JOURNAL OF VIROLOGY, Sept. 1983, p. 642–648 Vol. 47, No. 3
0022-538X/83/090642-07$02.00/0
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Complete Nucleotide Sequence of the Nucleoprotein Gene of Influenza B Virus

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Received 6 April 1983/Accepted 3 June 1983

A DNA copy of influenza B/Singapore/222/79 viral RNA segment 5, containing the gene coding for the nucleoprotein (NP), has been cloned in *Escherichia coli* plasmid pBR322, and its nucleotide sequence has been determined. The influenza B NP gene contains 1,839 nucleotides and codes for a protein of 560 amino acids with a molecular weight of 61,593. Comparison of the influenza B NP amino acid sequence with that of influenza A NP (A/PR/8/34) reveals 37% direct homology in the aligned regions, indicating a common ancestor. However, influenza B NP has an additional 50 amino acids at its N-terminal end. As is the case with influenza A NP, influenza B NP is a basic protein, with its charged residues relatively evenly distributed rather than clustered. The structural homology suggests functional similarity between the NP of influenza A and B viruses.

Influenza viruses are classified into type A, type B, or type C viruses based on the lack of serological cross-reactivity between their internal components, particularly nucleoprotein (NP) and matrix (M) proteins (39). All virus isolates belonging to a single type possess the cross-reactive M and NP proteins, although their surface antigens, hemagglutinin (HA) and neuraminidase (NA), may vary drastically. In addition, there are major differences in the epidemiology and host range among these three types. For example, only type A viruses, although species specific, can infect and produce disease both in humans and in various animal species in nature. Type B and type C viruses, on the other hand, are primarily restricted to humans (see reference 21), although occasionally they have been isolated from animal hosts (41). Type A viruses are known to undergo both antigenic shift and drift, whereas B and C viruses are subject only to antigenic drift. Furthermore, the antigenic variation observed among type A viruses is more pronounced than that observed in type B viruses, which in turn undergo more variation than type C viruses (20, 21). Additionally, genetic exchange yielding stable viruses of mixed genotypes has not been observed to occur between viruses of different types (14).

The molecular basis of these biological differences between the three types remains undefined. Clearly, an understanding of the structure of the genes as well as the structure and function of the gene products of all three types of viruses will be required before their differences in epidemiology and species specificity can be understood. Recently, cDNA cloning and DNA sequencing have permitted the determination of complete nucleotide sequences and predicted amino acid sequences of protein products of all eight RNA segments from one or more type A strains (see reference 34). Similarly, sequences of the HA, NA, M, and nonstructural (NS) genes of type B viruses have been determined (3, 4, 16, 28, 35). However, little is known about the structure or function of the influenza B NP (B NP), although the primary structure of the NP gene of two influenza A strains has been determined (12, 33, 36).

In both A and B types, NP appears to have a multifunctional role. First, it is the major component of the helical ribonucleoprotein (RNP) core (24), and it has been suggested that each influenza A NP (A NP) molecule may bind to ca. 20 bases (b) of RNA (5). Second, the RNP complexes in association with the polymerase proteins are active in transcription (2, 26). Temperature-sensitive mutants of influenza A virus defective in the NP gene fail to synthesize mRNA or virion RNA (vRNA) in infected cells at nonpermissive temperatures (15, 30, 31). Also, monoclonal antibodies to A NP inhibit viral transcription in vitro, suggesting that NP may be involved in the process of transcription (40). Furthermore, NP may be important in binding the RNP complex to M protein and thus, may have an important role in the process of budding and virion assembly (23). Evidence of intracistronic complementation between temperature-sensitive mutants defective in the NP gene also supports its multifunctional character

