Phylogenetic analysis of the three polymerase genes (PB1, PB2 and PA) of influenza B virus

Yasuaki Hiromoto,1 Takehiko Saito,1 Stephen E. Lindstrom,1 Yixing Li,1 Reiko Nerome,1 Shigeo Sugita,2 Masayoshi Shinjoh1 and Kuniaki Nerome1

1 Department of Virology I, National Institute of Infectious Diseases, 23-1, Toyama 1-chome, Shinjuku-ku, 162-8640 Tokyo, Japan
2 Epizootic Research Station, Equine Research Institute, Japan Racing Association, 1400-4 Shiba, Kokubunji-machi, Shimotsuga, 329-04 Tochigi, Japan

Phylogenetic patterns of the three polymerase (PB2, PB1 and PA) genes of a total of 20 influenza B viruses isolated during a 58 year period, 1940–1998, were analysed in detail in a parallel manner. All three polymerase genes consistently showed evolutionary divergence into two major distinct lineages and their amino acid profiles demonstrated conserved lineage-specific substitutions. Dendrogram topologies of the PB2 and PB1 genes were very similar and contrasted with that of the PA gene. It was of particular interest to reveal that even though the PA gene evolved into two major lineages, that of three recent Asian Victoria/1/87-like strains formed a branch cluster located in the same lineage as that of recent Yamagata/16/88-like isolates. Differences in the phylogenetic pathways of three polymerase genes were not only a reflection of genetic reassortment between co-circulating influenza B viruses, but also an indication that the polymerase genes were not co-evolving as a unit. As a result, comparison of the phylogenetic patterns of the three polymerase genes with previously determined patterns of the HA, NP, M and NS genes of 18 viruses defined the existence of eight distinct genome constellations. Also, similar phylogenetic profiles among the PA, NP and M genes, as well as between the PB2 and PB1 genes, were observed, suggesting possible functional interactions among these proteins. Completion of evolutionary analysis of the six internal genes and the HA gene of influenza B viruses revealed frequent genetic reassortment among co-circulating variable strains and suggested co-dependent evolution of genes.

Introduction

Influenza type A and type B viruses are included in the family Orthomyxoviridae based on biological, antigenic, genetic and structural similarities. However, they vary in their host range, genetic coding strategies and evolutionary patterns (Lamb & Krug, 1996; Murphy & Webster, 1996). In contrast to influenza A viruses, which infect a variety of animals and are divided into several antigenic subtypes, influenza B virus has not been found to naturally infect animals other than humans and is not divided into antigenic subtypes. However, evolutionary analyses of the HA gene of influenza B virus have characterized this gene to have evolved into two antigenically distinct lineages, including B/Yamagata/16/88-like and B/Victoria/2/87-like viruses (Kanegae et al., 1990; Lindstrom et al., 1999; Nerome et al., 1998; Rota et al., 1992, 1990). Previous reports on the evolutionary characteristics of the NP, M and NS genes of influenza B virus isolates have demonstrated multiple lineages (Jambrina et al., 1997; Lindstrom et al., 1999; Yamashita et al., 1988) and frequent reassortment among co-circulating viruses resulting in marked protein variability (Lindstrom et al., 1999). Although characterization of the phylogenetic patterns of human influenza A H3N2 virus genes has also provided evidence for co-circulation of minor branch clusters and reassortment among co-circulating strains (Lindstrom et al., 1998b; Xu et al., 1996), protein variability resulting from reassortment of the internal genes is less pronounced than with influenza B viruses as these genes were shown to evolve essentially in a single major lineage (Buonagurio et al., 1986; Ito et al., 1991; Lindstrom et al., 1998b; Shu et al., 1993; Xu et al., 1996).
The functional ribonucleoprotein (RNP) complex of influenza viruses responsible for transcription and replication of viral RNA exists as a heterotrimer polymerase complex consisting of the PB2, PB1 and PA proteins (Detjen et al., 1987; Digard et al., 1989; Honda et al., 1990; Kato et al., 1985) bound to viral RNA and the nucleoprotein (NP) (Jambrina et al., 1997; Krug et al., 1989). Extensive analysis of protein subunits of the RNP complex of type A viruses has resulted in a fairly detailed understanding of the functional role of each subunit. Briefly, the PB1 protein has been shown to possess RNA polymerase catalytic activity (Biswas & Nayak, 1994; Braam et al., 1983; Kobayashi et al., 1996; Nakagawa et al., 1996; Toyoda et al., 1996b), whereas the PB2 protein is responsible for the binding, cleavage and recruitment of cellular mRNA cap-1 structures essential for viral mRNA synthesis (Braam et al., 1983; Nakagawa et al., 1995; Ulmanen et al., 1983). Although the PA protein has been shown to be essential in influenza virus gene expression, its function has not been fully elucidated. However, experiments have provided evidence indicating that the PA protein is required for switching from mRNA synthesis to cRNA synthesis during virus replication (Mahy, 1983; Nakagawa et al., 1996) and the ability to induce generalized proteolysis has been demonstrated (Sanz-Ezquerro et al., 1995, 1996).

