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Direct Evidence that the Poly(A) Tail of Influenza A Virus mRNA Is Synthesized by Reiterative Copying of a U Track in the Virion RNA Template

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The poly(A) tail of influenza virus mRNA is thought to be synthesized by reiterative copying of the U track near the 5′ end of the virion RNA template. This has been widely accepted as a plausible hypothesis, but until now there has been no direct experimental evidence for it. Here, we report such direct evidence based on the fact that (i) replacing the U track with an A track directs synthesis of products with poly(U) tails, both in vitro and in vivo, and (ii) interrupting the U track abolishes polyadenylation in vitro.

The influenza A virus contains eight segments of single-stranded RNA of negative polarity (7). Virion RNAs (vRNAs) are templates for the synthesis of both cRNA and mRNA. cRNA synthesis is initiated by primer-independent transcription, giving rise to a complete copy of vRNA (3, 4). In contrast, mRNA transcription is initiated by a capped RNA primer, derived from host mRNA by the influenza virus polymerase complex (9). Transcription of mRNA is terminated at a track of five to seven U residues near the 5′ end of the vRNA, where polyadenylation occurs (13). Instead of transcribing the 5′ end of the vRNA, the RNA polymerase, it has been suggested, pauses on this U track and reiteratively copies it (14).

Initially, a base-paired panhandle structure (1) was proposed as the key element for the pausing of the RNA polymerase prior to polyadenylation (13). Early in vivo work supported this idea by showing that the proposed panhandle structure is essential for gene expression (6). However, the discovery of a strong polymerase binding site at the 5′ end of the vRNA suggested another model for polyadenylation (2, 17). In this polyadenylation model, it is proposed that the RNA polymerase remains bound to the 5′ end of the vRNA throughout transcription. Inevitably, at the end of transcription, the RNA polymerase cannot transcribe through the site to which it is bound. As a result, the RNA polymerase pauses at the U track and polyadenylates the mRNA. Results from recent in vitro polyadenylation studies support this newer model (10–12).

The reiterative copying model of Robertson and colleagues (14) explained how a short U track could give rise to an mRNA with a long poly(A) tail. This hypothesis became widely accepted as a plausible model for the polyadenylation of influenza virus mRNA and mRNAs synthesized by other negative-stranded viruses, such as vesicular stomatitis virus (15). Unfortunately, there is no direct experimental evidence yet available to show definitively that the U track is the template for poly(A) synthesis. In particular, a model in which the U track participates indirectly in polyadenylation has not been excluded. Thus, the U track might act as a pausing signal for transcription, allowing the influenza virus RNA polymerase to become a template-independent poly(A) polymerase. Previously, the U track was shown to be important for the expression of a model chloramphenicol acetyltransferase (CAT) reporter gene (5, 6), suggesting that the U track is involved in polyadenylation. These in vivo studies, however, could not determine whether the U track was the direct template for reiterative copying or was acting indirectly as a signal in stimulating the RNA polymerase to perform a template-independent polyadenylation. Therefore, the precise role of the U track in polyadenylation remains to be demonstrated.

Here, we investigate the precise function of the U track of the vRNA in polyadenylation. A T7 RNA polymerase-transcribed short vRNA-like template, with either the wild-type U track (Fig. 1) or a mutated U track (see below), was tested in the recently developed in vitro polyadenylation assay (11). Unless otherwise stated, about 1 μg of vRNA-like templates was transcribed by micrococcal nuclease-treated RNA polymerase (16) in 5-μl reaction mixtures containing 500 μM UTP, 500 μM CTP, 500 μM GTP, 25 μM ATP, 2 μCi of [α-^{32}P]ATP (3,000 Ci/mmol), 0.5 mM adenylyl (3′→5′) guanosine, 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 10 U of placental RNase inhibitor. After incubation at 30°C for 3 h, transcription products were analyzed on a 16% polyacrylamide gel in 7 M urea.