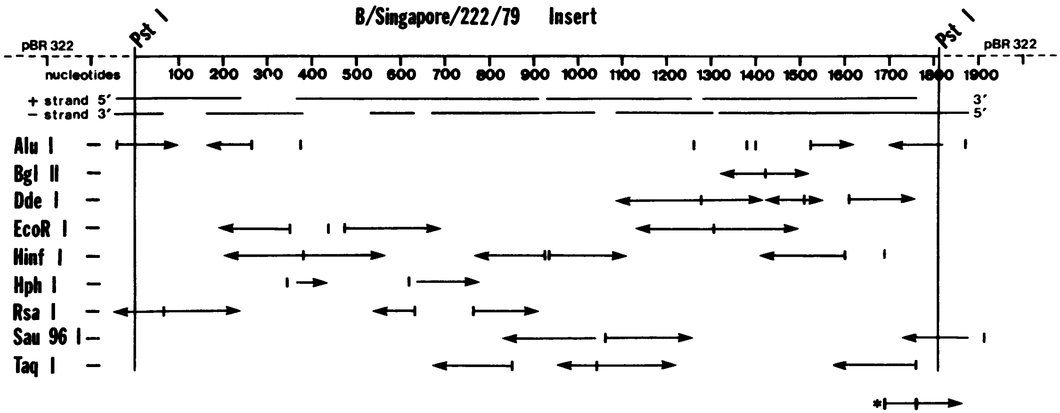

FIG. 1. Endonuclease restriction enzyme cleavage map and strategy used for sequencing of the cloned B/Sing NP DNA. The insert DNA was characterized by multiple restriction endonuclease digestions, mapping the sites relative to one another. DNA sequencing was carried out by using 5' singly end-labeled fragments by the methods of Maxam and Gilbert (18, 19). Approximately 85 to 90% of both strands were sequenced, and all restriction sites used in sequencing were sequenced through an intact DNA segment cut at a different position. The sequence at the 5' end of the viral RNA segment was determined by using a primer extension procedure (11). Total influenza B vRNA was hybridized to a 5' singly end-labeled DNA fragment of clone B1-28. This primer was extended by reverse transcriptase, and the resulting cDNA was purified and sequenced. The solid line along the top shows the cloned insert, with the broken line representing sequences from pBR322. Listed along the left side are the restriction endonuclease sites which were used for labeling with T4 polynucleotide kinase, as indicated by vertical bars, and sequenced in the direction of the arrows. Both strands are also represented showing their overlapping sequenced regions. The asterisk shows the *HinF* I to *TaqI* fragment used in the primer extension.

(31). In this report, we present the complete nucleotide sequence of the NP gene and the predicted amino acid sequence of the NP polypeptide of a type B influenza virus (B/Singapore/222/79 [B/Sing]) and a comparison of the sequence homology and diversity with type A NP.

Virus from the strain B/Sing was obtained from Alan Kendal, Centers for Disease Control, and grown in 10-day-old embryonated chicken eggs. Virus-specific genomic RNA was isolated from purified virions, enriched for specific RNA segments by fractionation in sucrose velocity gradients, and used for cDNA cloning (7).

The genes of influenza B/Sing were cloned into the *PstI* restriction site of pBR322 by procedures previously described (7, 13). Briefly, the vRNA enriched in the 4th and 5th largest segments was reverse transcribed by using a synthetic dodecanucleotide primer (5'-dAGCAGAAGCAGA-3') complementary to the common 3' ends of influenza B vRNA segments (9). Full length cDNA copies were isolated on 1.4% alkaline agarose gels and converted into double-stranded DNA by using the foldback loop at the 3' end as a self-primer. The double-stranded DNA fragments were treated with S1 nuclease, size fractionated on neutral agarose gels, and cloned into the *PstI* site of pBR322 with deoxyguanine:deoxycytosine tailing. *Escherichia coli* 294 was transformed with the recombinant plasmids and screened for tetracycline resistance and ampicillin sensitivity.

Cloned DNAs were grouped by insert size and restriction enzyme analyses (7). Groups were identified by hybridization to specific vRNA segments. Clone B1-28, containing an insert of ca. 1.8 kilobases (kb), was identified as a clone of the B NP gene on the following basis. (i) It hybridized exclusively to segment 4 vRNA which has been shown to code for NP (22); (ii) segment 5 vRNA, on the other hand, known to code for the HA gene (22, 32) hybridized exclusively to DNA of clones B1-61 and B2-6 and not to the B1-28 DNA; and (iii) neither B1-28, B1-61, nor B2-6 hybridized to the vRNAs of segments 1, 2, 3 (polymerase genes), or 6, known to code for the NA gene (22, 32). Additionally, the nucleotide sequence of B1-61 insert DNA (unpublished data) is over 90% homologous to the reported sequence of the HA gene of influenza B/Lee/40 (16).

A partial restriction endonuclease cleavage map of the cloned B NP gene was determined experimentally and is shown in Fig. 1. This served as the basis for preparing 5' end-labeled fragments used in DNA sequencing.