It is generally believed that the functional roles of the PB2, PB1 and PA proteins of type B viruses are essentially similar to those of type A viruses based on structural and functional analysis (Akoto-Amanfu et al., 1987; Jambrina et al., 1997; Kemdirim et al., 1986). Also, Jambrina et al. (1997) recently reported that all polymerase proteins and the NP protein of influenza B viruses are essential for replication of a model RNA template. Moreover, although the RNP complex of type A and type B viruses demonstrates type-specific subunit interactions, both RNA polymerase complexes were capable of binding and synthesizing heterotypic templates, albeit at reduced frequencies (Jambrina et al., 1997; Stevens & Barclay, 1998).

Although the complete nucleotide sequence of influenza B virus polymerase genes has been determined (DeBorde et al., 1988; Jambrina et al., 1997; Kemdirim et al., 1986), little is
known of the evolutionary characteristics of these genes. In the
present study, we investigated the phylogenetic patterns of the
PB2, PB1 and PA polymerase genes of influenza B viruses
isolated from 1940 to 1998 and analysed deduced protein
variability among predicted proteins of these genes. Addi-
tionally, in order to understand the evolutionary relation-
ships among the six internal genes and the HA gene,
phylogenetic patterns of the three polymerase genes were
compared in a parallel fashion with those previously de-
termined for the NP, M, NS and HA genes of influenza B virus
(Lindstrom et al., 1999). Evolutionary characteristics of the
polymerase proteins of influenza type A and B viruses are also
compared.

Methods

- **Viruses.** The influenza B viruses analysed in this study included
  B/Lee/40, B/Ann Arbor/1/66, B/Singapore/222/79, B/Norway/
  1/84, B/Ibaraki/2/85, B/Victoria/2/87, B/Aichi/5/88, B/Yamagata/
  16/88, B/Panama/45/90, B/Beijing/184/93, B/Guangdong/08/93,
  B/Mie/1/93, B/Guangdong/05/94, B/Harbin/07/94, B/Beijing/
  243/97, B/Henan/22/97, B/Chiba/447/98, B/Shiga/51/98, B/Shiga/
  T30/98 and B/Yamanashi/166/98, all propagated in 11-day-old fertile
  hen's eggs. Virus abbreviations are listed in Table 1.

- **RNA extraction and nucleotide sequences.** Viral RNAs were
  extracted from allantoic fluid using an RNA extraction kit
  (RNeasy; Qiagen). Amplification of each RNA segment was carried
  out by RT–PCR, as described previously (Lindstrom et al., 1999).
  After agarose electrophoresis of RT–PCR products and subsequent
  purification with QIAquick Gel Extraction kits (Qiagen), amplicons
  were sequenced directly using a PRISM Ready Reaction Dye Deoxy Terminator Cycle
  Sequencing kit with AmpliTaq DNA Polymerase FS (Perkin Elmer
  Applied Biosystems). Samples were electrophoresed on Perkin Elmer
  Applied Biosystems model 310 and 377 DNA automated sequencers.
  GenBank accession numbers and abbreviations of all nucleotide sequence
  data determined in the present study, as well as previously reported
  nucleotide sequences (Akoto-Amanfu et al., 1987; DeBorde et al.,
  1987, 1988; Jambrina et al., 1997; Kemdirim et al., 1986), are shown in Table 1.

