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Influenza A Virus NEP (NS2 Protein) Downregulates RNA Synthesis of Model Template RNAs

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The influenza A virus NEP (NS2) protein is an structural component of the viral particle. To investigate whether this protein has an effect on viral RNA synthesis, we examined the expression of an influenza A virus-like chloramphenicol acetyltransferase (CAT) RNA in cells synthesizing the four influenza A virus core proteins (nucleoprotein, PB1, PB2, and PA) and NEP from recombinant plasmids. Influenza A virus NEP inhibited drastically, and in a dose-dependent manner, the level of CAT expression mediated by the recombinant influenza A virus polymerase. This inhibitory effect was not observed in an analogous artificial system in which expression of a synthetic CAT RNA is mediated by the core proteins of an influenza B virus. This result ruled out the possibility that inhibition of reporter gene expression was due to a general toxic effect induced by NEP. Analysis of the virus-specific RNA species that accumulated in cells expressing the type A recombinant core proteins and NEP showed that there was an important reduction in the levels of minireplicon-derived vRNA, cRNA, and mRNA molecules. Taken together, the results obtained suggest a regulatory role for NEP during virus-specific RNA synthesis, and this finding is discussed regarding the biological implications for the virus life cycle.

The influenza A virus RNA segment 8 directs the synthesis of two mRNAs in infected cells (14, 17). One of them is colinear with the viral RNA segment and codes for NS1 protein, and the other one is derived by splicing from the NS1 mRNA and is translated into a protein of 121 amino acids (19). These two polypeptides share the 10 N-terminal residues, whereas the rest of the coding sequences are translated from different open reading frames. The 121-amino-acid protein localizes to the cell nucleus (10, 32), and it was originally considered to be a nonstructural protein and was named "nonstructural protein 2" (NS2) (14, 17). More recently, however, it has been established that the protein is present in purified virions, where it interacts with the virus matrix (M1) protein (31, 34).

The functional templates for influenza virus-specific RNA synthesis are RNP complexes (reviewed in reference 18). These complexes are made up of the viral genomic RNA associated with four virus-encoded proteins: the nucleoprotein (NP), which encapsidates the RNA, and the three subunits (PB1, PB2, and PA) of the viral polymerase. Replication and transcription of the influenza virus genome involve the synthesis of three different RNA species: (i) the negative-sense genomic RNAs (vRNAs); (ii) the cRNAs, which are complementary to the vRNAs and serve as templates for the synthesis of new vRNAs, and (iii) the mRNAs, which are capped and polyadenylated. Synthesis of virus-specific RNAs occurs in the cell nucleus (12, 15), and the newly synthesized RNPs accumulate in this cell compartment at early times postinfection. Later in infection, the RNP complexes are transported out of the nucleus, since packaging of RNPs into virions occurs in the cytoplasm (reviewed in references 30 and 33). A number of pieces of evidence indicate that the M1 protein is directly involved in the nuclear export of RNPs (33) and more recent data support a role for NS2 as well during this process (25). In fact, it was shown that NS2 contains a nuclear export signal and that microinjection of anti-NS2 antibodies prevents the nucleocytoplasmic transport of RNPs in infected cells (25). Based on this activity, O'Neill and collaborators (25) proposed renaming the NS2 polypeptide "nuclear export protein" (NEP).

In addition to the role of NEP during export of RNPs, studies of the reassortant virus Wa-182, which contains a mutated NEP gene, have suggested an ill-defined role for the protein during the process of virus RNA replication (23). Infection with virus Wa-182 was found to result in aberrant replication of the PA gene, which leads to the accumulation of subgenomic RNAs derived from this RNA segment and to the generation of defective interfering (DI) particles in just a single high-multiplicity passage (22–24). The effect is not confined to the PA gene, since accumulation of subgenomic PB2 and PB1 genes has also been observed upon serial passage of the mutant virus (24). Other experimental approaches, however, have failed to detect direct involvement of NEP in replication and/or transcription of the viral RNA. Huang et al. (13) were the first to demonstrate that a synthetic negative-sense chloramphenicol acetyltransferase (CAT) RNA containing the influenza A virus RNA promoter could be amplified and transcribed in mammalian cells that expressed the four RNP protein components from cDNAs. In the same report, it was shown that coexpression of NEP had no effect on the level of CAT activity detected in transfected cells. Similarly, Enami et al. (6), using an analogous artificial system, failed to detect any effect of NEP on expression of a synthetic CAT RNA.

