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Virology 351 (2006) 210–217

VIROLOGY

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Influenza virus inhibits RNA polymerase II elongation

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Received 9 February 2006; accepted 7 March 2006
Available online 19 April 2006

Abstract

The influenza virus RNA-dependent RNA polymerase interacts with the serine-5 phosphorylated carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (Pol II). It was proposed that this interaction allows the viral RNA polymerase to gain access to host mRNA-derived capped RNA fragments required as primers for the initiation of viral mRNA synthesis. Here, we show, using a chromatin immunoprecipitation (ChIP) analysis, that similar amounts of Pol II associate with Pol II promoter DNAs in influenza virus-infected and mock-infected cells. However, there is a statistically significant reduction in Pol II densities in the coding region of Pol II genes in infected cells. Thus, influenza virus specifically interferes with Pol II elongation, but not Pol II initiation. We propose that influenza virus RNA polymerase, by binding to the CTD of initiating Pol II and subsequent cleavage of the capped 5′ end of the nascent transcript, triggers premature Pol II termination.

Keywords: Influenza A virus; RNA-dependent RNA polymerase; RNA polymerase II; Carboxy-terminal domain (CTD); Transcription

Introduction

The synthesis of eukaryotic mRNAs is performed by RNA polymerase II (Pol II) in the cell nucleus. There is increasing evidence suggesting that mRNA processing events, i.e., capping, the removal of intron sequences by splicing, and the addition of a poly(A) tail at the 3′ end, are performed cotranscriptionally on the nascent transcript (Bentley, 2005; Proudfoot et al., 2002). The addition of the cap structure is known to occur soon after transcription initiation, around the time the transcript reaches a length of 25–30 nucleotides (Jove and Manley, 1984; Rasmussen and Lis, 1993). The components of the capping apparatus are recruited by binding to the phosphorylated carboxy-terminal domain (CTD) of the large subunit of Pol II (McCracken et al., 1997). The CTD, which is unique to Pol II, is composed in mammalian cells of 52 heptad repeats (YSPTSPS consensus sequence), ending with a unique 10 amino acid sequence. The CTD plays a key role in coupling Pol II transcription and RNA processing events. It binds not only the capping apparatus but also factors required for splicing and 3′ end processing. During the transcription cycle, the CTD undergoes extensive phosphorylation and dephosphorylation events (Palancade and Bensaude, 2003). Phosphorylation of serine-5 within the heptad repeat is associated with transcription initiation, and it plays a role in recruitment and stimulation of capping enzymes. As Pol II enters elongation, serine-2 phosphorylation increases. This change in the phosphorylation pattern is coupled with the release of 5′ end processing factors and the recruitment of 3′ end processing factors.

The influenza virus RNA-dependent RNA polymerase (RdRp), a complex of three subunits, PB1, PB2, and PA, is dependent on activities associated with the Pol II transcription apparatus (Fodor and Brownlee, 2002; Lamb and Krug, 2001). For the initiation of viral mRNA synthesis, it requires capped RNA fragments about 9–17 nucleotides in length that are derived from host mRNAs. These fragments are generated by an endonucleolytic activity associated with the viral RdRp. The capped fragments act as primers for the viral RdRp, and they become incorporated at the 5′ end of the viral mRNA transcripts. For this reason, viral mRNA synthesis is blocked in the presence of the Pol II inhibitor α-amanitin (Lamb and Choppin, 1977; Mark et al., 1979). Furthermore, two of the viral mRNAs are processed by the cellular splicing apparatus that is also known to be associated with Pol II transcription (Lamb and Horvath, 1991). Therefore, it was proposed that viral transcription might be coupled to Pol II transcription (Fodor et al., 2000). In fact, it was shown that the trimeric viral RdRp complex binds

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0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.03.005

to the CTD of Pol II (Engelhardt et al., 2005). Here, we were interested to further elucidate the details of this interaction with an emphasis on the effects this interaction might have on the activities of Pol II.

Results

Influenza virus RdRp associates with the promoter region of Pol II genes

It has been proposed that the viral RdRp, in order to gain access to nascent capped Pol II transcripts, binds to the CTD of an initiating Pol II that is serine-5 phosphorylated in its CTD (Engelhardt et al., 2005). This proposal was based on coimmunoprecipitation and copurification experiments, as well as colocalization studies using immunofluorescence microscopy. To obtain further evidence for the interaction between the viral RdRp and Pol II, we performed a chromatin immunoprecipitation (ChIP) assay using lysates from HeLa cells that were either infected with influenza A/WSN/33 virus or mock infected.