The complete nucleotide sequence of the mRNA sense strand of the B NP gene is shown in Fig. 2. The first four nucleotides from the

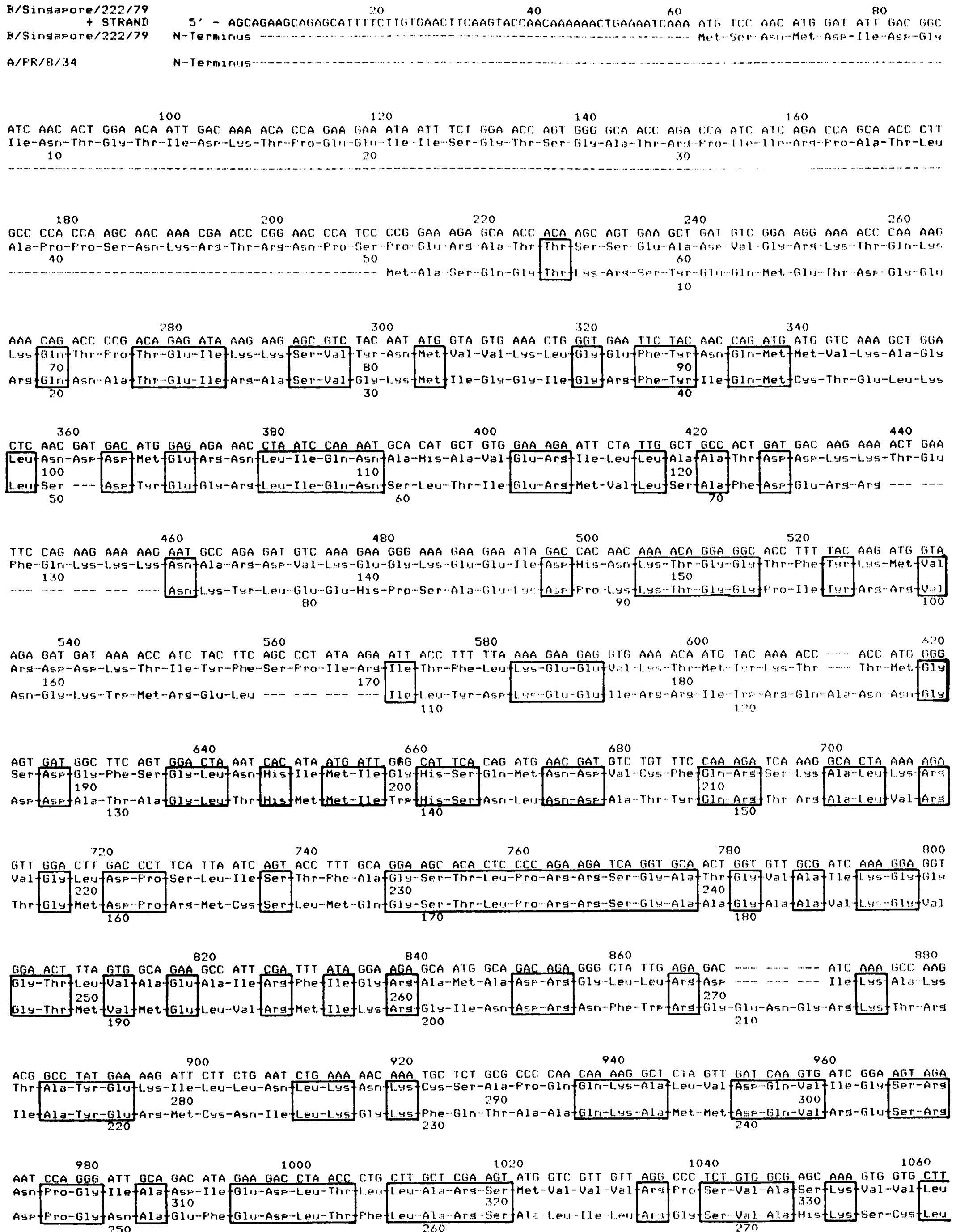

FIG. 2

synthetic dodecanucleotide primer used to reverse transcribe the virus-specific RNA were missing from the cloned DNA, presumably lost during manipulation of the transcribed DNA. The sequence from nucleotides 5 to 1,756 was obtained from the insert of clone B1-28, which represents the complete coding region for the NP polypeptide. The sequence from nucleotides 1,737 to 1,839 was obtained by the primer extension of a DNA fragment (nucleotides 1,664 to

