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Characterization of the RNA-Fork Model of Virion RNA in the Initiation of Transcription in Influenza A Virus

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It has been shown that both 3′ and 5′ conserved termini of influenza A virus virion RNA are involved in the initiation of transcription. An RNA-fork model has been proposed, according to which there is a crucial double-stranded region formed by complementary bases at positions 10 to 12 of the 3′ terminus and bases at positions 11′ to 13′ of the 5′ terminus, which are extended by 2 or 3 segment-specific base pairs. The two termini at positions 1 to 9 and 1′ to 10′ in the 3′ and 5′ termini, respectively, are in a single-stranded conformation. Here we further characterize this model, focusing on the individual roles of the proposed duplex region and the proposed two single-stranded ends. Residues within the conserved 5′ terminus that are involved in the initiation of transcription were determined. Single, double, and triple mutations in the proposed duplex region provided further evidence that, for the initiation of transcription in vitro, the duplex RNA is more important than the actual sequence of these residues, although some restrictions in sequence were apparent. On the other hand, there was evidence that base pairing is not required at residues 1 to 7. We propose that the 5′ terminus of virion RNA should be treated as an integral part of the virion RNA promoter and discuss a possible mechanism for the recognition of the virion RNA promoter by the influenza A virus RNA polymerase complex.

The influenza A virus contains eight segments of single-stranded RNA of negative polarity. The eight RNA segments encode at least 10 different viral proteins. These include PB1, PB2, and PA polymerase proteins, which form a complex and are responsible for transcription and replication of the viral genome. During the life cycle of the virus, the viral genome (vRNA) is transcribed into mRNA and replicated into complementary RNA (cRNA). This, in turn, is used as a template to synthesize vRNA (30, 32, 33, 36, 42). mRNA synthesis is primed by capped RNA fragments which are cleaved from host cell mRNAs by an endonuclease activity associated with the polymerase complex (1, 6–8, 34, 47). As a consequence, viral mRNAs contain a cap structure and a sequence of 9 to 15 heterologous nucleotides at their 5′ ends (11, 14, 34, 46). mRNA synthesis terminates at a sequence of 5 to 7 uridines 15 to 17 nucleotides (nt) from the 5′ end of vRNA templates, followed by the addition of a poly(A) tail (37, 39). In contrast with mRNA, cRNA is a full-length copy of vRNA. It is neither capped nor polyadenylated and functions as a template for vRNA synthesis (54). Both cRNA and vRNA have a pppA at their 5′ terminus, which implies that the initiation of their synthesis occurs without a primer (23, 24).

The control of transcription and replication of viral RNA, as well as the functions of viral proteins in this control, is not well understood. PB1 is probably the polymerase itself (3, 9, 48, 56). PB2 is a cap-binding protein (4, 5, 56, 57), and it is probably also involved in the recognition and binding of the vRNA promoter (19). The function of PA is unknown, although genetic evidence suggests a role in replication (35, 41). It is essential, along with PB1 and PB2, for the activity of a recombinant polymerase complex (28). Viral nucleoprotein, which is associated with viral RNA, might be involved in the switch of transcription to replication by preventing premature termination during cRNA synthesis (2, 53).

The question of what factors determine whether the polymerase complex synthesizes mRNA or cRNA remains open. However, significant progress has been achieved in the characterization of the RNA signals involved in regulation. Sequencing of influenza A virus RNA segments revealed that all segments contain 12 and 13 conserved nucleotides at their 3′ and 5′ ends, respectively (13, 49). These conserved nucleotides, together with two or three additional base pairs specific for each segment, are partially complementary and form a partially double-stranded, panhandle structure in infected cells and virions (26, 27). It has been shown, by a reverse genetics approach, that the 26 3′-terminal and the 22 5′-terminal nucleotides of influenza A virus RNA are sufficient to provide the signals for RNA transcription, RNA replication, and packaging of RNA into influenza virus particles (40). To determine the properties of the vRNA promoter, several in vitro systems have been established, which have allowed transcription of model RNA templates by the polymerase complex isolated from virions by different techniques (44, 45, 50–52). These studies showed that 12 to 14 nt at the 3′ end of vRNA were sufficient for activity of the vRNA promoter. Apparently no other sequences were involved.

More recently, however, it has become evident that the 5′ terminus of vRNA plays an important role in the regulation of transcription as well. Specifically, the enzymatic activity of recombinant polymerase was strictly dependent on the presence of a template RNA containing 3′ and 5′ viral sequences (22). It was also established that the 5′ end was involved in the binding of the polymerase complex, and the residues important for binding were determined (18, 55). Direct evidence for the involvement of the 5′ end in transcription came from an in vitro mutagenesis study, showing that 3′-end mutants with reduced activities transcription, were significantly stimulated by

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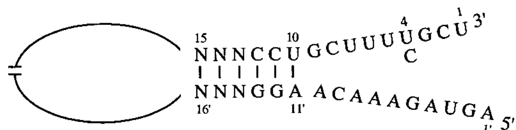

FIG. 1. RNA-fork model for the initiation of vRNA transcription. Watson-Crick base pairs are indicated by vertical lines. The U, C alternative at position 4 of the 3′ terminus is a naturally occurring sequence variation within the otherwise conserved nucleotides (13, 17, 49, 58). The numbering of nucleotide residues starts from the 3′ end in the 3′-terminal sequence and from the 5′ end in the 5′-terminal sequence. The nucleotide numbers of the 5′ end are indicated by a prime to distinguish them from nucleotides of the 3′ end.

5′ ends carrying complementary mutations (18). This study resulted in the proposal of an RNA-fork model for the initiation of influenza virus transcription (Fig. 1). We have now characterized this RNA-fork model further to gain insight into the RNA signals involved in the control of the initiation of transcription.

