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The Influenza Virus Panhandle Is Involved in the Initiation of Transcription

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The role of the influenza A virus panhandle structure formed from the 3′- and 5′-terminal nucleotides of virion RNA segments was studied in both an RNA polymerase binding assay and an in vitro transcription assay. Despite recent indications that promoter activity is simply a function of the 3′-terminal sequence of virion RNA, our results show that both 3′- and 5′-terminal sequences are involved in the initiation of transcription. We propose a new model for the initiation of transcription which has implications for the mechanisms by which influenza virus transcription, replication, and polyadenylation may be regulated in the infected cell.

All eight negative-sense single-stranded RNA segments of influenza A viruses have conserved sequences of 12 and 13 nucleotides (nt) at their 3′ and 5′ ends (3, 22), respectively, which are partially complementary and can form a panhandle structure in isolated virions and infected cells (12, 13). It has been suggested that the panhandle structure can serve as a regulatory signal for transcription and replication, as well as for packaging of RNA into virus particles (13). There is experimental evidence that the panhandle is a *cis*-acting signal for polyadenylation (17), and a recent study indicates that it may be needed for the endonuclease activity of the RNA polymerase complex (8). In vitro studies with synthetic RNA templates showed that the virion RNA (vRNA) promoter was located at the 3′ end of vRNA segments, suggesting that the panhandle structure was not required for transcriptional activity (17, 19, 20, 23–25). However, all these studies used RNA polymerase isolated from virions which may have contained residual vRNA 5′-terminal sequences capable of activating RNA polymerase. The only study with recombinant RNA polymerase used synthetic RNA template with both 3′- and 5′-terminal sequences (14). In order to investigate promoter functions and the role of the panhandle structure in vitro, we have now studied the role of the vRNA 5′-terminal sequence in both RNA polymerase binding and transcription.

We reported recently that nt 9 to 12 of the influenza A virus vRNA promoter at the 3′ end of vRNA segments were involved in influenza virus RNA polymerase binding (6). Since the 3′ end is known to form a panhandle with the 5′ end, we were interested in the role, if any, of the conserved 13 nt at the 5′ end in RNA polymerase binding. To test this, we used a photochemical cross-linking assay (6). Strong binding of the RNA polymerase complex to a synthetic vRNA 5′-end sequence was observed (Fig. 1A, lane 1). Immunoprecipitation of the cross-linked products by specific polyclonal antisera to the RNA polymerase subunits (PB1, PB2, and PA) showed that all three were cross-linked (Fig. 1A, lanes 2 to 4). The specificity of binding was tested in a competition experiment using homologous, 19-nt-long vRNA 5′-end sequence and heterologous, U_{15} and $tRNA^{Phe}$ competitors (Fig. 1B). The data suggest that the RNA polymerase binds the 5′-end region 100 to 1,000 times more efficiently than an unrelated U_{15} or tRNA sequence.

To identify the nucleotide residues of the 5′-end sequence involved in RNA polymerase binding, mutant 5′-end sequences carrying single transversions at each position of the 13-nt-long conserved sequence were tested in cross-linking (Fig. 2A). Mutations at positions 1′ to 3′ and 9′ (see Fig. 3A for nomenclature) severely decreased the efficiency of binding. Mutations at all other positions (4′ to 8′ and 10′ to 13′) either altered the binding efficiency or subtly altered the pattern of cross-linked products, although at these positions the effect was less marked than at positions 1′ to 3′ and 9′. To confirm that the binding of mutants is sequence specific, they were tested in a competition experiment with the wild-type 5′-end sequence as a homologous competitor and with the mutants as heterologous competitors (Fig. 2B). Consistent with the previous results, mutants which showed severely decreased efficiencies of binding (Fig. 2A) failed to outcompete the wild-type 5′-end sequence (mutations at positions 1′ to 3′ and 9′). In addition, mutants with mutations at positions 7′, 8′, and 11′ did not compete presumably because of their decreased efficiency of binding. All the other mutants (mutations at positions 4′ to 6′, 10′, 12′, and 13′) competed like the wild-type 5′-end sequence did. Taking the results of the binding assay and the competition experiment together, positions 1′ to 3′, 9′, and possibly 7′, 8′, and 11′ are the most important for efficient polymerase binding. These and previous cross-linking results on the 3′-terminal sequence (6) strongly suggest that both strands of the panhandle are involved in RNA polymerase binding and, therefore, may have an important role in the initiation of transcription.

