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(RESEARCH ARTICLE)

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An insight into the active sites of the catalytic basic protein subunit PB1 of the RNA polymerase of human influenza viruses

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Abstract

RNA polymerase from human influenza viruses is a heterotrimeric enzyme and performs the crucial functions of both transcription and replication for multiplication of the viruses in human cells. The heterotrimeric enzyme is made up of two basic protein subunits (PB1 and PB2) and an acidic protein subunit (PA). All the three subunits perform well-defined function(s) in the transcription and replication processes in the human cells. The basic protein subunit PB1 is shown to possess the polymerization activity, whereas the PB2 and the PA subunits are found to be involved in the cap-snatching and proofreading (PR) activities, respectively. The polymerase activity in the catalytic subunit, PB1, is found to be an RNA-dependent RNA polymerase (RdRp). Multiple sequence alignment (MSA) analysis of the PB1 subunits from all the three human influenza viruses, A, B and C shows large number of highly conserved peptides, amino acid motifs and invariant amino acids. Site-directed mutagenesis (SDM) analysis and X-ray crystallographic data have shown that two completely conserved motifs, viz. –GDN- and –SDD-, are involved in binding to the catalytic Mg^{2+} ion. These data are in close agreement with the MSA analysis data of the polymerases from all the three human influenza viruses. Furthermore, two highly conserved polymerase catalytic regions are identified in the PB1 subunits by sequence similarity to other DNA/RNA polymerases and hence, are proposed to function in the nucleotidyl transfer activities. Presence of the two catalytic regions suggest that the polymerase may function in a dual mode, i.e., in phase I, in association with the cap-snatching subunit PB2, it could be involved in the synthesis of mRNAs (transcription mode) and once enough proteins are made from the mRNAs, in the second phase, in association with PR exonuclease subunit PA, it could switch to the replication mode to synthesize error-free, exact copies of the viral genome. For both the activities, it could use the same invariant catalytic Mg^{2+}-binding –GDN- and –SDD- motifs.

Keywords: Human Influenza viruses; RNA polymerase; Polymerase Basic Protein subunit PB1; Catalytic Metal-binding motifs; Polymerase active sites; Mechanism of action

1. Introduction

A large number of human and animal viral pathogens belong to RNA viruses. They cause major global health-care crisis and unprecedented economic losses. Attempts have been made to prepare vaccines and synthesize novel antivirals to contain the spread of these viruses. Whereas the vaccines are targeted mainly to the viral surface protein(s), the antivirals are targeted to the crucial enzymes that are involved in the lifecycle of these viruses. As all RNA viruses employ the crucial enzyme, viz. the RdRp for their multiplication in human cells, most of the antiviral drugs are targeted to this enzyme. As the RdRps perform both the transcription and replication processes in these RNA viruses (except retro viruses), the RdRps have been the main target for antiviral drug development to control the spread of RNA viral pathogens, in general. However, these efforts are hindered by limited structural information on the RdRp catalytic core(s) and its catalytic mechanism. In this analysis, attempts are made to understand the catalytic core(s) of this polymerase from all the three human influenza viruses and its possible catalytic mechanism.

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Influenza, commonly known as "the flu", is an infectious disease caused by influenza viruses which also belong to (-) strand RNA viruses. Influenza viral infection is an airborne, highly contagious disease that generally causes acute respiratory illness resulting in variable degrees of systemic symptoms from mild fatigue to respiratory failure and death. Therefore, influenza viral infections have become a major public health-care concern worldwide. The influenza viruses are enveloped viruses of 150–200 nm diameter and belong to the family, Orthomyxoviridae. Out of the four genera (A-D) only influenza A, B and C viruses infect humans and the D virus infects livestock. Among them, infection by influenza A virus is the most dangerous and is the causative agent for the worldwide flu pandemics. For example, influenza A virus has caused four pandemics so far that have occurred in the last 100 years; in 1918, 1957, 1968 and 2009. Both influenza B and A viruses are also reported to cause seasonal epidemics each year and affect approximately 5–10% of the adult and 20–30% of the pediatric population. According to the World Health Organization, influenza epidemics lead to 3–5 million cases of severe illness and ~2,90,000–6,50,000 respiratory deaths each year (This estimate does not take into account of deaths from other diseases such as cardiovascular disease, which can be influenza-related). While influenza A virus shows animal to human transmissions, influenza B and C viruses show very limited host range and appear predominantly in humans [1]. Out of the three human influenza viruses, only the influenza A and B viruses cause substantial morbidity and mortality in humans, whereas the influenza C virus does not cause epidemics/pandemics, but is involved in sporadic outbreaks, causing only mild upper respiratory infections [2]. In contrast to influenza A virus, influenza B and C viruses do not have animal reservoirs, [3] suggesting the pandemic nature of the influenza A virus. Their genomic structure, surface proteins, serotypes, transcription and replication processes are already described by Palanivelu [4].

1.1. Salient Features of the Human Influenza Viral RNA Polymerases

Influenza viruses are (-) strand RNA viruses and replicated by the viral RNA (vRNA) polymerase in the host cell nucleus. Within a viral ribonucleotide particle (vRNP), a single RNA polymerase captures both the 3′- and 5′-termini of the viral RNA (vRNA) and forms a pseudo-circularized structure of the vRNA. The viral RNA polymerase is a heterotrimer and all three subunits are shown to perform well-defined function(s) in viral transcription and replication processes. For example, the PB1 subunit performs the polymerizing function, the PB2 subunit performs the cap-snatching function for priming the mRNA transcriptions [4] and the PA subunit has been suggested to perform the PR exonuclease function [5].

Though the RNA viruses are highly divergent in nature, but their key enzyme, i.e., the RdRp, is structurally and functionally very similar. For example, all the RdRps exhibit a characteristic cupped right-hand structure with the three major domains, viz. fingers, palm and thumb, similar to DNA-dependent DNA polymerases (DdDps) and many DNA-dependent RNA polymerases (DdRps). The X-ray crystallographic structures of the complete heterotrimeric viral polymerase have been reported by many investigators [6-8]. The X-ray structures revealed the PB1 subunits of the flu polymerases also showed the canonical closed right-handed fold, possessing the typical fingers, palm, and thumb domains [6-8]. Furthermore, the polymerase forms a compact structure with PB1 at its centre, capped on one face by PB2 and clamped between the two globular domains of the PA [6-8] (Fig. 1)

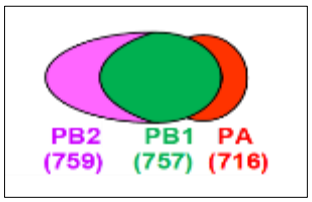
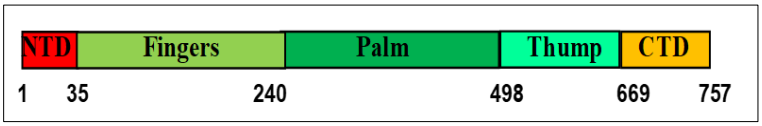


Figure 1 A schematic diagramme showing the various subunits of the influenza viral polymerase. (The number of amino acids are given in brackets and are from influenza A virus)



Adapted from Pflug et al. and Fan et al. [6,8]

Figure 2 Domain Architecture of the PB1 catalytic subunit of the RNA polymerase from human influenza A virus.

NTD, N-terminal domain; CTD, C-terminal domain; (K235, K237 and R239 form the finger priming loop); Palm (K308, K480 and K481, G304, D305, N306, S444, D445, D446; the Mg^{2+}- binding -^{304}GDN- and -^{444}SDD- motifs are in the palm domain)

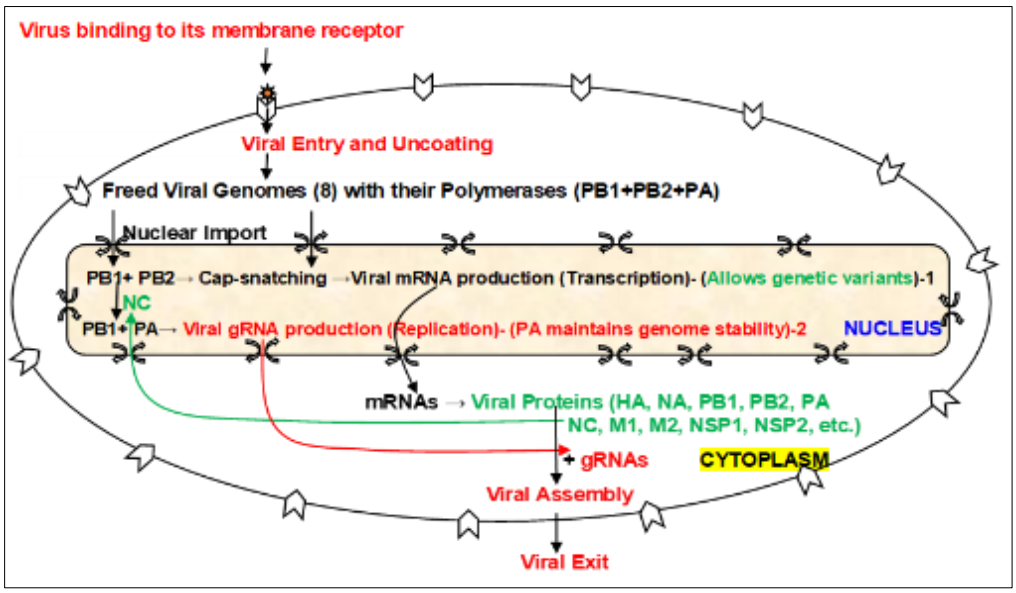
As discussed elsewhere, the influenza viral RNA polymerase (EC 2.7.7.48) performs the crucial steps in the viral multiplication, i.e., it involves both in the transcription and replication processes. As RdRps are not found in mammalian cells, it is suggested that they are an excellent target for designing antiviral compounds.

1.2. Influenza Viral Transcription and Replication

Initiation of transcription is primer-dependent and the primer with the cap is snatched from nascent host mRNAs by the PB2 subunit of the polymerase by a process known as 'cap-snatching'. With the capped primer, the polymerase goes for elongation and at the end of the elongation process, the transcription is terminated at a track of five to seven U residues (a poly-U track) near the 5′-end of the vRNAs, where polyadenylation occurs by a stuttering mechanism [9]. Now the 5'-capped and 3'-tailed viral mRNAs are ready for translation by the host ribosomes in the cytoplasm.

Unlike transcription, the replication is an end-to-end synthesis of the vRNAs. The vRNAs are synthesized in two steps: in the first step, the complementary RNAs (cRNAs; (+) strands) are synthesized using the vRNAs, as templates, and in the second step, the progeny vRNAs, (-) strands, are produced using the cRNAs as templates. In the context of the polymerase, the ten nucleotides at the 5′-termini of both vRNAs and cRNAs fold back into a hook-like structure which is bound tightly in a site formed by the fingers domain of the PB1 subunit and C–terminal of the PA subunit (PA-C) [6]. The initiation of replication starts with the synthesis of a ppp**A**p**G** dinucleotide to the opposite nucleotide positions $\mathbf{U_1}$-$\mathbf{C_2}$ of the 3′-vRNA terminus. This process requires support from the priming loop, a flexible region of the PB1 subunit that protrudes into the active site of the polymerase and controls the position of the template and initial dinucleotides. It is interesting to note, that for the synthesis of cRNAs and the subsequent synthesis of progeny vRNAs, the polymerase does not follow the transcription signals and thus, the same RdRp acts in different modes during the replication and transcription processes.

Figure 3 shows a schematic diagramme and suggested role(s) of the other two subunits in the transcription, replication processes. The PB2 subunit involves in the cap-snatching for initiation of transcription and the PA subunit is involved in PR and thus, maintaining genome stability. The replication mode is dependent on intracellular nuclear capsid protein (NC) concentrations. In this mode, the polymerase replicates the whole viral genome without recognizing the transcriptional signals, and the replicated genome is not caped or polyadenylated.



gRNA, genomic RNA; NC, Nuclear capsid protein (moves from the cytoplasm to nucleus through the nuclear pores), HA, Hemagglutinin, NA, Neuraminidase, PB1, PB2 & PA, polymerase subunits; M1, Matrix protein, M2; Ion channel; NSP1 & NSP2 (NEP), Non-structural proteins 1 & 2. NEP, Nuclear export protein.

Figure 3 A schematic diagram showing the proposed role(s) of each of the polymerase subunits in the production of viral mRNAs and gRNAs

In this communication, the PB1 subunit of the RNA polymerase from all the three human influenza viruses are analyzed for their active sites and reported. The catalytic metal-binding motifs already confirmed by SDM experiments and X-ray crystallographic techniques are further corroborated by the MSA analysis. Furthermore, the MSA analysis has also shown the possible catalytic amino acids involved in the nucleotide transfer reactions during the polymerization reactions. Based on these results, a catalytic mechanism for the human influenza viral polymerases is proposed.

2. Material and methods

Protein sequence data of the RNA polymerase basic protein subunit, PB1, from all three human influenza viruses, A, B and C were obtained from PUBMED and SWISS-PROT databases. The advanced version of Clustal Omega was used for protein sequence analysis. The highly conserved motifs identified by the bioinformatics analysis, and along with the data already available from biochemical, SDM and X-ray crystallographic analyses on the PB1 subunits, the possible amino acids that make up the polymerase active sites are proposed. Expasy tool was used for pI calculations of the PB1 subunits of the RNA polymerases.

3. Results and discussion

3.1. MSA Analysis of the Polymerase PB1 subunit of Influenza Virus A

Figure 4 shows the MSA of the PB1 subunits of the polymerase from several subtypes of the human influenza A virus (only the required regions for the discussions are shown here). The H1N1 subtype of the influenza A virus (Puerto Rico, 1934) is used as the standard and it showed a theoretical pI of 9.39. It is clear from Figure 4 that the PB1 subunits of influenza A viral strains from different regions are highly conserved from N- to C-terminals. Strikingly, five highly conserved motifs are identified in all the PB1 subunits of the human influenza A viral subtypes: Motif I (Finger priming loop) –^{235}**K**L**K**R**R**-; Motif II –^{303}T**GDN**^{306}-; Motif III –^{403}LSPG**M**M**M**^{409}G**M**F^{412}-; Motif IV –^{438}WDGLQS**S**^{444}**DD**FALI^{450}- and Motif V (end of the palm domain) –^{474}GINMSKK**K**^{481}S**Y**^{483}I- (highlighted in yellow, dark and light green). Several mutations in the above 5 conserved motifs from I to V of the PB1 protein were analyzed for their ability to replicate in human and chicken cells [10]. They found that replacement of the invariant amino acids, **S**^{444}→P, **D**^{445}→G and **D**^{446}→N in motif IV -(SDD-), and **K**^{481}→E/T/N mutants in motif V, abolished polymerase activity completely. Furthermore, by minireplicon assays, they confirmed that any mutation altering the –SDD- sequence rendered the polymerase nonfunctional. Moreover, in human cell replication assays, they found that the **Y**^{483}→H mutation showed only 0.5% of the activity, the **N**^{306}→T of -GDN^{306}- motif mutant showed only 2.4% of the activity and the **M**^{409}→T mutant showed no activity. They suggested that such conserved residues comprising the polymerase modules may serve as potential targets for anti-influenza drugs that can attenuate infection by inhibiting influenza polymerase activity and hence, viral replication. In contrast, the natural mutation **K**^{480}→R (Motif V) observed in several pandemics causing H1N1 human and swine influenza viruses, slightly increased transcription/ replication activity relative to the wild-type. The proposed amino acids in the catalytic regions and the highly conserved fingertip priming loop are highlighted in yellow. A –YAQ- triad (highlighted in yellow) was observed and a similar –YAN- triad was found to be involved in NTP selection in Severe acute respiratory syndrome-related Coronaviruses -(SARS-CoVs). The nuclear localization signals (NLSs) are highlighted in orange. The C-terminal ends in the triad -RQK in all.

