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# Striking Conformational Similarities between the Transcription Promoters of Thogoto and Influenza A Viruses: Evidence for Intrastrand Base Pairing in the 5′ Promoter Arm

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**In the accompanying report, we describe an in vitro polymerase assay based on reconstituted Thogoto virus (THOV) cores which provided evidence of a double-stranded vRNA promoter consisting of both the 3′ and 5′ sequences of vRNA (M. B. Leahy, J. T. Dessens, and P. A. Nuttall, J. Virol. 71:8347–8351, 1997). This system was used to investigate further the THOV vRNA promoter structure by using short, synthetic vRNA promoters. The results obtained show that interstrand base pairing between residues 10 and 11 of the 3′ promoter arm with residues 11 and 12 of the 5′ promoter arm, respectively, is important for promoter activity. In addition, intrastrand base pairing between residues 2 and 3 with residues 9 and 8 of the 5′ promoter arm, respectively, was shown to be involved in promoter activity, while no evidence of intrastrand base pairing between residues 2 and 9 of the 3′ promoter arm was obtained. These observations are consistent with a hook-like structure in the 5′ promoter arm of the THOV promoter. The THOV cores were able to transcribe an influenza A virus (FLUA) vRNA-like promoter, as well as hybrid THOV-FLUA promoters. Hence, the THOV and FLUA vRNA promoters appear to be both structurally and functionally similar.**

Thogoto virus (THOV) is the type species of a newly recognized genus in the family *Orthomyxoviridae* (12). In contrast to influenza viruses, THOV is transmitted by ticks (11) and has a surface glycoprotein similar to that of baculoviruses (9). However, the virus is structurally and genetically similar to influenza viruses and has a genome consisting of six negative-sense, single-stranded RNA segments (2, 13, 14). THOV gene products are related to the influenza virus polymerase proteins PB1 (7), PA (13), and PB2 and nucleocapsid protein (14). Like that of influenza A virus (FLUA), each THOV segment possesses conserved regions of semicomplementary nucleotides at the 3′ and 5′ termini (7, 13, 14) and mRNA synthesis is primed by host-derived cap structures (1, 8, 14). Moreover, we recently reported that both the 3′ and 5′ sequences of virion RNA (vRNA) are required for vRNA promoter activity (8). There are several similarities between the THOV and FLUA vRNA promoters (Fig. 1A), namely, (i) potential base-paired regions of various lengths between the 3′ and 5′ promoter arms; (ii) potential unpaired regions in the first 9 and 10 residues of the 3′ and 5′ promoter arms, respectively; (iii) a putative $(U)_6$ polyadenylation signal at nucleotide positions 17 to 22 of the 5′ promoter arm; and (iv) a high level of sequence conservation. By analogy to FLUA, different models for the structure of the THOV vRNA promoter can be constructed. The classical panhandle model (Fig. 1B) involves interstrand base pairing throughout the length of the promoter. In contrast, the forked-RNA model (Fig. 1C) does not involve interstrand base pairing between the first 9 and 10 residues of the 3′ and 5′ promoter arms, respectively (4, 5). The corkscrew model (Fig. 1D) is an extension of the forked-RNA model involving intrastrand, as well as interstrand, base pairing (3). In this investigation, we set out to test the relative merits of these models by using a recently developed in vitro polymerase assay based on reconstituted THOV cores and short, synthetic model RNAs constituting 3′ and 5′ vRNA-like sequences (8).

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## MATERIALS AND METHODS

**Preparation of viral cores.** THOV cores were prepared as described previously (8). Briefly, monolayers of BHK-21 (baby hamster kidney) cells were infected with Thogoto/SiAr/126/72 virus at approximately 0.05 PFU/cell. Media were harvested 30 h postinfection and clarified, and then the virus was pelleted by centrifugation at 80,000 × *g* through a 33% (vol/vol) glycerol cushion, resuspended in TMN buffer (100 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 5% glycerol and 1% Nonidet P-40, and incubated for 30 min at room temperature. The disrupted virus suspension was loaded onto a discontinuous glycerol gradient (66 and 33%, vol/vol) in TMN buffer and centrifuged for 2 h at 15°C at 80,000 × *g*. The interface was collected, loaded onto a 33% (vol/vol) glycerol cushion in TMN buffer, and centrifuged for 1 h at 15°C and 80,000 × *g*. Pelleted cores were dissolved in TMN with 50% glycerol and frozen until use.