We speculated that if the panhandle were involved in the initiation of transcription, then the mutant promoters (3′-terminal sequences) exhibiting low activity might be rescued by using 5′-end sequences which restore the structure of the panhandle (Fig. 3A). A previous in vitro limited mutational analysis of the vRNA promoter using viral cores treated with micrococcal nuclease had showed that only 3 nt (9 to 11) were crucial for transcription and that mutations at other positions had much smaller effects on RNA polymerase activity (24). We have now introduced all three possible mutations at each of

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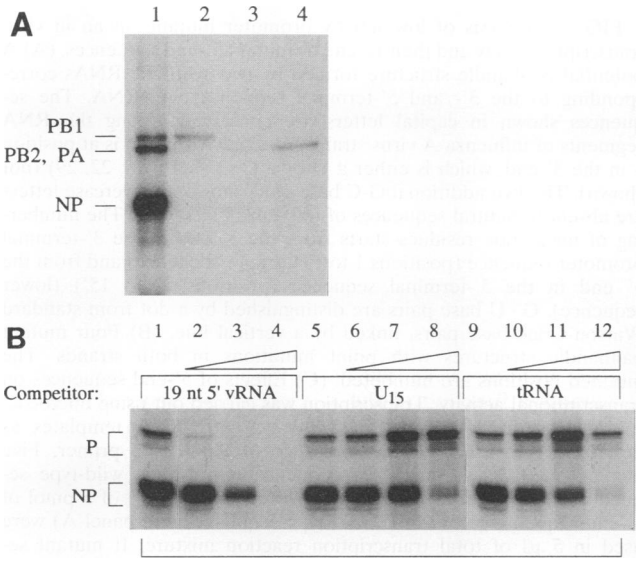


FIG. 1. The influenza A virus RNA polymerase complex specifically binds to the conserved 5′-end sequences of vRNA segments. (A) Viral core proteins cross-linked to vRNA 5′-end sequence (lane 1) and immunoprecipitation of cross-linked products with specific polyclonal antisera to PB1 (lane 2), PB2 (lane 3), and PA (lane 4). The cross-linking and immunoprecipitation were performed as described previously (6), using micrococcal nuclease-treated viral core (of influenza X-31 virus) as the source of RNA polymerase and synthetic 13-nt-long 5′-end ^{32}P-labelled RNA (7) corresponding to the terminal 13 conserved nt of the vRNA 5′ end (5′ AGUAGAAACAAGG 3′). Before immunoprecipitation, the cross-linked products were treated with 1% sodium dodecyl sulfate (SDS) to disrupt the polymerase complex (6). Under these conditions, the antisera precipitated the individual subunits of the RNA polymerase complex with no apparent cross-reaction with the other two subunits. The cross-linked products and immunoprecipitates were analyzed on SDS–8% polyacrylamide gels. The positions of the polymerase subunits (PB1 and the comigrating PB2 and PA) and nucleoprotein NP (which is the most abundant protein of the viral core and binds RNA nonspecifically) are indicated. (B) Competition experiment showing the specificity of binding of the RNA polymerase to the vRNA 5′-end sequence. Micrococcal nuclease-treated viral core was reconstituted with and cross-linked (6) to a synthetic 19-nt-long RNA sequence corresponding to the terminal 19 residues (5′ AGUAGAAACAAGGGUAUUU 3′) at the 5′ end of vRNA encoding NP (of influenza X-31 virus) alone (lanes 1, 5, and 9) or in the presence of increasing amounts of homologous competitor (1 [lane 2], 10 [lane 3], and 100 [lane 4] pmol), synthetic U_{15} sequence (1 [lane 6], 10 [lane 7], and 100 [lane 8] pmol), or *Saccharomyces cerevisiae* $tRNA^{Phe}$ (Boehringer) (1 [lane 10], 10 [lane 11], and 100 [lane 12] pmol). The positions of the RNA polymerase subunits (P) and the nucleoprotein (NP) are indicated.

