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The N-Terminal Extension of the Influenza B Virus Nucleoprotein Is Not Required for Nuclear Accumulation or the Expression and Replication of a Model RNA

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The nucleoprotein (NP) of influenza B virus is 50 amino acids longer at the N-terminus than influenza A virus NP and lacks homology to the A virus protein over the first 69 residues. We have deleted the N-terminal 51 and 69 residues of the influenza B/Ann Arbor/1/66 virus NP and show that nuclear accumulation of the protein is unaffected. This indicates that the nuclear localization signal is not located at the extreme N terminus, as in influenza A virus NP. To determine if the N-terminal mutants could support the expression and replication of a model influenza B virus RNA, the genes encoding the subunits of the viral RNA-dependent RNA polymerase (PA, PB1, and PB2) were cloned. Coexpression of NP and the P proteins in 293 cells was found to permit the expression and replication of a transfected model RNA based on segment 4 of B/Maryland/59, in which the hemagglutinin-coding region was replaced by a chloramphenicol acetyltransferase gene. The expression and replication of the synthetic RNA were not affected by the replacement of NP with NP mutants lacking the N-terminal 51 or 69 residues, indicating that the N-terminal extension is not required for transcription or replication of the viral RNA. In addition, we report that the influenza B virus NP cannot be functionally replaced by type A virus NP in this system.

Influenza A and B viruses have segmented genomes, each comprising eight negative-strand RNAs. The virion RNAs are complexed with nucleocapsid (NP) and subunits of the RNA-dependent RNA polymerase (PA, PB1, and PB2) to form viral ribonucleocapsids (vRNPs) (18). After the entry and uncoating of the virus, vRNPs enter the cell nucleus, where transcription or replication of the viral RNA takes place. The NP facilitates the nuclear import of vRNPs by interacting with proteins belonging to the karyopherin α family of nuclear transport factors (NPI-1 and NPI-3) (27–29). In addition, the NP of influenza A virus has been implicated in the determination of host range (32), the initiation of viral mRNA synthesis (3), RNA elongation (11), and the switch between mRNA and cRNA synthesis (antitermination) (5).

The region of the influenza A virus NP responsible for RNA binding has been mapped to the N-terminal third of the protein, specifically between residues 1 and 77 and 79 and 180 (1, 17). Recently, it was reported that the N terminus of influenza B virus NP also contains a nonconventional nuclear localization signal (NLS) (25, 34). Significantly, influenza B virus NP lacks homology to the A virus protein at the N terminus. For all influenza B virus isolates for which the sequences are available, the NP sequence diverges markedly in this region compared to influenza A virus NP and lacks classical mono- and bipartite NLSs such as those described for the large T antigen of simian virus 40 and nucleoplasmin, respectively (26). It is therefore of interest to investigate the function of the conserved N-terminal extension of influenza B virus NP, and in particular to determine if this region contains an NLS.

To assess the role of the N-terminal residues of influenza B virus NP, the cDNA for the B/AA/1/66 NP gene was amplified by reverse transcription-PCR (RT-PCR) with primers based on the published sequence (9). Influenza B/AA/1/66 virus was grown at 34°C in 10-day-old embryonated chicken eggs and purified from the allantoic fluid 48 h postinfection by centrifugation through a 30% sucrose cushion. The virus was resuspended in TMK (10 mM Tris-HCl [pH 7.4], 1.5 mM MgCl₂, 10 mM KCl), lysed by the addition of sodium dodecyl sulfate to 0.3%, and then digested with 2 U of proteinase K (GIBCO/BRL) for 10 min at 56°C. The viral RNA was then purified by phenol extraction and precipitated with ethanol. Approximately 200 ng of viral RNA was used for reverse transcription with 100 ng of an oligonucleotide (NPstart; 5′-gcgcccagacctATCAAAAT GTTCACACATG 3′) annealing to residues 53 to 70 of the segment 5 RNA which encodes the NP (numbering for all oligonucleotides listed here is of the positive-sense RNA). Reverse transcription was performed with a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, the four dNTPs (1 mM each), 30 U of human placental RNase inhibitor (Pharmacia), and 5 U of avian myeloblastosis virus reverse transcriptase (GIBCO/BRL). The product was amplified by PCR with NPstart and an oligonucleotide (NPstop; 5′-gcgcccagctgacGT GCTTATAAAATCCAG 3′) annealing to residues 1730 to 1747 with Vent DNA polymerase (New England Biolabs). The NP gene was then cloned on a HindIII-SalI fragment into mammalian expression vector pcDNA3 (Invitrogen) under the
control of the human cytomegalovirus immediate-early pro-
moter/enhancer, generating plasmid pcDNA3-NP.