**Preparation of model RNA templates.** Short RNA templates were transcribed with T7 RNA polymerase from partial DNA duplexes that consisted of an upstream double-stranded T7 RNA polymerase promoter region and a 5′-terminal overhang corresponding to the transcribed sequence as described previously (8).

**In vitro transcription with viral cores.** Viral cores (1 to 2 μg) were incubated with template RNA in the presence of 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM GTP, 0.5 mM UTP, 0.1 mM CTP, 1 μM [$\alpha$-$^{32}$P]CTP (specific activity, 800 Ci/mmol, Amersham), 20 U of RNAguard, and 1 mM ApG primer (Sigma) in a total reaction volume of 20 μl for 2 h at 37°C as described previously (8). Reactions were analyzed by 20% polyacrylamide gel electrophoresis in the presence of 7 M urea.

## RESULTS

**Interstrand base pairing.** Mutations were introduced in the 3′ promoter arm that would destroy potential base pairing with nucleotides in the opposite promoter arm. Such changes would reduce promoter activity if the terminal nucleotides were required to form a classic panhandle structure. Subsequently, potential base pair-restoring mutations were introduced into the 5′ promoter arm that rescue promoter activity if the base pairing is genuine (Fig. 2A). Mutations of residues 10 and 11 of the 3′ promoter arm (mutants 5 and 7, Fig. 2A) abolished

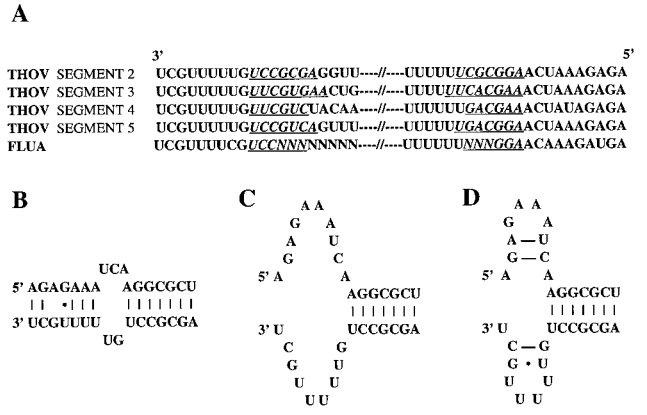


FIG. 1. Terminal sequences of THOV vRNA and structural models of the THOV vRNA promoter. (A) Terminal sequences of THOV segments 2 (7), 3 (11), 4 (8), and 5 (12) and that of a typical FLUA vRNA. Potential base-paired regions between the 3′ and 5′ promoter arms are underlined and in italics. The classical panhandle model (B), the forked-RNA model (C), and the corkscrew model (D) based on THOV segment 2 are depicted.

promoter activity (Fig. 2B, lanes 5 and 7). However, activity could be rescued by base pair-restoring mutations of nucleotides 11 and 12 of the 5′ promoter arm (Fig. 2B, lanes 6 and 8; mutants 6 and 8, Fig. 2A). Mutations at position 5 had no effect on promoter activity, whereas mutations at position 6 resulted in 30% activity compared to the wild type (Fig. 2B, lanes 1 and 3; mutants 1 and 3, Fig. 2A). Rescue mutations of residues 5 and 6 on the 5′ promoter arm had little effect (Fig. 2B, lanes 2 and 4; mutants 2 and 4, Fig. 2A). These results do not conform to the panhandle model. Mutation of residue 6 resulted in slightly reduced promoter activity. As promoter activity relies not only on the ability of the RNA to fold correctly but also on its ability to correctly bind to the polymerase complex, the mutation of residue 6 may have affected binding.