First, we performed ChIP using an antibody against the PA subunit of the viral RdRp to examine whether Pol II promoter DNA is present in complexes containing viral RdRp. Significant levels of the dihydrofolate reductase (DHFR) promoter DNA were immunoprecipitated from infected cells (Fig. 1A). Similar results were observed for the promoter regions of the β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, both transcribed by Pol II (Figs. 1B and C). In contrast, no significant levels of promoter DNA of the 45S rRNA or the 7SK RNA genes, transcribed by RNA Pol I or RNA Pol III, respectively, could be immunoprecipitated (Figs. 1A–C).

We also performed ChIP with an antibody that recognizes the N-terminal region of the large subunit of Pol II (N20). This antibody precipitated complexes containing promoter DNAs of the DHFR, β-actin, and GAPDH genes, but not promoter DNAs of the 45S rRNA and 7SK RNA genes, as expected. Interestingly, similar amounts of Pol II promoter DNAs were precipitated from both infected and uninfected cells, showing that viral infection does not interfere with RNA Pol II promoter recognition at 3 h post-infection.

Taken together, these results show that the influenza virus RdRp is associated with promoter regions of Pol II genes, but not with promoter regions of Pol I and Pol III genes. This is consistent with a previous study showing that the viral RdRp interacts with the serine-5 phosphorylated CTD of Pol II (Engelhardt et al., 2005).

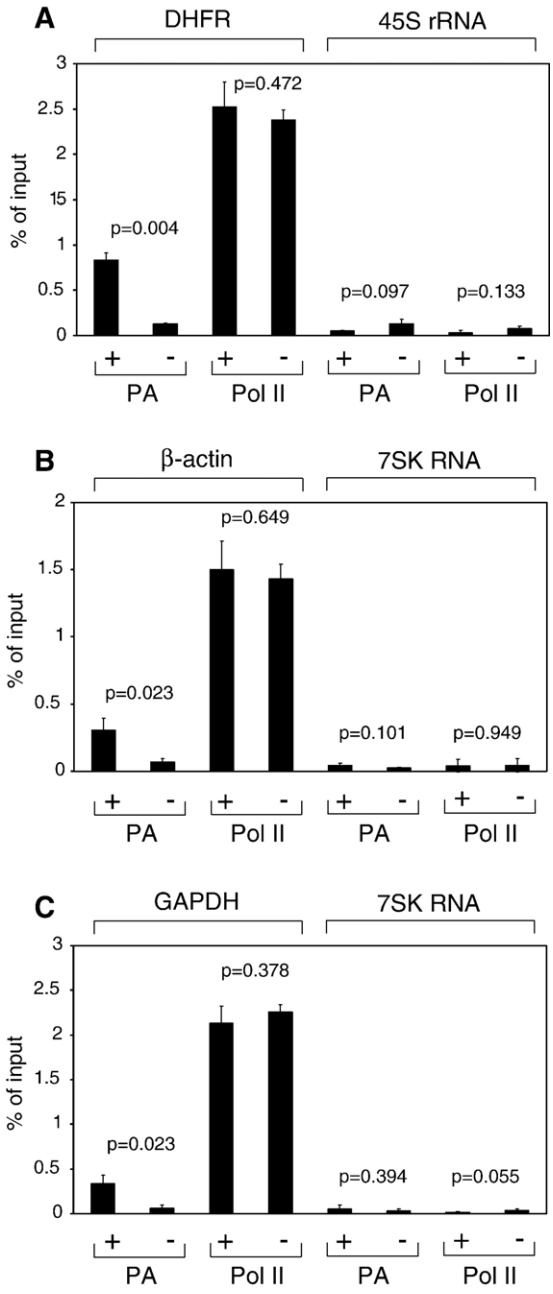

Fig. 1. Influenza virus RdRp associates with Pol II promoter DNA. ChIP was performed with an antibody against the PA subunit of the viral RNA polymerase (PA) (Engelhardt et al., 2005) or with the N20 Pol II-specific antibody (Pol II) (Santa Cruz) using lysates from influenza virus-infected (+) or mock-infected (−) HeLa cells. ChIP without specific antibody served as a negative control. Quantitation was performed by real-time PCR using primers specific for the promoter regions of the DHFR (panel A), 45S rRNA (A), β-actin (B), GAPDH (C), or 7SK RNA (B and C) genes. The amount of immunoprecipitated DNA was expressed as percentage of the input DNA. The average and standard deviations of three PCR repeats of a representative ChIP are shown. A two-tailed unpaired Student's *t* test was performed.