First, we tested whether the RNA polymerase uses the U track of the vRNA as a template for synthesizing a poly(A) tail, since the addition of a poly(A) tail could, in theory (see above), be due to a nontemplated polyadenylation activity of the RNA polymerase. If polyadenylation occurs by reiterative copying of the U track, replacing the U_6 track with an A_6 track might result in the synthesis of transcription products with poly(U) tails. As shown in Fig. 2A, lane 1, the transcription products from the wild-type template (U_6), labelled with [α-^{32}P]ATP, run as a high-molecular-weight polyadenylated mRNA smear and a major cRNA band as described previously (10, 11). However, when the mutant A_6 template was tested in a transcription reaction which contained [α-^{32}P]ATP, only the major cRNA band was observed (Fig. 2A, lane 2). This suggests that the mutant A_6 template failed to produce polyadenylated mRNA. However, transcription products with poly(U) tails, unlike polyadenylated mRNA, would incorporate only a limited number of [α-^{32}P] ATP residues. Therefore, transcription products with poly(U) tails might not easily be detected with [α-^{32}P]ATP. When [α-^{32}P]UTP was used as a substrate instead, a high-molecular-weight smear from the mutant A_6 template was then clearly observed (Fig. 2A, lane 4), suggesting that poly(U)-tailed transcripts were synthesized. By contrast,

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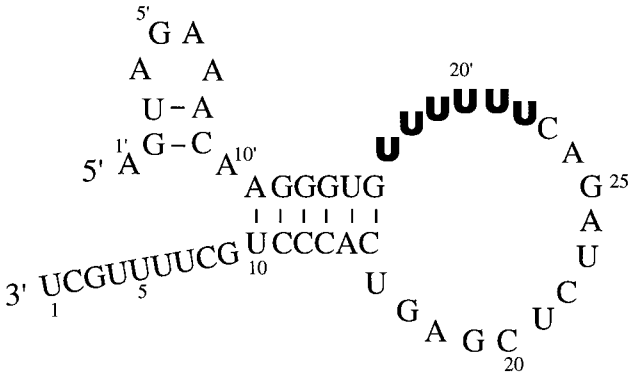

FIG. 1. vRNA-like template with the wild-type conserved terminal sequences used in the in vitro influenza virus transcription reactions. The Watson-Crick base pairs in the RNA hook model (12) derived from an earlier RNA fork model (2) are shown. The nucleotide numbers starting at the 5′ end are indicated by a prime to distinguish them from nucleotide numbers starting at the 3′ end. The U_6 polyadenylation site is shown in bold. (Modified from reference 12 with permission.)

the high-molecular-weight product derived from the wild-type (U_6) template was not detected when [α-^{32}P]UTP was used (Fig. 2A, lane 3). These experiments showed that poly(U)-tailed transcripts were specifically synthesized from the mutant A_6 template. The relative yields of mRNA to cRNA band from the wild-type template (Fig. 2A, lane 1) and the mutant A_6 template (Fig. 2A, lane 4) were 3 and 2.4%, respectively (mean of two experiments, as determined by PhosphorImager analysis). This suggested that the mutant A_6 template is almost as efficient as the wild-type template for the synthesis of homopolymeric-tailed products.

To confirm that the high-molecular-weight smear from the mutant A_6 template (Fig. 2A, lane 4) was poly(U)-tailed RNA, RNA products isolated from the smear were cloned and three clones were sequenced. The clones were found to contain poly(U) sequences of up to 97 nucleotides (Fig. 2B; a clone with 48 U residues). In all sequenced clones, the poly(U) tail started at the expected position, residue 28 (Fig. 1). These results showed that the identity of the homopolymeric tail [poly(A) or poly(U)] was dependent on the sequence of the homopolymeric track (U_6 or A_6) of the vRNA template. Obviously, these studies indicate that the U track of the vRNA acts directly as a template for the RNA polymerase and exclude the hypothesis that polyadenylation is a template-independent process. Furthermore, since reiterative copying still occurred, even when the U track was mutated to an A track, it follows that the sequence of the U track itself is not important for pausing the RNA polymerase in polyadenylation. Attempts at synthesizing transcription products with poly(C) or poly(G) tails were unsuccessful (data not shown), suggesting that a G or C track was not a favorable template for reiterative copying. However, we cannot exclude the possibility that trace amounts of transcription products were synthesized which were below our limit of detection.