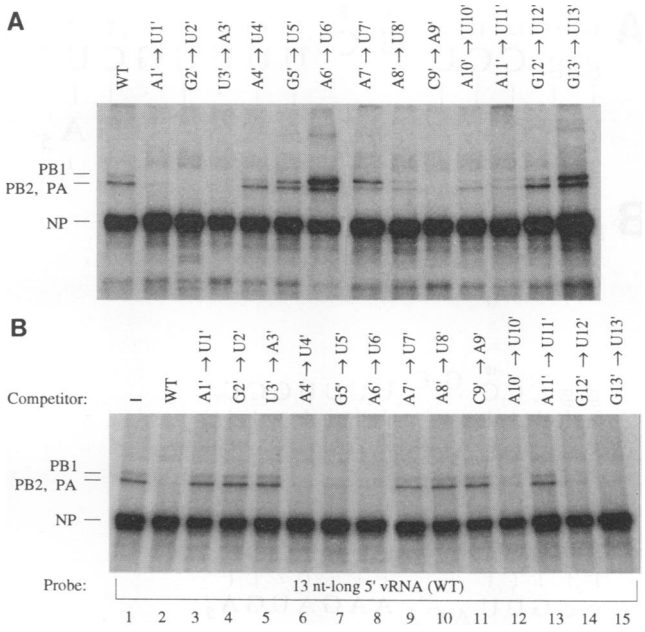


FIG. 2. Identification of nucleotide residues of the conserved vRNA 5′ end involved in RNA polymerase binding. (A) Binding of the RNA polymerase to wild-type and mutant 5′-end sequences. Viral core proteins were cross-linked to 13-nt-long synthetic 5′-end ^{32}P-labelled RNAs (wild-type [WT] or with a point mutation at the position indicated) and then analyzed on SDS–8% polyacrylamide gels (6). After purification (6), the wild-type and mutant synthetic RNAs were quantitated by spectrophotometry at A_{260} and equal amounts of the RNAs were used in ^{32}P labelling and cross-linking. The signal of cross-linked nucleoprotein, which is known to bind to RNA independently of RNA sequence, confirms that all reaction mixtures contained RNA with a similar specific activity. The results were reproducible and were observed in two other experiments. The positions of the cross-linked polymerase subunits (PB1, PB2, and PA) and nucleoprotein (NP) are marked. (B) Binding of RNA polymerase to wild-type 5′-end sequence in the presence of mutant 5′-end competitors. Viral core proteins were cross-linked to a 13-nt-long synthetic 5′-end ^{32}P-labelled RNA (wild type [WT]) alone (lane 1), in the presence of 10 pmol of homologous competitor (lane 2) or 10 pmol of mutant 5′-end sequence with a point mutation at the position indicated (lanes 3 to 15). The positions of PB1, PB2, PA, and NP are indicated.

positions 9 to 12 in the promoter and tested their effects in a transcription assay with ApG as a primer and the 14-nt-long synthetic model RNA templates as described before (24). Mutations U10→A10 (the uracil at position 10 changed to adenine), U10→G10, C11→A11, and C11→G11 showed significantly reduced transcriptional activity (Fig. 3C, compare lane 2 with lanes 4, 7, 10, and 13), confirming our previous results (24). On the other hand, the mutations U10→C10 and C11→U11 and all possible mutations at positions 9 and 12 showed weak or insignificant inhibition of transcription (results not shown). The reason for the discrepancy between the new results with G9→C9 and the previously reported inhibition (24) is unknown.

