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Mutational Analysis of Influenza B Virus RNA Transcription In Vitro

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The roles of the 3′- and 5′-terminal nucleotides and the panhandle structure of influenza B virus virion RNA were analyzed in vitro by transcription of model RNA templates with influenza B virus RNA polymerase. The results suggest that the stability of the panhandle and breathing of the extreme ends of the panhandle are important factors for efficient transcription. Influenza B virus polymerase appears to be more tolerant of mutations in the panhandle structure than influenza A virus polymerase. This is consistent with the greater degree of heterogeneity observed naturally in the 3′-terminal nucleotides of the virion RNA of influenza B virus than in influenza A virus.

Influenza B virus remains the cause of a significant number of illnesses associated with influenza virus infections, especially among children (10, 18). Influenza B viruses isolated in nature show differences from A virus in epidemiology, pathogenicity, and evolutionary pattern (25, 29). This virus is unique in its diversity of coding strategies, and significant progress has been made in understanding its translational control (12, 14, 23). However, little is known about the *cis*- and *trans*-acting elements involved in transcription and replication of its genome.

The genome of influenza B virus consists of eight single-stranded negative-sense RNA segments as in A virus. Each segment carries 30 to 60 nucleotides (nt) and 30 to 100 nt at the 3′ and the 5′ noncoding regions, respectively. As in A viruses, the extreme 3′ and 5′ termini are very well conserved in all eight RNA segments (4, 26). The inverted complementarity of the segment-specific sequences adjacent to the conserved termini allows both the 3′ and the 5′ ends to form an extended panhandle structure. However, more heterogeneity was observed in the extreme termini of B virus than in A virus RNAs (26). For example, while there are 12 and 13 conserved nucleotides at the 3′ and the 5′ ends, respectively, in A viruses, there are only 9 and 10 nt at the corresponding ends in B viruses. The differences in sequences and lengths of the noncoding regions may contribute to the specificities between A and B viruses in transcription, replication, and encapsidation.

In the present communication, the *cis*-acting signals in influenza B virus transcription were analyzed by using the active polymerase prepared from the B virus and short RNA templates derived from the 3′ and the 5′ ends of the influenza B virion RNA (vRNA). A similar approach has already been well advanced in influenza A viruses in dissecting RNA signals in vitro (5, 6, 9, 19, 20, 22) and in vivo by the reverse genetic approach (8, 15, 16, 19). As an initiative to study promoter specificity between the two influenza virus types, we also compared the cross-reactivity between the polymerase and RNA templates derived from both viruses.

Transcription was carried out in the presence of the ApG primer, essentially as before (21, 22), using micrococcal nuclease (MN)-treated viral core and RNA templates. RNA templates were synthesized by transcription of a partial DNA duplex with T7 RNA polymerase. After T7 transcription, the RNAs were resolved on 20% polyacrylamide gels in 7 M urea, visualized by staining with toluidine blue, and purified by elution from the gel. Trypsinized whole influenza B virus (B/HK/HG, a reassortant between B/Lee/40 and B/HK/73 [1]) was kindly provided by W. G. Laver, John Curtin Medical School, Australian National University, Canberra, Australia, and G. G. Brownlee, University of Oxford, Oxford, England. Trypsin inhibitor was added, the mixture was centrifuged, and viral pellet was resuspended for lysis with 1% Nonidet P-40 and digestion with MN. The presence of Nonidet P-40, 0.2% in the whole transcription reaction mixture, does not affect the efficiency of transcription (data not shown). Transcription was carried out in the presence of [α-^{32}P]CTP for 2 h at 30°C. The RNA was extracted with a phenol-chloroform mixture, ethanol precipitated in the presence of carrier RNA, and analyzed on a 20% polyacrylamide gel in 7 M urea. Labeled RNA products were estimated by densitometric scanning of the exposed X-ray films.

