The Polyadenylation Signal of Influenza Virus RNA Involves a Stretch of Uridines Followed by the RNA Duplex of the Panhandle Structure

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Appropriate RNAs are transcribed and amplified and proteins are expressed after transfection into cells of in vitro-reconstituted RNA-protein complexes and infection with influenza virus as the helper. This system permits us to study the signals involved in transcription of influenza virus RNAs. For the analysis we used a plasmid-derived RNA containing the reporter gene for chloramphenicol acetyltransferase (CAT) flanked by the noncoding sequences of the NS RNA segment of influenza A/WSN/33 virus. Mutations were then introduced into both the 5' and 3' ends, and the resulting RNAs were studied to determine their transcription in vitro and their CAT expression activity in the RNA-protein transfection system. The results reveal that a stretch of uninterrupted uridines at the 5' end of the negative-strand RNA is essential for mRNA synthesis. Also, a double-stranded RNA "panhandle" structure generated by the 5'- and 3'-terminal nucleotides appears to be required for polyadenylation, since opening up of these base pairs diminished mRNA synthesis and eliminated expression of CAT activity by the mutant RNAs. Finally, it was shown that this double-stranded RNA structural requirement is not sequence specific, since a synthetic GC clamp can replace the virus-coded RNA duplex. The data suggest that the viral RNA polymerase adds poly(A) by a slippage (stuttering) mechanism which occurs when it hits the double-stranded RNA barrier next to the stretch of uridines.

Influenza viruses, members of the orthomyxoviruses, are negative-strand RNA viruses with a segmented genome (12). The viral RNA genome is replicated in the nucleus of target cells, and three different modes of RNA synthesis have been distinguished (reviewed in reference 9): (i) transcription of mRNA from virion RNA (vRNA) templates, which is primed by short capped mRNA fragments derived from the host; (ii) synthesis of full-length complementary RNA (cRNA) from the vRNA template; and (iii) replication of cRNA to yield new copies of genomic vRNA.

Sequence analysis of influenza A virus RNAs has revealed distinctive features. There is extensive conservation of the terminal sequences of vRNA (and thus cRNA); the 12 nucleotides at the 3' terminus show only a single base difference in half of the genome segments, and the 13 5'-terminal nucleotides are completely conserved. A second aspect of influenza A virus RNA segments is the extensive inverted complementarity between the termini, extending beyond the conserved nucleotides. It had been predicted that this complementarity, by base-pairing, could result in a panhandle structure of vRNA (1, 16, 21), and the existence of panhandle structures in purified virus as well as in infected cells has been confirmed by psoralen cross-linking experiments (7). Both these primary and secondary structural features have been postulated to be involved in transcription and replication of viral RNAs. However, until recently this could not be proven experimentally.

We have now developed a system, based on purified viral polymerase proteins and synthetic RNAs, which enables us to address these questions both in vitro (13) and in vivo (2, 8, 11). In vitro, we were able to show that the highly conserved 3' terminus of influenza A virus genome segments serves as a promoter for the transcription of viral RNA polymerase (13). However, we could not establish whether the panhandle structure plays a role in transcription; the in vitro system required just the 3' noncoding sequences of vRNA, and only cRNA was made under the conditions used. In order to investigate promoter functions and the role of the panhandle structure, it is necessary to study replication in vivo with mutagenized RNAs.

Our recent success in developing an in vivo system which allows introduction of modified RNAs into the genome of a negative-strand RNA virus (2, 11) now allows the use of mutagenesis to study the signals required for influenza virus mRNA transcription. The procedure is based on the preassembly of RNA, transcribed from plasmids, with purified influenza virus polymerase complexes (6, 13) into a ribonucleoprotein (RNP) complex which can be transfected into influenza virus-infected cells. The RNA is transcribed, replicated, and packaged by the virus, and the genetic information on the RNA is expressed (2, 11). The system only requires that the 5' and 3' noncoding sequences of vRNA be present on the synthetic RNA, and mutagenesis of these flanking sequences now permits study of their function in transcription, replication, and packaging.

In this article we present studies on the role of the panhandle structure, specifically of the base-paired nucleotides, in the synthesis of viral mRNA. Using constructs which direct synthesis of RNA containing a chloramphenicol acetyltransferase (CAT) gene between mutagenized influenza virus noncoding sequences derived from the NS genome segment 8, we show that an RNA duplex juxtaposed to the uridine stretch at the 5' end is necessary for mRNA synthesis to occur. Previously the site of poly(A) addition of