- **Phylogenetic analysis and evolutionary rates.** Phylogenetic
trees were constructed using the neighbour-joining method (Gojobori et
al., 1982; Nei & Gojobori, 1986; Saikou & Nei, 1987) and bootstrap
analysis (n = 500) (Felsenstein, 1985) to determine the best fitting tree
for each gene. Nucleotide distance matrices were estimated by the three-
parameter method based on the total number of nucleotide substitutions
(Gojobori et al., 1982). Dendrograms were also constructed by maximum-
parsimony and maximum-likelihood methods using PHYLIP (Phylogeny
Inference Package) software version 3.57c (Felsenstein, 1995) to evaluate
the consistency of tree topologies. Evolutionary rates were calculated
as described previously (Lindstrom et al., 1998a).

Results

**Phylogenetic characterization of the PB2, PB1 and PA
genes**

Evolutionary analysis of the three polymerase genes of the
RNP complex of influenza B viruses included sequences of 20
strains isolated from 1940 to 1998. Phylogenetic trees of the

![Fig. 1. Phylogenetic tree of the PB2 gene of influenza B viruses isolated from 1940 to 1998. This tree was constructed by the neighbour-joining method. Internal branching probabilities were determined by bootstrap analysis using 500 replications and are indicated by a percentage value on each branch. Viruses belonging to the Victoria-like HA lineage are indicated with an asterisk (*). Dendrograms of the PB2 gene were also constructed by maximum-parsimony and maximum-likelihood methods using PHYLIP software version 3.57c (Felsenstein, 1995) (not shown) and were identical to that shown.](image1)

![Fig. 2. Phylogenetic tree of the PB1 gene of influenza B viruses isolated from 1940 to 1998. This tree was constructed by the neighbour-joining method. Internal branching probabilities were determined by bootstrap analysis using 500 replications and are indicated by a percentage value on each branch. Viruses belonging to the Victoria-like HA lineage are indicated with an asterisk (*). Dendrograms of the PB1 gene were also constructed by maximum-parsimony and maximum-likelihood methods using PHYLIP software version 3.57c (Felsenstein, 1995) (not shown) and were identical to that shown.](image2)
It appeared that the PB2 genes were evolving in a lineages, demonstrating high bootstrap confidence values of lineages I and II have since clearly evolved into two distinct (lineage II) were somewhat low (65 and 43%, respectively), lineages I and II have since clearly evolved into two distinct lineages, demonstrating high bootstrap confidence values of 99–100%. It appeared that the PB2 genes were evolving in a manner similar to that of the HA gene (Lindstrom et al., 1999), with YAM88 and VIC87 belonging to lineages I and II, respectively. In fact, with the exception of NOR84, the evolutionary locations of the PB2 genes of all isolates examined were consistent with the HA gene. Moreover, divergent PB2 genes were observed to have co-circulated in 1998 in Japan as demonstrated by isolates of lineage I (SHI3098, YAN98) and lineage II (SHI5198, CHI98).

Interestingly, characterization of the PB1 gene of influenza B virus revealed that, in contrast to the earliest divergent PB2 genes, the PB1 gene of SIN79 was located in lineage II together with that of NOR84. The confidence levels of these earliest divergent branches for lineages I and II were estimated to be 100 and 94%, respectively (Fig. 2). However, the phylogenetic locations of the PB1 genes of the remaining isolates were essentially identical to those of the PB2 genes. As illustrated, the PB1 genes of YAM88 and VIC87 belonged to lineage I and lineage II, respectively. Furthermore, phylogenetically distinct genes of 1998 viruses formed branch clusters in lineages I (YAN98, SHI3098) and II (SHI5198, CHI98).

Through analysis of the PA gene of B virus isolates (Fig. 3), it became apparent that the PA genes of viruses isolated before 1994 were evolving in a similar manner to the PB2 gene. Like the PB2 gene, the PA genes of SIN79 and NOR84 were the earliest divergent genes of lineages I and II, respectively, and evidently diverged from a putative gene similar to that of AA66, with branch confidence probabilities of 100%. Additionally, the phylogenetic locations of the PA genes of viruses subsequently isolated until 1994 were similar to those of the PB2 and PB1 genes. In contrast to the PB2 and PB1 genes, however, the PA genes of viruses isolated in China and Japan in 1997 and 1998 were all located in lineage I, forming two minor clades, I-i (BEI97, HEN97, CHI98, BEI97) and I-ii (SHI3098, YAN98). Thus, unlike the PB2 and PB1 genes, distinct PA genes of both lineages were shown not to have co-circulated in 1998 in Japan.