How does a short homopolymeric track of U or A residues serve as a template for a long homopolymeric tail? Inevitably, a polymerase slippage mechanism has to occur, as previously proposed (14). In polymerase slippage, the RNA polymerase reiteratively transcribes a short homopolymeric track from the template, presumably by repeated cycles of melting the RNA-RNA hybrid, backward slippage of the nascent mRNA strand relative to the vRNA template, reannealing, and subsequent elongation. If there is such a mechanism, then interrupting the

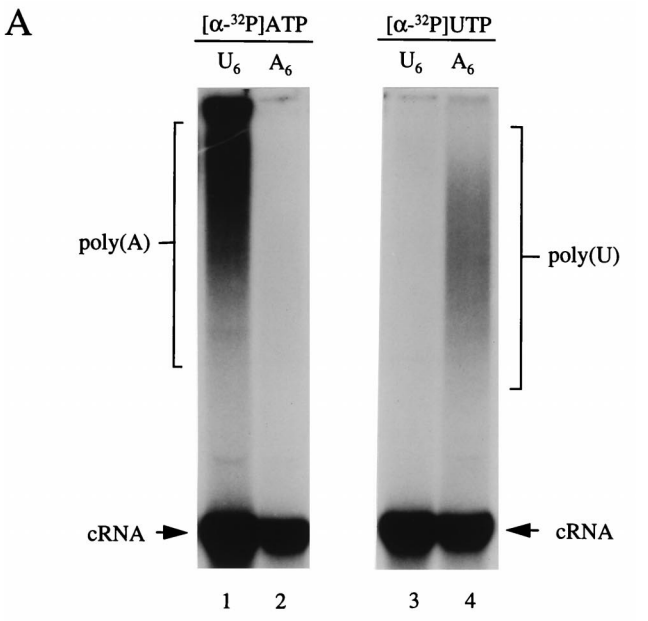

FIG. 2. Mutation of the U_6 track to an A_6 track of a vRNA-like template. (A) Wild-type vRNA (U_6 [lanes 1 and 3]) and mutated RNA (A_6 [lanes 2 and 4]) templates are tested in transcription reactions in the presence of [α-^{32}P]ATP (lanes 1 and 2) or [α-^{32}P]UTP (lanes 3 and 4). The cRNA, the polyadenylated mRNA, and the polyuridylated products are indicated. The signal at the origin is thought to be due to transcription products derived from residual endogenous vRNA. When [α-^{32}P]UTP was used in the transcription reaction, the concentration of ATP was increased to 500 μM and the concentration of UTP was reduced to 25 μM. The mobility of the high-molecular-weight smear varies in different gels (lanes 1 and 4). (B) The poly(U) sequence (n = 48) of a cloned RT-PCR product which is derived from the high-molecular-weight transcription product of the A_6 mutant (panel A, lane 4). The high-molecular-weight transcription product was eluted from the polyacrylamide gel, reverse transcribed, and amplified by PCR as described previously (11), except that the 5′ GC-clamped T_{20} primer was replaced by a GC-clamped A_{20} primer (5′-GCCCCGGGATCCA$_{20}$-3′).

U track should inhibit polyadenylation by hindering or preventing the realignment of mRNA and vRNA template necessary for reiterative copying. To test this hypothesis, the U track was interrupted by inserting a nucleotide (G, A, or C) in the middle of the U track or by mutating individual nucleotides (U→A) within the U track. As shown in Fig. 3, either inserting a nucleotide (Fig. 3A) or mutating individual nucleotides (Fig. 3B) within the U track severely inhibited the polyadenylation activities of all mutants (Fig. 3A, lanes 2 to 4; Fig. 3B, lanes 2 to 7). The inhibition of polyadenylation by such mutations can best be understood if one considers a particular example. Thus, the realignment of a mutated template (e.g., UUUAUUU) with its product (AAAUAAA) to form an RNA duplex would