The wild-type influenza B vRNA template used in this study represents sequences found in the majority of influenza B vRNA segments (Fig. 1). Mutations were introduced at the conserved nucleotides at the 3′ end of the vRNA (Fig. 1b). Positions 9, 10, and 11 have been reported to be important for influenza A virus polymerase activity (22); therefore, mutations at the same locations were tested for the influenza B vRNA templates. ApG-dependent transcription was carried out with different combinations of polymerase and RNA templates (Fig. 2).

In summary, polymerases from both A virus and B virus were active in transcribing both cognate and noncognate wild-type RNA templates (Fig. 2, lane 2). Both polymerases showed a similar spectrum of activity toward mutants in position 9 (Fig. 2, lanes 3, 4, and 5). The C9 mutant showed much decreased activity (5 to 20% of wild type), whereas the U9 and A9 mutants showed variable but, in general, reduced activity with the two different polymerases. Greater sensitivity was observed toward B polymerase than A polymerase by the U9 and A9 mutants (Fig. 2b, lanes 3 and 5). However, much clearer discrepancies in transcription efficiency were observed with specific mutants at positions 10 and 11.

First, influenza B virus polymerase was active toward the G10 mutant (102% of the wild-type influenza B virus; Fig. 2b,

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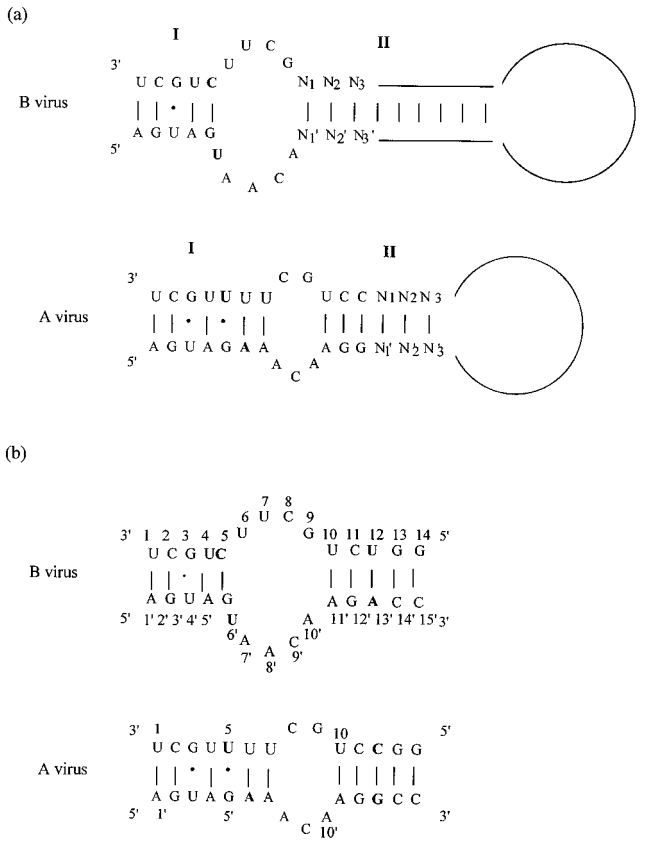