**Comparison of predicted amino acid sequences**

PB2 genes of all strains investigated here contained a conserved open reading frame (ORF) of 2313 nt encoding a predicted polypeptide of 769 aa, which was consistent with that previously described (DeBorde et al., 1988; Jambrina et al., 1997). Comparison of deduced amino acids of PB2 proteins (Table 2) revealed lineage-specific amino acid variability among PB2 proteins of lineages I and II, although proteins of lineage I demonstrated lower variability than those of lineage II. PB2 proteins of lineage I were found to contain two amino acid changes at positions 301 (A to T) and 641 (I to V), whereas those of lineage II contained six differences at positions 115 (K to R), 383 (M to L), 397 (K to R), 468 (L to S), 639 (R to K) and 641 (M to V). Curiously, similar amino acid substitutions occurred independently in both lineages resulting in a valine residue at position 641. Regardless of this, phylogenetically distinct viruses isolated in Japan in 1998, were observed to differ by six conserved amino acids (0.8%).

Determined nucleotide sequences of PB1 genes of influenza B viruses all contained a conserved ORF encoding a deduced protein of 752 aa, supporting the characterized PB1 ORF of LEE40 (Kemdirim et al., 1986). As shown in Table 2, conserved protein variability among PB1 proteins contrasted with that of the PB2 protein as isolates of lineage I showed higher amino acid variability than those of lineage II. Seven conserved lineage-specific changes were found in the PB1 proteins of lineage I, whereas only two differences were found in those belonging to lineage II. Accordingly, the PB1 proteins of distinct viruses of 1998 varied by nine conserved amino acids (1.2%).

Deduced nucleotide sequences of RNA segment 3 determined in this study all contained a conserved ORF encoding a PA protein of 726 aa. Interestingly, a three nucleotide deletion in the non-coding region of the 5’ end of the genomic RNA was observed in isolates of lineage I (1993–1998), which was similar to that found in B/Panama/45/90 (Jambrina et al., 1997). As shown in Table 2, PA proteins of influenza B viruses showed higher amino acid variability than the PB2 or PB1 proteins. A total of nine lineage-specific amino acid changes was observed in the PA proteins of lineage I, whereas those of lineage II contained four amino acid differences. As a result, the PA protein of the most recent strain of lineage II (GUA94) differed from 1998 isolates of lineage I by 17 aa (2.3%) or 18 aa (2.5%). Since recent viruses from 1998 were observed to form two branch clusters within lineage I, variability among
Table 2. Conserved amino acid variation of the polymerase proteins of influenza B viruses isolated from 1940 to 1998 (deduced amino acid differences in the PB2, PB1 and PA proteins)

<table>
<thead>
<tr>
<th>Lineage</th>
<th>LEE40</th>
<th>A400</th>
<th>SIN79</th>
<th>YAM88</th>
<th>PAN90</th>
<th>BEI83</th>
<th>MIE93</th>
<th>HAR94</th>
<th>SHI3098</th>
<th>YAN98</th>
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</table>

Table 3. Evolutionary rates of human influenza virus PB2, PB1 and PA genes

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Nucleotide evolutionary rate (ns/site/year × 10^{-3})</th>
<th>Protein evolutionary rate (aas/site/year × 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza B Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.76</td>
<td>1.20</td>
</tr>
<tr>
<td>II</td>
<td>0.76</td>
<td>0.83</td>
</tr>
<tr>
<td>Human H3N2 influenza virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.27</td>
<td>1.41</td>
</tr>
</tbody>
</table>

* Nucleotide evolutionary rates were based on the entire gene segment of each gene. ND, Not detectable.

these viruses was more limited, with PA proteins of SHI5198 (I-i) and SHI3098 (I-ii) differing by only one conserved amino acid (0.1%). It should be noted that a deletion of three nucleotides resulting in an amino acid deletion at position 486 (V) of a previously sequenced PA gene of SIN79 (Akoto-Amanfu et al., 1987) was not observed in any other viruses examined in this study.