Viral infection affects Pol II distribution on Pol II genes

Next, we performed ChIP with the Pol II-specific N20 antibody, or with no antibody as a negative control, to examine the distribution of Pol II along the β-actin gene. ChIP was performed on both infected and uninfected cells. Statistical analysis of five independent ChIP experiments showed that viral infection did not affect Pol II density in the promoter region of the β-actin gene (Fig. 2A, left panel, also see Supplementary Table 1). In contrast, there was about a 2-fold decrease in RNA Pol II densities in the β-actin coding regions and 3′ UTR in infected cells compared to uninfected cells. These differences were statistically highly significant (Fig. 2A,

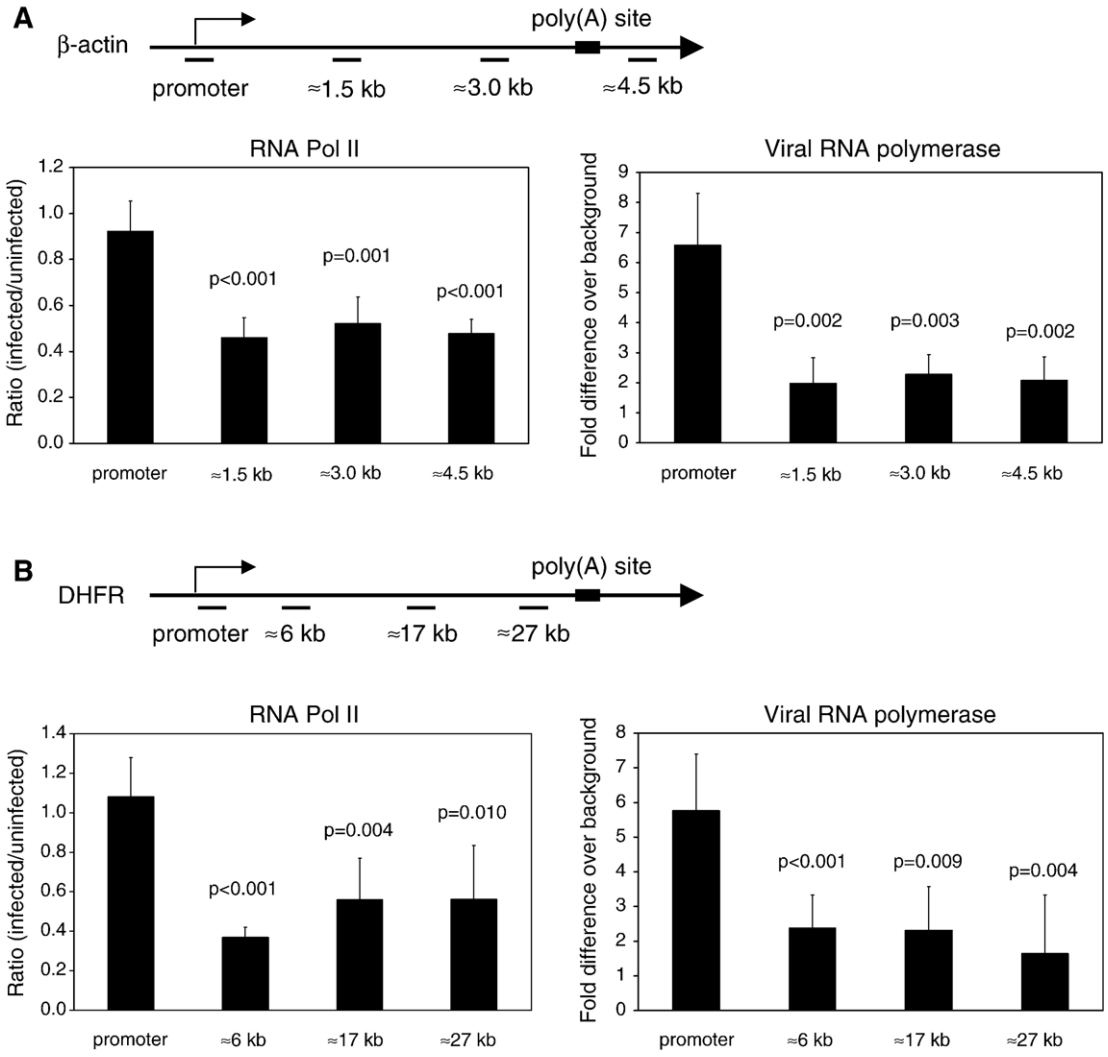

Fig. 2. Statistical analysis of the distribution of Pol II and viral RdRp along Pol II genes. ChIP was performed with the N20 Pol II-specific antibody (left panels) or with an antibody against the PA subunit of the viral RNA polymerase (right panels) using lysates from influenza virus-infected or mock-infected HeLa cells. ChIP without specific antibody served as a negative control. Quantitation was performed by real-time PCR using primers specific for the promoter or downstream regions of the β-actin (A) and DHFR (B) genes. A summary of data from five independent ChIP experiments with the N20 or PA-specific antibody is shown. In each experiment, ratios of the concentrations of DNA immunoprecipitated from infected and uninfected cells were calculated for each gene region tested. The average and standard deviations of the five ratios are shown. A two-tailed unpaired Student's *t* test was performed to assess whether the ratios in the downstream regions were significantly different from the ratio in the promoter region. Schematic diagrams of the β-actin (A) and DHFR (B) genes are shown. DNA fragments for PCR amplification are shown as bars under the genes and their approximate positions relative to the site of initiation are indicated in kilobases (kb).

left panel, also see Supplementary Table 1). It should be noted that there was about 5- to 10-fold more Pol II associated with the promoter–proximal region than with the downstream regions in uninfected cells (see Supplementary Table 1). This observation is consistent with the results of a previous report showing about an 8-fold enrichment of Pol II at the promoter region (Cheng and Sharp, 2003).

In parallel, we also analyzed the distribution of the viral RdRp along the β-actin gene by ChIP performed with the PA-specific antibody or no antibody as a negative control. ChIP was performed on both infected and uninfected cells. We found that the viral RdRp accumulated at the promoter region of the gene, with much lower levels of viral polymerase associated with the downstream regions (Fig. 2A, right panel, see also Supplementary Table 1). This finding is consistent with the previous observation that the viral RdRp preferentially binds to serine-5 phosphorylated CTD which is associated with initiating Pol II (Engelhardt et al., 2005). In contrast, the viral RdRp exhibited no detectable affinity for the serine-2 phosphorylated CTD known to be associated with elongating Pol II.

