Selected host cell capped RNA fragments prime influenza viral RNA transcription *in vivo*

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ABSTRACT

Influenza viral RNA transcription *in vitro* is primed by capped RNA fragments cleaved from capped RNAs by a viral endonuclease. The present study was undertaken to determine whether the specificities of the viral endonuclease and transcriptase observed in *in vitro* studies are also observed in the infected cell. The NS (nonstructural) gene of influenza WSN virus was cloned in pBR322 by using a double-stranded DNA containing a cDNA copy of both virion RNA (vRNA) and *in vivo* viral mRNA. We determined the 5' terminal sequence of the particular NS viral mRNA molecule which was cloned and also the 5' terminal sequences of the entire population of *in vivo* NS viral mRNAs synthesized in two different cell lines. For the latter determination we used a restriction fragment from the cloned DNA for the reverse transcriptase-catalyzed extension of total *in vivo* viral mRNA. The results indicate that *in vivo* and *in vitro* viral RNA transcription are similar in two important respects: (i) transcription initiates not with an A residue directed by the 3' terminal U of the vRNA, but with a G residue directed by the 3' penultimate C of the vRNA; and (ii) capped RNA fragments containing a 3' terminal A residue are preferentially used as primers, thereby generating an AG sequence in the viral mRNA complementary to the 3' terminal UC of the vRNA. Actually, for *in vivo* transcription, a subset of A-terminated capped fragments, namely those containing a 3' penultimate C residue, are the preferred primers. The latter specificity had not been observed in previous *in vitro* studies.

INTRODUCTION

Influenza virus employs a unique mechanism for the initiation of the synthesis of its messenger RNA (mRNA). The basic mechanism was worked out in *in vitro* studies using the virion-associated transcriptase. It was shown that heterologous capped RNAs strongly stimulate viral RNA transcription *in vitro* (1,2,3). The capped RNAs are cleaved 10-13 nucleotides from their 5' ends by a virion-associated cap-dependent endonuclease, generating fragments with 3' terminal hydroxyl groups (4). Cleavage occurs preferentially, or almost exclusively, at purine residues, yielding A- and G-terminated capped fragments. Most of these capped RNA fragments were shown to be linked to one or more G residues in transcriptase reactions

containing labeled GTP as the only ribonucleoside triphosphate, indicating that they are most likely the actual primers initiating viral RNA transcription (4). The results suggested that this G incorporation is directed by the penultimate C residue at the 3' end of the eight virion RNA (vRNA) templates (4), all of which have the sequence 3' UCG (5). Consistent with this, a C residue was incorporated onto the primer fragments after the G. Because the 3' terminal U residue of the vRNA apparently does not direct the incorporation of an A residue, the only way that an A residue could appear in the viral mRNA opposite the 3' terminal U of the vRNA would be if the primer fragment used to initiate mRNA synthesis had an A residue at its 3' terminus. It was concluded that this is what in fact usually occurs, because primer fragments with a 3' terminal A residue were used more effectively for G incorporation than fragments with a 3' terminal G residue (4). Consequently, influenza viral mRNAs synthesized in vitro would contain an AGC sequence complementary to the 3' end of the vRNA.

The situation concerning in vivo viral RNA transcription, however, is much less clear. Priming by capped RNAs also occurs in vivo because the viral mRNAs synthesized in the infected cell contain 10-15 nucleotides at their 5' ends, including the cap, which are not viral-coded (6,7,8). This host-donated sequence is heterogeneous for most of its length (7,8), indicating that multiple different host capped RNAs almost certainly serve as primers. The question is how much heterogeneity occurs at the position opposite the 3' terminal U of the vRNA. A few of the viral mRNAs coding for various viral proteins apparently contain a base other than A at this position (7,8), indicating that transcription in vivo, like that in vitro, most probably initiates not with an A residue directed by the 3' terminal U of the vRNA template, but rather with a G residue directed by the 3' penultimate C of the vRNA. One report (8) has claimed that the position in in vivo viral mRNA (coding for the viral membrane protein) opposite the 3' terminal U of the vRNA is completely heterogeneous, consistent with a complete lack of preference for the utilization of A-terminated host cell capped RNA fragments as primers in the infected cell. This would mean that in vivo the viral endonuclease cleaves capped host cell RNAs at both purines and pyrimidines and that all of the resulting fragments are used indiscriminately as primers by the viral transcriptase. In contrast, another study (19) has presented some evidence suggesting that the in vivo viral mRNA coding for the NS₂ viral protein contains predominantly an A at the position opposite the 3' terminal U of the vRNA.