Evolutionary rates

Evolutionary rates of the polymerase PB2, PB1 and PA genes were calculated based on the total number of nucleotide and amino acid changes. As shown in Table 3, respective rates of change for lineages I and II of each gene were calculated separately. For comparison, the rates of change of the polymerase genes of human influenza A H3N2 viruses were also calculated based on previously published sequences of viruses isolated in 1968–1997. Both lineages of the PB2 gene of B viruses were estimated to be evolving at a rate of approximately 0.76 × 10^{-3} nucleotide substitutions per site per year (ns/site/year) which was slower than that of human influenza A H3N2 viruses (1.27 × 10^{-3} ns/site/year). However, the corresponding rates of change of the PB2 protein of each lineage of influenza B virus varied considerably, from no detectable change in lineage I to 0.14 × 10^{-3} amino acid.
changes per site per year (aas/site/year) in lineage II. The rate of substitution in the PB2 protein of type A viruses was considerably higher (0.40 × 10⁻³ aas/site/year).

The PB1 genes of influenza B viruses belonging to lineages I and II were evolving at somewhat variable rates of 1.20 × 10⁻³ ns/site/year and 0.83 × 10⁻³ ns/site/year, respectively. Differences in evolutionary rates were more evident at the protein level with PB1 proteins of lineage I (0.57 × 10⁻³ aas/site/year) changing markedly faster than those of lineage II (0.01 × 10⁻³ aas/site/year). These rates were noticeably faster and slower, respectively, than that estimated for influenza H3N2 viruses (0.12 × 10⁻³ aas/site/year).

Lineages of the PA gene of type B viruses were calculated to be evolving at similar rates of 1.24 × 10⁻³ ns/site/year (lineage I) and 1.16 × 10⁻³ ns/site/year (lineage II), which were comparable with that of influenza A H3N2 viruses (1.46 × 10⁻³ ns/site/year). At the amino acid level, the PA proteins also evolved at similar rates of 0.26 × 10⁻³ aas/site/year (lineage I) and 0.37 × 10⁻³ aas/site/year (lineage II), which were somewhat slower than that of type A human H3N2 viruses (0.49 × 10⁻³ aas/site/year).

Discussion

Phylogenetic analysis of the PB2, PB1 and PA genes of influenza B virus isolates revealed a number of noteworthy features concerning the evolution of the polymerase genes. All three polymerase genes demonstrated divergent evolution into two distinct lineages which was consistent with phylogenetic divergence described for the HA (Kanegae et al., 1990; Lindstrom et al., 1999; Nerome et al., 1998; Rota et al., 1992, 1990), NA (Burmeister et al., 1993) and internal NP, M and NS genes (Jambrina et al., 1997; Lindstrom et al., 1999; Yamashita et al., 1988) of influenza B virus. Phylogenetic divergence into two distinct major lineages was thus confirmed for all eight RNA segments of influenza B virus. Distinct evolutionary pathways among the three polymerase genes were indicative of genetic reassortment of these genes among variable influenza B viruses. Also, it was apparent that the three polymerase genes encoding the interacting subunits of the polymerase RNP complex appeared not to be evolving as a unit, supporting previous analysis of the polymerase genes of influenza A virus of various host animals (Gorman et al., 1990).

Similarly to previous analysis of the HA, NP, M and NS genes (Lindstrom et al., 1999), nucleotide substitution rates estimated for the polymerase genes of influenza B virus appeared to be slightly slower than those of human influenza A H3N2 virus. Protein evolutionary rates of divergent lineages of the polymerase proteins, in particular the PB1 protein, tended to differ considerably, suggesting variable functional constraints or other evolutionary pressures on these proteins. Genetically variable PA proteins of type B virus demonstrated higher variability than the PB1 or PB2 proteins which was consistent with the observed variability among polymerase proteins of influenza A viruses (Gorman et al., 1990; Kawaoaka et al., 1989; Okazaki et al., 1989). Most lineage-specific amino acid substitutions were located in the N-terminal half of the PA protein which may have significance as most host-specific substitutions observed in the PA protein of influenza A viruses were also located in this region (Okazaki et al., 1989). Additionally, the N-terminal 247 aa region of the PA protein influenza A virus has demonstrated induction of proteolytic activity and contains the signal for nuclear localization (Sanz-Ezquerro et al., 1995, 1996). It was interesting to note that four of nine conserved amino acid substitutions in the PB1 protein of type B viruses were located in regions implicated in recognition of the 5’ and 3’ ends of vRNA and the cRNA panhandle of influenza A virus (Gonzalez & Ortin, 1999a, b).