3.1.1. CLUSTAL O (1.2.4) MSA of the PB1 catalytic subunits of the RdRp from influenza A viruses

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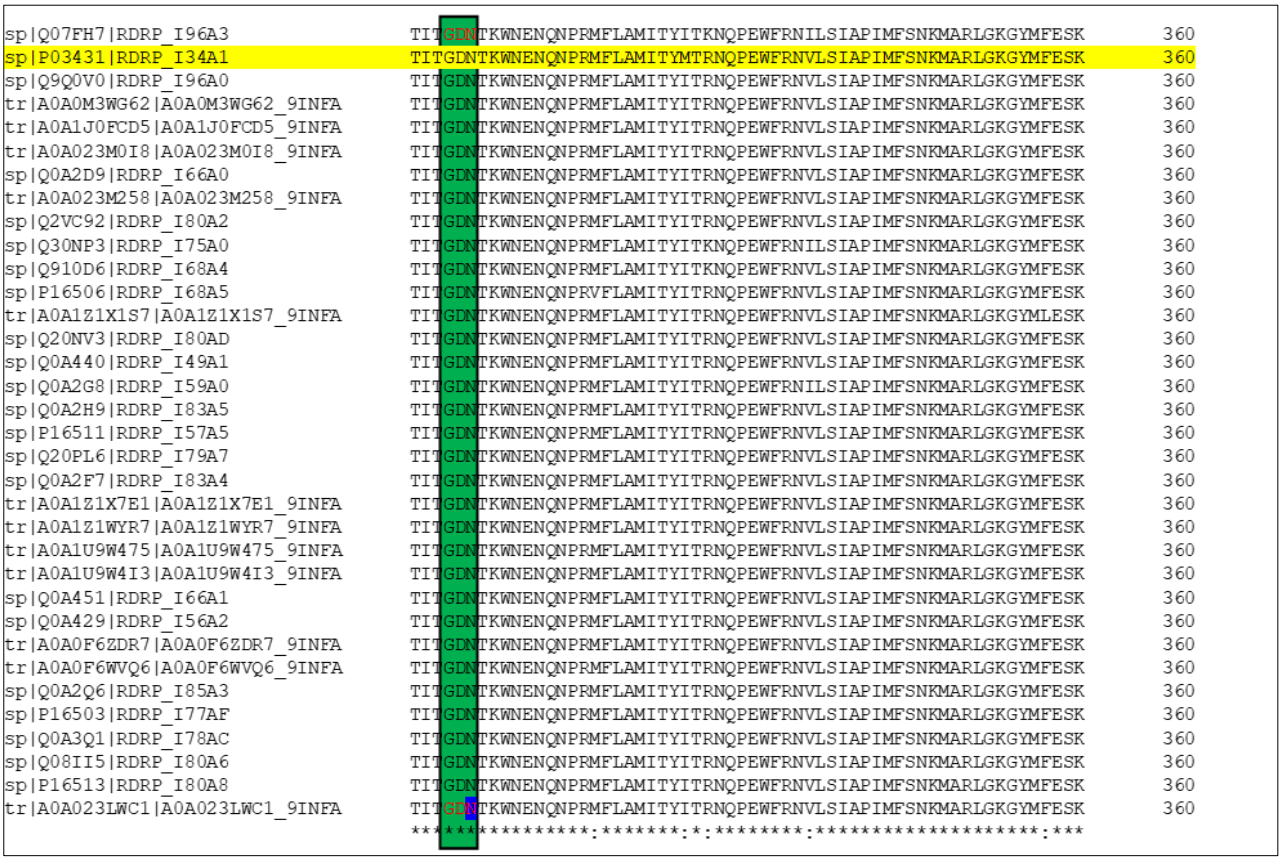
N-terminal
Fingers
NLS
FPLoop

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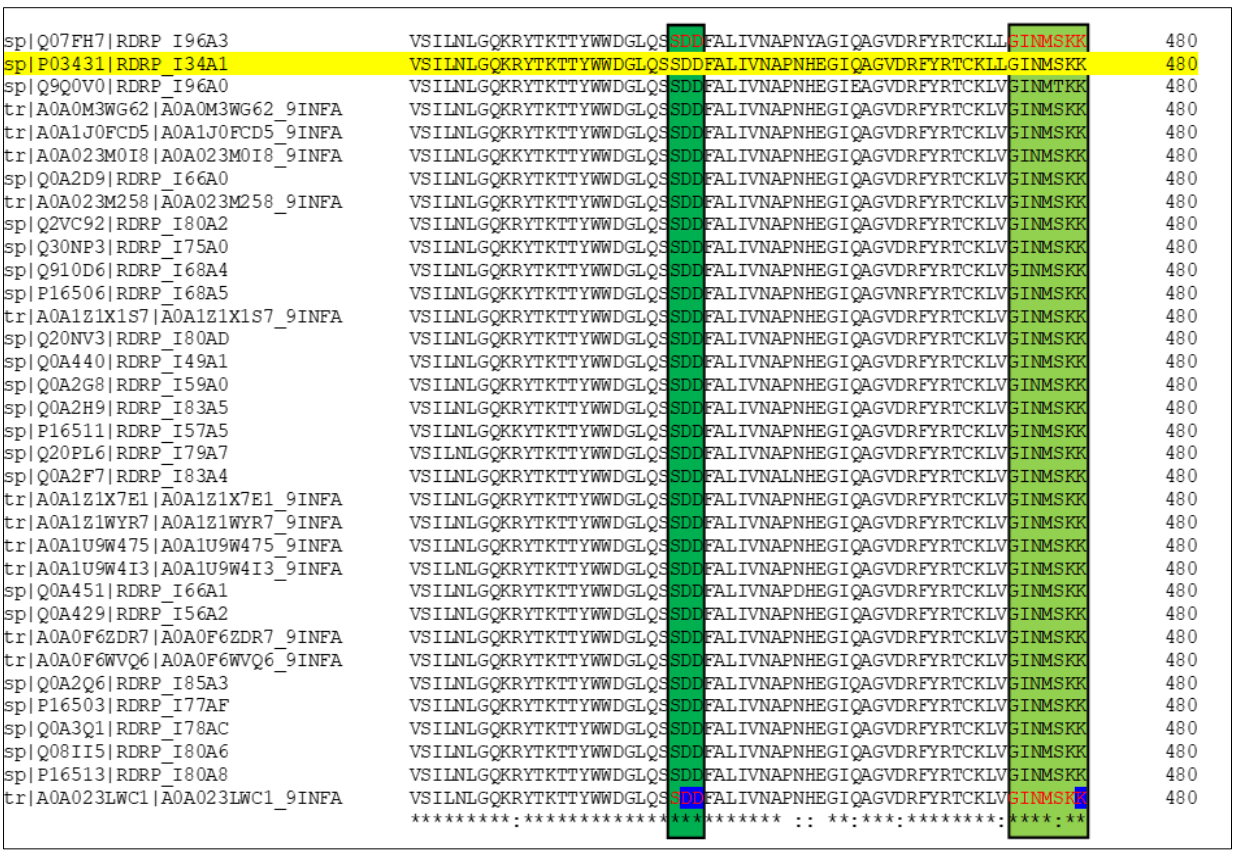
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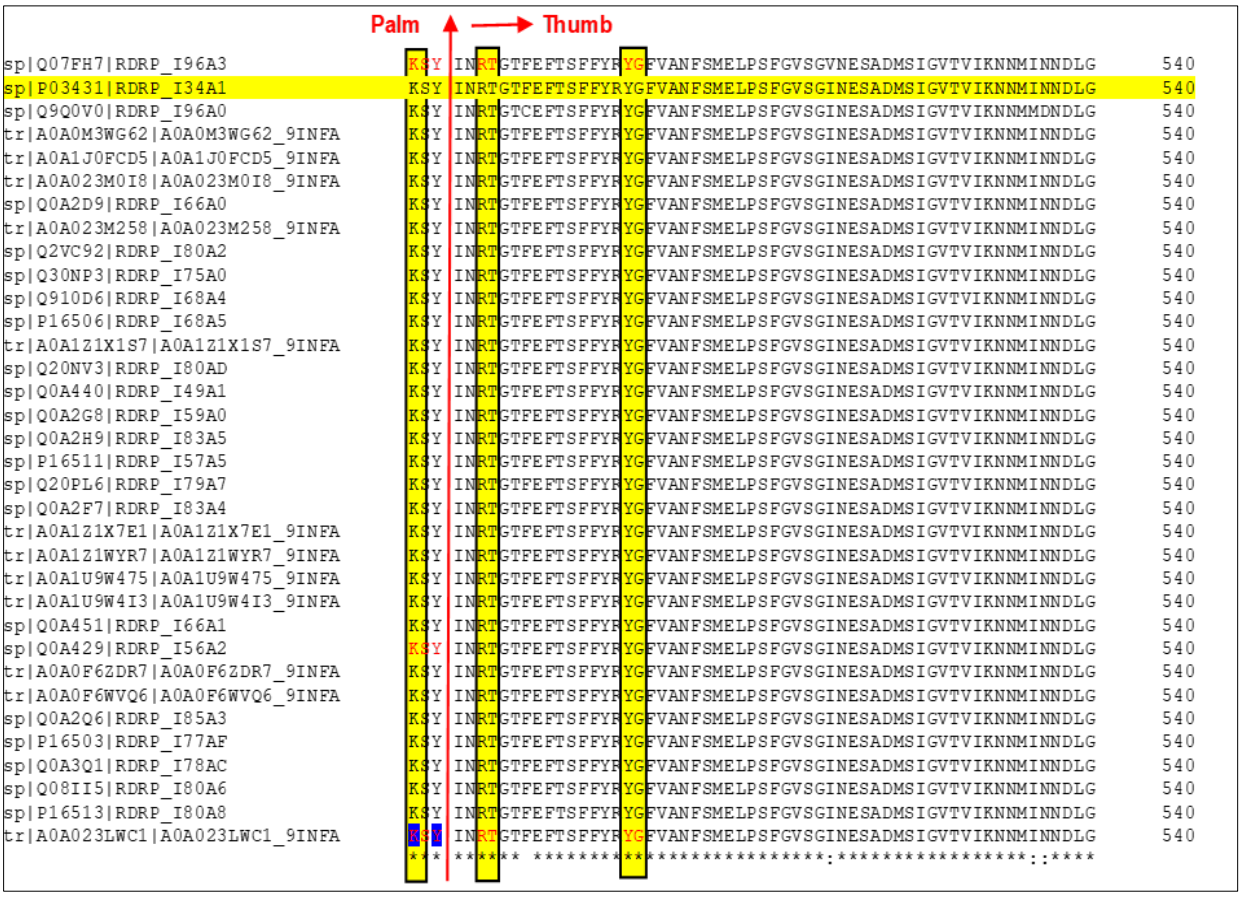
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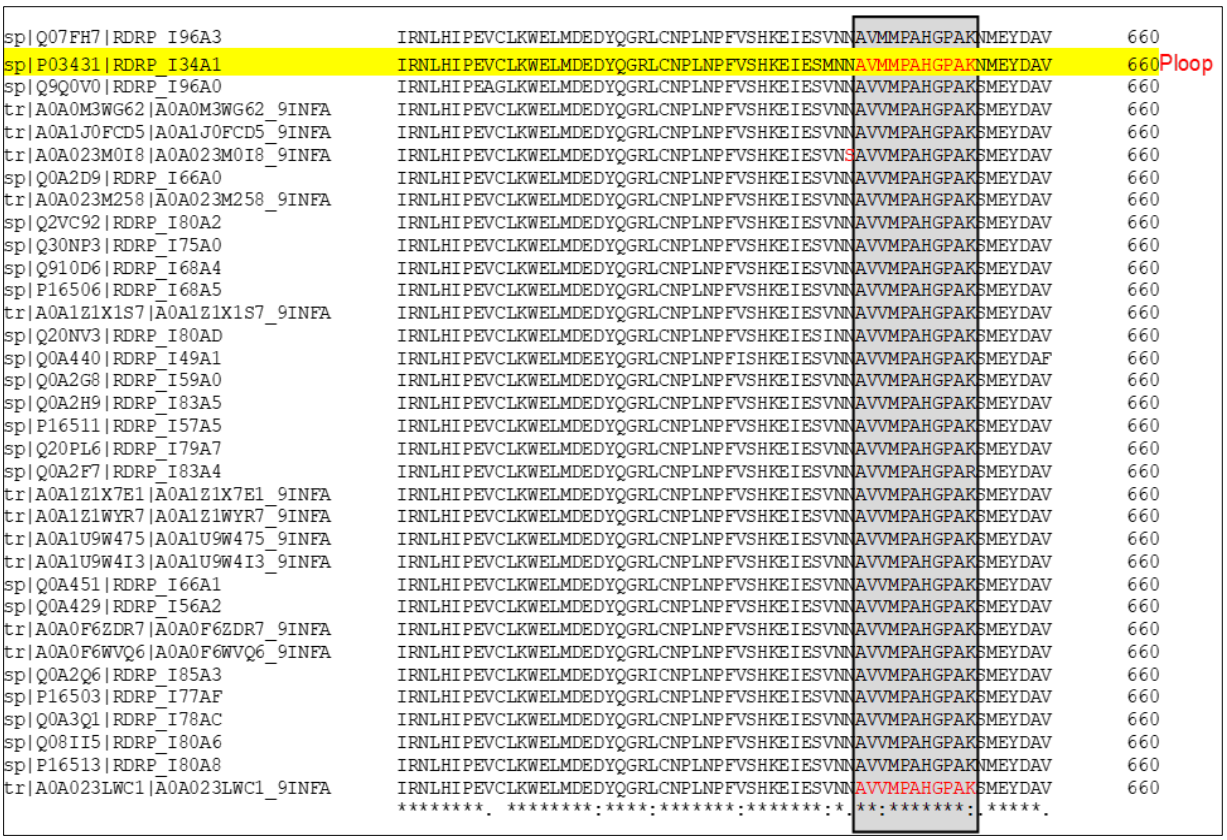
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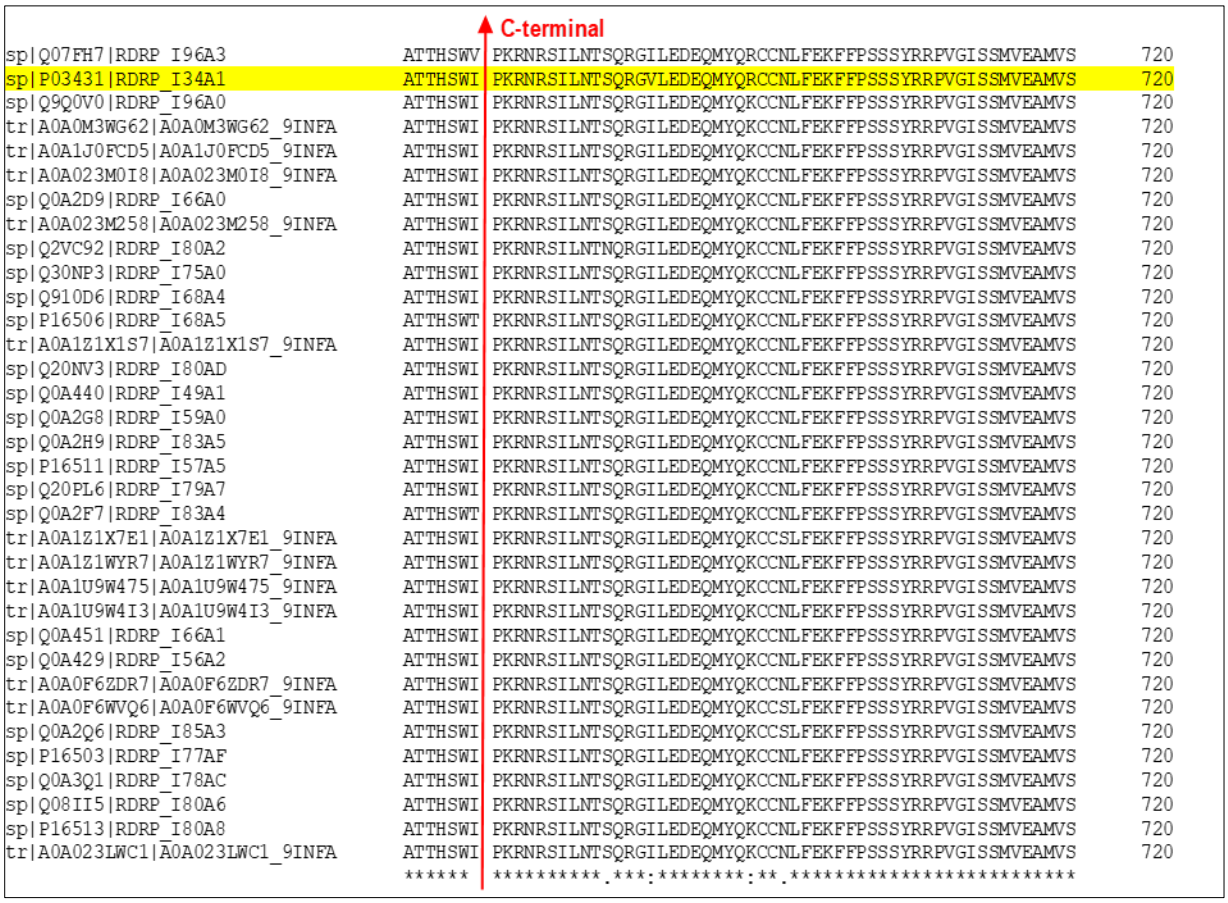
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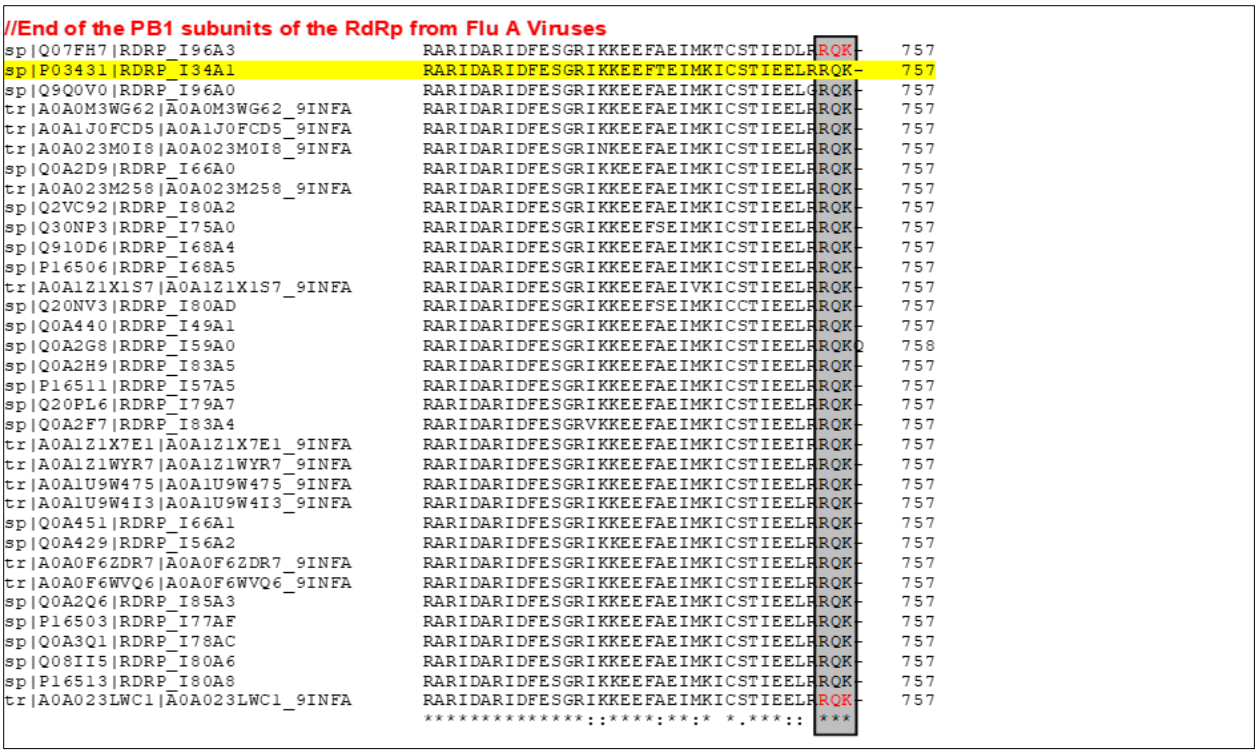
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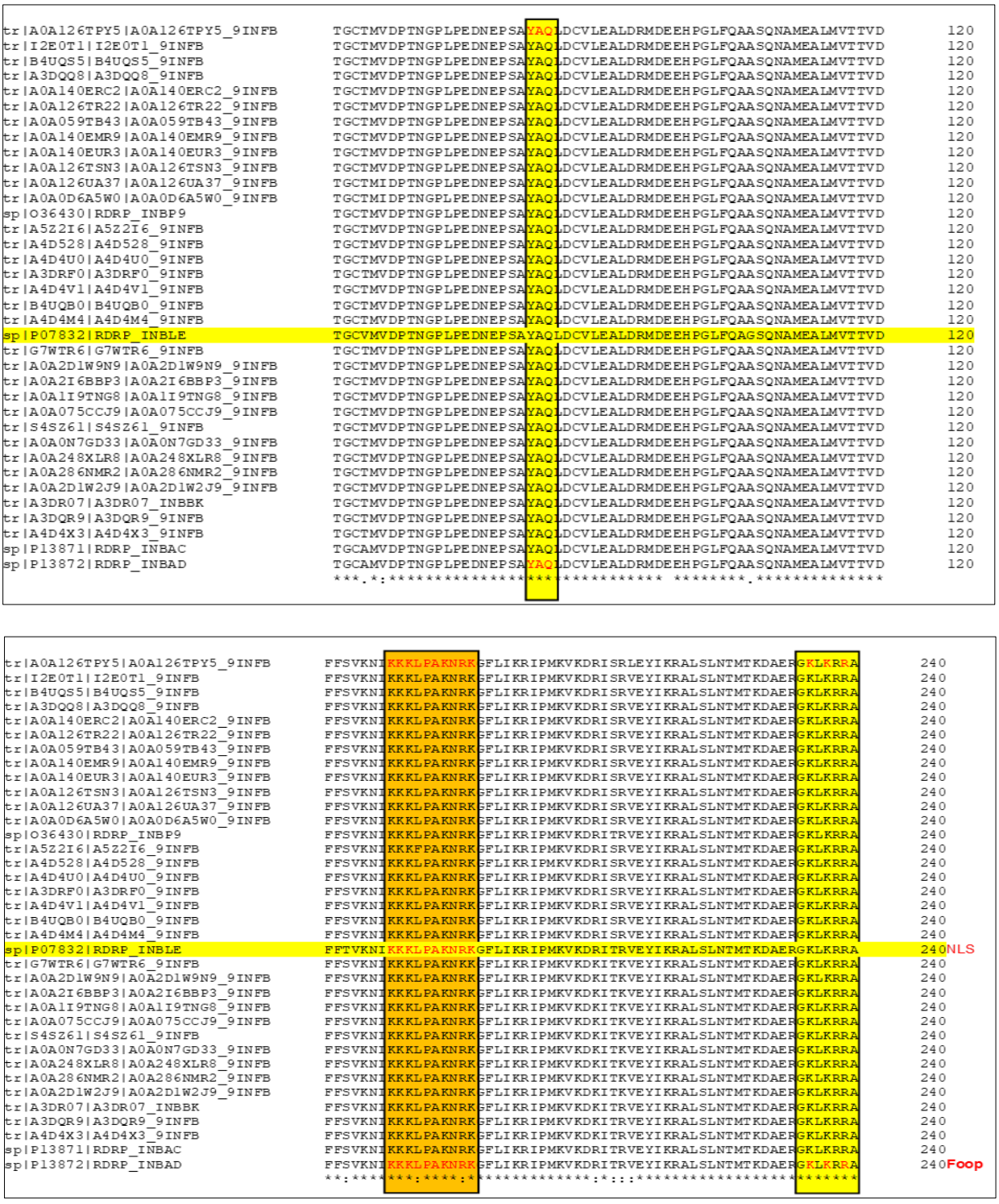
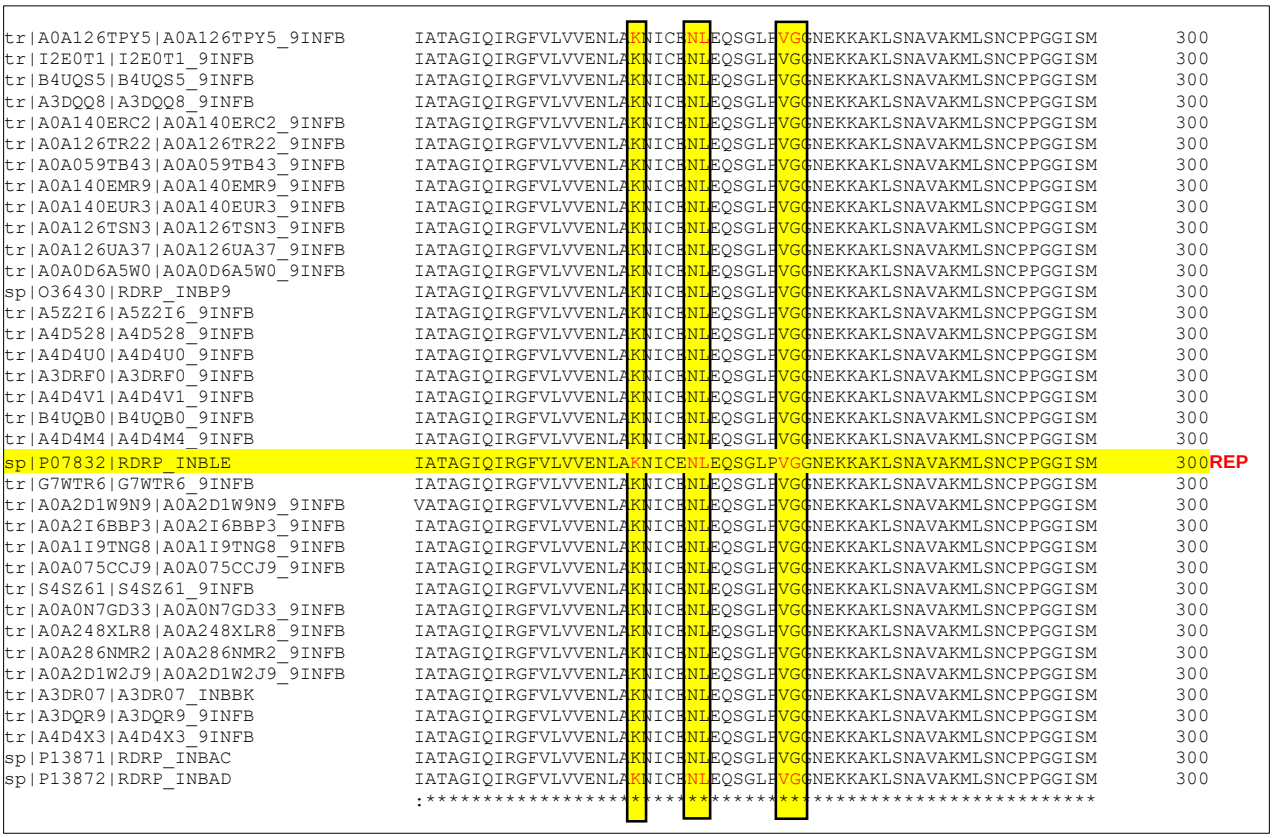
Figure 4 MSA of the PB1 catalytic subunits of the RdRp from human influenza A virus