Next, we made deletions which removed the N-terminal extension (NPΔ51) and all of the nonhomologous sequence at the N terminus (NPΔ69) (Fig. 1). The NPΔ51 mutant lacks amino acid residues 2 to 51 and was generated by PCR with oligonucleotide 5′-gcgcgcaagcttATCAAAATGGTAGTGAAACTGGGT 3′ and NPstop, with pcDNA3-NP as the template. The product was cloned into pcDNA3 as described for NP, giving pcDNA3-NPΔ51. Mutant NPΔ69 lacks amino acids 2 to 69 and was generated with oligonucleotide 5′-gcgcgcaagcttATCAAAATGGTAGTGAAACTGGGT 3′ and NPstop, giving pcDNA3-NPΔ69. We also deleted further into the protein (residues 2 to 82) by PCR with oligonucleotide 5′-gcgcgcaagcttATCAAAATGGTAGTGAAACTGGGT 3′ and NPstop, giving pcDNA3-NPΔ82. Proteins of the expected size were synthesized in in vitro translation reactions with T7 run-off transcription of SmaI-linearized templates in the presence of [35S]methionine according to the manufacturer’s instructions. Proteins were resolved on an SDS–10% PAGE gel and visualized by autoradiography. Numbers refer to the sizes in kilodaltons of protein standards.

Our data indicate that the N-terminal extension of influenza B virus NP does not contain the sole NLS. The region of the influenza A virus NP responsible for nuclear accumulation has been mapped by Wang et al. (34) to residues 1 to 13 and 25 to 35. However, the authors of both reports concede that mutants lacking part or all of the NLS still possess NLS activity, since they can target a normally cytoplasmic protein to the nucleus (25, 34). The identification of the sequence(s) responsible for the nuclear accumulation of the influenza B virus NP awaits further mutagenesis of the protein.

Earlier results with influenza A virus NP had suggested that a motif which determines the accumulation of NP in the nuclei of Xenopus oocytes was located between residues 327 and 345 (8). This region is conserved in the NP of influenza B/AA/1/66 virus; however, its importance in determining the nuclear accumulation of type A virus NPs in mammalian cells is contested (25, 34). The identification of the sequence(s) responsible for the nuclear accumulation of the influenza B virus NP awaits further mutagenesis of the protein.

A plasmid-based system to study the expression and replication of influenza B virus RNAs. A number of systems to study the expression and replication of influenza virus RNAs have been described. Luytjes et al. (21) first reported that RNP complexes reconstituted in vitro with purified NP and P proteins and a synthetic influenza virus RNA containing a cat gene could give rise to CAT activity following transfection into helper virus-infected cells. It has since been shown that functional RNP complexes can be reconstituted in vivo, since cells which supply NP and the P proteins in trans from plasmids, vaccinia virus, or simian virus 40 recombinants, can support the expression and replication of a transfected model influenza A virus RNA (6, 10, 12, 15, 22, 38). To establish such a system for
FIG. 3. Localization of NP and NP deletion mutants in MDCK cells. MDCK cells (American Type Culture Collection; CCL 34) were grown on glass coverslips to 50 to 70% confluency and were transfected with 5 μg of pcDNA3-NP, pcDNA3-NPΔ51, pcDNA3-NPΔ69, or pHMG-NP (encoding A/PR/8/34 NP), by using 30 μg of Pfx-2 lipofection reagent (Invitrogen) in serum-free Eagle’s minimal essential medium. Twenty-four or forty-eight hours after transfection the cells were fixed and permeabilized with −20°C absolute ethanol for 5 min and then analyzed by indirect immunofluorescence with a mouse anti-B virus NP MAb (MAS774b; Harlan Sera-lab) and an anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate. Samples were mounted with Mowiol 40-88 and 1,4-diazabicyclo[2.2.2]octane (Aldrich) and analyzed with a Zeiss Axioplan fluorescence microscope and a 100× oil immersion lens. The same localization of NP or the NP deletion mutants was observed if the cells were fixed with 3% (wt/vol) paraformaldehyde and permeabilized with 0.1% (vol/vol) Triton X-100 (data not shown).
influenza B virus, the genes encoding PA, PB1, and PB2 were cloned from B/AA/1/66 into pcDNA3.