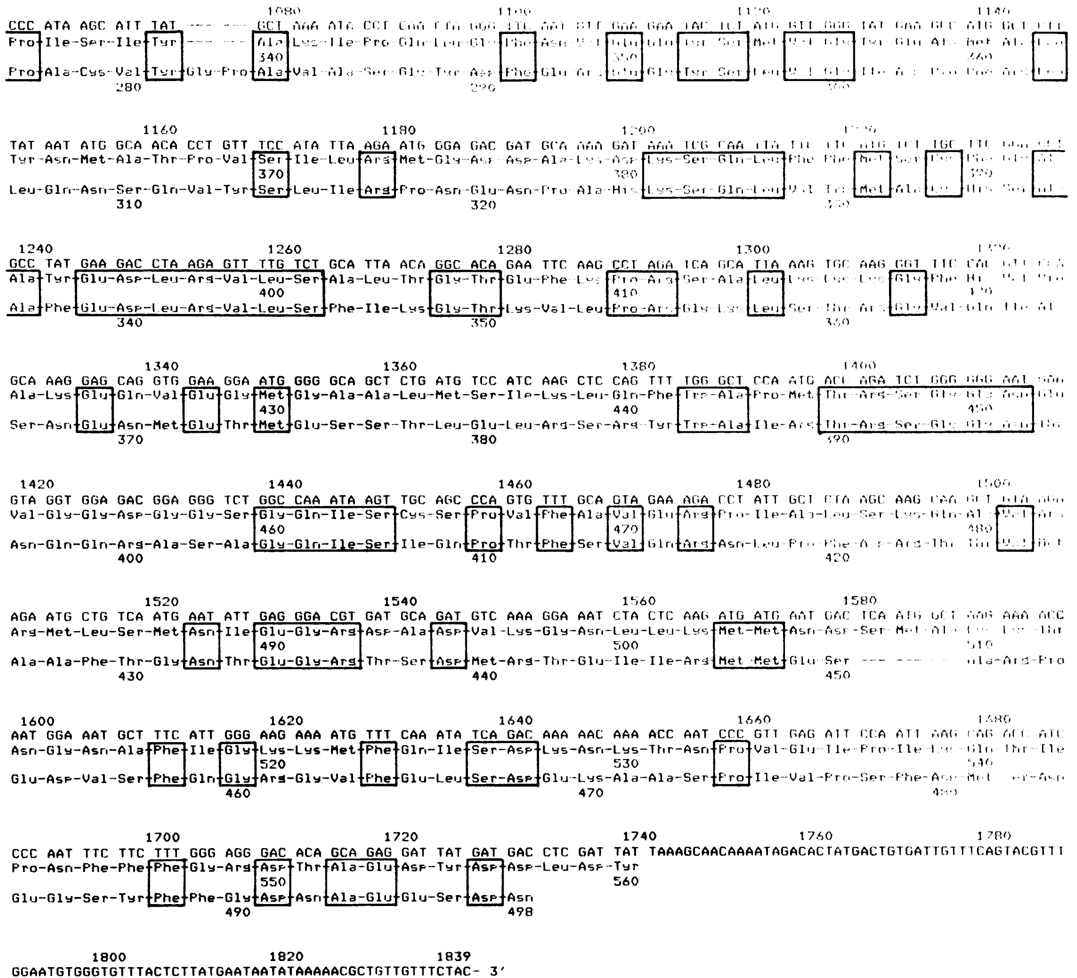

FIG. 2—*Continued*

FIG. 2. Complete nucleotide sequence of the NP gene (plus-sense strand) and the deduced amino acid sequence of the NP polypeptide of influenza B/Sing. The first 12 nucleotides represent the synthetic primer used to reverse transcribe the virus-specific RNA. Nucleotides are numbered from the 5′ end of the plus-strand. Also shown in alignment with B NP is the amino acid sequence of A/PR/8/34 NP (36). Boxes are drawn to show regions of direct amino acid homology. Dashes indicate where deletions were placed to bring the two sequences into maximum alignment. The precise location of substitution, addition, or deletion of nucleotides is not known, therefore, amino acid deletions were placed in regions only to satisfy the criteria of maximum amino acid homology. Both amino acid sequences are numbered starting from their N-terminal end.

1,732) labeled at the 5′ end. The B NP gene segment contains 1,839 nucleotides and has an open reading frame from the first initiation codon (AUG) at position 60 to 62 to a termination codon (UAA) at position 1,740 to 1,742. This open reading frame of 1,680 nucleotides could code for a polypeptide of 560 amino acids, leaving 97 and 59 noncoding nucleotides at the 3′ and 5′ ends, respectively, of the plus-sense DNA strand. There are no other open reading frames in either strand which could code for more than 125 amino acids. A tract of five adenine residues at position 1,819 to 1,823 probably represents the polyadenylation site for the virus mRNAs as has been reported for influenza A viruses (25). The B NP gene has a calculated base composition of 35.2% uridine, 22.3% adenine, 22.9% cytosine and 19.6% guanine, reflecting the fact that adenine is more commonly used in the third codon positions than guanine. The frequency of the cytosine-guanine dinucleotide as well as the use of cytosine-guanine-containing codons is low, as is commonly seen in other virus and eukaryotic genes (29).