Figure 5 shows the MSA of the catalytic subunits PB1 of the polymerase in different human influenza B viral strains from different regions. The standard strain, Lee 1948, showed a theoretical pI of 8.78. The subunits from various strains are completely conserved for the entire sequence with only a few amino acid changes (Fig. 5). The metal-binding regions are highlighted in dark green and the proposed amino acids in the catalytic regions and the highly conserved fingertip priming loop are highlighted in yellow. An invariant direct repeat is observed in influenza B viral strains and is marked by arrows. All the five motifs identified in influenza A viral strains are also highly conserved in the B virus: Motif I –KLKRR- Motif II $-^{303}$TGDN^{306}-; Motif III $-^{403}$LSPGMMMGMF^{412}-; Motif IV $-^{438}$WDGLQSSDDFAL*F*^{450}-; Motif V–^{474}GINMSKKKSYC^{484}- (highlighted in dark and light green) suggesting their importance in the polymerase function. The NLS region is highlighted in orange. Interestingly, the cold-adapted and wild-type strains differ only in three amino acids and are marked with stars. Among them, two are conservative replacements, whereas only one is a non-conservative type (Y→H), which is found at the C-terminal end. In influenza B virus, also the completely conserved $-^{22}$YAQ- motif (highlighted in yellow) is found as in influenza A viruses, suggesting its possible importance as in SARS-CoV-2 (YAN). Besides, a zinc-binding domain is also found in the PB1 subunits of the B viral strains in the mRNA catalytic region.