To attempt the rescue of position 10 and 11 mutants (U10→A10, U10→G10, C11→A11, and C11→G11), which are the only mutants with significantly reduced activities, we tested the effects of adding to the transcription assay equimolar amounts of various 5′-end sequences in order to restore base pairing in the panhandle (Fig. 3B). Fig. 3C shows that the activities of the two different position 10 mutants (U→A and U→G) were partially rescued by adding mutant 5′-end sequences (41 and 68% of the wild-type activity, respectively, compare lane 4 to 5 and lane 7 to 8), while the activities of the two different position 11 mutants (C→A and C→G) were almost completely restored to wild-type levels (94 and 87% of the wild-type, respectively, compare lane 10 to 11 and lane 13 to 14). In control experiments, 5′-end sequences alone produced no signal (results not shown).

These results demonstrate that for the initiation of transcription in vitro, the base pairing between the 3′ and 5′ ends at positions U10-A11′ and C11-G12′ (Fig. 3A), rather than the sequence at these positions, is important. It is very likely that these 2 bp are extended by the formation of further base pairs in the panhandle, including the conserved C12-G13′ base pair

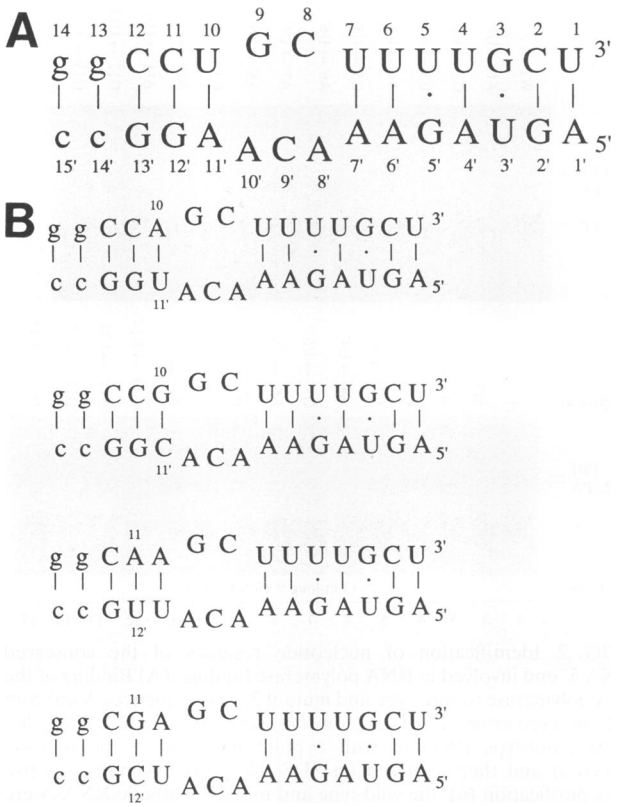
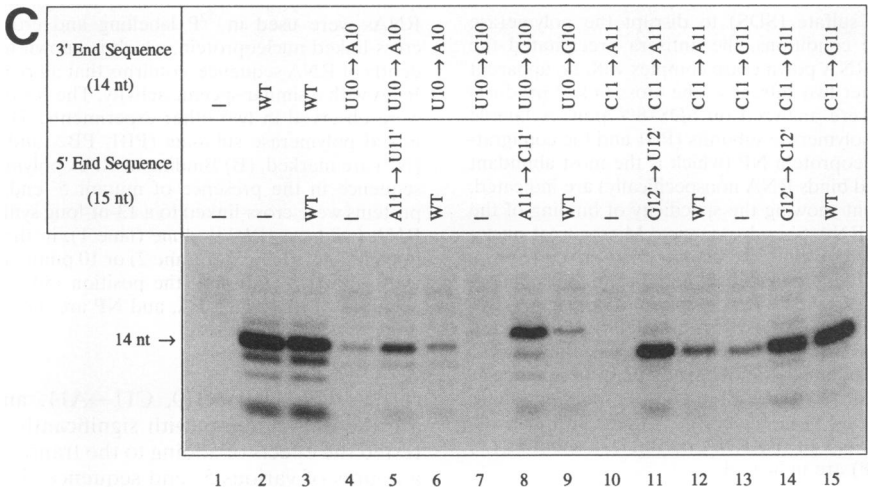