MATERIALS AND METHODS

Preparation of micrococcal nuclease-treated virus cores. Influenza A virus, strain X-31, a reassortant of A/HK/68 and A/PR/8/34, was supplied by Evans Medical Limited, Liverpool, United Kingdom. Viral cores were isolated by glycerol step gradient centrifugation from virus disrupted with Triton X-100 and lysolecithin and were treated with micrococcal nuclease (Sigma) to degrade RNA (50).

Preparation of RNA templates. Short RNA templates corresponding to the 3′ (14 nt) and 5′ (15 nt) ends of vRNA, wild type or mutants, were synthesized chemically on an Applied Biosystems synthesizer by standard RNA synthesis methods (20), starting from 2′-*O*-*t*-butyldimethylsilyl-protected phosphoramidites (Peninsula Chemicals). After deblocking by a mixture of anhydrous ethanolamine (Romil Chemicals) and anhydrous ethanol (1:1) at 60°C for 3 h, followed by treatment with 1 M tetrabutylammonium fluoride in tetrahydrofuran (Aldrich) at 33°C for 24 h and *n*-butanol precipitation, the RNA was purified by 18% polyacrylamide gel electrophoresis in 7 M urea. The RNA was located by UV shadowing, eluted in H_2O for 14 to 16 h at 4°C, and desalted on NAP-10 columns (Pharmacia). The RNA was freeze-dried and dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA, and the concentration was determined by measuring the A_{260}. Synthetic RNAs were analyzed for purity by 5′ phosphate labelling with [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by 18% polyacrylamide gel electrophoresis in 7 M urea.

In vitro transcription assay. Virus cores treated with micrococcal nuclease (about 4 to 5 ng of polymerase proteins [52]) were incubated with 5 pmol of RNA corresponding to the 3′ end of vRNA and 5 pmol of RNA corresponding to the 5′ end of vRNA in the presence of 50 mM Tris-HCl (pH 7.4)–50 mM KCl–10 mM NaCl–5 mM $MgCl_2$–1 mM dithiothreitol–1 mM ATP–0.5 mM GTP–0.5 mM UTP–0.1 mM CTP–1 μM [α-^{32}P]CTP (800 Ci/mmol)–1 U of human placental RNase inhibitor (Promega), with 1 mM ApG (Sigma) or other primers, as indicated in the figure legends, in a total reaction volume of 5 μl. After incubation at 30°C for 2 to 3 h, the RNA was phenol-chloroform extracted, ethanol precipitated with 10 μg of yeast carrier RNA (BDH), and analyzed on an 18% polyacrylamide gel in 7 M urea. When radiolabelled capped RNA was used as a primer in transcription, the reaction mixture was modified by replacing micrococcal nuclease-treated virus cores with untreated viral cores and increasing the CTP concentration to 0.5 mM. No [α-^{32}P]CTP was added. The final amount of radiolabelled capped RNA primer in the transcription mixture was about 2 to 3 fmol (about 10^4 dpm). After 2 to 3 h of incubation at 30°C, 10 μl of 90% formamide was added to stop the reaction, and the reaction mixture was heated to 95°C for 3 min and loaded on an 18% polyacrylamide gel in 7 M urea. Gels were exposed to X-ray films, and RNA products were estimated by phosphorimage analysis (Molecular Dynamics). To assess the effects of mutations, the mean values from three experiments were calculated.

Preparation of capped RNA primer. An 11-nt RNA sequence with a 2′-O-methyl group on the 5′-terminal G (5′ $_{HO}$GmAAUACUCAAG$_{OH}$ 3′) was synthesized chemically (see above) and converted to a 5′-diphosphorylated derivative by chemical modification by adapting a method (21, 38) for the synthesis of ATP analogs from adenosine (unpublished data; details available from the authors). The 5′-diphosphorylated RNA was capped at its 5′ end by using 1.5 U of guanylyltransferase (Gibco BRL) in the presence of 50 mM Tris-HCl (pH 7.8)–1.25 mM $MgCl_2$–6 mM KCl–2.5 mM dithiothreitol–0.5 μg of bovine serum albumin–20 U of human placental RNase inhibitor (Promega)–0.1 mM *S*-adenosylmethionine (Fermentas)–1.2 μM [α-^{32}P]GTP (3,000 Ci/mmol) in a 5-μl reaction volume at 37°C for 1 h. The radiolabelled capped RNA (5′ m^{7}G^{32}pppGmAAUACUCAAG$_{OH}$ 3′) was purified by polyacrylamide gel electrophoresis, localized by autoradiography, and eluted in H_2O for 14 to 16 h at 4°C. Then 20 μg of yeast carrier RNA was added to the eluent, followed by ammonium acetate to 2 M final concentration, and the RNA was precipitated with 3 volumes of ethanol. After the RNA pellet was washed with 75% ethanol, the RNA was dissolved in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The final concentration of radiolabelled capped RNA was 10 nM, as estimated by scintillation counting.

RNase T_1 analysis of transcription products. Radiolabelled RNA products were localized on the gel by autoradiography and eluted in 1 ml of H_2O for 14 to 16 h at 4°C. After desalting on an NAP-10 column (Pharmacia) and freeze-drying, the RNA was dissolved in H_2O. Partial RNase T_1 digestion was carried out (15) in 20 mM sodium citrate (pH 5.0)–7 M urea–1 mM EDTA–0.25 mg of tRNA per ml–0.025% xylene cyanol–bromophenol blue with 500 U of RNase T_1 (Boehringer) for 15 min at 50°C in a final volume of 15 μl. RNA fragments of RNase T_1 digestion were analyzed by 25% polyacrylamide gel electrophoresis in 7 M urea.