FIG. 1. (a) Sequences and potential panhandle structures of the influenza A and B vRNAs. Both influenza A and B vRNAs can form a panhandle structure by partial base pairing between the 3′- and 5′-terminal sequences. The panhandle can be divided into structures I and II, which are joined together by a loop structure. The number of nucleotides conserved in all eight RNA segments at the 3′ and the 5′ ends are 12 and 13 nt, respectively, for influenza A virus. In influenza B virus, however, only 9 and 10 nt are absolutely conserved at the 3′ and the 5′ ends, respectively. Panhandle structure II is usually extended in variable lengths, usually with 8 to 10 bp in various influenza B virus strains. Only limited variation is observed in nature according to Stoeckle et al. (26). The variations occur at position 10 of the 3′ end (N1-N1′); U10-A11′ for the HA, NA, NS, NP, and M proteins; and C10-N11′ for PA, PB1, and PB2. At position 11 (N2-N2′), the variations are C11-G12′ for HA, NA, and NS, and G11-C12′ for NP and the M segment. At position 12 (N3-N3′), the variations are U12-A13′ for HA, NA, NS, and NP, and C12-G13′ for M. Beyond this region, more heterogeneity is found among RNA segments. Natural variation is observed at position 4 (U and C) in A virus and at position 10 (U and C) in B virus. This variation in B virus involves a mismatch in the 10-11′ base pair (C10-C11′) (3, 11). In rare cases, the mismatch is more extensive and includes even the 11-12′ base pair (4). (b) Potential panhandle structure formed by two synthetic RNAs (14-nt long 3′ RNA and 15-nt long 5′ RNA) used in this study which correspond to the 3′- and 5′-terminal sequences. The conserved sequences among RNA segments are shown. The numbering starts from the 3′ end (positions 1 to 14) and from the 5′ end (positions 1′ to 15′). The nucleotides in influenza B vRNA at positions 10, 11, and 12 of the 3′ end and positions 11′, 12′, and 13′ of the 5′ end represent the sequence most frequently observed in the eight RNA segments. The differences in nucleotides between A and B virus RNAs are shown in boldtype.

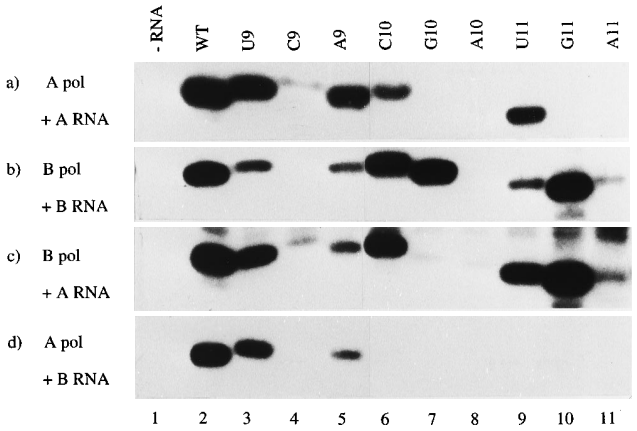

FIG. 2. Transcription of positions 9, 10, and 11 mutant RNAs in the presence of ApG dinucleotide primer. Polymerases were prepared from both A and B viruses by MN treatment (21, 22). T7 RNA transcripts, corresponding to the 3′-end vRNA conserved sequences of the respective A and B viruses, were mixed with polymerase for the transcription reaction. The low activity of the A polymerase toward influenza A vRNA mutants C9, A10, and A11 confirms our earlier observation (22), and the low activity toward A10, G10, A11, and G11 is consistent with the results of others (5). Whether the discrepant results with the C9 mutant (5) are due to different methods of RNA preparation is not known. Lanes 1, no RNA; lanes WT, wild-type influenza A or B virus as indicated on the left.

lane 7), whereas it was virtually inactive toward the influenza A virus G10 mutant (10% of the wild-type activity; Fig. 2c, lane 7). In contrast, A polymerase was inactive toward both B and A virus G10 mutants (<5% of the wild-type influenza A virus; Fig. 2a and d, lane 7). Second, B polymerase was active toward G11 mutants (105% of the wild-type influenza A and B viruses; Fig. 2b and 2c, lanes 10). However, A polymerase was inactive toward the same mutants (<5% of the wild type; Fig. 2a and 2d, lanes 10). Third, A polymerase was active toward the U11 mutant (45% of the wild type; Fig. 2a, lane 9) but inactive toward the influenza B virus U11 mutant (<5% of the wild type; Fig. 2d, lane 9). Fourth, with the exception of the U9 and A9 mutants, A polymerase was unable to transcribe all other mutants of the influenza B virus promoter (Fig. 2d). Overall, B polymerase was more tolerant of some mutations involved in the panhandle structure at positions 10 and 11 than was A polymerase (e.g., C10, G10, and G11 mutants in Fig. 2b, lanes 6, 7, and 10; 95%, 102%, and 105% of the wild type, respectively) and showed consistently higher activity toward these mutants in every combination of polymerase and RNA (compare Fig. 2a and d for A polymerase with Fig. 2b and c for B polymerase). This finding is consistent with the heterogeneity observed with the influenza B vRNA at positions 10 and 11 (3, 26). That is, sequence conservation at the 3′ end of influenza B vRNA is confined within the first 9 nt compared with 12 nt in A virus vRNA (Fig. 1a).