The PA gene was amplified by reverse transcription from B/AA/1/66 viral RNA and PCR with oligonucleotides 5′ gecgeggaattGCCTAATGGGATATTTT 3′ and PA stop (5′ gecgeggaattCTCTCCATCTCATCCAT 3′), which anneal to residues 24 to 41 and 2199 to 2116, respectively. The PB1 gene was amplified with oligonucleotides PB1 start (5′ gecgeggaattTTAAGATGAATATATAATTCC 3′) and PB1 stop (5′ gecgeggaattCGAAGCTATATGTTGCCC 3′), which anneal to residues 16 to 35 and 2269 to 2286, respectively. Both the PA and PB1 RT-PCR products were cloned into EcoRI-SalI fragments into pcDNA3. We were unable to amplify the full-length PB2 gene from B/AA/1/66 viral RNA by RT-PCR; therefore, the gene was cloned in two halves, by making use of primers PB2 start (5′ gecgeggaattTTCAGAGATGACATTGG CC 3′ [anneals to residues 18 to 35]) and PB26 (5′ gecgeg-gaattTTCCTTCTCTGCC [1103 to 1118]) and primers PB27 (5′ gecgeggaattTTTATATTAGCTCAAGGC 3′ [2324 to 2342]). Oligonucleotides PB26 and PB27 introduce a silent change (G → A at position 1115) to generate an EcoRI site. The product of RT-PCR with PB2 start and PB26 was first cloned into the EcoRI site of pcDNA3, and then the PB27 and PB2 stop RT-PCR product was cloned into this plasmid on an EcoRI-SalI fragment.

To confirm that the cloned cDNAs for the B/AA/1/66 NP and P genes encode proteins of the expected size, in vitro translation reactions were performed with T7 transcripts from cloned cDNAs. PA, PB1, and PB2 were cotransfected into 293 cells and the cells were transfected with 0.5 μg of pcDNA3-PA, 0.5 μg of pcDNA3-PB1, and 1 μg of pcDNA3-NP by using 20 μg of Lipofectamine ( Gibco/BRL) in serum-free Eagle’s minimal essential medium (EMEM) according to the manufacturer’s instructions. At time zero and at 3, 6, 9, 12, and 24 h after transfection of the plasmids a separate mixture of 1 μg of HABCAT RNA and 20 μg of Lipofectamine was added. HABCAT RNA was synthesized in vitro in a 25-μl reaction mixture containing 1 μg of Hind-linearized pT3HABCAT (4), 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 8 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, the four dNTPs (1 mM each), 30 U of human placental RNase inhibitor, and 50 U of T3 RNA polymerase. After incubation at 37°C for 1 h, 2 U of RQ1 RNase-free DNase (Promega) was added to remove the template and the RNA was extracted with phenol–chloroform and precipitated with ethanol. After 24 h at 37°C the cells were supplemented with 1 ml of EMEM containing 10% heat-inactivated fetal calf serum. Forty-eight hours posttransfection the cells were harvested into 100 μl of 250 mM Tris-HCl (pH 7.5) and lyzed by freezing and thawing three times. Lysates (50 μl) were then processed for the detection of CAT as described elsewhere (21).

**FIG. 4.** Expression of HABCAT RNA in cells supplying PA, PB1, PB2, and NP in trans. Approximately 10⁶ 293 cells in 35-mm-diameter dishes were transfected with 0.5 μg of pcDNA3-PA, 0.5 μg of pcDNA3-PB1, 0.5 μg of pcDNA3-PB2, and 1 μg of pcDNA3-NP by using 20 μg of Lipofectamine ( Gibco/BRL) in serum-free Eagle’s minimal essential medium (EMEM) according to the manufacturer’s instructions. At time zero and at 3, 6, 9, 12, and 24 h after transfection of the plasmids a separate mixture of 1 μg of HABCAT RNA and 20 μg of Lipofectamine was added. HABCAT RNA was synthesized in vitro in a 25-μl reaction mixture containing 1 μg of Hind-linearized pT3HABCAT (4), 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 8 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine, the four dNTPs (1 mM each), 30 U of human placental RNase inhibitor, and 50 U of T3 RNA polymerase. After incubation at 37°C for 1 h, 2 U of RQ1 RNase-free DNase (Promega) was added to remove the template and the RNA was extracted with phenol–chloroform and precipitated with ethanol. After 24 h at 37°C the cells were supplemented with 1 ml of EMEM containing 10% heat-inactivated fetal calf serum. Forty-eight hours posttransfection the cells were harvested into 100 μl of 250 mM Tris-HCl (pH 7.5) and lyzed by freezing and thawing three times. Lysates (50 μl) were then processed for the detection of CAT as described elsewhere (21).

N-terminal deletions in influenza B virus NP do not affect the expression of HABCAT RNA. To determine if the N-terminal extension plays a role in the ability of NP to support the expression of a model influenza B virus RNA, 293 cells were transfected with HABCAT RNA, the polymerase, and plasmids for the expression of either NP, NPΔ51, or NPΔ69. Wild-type levels of CAT conversion were observed when NPΔ51 or NPΔ69 was supplied (Fig. 5). We also obtained approximately equal levels of CAT conversion when pcDNA3-NP, pcDNA3-NPΔ51, and pcDNA3-NPΔ69 were supplied at a range of suboptimal amounts while keeping the concentrations of the P clones and HABCAT RNA the same (data not shown). This indicates that the N-terminal extension of influenza B virus NP is not involved in the binding of NP to the RNA or the initiation and elongation of transcription.