**Intrastrand base pairing on the 5′ promoter arm.** Mutations that destroy possible base pairing and potentially reduce promoter activity were again introduced, but in this experiment, rescue was attempted with base pair-restoring mutations on the same promoter arm (Fig. 3A). Mutation of residue 3 of the 5′ promoter arm from an adenine to a uracil abolished activity (Fig. 3B, lane 3; mutant 3, Fig. 3A). Activity was rescued by a complementary mutation of residue 8 of the 5′ strand from a uracil to an adenine. The nearly wild-type activity resulting from this mutant was due to the restoration of a base pair rather than simply the mutation of residue 8 because synthetic promoters with an A residue at position 8 alone are not transcriptionally active (data not shown). Mutation of residue 2 of the 5′ promoter arm also abolished activity (Fig. 3B, lane 1; mutant 1, Fig. 3A), which was indeed partially rescued by the base pair-restoring mutation of residue 9 on the same promoter arm (Fig. 3B, lane 2; mutant 2, Fig. 3A). A similar outcome was obtained with mutations at positions 2 and 3 and their respective rescues (Fig. 3B, lanes 5 and 6, respectively; mutants 5 and 6, Fig. 3A). These results indicate that intrastrand base pairing in the 5′ promoter arm of the THOV vRNA promoter is important for promoter activity. Rescue of promoter activity impaired by mutation of residue 2 was significantly less effective than that of residue 3 (Fig. 3B, lanes 2 and 4), possibly as a result of altered binding affinities introduced by the mutations.

**Intrastrand base pairing on the 3′ promoter arm.** Residue 2 of the 3′-like synthetic RNA template was mutated, and rescue was tested with a base pair-restoring mutation of residue 9