RESULTS

Systematic investigation of sequence requirements in the vRNA 5′-terminal sequence for initiation of transcription. We previously reported (18) that a C-11 → A-11 3′-end mutant is inactive in transcription (less than 10% of wild-type activity) unless rescued by a compensatory 5′-end mutant (G-12′ → U-12′) (Fig. 2A, sequence 1). We have now prepared a set of double mutants of the 5′-terminal sequence which contain the compensatory G-12′ → U-12′ mutation and an additional point mutation at each of the other 12 conserved nucleotides (Fig. 2A, sequences 2 to 13). Because the C-11 → A-11 3′-end mutant is active only in the presence of the 5′-terminal sequence carrying the G-12′ → U-12′ compensatory mutation, the effects of these additional mutations assay the importance of residues 1′ to 13′ of the 5′ end without interference from endogenous RNA.

Figure 2B shows that mutations at positions 4′ to 6′ and 10′ of the 5′ end did not interfere with transcriptional activity (lanes 5 to 7 and 11), which is in good agreement with previous results showing that these residues are not involved in the binding of the polymerase complex (18). Mutations at all other positions inhibited transcriptional activity either almost completely (positions 2′, 3′, 7′, 8′, 9′ and 13′; Fig. 2B, lanes 3, 4, 8, 9, 10, and 13, respectively) or partially (positions 1′ and 11′; Fig. 2B, lanes 2 and 12, respectively). The decreased activity of position 1′ to 3′, 7′ to 9′, and 11′ mutants is probably due to the decreased efficiency of binding of these mutants to the polymerase complex (18). On the other hand, the decreased activity of the position 13′ mutant might be the result of the disrupted C-12–G-13′ base pair, which, according to the RNA-fork model (Fig. 1), is required for the initiation of transcription (18). This may also partly explain the decreased activity of the A-11′ → U-11′ mutant (Fig. 2B, lane 12), which has a disrupted base pair at a critical double-stranded region of the RNA fork, as well as a low efficiency of binding to the polymerase. The activity of the 12 mutants relative to the parent sequence 1 is summarized in Fig. 2A.

Having identified 5′-end mutants which showed low activity in transcription, we then tested the effects of introducing complementary mutations into the 3′ end. Figure 3 shows that mutations at positions 1 to 3 and 7 of the 3′ end which theoretically restored the base pairing (sequences 15 to 18; Fig. 3A and B) did not stimulate the activity (compare activities of sequences 15 with 2, 16 with 3, 17 with 4, and 18 with 8 in Fig. 2 and 3), suggesting that in that region, features other than a double-stranded structure are important for the RNA polymerase. On the other hand, mutation C-12 → A-12 (sequence 20, Fig. 3A and B) fully restored the activity, consistent with the RNA-fork model, which predicts that base pairing is critical at this position. Mutation U-10 → A-10 (sequence 19, Fig. 3A and B) did not stimulate the activity, probably because a