We first speculated about why the influenza B virus polymerase is tolerant to the G10 mutation in cognate RNA and not to the same mutation in noncognate RNA (compare Fig. 2b, lane 7, with 2c, lane 7). The possibility is that the sequence variations at positions 5 and 12 between the A and B vRNA 3′ ends play a role in this discrimination (Fig. 1b). Therefore, the G10 mutation was coupled either with the C5 or U12 mutation and the activities of these mutants were tested with the B polymerase (Fig. 3). The low activity of B polymerase to mutant A/10G was rescued in mutant A/G10U12 (85% of the wild type; Fig. 3, lanes 3 and 6). However, the activity was not rescued by coupling with the C5 mutation (mutant A/C5G10; Fig. 3, lane 5; <15% of wild type). This confirms that position 12, rather than position 5, is important for the observed discrimination.

Since the 3′ and the 5′ ends of the vRNAs are expected to form a panhandle structure (Fig. 1), we speculated whether the low activity of particular mutants (e.g., A10, A11, and U11 in Fig. 2) is due to destabilization of the panhandle. We tested, therefore, if restoration of the panhandle by the addition of the 5′-end RNA fragment could rescue the activity. Transcription of the 3′ RNA by B polymerase was carried out both in the

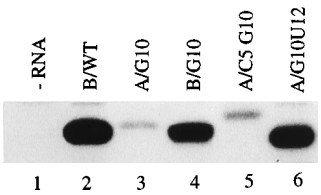

FIG. 3. Effect on transcription of coupling single-base mutations. B polymerase was used to transcribe mutant G10 RNA, either alone or coupled with mutations at position 5 or 12. The slightly slower mobility of mutant A/C5G10 (lane 5) than mutant A/G10 (lane 3) is probably due to a sequence-specific effect caused by a greater retardation of G residues in dense gels (24).

absence and in the presence of the 5′ RNA to form the panhandle structure (Fig. 4). The rescue of activity was observed when the added 5′ RNA fragment complemented the base pair A10-U11′ (54% of the wild type; Fig. 4a, compare lanes 7 and 8) and the base pair A11-U12′ (80% of the wild type; Fig. 4a, compare lanes 10 and 11). Similarly, the low activity of mutant B/U11 was rescued by the addition of the 5′ RNA that restores the U11-A12′ base pair (60% of the wild type; Fig. 4b, compare lanes 6 and 7). However, the wild-type 5′ RNA fragment which does not restore the base pair with the 3′ RNA did not rescue the activity (Fig. 4a, lanes 9 and 12). These results are consistent with the importance of the panhandle in initiation. This conclusion is further confirmed by experiments with the 3′ triple mutants B/A10A11A12 and B/A10U11A12, for which the activity of these transcriptionally inactive mutants was restored by the 5′ RNA carrying the complementary triple mutations U11U12U13 and U11A12U13, respectively (Fig. 5; compare lanes 3 and 4 with lanes 5 and 6).