FIG. 3. Analysis of low-activity promoter mutants in an in vitro transcription assay and their rescue by mutant 5′-end sequences. (A) A potential panhandle structure formed by two synthetic RNAs corresponding to the 3′- and 5′-terminal sequences of vRNA. The sequences shown in capital letters are conserved among the RNA segments of influenza A virus strains. The only variation is at position 4 in the 3′ end, which is either a U or a C (3, 5, 16, 18, 22, 29) (not shown). The two additional G-C base pairs shown in lowercase letters are absent in natural sequences of influenza X-31 virus. The numbering of nucleotide residues starts from the 3′ end in the 3′-terminal promoter sequence (positions 1 to 14) (upper sequence) and from the 5′ end in the 5′-terminal sequence (positions 1′ to 15′) (lower sequence). G · U base pairs are distinguished by a dot from standard Watson-Crick base pairs, linked by a vertical line. (B) Four mutant panhandle structures with point mutations in both strands. The mutated positions are numbered. (C) Effects of 5′-end sequences on transcriptional activity. Transcription was carried out using micrococcal nuclease-treated viral cores and synthetic RNA templates as described before (24) in the presence of ApG as a primer. Five picomoles of 3′-end promoter sequence (14-nt-long, wild-type sequences as in panel A) in the presence or absence (−) of 5 pmol of 5′-end sequence (15 nt long, wild-type sequence as in panel A) were used in 5 μl of total transcription reaction mixture. If mutant sequences were used, this is indicated over the lanes. After incubation at 30°C for 3 h, the RNA was phenol-chloroform extracted, ethanol precipitated, and analyzed on a 18% polyacrylamide gel in 7 M urea. Labelled RNA products were estimated quantitatively by laser densitometry of the exposed X-ray films, and the average values from three independent experiments were calculated. The identities of transcription products were confirmed by further T_1 RNase digestion and analysis on 25% acrylamide gels in 7 M urea after elution from the gel (results not shown). The reason for the slightly anomalous mobility of the transcript of the U10→G10 mutant is unknown.



and the 1 to 3 vRNA segment-specific base pairs (12, 13). Surprisingly, the addition of wild-type 5′-end sequence also resulted in significantly increased activity for the C11→G11 mutant with a G11-G12′ mismatch (Fig. 3C, compare lanes 13 to 15), while the other three mutants were slightly stimulated (compare lanes 4 to 6, 7 to 9, and 10 to 12). By contrast, a 13-nt-long wild-type 5′-end sequence lacking the two C residues at its 3′ end and therefore unable to form the G13-C14′ and G14-C15′ base pairs (Fig. 3A) was unable to stimulate the activity of the 14-nt-long C11→A11 and C11→G11 mutants significantly. On the other hand, a 13-nt-long mutant 5′ end sequence with G12′→U12′ mutation partially rescued the activity of the 14-nt-long C11→A11 and C11→G11 mutants, presumably by restoring a A11-U12′ base pair or a G11 · U12′ non-Watson-Crick base pair (results not shown). Overall, these results imply that if the double-stranded RNA region (at residues 10 to 13 or 15) is stabilized by a sufficient number of base pairs, a mismatch in this region may be compatible with transcriptional activity.

A multiple mutation of the 3′-end sequence (U_{4-7}→A_{4-7}) did not affect the transcriptional activity and a mutant 5′ end ($AGAA_{4-7'}$→$U_{4-7'}$), which restored the base pairs of the panhandle at positions A_{4-7}, had no effect on activity (data not shown), suggesting that base pairing is not required at positions 4 to 7. In fact, U at position 4 is often replaced by C in the 3′-end sequence in some of vRNA segments (3, 5, 16, 18, 22,

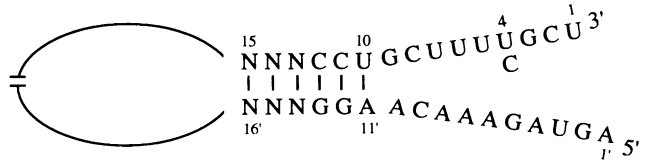


FIG. 4. An RNA-fork model for the initiation of transcription. See text for details.