3.1.2. CLUSTAL O (1.2.4) MSA of the PB1 catalytic subunits of the RdRp from human influenza B viruses

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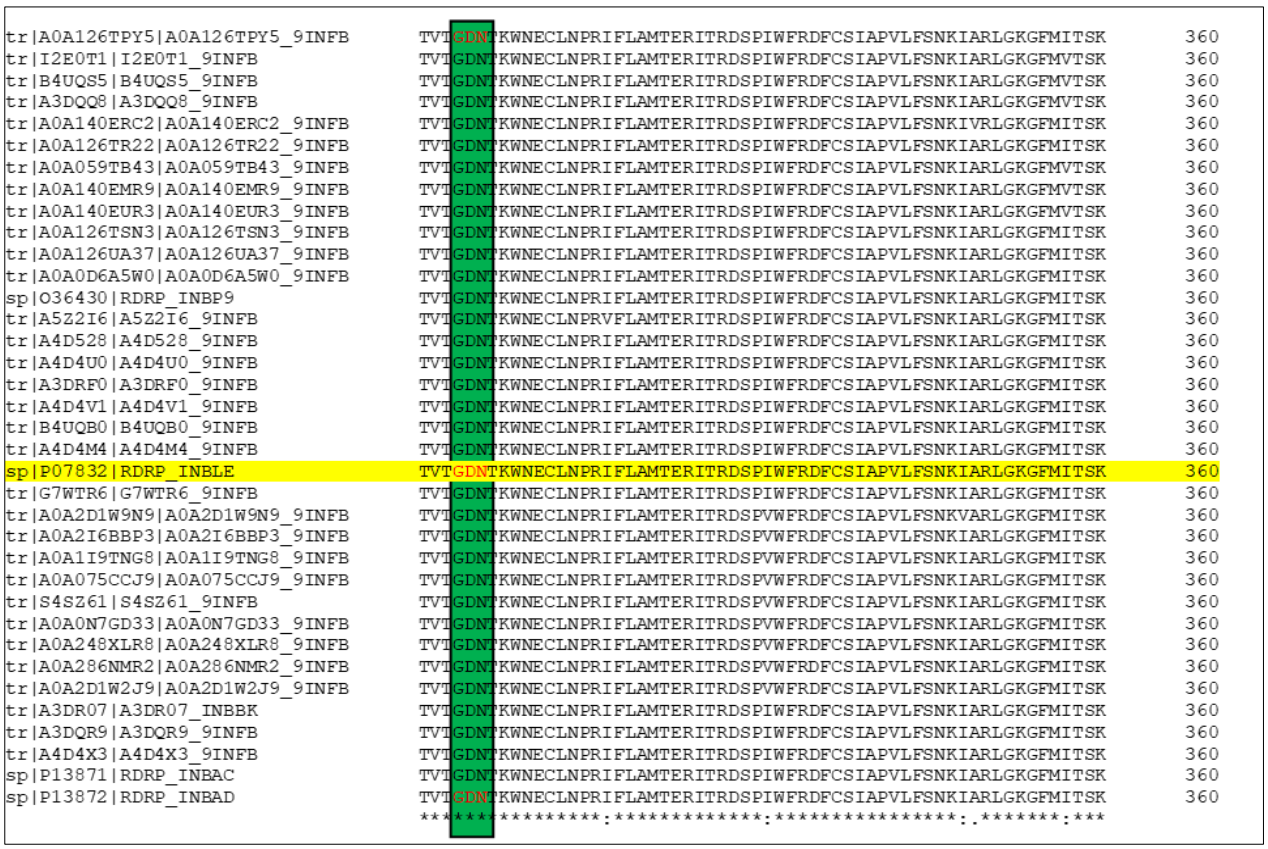
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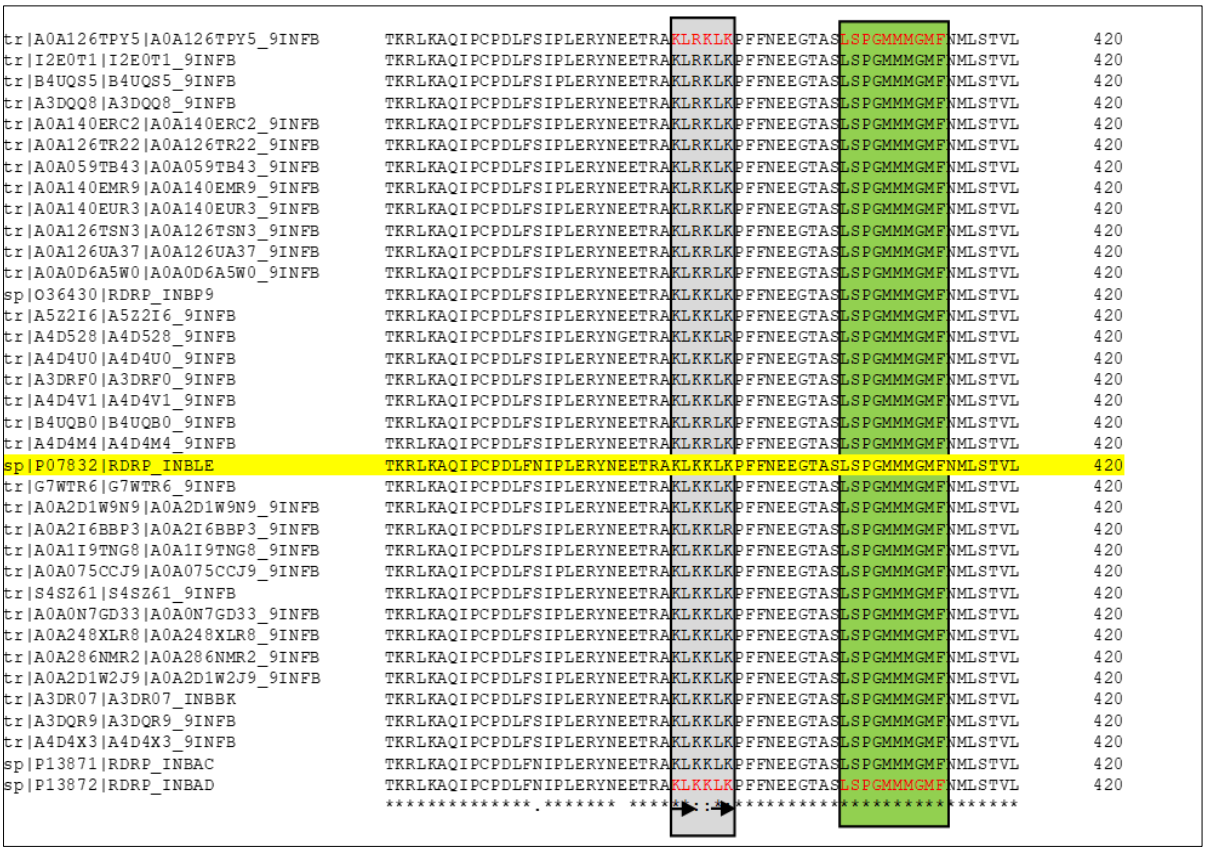
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tr|I2E0T1|I2E0T1_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEKCMEGINDFYRTCKLLGINMSKKK 480
tr|B4UQS5|B4UQS5_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A3DQQ8|A3DQQ8_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A140ERC2|A0A140ERC2_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEACMEGINDFYRTCKLLGINMSKKK 480
tr|A0A126TR22|A0A126TR22_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEACMEGINDFYRTCKLLGINMSKKK 480
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tr|A0A140EMR9|A0A140EMR9_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEACMEGINDFYRTCKLLGINMSKKK 480
tr|A0A140EUR3|A0A140EUR3_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEACMEGINDFYRTCKLLGINMSKKK 480
tr|A0A126TSN3|A0A126TSN3_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEEACMEGINDFYRTCKLLGINMSKKK 480
tr|A0A126UA37|A0A126UA37_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A0D6A5W0|A0A0D6A5W0_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
sp|O36430|RDRP_INBP9 GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A5Z2I6|A5Z2I6_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A4D528|A4D528_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A4D4U0|A4D4U0_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A3DRF0|A3DRF0_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A4D4V1|A4D4V1_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|B4UQB0|B4UQB0_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A4D4M4|A4D4M4_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
sp|P07832|RDRP_INBLE GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|G7WTR6|G7WTR6_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGVNMSKKK 480
tr|A0A2D1W9N9|A0A2D1W9N9_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A2I6BBP3|A0A2I6BBP3_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A1I9TNG8|A0A1I9TNG8_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A075CCJ9|A0A075CCJ9_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGVNMSKKK 480
tr|S4SZ61|S4SZ61_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGVNMSKKK 480
tr|A0A0N7GD33|A0A0N7GD33_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMKGINDFYRTCKLLGINMSKKK 480
tr|A0A248XLR8|A0A248XLR8_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
tr|A0A286NMR2|A0A286NMR2_9INFB GVAALGIKNIGNKEYLWDGLQSSDDFALFVNAKDEETCMEGINDFYRTCKLLGINMSKKK 480
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tr|I2E0T1|I2E0T1_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
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tr|A0A140ERC2|A0A140ERC2_9INFB      SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|A0A126TR22|A0A126TR22_9INFB      SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|A0A059TB43|A0A059TB43_9INFB      SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
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tr|A0A0D6A5W0|A0A0D6A5W0_9INFB      SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
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tr|A5Z2I6|A5Z2I6_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
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tr|A4D4U0|A4D4U0_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|A3DRF0|A3DRF0_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|A4D4V1|A4D4V1_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|B4UQB0|B4UQB0_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
tr|A4D4M4|A4D4M4_9INFB              SYCNETGMFEFTSMFYRDGFVSNFAMEIPSFGVAGVNESADMAIGMTIIKNNMINNGMGP      540
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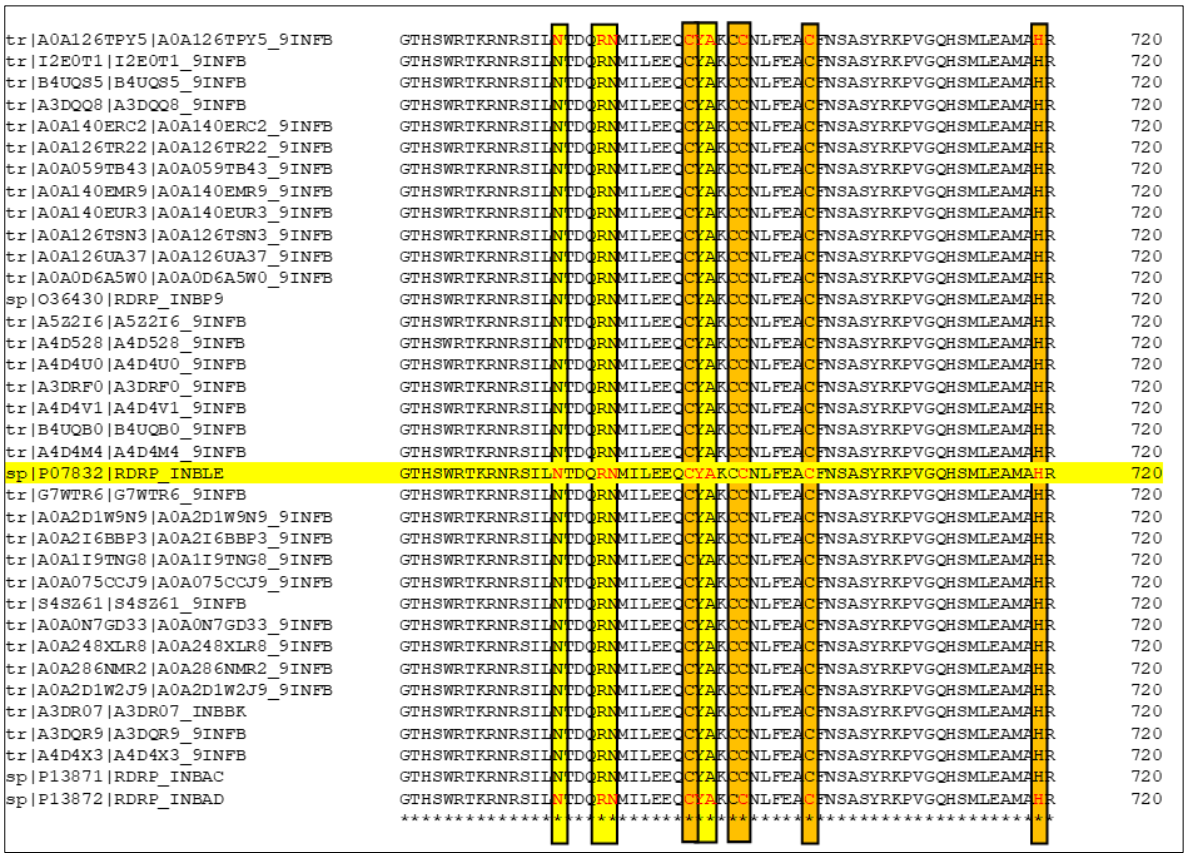
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tr|A0A0D6A5W0|A0A0D6A5W0_9INFB GTHSWRTKRNRSILNTDQRNMILEEQCYAKCCNLFEACFNSASYRKPVGQHSMLEAMAHR 720
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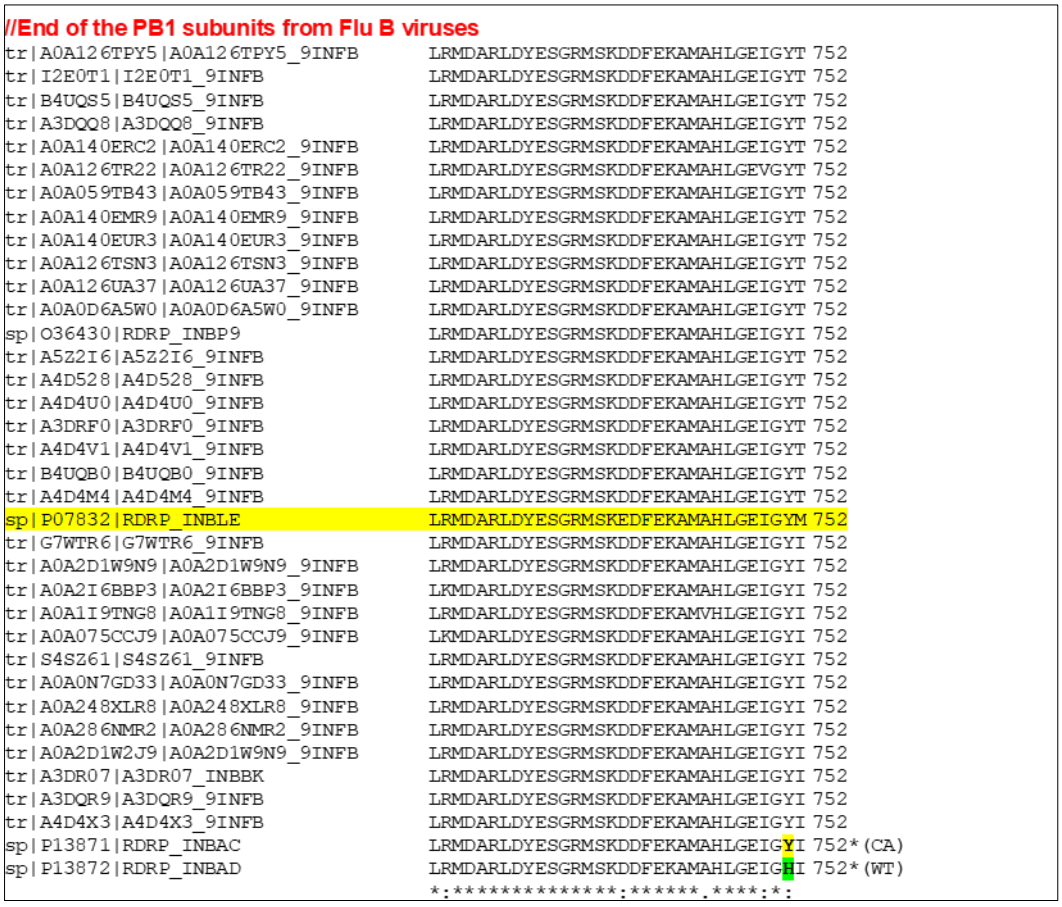
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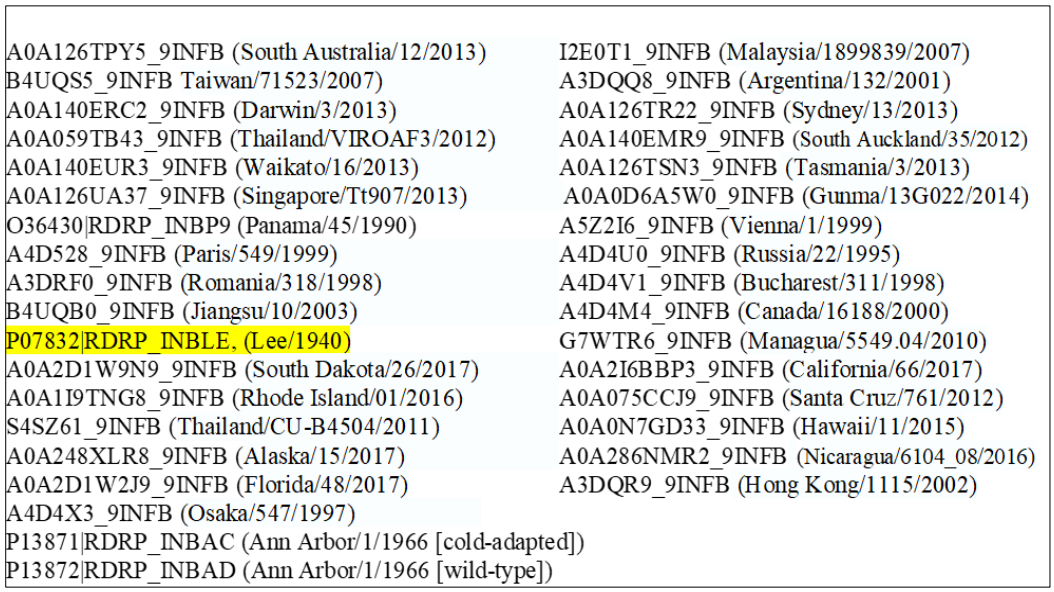
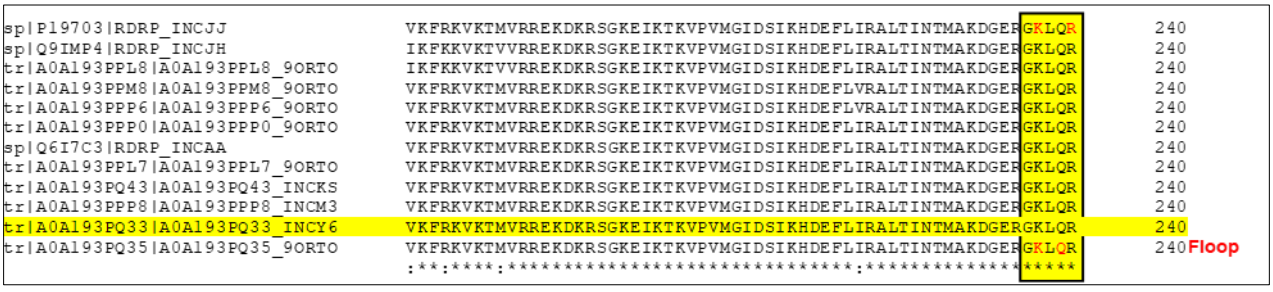
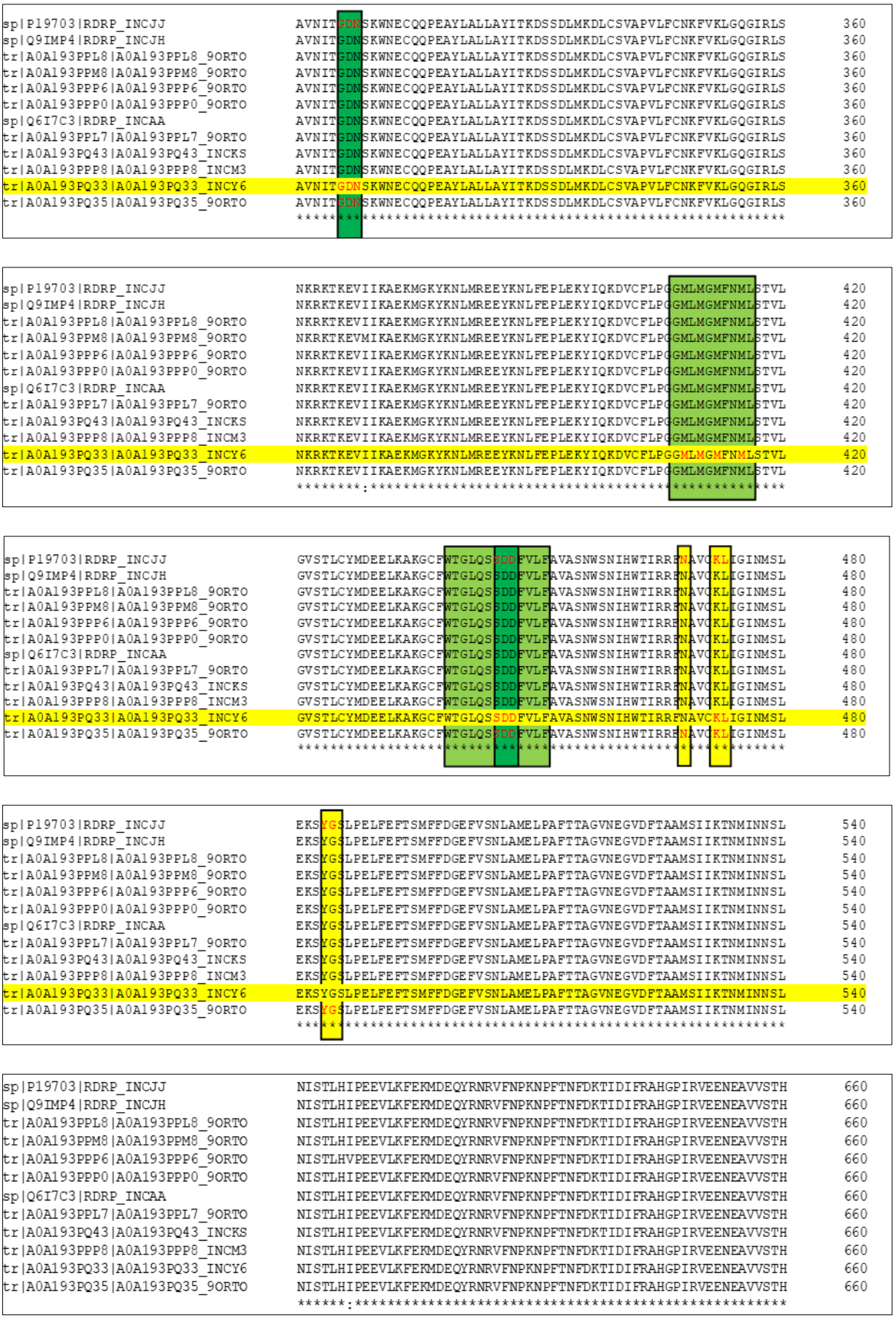