It is important to resolve this issue in order to determine whether the specificities of the viral endonuclease and transcriptase are the same in vitro and in vivo. Further, if an A residue were predominantly found at the position opposite the 3' terminal U of the vRNA, then it would be conceivable that the same initiation step could be used for the synthesis of both viral mRNA and the full-length transcripts that are the putative templates for vRNA synthesis. Here, we will show conclusively that there is a strong preference for A-terminated capped RNA fragments as primers in vivo as well in vitro. Further, we unexpectedly found that in vivo there is also a preference for the utilization of fragments containing a 3' penultimate C residue, a specificity which was not observed previously in in vitro studies.

MATERIALS AND METHODS

RNA Preparations. Virion RNA (vRNA) was extracted from purified influenza WSN virus as previously described (10,11). Viral mRNA synthesized in infected MDCK (canine kidney) or chicken embryo fibroblast (CEF) cells was isolated as described previously (10,11). Briefly, cycloheximide (100 µg/ml) was added to the infected cells at 2.5 hours after infection to increase the amount of viral mRNA produced, the cells were collected three hours later, and the cytoplasmic poly A(+) RNA was isolated by two successive chromatographies on oligo dT cellulose.

Cloning of the NS viral RNA segment. DNA complementary to the vRNA segments was synthesized using reverse transcriptase and a dodecanucleotide primer AGCAAAAGCAGG (Collaborative Research, Inc.) which is complementary to the 3' end of all the vRNA segments. Complementary DNA (cDNA) to poly A(+) RNA from the cytoplasm of infected MDCK cells (prepared as described above) was synthesized using oligo dT₁₂₋₁₈ as primer. The total cDNA of the two preparations was fractionated by electrophoresis on a 3% polyacrylamide gel containing 7 M urea. The cDNAs corresponding to the smallest genome segment (NS segment) were eluted, and the two complementary cDNAs were annealed to each other for 3 hours at 68 C in 0.3 M NaCl, 1 mM EDTA, 10 mM Hepes, pH 7.8. This strategy for obtaining viral double-stranded DNA has been employed by others (12,13,14). The double-stranded NS DNA was tailed with about 30 dC residues by incubation with calf thymus terminal deoxynucleotidyl transferase in the presence of 50 mM Hepes, pH 7.8, 1 mM MnCl₂, 100 µM dCTP, 1 mM DTT, 50 mM KCl, 100 µg/ml BSA. The plasmid pBR322 was digested with Pst I, and tailed with about 20-30 dG residues using

terminal transferase. The NS double-stranded DNA (tailed with C's) and the pBR322 (tailed with G's) were annealed to each other for 3 hours at 68 C, and after a two-fold dilution with water was used directly to transform *E. coli* HB 101. Transformants were selected for tetracycline resistance and ampicillin sensitivity, and were screened by the colony filter hybridization method (15) using ^{32}P -labeled cDNA (to vRNA) as probe. The transformant containing the largest insert at the Pst I site was shown to contain NS-specific sequences by a Southern blot (16) using ^{32}P -labeled NS cDNA as probe. DNA isolated from this clone (NS-10) was used for all the experiments described here.

Sequencing and primer extension. DNA sequence analysis was carried out using the Maxam and Gilbert method (17). A 18 base pair (bp), Hind III-Bam HI restriction fragment of the DNA from the NS-10 clone (see Figure 1) was used as primer for the reverse transcriptase-catalyzed extension of *in vivo* NS viral mRNA. After complete digestion of NS-10 DNA with Hind III followed by treatment with calf intestine alkaline phosphatase, the DNA fragments were labeled at their 5' ends with ^{32}P using T4 polynucleotide kinase, and were then cleaved with Bam HI. This digest was electrophoresed on a 10% polyacrylamide gel, and the 18 bp fragment was eluted and purified

Figure 1. Nucleotide sequence of the Taq I fragment of NS-10 DNA corresponding to the 5' end of the viral mRNA. Base pairs in boxes are those which differ from the published sequence of the NS gene of A/PR/8/32 (18). The 4 nucleotides designated N and underlined with dashes could not be determined unambiguously.

by DEAE-cellulose chromatography. This DNA fragment and the viral mRNA preparation were dissolved in 10 μ l of water, and were heated at 100 C for 2 minutes and fast-cooled in a dry ice-ethanol bath. After addition of deionized formamide to 80%, the mixture was hybridized at 42 C for 4 hours in 0.4 M NaCl, 2 mM EDTA, 10 mM Pipes, pH 6.4 in a final volume of 100 μ l. After four ethanol precipitations, the primer was extended for 60 minutes at 37 C with 80 units of reverse transcriptase in 50 mM Tris - HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT and 600 μ M of each of the deoxynucleoside triphosphates in a final volume of 400 μ l. The reaction was terminated by the addition of NaOH to 0.1 N, the mixture was incubated for 10 minutes at 68 C and then neutralized. DNA was isolated by phenol-chloroform extraction, Sephadex G-50 chromatography, and ethanol precipitation. After heating and fast-cooling, the extended primer was separated from the unextended primer by electrophoresis on a 10% polyacrylamide gel containing 9 M urea. The extended primer was eluted, chromatographed on DEAE-cellulose and sequenced.