To confirm these results, we performed a similar analysis on another Pol II gene, the DHFR gene (Fig. 2B, also see Supplementary Table 2). Essentially, we observed the same results showing that there is a significant decrease in Pol II densities in the coding region of the DHFR gene in the infected cells compared to mock-infected cells. Also, the viral RdRp

preferentially accumulated in the promoter region as observed for the β-actin gene (Fig. 2B, right panel).

Viral RdRp requires Pol II initiation but not elongation

The findings described above suggest that the viral RdRp might have an inhibitory effect on Pol II elongation. However, as viral mRNA production is dependent on Pol II-associated activities (Lamb and Choppin, 1977; Mark et al., 1979), we were interested to examine whether viral transcription depends on Pol II elongation. For this, we used the kinase inhibitor DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) which inhibits RNA Pol II elongation by preventing CTD hyperphosphorylation but does not interfere with transcription initiation and capping (Giardina and Lis, 1993; Medlin et al., 2003; Yamaguchi et al., 1998). As shown in Fig. 3A (lane 4), viral primary transcription was not affected by DRB treatment. However, viral replication (cRNA and vRNA synthesis) and secondary mRNA transcription appeared to be strongly inhibited by DRB treatment, in agreement with previously published work describing the inhibition of influenza virus infection by DRB (Tamm et al., 1954; Tamm and Tyrrell, 1954). This pattern of transcription is strongly reminiscent of that obtained following treatment of infected cells with cycloheximide (Vreede et al., 2004), an inhibitor of protein expression. As viral replication and secondary transcription rely on the expression of viral proteins (Barrett et al., 1979; Hay et al., 1977), we speculated that the inhibition of these processes could be caused by the inhibition of viral protein synthesis in DRB-treated cells. In fact, it appears that DRB treatment inhibits viral protein synthesis possibly by the inhibition of downstream events after viral transcription, i.e., packaging of viral mRNAs into translation-competent ribonucleoprotein complexes (P. Digard, personal communication). In order to examine whether this was the case, we pre-expressed the viral RdRp subunits (3P) and the nucleoprotein (NP), the minimal set of viral proteins required for viral RNA synthesis prior to viral infection. When these transfected 293T cells were then infected as before (Fig. 3A), we observed significant levels of all three viral RNA species (lane 3). Although we do not fully understand every aspect of the effects of DRB on the Pol II transcription cycle and RNA processing, there is ample evidence demonstrating that DRB blocks Pol II elongation by preventing serine-2 phosphorylation of the CTD (Giardina and Lis, 1993; Medlin et al., 2003, 2005; Yamaguchi et al., 1998). Since we were able to demonstrate synthesis of capped viral mRNAs in the presence of DRB (Fig. 3, lane 3), our results clearly show that the viral RdRp is able to utilize capped RNA primers obtained in the absence of elongating Pol II.