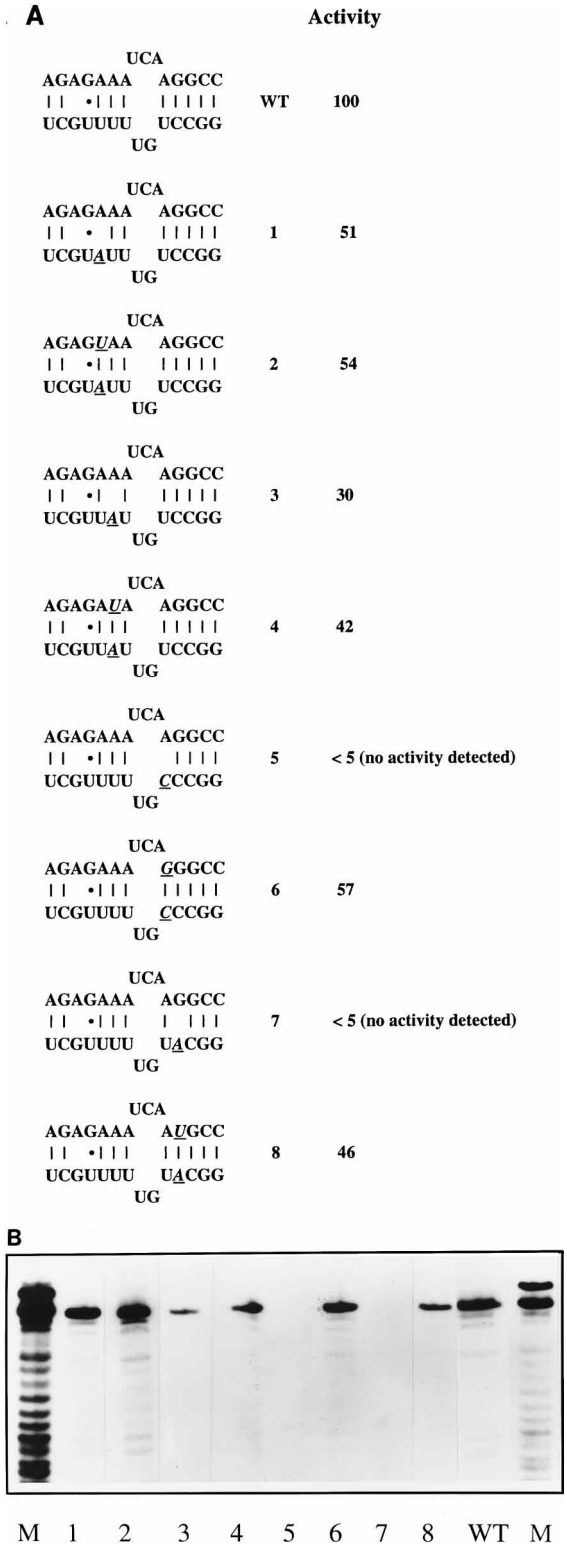


FIG. 2. Mutations introduced into the THOV vRNA promoter to test interstrand base pairing. (A) Models for the THOV wild type (WT) and vRNA promoter mutants 1 to 8 (mutations are underlined and in italics) and relative transcription activities determined by densitometry of the autoradiograph and normalization for incorporated-label content (limit of detection, <5%). (B) In vitro transcription reactions with the promoters described in A. Lanes 1 to 8 are mutants 1 to 8, respectively. Transcription products were fractionated in 22% polyacrylamide–7 M urea gels and visualized by autoradiography of the dried gels. M denotes lanes in which end-labelled 14- and 15-nt markers were loaded.

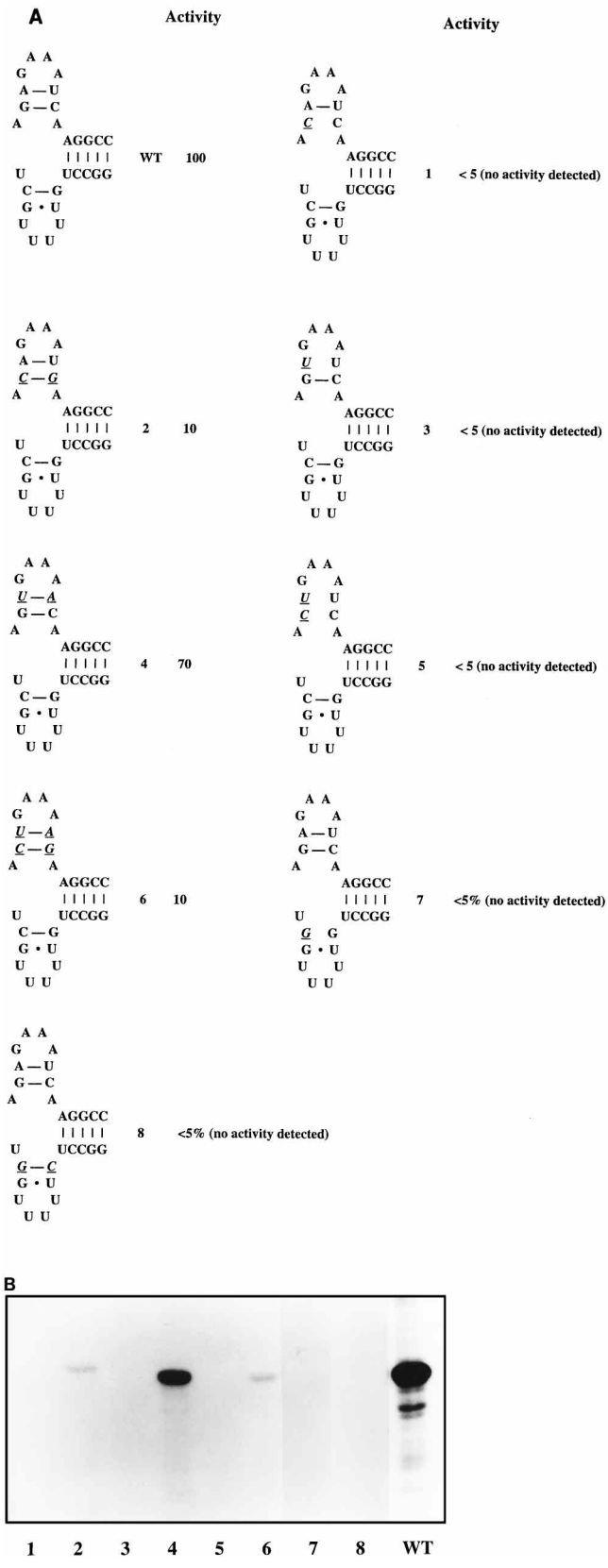


FIG. 3. Mutations introduced into the THOV vRNA promoter to test intrastrand base pairing. (A) Models for the THOV wild type (WT) and vRNA promoter mutants 1 to 8 (mutations are underlined and in italics) and relative transcription activities (limit of detection, <5%). (B) In vitro transcription reactions with the promoters described in A. Lanes 1 to 8 are mutants 1 to 8, respectively. Transcription products were fractionated in 22% polyacrylamide–7 M urea gels and visualized by autoradiography of the dried gels.