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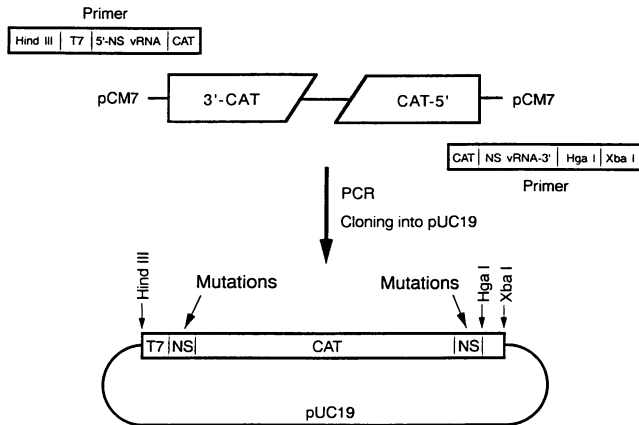
FIG. 1. Strategy for construction of derivatives of pIVACAT1. Plasmid pCM7, containing the entire coding region of the CAT gene, was used as the template in PCR. One primer had the T7 promoter and the 5' nontranslated region of the influenza virus NS gene. The other primer contained the 3' nontranslated region of the influenza virus NS gene and a unique site for the restriction enzyme HgaI. The PCR product was then cloned into pUC19 via its terminal HindIII and XbaI sites. Mutations in the 5'-terminal or the 3'-terminal regions of the insert for pIVACAT5, -6, -7, -8, -10, -11, -12, -13, -15, -18, -19, -20, -21, and -22 were introduced by using appropriate primers during the PCR. Methods used were as described for the construction of pIVACAT1 (11).

influenza virus RNAs was identified as the stretch of uridines located close to the 5' end of the vRNA (4, 17). We also show that RNA with a displaced uridine stretch is not functional and that introduction of a new base-pairing region, adjacent to the uridine stretch, reestablishes significant levels of mRNA transcription from the mutant RNA. This suggests that the polyadenylation signal of influenza virus consists of a uridine stretch in juxtaposition to an RNA duplex (panhandle) made up of the 3' and 5' termini of vRNA.

MATERIALS AND METHODS

Cells and viruses. All RNP transfection experiments were performed with MDBK cells (22). Influenza viruses A/PR/8/34 and A/WSN/33 were grown in embryonated eggs and MDBK cells, respectively (15, 22).

Construction of plasmids. The structure of all plasmids is similar to that of pIVACAT1 (influenza virus/A/CAT1), which contains the CAT gene flanked by the noncoding sequences of the influenza A virus NS RNA (11). Mutagenesis of the influenza A virus noncoding sequences was achieved by the polymerase chain reaction (PCR) with the CAT plasmid pCM7 as the template (Pharmacia). Figure 1 shows a diagram of the primers used and the PCR strategy for constructing the double-stranded DNA (dsDNA) inserts. The PCR products were digested with HindIII and XbaI and cloned into pUC19 plasmids. Recombinant plasmids were sequenced and, when found to be correct, used for T7 polymerase transcription. As an example of this strategy, for the construction of plasmid pIVACAT10, the following two primers were used: primer 25, 5'-AAGCTTTAAGCTTAA TACGACTCACTATAAGTAGAAACAAGGGTGATCG ATCGCGCGTTTTTTCAGATCTATTACGCCCCGC CCTGCCACTC-3', and primer 13, 5'-TCTAGAATCTAGA CGCCCTGCAGCAAAAGCAGGGTGACAAAGACAT

AATGGAGAAAAAAATCACTGGA-3'. For pCAT1-BalI/SalI, the following two oligonucleotides were synthesized and used to insert a 53-nucleotide-long fragment between the PstI and the BalI sites of pIVACAT1: 5'-GTCGACAGC ATTGGAACGTCGGAGAAACTACTCCCAAGAAGCT AAGAATGGTCC-3' and 5'-GGACCATTCTTAGCTTCTTG GGAGTAGTTTCTCCGACGTTCCAATGCTGTCGACT GCA-3'.

T7 polymerase transcription. The plasmid DNAs were digested with restriction enzyme HgaI or SalI and transcribed by T7 RNA polymerase (2,13).

RNP transfection. RNA obtained from T7 polymerase transcription was used for RNP transfections as described before (11). Briefly, in all cases, MDBK cells were first infected with A/WSN/33 seed virus at a multiplicity of infection of approx. 1 (1 h), then treated with a DEAE-dextran solution for 30 min as described before, and subsequently RNP-transfected with a mixture of 1 μg of RNA and 5 μl of purified viral nucleoprotein and polymerase proteins in buffer and phosphate-buffered saline-gelatine. IVACAT RNAs were preheated for 60 s at 100°C to prevent possible formation of aggregates prior to RNP assembly.

In vitro transcription and CAT assay. In vitro transcriptions were done by the method of Parvin et al. (13). RNA (500 ng) derived from T7 polymerase transcription was incubated with 5 μl of purified influenza virus polymerase proteins (13) for 2 h at 30°C in the presence of 0.4 mM ApG primer. RNA was phenol extracted twice and subsequently ethanol precipitated with 10 μg of dextran as the carrier. Half of the final material was loaded onto a 4% acrylamide gel containing 7.7 M urea. The CAT assay was performed with extracts of RNP-transfected cells as described before (11).