We have also established an artificial system in which a

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synthetic influenza virus-like CAT RNA is replicated and transcribed by influenza A virus recombinant proteins (20). Expression of the recombinant proteins is achieved in COS-1 cells, which are first infected with a vaccinia virus recombinant that expresses the T7 RNA polymerase (vTF7–3) (7) and then transfected with four pGEM-3-derived plasmids, each encoding one of the RNP components of virus strain A/Victoria/3/75 (5). We have also expanded the usefulness of this system by showing that the CAT RNPs synthesized in the transfected cultures could be packaged into virus-like particles (VLPs) when the cells expressed all viral structural proteins from recombinant plasmids (9, 21). It was during setting up and optimization of this VLP formation system that it was observed that cells transfected with increasing amounts of an NEP-encoding plasmid displayed a reduced level of expression of CAT enzyme (9, 21). This result suggested a role for NEP in replication and/or transcription of the CAT RNA. Here, we have examined this effect in further detail and demonstrate that NEP inhibits, in a dose-dependent manner, RNA synthesis of model virus-like RNAs.

We first determined whether the NEP inhibitory effect detected in the VLP formation system (9, 21) could be observed in cells expressing only the four RNP protein components. Thus, COS-1 cells were infected with the vaccinia virus vTF7–3 (7) and transfected with DNA mixtures that contained (i) the four pGEM-derived plasmids encoding the influenza A virus RNP components (21), (ii) different amounts of plasmid pGEM-NS2, which includes a cDNA copy of the A/Victoria/3/75 NEP gene under the control of the T7 promoter of plasmid pGEM-3 (20), and (iii) plasmid pCATCA-18 (8). Plasmid pCATCA-18 contains an influenza virus-like CAT gene flanked by a T7 RNA polymerase promoter and the hepatitis delta ribozyme, such that, in vTF7–3-infected cells, it directs the synthesis of a negative-sense influenza A virus model CAT RNA identical to that produced in vitro by plasmid pIVACAT1/S (16, 29). As can be observed in Fig. 1A, transfection of plasmid pGEM-NS2 inhibited, in a dose-dependent manner, the CAT signal detected in cultures expressing the recombinant polymerase. A significant inhibitory effect (threefold reduction) was observed during transfection with as little as 300 ng of the NEP-encoding plasmid, and a drastic reduction (ranging from 10- to 100-fold depending on the experiment) was observed during transfection with 1 to 2 μg of the same plasmid. The inhibitory effect was specific for the NEP-encoding plasmid, since no significant alteration of the reporter gene activity was observed during transfection with 1 μg of pGEM-derived plasmids encoding the influenza A virus HA or M2 proteins instead of the plasmid pGEM-NS2 (9; data not shown). The extracts of the pGEM-NS2 transfected cells were examined by Western blotting with an anti-NEP serum (Fig. 1B). The NEP protein detected in the transfected cultures comigrated with the protein present in influenza virus-infected cells, and its intracellular concentration increased concomitantly with the amount of transfected plasmid. Importantly, the amount of NEP present in the cultures in which CAT expression was drastically inhibited (during transfection with 1 or 2 μg of the NEP-encoding plasmid) was similar to that present in MDCK cells infected with the influenza virus A/Victoria/3/75 strain, a result which indicated that the NEP levels reached in transfected cells were in the physiological range. It should be mentioned that the NEP band detected in the transfected cell extracts appears to migrate as a double band. Although this phenomenon has not been investigated in great detail, it is suggested that it may be due to protein degradation in these particular samples, since the double band has not been observed consistently in transfected cultures (data not shown). Based on the results shown in Fig. 1, it was concluded that the plasmid pGEM-NS2 alone inhibited the CAT gene expression mediated by the recombinant influenza A virus polymerase.