It should be noted that the polymerase used in the present study was prepared by digestion of the influenza virus core with MN. Previous studies with influenza A virus showed that binding of the influenza virus polymerase complex to the panhandle protects the 3′ and the 5′ ends of the virion RNAs from digestion. This observation is supported by cross-linking studies (5, 7), transcription of the residual RNA fragments after nuclease digestion (21), and by gel retardation assays (27). The presence of the 5′-end RNA fragments in the polymerase preparation could explain why, using the B polymerase, the A/G10 mutant is inactive, whereas the activity is rescued in the A/G10U12 double mutant. A/G10 mutant RNA is not expected to form a stable panhandle with the 5′ B RNA fragment because of a double mismatch (G10-A11′ and U12-G13′), hence the low activity (Fig. 1a, legend). However, the U12-A13′ base pair is restored in the A/G10U12 double mutant, which rescues the activity. Considering the less-critical nature of the 10-11′ base pair (see below), this result suggests a critical role for the 12-13′ base pair in template activity. The importance of the panhandle on influenza B virus RNA transcription is consistent with and further extends similar findings with influenza A virus (5, 6).

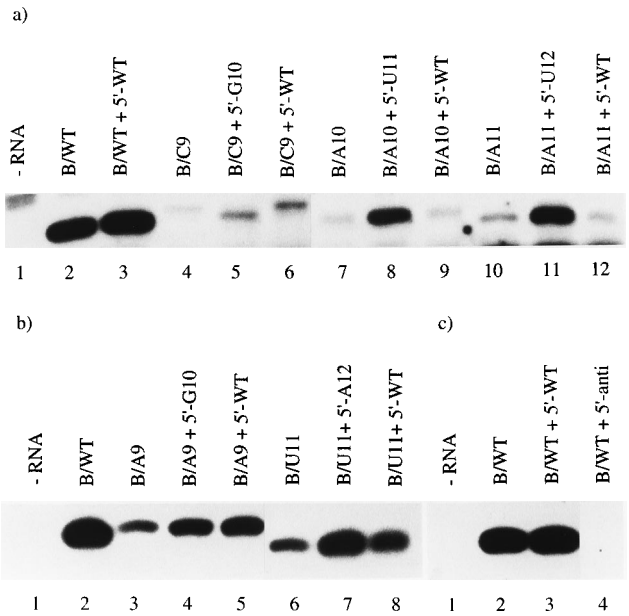

FIG. 4. Effect of 5′-end RNA on transcription of 3′ vRNA. Influenza B virus polymerase was used to transcribe the vRNA templates of B virus. (a) and (b) Rescue of transcription activity of RNAs carrying mutations at positions 9, 10, or 11 by 5′-end RNAs restoring the panhandle structure. (c) Effect of antisense RNA on transcription. The sequence of the 5′ antisense RNA (5′-anti) versus the 3′ vRNA is 5′ AGCAGAAGCAGACC 3′.

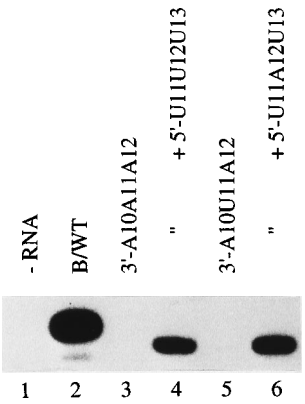

FIG. 5. The activities of triple mutants were rescued by 5′ RNAs that restore panhandle formation. Influenza B virus polymerase was used to transcribe the influenza B vRNA templates.

The activity of the B/C9 mutant was only marginally rescued (17% of the wild-type influenza B virus) by C9-G10′ base-pair formation by the addition of the 5′ G10 RNA (Fig. 4a, compare lanes 4 and 5). The rescue of mutant B/A9 activity by the 5′ U10 RNA is noticeable (about a twofold increase), but the activity is still much lower than that of the wild-type influenza B virus (about 40%) (Fig. 4b, compare lanes 2, 3, and 4). This observation is consistent with the results seen with mutant U9 which extends panhandle structure II by an extra base pair (U9-A10′) but shows much lower activity than the wild type (Fig. 2b, lane 3).