(Fig. 3A, mutants 7 and 8, respectively). The involvement of the residue at position 3 was not tested, as this nucleotide can only form a non-Watson-Crick base pair with the residue at position 8 (Fig. 1D and 3A). As residue 2 was mutated from C to G, ApC instead of ApG was used to prime these reactions. Mutation of residue 2 fully abolished promoter activity; however, no rescue of activity was observed by the base pair-restoring mutation at position 9 (Fig. 3B, lanes 7 and 8, respectively). Surprisingly, the same results were obtained when the reactions were primed with globin mRNA (data not shown).

**Transcription of influenza virus-like templates.** Synthetic model RNAs were synthesized corresponding to the 3′ and 5′ ends of FLUA vRNA with the base-paired regions normalized to allow heterologous combinations with THOV (Fig. 4A). The FLUA promoter (Fig. 4A, construct 4) and both hybrid FLUA-THOV promoters (Fig. 4A, constructs 2 and 3) were transcriptionally active in the THOV polymerase assay. THOV cores transcribed FLUA-like templates with 50% activity (Fig. 4B, lane 4). When the THOV-FLUA combinations were used, levels of activity were reduced to approximately 10% compared to that of the THOV vRNA promoter (Fig. 4B, lanes 2 and 3). These results support the observation that interstrand base pairing between the first 9 and 10 residues of the 3′ and 5′ promoter arms, respectively, of the promoter is not required for activity and indicate that the THOV and FLUA vRNA promoters are not only structurally but also functionally similar.

## DISCUSSION

Sequence analyses of the THOV vRNA terminal nucleotides have shown that the conserved 12 and 13 nucleotides at the 3′ and 5′ termini, respectively, show a high degree of homology with those of FLUA (7, 14). THOV cores were able to transcribe FLUA-like synthetic RNAs with activity (50%) comparable to that of THOV-like templates (Fig. 4B, lanes 1 and 4). Even more striking were the results obtained with chimeric promoter structures formed by annealing the 3′ FLUA-like template with the 5′ THOV-like RNA and vice versa. These were transcribed by THOV viral cores, albeit with reduced promoter activity (Fig. 4, lanes 2 and 3). The ability of the THOV polymerase complex to recognize and transcribe such structures indicates a very close conformational similarity between the promoter regions of THOV and FLUA.

While the promoter regions of the THOV and FLUA vRNAs show marked sequence similarity, there are notable differences. The residue at position 5 of the 3′ promoter arm, which is absolutely required for FLUA promoter activity, could be mutated with no loss of THOV promoter activity (Fig. 2B, lane 1). Likewise, the exact nature of nucleotide 10 of the 3′ terminus was less important for THOV promoter activity than for FLUA promoter activity (6). It may be that the requirements for such sequence-specific interactions are more stringent for the FLUA RNA polymerase complex than for the cognate THOV enzymes.

The results presented here provide experimental evidence for a model of THOV vRNA promoter structure novel among orthomyxoviruses involving intrastrand base pairing in the 5′ promoter arm but, while not proven, probably not in the 3′ promoter arm. In fact, this model is in full agreement with a hook-like model for the THOV vRNA promoter previously