RESULTS

Sequence of the cloned NS DNA corresponding to the 5' end of the viral mRNA. The estimated size of the influenza virus-specific Pst I insert of the NS-10 clone as determined by electrophoresis on a 4% acrylamide gel in comparison to Hae III restriction fragments of ϕ X174 DNA is 950 bp (data not shown). This is consistent with this insert being a full-length copy of the NS viral gene segment. The entire 5' end of the viral mRNA is certainly represented in the Pst I insert, as shown by the sequence of one of the three Taq I restriction fragments of the insert (Figure 1). This sequence indicates the existence of several restriction enzyme sites and hence several possible restriction fragments suitable for primer extension of NS viral mRNA. The 18 bp Hind III-Bam HI restriction fragment was chosen for primer extension.

It is of interest to note that in the viral-coded region this part of the NS sequence of the WSN influenza A strain differs in only 7 nucleotides (shown by boxes) from the sequence of the closely related virus strain A/PR/8/34 (18). If the same frequency of differences occurred in the rest of the NS gene, there would only be about a 4% difference in nucleotide sequence between the NS genes of WSN and PR8 virus. This would be the smallest difference in nucleotide sequence between NS genes of different influenza virus strains (18).

The NS-10 insert also contained additional nonviral nucleotides at its end (the end corresponding to the 5' end of the viral mRNA). These additional nucleotides almost certainly represent the host cell primer sequence found at the 5' end of one of the *in vivo* NS viral mRNA molecules. In the original DNA duplex inserted into pBR322 the nucleotides in the nonviral region were unpaired:

Therefore, it was necessary for bacterial DNA repair enzymes to fill in the second strand. If nucleotide X were a T (i.e., the viral mRNA had an A at this position), then the insert should have an A-T base pair at this position. If, on the other hand, nucleotide X were not a T (i.e., the viral mRNA had a base other than A at this position), then the insert could have a base pair other than A-T at this position. The fact that the insert contained a G-C base pair at this position (Figures 1 and 2) is thus evidence that the particular NS viral mRNA that was cloned contains a G at this position. This observation, also previously made by others (7,8), indicates that the base opposite the 3' terminal U of the vRNA is not viral coded but is supplied by the host cell RNA primer and that consequently viral RNA transcription *in vivo*, like that *in vitro*(4), initiates with a G residue directed by the 3' penultimate C of the vRNA. It must, however, be emphasized that this cloned DNA is derived from an individual molecule of NS viral mRNA and that the majority of the NS viral mRNA molecules could differ from the particular molecule that was cloned (see later). The length of the host primer sequence in the NS-10 clone is 12 nucleotides.

The 5' terminal sequences of the total population of NS viral mRNAs synthesized in the infected cell. During viral RNA transcription *in vitro*, A-terminated capped RNA fragments are preferentially used as primers, thereby generating an AGC sequence in the viral mRNA complementary to the 3' terminal UCG of the vRNA (4). These results lead to the prediction that when transcription occurs in the presence of a heterogeneous population of capped RNAs, as occurs *in vivo*, most of the viral mRNAs would again contain this AGC sequence. However, the previous report directly examining this region of *in vivo* viral mRNA presented data against this prediction (8).

To resolve this issue, the NS viral mRNAs synthesized in two different

Figure 2. Sequencing gel (8%) of the 5' terminal Hind III-Pst I fragment of NS-10 DNA. The Hind III fragments of NS-10 were labeled at their 5' ends as described in Materials and Methods and then digested with Pst I. The 90 bp fragment derived from the end corresponding to the 5' end of the viral mRNA was isolated by gel electrophoresis on a 10% gel and sequenced. The nucleotides are numbered such that 1 corresponds to the 3' terminal U of the vRNA. Positive numbers are in the viral-coded region and negative numbers are in the host-coded region (i.e., the sequence GAGGCCTCCG extends from -1 to -11). The bands from position -12 to the top of the gel correspond to the C tails of the clone.