In contrast, inhibition of Pol II by α-amanitin, which blocks both Pol II initiation and elongation, abolished the accumulation of all three types of viral RNA transcripts in infected cells (Fig. 3B, compare lanes 2 and 3). The only RNA detected under this condition was the vRNA introduced into the cell via viral infection (input vRNA). Pre-expression of the viral RdRp and NP (3P/NP) prior to α-amanitin treatment and viral infection resulted in the accumulation of cRNA and vRNA showing that Pol II activity is essential only for viral mRNA transcription, but not cRNA and vRNA synthesis (lane 5). The lack of cRNA and vRNA accumulation in the absence of pre-expressed viral RdRp and NP (lanes 3 and 4) is not due to an inhibition of replication. Rather, the lack of viral protein synthesis, caused by the inhibition of mRNA transcription, results in insufficient quantities of viral RdRp and NP to prevent degradation of and to replicate cRNA synthesized by the input viral RdRp (Vreede et al., 2004). In order to exclude the possibility that α-

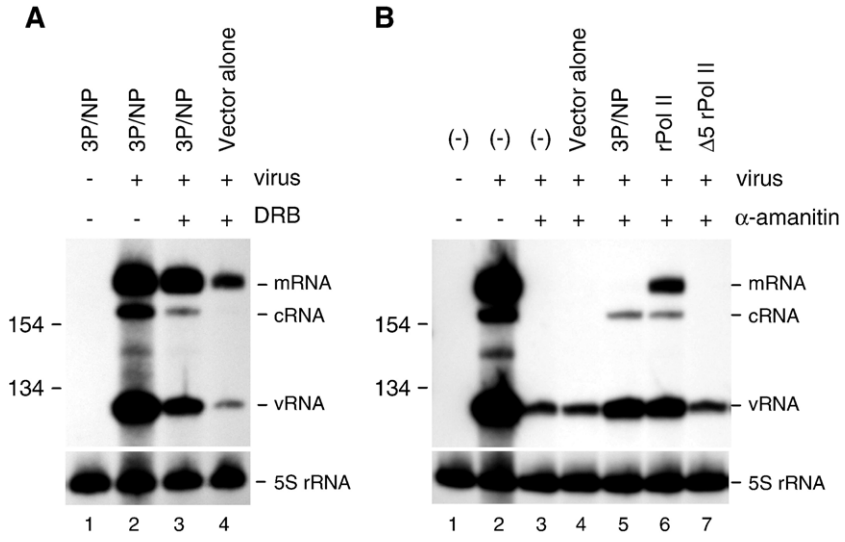

Fig. 3. The effect of DRB and α-amanitin on viral RdRp activity. 293T cells in 35-mm dishes were transfected with 1 μg of each of pcDNA-PB1, -PB2, -PA, and -NP (3P/NP), 4 μg of pcDNA3A (Vector alone) (Fodor et al., 2002), 4 μg of constructs expressing α-amanitin resistant Pol II (rPol II and Δ5 rPol II) (Gerber et al., 1995), or mock transfected (−). At 22 h post-transfection, DRB (A) or α-amanitin (B) was added to a final concentration of 250 μM or 20 μg/ml, respectively. Cells were incubated for a further 1 h (A) or 5 h (B) before infection with influenza A/WSN/33 virus at an MOI of 5. Cells were harvested 3 h post-infection and RNA was isolated using TRIzol (Invitrogen). Viral RNA species were analyzed by an NA gene-specific primer extension assay (Vreede et al., 2004). 5S rRNA was used as an internal control. Size markers in nucleotides are shown on the left.

amanitin directly interfered with transcriptional activity of the viral RNA polymerase, we used an α-amanitin-resistant clone of Pol II (Gerber et al., 1995) (lane 6). Under these conditions, we observed significant levels of all three viral RNA products, indicating that the inhibition of viral mRNA synthesis in the presence of α-amanitin is due to the inhibition of Pol II function. However, deletion of the CTD of the α-amanitin-resistant Pol II clone, abolished this activity (lane 7). Taken together, these results show that viral transcription is dependent on functional Pol II transcription.