TABLE 1. Amino acid composition of A and B NP

Amino acids	Frequency (%)		
	B/Sing (%)	A/PR/8/34 (%)[a]	Avg protein (%)[a]
Ala	46 (8.2)	39 (7.8)	(8.6)
Arg	31 (5.5)	49 (9.8)	(4.9)
Asn	27 (4.8)	25 (5.0)	(4.3)
Asp	34 (6.1)	23 (4.6)	(5.5)
Cys	5 (0.9)	6 (1.2)	(2.9)
Gln	18 (3.2)	21 (4.2)	(6.0)
Glu	29 (5.2)	36 (7.2)	(3.9)
Gly	45 (8.0)	41 (8.2)	(8.4)
His	5 (0.9)	6 (1.2)	(2.0)
Ile	38 (6.8)	26 (5.2)	(4.5)
Leu	38 (6.8)	32 (6.4)	(7.4)
Lys	50 (8.9)	21 (4.2)	(6.6)
Met	27 (4.8)	25 (5.0)	(1.7)
Phe	22 (3.9)	18 (3.6)	(3.6)
Pro	25 (4.5)	17 (3.4)	(5.2)
Ser	38 (6.8)	39 (7.8)	(7.0)
Thr	35 (6.3)	29 (5.8)	(6.1)
Trp	1 (0.2)	6 (1.2)	(1.3)
Tyr	13 (2.3)	15 (3.0)	(3.4)
Val	33 (5.9)	24 (4.8)	(6.6)
Total	560 (100)	498 (100)	(100)

[a] Amino acid compositions of A/PR/8/34 NP and an average protein were obtained from Winter and Fields (36) and Dayhoff et al. (8), respectively.

The B/Sing NP gene is 274 nucleotides longer than the A NP gene (1,839 versus 1,565; see reference 36). These extra nucleotides are present in both the 5′ noncoding (97 b versus 45 b) and the 3′ noncoding (59 b versus 26 b) regions as well as in the coding region (1,680 b versus 1,494 b) of the B NP gene. The sequence data show that RNA segments of influenza B virus, excluding the three unstudied polymerase RNA segments, are all larger than the corresponding RNA segments of influenza A virus. However, since these extra sequences are present in most cases in both coding and noncoding regions, their significance remains unclear. These data also show that the segment 4 RNA of B/Sing encodes the NP gene and, therefore, agree with the gene assignment of three other influenza B strains, namely B/Lee/40, B/Mass/1/71, and B/Md/59 (24). Furthermore, our data also show that the B/Sing NP gene (1,839 nucleotides) is smaller than the B/Lee HA gene (1,882 nucleotides) as was predicted by the migration pattern of glyoxalated RNAs (22).

TABLE 2. Amino acid homology between regions of B NP and A/PR/8 NP[a]

Amino acid region of B NP	% Homology to A/PR/8 NP
51–150	31.5
151–250	45.8
251–350	46.0
351–450	38.0
451–550	25.8

[a] The sequence homology was determined for every 100 amino acids beginning with amino acid residue 51 (Fig. 2). Since amino acids corresponding to the N-terminal 50 amino acids of B/Sing NP are not present in A/PR/8 NP, they are not included in the calculation. Also unused were amino acids corresponding to areas of addition or deletion created to obtain optimum alignment of the two amino acid sequences.