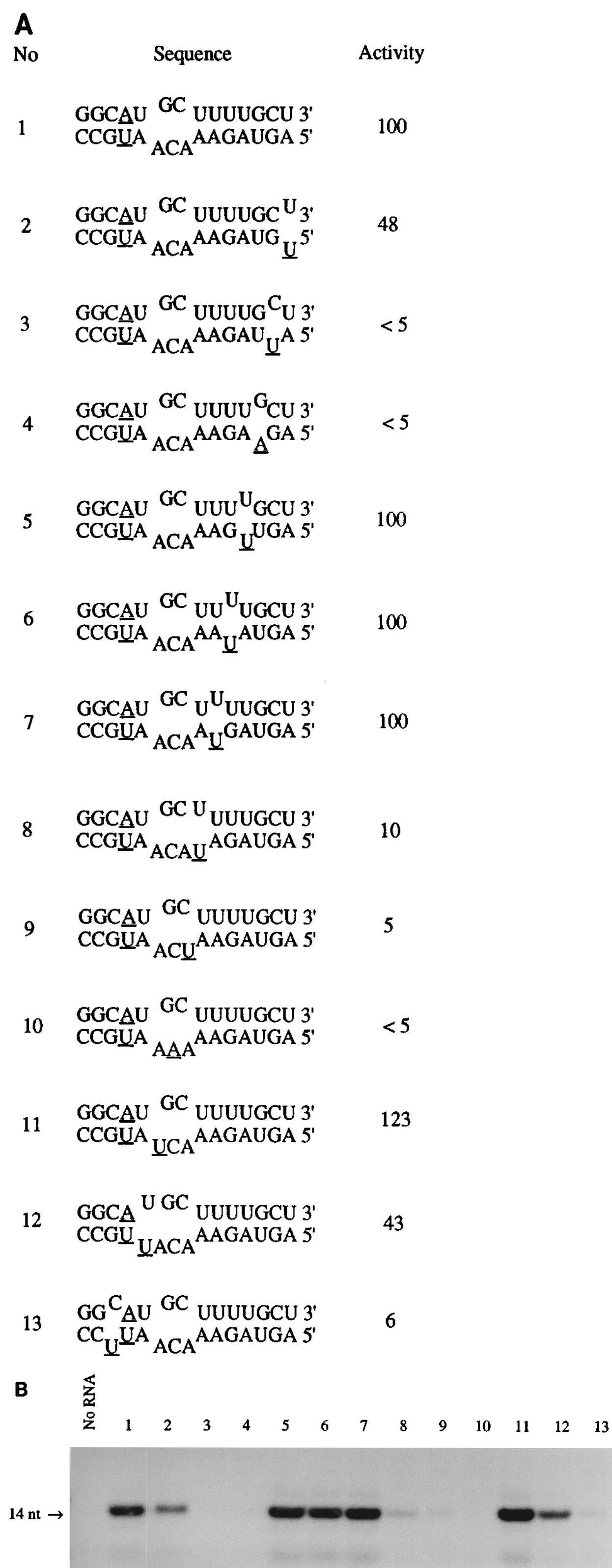

FIG. 2. Effects of point mutations in the conserved vRNA 5′-terminal sequence on the initiation of transcription. (A) Potential mutant panhandle structures formed by two synthetic RNAs corresponding to the 3′- and 5′-terminal sequences of vRNA. The mutated positions are indicated by underlining. The first 12 nucleotide residues of the 3′ end (upper sequence) and the first 13 nucleotide residues of the 5′ end (lower sequence) are conserved among the RNA segments of influenza A virus strains (13, 17, 49, 58). The average relative activity of mutants is shown next to the sequences. The activity of the base pair mutant sequence 1 (C-11 → A-11, G-12′ → U-12′), which does not differ significantly from that of the wild type (18), was taken as 100%. (B) Effects of point mutations on transcriptional activity. In vitro transcription was carried out with micrococcal nuclease-treated virus cores, synthetic RNA templates, and ApG primer as described in Materials and Methods. The size of the transcription products is indicated (14 nt). In this and following figures, the lane numbers correspond to the sequence numbers in panel A.

U-11′ is not fully compatible with polymerase binding (18). We did not study the other two low-activity 5′-end mutants (sequences 9 and 10, Fig. 2A and B) because these have mutations in the bulge region of the classical panhandle model (positions A-8′ and C-9′).

Although the use of a mutant template (C-11 → A-11) to eliminate the interference from endogenous RNA allowed us to carry out this detailed study of the function of the 5′ end in the initiation of transcription, we cannot exclude the possibility that this additional mutation might have affected our results to some extent. However, this is unlikely because of the agreement between the results of the binding (18) and transcription (Fig. 2) assays. To test whether the additional G-12′ → U-12′ mutation interferes with the binding of the RNA polymerase complex, we examined all the 5′-end double mutants used above (sequences 2 to 13) in photochemical cross-linking (19). We observed no significant difference in binding between the single and double mutants (results not shown) except for the position 11′ mutant, which exhibited more efficient binding. We assume that this is probably due to the difference between the length of the single mutants tested before (13 nt long) (18) and the double mutants used here (15 nt long) (see Fig. 2A).

Role of vRNA 3′-terminal sequence in initiation of transcription. We reported previously (19) that residues 1 to 8 of the 3′ end were not crucial for the binding of the RNA polymerase. In addition, individual point mutations in the 3′ end (at residues 1 to 9) showed only insignificant or weak inhibition when studied with dinucleotide primer (18, 51). To gain further insight into the role of the 3′ end, we have now tested multiple mutations at positions 1 to 9 of the proposed single-stranded region of the 3′ end (Fig. 4A) and studied their effects on the initiation of transcription (Fig. 4B). Figure 4B shows that some multiple mutations did not inhibit activity significantly, while other multiple mutations resulted in increased activity. At least six or seven residues had to be mutated simultaneously to obtain significant inhibition (Fig. 4, sequences 26, 27, and 28). It is interesting that some point mutations appear to be inhibitory in the context of one set of multiple mutations and neutral in the context of others. For example, the C-8 → A-8 and G-3 → C-3 mutations appear to be fairly neutral in sequences 24 and 25, respectively, but when they are present together, significant inhibition is observed (Fig. 4, sequence 26).

Overall, the results of this experiment confirmed that the sequence of the single-stranded 3′ end is less important than that of the 5′ end and that base pairing at positions 1 to 9 is not required for the initiation of transcription in vitro, as some of the multiple mutations disrupted the potential base pairing at residues 1 to 7 between the 3′ and 5′ ends and still exhibited high activity (sequences 22 to 25, 31, 32, and 33). On the other hand, as some multiple mutations inhibited transcription (sequences 26, 27, and 28), it is obvious that this sequence contains important signals for the polymerase.

Effects of triple mutations at conserved positions 10 to 12 of the vRNA 3′-terminal sequence on transcription. Figure 4 showed that mutations in the single-stranded region of the

A

No	Sequence	Activity
14	GGCAU GC UUUUGCU 3' CCGUA ACA AAGAUGA 5'	100
15	GGCAU GC UUUUGCA 3' CCGUA ACA AAGAUGU 5'	38
16	GGCAU GC UUUUGAU 3' CCGUA ACA AAGAUUA 5'	<5
17	GGCAU GC UUUUUCU 3' CCGUA ACA AAGAAGA 5'	<5
18	GGCAU GC AUUUGCU 3' CCGUA ACA UAGAUGA 5'	8
19	GGCAA GC UUUUGCU 3' CCGUU ACA AAGAUGA 5'	35
20	GGAAU GC UUUUGCU 3' CCUUA ACA AAGAUGA 5'	130