RNA extraction, RNase protection assay, and PCR. After RNP transfection, cells were harvested at 6 h postinfection (p.i.) and total RNA was extracted with guanidinium isothiocyanate, followed by equilibrium centrifugation in cesium chloride solutions (18). The RNA was precipitated with ethanol, and the CAT-specific mRNA and cRNA were detected in RNP-transfected cells with an RNase protection assay kit as described in the manufacturer's instructions (Ambion, Inc., Austin, Tex.). Briefly, 20 μg of total RNA isolated from RNP-transfected cells was hybridized with 10^5 cpm of [α-^{32}P]-UTP-labeled pIVACAT1-specific RNA probe (see Fig. 4) in 20 μl of buffer containing 80% formamide, 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4], 400 mM sodium acetate (pH 6.4), and 1 mM EDTA, and the mixture was incubated at 45°C for 12 h. Then, 200 μl of RNase digestion buffer containing 0.5 U of RNase A and 100 U of RNase T_1 per ml was added, and the reaction mixture was incubated at 37°C for 30 min. After RNA extraction, samples were loaded on a 6% polyacrylamide gel containing 7 M urea and electrophoresed.

The RNA from the guanidinium isothiocyanate extraction was also used as the template for the PCR analysis. cDNA synthesis was carried out as described in the manufacturer's instructions with RNase H-minus reverse transcriptase (Bethesda Research Laboratories). The reaction mixture contained 10 μg of total RNA and 0.2 μg of primer in a 25-μl volume. Primer A (CAGCTGCAGTAGAAACAAGG) is 20 bases long and contains sequences complementary to the first 13 nucleotides of the 3' terminus of the cRNA. Only viral cRNA should be copied by this primer. In order to remove the RNA template, the cDNA products were treated with alkali (0.3 N NaOH, 95°C, 10 min). For the PCR, a second primer (CGCCCCCGTTTTCACCATGGGC), span-
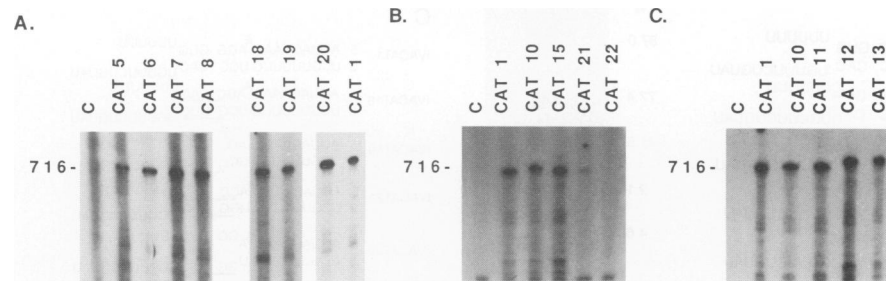
FIG. 2. In vitro transcription of IVACAT1 and mutant RNAs with purified influenza virus proteins. RNAs derived from pIVACAT1 and mutant plasmids were used as templates for in vitro transcription under standard conditions (13) (see text). The labeled products were analyzed on 4% polyacrylamide gels. The 3'- and 5'-terminal sequences of IVACAT1 RNA and of mutant RNAs are shown in Fig. 3. In vitro transcription of samples shown in a single panel was done at the same time on the same gel for analysis. The RNAs used are indicated. The size (in nucleotides) of a marker RNA is indicated. C indicates the presence of polymerase only; no RNA template was provided. Expression levels were estimated by scanning the bands on the X-ray film.

ning the region from +501 to +522 of the CAT gene, was used. The PCR was run as described previously (10) in the presence of 5'-end-labeled primer B with Taq DNA polymerase (Perkin Elmer Cetus). PCR products were collected by ethanol precipitation and analyzed on an 8% polyacrylamide gel containing 7 M urea.

RESULTS

In vitro transcription of mutant RNAs. We have recently shown that it is possible to express synthetic RNAs in cells infected with influenza virus as the helper (2, 11). With this system, it was found that the signals required for transcription, polyadenylation, replication, and packaging of influenza virus RNA are located in the 5'- and 3'-terminal noncoding sequences of the viral RNAs. We then decided to use this system to further dissect the role of the primary and secondary structural features of the noncoding terminal regions of the viral RNA in transcription. A series of mutant plasmids were constructed from plasmid pIVACAT1 (11). From these plasmids, after transcription with T7 polymerase, RNAs were obtained which contain the CAT gene (in negative polarity) flanked by the 5'- and 3'-terminal noncoding sequences of the influenza virus NS gene. Mutations were introduced into the 5'-terminal and 3'-terminal regions of the RNAs. Before using the mutant RNAs in RNP transfections, we tested each of them in the in vitro transcription system (Fig. 2) with purified influenza virus proteins as described by Parvin et al. (13). IVACAT5, -6, -7, and -8 RNAs differ by changes in the 5' terminus, and not unexpectedly, the level of in vitro transcription was similar for all of these mutant RNAs (Fig. 2A; for precise sequences of the mutant RNAs, see Fig. 3). This is in accordance with our earlier finding (13) that the viral polymerase-mediated in vitro transcription of synthetic RNAs required only an intact 3' end and not a virus-derived 5' end. Although RNAs IVACAT18, -19, and -20 have mutations at the 3' terminus, their ability to be a template for the in vitro influenza virus polymerase was not lowered (Fig. 3A). This finding is most likely due to the fact that the 11 terminal nucleotides of the wild-type (IVACAT1) RNA are conserved in these mutants (see Fig. 3 for precise sequences). RNAs IVACAT10 and -15 also retain an unchanged 3' terminus, and they appeared to show a level of in vitro transcription similar to that of IVACAT1 RNA (Fig. 2B). In contrast, the low level of transcription of IVACAT21 and -22 RNAs (less than 10% of that of IVACAT1 RNA) is most likely due to the base change