cell lines, MDCK and CEF cells, were isolated, and the sequence at the 5' ends of the NS viral mRNAs was determined by reverse transcriptase-catalyzed primer extension using the 18 bp Hind III-Bam HI fragment of the NS-10 clone as primer. As shown in Figures 3 and 4, the extended primer copied from the NS viral mRNA synthesized in both MDCK and CEF cells contains predominantly, or exclusively, a T at position 1 (position 1 corresponds to the 3' terminal U of vRNA), indicating that most, or almost all, of the NS viral mRNAs contain an A residue opposite the 3' terminal U of the vRNA template. Consequently, A-terminated capped fragments are predominantly used as primers *in vivo* as well as *in vitro*, thereby generating an AGC sequence in the viral mRNA complementary to the UCG at the 3' end of vRNA. In addition, the extended primer copied from the viral mRNA from both cell lines contains predominantly a G at position minus 1. This indicates that the cell-

Figure 3. Sequencing gel (8%) of the cDNA copy of the 5'-terminal region of NS viral mRNA synthesized in MDCK cells.

ular capped fragments which were predominantly used as primers in both MDCK and CEF cells contain the sequence CA at their 3' ends. The rest of the host-donated primer sequence is heterogeneous.

DISCUSSION

The mechanism of initiation of influenza viral RNA transcription in the infected cell is similar in most respects to the mechanism established for *in vitro* transcription using various capped RNAs as primers. First, both *in vitro* (4) and *in vivo*, transcription initiates with a G residue directed by the 3' penultimate C residue of the vRNA. For *in vivo* transcription, the evidence is that the 3' terminal U residue of vRNA is not always represented

Figure 4. Sequencing gel (8%) of the cDNA copy of the 5' terminal region of NS viral mRNA synthesized in CEF cells.

by an A-T base pair in DNAs cloned from various viral RNA segments when one of the strands in the original duplex used for cloning was a cDNA copy of in vivo viral mRNA (7,8; present study). This indicates that the residue at this position in viral mRNA is most likely not viral coded. Because the 3' penultimate C residue of vRNA is apparently always represented by a G-C base pair in the cloned DNAs, it can be concluded that this G residue in viral mRNA is the first base directed by the 3' end of the vRNA.

Figure 5 shows a summary of the sequences at the ends of the various clones which have been analyzed (including the one reported here). Four of the cloned DNAs contain an A residue opposite the 3' terminal U of the vRNA, two contain a G at this position, and one contains a U at this position. From the results of the primer extension experiments reported here, it is

clear that the primer sequences containing G or U at this position are minor species. In particular, the U-containing species should be very minor. The virion endonuclease *in vitro* cleaves predominately, usually exclusively, at purine residues (4). In fact, cleavage at U residues occurred only under special circumstances, such as when the ribopolymer $m^7GpppGmC(U)_n$ was used as primer (19). It is therefore likely that the only reason that the U-terminated host primer sequence shown in Figure 5 was produced is that this particular capped cellular RNA did not contain a purine residue at the usual viral endonuclease cleavage sites (10 to 13 nucleotides from the cap). This would be expected to be an infrequent event in the cell. All of the clones except one (the HA clone-Udorn-MDCK cells) contain a cellular primer sequence 13 nucleotides long or less. These are the expected lengths because the virion endonuclease *in vitro* has been shown to cleave at a pos-

Figure 5. Comparison of host-derived sequences at the 5' ends of influenza viral mRNA determined by sequencing of cloned cDNAs. The proteins encoded by the cloned segments are indicated on the right: NS (nonstructural protein), HA (hemagglutinin), M (membrane protein). The influenza A strain and the cell line used for preparation of the viral mRNA are indicated in parentheses. The (WSN-MDCK) data are from the sequence reported here. The (Udorn-MDCK) data are from Dhar et. al. (7) and the (FPV-CEF) data are from Caton and Robertson (8).

ition 10 to 13 nucleotides from the cap (4) and because reverse transcriptase may not always copy the entire 5' terminus of the in vivo viral mRNA segments. The 5' sequence of the one longer primer sequence in Figure 5 contains two copies of a 5-nucleotide sequence (AGCAA) complementary to the 3' end of the vRNA, suggesting that copying errors occurred during the cloning procedure. Errors in the region of the nonviral sequence are not unexpected because the nucleotides in this region were unpaired in the original duplex inserted into pBR322, thereby requiring faithful completion of the double-strands by bacterial DNA repair enzymes.