B

No RNA 14 15 16 17 18 19 20

14 nt →

FIG. 3. Attempted rescue of the activity of 5′-end mutants by introducing complementary mutations into the 3′ end. (A) Potential mutant panhandle structures formed by two synthetic RNAs corresponding to the 3′- and 5′-terminal sequences of vRNA. Sequences 2, 3, 4, 8, 12, and 13 (Fig. 2A) were modified by introducing a complementary mutation into the 3′ end opposite the mutated residues in the 5′ end so as to restore base pairs according to the structure of the classical panhandle, to form sequences 15 to 20, respectively. Mutated positions are underlined. (B) Effects of complementary mutations in the 3′ end on transcriptional activity. In vitro transcription was carried out with micrococcal nuclease-treated virus cores, synthetic RNA templates, and ApG (lanes 14 and 17 to 20), UpG (lane 15), or ApU (lane 16) as a primer. The reason for using UpG and ApU with sequences 15 and 16 was to maintain the base pairing between the two 3′-terminal nucleotides in the 3′ end and the dinucleotide primer. The ability of a dinucleotide to prime transcription efficiently has been shown to be dependent on the number of base pairs it can form with the template (25, 31, 51). Relative activities were corrected according to the number of ^{32}P-labelled C residues in transcription products. The size of the transcription products is indicated (14 nt). The reason for the different mobilities of transcription products of equal length is that their sequence differs.

vRNA 3′ end (positions 1 to 9) are fairly well tolerated by the RNA polymerase (see Fig. 1). On the other hand, transversions at positions 10 and 11 inhibited promoter activity severely, but these mutants were significantly stimulated when sequences corresponding to the 5′ end and carrying complementary mutations were added to the transcription reaction mix (18). By contrast, 3′-terminal sequences with transitions at positions 10 and 11 and all three possible mutants at position 12 showed activity similar to that of the wild type even in the absence of 5′ ends carrying complementary mutations. We proposed (18) that some nondisruptive mismatches in the dou-

A

No	Sequence	Activity
21	GGCCU GC UUUUGCU 3' CCGGA ACA AAGAUGA 5'	100
22	GGCCU GC AAA UGCU 3' CCGGA ACAAAG AUGA 5'	295
23	GGCCU GC AAAA GCU 3' CCGGA ACAAAGA UGA 5'	156
24	GGCCU G AAAAA GCU 3' CCGGA ACAAAGA UGA 5'	77
25	GGCCU GC AAAAC CU 3' CCGGA ACAAAGAU GA 5'	155
26	GGCCU G AAAAAC CU 3' CCGGA ACAAAGAU GA 5'	25
27	GGCCU CA AAAA GCU 3' CCGGA ACAAAGA UGA 5'	20
28	GGCCU CA AAAAC CU 3' CCGGA ACAAAGAU GA 5'	9
29	GGCCU CA UUUUGCU 3' CCGGA ACA AAGAUGA 5'	64
30	GGCCU CAA UUUGCU 3' CCGGA ACAA AGAUGA 5'	78
31	GGCCU GC UUUUG GA 3' CCGGA ACA AAGAU GA 5'	146
32	GGCCU GC UUUU CAG 3' CCGGA ACA AAGA UGA 5'	221
33	GGCCU GC UUU ACAG 3' CCGGA ACA AAG AUGA 5'	216

B

No RNA 21 22 23 24 25 26 27 28 29 30 31 32 33

14 nt →

FIG. 4. Effects of multiple mutations in the conserved vRNA 3′-terminal sequence on the initiation of transcription. (A) Potential mutant panhandle structures formed by two synthetic RNAs corresponding to the 3′- and 5′-terminal sequences of vRNA. The mutated positions are indicated by underlining. (B) Effects of multiple mutations on transcriptional activity. In vitro transcription was carried out with micrococcal nuclease-treated virus cores and synthetic RNA templates as described in Materials and Methods. For priming, ApG (lanes 21 to 30), UpC (lane 31), or CpU (lanes 32 and 33) was used. See also the legend to Fig. 3B.