In order to understand the phylogenetic relationships among gene segments of influenza B virus, the evolutionary patterns of the three polymerase genes were compared with those of the internal NP, M, NS and surface HA genes recently described by Lindstrom et al. (1999) (Table 4). As shown in Table 4, evolution of the HA and internal gene segments of influenza B virus was characterized by genetic reassortment of phylogenetically divergent genes. Indeed, comparison of 18 viruses isolated following phylogenetic divergence revealed eight distinct genome constellations, whereas only five isolates (YAM88, PAN90, BEI93, MIE93, HAR94) contained all gene segments of a single lineage corresponding with the Yamagata/16/88-like HA lineage. It was previously demonstrated that the NP, M and NS genes evolved independently of the HA gene (Lindstrom et al., 1999). Therefore, it was noteworthy to observe that while the evolutionary pathway of the PA gene was similar to those of the NP and M genes (Lindstrom et al., 1999), dendrogram topologies of the PB2 and PB1 genes were, in contrast, quite similar to that of the HA gene. Thus, despite reassortment among co-circulating viruses, it appeared that the six gene segments encoding the internal proteins of influenza B virus demonstrated three general evolutionary patterns including: (i) PB2 and PB1 genes; (ii) PA, NP and M genes; and (iii) the NS gene.

Similarities among the evolutionary profiles of the internal genes led to the suggestion of possible functional association between the PA, NP and M proteins as well as between the PB2 and PB1 proteins. Similar phylogenetic patterns of the PA and NP genes of type B virus are supported by analysis of those of type A virus based on host-specific evolutionary pathways (Gorman et al., 1990). Protein interaction experiments involving the proteins of the RNP complex of influenza A viruses show that the PB1 protein may associate independently with the PB2 and PA proteins (Biswas & Nayak, 1996; Digard et al., 1989; Gonzalez et al., 1996; Perez & Donis, 1995; Toyoda et al., 1996a; Zürcher et al., 1996) supporting phylogenetic evidence for association of the PB1 and PB2 proteins of influenza B virus observed here. Still, the question arises as to why the PB1 and PB2 polymerase genes might be co-evolving with the HA gene. Although non-coding terminal
Evolution of influenza B polymerase genes

Table 4. Evolutionary profiles of influenza B virus genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>HA*</th>
<th>PB2</th>
<th>PB1</th>
<th>PA</th>
<th>NP*</th>
<th>M/BM2*</th>
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</table>

* Evolutionary profiles of the HA, NP, M and NS genes were determined previously (Lindstrom et al., 1999).

sequences were not compared in this study, segment-specific nucleotides in the non-coding regions of the genome segments have been shown to influence interaction specificity with the RNA polymerase complex of influenza A and B viruses (Lee & Seong, 1996, 1998; Stoeckle et al., 1987). Thus, the possibility that genetic linkage of functionally unrelated proteins may be a result of selective packaging of RNA segments during virus assembly must also be considered.

Phylogenetic characterization of all six internal genes of influenza B viruses provided evidence suggesting that genetic reassortment among co-circulating divergent lineages of the internal genes may not be a random process but instead involves mechanisms which lead to selective reassortment of genes. Future investigation into specific protein interactions as well as terminal non-coding sequence variability of genetic RNA segments may provide evidence for understanding co-evolutionary patterns of influenza B virus genes. Genetic characterization of the internal genes of influenza B virus allows for subsequent analysis of the functional significance of lineage-specific amino acid changes in the internal proteins of influenza B viruses to be undertaken through construction of viruses with novel genome constellations by genetic reassortment or reverse genetics techniques.

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Washington, Seattle, WA, USA.

3.57c, Distributed by the author. Department of Genetics, University of

Virology nucleocapsids in the infected cell are in the form of a complex.
molecular structure of RNA polymerase from influenza virus A

Journal of Biochemistry polymerase structure.

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Evolution of influenza B polymerase genes


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