Figure 5 MSA of the PB1 catalytic subunits from different strains of human influenza B virus
CA, cold adapted; WT, wild type

Figure 6 shows the MSA of the catalytic subunits PB1 of the polymerase of different human influenza C viral strains from various regions. The theoretical pI of the standard Yamagata 1964 strain is 8.82 and highlighted in yellow. The PB1 subunits are completely conserved for the entire sequence with only a few changes and these subunits from the C virus are the most conserved among the three human influenza viruses (Fig. 6). The metal-binding regions are highlighted in dark green and the proposed amino acids in the catalytic regions and the highly conserved fingertip priming loop are highlighted in yellow. All the five motifs identified in influenza A and B viral strains are also highly conserved in the C virus: Motif I –KLQRR- (the middle K found in A and B viruses is replaced by Q) Motif II –^{305}TGDN-; Motif III –^{405}PGGMLMGMF-; Motif IV –^{439}WTGLQSSDDFVLF-; Motif V -^{475}GINMSLEKSYG– (highlighted in dark and light green). The NLS region is highlighted in orange. In striking contrast, the –YAQ- motif is not observed in the C viral strains.

3.1.3. CLUSTAL O (1.2.4) MSA of BP1 catalytic subunits of the RdRP from Flu C Viral strains

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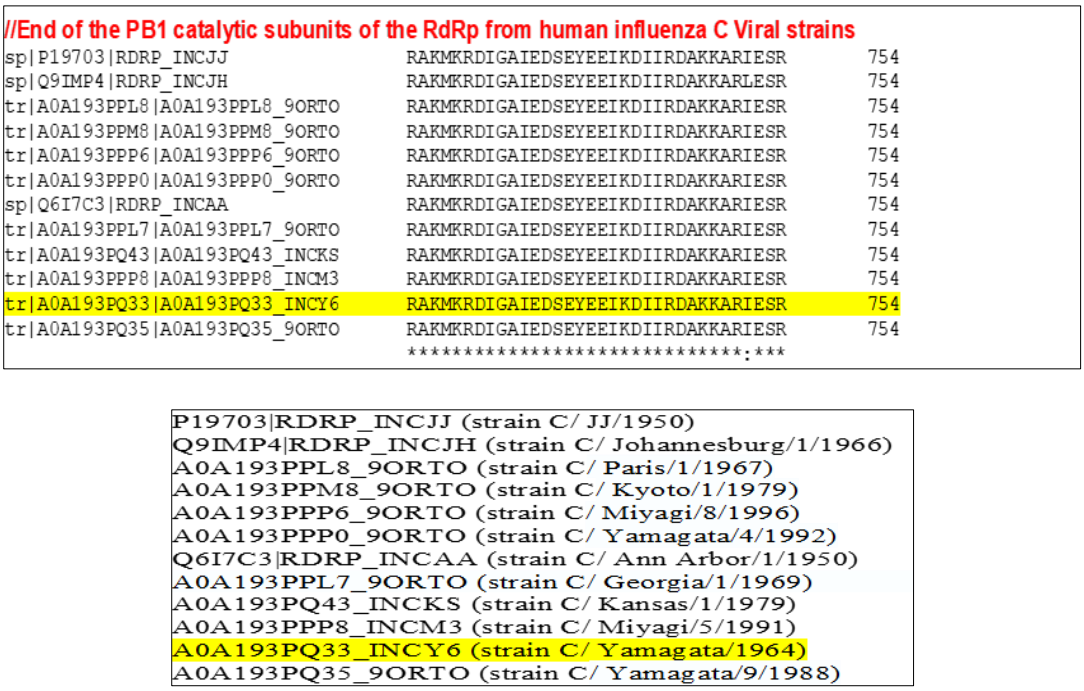


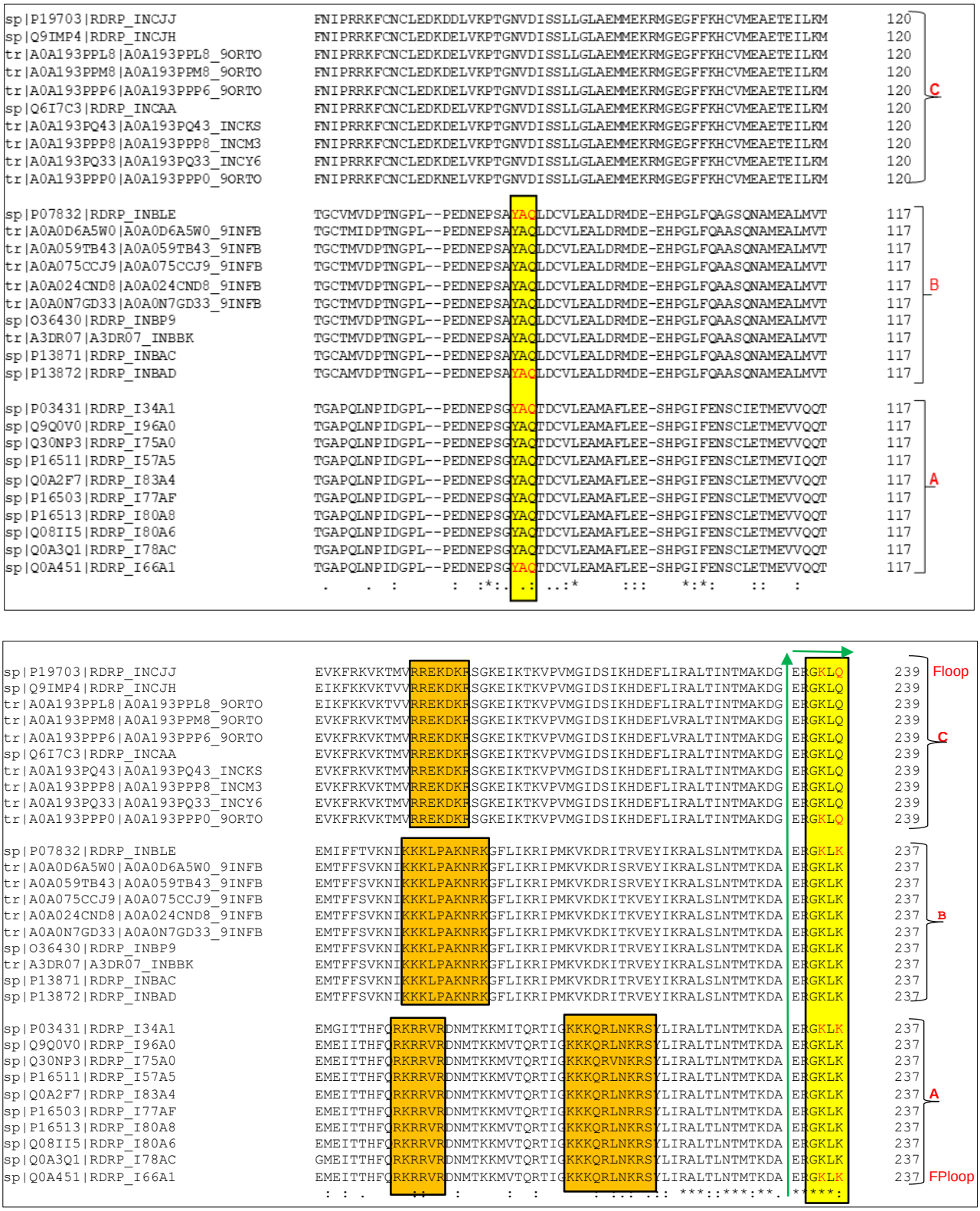
Figure 6 MSA of the PB1 catalytic subunits of the polymerase from different human influenza C viral strains

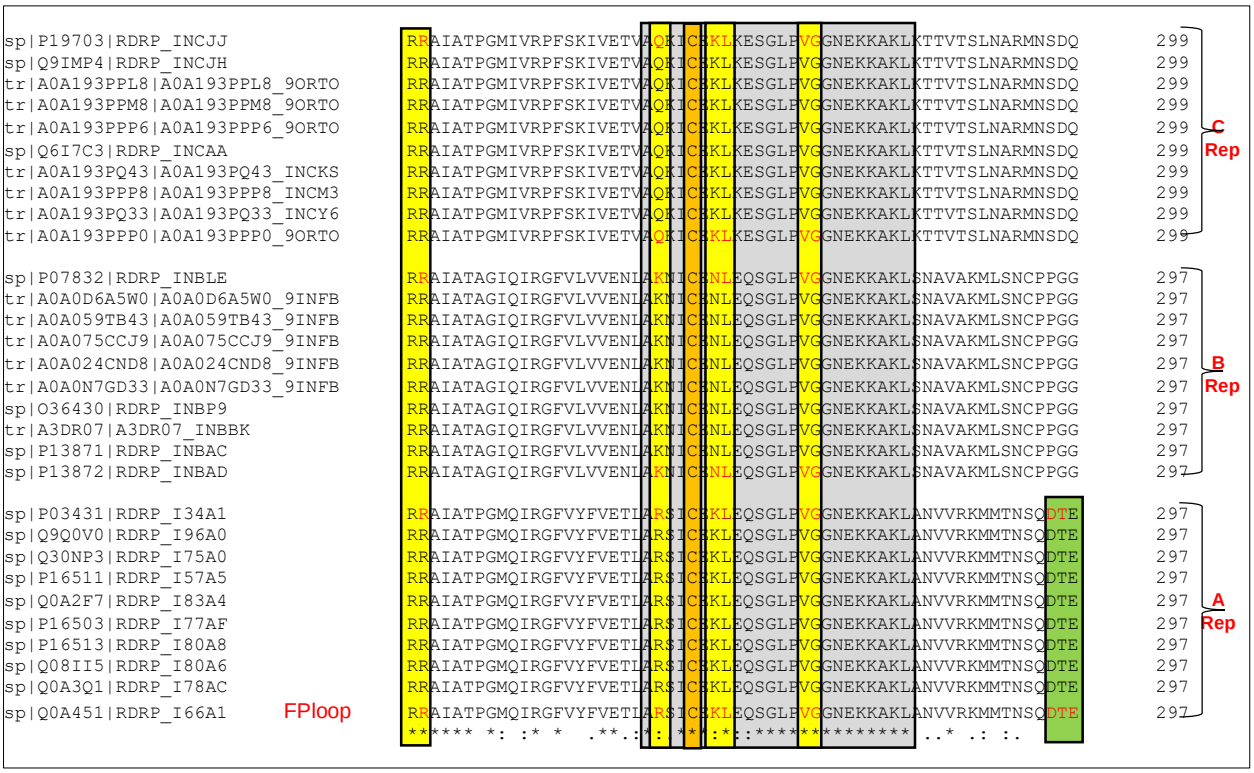
Figure 7 shows the 'Mix and Match' MSA of the PB1 catalytic subunits from all the three human influenza viruses. Only a few peptides show complete alignment. Interestingly, all the five motifs, Motif I $-^{235}$KLK/QRR- Motif II $-^{305}$TGDN-; Motif III $-^{405}$PGGMLMGMF-; Motif IV $-^{439}$WTGLQSSDDFVLF-; Motif V $-^{475}$GINMSLEKSYG– (highlighted in dark and light green) are highly conserved and aligned in all. Furthermore, a striking conservation and alignment is found in the proposed replication catalytic core in all. The proposed replication catalytic pair is highly conserved in all except an –NL- pair is found in B virus instead of a –KL- pair found in A and C viruses. The finger-priming loop in Motif-I is highly conserved in all except a Q replaces the K in C virus. However, the proposed transcription catalytic region is mostly found in the thumb regions. In contrast to the finger-priming loop which is highly conserved in all the three viruses (highlighted in yellow), the suggested priming loop (highlighted in grey) is not conserved in all but for 4 amino acids. It is interesting to note that both the catalytic metal-binding sites are preceded by an invariant hydroxy amino acid, **T/S** in all the three human influenza viruses, suggesting its possible role in metal-binding. Three striking differences among the subunits are, the –YAQ- triad, the –KLKKLK- repeat and a zinc-binding motif (ZBM). The PB1 subunit of the B virus shows a typical ZBM (highlighted in orange) in the mRNA catalytic region and a –KLK- triad repeat (highlighted in grey) preceding the Met cluster at the template entry site and the -YAQ- in the N-terminal region (Fig. 7). The PB1 subunit of the A viruses show two additional metal-binding sites, one in the replication and the other in the mRNA catalytic regions.

The –YAQ- triad is found only in influenza A and B viruses which is similar to the invariant -YAN^{691}- found in SARS-CoV-1, SARS-CoV-2 and other SARS-related CoVs. It is located in the palm domain and suggested to be involved in the nucleotide selection [11,12]. The N/Q of the triad –YAN/Q- with the dual hydrogen bonding capacity can recognize the 2'-OH group of incoming NTPs and thus, discriminate NTPs and dNTPs during RNA synthesis. It is interesting to note that the YAN/Q type of motif is also found in the RdRps of Dengue, Zika, and Yellow Fever viruses too (data not shown) (11). However, it is intriguing to know that a similar triad is not found in the influenza C virus. The NLSs are found in the N-terminal region in all the three viruses and is highlighted in orange and the metal-binding regions are highlighted in dark green.