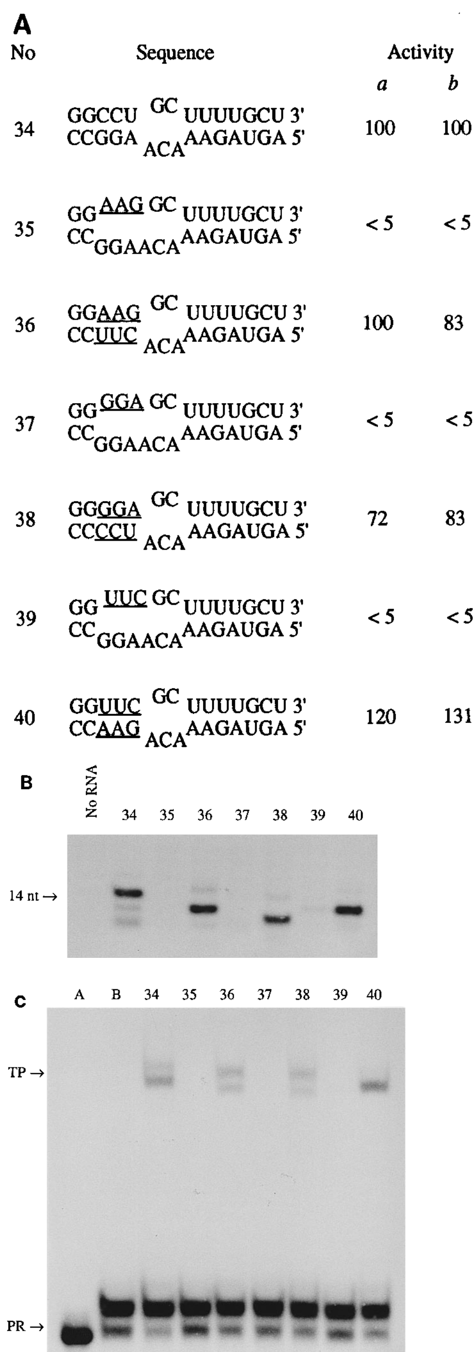

FIG. 5. Effects of triple mutations in the conserved double-stranded region of the RNA fork on transcription. (A) Potential mutant panhandle structures formed by two synthetic RNAs corresponding to the 3′- and 5′-terminal sequences of vRNA. In sequences 36 and 38, transversions were introduced at positions 10 to 12 and 11′ to 13′, mutating C ↔ A, U ↔ G and C ↔ G, U ↔ A, respectively. In sequence 40, transitions were introduced, mutating C ↔ U, G ↔ A. The relative activities indicated in column a were the activities when ApG was used as a primer, and those in column b represent the activities achieved with capped RNA as a primer. (B) Effects of triple mutations on the initiation of ApG-primed transcription. In vitro transcription was carried out with micrococcal nuclease-treated virus cores, synthetic RNA templates, and ApG primer as described in Materials and Methods. See also the legend to Fig. 2B. (C) Effects of triple mutations on the initiation of capped-RNA-primed transcription. In vitro transcription was carried out with virus cores, synthetic RNA templates, and a capped ^{32}P-labelled RNA primer as described in Materials and Methods. Micrococcal nuclease-treated virus cores were replaced by untreated cores in order to avoid background signals originating from endogenous RNA fragments (escaping digestion by micrococcal nuclease) and interfering with the specific signal from the added RNA templates. If untreated cores are used, the polymerase transcribes the added short RNA templates as well as the endogenous full-length RNA, which, because of its size, does not enter the 18% polyacrylamide gel (not shown) and does not interfere with the signal of the short RNA templates (48a). Lane A, capped RNA primer; lane B, capped RNA primer in the absence of added RNA template; lanes 34 to 40, capped RNA primers elongated in the presence of added RNA templates. TP, transcription product; PR, primer.

ble-stranded region may be acceptable for the polymerase if the structure is stabilized by a sufficient number of base pairs.

To characterize the double-stranded region further, we introduced triple mutations at positions 10 to 12 of the 3′ end (Fig. 5A). The three triple mutants tested showed severe inhibition of activity (<5% of wild type) (Fig. 5B, lanes 35, 37, and 39). However, introduction of complementary triple mutations into the 5′ end resulted in restoration (Fig. 5B, lanes 36 and 40) or significant stimulation (lane 38) of activity.

Effects of triple mutations in capped-RNA-primed transcription. We tested the triple mutants described above (Fig. 5A) by using a capped RNA instead of ApG to prime transcription, since such a primer more closely represents the in vivo situation. Chung et al. (12) characterized a capped 11-nt RNA fragment (m^{7}GpppGmAAUACUCAAG$_{OH}$) capable of directly priming influenza virus transcription. This capped RNA primed transcription without the need for its further cleavage, with efficiency similar to that obtained with a long RNA, confirming that cleavage and elongation could be uncoupled (9, 56). We synthesized an 11-nt RNA of the same sequence ($_{HO}$GmAAUACUCAAG$_{OH}$) and modified its 5′ end by adding a diphosphate by chemical phosphorylation to allow capping with guanylyltransferase, which needs 5′-di- or triphosphate (see Materials and Methods). Alternative methods for preparing this same ^{32}P-labelled capped oligonucleotide by SP6 RNA polymerase transcription of *Sma*I-digested pGEM-7Zf(+) plasmid DNA (Promega) and subsequent capping by guanylyltransferase have been described (12).

The RNA with the ^{32}P-labelled cap structure (see Table 1, line A) was used as the primer in transcription with the triple mutants (Fig. 5A). Addition of wild-type template to the transcription mixture with capped RNA primer resulted in a shift of the primer by about 14 nt, which corresponds to the length of the template, indicating that the polymerase can recognize and use the capped RNA as a primer to initiate transcription on the model RNA template (Fig. 5C, lane 34). When 3′-end sequences carrying triple mutations at positions 10 to 12 were used as templates in the presence of wild-type 5′-end sequence (Fig. 5C, lanes 35, 37, and 39), no elongation of the primer was observed. However, upon addition of 5′-end sequences carrying complementary triple mutations, the mutant templates were elongated similarly to the wild type (Fig. 5C, lanes 36, 38, and 40). This confirms the previous results with the ApG primer (Fig. 5B) and also suggests that ApG acts by a mechanism similar to that of capped RNA.

As observed before (12), elongation of the capped RNA primer was not quantitative. A large proportion of the primer was elongated by only one nucleotide (Fig. 5C, lanes B and 34 to 40), probably because of premature termination, as described earlier (12, 22, 51). To characterize this added single nucleotide, we performed single-base addition experiments (results not shown), obtaining results essentially similar to those of others (12, 29).

While the ApG-primed transcription products appeared mainly as single bands (Fig. 5B, lanes 34, 36, 38, and 40), with the capped RNA primer, we observed doublets (Fig. 5C, lanes

34, 36, 38, and 40). There were several possible explanations for the origin of these doublets: (i) the polymerase can initiate opposite the second or third nucleotide of the template, producing a mixture of RNAs of two different sizes (47); (ii) there is a nonspecifically added single nucleotide at the 3′ end of the transcription products. Because the same templates, in ApG-primed transcription, produced only one major transcription product, we rejected the hypothesis that the doublets are due to the heterogeneity in the templates. To test the origin of the doublets and also to characterize the transcription products further, we gel isolated the transcription products and performed a partial RNase T_1 digestion. Figure 6 shows that both RNA components of the doublet degrade to give partial T_1 fragments of the same length, indicating that the difference between the two products forming the doublet is at their 3′ end. This excludes hypothesis i above, that the polymerase initiates transcription opposite both the second and third nucleotide in the template. This is further confirmed by the observation that partial RNase T_1 digestion produces 11-nt- but no 12-nt-long capped RNA fragments. Thus, initiation with the capped RNA occurs exclusively opposite the third base in the template, so that the first added nucleotide to the primer is CTP. If priming occurs opposite the second base, the first added nucleotide would be GTP, and in RNase T_1 digestion, a 12-nt capped RNA fragment should be visible. We conclude that the reason for the presence of doublets is hypothesis ii above, and there is one added nucleotide at the 3′ end. The enzyme activity responsible for this may be the same activity which adds one additional nucleotide to the capped primer (see above).