The predicted B/Sing NP polypeptide is 560 amino acids in length (Fig. 2), with a calculated M_r of 61,593, which is slightly less than the 66,000 estimated in gels (22). Of the 560 amino acids of B NP, 86 residues are basic (Arg, 31; Lys, 50; His, 5) and 63 residues are acidic (Asp, 34; Glu, 29; Table 1). B NP is a basic protein with a net charge of +20.5 at pH 7.0, assuming a charge of +1 for each arginine and lysine, −1 for each glutamic acid and aspartic acid, and +0.5 for each histidine, and, therefore, possesses a greater net positive charge than A NP, with a charge of +14 (36). The amino acid composition (Table 1) shows that B NP is remarkably rich in lysine and methionine but poor in histidine, cysteine, and tryptophan when compared with that of a statistically average protein (8). When compared with A/PR/8/34 NP (36), the B NP polypeptide is 62 amino acids longer than A NP (Fig. 2). Seven regions of deletion or addition or both of amino acids were required for optimum alignment of the two sequences, leaving influenza B NP with an extra three residues at its C-terminal end and 50 residues at its N-terminal end. The amino acid composition (Table 1) shows that both A and B NP are rich in basic amino acids and in methionine but low in cysteine as expected for soluble globular proteins. Only one of the cysteine residues appears conserved between B NP (amino acid 389) and A NP (amino acid 333), supporting the finding of Selimova et al. that disulfide bonds are not important in the formation of the secondary structure of NP (27). This is in sharp contrast to the conservation of cysteine residues between influenza A and B viruses in the HA and NA proteins (A. R. Davis, T. J. Bos, and D. P. Nayak, Proc. Natl. Acad. Sci., in press; 16, 28). The distribution and composition of basic amino acids are different between A and B NP. For example, B NP is rich in lysine and low in arginine, whereas the reverse is true for A NP. Both A and B NPs are low in histidine, and B NP contains only one tryptophan residue. It remains to be seen whether this divergence in the composition of basic amino acids between B and A NP has any structural or functional significance or provides the basis for any differences in biologi-

cal specificity. As with A NP, B NP does not possess large clusters of charged residues. Rather, these are evenly distributed along the length of the polypeptide, suggesting multiple contact points between each NP molecule and the viral RNA. Since B NP has a greater net positive charge than A NP, it may bind more tightly to the negatively charged vRNA. Relative nuclease sensitivity of B RNP versus A RNP may determine whether the greater positive charge in B NP provides an increased protection of viral RNA segments by B NP.

Comparison of the amino acid sequence homology between B and A NP shows that some parts of the sequence are remarkably conserved, whereas other areas are quite divergent. The conservation of sequence is most striking in the central region of the protein (amino acid 150 to 350) which contains nearly 50% direct homology (Table 2). This region also contains a direct 10 amino acid residue homology (amino acid 230 to 239; B NP) between A and B NPs. This homology is remarkable because such a large stretch of homology is not present even in the highly conserved hydrophobic region at the N terminus of HA 2 of A and B HAs (16, 37). This region then may provide some critical structure for the function of both A and B NP, such as a nucleation point in RNP formation or a critical contact with RNA or with M or polymerase (P) proteins. The entire aligned region (amino acids 51 to 557) contains 37% direct homology (184/492 residues). Since 43% of the differences (133/308 residues) are of conservative nature, i.e., basic, acidic, polar, or nonpolar amino acid replaced by similar residue, the combined structural homology between A and B NP is 64%. The most remarkable difference in the primary structure of A and B NP is the presence of 50 additional amino acids at the N terminus (Fig. 2), making it unique in that respect among the influenza virus proteins, except for influenza B NS1, which is 51 amino acids longer than influenza A NS1 (1, 38). Whether these extra sequences are involved in providing functional type specificity remains to be seen.

The proteins coded by the five genes (HA, NP, NA, M, and NS) of influenza B virus that have been completely sequenced show that influenza B proteins possess structural features similar to those of influenza A viral proteins, attesting for their functional similarity. Among these viral proteins, NP shows the greatest amino acid homology between the types. However, diversity in the sequence between influenza A and B proteins is far greater than that observed among the corresponding proteins of influenza A virus strains, suggesting that influenza A and B viruses have undergone similar but independent evolutionary pathways and that none of these five RNA segments have been recently acquired by reassortment between influenza A and B viruses. Since stable recombinants between A and B viruses have not been observed even under laboratory conditions (14), it is likely that these viral proteins are functionally incompatible with the genes and gene products of another type. Furthermore, since envelope proteins can form pseudotypes with divergent viruses (6), the functional type specificity is likely to be provided by the internal components such as polymerases and NPs, etc. Since active influenza proteins, including NPs, now can be expressed from cloned cDNAs in eukaryotic cells (17), the function of these proteins as well as chimeric proteins (constructed by joining two or more DNAs) can now be tested by intertype complementation and should provide insight into their functional specificity and incompatibility with each other.

We thank N. Sivasubramanian for his helpful discussion and advice.

This paper was supported by grant PCM 7823220 from the National Science Foundation, by Public Health Service grant RO1 AI16348 from the National Institute of Allergy and Infectious Diseases, and by a biological research support grant BRSG RR 05304 from the National Institutes of Health.

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