Interestingly, a completely conserved peptide with 3 to 4 Met residues, -^{406}G**M**M**M**G**M**X$_2$**M**L- (MxMxMX$_2$ML) is found in all the three human influenza viruses. This characteristic methionine-rich loop (-^{406}GMMM^{409}GMF), is found at the template entry site [Pflug et al. [6]. It is interesting to note that a similar characteristic, highly conserved methionine-rich peptide –^{897}GH**M**LD**M**YSV**M**L- or (-X$_2$MX$_2$MX$_3$M-) is also found in the RdRps of SARS-CoV-1, SARS-CoV-2 and other SARS-related CoVs. This methionine-rich motif at the template entry site is suggested to be involved in stabilizing the base pair between the template and the incoming NTP [6, 11].

3.1.4. CLUSTAL O (1.2.4) Mix and Match MSA of the PB1 catalytic subunits of the RNA Polymerases all three human influenza viruses

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B Rep
A Rep
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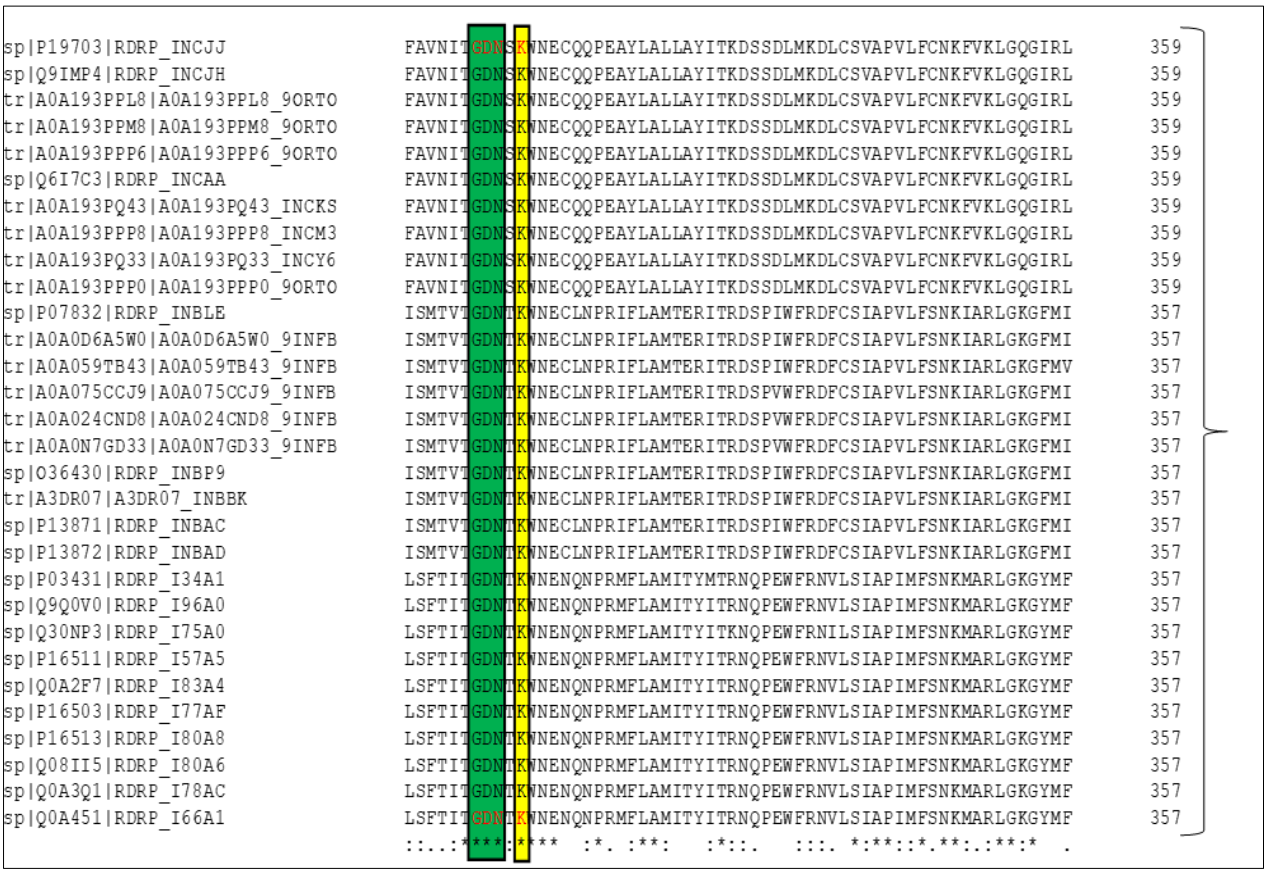

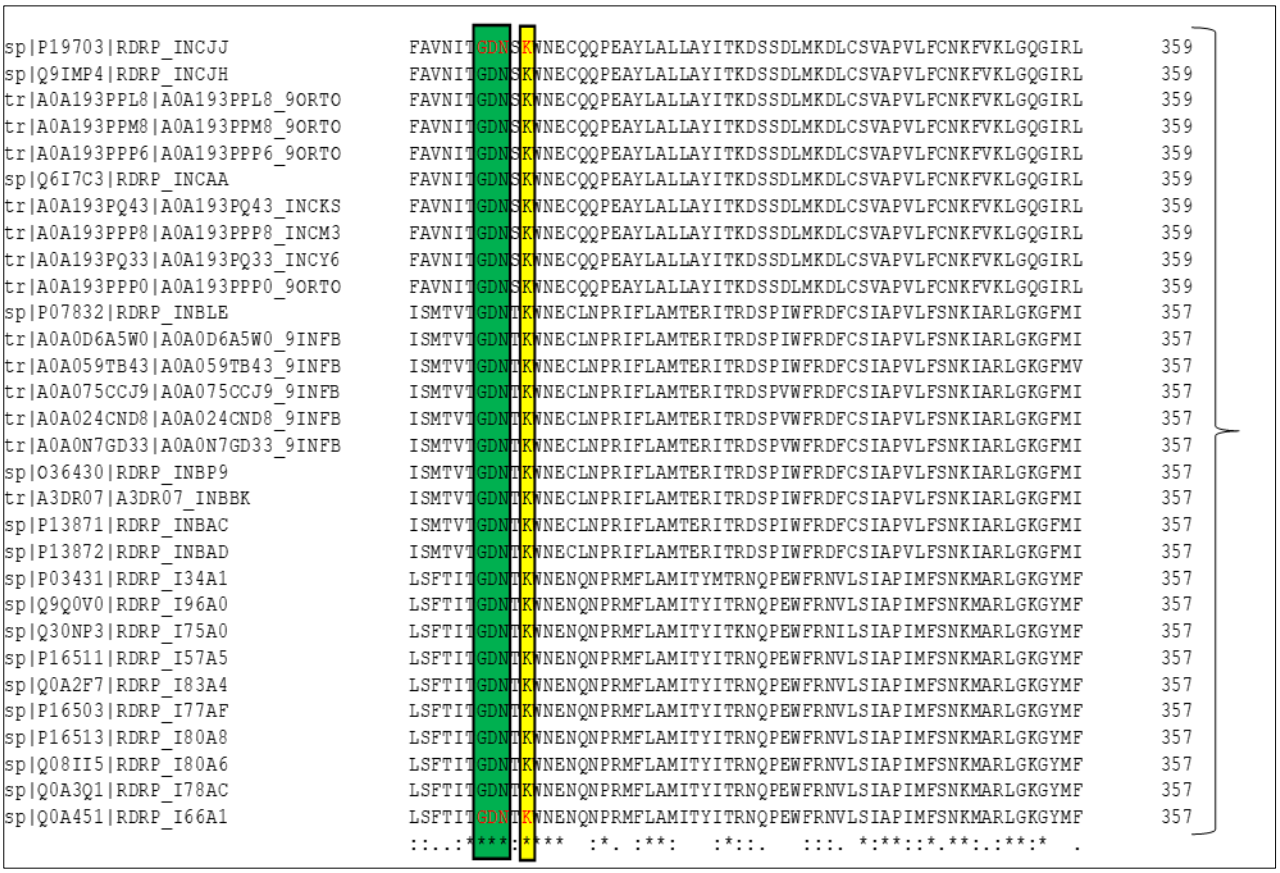
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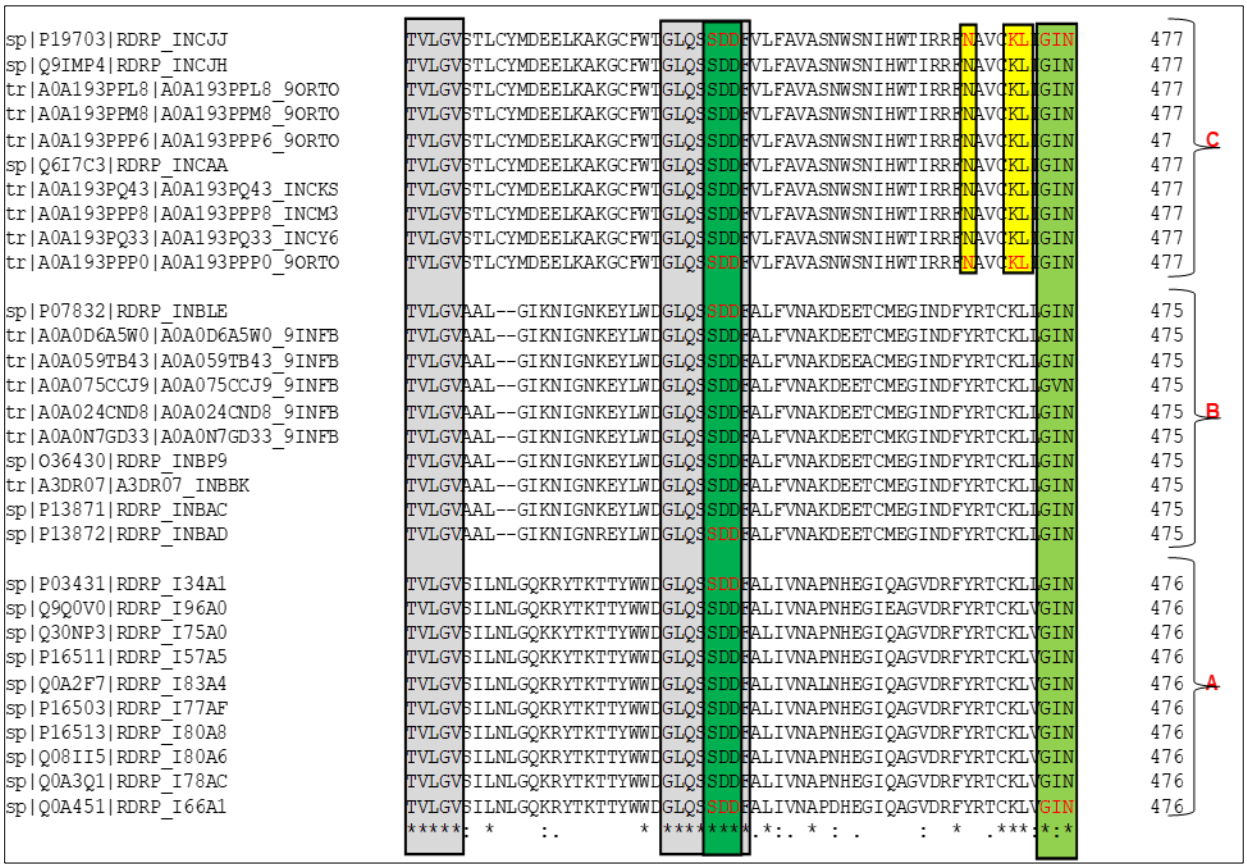
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sp|P13872|RDRP_INBAD
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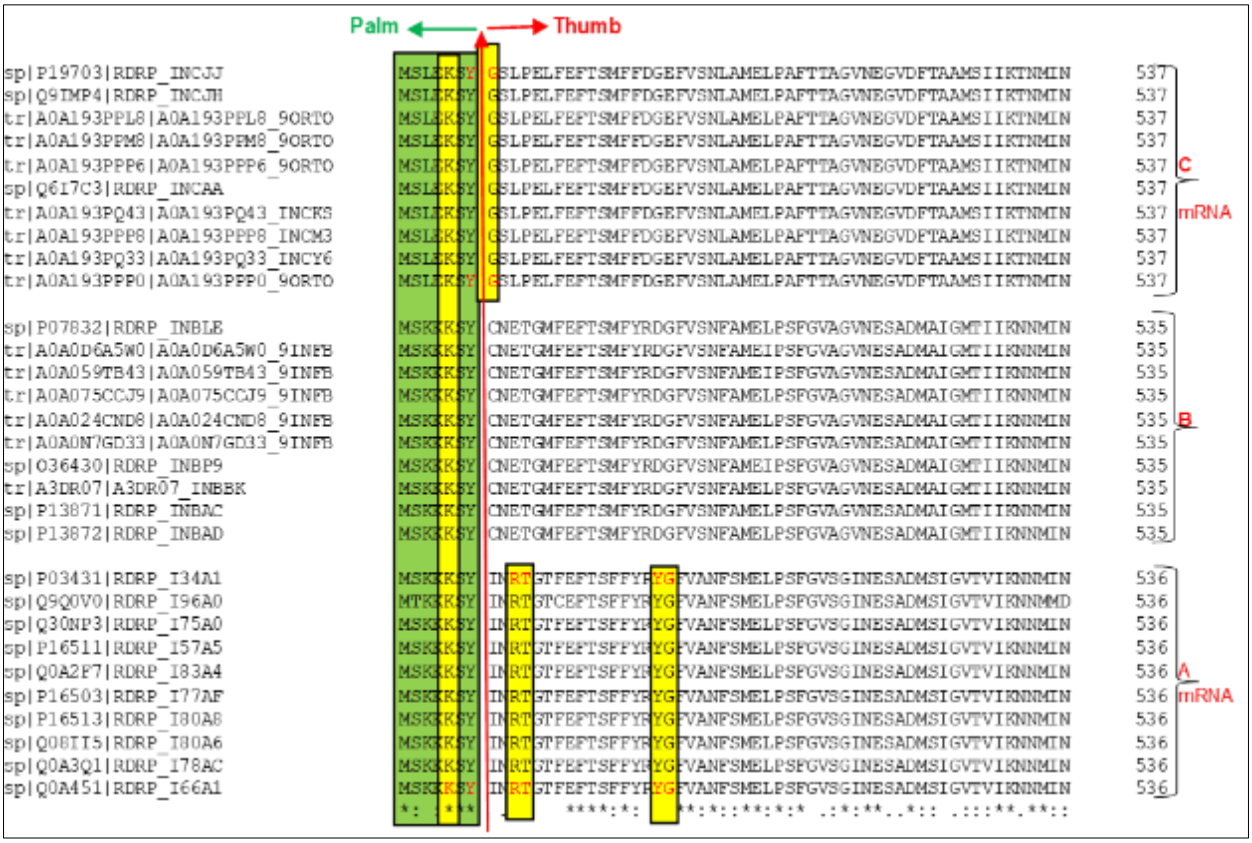
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tr|A3DR07|A3DR07_INBBK
sp|P13871|RDRP_INBAC
sp|P13872|RDRP_INBAD
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mRNA