The partial RNase T_1 fragments also confirmed the positions of G residues in the transcription products, providing evidence for the authenticity of their sequence. The wild-type product produced capped fragments of 21, 20, 17, and 11 nt (Fig. 6, lanes 34F and 34S), while the mutants produced capped fragments of 17 and 11 nt (lanes 36F, 36S, 38F, and 38S) or 19, 17, and 11 nt (lanes 40F and 40S), as expected (Table 1). The partial T_1 digestion of the gel-isolated primer extended by one nucleotide resulted in a fragment (lane B) carrying a 3′-phosphate, which runs faster than the primer of the same sequence but with a 3′-hydroxyl group.

DISCUSSION

We report here the further characterization of an RNA-fork model for the initiation of influenza A virus transcription (Fig. 1). Our model was originally based on two lines of evidence: (i) the influenza A virus RNA polymerase complex exhibits specific binding activity for both 3′ and 5′ conserved termini of vRNA segments, and (ii) vRNA 5′-terminal sequences significantly affected the ability of 3′-terminal sequences to serve as templates in transcription in vitro (18).

At first, we focused on the proposed single-stranded 5′ terminus. We have now tested 5′-terminal sequences with individual point mutations at each of the conserved positions in transcription in vitro to establish which residues are involved in the initiation of transcription. We have chosen an indirect approach with a mutant 3′ end which is active only in the presence of a 5′ end with a complementary mutation, to eliminate interference by residual endogenous viral RNA (which escaped digestion by micrococcal nuclease) in the RNA polymerase preparations. Mutations at all residues identified previously (18) as important for binding in our photochemical cross-linking assay were inhibitory in transcription, including residues 1′ to 3′, with residue 1′ being apparently less important than residues 2′ and 3′. The transcription assay (Fig. 2) also confirmed the importance of residues 7′ to 9′ and 11′ but, contrary to an independent study using modification interference analysis (55) and in agreement with the cross-linking assay, failed to detect position 10′ as important.

TABLE 1. Analysis of capped-RNA-primed transcription products by partial RNase T_1 digestion[a]

No.	Sequence of capped RNA primer and transcription products
A	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{G}}_{OH}$
B	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{Gp}}$↓$\overset{12}{\text{N}}_{OH}$
34	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{Gp}}$↓CpApApApAp$\overset{17}{\text{Gp}}$↓CpAp$\overset{20}{\text{Gp}}$↓$\overset{21}{\text{Gp}}$↓Cp$\overset{23}{\text{C}}_{OH}$
36	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{Gp}}$↓CpApApApAp$\overset{17}{\text{Gp}}$↓CpCpUpUpCp$\overset{23}{\text{C}}_{OH}$
38	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{Gp}}$↓CpApApApAp$\overset{17}{\text{Gp}}$↓CpUpCpCpCp$\overset{23}{\text{C}}_{OH}$
40	m^{7}G^{32}pppGm pApApUpApCpUpCpApAp$\overset{11}{\text{Gp}}$↓CpApApApAp$\overset{17}{\text{Gp}}$↓Cp$\overset{19}{\text{Gp}}$↓ApApCp$\overset{23}{\text{C}}_{OH}$

[a] RNase T_1 cleavage sites are indicated by arrows. The sizes of the capped RNA fragments are marked.

When we attempted to rescue the activity of the 5′-end mutants (positions 1′ to 3′ and 7′) by introducing complementary mutations into the 3′ end, we did not observe significant stimulation of activity (Fig. 3). This further suggests that base-pairing between the 3′ and 5′ ends is not required at these positions and that the two termini do not function as an RNA duplex. Mutation at position 13′ decreased the efficiency of transcription severely because this mutation disrupted a base pair in the proposed duplex region of the RNA fork. When we reformed the base pair by introducing a complementary mutation into the 3′ end, full activity was observed.

The finding that the U-10 → A-10 mutation in the 3′ end did not stimulate the activity of the A-11′ → U-11′ 5′-end mutant (43% versus 35%) was somewhat surprising (Fig. 3), because these bases form part of the duplex region of the RNA fork.

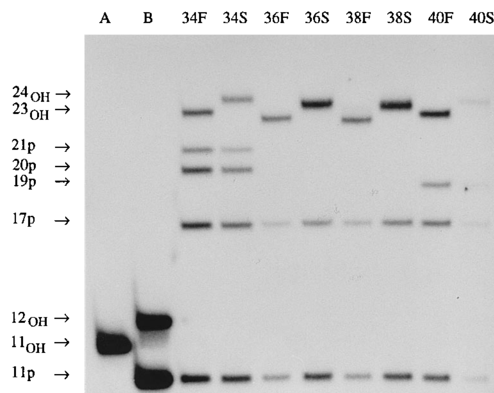

FIG. 6. Partial RNase T_1 analysis of capped-RNA-primed transcription products. Transcription products forming a doublet (Fig. 5C, lanes 34, 36, 38, and 40), capped RNA primer (Fig. 5C, lane A), and capped RNA primer presumably elongated by one nucleotide (Fig. 5C, lane B) were gel isolated and treated with RNase T_1 as described in Materials and Methods. The two bands of doublets (faster band [F] and slower band [S]) were isolated separately. The T_1 digests were analyzed on 25% polyacrylamide gels in 7 M urea. Lane A, capped RNA primer after T_1 digestion; lane B, capped RNA primer elongated by one nucleotide after T_1 digestion; lanes 34F, 34S, 36F, 36S, 38F, 38S, 40F, and 40S, transcription products isolated from gel shown in Fig. 5C, lanes 34, 36, 38, and 40, respectively (S, slower band; F, faster band of the doublet). The size of RNAs is indicated. For the sequence and size of expected RNA fragments of T_1 digestion of wild-type and triple mutants, see Table 1. 24_{OH} marks the position of undigested 34S, 36S, 38S, and 40S.