These combinations of the 3′ and the 5′ RNAs are expected to extend the panhandle (base pairs C9-G10′, A9-U10′, and U9-A10′ in panhandle structure II [Fig. 1b]) by an extra base pair at position 9, and yet, they fail to rescue the low activity of position 9 mutants. Moreover, the effect appears to be nonspecific, because low-level stimulation was also observed by the wild-type 5′ RNA (Fig. 4a, lane 6, and Fig. 4b, lane 5). This suggests that, as proposed in the legend to Fig. 1, the nucleotide at position 9 remains part of the loop structure in the influenza B vRNA and may be important for interaction with the polymerase. To confirm the role of this loop structure, we tested the effect of the 5′ antisense RNA on transcription. The perfect panhandle formation by this antisense RNA abolished the activity completely (Fig. 4c, compare lanes 2 and 4). This means that, although B polymerase recognizes the panhandle structure as an important element, the flexibility or breathing

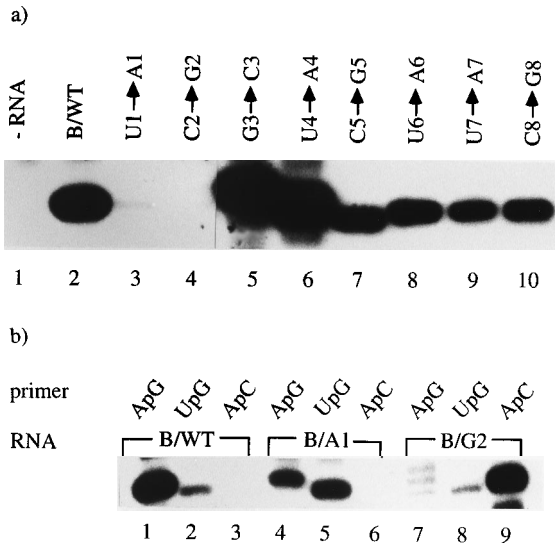

FIG. 6. (a) ApG-primed transcription of influenza B vRNA mutants at the 3′ end. (b) Transcription of position 1 and 2 mutants, using dinucleotide primers UpG, ApC, and ApG.

of the 3′ and 5′ ends, as mediated by the loop structure, is critical for efficient initiation of transcription.

We also generated point mutants in all conserved nucleotides at the 3′ end of influenza B vRNA and tested them in ApG-primed transcription with B polymerase (Fig. 6). Mutations at positions 5, 6, 7, and 8 all caused about 50% reduction of the templated activity (62, 59, 45, and 48% of wild-type activity, respectively). Interestingly, upregulation of template activity (1.5- to 2-fold increase) was observed with mutants at positions 3 and 4 (mutants C3 and A4; Fig. 6a, lanes 5 and 6). It is worth noting that similar upregulation was also observed in vitro with influenza A virus template mutants at the same positions (22). Furthermore, the position 4 mutation could be introduced into the influenza A virus genomic RNA and rescued in infectious virus particles, and the control of transcription and replication was shown to be influenced by the nature of this nucleotide (unpublished results). It would be interesting to see if similar observations could be made in influenza B virus as well. The lack of activity at positions 1 and 2 (Fig. 6a, lanes 3 and 4) is not due to the intrinsic loss of activity but rather to the lack of priming with the dinucleotide ApG. In fact, the activities of mutants B/A1 and B/G2 were rescued by using the complementary primers UpG and ApC, respectively (44 and 85% of the wild-type activity, respectively; Fig. 6b, lanes 5 and 9).