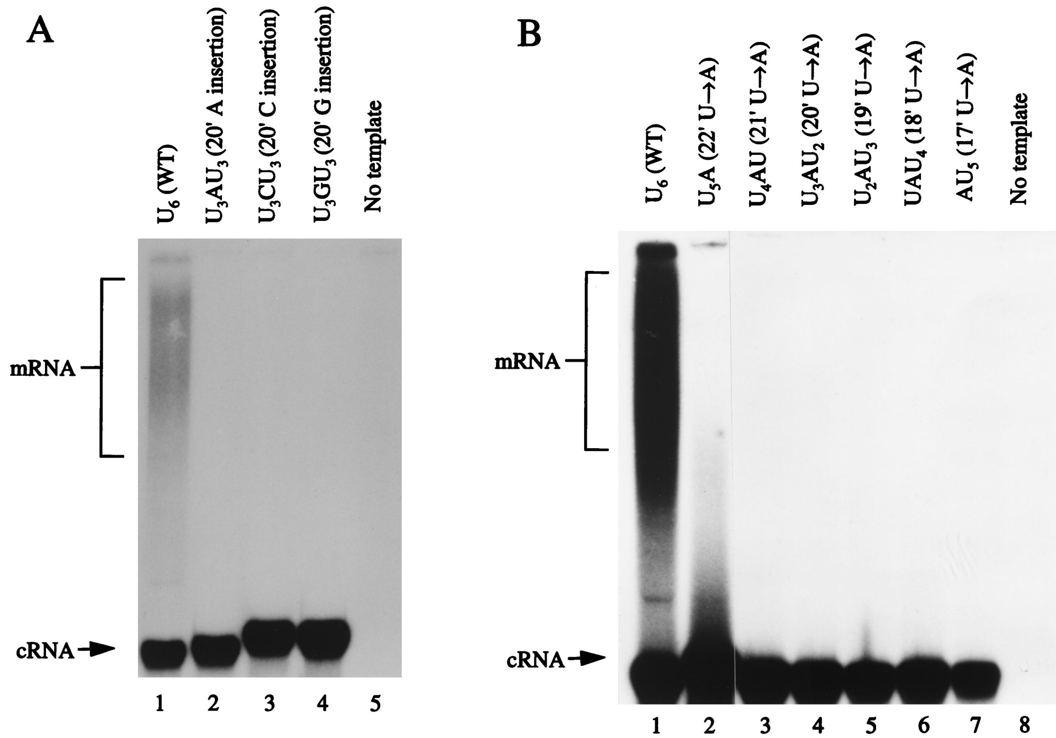

FIG. 3. Effect of interrupting the U track of the vRNA on polyadenylation activity in vitro. (A) The U$_6$ track is interrupted by inserting a nucleotide (A, C, or G) in the middle. Lane 1, wild-type (WT) RNA (U$_6$); lanes 2 to 4, RNA mutants; lane 5, no template. (B) RNA templates with a single point mutation (U→A) within the U$_6$ track. Lane 1, wild-type (WT) RNA; lanes 2 to 7, point mutants; lane 8, no template. For the numbering scheme, see Fig. 1.

not be favored, because the RNA duplex would be thermodynamically unstable due to A:A and U:U mismatches. Our results (Fig. 3) are consistent with previous in vivo findings that vRNA templates with interrupted U tracks showed a dramatic reduction in gene expression (6). Among all mutants tested (Fig. 3B), only the mutant U$_5$A template which contains five consecutive uridine residues showed some residual activity (<10%) in polyadenylation. This finding agrees with previous data that the minimum length of the U track for gene expression is five (5). The lack of mRNA production from the mutant AU$_5$ template (Fig. 3B, lane 7), which also contains five consecutive uridine residues, may be due to the U track of this template starting at position 18′ instead of position 17′. This also agrees with a previous finding that the position of the U track relative to the 5′ end of the vRNA is important for polyadenylation (5).

