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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.
control of the human cytomegalovirus immediate-early promoter/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′gccggaagtATCAGAAATGGAAGGGGACACCACAGC3′ 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′gccggaagtATCAGAAATGGAAGGGGACACCACAGC3′ and NPstop, giving pcDNA3-NPΔ69. We also deleted further into the protein (residues 2 to 82) by PCR with oligonucleotide 5′gccggaagtATCAGAAATGGAAGGGGACACCACAGC3′ and NPstop, giving pcDNA3-NPΔ82. Proteins of the expected size were synthesized in vivo translation reactions with T7 run-off transcripts from Smal-linearized pcDNA3-NP, pcDNA3-NPΔ51, pcDNA3-NPΔ69, and pcDNA3-NPΔ82 (Fig. 2A).

N-terminal deletions in influenza B virus NP do not affect nuclear accumulation. Plasmids for the expression of NP, NPΔ51, NPΔ69, and NPΔ82 were transfected into MDCK cells, and the proteins were visualized by indirect immunofluorescence with a mouse anti-B virus NP monoclonal antibody (mAb) and an anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate. We were unable to detect NPΔ82 with either of two different anti-B virus NP mAbs (data not shown), which may indicate that the protein is unstable when expressed in mammalian cells. In cells fixed 24 h posttransfection, NP, NPΔ51, and NPΔ69 were found to accumulate mostly in the nucleus (Fig. 3). However, in cells fixed 48 h posttransfection NP, NPΔ51, and NPΔ69 were distributed mostly in the cytosol. This pattern was also observed upon transfection of plasmid pHMG-NP, which contains the influenza A/PR/8/34 virus NP gene under the control of a mouse hydroxymethylglutaryl-coenzyme A reductase promoter (kindly supplied by J. Pavlovic, University of Zürich, Zürich, Switzerland) (Fig. 3), and is consistent with observations that NP expressed in the absence of other virus proteins is capable of shuttling between the nucleus and cytosol (25, 35).

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 separately by Neumann et al. (25) to residues 1 to 38. In both cases, the N-terminal residues have been shown to possess NLS activity, since they can target a normally cytoplasmic protein to the nucleus (25, 34). However, the authors of both reports concede that mutants lacking part or all of the NLS still enter the nucleus, indicating that other, perhaps weaker, NLSs exist in 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. 1. Alignment of the amino acid sequences of the influenza A/PR/8/34 and B/AA/1/66 virus NPs at the N-termini. The alignment was generated with the FASTA program (Genetics Computer Group, University of Wisconsin) and predicts 37.7% identity and 76.2% similarity of the proteins in a 496-amino-acid overlap. The N-terminal deletions in the type B virus NP made in this study, vertical lines denote amino acid identity, and colons refer to conservative changes.

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the influenza B/AA/1/66 virus NP and NP deletion mutants (A) and of influenza B/AA/1/66 virus polymerase proteins (B) synthesized in vitro. Rabbit reticulocyte lysates (Promega) were primed with 50 ng of T7 transcripts from Smal-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.
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/834 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 Axiosvert 135 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 PAsstart (5' gcgegcgaattGCCCATATGGAATATTTT 3') and PAsstop (5' gcgegcgtgacTTCTTCTTTCACTCATCATCAT 3'), which anneal to residues 24 to 41 and 2199 to 2116, respectively. The PB1 gene was amplified with oligonucleotides PB1sstart (5' gcgegcgaattTTTAAGATGAAATAATAC 3') and PB1stop (5' gcgegcgtgacGAAAGCTTATATGTGCCC 3'), which anneal to residues 16 to 35 and 2269 to 2286, respectively. Both the PA and PB1 RT-PCR products were cloned on 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 PB2start (5' gcgegcgaattTCTCAAGATGACATTTGGCC 3' [anneals to residues 18 to 35]) and PB2stop (5' gcgegcGAAAttTCTCTTCTTCCC 3' [1103 to 1118]) and primers PB2start (5' gcgegcGaattTTCAAGATGACATTTGGCC 3' [3234 to 3242]). Oligonucleotides PB26 and PB27 introduce a silent change (G→A at position 1115) to generate an EcoRI site. The product of RT-PCR with PB2start and PB2stop was first cloned into the EcoRI site of pcDNA3, and then the PB2 and PB2stop 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 Smal-linearized pcDNA3-NP, pcDNA3-PA, pcDNA3-PB1, and pcDNA3-PB2. With the exception of PB2, the electrophoretic mobilities of the proteins correlated with their predicted molecular weights (Fig. 2B). The PB2 protein migrated at a rate below that expected on the basis of its predicted molecular weight; however, this has also been noted for the PB2 protein of B/Panama/45/90 (13). Proteins corresponding in size to the influenza B/AA/1/66 virus NP and P proteins were approximately equal levels of CAT conversion when pcDNA3-NP, pcDNA3-NP, pcDNA3-NP, and pcDNA3-PB1 were cotransfected into 293 cells and the cells were transfected at intervals thereafter with a synthetic influenza B virus PB2 were cotransfected into 293 cells and the cells were transmids pcDNA3-NP, pcDNA3-PA, pcDNA3-PB1, and pcDNA3-PB2. With the exception of PB2, the electrophoretic mobilities of the proteins correlated with their predicted molecular weights (Fig. 2B). The PB2 protein migrated at a rate below that expected on the basis of its predicted molecular weight; however, this has also been noted for the PB2 protein of B/Panama/45/90 (13). Proteins corresponding in size to the influenza B/AA/1/66 virus NP and P proteins were approximately equal levels of CAT conversion when pcDNA3-NP, pcDNA3-PA, pcDNA3-PB1, and pcDNA3-PB2 were cotransfected into 293 cells and the cells were transfected at intervals thereafter with a synthetic influenza B virus RNA (HABCAT). The HABCAT RNA is based on segment 4 encoded in a preparation of purified radiolabeled B/AA/1/66 viral RNA and PCR with oligonucleotides PA and PB1 RT-PCR products were cloned on EcoRI-SalI site. The product of RT-PCR with PB2start and PB2stop was first cloned into the EcoRI site of pcDNA3, and then the PB2 and PB2stop RT-PCR product was cloned into this plasmid on an EcoRI-SalI fragment.