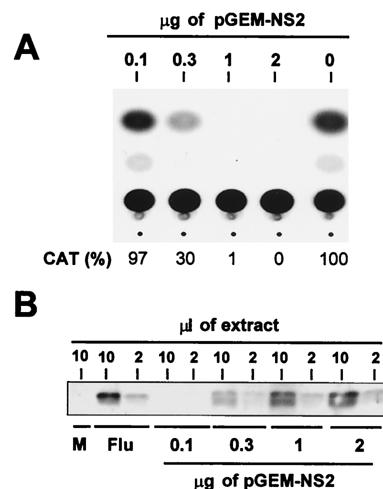

FIG. 1. Plasmid pGEM-NS2 inhibits expression of a model CAT RNA. (A) COS-1 cell cultures (10^6 cells) were infected with vaccinia virus vTF7–3 and transfected with a DNA mixture containing liposomes and plasmids pGEM-NP (2 μg), pGEM-PB1 (0.6 μg), pGEM-PB2 (0.6 μg), pGEM-PA (0.1 μg), and pCATCA-18 (0.5 μg) (see text for details) and the indicated amounts (expressed in micrograms) of plasmid pGEM-NS2, following the protocol detailed previously (21). After 24 h of incubation, cell extracts were prepared and tested for CAT activity with [^{14}C]chloramphenicol and thin-layer chromatography as described by Mena et al. (21). CAT activity values were expressed as a percentage of the activity obtained in the sample that was not transfected with plasmid pGEM-NS2. (B). Extracts from the cultures transfected with different amounts of plasmid pGEM-NS2 and from MDCK cells that had been either mock infected (M) or infected with the influenza virus A/Victoria/3/75 (Flu) (multiplicity of infection of 5) for 24 h were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with a serum raised against a bacterially expressed, purified His-tagged virus strain, A/Victoria/3/75 NEP. As indicated in the figure, two doses (2 and 10 μl) of each of the cell extracts were loaded into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel.

It was then necessary to determine whether the effect displayed by plasmid pGEM-NS2 was due to NEP, to the NEP mRNA, or to an artifactual inhibition caused by the plasmidic DNA sequences. It should be indicated that the nucleotide sequences that flank the NEP open reading frame in plasmid pGEM-NS2 are untranslated sequences derived from influenza virus RNA segment 8. In fact, there are 26 influenza virus-derived noncoding nucleotides (nt) preceding the NEP ATG initiation codon and 8 nt following the NEP stop codon (Fig. 2). The 26 noncoding nt that precede the NEP initiation codon are exactly complementary to the 3′-end sequences of the CAT RNA synthesized from plasmid pCATCA-18 in the transfected COS-1 cells, whereas the 8 nt that follow the NEP stop codon do not bear any resemblance to those following the