in position 11 at the 3' end (Fig. 2B). This suggests that the promoter signal for optimal in vitro transcription consists of the 11 3'-terminal sequences present in IVACAT1 RNA. IVACAT10, -11, -12, and -13 all retain at least 15 unchanged nucleotides at the 3' end compared with the control IVACAT1 RNA (Fig. 2C). Their level of in vitro transcription was comparable to that of control IVACAT1 RNA. As shown in Fig. 2, all mutant RNAs except IVACAT RNAs 21 and 22 revealed significant transcription levels. Their in vitro template activities were not less than 80% of that of IVACAT1 RNA, and we thus conclude that mutations in these RNAs do not interfere with promoter signals for in vitro transcription as long as the 3'-terminal 11 nucleotides are retained.

Requirement of a panhandle structure for the expression of CAT activity. As mentioned, Hsu et al. (7) have shown that the 3'- and 5'-terminal sequences of the influenza virus RNA segments may form a panhandle structure in vivo. Sequence analysis of the different vRNAs suggested that these panhandle (RNA duplex) structures could be formed by basepairing of 15 to 17 nucleotides at the 3' and 5' ends (1, 21). However, the in vitro transcription experiments described above did not allow us to define a function for these panhandle structures. In order to answer this question, the in vivo RNP transfection system was used. Mutant plasmids pIVACAT5, pIVACAT6, pIVACAT7, and pIVACAT8 were transcribed with T7 polymerase to produce RNAs which differed from wild-type IVACAT1 RNA by one, two, three, or four mutations, respectively, in the 5' panhandle-forming sequence (Fig. 3A). These changes resulted in a lowering of the number of RNA duplex-forming nucleotides in the panhandle. The mutant RNAs were RNP-transfected in the presence of helper virus, and the levels of CAT activity were measured (Fig. 3A). Opening up the panhandle by 1 bp (IVACAT5, lane 1) did not lead to a significant drop in CAT activity (87 versus 77% conversion). Further opening by introducing two mutations (IVACAT6, lane 2) resulted in somewhat reduced (41% conversion) but still high levels of CAT activity. However, the introduction of three (IVACAT7, lane 3) and four (IVACAT8, lane 4) mutations, which would reduce the size of the panhandle by 3 and 4 bp, respectively, greatly reduced the CAT activity in this assay (less than 5% conversion), although it was still above the background level (lane 5).

In order to prove that the level of CAT activity correlates with the amount of CAT-specific mRNA present in transfected cells, we performed an RNase protection assay. As shown in Fig. 4, a minus-sense probe (262 nucleotides) was
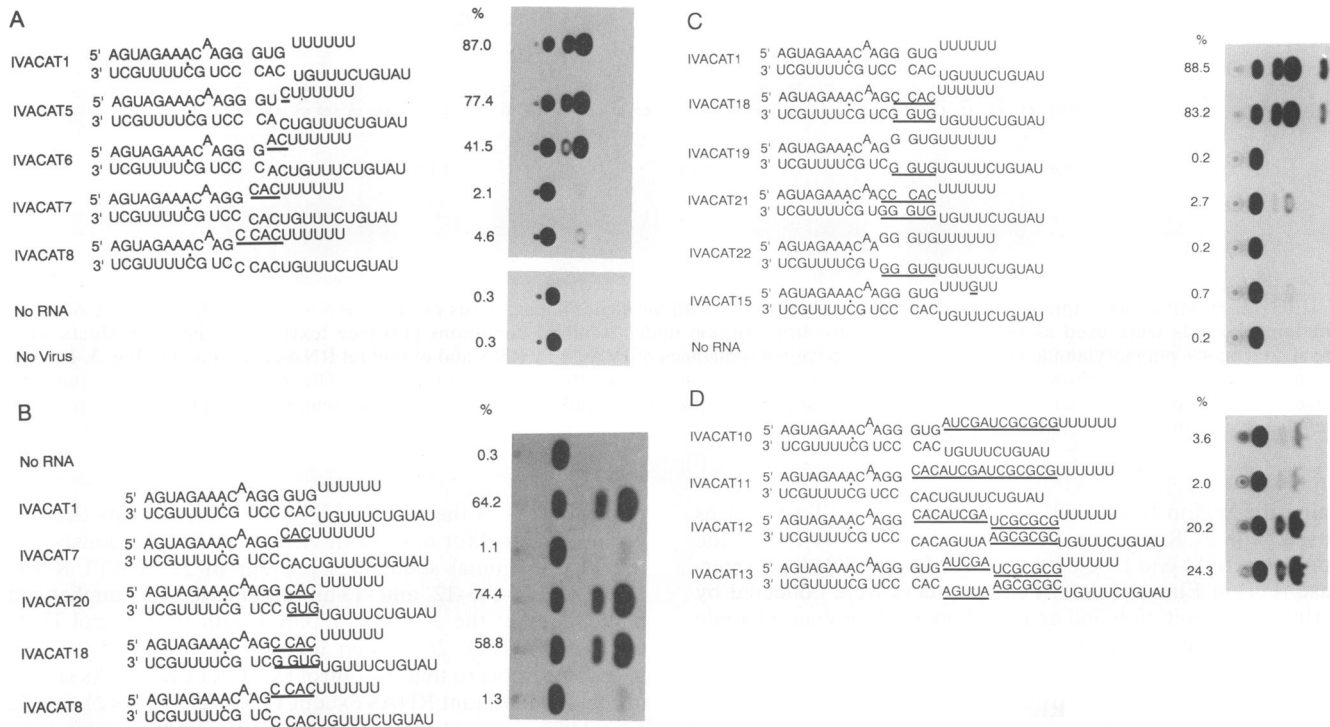
FIG. 3. CAT expression levels of IVACAT1 and mutant RNAs following RNP transfection. IVACAT1 RNA and mutant RNAs were obtained from appropriate plasmids by T7 polymerase transcription. RNAs were then RNP-transfected into helper virus-infected cells, and the level of CAT activity was determined as described previously (11). The terminal sequences are indicated and shown in the form of a postulated panhandle by the folding program of Zuker and Stiegler (23). The A-C mismatch of the panhandle in position 8 from the ends is indicated by a dot. The bulged A in position 10 at the 5' end is highlighted. Positions in the RNA mutants which differ from the sequence of IVACAT1 RNA are underlined. Percentage values indicate the percent conversion of chloramphenicol to its acetylated products. Within each panel (A through D), experiments were all done at the same time with the same batch of cells and the same preparation of purified influenza virus proteins. Also, CAT assays for the samples shown in each panel were all done at the same time. The RNAs used for RNP transfection are indicated.