29) and would destabilize the RNA duplex at residues 1 to 7 (Fig. 3A).

The addition of wild-type 5′-end sequence to wild-type 3′-end sequence had no effect on transcriptional efficiency (Fig. 3C, lane 3). This result suggests that there are significant levels of free 5′ ends still associated with the polymerase in the micrococcal nuclease-treated core. To address this question, a study of the residual RNA content of micrococcal nuclease-treated viral core was performed, which suggested that it contained RNA fragments (14 to 18 nt long) corresponding to the 3′- and 5′-end sequences of vRNA probably protected by the polymerase during nuclease treatment (results not shown). As in our assays with micrococcal nuclease-treated core, we use an excess of added model RNAs over endogenous RNAs, we assume that the results with mutants are not significantly altered by the presence of endogenous wild-type RNA. In a previous study (17), the introduction of a double mutation at positions 11 and 12 of the 3′ end of a vRNA-like chloramphenicol acetyltransferase construct resulted in greater than 90% loss of in vitro transcriptional activity. Although the introduction of compensatory mutations in the 5′ end did not restore wild-type activity, it appears from their results (Fig. 2C of reference 17) that there was some increased activity, which was not noted by these researchers (17).

On the basis of the above results, we propose a new model for the initiation of influenza virus RNA transcription in which both strands of the influenza virus panhandle are involved (Fig. 4). This "RNA-forked" structure is partly double stranded (residues 10 to 13 or 15, depending on the number of additional segment-specific base pairs) and partly single-stranded (the two ends). In addition to base pairing, the duplex region is stabilized by protein-RNA or protein-protein interactions dependent on the RNA polymerase subunits (13, 26). We postulate that there are one or more binding sites for polymerase subunit(s) on the 5′ single-stranded region and on the duplex region. The presence of a single-stranded 3′ end provides a template for the initiation of transcription. Previously we identified a regulatory site at residues 1, and possibly 2 to 4 of the 3′ end (24), which have been shown not to be involved in polymerase binding (6). Although we favor the presence of single-stranded ends, we cannot exclude the traditional model based on base pairs between residues 1 to 7 and 1′ to 7′ (Fig. 3A). The new model is further supported by an in vivo study suggesting that important promoter signals reside at positions 6 to 14 with respect to the 3′ end but not at the 3′ terminal base (30).

The model suggests novel mechanisms for the control of mRNA and cRNA synthesis. The synthesis of influenza virus mRNA and cRNA differs in both the initiation and termination steps. Whereas mRNA is capped, prematurely terminated, and polyadenylated (2, 4, 15), cRNA is a full-length copy of the vRNA and is neither capped nor polyadenylated (10, 11). The mechanism by which initiation switches from mRNA synthesis to predominantly cRNA synthesis during the viral life cycle is unknown. The presence of an RNA polymerase binding site in the 5′-end sequence of the RNA-forked structure could facilitate this switch.

For the initiation of mRNA synthesis, the PB2 subunit of the RNA polymerase generates capped RNA primers by endonucleotic cleavage of host cell pre-mRNA in the nucleus of the infected cell (21, 27, 28). A specific interaction of the RNA polymerase with the 3′ and 5′ ends of vRNA might be required for the endonuclease activity of PB2 (8) and, therefore, for the initiation of mRNA synthesis. If the RNA polymerase remained attached to the binding site in the 5′-end sequence of the template vRNA during transcription, steric hindrance would inevitably cause premature termination at the U stretch near the 5′ end. Furthermore, continued synthesis by the RNA polymerase would lead to the synthesis of a poly(A) tail as a result of reiterative copying of the same U stretch.

On the other hand, a regulatory protein, e.g. NP (1) or NS1 (9), may modify the structure of the panhandle or the binding of the RNA polymerase which would prevent PB2 generating capped RNA primers. This might then allow primer-independent initiation of cRNA synthesis by the RNA polymerase acting either on the modified panhandle or a single 3′ terminus. Moreover, the absence of the RNA polymerase from the 5′-end sequence would result in the synthesis of full-length cRNA.

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