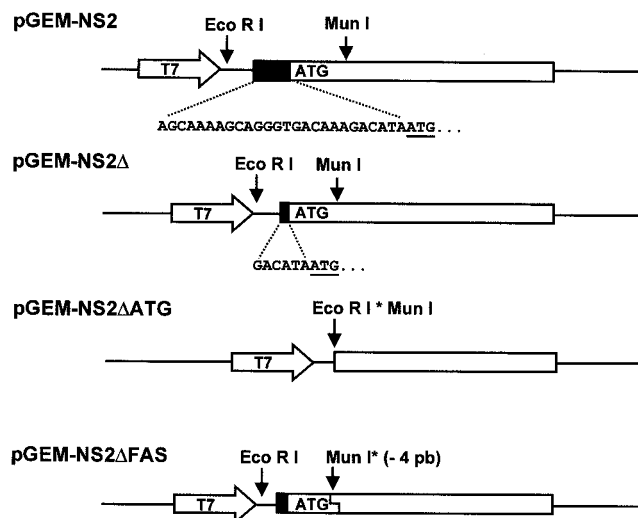

FIG. 2. Schematic representation of the plasmids containing NEP gene sequences. Plasmids pGEM-NS2 and pGEM-NS2Δ have been described previously (9, 21). For these two plasmids, the 5′-end influenza virus noncoding nucleotide sequences that are located upstream of the NEP initiation codon are shown. Plasmid pGEM-NS2ΔATG was obtained by removing the nucleotide sequences (69 bp) between the *Eco*RI and *Mun*I restriction sites present in plasmid pGEM-NS2Δ. Plasmid pGEM-NS2Δ was linearized with restriction enzyme *Mun*I, treated with mung bean nuclease, and religated to yield plasmid pGEM-NS2ΔFAS, which lacks 4 bp compared to plasmid pGEM-NS2Δ. Open arrow, T7 RNA polymerase promoter; ■, nontranslated influenza virus-derived sequences at the 5′ ends of the NEP mRNA; □, nucleotide sequences coding for the NEP gene; —, pGEM-3 derived sequences; ATG, NEP initiation codon.

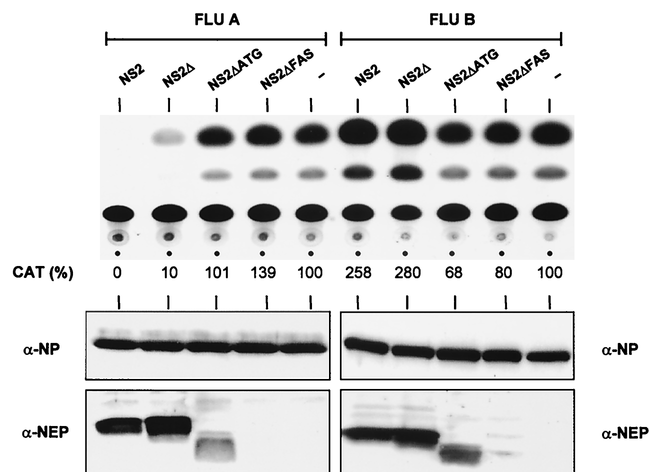

FIG. 3. Effect of the pGEM-NS2-derived plasmids on CAT expression mediated by the influenza A and B virus recombinant polymerases. In section FLU A, COS-1 cell cultures were infected with vTF7–3 and transfected with mixtures including the plasmids encoding the influenza A virus RNP protein components, plasmid pCATCA18, and the indicated pGEM-NS2-derived plasmid (at a concentration of 2 μg) as described in the legend to Fig. 1. The sample "−" was transfected only with the plasmids encoding the four RNP protein components and the CAT RNA. The cell cultures were collected and tested for CAT activity as indicated in Fig. 1, and the CAT activity values obtained were expressed as a percentage of the activity obtained with the sample "−." The lower panels show a Western blotting analysis of the corresponding cell extracts probed with antibodies that recognized the NP (α-NP) or NEP (α-NEP) of the virus strain A/Victoria/3/75. In section FLU B, COS- 1 cell extracts were processed as indicated above for the FLU A cultures, except that the cells expressed a type B influenza virus-like CAT RNA and the RNP components of virus strain B/Panamá/45/90. The plasmids used to produce the type B RNP components were pribo-NSBCAT (0.5 μg), pGB-NP-7 (2 μg), pGB-PB1–89.1 (0.6 μg), pGB-PB2–2 (0.6 μg), and pGB-PA-4 (0.1 μg) (described in reference 16). The lower panels show a Western blotting analysis of the corresponding cell extracts probed with antibodies that recognized influenza B virus NP (α-NP) or A/Victoria/3/75 NEP (α-NEP).