On the other hand, A-11′ may be required for polymerase binding, suggesting that not all base pairs at positions 10 to 11′ are compatible with wild-type activity. We previously observed only 41 and 68% of wild-type activity with an A-10–U-11′ and a G-10–C-11′ base pair, respectively (18), suggesting that a C-11′ may be better for the binding of the polymerase complex than a U-11′. Interestingly, a triple mutant (sequence 38) with an A-10–U-11′ base pair showed 72% of wild-type activity, while two other triple mutants with G-10–C-11′ and C-10–G-11′ base pairs (sequences 36 and 40) showed wild-type activity. This suggests that the effect of a base pair mutation on transcriptional activity may also depend on the other base pairs of the duplex. In contrast to the situation with the U-10–A-11′ base pair, the other two conserved base pairs (C-11–G-12′ and C-12–G-13′) proved to be less specific, since all mutations at these positions resulted in activity comparable to that of the wild type. Replacement of the C-11–G-12′ base pair with A-11–U-12′ or G-11–C-12′ resulted in 94 and 87% of wild-type activity (18), respectively. A double mutant with A-11–U-12′ and A-12–U-13′ base pairs (sequence 20) exhibited 130% of wild-type activity. Overall, our results with single, double, and triple mutations in the proposed duplex region of the RNA fork confirm our previous suggestion that, for the initiation of transcription in vitro, the secondary structure in this region is more important than the actual sequence of residues.

We compared the activity of different triple mutants in transcription reactions with either ApG or a capped RNA primer (Fig. 5). No significant differences were observed. In addition, we tested all the 5′-end mutants (Fig. 2A) with a capped RNA primer and observed essentially the same activities as with ApG (data not shown). This suggests that ApG probably functions by a mechanism similar to that of capped RNA and a reaction primed with ApG probably mimics mRNA synthesis.

The study of multiple mutations in the proposed single-stranded region of the 3′ end (Fig. 4) confirmed our previous findings that individual point mutations in dinucleotide-primed transcription had no crucial effect on transcriptional activity in this region (51). At least six or seven residues had to be mutated to achieve inhibitory effect. A study of residues 1 and 2 (Fig. 4, sequences 31 and 32) failed to confirm the previous suggestion that these residues are important (51). These results suggest that transcriptional activity is not strictly dependent on the sequence of this region. On the other hand, deletions or insertion in the U-rich region of the single-stranded 3′ termini resulted in a strong inhibition or stimulation of activity, in some cases causing premature termination (51). This may suggest that the length of this sequence is the important factor for the initiation of transcription.

The fact that this sequence is conserved indicates its importance, perhaps, for a function other than the initiation of vRNA transcription. The vRNA 3′ end, after replication of vRNA into cRNA, functions as the 5′ end of cRNA. It was shown recently that influenza virus polymerase complex can bind cRNA 5′ end as well as vRNA 5′ end, and in addition the binding was strictly dependent on some of the conserved residues (55). We also observed efficient binding of polymerase complex to an 18-nt-long RNA representing the 5′ end of cRNA in a photochemical cross-linking assay (results not shown). In spite of the differences between the two sequences, the binding was as efficient as to the vRNA 5′ end and, in competition, exhibited the same specificity (results not shown). Alternatively, we cannot exclude that the 3′ end of vRNA may contain important signals for packaging vRNA into virions.

In summary, we propose that vRNA 5′ end should be treated as an integral part of the vRNA promoter. We envisage the following mechanism for the recognition of the vRNA promoter and the initiation of transcription. Initially, the polymerase complex recognizes and binds to the 5′ end of the RNA in a single-stranded conformation. Once bound to the 5′ end, the affinity of the polymerase for the single-stranded 3′ end will be enhanced because of the synergistic effect caused by base pairing in the duplex region of the RNA fork (Fig. 1). Thus, three mechanisms, (i) binding of the polymerase to the 5′ single-stranded end, (ii) binding of the polymerase to the single-stranded 3′ end, and (iii) base complementarity of the RNA duplex of the fork, all play a part. We suggest that residues 1′ to 10′ of the 5′ end would be masked by the bound polymerase, preventing these bases from forming pairs with the bases of the 3′ end. Rather, the single-stranded 3′ terminus will be positioned at the catalytic site of the polymerase to allow initiation of transcription. We assume that interactions between the 3′ and 5′ ends are not sufficient by themselves to stabilize the partially complementary double-stranded structure of vRNA termini (27).

Our finding that base pair replacements are possible in the conserved duplex region of the RNA fork with minimal or no interference with promoter activity may serve as a theoretical basis for developing new attenuated vaccines against influenza. If a virus with a mutated base pair is viable and attenuated, the probability of a revertant's arising from such a virus would be minimal. If a reversion occurs at one of the bases of the mutated base pair, this would be eliminated before a complementary reversion could occur at the other base because of the predicted inability of such an RNA to replicate. It is now feasible to introduce site-specific mutations into the viral genome and rescue mutant genotypes (16), and positive results in obtaining attenuated viruses by related approaches have been reported (10, 43).

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