**FIG. 5.** Expression of HABCAT RNA in cells supplying PA, PB1, PB2, and NP in trans. Approximately 10⁶ 293 cells in 35-mm-diameter dishes were transfected with 0.5 μg of pcDNA3-PA, 0.5 μg of pcDNA3-PB1, 0.5 μg of pcDNA3-PB2, and 1 μg of either pcDNA3-NP, pcDNA3-NPΔ51, pcDNA3-NPΔ69, pHMG-NP, or pcDNA3 by using 20 μg of Lipofectamine. Immediately after, a separate mixture of 1 μg of HABCAT RNA and 20 μg of Lipofectamine was added.

The influenza B virus plasmid-based system can be used to study the replication of viral RNA. It has been reported that cells which supply the influenza A virus NP and P proteins in
trans are capable of synthesizing viral RNA from a transfected model cRNA template (12). In order to determine if the cloned influenza B virus NP and P proteins can synthesize viral RNA, 293 cells were cotransfected with plasmids for the expression of the NP and P proteins and a synthetic RNA corresponding to the cRNA intermediate of HABCAT RNA replication. We were able to detect CAT activity in the transfected cells, at levels comparable to those achieved with negative-sense HABCAT RNA (Fig. 6). A low level of CAT activity could be detected if the HABCAT cRNA was cotransfected into cells with pcDNA3 in place of the NP and P plasmids, indicating that the RNA can be weakly translated. The ability to detect elevated levels of CAT in the cells supplying NP and P proteins suggests that viral RNA was synthesized from the input RNA and was subsequently transcribed to give mRNA. We observed that deletion of the N-terminal 51 or 69 residues of the NP did not affect its ability to support the replication of the transfected model RNA in this system (Fig. 6).

Influenza B virus NP cannot be functionally replaced by type A virus NP. We also investigated if the influenza A virus NP is capable of replacing the type B virus NP in the plasmid-based system by supplying pHMG-NP in place of pcDNA3-NP. Plasmid pHMG-NP encodes the A/PR8/34 NP and has been used to drive the expression of an influenza A virus model RNA in the plasmid-based system described by Pleschka et al. (30). No CAT activity was detected in the transfection by using pHMG-NP, the influenza B virus P clones, and either HABCAT RNA (Fig. 5) or the cRNA intermediate of HABCAT replication (Fig. 6), indicating that the influenza A virus NP cannot form functional RNP complexes with the B virus polymerase proteins and a model influenza B virus RNA. It is known, however, that the four influenza A virus core proteins can form functional RNPs with a model influenza B virus RNA, whether this complex is reconstituted in vitro (19, 24) or in vivo (13). The finding that type A and B virus RNPs are not interchangeable is consistent with the observations of Jambrina et al. (13) and indicates that there are type-specific interactions between NP and the P proteins that are essential for the expression and replication of the virus genome. This notion is supported by the finding that natural reassortment of the NP and P genes of influenza A and B viruses is not observed (14, 23). We consider this surprising, as the sequences of the type A and B virus RNPs have 37.7% identity and 76.2% similarity over a 496-amino-acid overlap. It seems unlikely that the N-terminal extension of influenza B virus NP is involved in type-specific interactions with the type B virus P proteins, since its removal does not affect the activity of the RNP complex.

It is possible that the N-terminal extension of type B virus NP has a role in the specific incorporation of vRNPs into influenza B virus particles. It has been reported that, while type A and B virus RNPs may exist in the same RNP complex in vivo, these phenotypically mixed forms are not incorporated into virions (33). Since the RNPs of type A and B viruses differ most at their N termini, the N-terminal extension may be involved in the selection of RNPs containing only type B virus NP.

The type-specific nature of the interaction of NP with the P proteins may be influenced by differences in the posttranslational processing of the influenza A and B virus RNPs. It is known that influenza A virus NP is modified by phosphorylation (2, 16) and by proteolytic cleavage (36). The extent of these modifications varies with the virus strain, the cell line on which the virus is grown, and the phase of the replication cycle (16, 36, 37). The type B virus NP is also proteolytically cleaved, but in a manner distinct from that in which influenza A virus RNPs are cleaved (37).

Posttranslational processing of the NP may also modulate the nuclear import and export of the protein. There is evidence that the nucleocytoplasmic shuttling of the influenza A virus NP may be controlled by phosphorylation, since protein kinase inhibitor H7 causes the redistribution of NP (expressed in the absence of other virus proteins) from the cytosol to the nucleus (25). So far nothing is known of the sites and extent of phosphorylation of type B virus NP, or whether the proteolytic processing of NP is relevant to its activity. The plasmid-based system described here may prove useful in assessing the importance of posttranslational modifications of the NP, and in identifying those regions of the influenza B virus NP that are involved in type-specific interactions with the P proteins.

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