The above experiments showed that the RNA polymerase uses the homopolymeric track (U$_6$ or A$_6$) as the template for the synthesis of the homopolymeric tail [poly(A) or poly(U)] in vitro. To validate our findings in vivo, a plasmid-based reverse genetics system for influenza virus was employed (8). A mutated version of a CAT vRNA expression plasmid, pPOLI-CAT-RT (8), was transfected into human 293 kidney cells to synthesize a model vRNA template containing a CAT gene in negative orientation, flanked by the 3′ and 5′ noncoding regions of segment 8 of influenza virus A/WSN/33. In the 5′ noncoding region of this mutated vRNA template, the U$_6$ track was mutated into an A$_6$ track. Four protein expression plas-

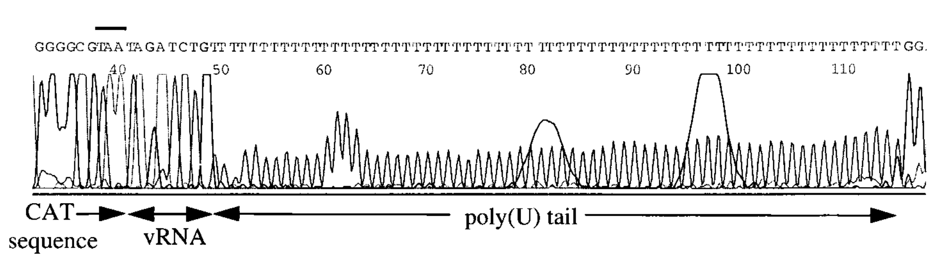

FIG. 4. The poly(U) sequence of a cloned RT-PCR product derived from the poly(U)-tailed CAT mRNA (n = 67). The CAT sequence, vRNA, and poly(U) tail are indicated, with a line drawn over the TAA translation terminator of CAT. Two broad artifact dye blobs centered on residues 82 and 97, respectively, overlap part of the poly(U) sequence. Plasmids pGT-h-PB1, pGT-h-PB2, pGT-h-PA, and pGT-h-NP, which express the PB1, PB2, PA, and NP proteins, respectively, under the control of the adenovirus 2 major late promoter, and pPOLI-CAT-RT (see the text) were generously supplied by Peter Palese. One microgram of each of the pGT-h-PB1, pGT-h-PB2, pGT-h-PA, and pGT-h-NP plasmids and the mutated pPOLI-CAT-RT plasmids were transfected into 293 cells in 30-mm-diameter dishes with 25 μl of DOTAP transfection reagent (Boehringer Mannheim). At 36 h posttransfection, RNA was isolated with TRIZOL reagent (Life Technologies). RNA (3 μg) was reverse transcribed, amplified by PCR, and cloned as described previously (11), except that the 5′ GC-clamped T$_{20}$ primer was replaced by a GC-clamped A$_{20}$ primer.

mids, which encode PB1, PB2, PA, and NP, were also cotransfected into the cell for the replication and transcription of the model CAT vRNA template. If poly(A) tails of influenza virus mRNA are synthesized by reiterative copying of the U track in vivo, a U_6 to A_6 mutation in the model vRNA template should result in the synthesis of poly(U)-tailed CAT transcripts. To detect the presence of poly(U)-tailed CAT transcripts in the 293 cells, total RNA was harvested at 36 h posttransfection, reverse transcribed, and amplified by PCR. The reverse transcriptase (RT) PCR products were cloned, and eight clones were sequenced. The clones contained poly(U) sequences of up to 73 nucleotides (Fig. 4; a clone with 67 U residues). These in vivo results thus confirmed the in vitro finding that the A track of the vRNA is a template for the synthesis of poly(U)-tailed mRNA. It is not known, however, whether this poly(U)-tailed mRNA has different properties (e.g., the mRNA stability and efficiency of translation) compared to a poly(A)-tailed mRNA. Further characterization of this novel form of mRNA is in progress.

In summary, we provide the first direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of the U track in the vRNA template. Mutating the U track of a vRNA-like template into an A track resulted in the synthesis of transcription products with poly(U) tails, both in vitro and in vivo. In addition, we also showed that vRNA templates with disrupted U tracks were not functional templates in polyadenylation, consistent with RNA polymerase slippage as the mechanism for poly(A) tail synthesis in influenza virus.

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