CAT gene stop codon. It could then be argued that hybrid RNA molecules (NEP mRNA transcripts hybridized to model CAT RNAs) were formed in transfected cells and that this phenomenon may be responsible for the inhibitory effect on CAT expression. Thus, we prepared plasmid pGEM-NS2Δ, in which 20 influenza A virus noncoding nt upstream from the NEP ATG codon were removed (Fig. 2) (9). In addition, plasmids pGEM-NS2ΔATG and pGEM-NS2ΔFAS, which contained alterations in the NEP open reading frame, were also prepared from plasmid pGEM-NS2Δ (Fig. 2). Plasmid pGEM-NS2ΔATG is 69 nt smaller than plasmid pGEM-NS2Δ and lacks the NEP ATG initiation codon and the following 21 codons of NEP. In plasmid pGEM-NS2ΔFAS, 4 bp were removed. This deletion causes a frameshift mutation in the NEP open reading frame so that the plasmid was predicted to direct the synthesis of a 26-amino-acid protein containing the N-terminal 19 residues of NEP and 7 amino acids derived from a different open reading frame.

All four of these plasmids (pGEM-NS2, pGEM-NS2Δ, pGEM-NS2ΔATG, and pGEM-NS2ΔFAS) were tested in parallel, at a concentration of 2 μg, for their capacity to alter CAT RNA expression mediated by the influenza A virus polymerase complex. The results of a representative experiment are shown in Fig. 3 (FLU A). The two plasmids that encoded the full-length NEP inhibited CAT expression, whereas the two plasmids containing alterations in the NEP open reading frame yielded CAT activity values similar to those observed in cells expressing only the four influenza A virus RNP protein components (Fig. 3, lane −). The extracts of the transfected cells were also examined by Western blotting to check for expression of NP and NEP (Fig. 3, FLU A, lower panel). As expected, the NEP polypeptide was detected in cells transfected with plasmids pGEM-NS2 and pGEM-NS2Δ, whereas no specific protein band was recognized by the anti-NEP serum in cells transfected with the plasmid encoding the frameshifted NEP. Unexpectedly, a fast-moving band was detected in cells transfected with plasmid pGEM-NS2ΔATG, which lacks the NEP ATG initiation codon. Although this protein band has not been examined in further detail, it is suggested to correspond to an NEP-derived polypeptide which would be translated from an internal in-frame NEP ATG codon. The results shown in Fig. 3 (FLU A) demonstrated that CAT inhibition was in fact mediated by NEP itself and not by the NEP mRNA or the DNA plasmidic sequences of plasmid pGEM-NS2.

As can be observed in Fig. 3 (FLU A, lower panel), no significant differences in the level of NP accumulation were observed among the various transfected cultures, suggesting that NEP was not exerting a general toxic effect on protein translation that would account for the reduction in the CAT

activity levels observed in the transfected cell cultures. Moreover, the accumulation levels of the PB2 and PA proteins in the transfected cultures were also examined by Western blotting, and only minor differences (less than twofold) between the different samples were observed (data not shown). Although we could not examine the accumulation levels of the third influenza virus polymerase component (protein PB1) because of the lack of an appropriate immunological reagent, these data strongly argue against the inhibitory effect observed in CAT expression being due to differences in the expression levels of the polymerase proteins. Despite these results, it was important to fully prove that the inhibition of CAT expression was not due to a nonspecific toxic effect of NEP. We have previously described the cloning of the four RNP components of the influenza B virus isolate B/Panamá/45/90 (16). The genes were cloned downstream of the T7 RNA polymerase promoter of plasmid pGEM-3, and we demonstrated that these plasmids lead to the expression of the four RNP components when transfected into vTF7–3-infected cells. Moreover, it was shown that the recombinant proteins synthesized in mammalian cells were competent to express a synthetic CAT RNA containing the influenza B virus RNA promoter (16). To determine whether A/Victoria/3/75 NEP had an inhibitory effect on the influenza B virus polymerase activity, mixtures containing the four plasmids encoding the type B RNP components, the various pGEM-NS2-derived plasmids, and plasmid pribo-NSBCAT were transfected into COS-1 cells (Fig. 3, FLU B). Plasmid pribo-NSBCAT is analogous to plasmid pCATCA18, but directs the synthesis of a type B influenza virus-like CAT RNA that is identical to the CAT RNA obtained from plasmid pT7NSBCAT upon in vitro transcription with T7 RNA polymerase (2, 16). None of the pGEM-NS2-derived plasmids displayed any inhibitory effect on the CAT expression levels reached in cells expressing the influenza B virus polymerase (Fig. 3, FLU B). Therefore, it was concluded that the type A NEP inhibited the homotypic virus polymerase, but not the heterotypic type B enzyme. This observation ruled out the possibility that the reduced levels of CAT expression detected in cells expressing the type A viral polymerase were the consequence of a general toxic effect of NEP. Moreover, the fact that NEP did not alter expression of the type B CAT RNA excluded the possibility that the inhibitory effects of NEP were mediated through the CAT gene nucleotide sequence or the CAT protein.