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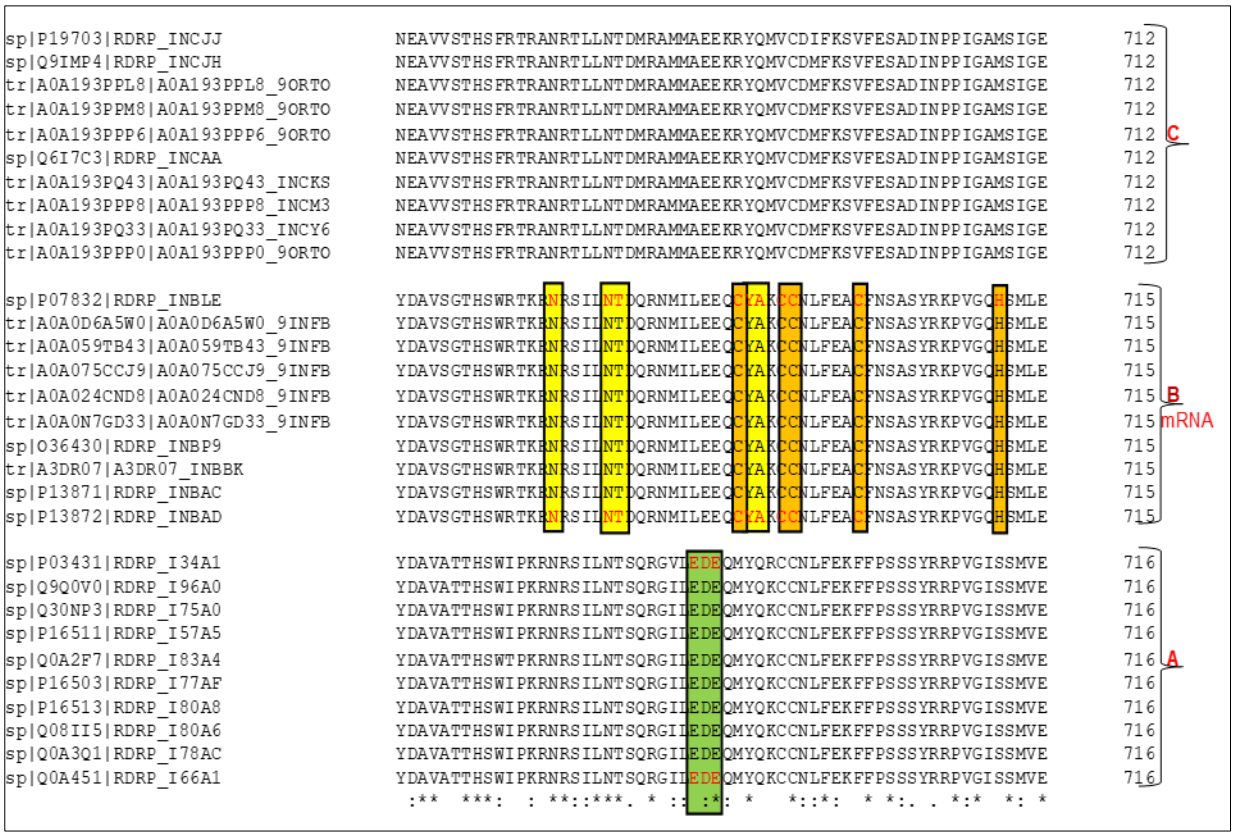
C

B

A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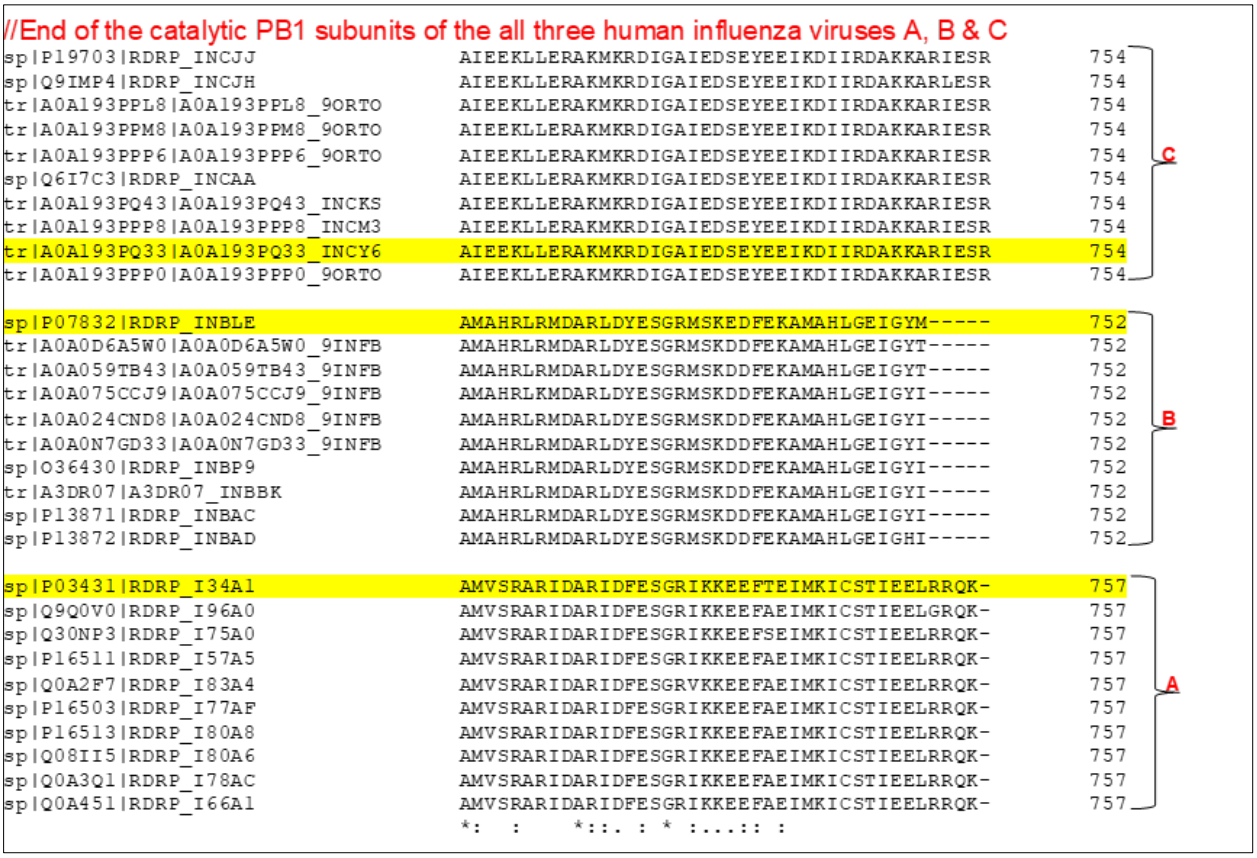
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C
B
A

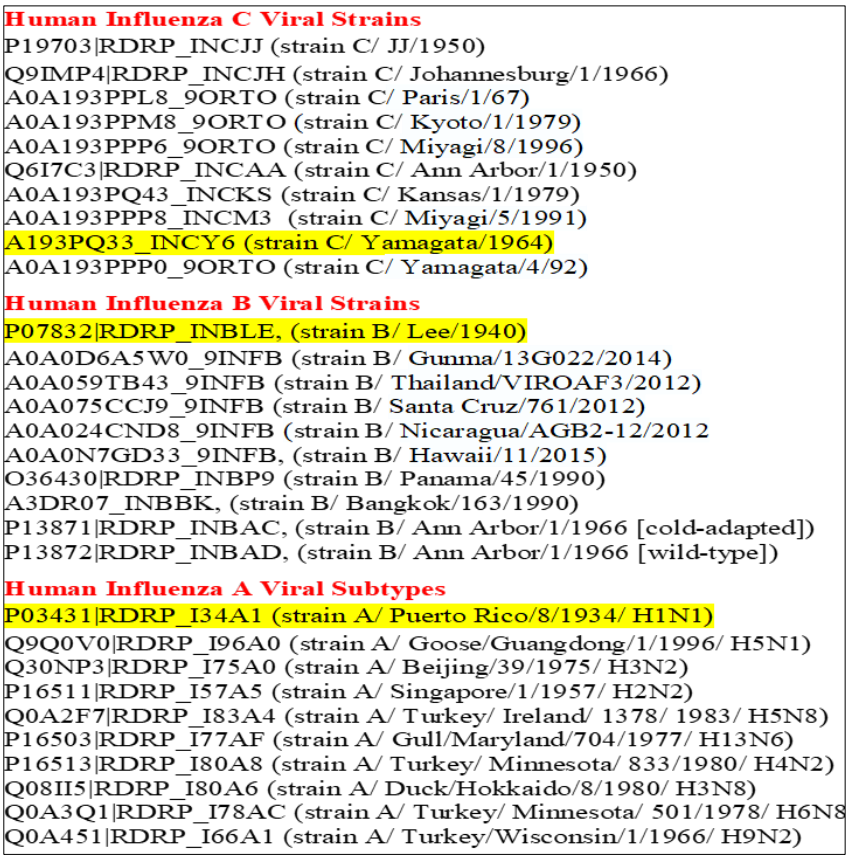

Figure 7 Mix and Match MSA of the PB1 subunits of the RNA polymerases of all the three human influenza viruses

3.2. Active site Analyses of the Catalytic Subunit PB1 of Human Influenza Polymerases

3.2.1. Analyses of Metal-Binding Sites in RdRps of RNA Viral Human Pathogens

Human RNA pathogens mostly possess either (+) strand or (–) strand RNA genomes. The (+) RNA strand viruses, including the pandemic causing SARS-CoVs, are known to use two invariant Ds, one from the –A/SDD- and the other from the –GDD- motifs for catalytic metal-binding and are confirmed by SDM experiments and X-ray crystallographic techniques. For example, Sankar and Porter [13] have shown that the point mutations generated by SDM experiments in the metal-binding motif -G**D**^{332}D- drastically affected the polymerization activity of RdRp of encephalomyocarditis virus, a small non-enveloped, (+) strand RNA virus (the causative agent of not only myocarditis and encephalitis, but also neurological diseases, reproductive disorders and diabetes in many mammalian species). Their results showed that all three amino acids of the –GDD- motif are important in maintaining the structure and function of the catalytic metal (Mg^{2+})-binding (the G in the –**G**DD- motif is known to generate tight bends in the structure) (Table 1).

Similar experiments have also shown that a slightly different –GDN- catalytic metal-binding motif is essential for catalysis in the (-) strand RNA viral pathogens. The (-) strand human RNA viral pathogens such as influenza, measles, mumps and respiratory syncytial viruses and Ebola virus (EBOV) also use Mg^{2+} as a co-factor for RdRp reaction. By MSA analysis, SDM experiments and minigenome assays [14] Schmidt and Hoenen, [14] have shown that -GDN- is the invariant motif in all the (-) strand RNA viral RdRps like Ebola, influenza, rabies, measles and mumps (Table 1). Furthermore, by SDM experiments, Schmidt and Hoenen [14] have confirmed that the triple mutation, –^{741}G**DNQ**-→ -^{741}G**AAA**- in Ebola viral RdRp abolished both the replication as well as transcription activities. In addition to the above findings, present MSA analysis has shown that all the (–) strand RNA viral pathogens possess the invariant –GDN- in their RdRps, indicating that this motif is absolutely essential for genome replication, transcription, or both. Tchesnokov et al. [15] found that the **D**^{742}→A mutant in the –G**D**^{742}N- motif of Ebola viral RdRp lacked the ability to extend the primer in the presence of Mg^{2+} and hence, the Ebola viral RdRp mutant D^{742}→A abolished the catalytic Mg^{2+}-binding activity which resulted in the absence of polymerase activity. Similar observations were made by Schnell and Conzelmann [16] on another human pathogenic (-) strand RNA virus, viz. the rabies virus. They found by SDM experiments that any modification of the –^{728}GDN- motif by conservative and non-conservative amino acids resulted in complete loss of the

RNA polymerase activity. In addition to this motif, the (–) strand RNA viral pathogens also possess a regular -G/SDD- motif as found in (+) strand RNA viral RdRps (Table 1) which provides the second D for the catalytic Mg^{2+}-binding.

Metal-Binding Sites in Human Influenza Viruses

Human influenza viruses, as typical (-) strand RNA viruses, possess both the invariant catalytic Mg^{2+}-binding –GDN- and -SDD- motifs in their RdRps (Table 1). These two metal-binding motifs were extensively analyzed by Biswas and Nayak [17] in human influenza A virus. They found that the –**DD**- in the –^{444}S**DD**- motif was absolutely critical for influenza A viral polymerase activity. When either of these –DD- residues were mutated, D^{445}→H/E, or D^{446}→Y/N/E, the mutated PB1 polymerase subunit became nonfunctional. Furthermore, the polymerase containing an active-site mutation D445→A/D446→A (also known as PB1a) of the –^{444}SDD- motif in influenza A virus has been used as a polymerase-negative strain in experiments [18]. When the -**S^{444}** of the above –**^{444}S**DD- motif was mutated to G, modifying the sequence to –**^{444}G**DD- as is found in (+) strand RNA viral RdRps, the polymerase activity was drastically reduced (1.9%), suggesting that the influenza A viral PB1 polymerase subunit cannot accommodate G in this position (Table 1). The second invariant metal-binding motif is the –GDN- motif in all (–) strand RNA viruses. When the -**D^{305}** in the –G**D^{305}**N- motif was mutated to a conservative amino acid Q, it showed only feeble polymerase activity, suggesting its involvement in the metal-binding. These findings were further corroborated by the SDM analysis of the -GDN- motif of Ebola virus RdRp, again a (–) strand RNA virus like influenza viruses as discussed elsewhere. Further insights were provided by the X-ray crystallographic analysis of the influenza A viral polymerase PB1 subunit by Pflug et al. [6]. They found that the conserved active site -D^{305} of –GD^{305}N- motif, together with -D^{445} and -D^{446} of the -^{444}SDD- motif coordinate binding to metal ions and promote catalysis [6, 15]. Thus, it is clear from the above SDM experiments, X-ray crystallographic and MSA analyses that the invariant -GDN- and –SDD- are the signature metal-binding motifs in all three human influenza viral RdRps (Table 1) [19].

3.2.2. Analyses of the Catalytic Core Regions of the Polymerase Subunit PB1

In addition to the two invariant catalytic Mg^{2+}-binding motifs, viz. –GDN- and -SDD-, (Fig. 7 and Table 1), all the three influenza viruses also possess two highly conserved catalytic cores. The catalytic cores consist essentially three components, viz. a template–binding pair and a catalytic amino acid for initiating catalysis by proton abstraction from 3'-OH and usually a basic amino acid at -4 to -5 position from the catalytic amino acid which has been proposed in nucleotide selection. Interestingly, two different catalytic cores are identified in human influenza viruses, suggesting one for the viral gRNA and the other for mRNA syntheses. An invariant -VG- pair is identified as the template–binding pair for the replication in all three human influenza viruses. However, the catalytic pair for the replication is found to be a –KL- in influenza A and C viruses and an -NL- for the influenza B virus. The nucleotide selection amino acid at -5 position is R/K/Q, respectively. It is interesting to note that the replication catalytic core of human influenza viruses are very similar to the other viral, prokaryotic and eukaryotic replicase enzymes (Table 2). The additional catalytic core identified in the PB1 subunits comprises of an –YG/A pair for the template-binding, a RT/NT/KL catalytic pair for initiating the catalysis and the nucleotide selection amino acid at -5 position is found to be K/N/N, respectively. The second catalytic core is similar to mRNA synthesis (Table 2), and hence could be involved in transcription.

The next conserved motif is a –KK- dyad (K^{480} and K^{481}), at the end of the palm domain in influenza A and B viruses. These two residues are found in the NTP entry site and are suggested to be involved in NTP binding [6]. This putative NTP tunnel directly leads to the tip of the putative priming loop which is composed of highly conserved basic residues R^{45}, K^{235}, K^{237} and R^{239} (the last three amino acids form the finger-tip subdomain), -K^{308} (of the –GDNTK^{308}-motif), and K^{480} and K^{481} (of the palm domain) [6]. The catalytic core encompasses the polymerase active site, which is connected to the exterior by several channels, including channels for the template entry and NTPs as well as for the exit of the template and product. Near the active site, the priming loop is a feature that is essential for primer-independent initiation on the vRNA templates, but that is not required for primer-dependent transcription and, surprisingly, also not for primer-independent initiation on the cRNA templates [20] as also proposed here.