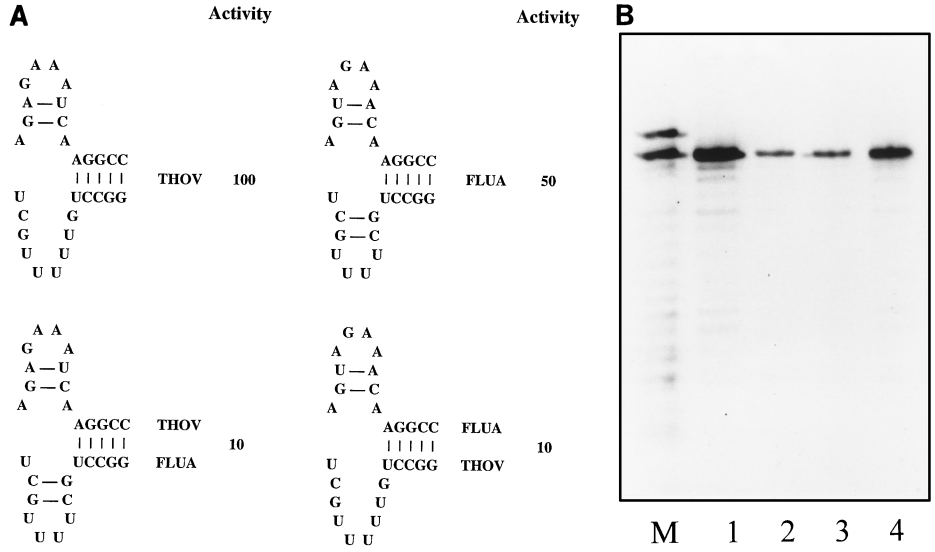


FIG. 4. In vitro transcription of THOV, FLUA, and hybrid THOV-FLUA vRNA promoters. (A) Models for the various vRNA promoters and relative transcription activities. (B) In vitro transcription reactions with the promoters described in A. Lanes 1 to 4 are constructs 1 to 4, respectively. Transcription products were fractionated in 22% polyacrylamide–7 M urea gels and visualized by autoradiography of the dried gels. M, see Fig. 2 legend.

predicted by computer analysis (15). Accepting the similarities between the FLUA and THOV promoters, our data address several apparent anomalies in data previously published for FLUA promoter requirements (10). In in vitro systems, it has been observed that mutations at positions 2, 3, 8, and 9 on the 5′ promoter arm of FLUA vRNA molecules abolish promoter activity. Attempts to rescue activity with complementary mutations in the 3′ promoter arm failed (5). It was assumed that the reduced promoter activity of these mutants was probably due to decreased efficiency of binding to the polymerase complex (5). If the RNA hook model is correct, these residues would be involved in intrastrand base pairing and mutations at positions 2, 3, 8, and 9 would destroy a possible 5′ hook structure. No attempts were made to rescue activity with complementary intrastrand mutations. In vivo analysis of the FLUA promoter has also resulted in some unexpected data (10). Mutations that increased base pairing between nucleotides 1 and 9 of the 3′ promoter arm with cognate residues of the 5′ promoter arm were considered to increase promoter activity. Surprisingly, single mutations at positions 3 and 8 which should restore interstrand base pairing resulted in reduced promoter activity (10). However, when constructs with double mutations at these positions were used, activity was increased. Coincidentally, intrastrand base pairing would have been restored by using this construct whereas intrastrand base pairing would have been destroyed by single mutations. It is possible, therefore, that a hook-like structure exists in the 5′ conserved nucleotides of FLUA vRNA molecules. The apparent lack of intrastrand base pairing in the 3′-terminal nucleotides of the THOV vRNA promoter, as opposed to that predicted for FLUA by Flick et al. (3), may result from the inability of THOV to form a second Watson-Crick base pair between the 3′ residues in positions 3 and 8 (Fig. 1D). In vitro studies of the FLUA vRNA promoter (5) do not show evidence of such 3′ intrastrand base pairing, which is inconsistent with the in vivo data reported by Flick et al. (3). However, many contradictions exist between data generated by in vivo and in vitro assays which may reflect the different experimental conditions, such as temperature or cellular factors (6). Ultimately, in vivo studies of the THOV vRNA promoter may shed more light on these discrepancies.

What might be the function of a hook structure in the THOV vRNA 5′ terminus? During replication, a 5′ hook structure would not exist in the cRNA molecule and would, instead, be found at the 3′ end. Moreover, the possible non-Watson-Crick base pair found in the vRNA 3′ terminus between residues 3 and 8 would not be present in the cRNA molecule, making the presence of a 5′ hook structure even less likely than that of a 3′ hook structure in vRNA molecules. As all of the signals necessary for replication, transcription, and packaging of the viral RNAs are thought to be present in the conserved terminal nucleotides of FLUA vRNA (16), it is tempting to speculate that the putative hook structure constitutes a switching mechanism or a component of a switching mechanism for such activities.

## ACKNOWLEDGMENTS

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