Discussion

It has been known for decades that the influenza virus RdRp depends on cellular functions associated with Pol II (Lamb and Choppin, 1977; Mark et al., 1979). Viral mRNA synthesis by the viral RdRp is initiated by capped RNA primers which are derived from Pol II transcripts. Recently, it was demonstrated that the viral RdRp interacts with the CTD of initiating Pol II, and it was proposed that this interaction is required for the viral RdRp to gain efficient access to 5′ cap structures of nascent Pol II transcripts (Engelhardt et al., 2005). In this paper, we provide evidence that the influenza virus RdRp is associated with Pol II, but not Pol I or Pol III, promoter DNA. We found that the viral RdRp is specifically associated with the promoter region of Pol II genes, with much lower levels being associated with the regions downstream of the promoter. This distribution of the viral RdRp reflects the higher densities of Pol II in the promoter region compared to the downstream regions and the fact that it preferentially binds to the serine-5 phosphorylated CTD of Pol II that is known to be associated with the promoter region. We also show that viral infection does not interfere with promoter recognition by Pol II, but it interferes with events downstream of Pol II promoters. More specifically, we found decreased amounts of Pol II associated with the internal gene regions in infected cells, suggesting that Pol II elongation is inhibited by influenza virus.

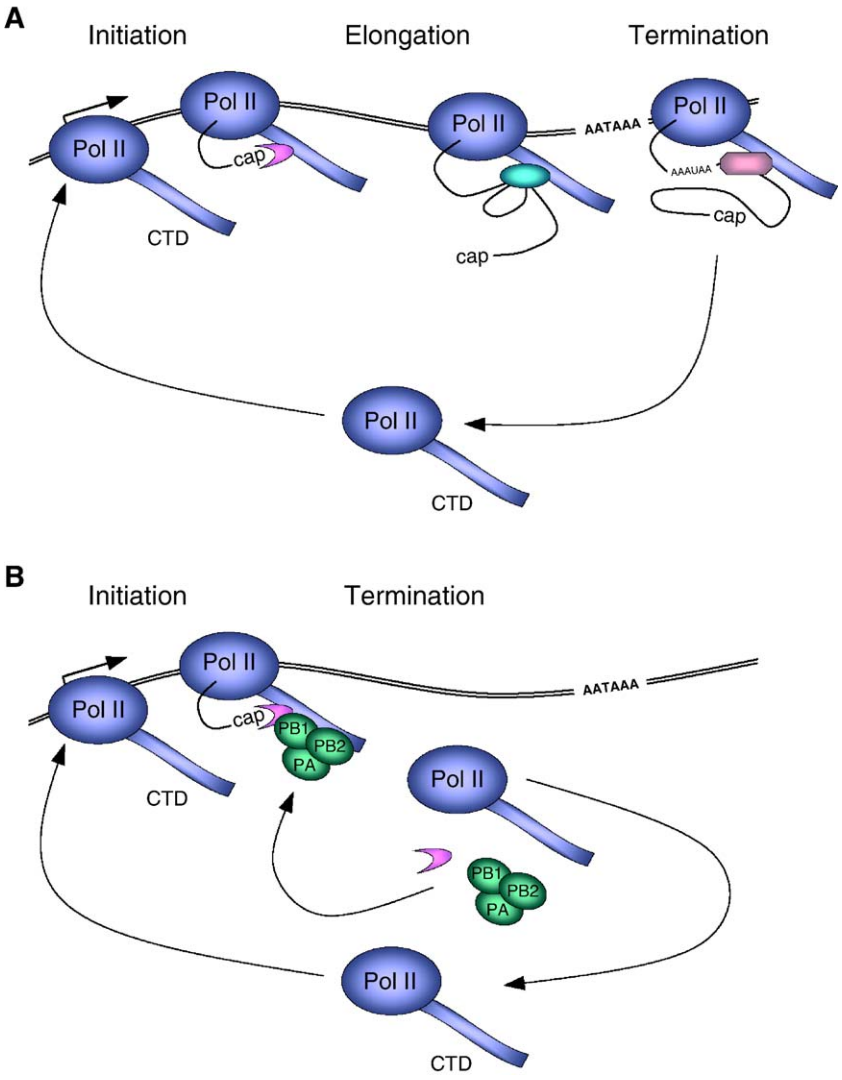

Fig. 4. Schematic model for Pol II transcription cycle in uninfected cells (A) and in cells infected with influenza virus (B). The capping, splicing, and 3′ end processing machineries associated with CTD are indicated. See text for details.