hybridized to RNA from RNP-transfected cells, and the level of CAT-specific cRNA and mRNA could be detected. Opening up of the panhandle (IVACAT19) or disruption of the uridine stretch (IVACAT15) (see below) dramatically reduced the level of mRNA but not that of CAT-specific cRNA molecules. This experiment suggests that the level of CAT activity adequately correlates with the level of mRNA molecules in RNP-transfected cells.

Is the panhandle structure sequence specific? The question then arises whether the panhandle structure is sequence specific or whether a dsRNA structure formed by different sequences would be biologically functional. A plasmid, pIVACAT20, was constructed from which, by using T7 polymerase, an RNA was transcribed which was identical to IVACAT7 RNA except for changes in the 3' nonconserved region. These compensatory mutations in the 3' end of IVACAT20 would restore a panhandle structure but with sequences which are different from those in IVACAT1 RNA. After RNP transfection, this RNA gave a CAT signal (74.4% conversion) similar to that obtained with IVACAT1 (64.2% conversion) (Fig. 3B). We introduced compensatory mutations into construct pIVACAT8, which had 4 bp open at the panhandle, giving pIVACAT18. Again, an RNA was generated that allowed formation of a panhandle structure through sequences that have not been shown to exist at the termini of any influenza virus RNA segment. After RNP transfection, this RNA expressed a CAT activity level

(58.8% conversion) similar to that of wild-type IVACAT1 RNA (64.2% conversion) (Fig. 3B). These results suggest that the presence of a panhandle structure rather than specific sequences in these nucleotide positions is important for CAT expression.

Several control experiments were performed. Opening of the panhandle in IVACAT8 RNA created a sequence at the 5' end not found in influenza virus RNAs. We thus reintroduced the "correct" 5' end in IVACAT19 and had the same nucleotides (GGUG) at the 3' end, thereby again creating a disrupted panhandle structure. As expected, IVACAT19 RNA, in contrast to IVACAT18 RNA, showed a low CAT activity level (0.2% conversion). RNAs IVACAT21 and IVACAT22 extended the structural analysis. Both RNAs have changes in the 3'-terminal region, and although IVACAT21 RNA could retain a perfect panhandle structure, both constructs showed a low level of CAT activity (2.7 and 0.2% conversion, respectively). Based on this result and the finding of the in vitro transcription experiment (Fig. 2B), we suggest that nucleotide 11 at the 3' end delineates the promoter signal for transcription and that changes in that position eliminate recognition by the polymerase.
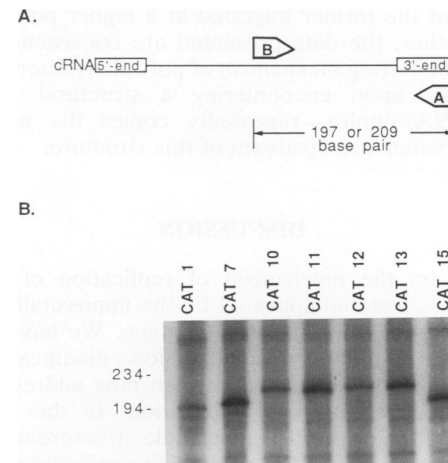
Polyadenylation signal of influenza virus RNA involves a uridine-rich region followed by an RNA duplex structure. Sequence comparison of influenza A virus RNA segments reveals that in each segment a stretch of five to seven uridines, starting 15 to 17 nucleotides from the 5' end, is

A.