All of the experiments presented above allowed us to conclude that NEP was in fact causing the reduction in CAT expression detected in transfected cells. However, we did not know whether NEP was negatively affecting transcription and/or replication of the CAT RNA. To determine the RNA synthesis step inhibited by NEP, we took advantage of the system described by Perales and Ortín (27) and later improved by the same group (26, 28). In these systems, accumulation of the different virus-specific RNA molecules produced in cells expressing a recombinant polymerase are analyzed by the RNase A protection assay. In the improved system, the model RNAs (either vRNA [313 nt] or cRNA [306 nt]) to be amplified by the recombinant polymerase are produced intracellularly in vTF7–3-infected cells from plasmids (pT7vNSΔCAT-RT, pT7cNSΔCAT-RT) that contain the model influenza A virus-like RNA sequences flanked by the T7 RNA polymerase promoter and the hepatitis delta virus ribozyme (26, 28). As can be observed in Fig. 4A, when a negative-sense model vRNA was used as template, expression of NEP had a negative effect on the level of accumulation of the cRNA and mRNA molecules. This inhibitory effect was not observed in cells transfected with plasmid pGEM-NS2ΔFAS. It should be noted that the reduction in mRNA levels detected in cells expressing NEP accounted for the reduction in CAT activity levels shown in Fig. 1 and 3. When the model RNA produced intracellularly was a positive-sense cRNA template, there was also a drastic reduction in the levels of vRNA and mRNA molecules only in the cells expressing NEP (Fig. 4B). Based on these results, it was concluded that NEP inhibits synthesis of the genome and the antigenome RNAs during virus RNA replication. Although the levels of minireplicon-derived mRNAs were also reduced in the NEP-expressing cells (Fig. 4A and B), it cannot be concluded that NEP inhibits virus mRNA synthesis, since this