Model for Pol II transcription cycle in cells infected with influenza virus

We propose a model for the Pol II transcription cycle in the presence of influenza virus infection (Fig. 4). In the absence of viral infection, RNA Pol II enters elongation after transcription initiation and capping, producing mature capped, spliced, and polyadenylated transcripts (Fig. 4A). However, in the presence of viral infection, Pol II is prevented from entering the elongation phase due to the viral RdRp binding to the CTD of initiating Pol II (Fig. 4B). We speculate that this could be due to the following reasons: (i) removal of the cap structure from the 5′ end of Pol II transcripts results in their degradation by the 5′-3′ exonuclease Xrn2. As the exonuclease catches up with the elongating Pol II, transcription is prematurely terminated, releasing Pol II from the template. This situation would be analogous to that recently described for termination of Pol II transcripts, induced by cotranscriptional cleavage, followed by degradation of the downstream cleavage product by Xrn2 (torpedo model) (West et al., 2004). Alternatively, (ii) the presence of the viral RdRp on the CTD of the initiating Pol II might physically prevent its normal functioning. The viral RdRp could compete with host factors normally binding to the CTD that are required for transcription elongation, and/or it could prevent phosphorylation of CTD by steric hindrance. Both mechanisms could result in Pol II pausing, terminating prematurely and possibly releasing the template. Our results, showing reduced Pol II densities in the coding, but not in the promoter region of Pol II genes, are consistent with this model.

According to this model, influenza virus infection leads to the inhibition of Pol II elongation but not initiation. Inhibition of Pol II initiation would be detrimental to the virus considering its requirement for capped RNA primers that are synthesized by Pol II. Viral mRNA synthesis is inhibited by α-amanitin, which blocks both Pol II initiation and elongation. However, viral transcription can be observed in the presence of DRB, which affects only Pol II elongation, but not initiation. The observation that viral transcription can proceed in the presence of DRB shows that nascent capped transcripts, which are possibly still associated with the Pol II transcriptional machinery, are substrates for the viral endonuclease. This suggests that the viral RdRp is not dependent on mature, fully processed mRNAs, and that the viral RdRp is able to access nascent transcripts at the site of their synthesis. In fact, fully processed mature mRNAs packaged in ribonucleoprotein complexes destined for rapid export from the nucleus might not be accessible for the viral RdRp. To overcome this potential problem, the virus may have evolved a mechanism to target nascent transcripts at the site of their synthesis when they are not yet associated with host proteins that might protect them from cap snatching.

Specific inhibition of Pol II elongation in cells infected with influenza virus could contribute towards the inhibition of the synthesis of host proteins, a phenomenon known as cell shut-off. During influenza virus infection, there is a progressive decline in the synthesis of cellular proteins leading to the almost exclusive synthesis of viral proteins (Beloso et al., 1992; Inglis, 1982; Zurcher et al., 2000). This could be explained, at least in part, by inhibition of host mRNA synthesis and processing. For example, it was proposed that cap snatching could result in the degradation of newly synthesized Pol II transcripts in the nucleus (Katze and Krug, 1984), or that the viral NS1 protein could inhibit mRNA processing leading to a block in mRNA export (Nemeroff et al., 1998; Shimizu et al., 1999). Our data extend these previous studies by suggesting additional mechanisms that could lead to a decrease in host mRNA levels and consequently to cell shut-off.

