphy SK, Parks GD. 1999. RNA replication for the paramyxovirus simian virus 5 requires an internal repeated (CGNNNN) sequence motif. *J Virol 73*:805–809.

Nudler E, Mustaev A, Lukhtanov E, Goldfarb A. 1997. The RNA–DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. *Cell 89*:33–41.

Pattnaik AK, Ball LA, LeGrone A, Wertz GW. 1995. The termini of VSV DI particles are sufficient to signal encapsidation, replication and budding to generate infectious particles. *Virology 206*: 760–764.

Pelet T, Curran J, Kolakofsky D. 1991. The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. *EMBO J 10*:443–448.

Pelet T, Delenda C, Gubbay O, Garcin D, Kolakofsky D. 1996. Partial characterization of a Sendai virus replication promoter and the rule of six. *Virology 224*:405–414.

Samal SK, Collins PL. 1996. RNA replication by a respiratory syncytial virus RNA analog does not obey the rule of six and retains a nonviral trinucleotide extension at the leader end. *J Virol 70*: 5075–5082.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Schnell MJ, Mebatsion T, Conzelmann K-K. 1994. Infectious rabies viruses from cloned cDNA. *EMBO J 13*:4195–4203.

Tapparel C, Maurice D, Roux L. 1998. The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: A motif (GNNNNN)3 is essential for replication. *J Virol 72*:3117–3128.

Vidal S, Curran J, Kolakofsky D. 1990. A stuttering model for paramyxovirus P mRNA editing. *EMBO J 9*:2017–2022.

Vulliemoz D, Roux L. 2001. "Rule of six": How does the Sendai virus RNA polymerase keep count? *J Virol 75*:4506–4518.

Zhang G, Campbell EA, Minakhin L, Richter C, Severinov K, Darst SA. 1999. Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Å resolution. *Cell 98*:811–824.