If the panhandle formation is important for the initiation of transcription, why then do mutants such as C10, G10, or G11, which disrupt the base pair, still show high activity? There are at least two possible explanations. First, the 5′ RNA fragments in the MN-digested core can form panhandle structures by base pair and activate the mutant 3′ RNAs. However, this does not explain the high activity of the C10 mutant (Fig. 2b, lane 6) since G11′ is not observed at the 5′ end of influenza B virus RNAs (3, 4, 11, 26). Moreover, limited sequence information of the extreme 5′ end of the vRNA precludes detailed interpretation of the role of the 5′ RNA. A second possibility is that, in influenza B virus, the base pairs at positions at 10 and 11 may be less critical in transcription than the base pairs at position 12 (and onwards). The rescue of activity by the restoration of a certain base pair was only partial (54, 60, and 80% of the wild type for mutants A10-U11′, U11-A12′, and A11-U12′, respectively; Fig. 4a), whereas the mismatch mutants C10, G10, and G11 all showed activities comparable to that of the wild type (95, 102, and 105%, respectively; Fig. 2b). It is possible that, even in the wild-type vRNA, the U10-A11′ base pair (Fig. 1) is destabilized because of its close proximity to the loop structure. Consistent with this interpretation is the fact that a mismatch is observed at the 10-11′ base pair in some RNA segments of influenza B virus (3). Similar studies in vitro with influenza A virus also observed the less-critical nature of the base pair at 10-11 (6). In vivo analysis with a chloramphenicol acetyltransferase reporter gene further supports this view. All four different mutations, with or without the additional base pair, were tested in vivo, and B virus was shown to accommodate the mismatch at the 10-11′ base pair and could tolerate a certain 11-12′ base-pair mismatch to a much greater extent than A virus (unpublished results).

Formation of perfect base pairs in the panhandle by the addition of antisense RNA abrogated the template activity of the 3′ end RNA. This suggests that the 3′ end of the influenza B vRNA needs to breathe for efficient initiation. The flexible nature of the extreme ends of the vRNA may allow the cap primer or ATP easy access to the 3′ end of the vRNA to initiate RNA synthesis for transcription and replication (13, 17). The breathing of the extreme end of the panhandle required at the initiation step is likely to be mediated by the loop structure. Presumably, partial destabilization of the distal part of panhandle structure II, including the base pair U10-A11′ (and C11-G12′) may help the RNA polymerase elongate the nascent RNA through this RNA duplex region. However, a mismatch in the central part of panhandle structure II (e.g., the base pair U12-A13′) causes a much greater destabilizing effect on the panhandle and cannot be tolerated.

Considering the flexible nature of the panhandle, whether the postulated interactions between the extreme 5′ and 3′ ends (panhandle structure I) really do exist in the ribonucleoprotein complex is not known. The weak interaction, if it does exist, should allow melting of the extreme 3′ end to make it available for initiation. Our result is consistent with an RNA-fork model that was proposed with influenza A virus and postulates an RNA duplex in panhandle region II and the single-stranded open structure at the 5′ and the 3′ ends (5, 6). We found in this report that the only nucleotide at the 3′ end of the vRNA that critically influences influenza B virus transcription was G9. If this nucleotide is directly involved in the polymerase-vRNA interaction, it is conceivable that the loop structure as well as the adjacent nucleotides in the panhandle may provide enough flexibility to this nucleotide for efficient interaction. It is worth noting in this vein that natural variation (U→C) is observed at position 10 of the 3′ ends of some influenza B virus RNA segments. This nucleotide abuts the loop and therefore may affect its conformation. Conceivably, its effect on polymerase interaction and transcription efficiency may contribute to segment-specific regulation of influenza B virus RNA transcription (28).

In conclusion, with the potential exception of position 9, none of the absolutely conserved nine nucleotides at the 3′ end of influenza B virus had a detrimental effect on transcription in vitro. The stability of the panhandle and the flexibility of its extreme ends are major factors influencing the initiation of transcription. Influenza B virus polymerase can better accommodate mismatch mutations in the distal part of the panhandle than influenza A virus polymerase. This finding is consistent with the increased heterogeneity observed at the 3′ ends of the influenza B vRNAs than of influenza A vRNAs. Major differences between influenza A and B viruses are found in the

length and sequence of the panhandle of the vRNAs. The detrimental effect of point mutations that destabilize the panhandle, as well as the minimal disturbance of transcriptional activity by replacing a base pair by a double mutation, suggests a role for the panhandle in maintaining evolutionary stability between influenza A and B viruses. Similarities and differences in RNA transcription between the influenza A and B viruses studied in this report are being tested and extended in vivo, using the reporter gene (2).

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