Materials and methods

Chromatin immunoprecipitation (ChIP) assay

HeLa cells grown in 10-cm cell culture dishes (~70% confluency) were either infected with influenza A/WSN/33 virus at a multiplicity of infection (MOI) of 5 or mock infected for 1 h at room temperature. Three hours post-infection, formaldehyde was added to the cell culture medium to a final concentration of 1%. Cross-linking reactions were stopped by the addition of glycine to a final concentration of 125 mM. Cells pooled from 3 dishes were resuspended in 1 ml cell lysis buffer [50 mM Tris–HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Igepal CA-630 (Sigma), and 1 Complete Mini, EDTA-free protease inhibitor cocktail tablet (Roche)/10 ml] and incubated for 10 min on ice. Nuclei were lysed in 1 ml of cell lysis buffer containing 200 mM NaCl and sonicated in a Bioruptor (Diagenode) for 15 min. 300 μl clarified cell lysates were incubated with various antibodies as indicated for 2 h at 4 °C, followed by the addition of protein A-Sepharose CL-4B beads (Sigma) and a further 2-h incubation at 4 °C. The beads were washed in 10 mM Tris–HCl, 1 mM EDTA and 0.1% Igepal CA-630 containing 150 mM NaCl (1 wash), 1 M NaCl (3 washes), or 0.5 M LiCl (3 washes). Complexes were eluted and cross-links reversed by the addition of 300 μl of elution buffer (50 mM Tris–HCl, pH 6.8, 200 mM NaCl, 1 mM EDTA, 1% SDS) and incubation at 65 °C for 12 h. 30 μl of cell extracts mixed with 300 μl elution buffer was also incubated at 65 °C for 12 h to obtain an "input" DNA sample. DNA was isolated by proteinase K treatment, extraction with phenol/chloroform and precipitation with ethanol.

Quantitative PCR analysis

PCR was performed by using the QuantiTect SYBR Green PCR Kit (Qiagen) and a Corbett Rotor-Gene RG-3000 cycler. Reactions were set up in triplicate, and data were analyzed by using the Rotor-Gene 6 software. A standard curve was obtained for each set of PCR primers by analyzing serial dilutions of the input DNA. The following sets of primers were used: DHFR-specific primers, promoter (ACCTGGTCGGCTGCACCT, TTGCCCTGCCATGTCTCG), ≈6.0 kb (AACAGAATCTGGTGATTATGGG, TACTGATCTCCACTATGAGA), ≈17.0 kb (GTTCTATAGTCACTGCATCTTAGTC, TGCTAATTCTGGTTGTTCAGTAAG), ≈27.0 kb (GAGTATGTTTCTGTCTTAGATTGG,

ATGAGAACCTGCTCGCTGAC) (Cheng and Sharp, 2003); β-actin-specific primers, promoter (CCAATCAGCGTGCGCCGTTCCGAA, CCTCCGAACTGGCGTGGGGTGT), ≈1.5 kb (GACCTGAGTCTCCTTTGGAAC, TAATACACACTCCAAGGCCGC), ≈3.0 kb (ACAATGTGGCCGAGGACTTTGA, ACACGAAAGCAATGCTATCAC), ≈4.5 kb (TGTTGCCCAGGCTGGAGTGTA, CCAGGCTTGGTTTCTTCTTGCGAG); GAPDH-specific primers, promoter (GCTCAGGCCTCAAGACCTTGGGC, GTCCCTAGCCTCCCGGGTTTCTCT) (Medlin et al., 2005); 45S rRNA-specific primers, promoter (GCGTTTTTGGGGACAGGTGTC, CGCGCATCCGGAGGCCCAACC); 7SK RNA-specific primers, promoter (GCCCCACCCATCTGCAAGGCATTC, GAGCGGTGAGGGAGGAAGGGGA).

RNA isolation and analysis of vRNA, mRNA, and cRNA by primer extension assay

RNA isolation and analysis were performed as described (Fodor et al., 2002; Vreede et al., 2004). To analyze NA-specific viral RNAs, the following primers were used: TGGACTAGTGGGAGCATCAT to detect NA vRNA and TCCAGTATGGTTTTGATTTCCG to detect NA mRNA and cRNA. The expected size of the primer extension products in nucleotides is 129 for the vRNA, 160 for the cRNA, and 169 to 177 (depending on the length of the capped primer) for the mRNA.

TCCCAGGCGGTCTCCCATCC (K. Hara, personal communication) was used as a primer to detect 5S rRNA.

Acknowledgments

We thank J. L. Corden, T. Deng, K. Hara, and S. Murphy for reagents; G. G. Brownlee and N. Proudfoot for the helpful discussions. This work was supported by the MRC (senior nonclinical research fellowship G117/457 to E. F. and cooperative grant G9826944). F. T. V. was supported by MRC programme grant G9523972 to G. G. Brownlee and A. Y. C. was supported by a studentship from the Croucher Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2006.03.005.

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