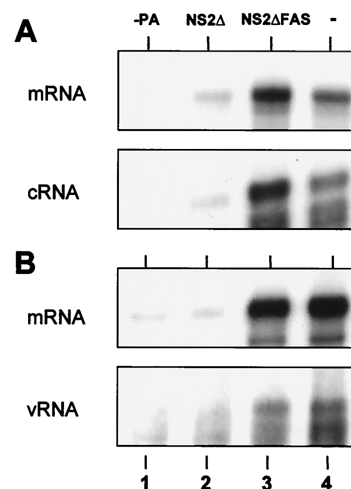

FIG. 4. Accumulation of virus-specific RNA products in cells expressing the recombinant influenza A virus polymerase. COS-1 cells were infected with vTF7–3 and transfected with different DNA mixtures as described in the legend to Fig. 1. The DNA mixtures contained plasmids encoding the influenza A virus NP, PB1, PB2, and PA proteins (samples 2, 3, and 4), or the mixture used lacked the plasmid encoding PA (sample 1). In addition, the DNA mixtures transfected in samples 2 and 3 contained the plasmids pGEM-NS2Δ and pGEM-NS2ΔFAS, respectively. All transfection mixtures also contained a plasmid analogous to plasmid pCATCA18 that directed the synthesis of either a negative-sense (pT7vNSΔCAT-RT, 313 nt) (A) or a positive-sense (pT7cNSΔCAT-RT, 306 nt) (B) model RNA template. At 24 h postinfection with vTF7–3, total RNA was obtained following cell lysis with TRIZOL reagent (Gibco-BRL) and fractionated into poly(A)$^+$ and poly(A)$^-$ RNAs by oligo(dT)cellulose chromatography. Aliquots of the poly(A)$^+$ fractions were then analyzed for the presence of mRNAs derived from the synthetic model RNA by the RNase protection assay (with the RPA III kit from Ambion) with a negative-sense labeled probe (mRNA panels). The poly(A)$^-$ fractions were self-annealed and treated with RNase A to select for vRNA-cRNA hybrids (see reference 27) and were then analyzed for the presence of cRNA (A [cRNA]) or vRNA (B [vRNA]) by the RNase A protection assay by using ^{32}P-labeled probes with predetermined polarity (28). The protected, labeled fragments were visualized after electrophoresis in a 4% sequencing gel and autoradiography. In all panels, the mobility of the slowest-migrating band was that expected for the different RNA products, as determined by comparison to DNA markers included in the gel (not shown).

effect could be a consequence of the reduction of vRNA molecules, which are the templates for mRNA synthesis.

As indicated above, other studies (6, 13) have failed to detect an effect of NEP on expression of synthetic CAT minireplicons. In these two reports, the expression of NEP was achieved through infection with a vaccinia virus recombinant containing the NEP gene. Considering that we have shown here that the RNA synthesis inhibition was NEP dose dependent, we speculate that the failure of the previous reports to detect an inhibitory effect of NEP was due to a low level of expression of the recombinant NEP.

It is shown here that the NEP inhibitory effect increases concomitantly with the level of accumulation of the protein and that the NEP concentrations needed to inhibit CAT RNA expression can be physiologically reached in influenza virus-infected cells (Fig. 1B). In cells infected with influenza virus, the viral proteins increase their concentration during the course of infection, and it is thus predicted that the major inhibitory effect of NEP would be reached late in the infectious cycle. We hypothesize that the inhibitory effect of NEP would contribute to render, later in infection, the RNPs quiescent, a fact that could facilitate packaging of the RNP complexes into the newly formed virions.

At this time, we can only speculate on the mechanism by which NEP is exerting its negative effect on virus RNA replication. It is known that influenza virus RNA replication takes place in the cell nucleus (12, 15), and as mentioned above, there are data that indicate that NEP is directly involved in the export of RNPs out of the nucleus (25). Thus, an explanation that would account for the effect observed in the minireplicon system would be that NEP, in the absence of other viral proteins, promotes the export of RNPs from the nucleus to the cytoplasm, so that the number of RNPs engaged in RNA replication is reduced. Nonetheless, that the inhibitory effect is occurring through binding of NEP to one of the RNP components or via a cellular factor required for polymerase function cannot be excluded.

Other negative-sense RNA viruses encode proteins, apart from the components of the nucleocapsid, that have regulatory roles in transcription and RNA replication in minigenome model systems. In particular, the NS1, NS2, and M2–2 proteins of human respiratory syncytial virus downregulate RNA transcription and replication (1, 4, 11), and the C protein of Sendai virus specifically inhibits RNA synthesis from the genomic promoter (3). It is thus tempting to speculate that influenza A virus NEP can be equivalent to these viral regulatory proteins.

In summary, it is shown here that influenza A virus NEP inhibits replication of model RNAs in cells expressing the influenza A virus RNP components from recombinant plasmids. Moreover, we have demonstrated that the inhibitory effect is not a consequence of toxicity induced by NEP and that it affects synthesis of vRNA as well as cRNA molecules in a virus-type-specific manner.

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