B.
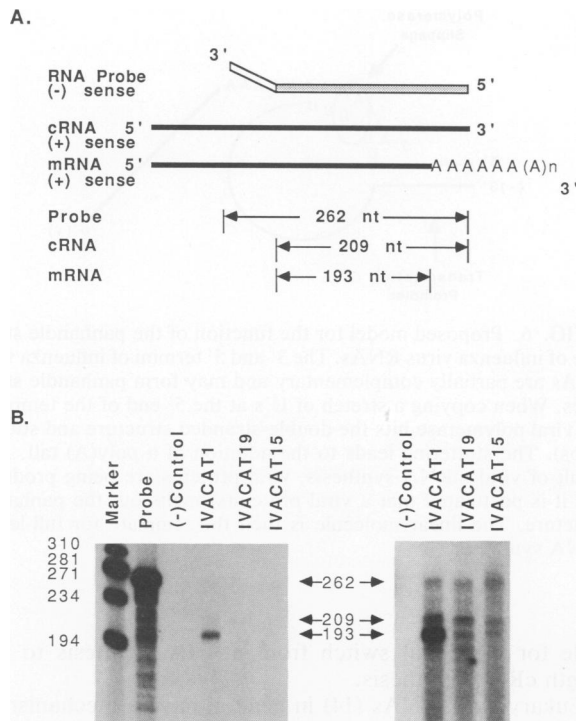
FIG. 4. Demonstration of CAT-specific mRNA in RNP-transfected cells. (A) Diagram illustrating the ribonuclease protection assay. The minus-sense RNA probe was derived from pCAT1-BalI/SalI by runoff transcription using T7 RNA polymerase and SalI-digested plasmid DNA. The open box represents the 53 extra nucleotides (nt) in the minus-sense RNA probe. Hybridization and RNA digestion using the 262-nt-long probe and cRNA and mRNA yield products of 209 and 193 nt, respectively. (B) Analysis of CAT-specific mRNA and cRNA molecules in RNP-transfected cells. The RNase protection assay was performed as described in Materials and Methods. Marker represents DNA markers which migrate 5% faster than corresponding RNA molecules (20). Probe represents the 262-nt-long minus-sense RNA used in the experiment. IVACAT1, IVACAT19, and IVACAT15 are RNA samples from RNP-transfected cells. The right portion of the panel represents the same lanes as the left portion, but with a fourfold-longer exposure (6 days). Arrows indicate positions of probes protected by cRNA or mRNA. The lower cRNA level in the IVACAT15 sample may reflect partial digestion of the hybrid by RNase due to a single mismatch between the probe and the cRNA which carries a U-to-G change in the U stretch of the poly(A) signal. Control represents probe treated with RNase only.

A.

B.

FIG. 5. Detection of cRNA in MDBK cells RNP-transfected with wild-type RNA and mutant RNAs. (A) PCR strategy used for detection. The cRNA was first copied by primer A, and the cDNA was then used as the template for PCR with both primers A and B (see Materials and Methods). (B) Analysis of PCR products on a denaturing 8% polyacrylamide gel. The expected lengths of PCR products are 197 and 209 bp, respectively, for RNP-transfected IVACAT1, -7, and -15 samples and IVACAT10, -11, -12, and -13 samples.

contiguous to the residues which could form the duplex structure of the panhandle. It was previously shown that these uridines are the site of poly(A) addition of influenza virus mRNAs (17). We were able to confirm this result by changing the poly(U) region in our model RNAs. IVACAT15 RNA has one U replaced with a G in the poly(U) stretch, and this RNA in an RNP transfection assay showed a low CAT signal (0.7% conversion) (Fig. 3C). Since other stretches of five or six uridines are present throughout the genome of influenza virus, we suggest that the viral signal for poly(A) addition consists of a uridine stretch juxtaposed to an RNA duplex structure. We have also made a construct in which the 6 U's in pIVACAT1 were replaced by 6 A residues (5). Also, this CAT construct did not give rise to signficant levels of CAT activity.

In order to provide further support for our hypothesis, we designed a series of mutants in which the six uridine residues were moved away from the panhandle-forming sequence. First, a stretch of 12 nucleotides was placed between the 5' nonconserved panhandle-forming nucleotides and the uridine stretch (pIVACAT10). A second similar plasmid was constructed in which, in addition, the three 5' nonconserved panhandle-forming nucleotides were mutated (pIVACAT11). As expected, neither RNA produced signficant CAT signals (less than 5% conversion) after RNP transfection (Fig. 3D). We then created mutants in which a new potentially base-paired structure downstream of the displaced uridine stretch was introduced. This was achieved by insertion of complementary sequences at the 3' end, forming a potential GC clamp adjacent to the poly(U) stretch (Fig. 3D). After RNP transfection, these mutant RNAs (IVACAT12 and IVACAT13) generated significantly higher CAT levels (20.2 and 24.3% conversion, respectively) than their counterparts lacking the clamp (IVACAT11 and IVACAT10, respectively), suggesting that the restored structure is responsible for this effect. Although the levels of CAT activity are not as high as for IVACAT1 RNA, this experiment again suggests that a dsRNA structure downstream of a poly(U) stretch allows the formation of functional mRNAs.