To test the activities of the cloned NP and P proteins, plasmids pcDNA3-NP, pcDNA3-PA, pcDNA3-PB1, and pcDNA3-PB2 were cotransfected into 293 cells and the cells were transfected at intervals thereafter with a synthetic influenza B virus RNA (HABCAT). The HABCAT RNA is based on segment 4 encoded in a preparation of purified radiolabeled B/AA/1/66 viral RNA and PCR with oligonucleotides PA and PB1 RT-PCR products were cloned on EcoRI-SalI site. The product of RT-PCR with PB2start and PB2stop was first cloned into the EcoRI site of pcDNA3, and then the PB2 and PB2stop RT-PCR product was cloned into this plasmid on an EcoRI-SalI fragment.

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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

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 clones, and the hemagglutinin coding region (4). We observed that chloroamphenicol acetyltransferase (CAT) activity could be detected if the cells were transfected with the plasmids and the RNA at the same time, but not if the RNA was added three or more hours after the plasmids (Fig. 4). The ability to detect CAT indicates that the transcribed RNA was reconstituted intracellularly into functional RNPs capable of synthesizing mRNA. The level of CAT activity obtained on transfection of the HABCAT RNA into cells supplying NP and the P proteins in trans was comparable to the levels achieved on transfection of the naked RNA into helper virus-infected cells (data not shown). The amounts of the model RNA and plasmids for the expression of NP and P proteins which yielded the highest levels of CAT conversion were pcDNA3-PA, 0.5 μg; pcDNA3-PA, 0.5 μg; pcDNA3-PB1, 0.5 μg; pcDNA3-PB2, 0.5 μg; pcDNA3-NP, 1 μg; and HABCAT RNA, 1 μg. No CAT activity was detected if the HABCAT RNA alone was transfected, or if any one of the four plasmids was omitted (data not shown). This is consistent with the observation by Jambrina et al. (13) that NP and the three P proteins are the minimum set of influenza B virus proteins required for the expression of a model RNA.

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 Hpal-linearized t3HABCAT (4), 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 8 mM MgCl₂, 2 mM dithiotreitol, 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 lysed by freezing and thawing three times. Lysates (50 μl) were then processed for the detection of CAT as described elsewhere (21).
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/PR/8/34 NP and has been used to drive the expression of an influenza A virus model RNA in the plasmid-based system described by Pleshchka 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 NP

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. This work was supported by a grant to W.S.B. from the Medical Research Council, United Kingdom (G9508170).

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FIG. 6. Replication of HABCAT RNA is not affected by removal of the N-terminal extension of influenza B virus NP. In order to synthesize the cRNA intermediate of HABCAT replication, the HABCAT cDNA was cloned under a T3 promoter in the reverse orientation to that in pT3HABCAT (4) by PCR with oligonucleotides 5′-gggcaatgaaagcagatc3′ and 5′-gggccgattcttaacctactaaaaAGCGAGGACAGGC 3′. 293 cells 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 cRNA and 20 μg of Lipofectamine was added.