In vitro and in vivo viral RNA transcription are also similar in the preferential, or almost exclusive, use of A-terminated capped RNA fragments as primers to initiate transcription. In this paper we present the evidence establishing that A-terminated host capped fragments are predominantly used as primers to initiate viral RNA transcription in vivo. Using an appropriate restriction fragment from the NS-10 DNA clone as a primer for the reverse transcriptase-catalyzed extension of the entire population of in vivo NS viral mRNAs from two different cell lines, an A was almost exclusively found in the viral mRNAs opposite the 3' terminal U of the vRNA. This result is in direct conflict with the results of Caton and Robertson (8). These workers used primer extension to analyze the 5' ends of the M (membrane protein) viral mRNAs (of fowl plague virus) synthesized in CEF and concluded that there was complete heterogeneity in these mRNAs at the position opposite the 3' terminal U of the vRNA. However, with their method of sequencing by dideoxy-chain termination, many artefactual bands occurred in several lanes of the sequencing gel, and as a consequence it was not possible to read unambiguously the 5' sequence in the viral mRNAs that is complementary to the 3' end of vRNA. It is therefore likely that the heterogeneity observed in the viral mRNAs at the position opposite the 3' terminal U of vRNA was an artefact. Support for our data comes from another group of workers. In a study in which the nucleotide sequence overlap of the NS₁ and NS₂ viral mRNAs (both coded by the NS viral gene segment) (Udorn virus strain) was analyzed by primer extension, Lamb and Lai (9) claimed that there was predominantly an A opposite the 3' terminal U of the vRNA. However, because the sequencing gel was run in order to read internal positions of the mRNA, it was difficult to read the terminal positions of the mRNA, and consequently some heterogeneity at the position opposite the 3' terminal U of the vRNA was not convincingly ruled out. After we completed the experiments reported here, we learned that Lamb and

coworkers (personal communication) analyzed the 5' ends of the M viral mRNAs (Udorn virus strain) synthesized in HeLa cells and found predominantly an A in these viral mRNAs at the position opposite the 3' terminal U of the vRNA. Thus, this is a general phenomenon, occurring with at least two different viral strains, with at least two viral mRNA segments and in at least three different cell lines.

The fact that A-terminated host capped RNA fragments are almost exclusively used as primers means that an AGC is generated in the viral mRNAs complementary to the 3' end of the vRNA. It is then conceivable that the same initiation step is used for the synthesis of both viral mRNA and the full-length transcripts that are the putative templates for vRNA synthesis. The latter transcripts, which comprise only a small fraction of the total viral transcripts in the infected cell, do not have poly A at their 3' end or a cap structure and host-derived sequence at their 5' end (20,21). The synthesis of these transcripts requires the synthesis of one or more virus-coded proteins. One function of this protein(s) would be to block the termination of transcription (17-22 nucleotides from the 5' end of vRNA) that occurs during viral mRNA synthesis (22). It is not yet known how the full-length transcripts are initiated. One possibility is that the newly synthesized virus-coded protein(s) enable the transcriptase to initiate RNA synthesis without a primer at the 3' terminal U of vRNA (instead of at the 3' penultimate C as occurs during viral mRNA synthesis). If this were the case, these transcripts would have a pppA 5' terminus. At present, the number of phosphates at the 5' end of the full-length transcripts is not known (21). The other alternative is that the same host cell RNA-primed initiation step is used for the full-length transcripts, followed by removal of the host-derived sequence by nucleolytic cleavage. Clearly, this would be feasible only if there were predominantly an A residue opposite the 3' terminal U of the vRNA, as we have found, thereby resulting in the proper AGC... sequence at the 5' end of the full-length transcripts.

Our results indicate that not all A-terminated host capped RNA fragments are used as primers for viral RNA transcription in vivo, but rather only those fragments with a CA 3' terminus are principally used. This specificity had not been observed in previous studies of transcription in vitro catalyzed by the virion-associated transcriptase as none of the capped RNA primers used in these in vitro experiments contained a CA sequence 10 to 13 nucleotides from the cap. Several possibilities emerge from this result. One possibility is that in the infected cell the in-

fluenza viral endonuclease preferentially cleaves capped host cell RNAs on the 3' side of CA sequences. Another possibility is that the influenza viral transcriptase in the infected cell preferentially utilizes CA-terminated capped fragments as primers. If the preferential usage of CA-terminated fragments were found to be unique to the in vivo situation, then it might be ascribed to the only apparent difference between in vitro and in vivo viral RNA transcription previously described, namely the presence of an m⁶A residue in the host-donated primer sequence on in vivo viral mRNA (6). Such a role for m⁶A would be consistent with the postulated role of m⁶A residues as recognition sites for cellular processing or splicing enzymes (23,24).

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