An additional control experiment was done to demonstrate that replication of full-length RNA is not eliminated by mutations which interfere with mRNA expression. Using PCR, we attempted to identify the presence of full-length cRNA in CAT RNP-transfected cells. The first primer was complementary to the 3' end of full-length plus-sense RNA, and the second primer was complementary to an internal site in the minus-sense CAT RNA. A positive PCR signal would thus suggest the presence of full-length complementary RNA in RNP-transfected cells. As shown in Fig. 5, a PCR signal was observed in IVACAT1, -7, -10, -11, -12, -13 and -15 RNP-transfected cells. Since the 3' ends of the cRNAs of IVACAT10,-11,-12, and -13 are 12 nucleotides longer than those of IVACAT1 and of the other mutant RNAs, the PCR

products of the former migrated at a higher position (Fig. 5B). Together, the data presented are consistent with the proposed stuttering mechanism of polyadenylation; the viral polymerase, upon encountering a structural barrier—a strong RNA duplex—repeatedly copies the nucleotides (uridines) which are upstream of this structure.

DISCUSSION

Studies on the mechanism of replication of influenza viruses have been complicated by the impossibility of site-specifically modifying the viral genome. We have recently been able to develop a system that allows modification of the influenza virus genome, and we can now address specific questions concerning viral replication. In this study we investigated the role of the panhandle structure of genomic RNA. A stretch of five to seven uridine residues starting 15 to 17 nucleotides from the 5' end of vRNA has been shown by Robertson et al. (17) to be the site of polyadenylation during transcription. Polyadenylation was thought to be the result of "stuttering" by the viral polymerase at these uridine residues (4). Since this stretch of uridines is directly adjacent to the panhandle in all RNA segments, the base-paired RNA has been suggested to be the cause of the stuttering (4, 7). However, no direct experimental evidence could be provided for this mechanism.

For the present study, we constructed a series of mutant RNAs which contain the reporter CAT gene flanked by the terminal noncoding sequences of genomic RNA segment 8, which encodes the NS proteins of influenza virus. By RNP transfection and determination of the levels of CAT expression we obtained data that strongly support the role of the panhandle structure in mRNA synthesis. Although we have not shown it for all constructs, the level of CAT activity correlates well with the level of CAT-specific mRNA levels in different RNP-transfected cells. We thus suggest that the level of CAT activity is an adequate test for determining poly(A)-containing mRNA levels. It should be noted, however, that we have not made attempts to measure the stability or the translatability of CAT-specific mRNA molecules.

Mutations that open up the panhandle structure in different CAT constructs increasingly diminished the expression of the reporter gene. Compensatory mutations which restored the panhandle structure but not the sequences resulted in mutants with wild-type expression levels. Placing the stretch of six uridines away from the panhandle or replacing it with six adenosine residues also reduced CAT expression levels. More signficantly, by providing a new base-pairing structure immediately downstream of the displaced uridine stretch, the expression of the reporter gene could be greatly enhanced compared with that of the control. Finally, we demonstrated that a single mutation in the uridine stretch dramatically reduced the CAT signal. Based on these experiments, our speculation is that the RNA duplex structure serves as a barrier for the viral polymerase and causes it to stutter (slip) at the uridine residues, leading to the addition of adenylates. We therefore suggest that one of the functions of the panhandle (made up of the 5' and 3' termini) is to provide a structural feature required for mRNA transcription (Fig. 6). We further speculate that a viral protein(s), possibly the nucleoprotein, accumulates in the cell as a result of mRNA transcription and that this protein(s) melts out the panhandle structure, resulting in a linear template. This accumulation of protein could thus be respon-
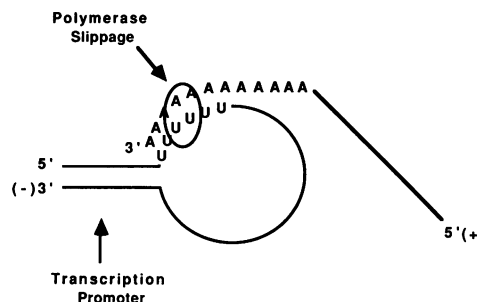

FIG. 6. Proposed model for the function of the panhandle structure of influenza virus RNAs. The 3' and 5' termini of influenza virus RNAs are partially complementary and may form panhandle structures. When copying a stretch of U's at the 5' end of the template, the viral polymerase hits the double-stranded structure and stutters (slips). The stuttering leads to the addition of a poly(A) tail. As a result of viral mRNA synthesis, viral proteins are being produced and it is postulated that a viral protein(s) melts out the panhandle structure. The linear molecule is then the template for full-length cRNA synthesis.

sible for a gradual switch from mRNA synthesis to full-length cRNA synthesis.

Eukaryotic mRNAs (14) in general have a mechanism of poly(A) addition that involves processing of RNA, and several models have been proposed for the formation of mRNAs by nonsegmented negative-strand RNA viruses (3, 19). Although we favor a slippage model for the influenza virus polymerase, our results do not exclude the possibility that the mRNA transcripts are actually cleaved at or near the poly(U) stretch and then polyadenylated. Another uncertainty of our model concerns the presence of the panhandle during initiation of transcription. We suggest that the polymerase stops (slips) at the barrier and adds poly(A) at that site. However, we do not understand how the polymerase "melts" this barrier at the start of transcription when it copies the 3' end of the vRNA.

We suggest that the physical structure which causes the viral polymerase to reiterate on its template is located at the panhandle. Whether this RNA duplex by ityself or any protein(s) specifically binding to an RNA duplex is responsible for this barrier remains to be investigated. From the in vitro and in vivo data, we can conclude that the nonconserved panhandle-forming nucleotides are not part of any transcription or replication promoter sequence. (This is not surprising, since by implication these nucleotides are different for each genome segment and all eight viral RNAs can replicate.) These nucleotides can even be removed as long as a compensatory RNA duplex, juxtaposed to the uridine stretch, is introduced. It thus seems likely that replication of RNA does not require the presence of a panhandle structure. This is also supported by the data involving PCR detection, which suggest that full-length cRNA molecules are present when mutant RNAs lacking a dsRNA structure adjacent to the U region are RNP transfected. It should be noted, however, that the PCR technique as used here is not quantitative and that we have not determined the level of full-length cRNA synthesis in these RNP-transfected cells. In other words, the promoter on the cRNAs, like its counterpart on vRNAs, is probably not dependent on the presence of the panhandle and synthesis of full-length RNAs most likely takes place from templates that are linear (not in panhandle formation).

Another result of our experiments concerns the length of the promoter for transcription. Based on the in vitro and in vivo experiments with IVACAT21 and IVACAT22 RNAs, we suggest that the first 11 3'-terminal nucleotides make up the promoter sequence. Apparently not all the conserved sequences at the termini are required for the promoter signals. A precise mutational analysis of the promoter region is being done at this time to shed further light on this question.

REFERENCES

1. Desselberger, U., V. R. Racaniello, J. J. Zazra, and P. Palese. 1980. The 3' and 5' end terminal sequences of influenza A, B, and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 8:315–328.
2. Enami, M., W. Luytjes, M. Krystal, and P. Palese. 1990. Introduction of site-specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA 87:3802–3805.
3. Gupta, K. C., and D. W. Kingsbury. 1982. Conserved polyadenylation signals in two negative-strand RNA virus families. Virology 12:951–961.
4. Hay, A. J., G. Abraham, J. J. Skehel, J. C. Smith, and P. Fellner. 1977. Influenza virus messenger RNAs are incomplete transcripts of the genome RNA. Nucleic Acids Res. 4:4197–4209.
5. Honda, A., P. Palese, and R. Schneider. Unpublished data.
6. Honda, A., K. Ueda, K. Nagata, and A. Ishihama. 1988. RNA polymerase of influenza virus: role of NP on RNA chain elongation. J. Biochem. 104:1021–1026.
7. Hsu, M.-T., J. D. Parvin, S. Gupta, M. Krystal, and P. Palese. 1987. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc. Natl. Acad. Sci. USA 84:8140–8144.
8. Huang, T.-S., P. Palese, and M. Krystal. 1990. Determination of influenza virus proteins required for genome replication. J.

Virol. 64:5669–5673.
9. Krug, R. M., F. V. Alonso-Caplen, I. Julkunen, and M. G. Katze. 1989. Expression and replication of the influenza virus genome, p. 89–142. In R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
10. Luo, G., M. Chao, S.-Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor. 1990. A specific base transition occurs on replicating hepatitis delta virus RNA. J. Virol. 64:1021–1027.
11. Luytjes, W., M. Krystal, M. Enami, J. D. Parvin, and P. Palese. 1989. Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59:1107–1113.
12. Palese, P. 1977. The genes of influenza virus. Cell 10:1–10.
13. Parvin, J. D., P. Palese, A. Honda, A. Ishihama, and M. Krystal. 1989. Promoter analysis of the influenza virus RNA polymerase. J. Virol. 63:5142–5152.
14. Proudfoot, N. J., and E. Whitelow. 1988. Termination and 3' end processing of eukaryotic RNA, p. 97–129. In D. M. Glover and B. D. Hames (ed.), Frontiers in molecular biology—transcription and splicing. IRL Press, Oxford.
15. Ritchey, M. B., P. Palese, and E. D. Kilbourne. 1976. The RNAs of influenza A, B, and C viruses. J. Virol. 18:736–744.
16. Robertson, J. S. 1979. 5' and 3' terminal nucleotide sequences of the RNA genome segments of influenza virus. Nucleic Acids Res. 6:3745–3757.
17. Robertson, J. S., M. Schubert, and R. A. Lazzarini. 1981. Polyadenylation sites for influenza virus mRNA. J. Virol. 38:157–163.
18. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. Schubert, M., J. D. Herman, and R. A. Lazzarini. 1980. Site on the vesicular stomatitis virus genome specifying polyadenylation and the end of the L gene mRNA. J. Virol. 34:550–559.
20. Sharmeen, L., and J. Taylor. 1987. Enzymatic synthesis of RNA oligonucleotides. Nucleic Acids Res. 15:6705–6711.
21. Skehel, J. J., and A. J. Hay. 1978. Nucleotide sequence of the 5' termini of influenza virus RNAs and their transcripts. Nucleic Acids Res. 5:1207–1219.
22. Sugiura, A., K. Tobita, and E. D. Kilbourne. 1972. Isolation and preliminary characterization of temperature-sensitive mutants of influenza virus. J. Virol. 10:639–647.
23. Zuker, M., and P. Stiegler. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. 9:133–148.