Table 1 Catalytic metal-binding sites in viral RNA-dependent RNA polymerases

Organism	Metal-biding site(s)	Method and Reference
RdRps (EC 2.7.7.48) of (+) Strand RNA Viruses		
SARS-CoV-2	-LSDD^{761}A- & -QGDD^{825}Y-	X-ray [21]
SARS-CoV-1	-LSD^{760}DA- & -QGDD^{825}Y-	SDM [22]
MERS-CoV	-LSDD^{762}A- & -QGDD^{826}Y-	By seq. similarity
Polio	---------- -YGDD^{329}-	X-ray [21]
Hepatitis-C	---------- -NGDD^{319}-	X-ray [21]
Dengue	-YADD^{533}TA- & -SGDD^{664}CV-	X-ray [23]
Zika	-YADD^{535}TA- & -SGDD^{666}CV-	By seq. similarity
Yellow Fever	-YADD^{284}TA- & -SGDD^{417}CV-	By seq. similarity
Rubella	-LGDD^{26}A- & -QGDD^{667}M-	By seq. similarity
Encephalomyocarditis	---------- -YG^{332}DDL-	SDM [13]
RdRps (EC 2.7.7.48) of (-) Strand RNA Viruses		
Ebola	-MG^{741}DNQC- & -HTSDD^{617}FG-	SDM [14]
Ebola	-MGD^{742}NQC- & ,,	SDM [15]
Rabies	-QGD^{729}NQV- & -LESDD^{1589}NI-	By seq. similarity
Nipah	-QGD^{832}NES- & -IDSDD^{1525}NI-	By seq. similarity
Mumps	-QGD^{779}NQA- & -SSGDD^{1195}KF-	By seq. similarity
Measles	-QGD^{773}NQT- & -AYGDD^{1256}DS- & -LIGDD^{1470}D- & -ESDED^{1598}V-	By seq. similarity
RdRps (EC 2.7.7.48) of (-) Strand Segmented RNA Viruses		
Influenza A	-TGD^{305}NTK- & -QSSDD^{446}FA-	SDM [17]
Influenza B	-TGD^{305}NTK- & -QSSDD^{445}FA-	By seq. similarity
Influenza C	-TGD^{307}NSK- & -QSSDD^{451}FV-	By seq. similarity

Adapted from Palanivelu [11]

Table 2 Catalytic region(s) in the viral, prokaryotic and eukaryotic (nuclear and organellar) polymerases

Polymerase Type	Catalytic Region(s)
SSU RNA and DNA Polymerases	
Viral T7 SSU RNA pol	-620WLAY-8GVTR-4SVTK631R1SVMTLAY8GS- [24]
Viral SP6 SSU RNA Pol	-612WDSI-8GITR-4SLTKK1PVMTLPY8GS-
Mitochondrial SSU RNA pol (Yeast)	-TR-4KVVKQ1TVMTNVY8GV--
Chloroplast SSU pol (ARATH)	-DR-4KLVKQ1TVMTSVY8GV-
E. coli DNA pol I (SSU)	-753QR-4RSAK758A1INFGLIY8GM- [25]
Initiation Subunits of Prokaryotic and Eukaryotic MSU DdRps (mRNAs)	
E. coli MSU RNAP β subunit	-539TR-8ERAGFEVRD1VHPTHY7GRV558-
S. cerevisiae MSU RNAP II Rpb2 subunit	-851FR-5SLFFRS1YMDQEKKY9GMSI870-
Human MSU RNAP II Rpb2 subunit	-806FR-5SVFYRS1YKEQESKK9GFDQ825-
Elongation Subunits of Prokaryotic and Eukaryotic MSU DdRps (mRNAs)	
E. coli MSU RNAP β' subunit	-833NSV-6DAVKVRS1VVSC5DTDFGVC12AHC15Y16G17RDL861-
S. cerevisiae MSU RNAP II Rpb1 subunit	-55DPR-6LGSIDRN1LKC4QTC7QEGMNEC14PGHF18G19HI84-
Human MSU RNAP II Rpb1 subunit	-59DPR-6QGVIERT1GRC4QTC7AGNMTEC14PGHF18G19HI88-
Active Site Regions in Prokaryotic and Eukaryotic DNA Polymerases#	
T7 DNA polymerase (Rep)	-622AYGVTR-4SVTK631R1SVMTLAY639G9SKEFG [26]
P. furiosus DNA polymerase	-478ILLDYR-5QKAIKL1LANSFY7GYYGYAK-
Yeast DNA polymerase α (Rep)	-933RVQCDIR-5QQALKL1TANSMY7GCLGYVN-
Human DNA polymerase α (Rep)	-939ILQYDIR-5QKALKL1TANSMY7GCLGFSY-
Human DNA polymerase δ (Rep) (Catalytic subunit)	-683RQVLDGR-5QLALKV1SANSVY7GFTGAQV-
Human DNA polymerase ε (Rep) (Catalytic subunit)	-801YDSLQ-4LAHKC1ILNSFY7GVVMR-
Yeast DNA polymerase ε (Rep) (Catalytic subunit)	-816YDSLQ-4LAHKV1ILNSFY831G8YVMR- [27]
Human DNA polymerase γ (Rep)	-917TTVGISR-4EHAKI1FNYGRIY8GAGQPFAER-
(+) Strand RNA Viruses- (SARS-Coronaviruses)	
RdRps (NSP12)	
SARS-CoV-1	-STMTNR-5QFHQKL1LKSIAATRGATVVIGTSKFY21GG597-
SARS-CoV-2	-STMTNR-5QFHQKL1LKSIAATRGATVVIGTSKFY21GG597- [13]
MERS-CoV	-STMTNR-5QYHQKM1LKSMAATRGATCVIGTTKFY21GG598-
(-) Strand RNA Viruses- (Human Influenza Viruses)	
PB1 Catalytic Subunits of the RdRps	
Human influenza A virus	-256ETLAR-5SICEKL1EQSGLPV8GGN- gRNA -477MSKKK-5SYINRT1GTFEFTSFFYRY13GFV- mRNA#
Human influenza B virus	-256ENLAK-5NICENL1EQSGLPV8GGN- gRNA -666RTKRN-5RSILNT1DQRNMILEEQCY13AKCCX5CF- mRNA#
Human influenza C virus	-258ETVAQK-4ICEKL1KESGLPV8GGN- gRNA -464IRRFN-4AVCKL1IGINMSLEKSY12GSL- mRNA#

Adapted from Palanivelu [11,27].

gRNA, genomic RNA; Rep, Replicase. *P. furiosus*, *Pyrococcus furiosus*; The amino acids confirmed by SDM and by other techniques in the active sites are highlighted in dark blue; *The catalytic region of the human influenza viruses is arrived at by sequence similarity with other DNA/RNA polymerases. In the human influenza viruses, two such possible catalytic regions are found: one similar to RNA polymerases may involve in transcriptions and the other one similar to SSU DNA/RNA polymerases and eukaryotic replicases may involve in viral genome replications.

Table 3 shows the effect of mutations in SDM and natural mutations in the conserved motifs of the PB1 catalytic subunit of the human influenza A virus and their effects on polymerase activity/ replicative ability in human cells.

Table 3 Effect of mutations by SDM and natural mutations in the conserved motifs of the polymerase subunit PB1

Metal-binding site	Polymerase activity (%)	Reference
Metal-Binding Site-1 (-^{444}SDD-)		
^{445}D/^{446}D→A	0	[18]
^{444}S→G	1.9	[17]
^{445}D→H/E	0	[17]
^{446}D→Y/N/E	0	[17]
^{444}S→P	0	[10]
^{445}D→G	0	[10]
^{446}D→N	0	[10]
Metal-Binding Site-2 (-^{304}GDN-)		
-^{305}D→Q	Feeble	[17]
-^{306}N→T	2.4	[10]
Metal-Binding Site-2 (-^{741}GDNQ-), Ebola virus- a (–) Strand RNA virus		
-^{741}GDNQ→^{741}GAAA	0	[14]
-^{742}D→A	0	[15]
Polymerase Active Site		
^{476}N→L/^{477}M→T (DM)	0	[10]
^{476}N→T/^{478}S→T (DM)	0	[10]
^{478}S→T/R	0	[10]
^{480}K→R	117.2	[10]
^{481}K→E/T/N	0	[10]
^{483}Y→H	0.5	[10]

4. Proposed Mechanism of Action of the Polymerase Catalytic Subunit PB1 of Human Influenza Viruses

It is interesting to note that all DNA and RNA polymerases, irrespective of their sources, possess not highly conserved active sites but also very similar 3D structures, suggesting a similar catalytic mechanism [4, 28,29]. The finding that the PB1 subunit alone could transcribe RNA templates *in vitro*, suggested it possessed all the required active sites to synthesize a new strand of RNA from vRNA templates and possesses similar active sites and 3D structure [6, 30, 31].

A minimal number of steps involved in the catalytic cycle of the DNA/RNA polymerases are i) NTP selection and Watson-Crick base pairing with the complementary nucleotide to the template, ii) Addition of the nucleotide to the growing primer, iii) Inorganic pyrophosphate formation and iv) Restoration of the active site and translocation.

The catalysis is initiated by the proton abstraction by the catalytic amino acid from the 3'-OH of the primer, which is followed by an electrophilic-nucleophilic attack between the α-phosphate of the Watson-Crick base-paired NTP at the active site and the 3′-Oxyanion of the growing primer, resulting in the phosphodiester bond formation with the subsequent release of pyrophosphate. The proton abstracted in the first step is transferred to the pyrophosphate resulting in the formation of inorganic pyrophosphate (pp_i). The active site is restored and translocated for the next addition.

Based on the data available, Figs. 8 and 9 describe plausible mechanisms of action of the PB1 polymerase subunits of the human influenza viruses.

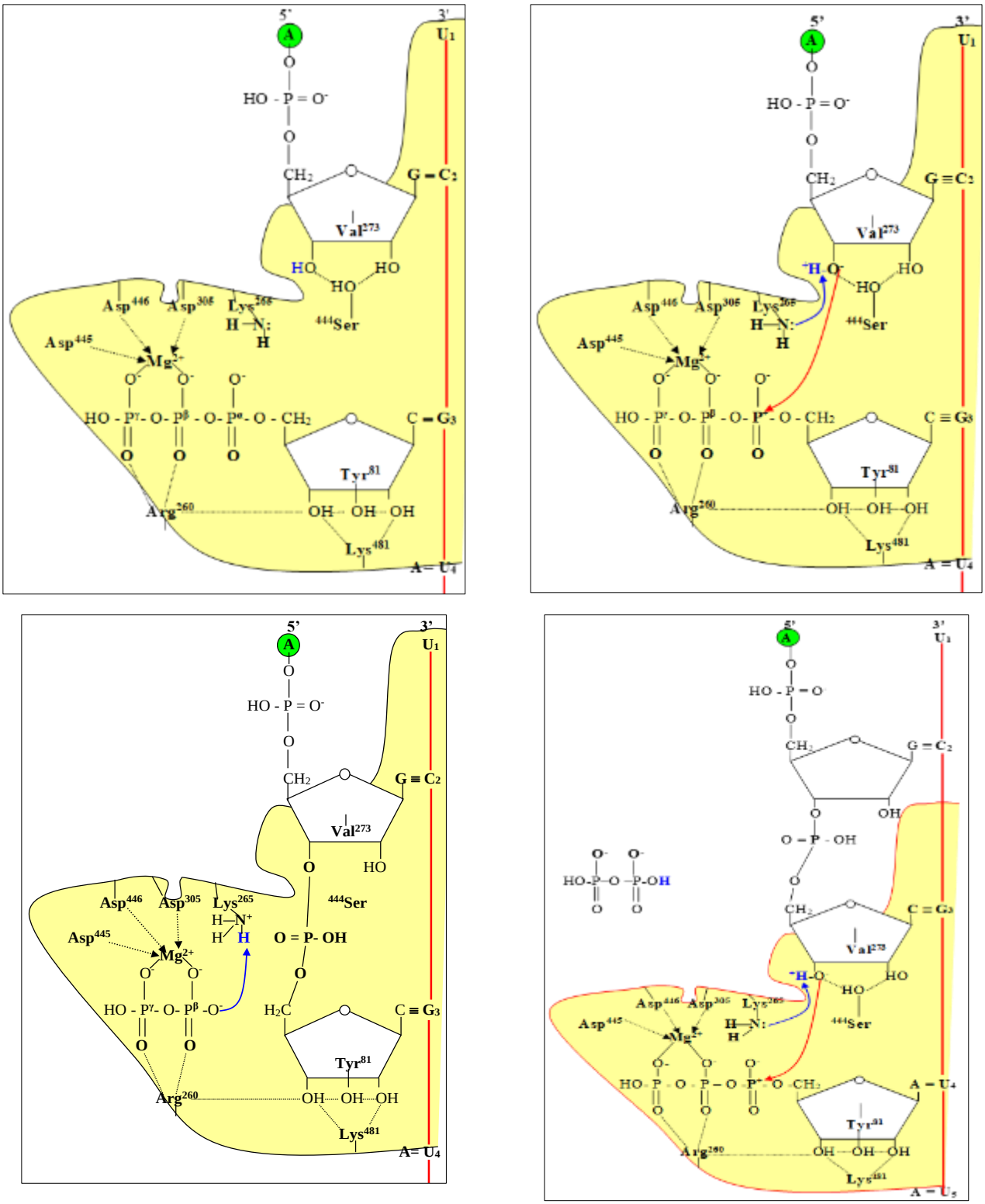

Figure 8 Proposed steps in the replication of viral genomic RNAs

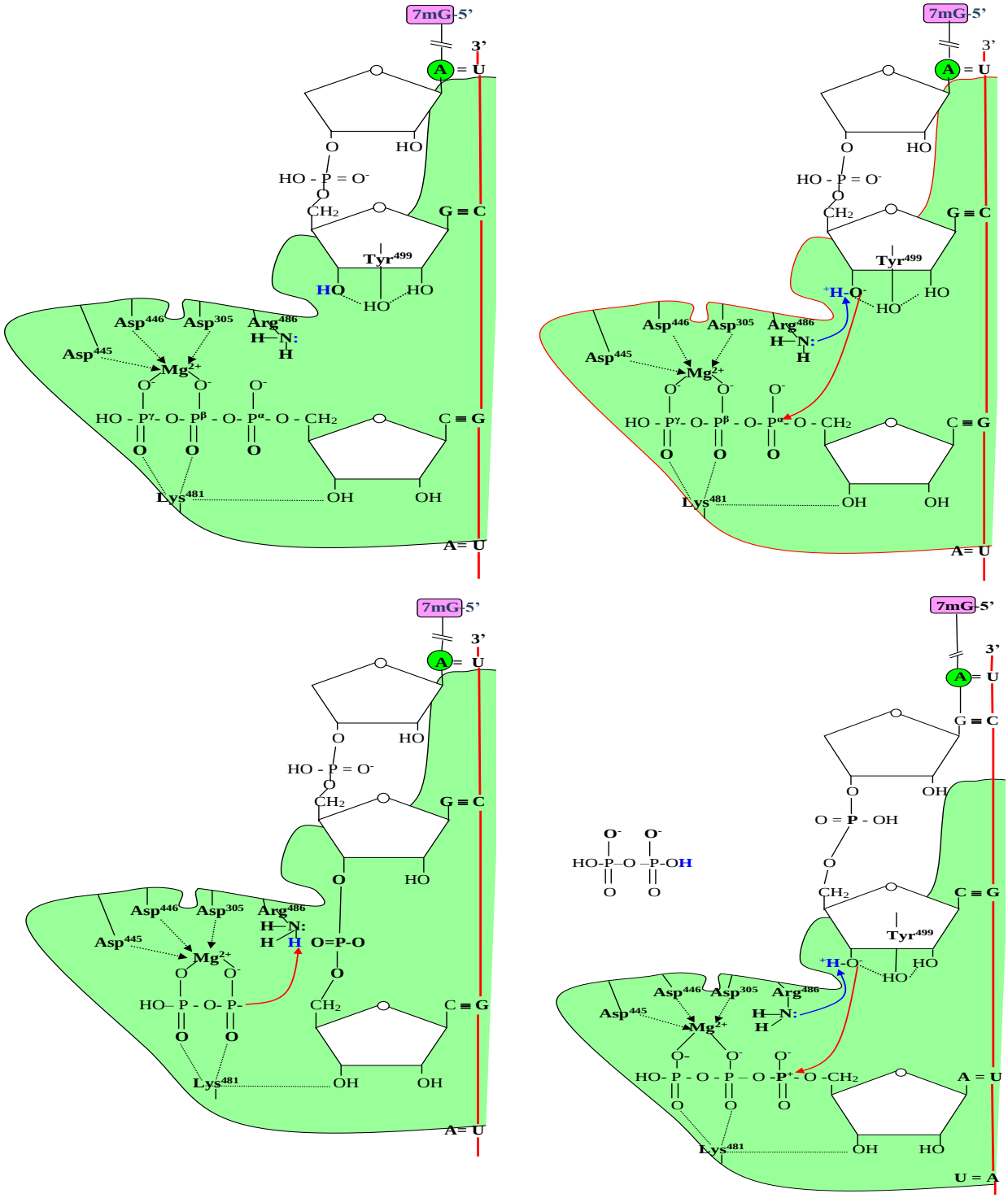

Figure 9 Proposed steps in the transcription of viral mRNAs

- **Step 1.** Enzyme with the Watson-Crick base-paired NTP at the Entry Site: In replication, the initiation step of RNA synthesis begins at the 3'- end of the vRNA template by means of a *primer-independent* mechanism. The primer-independent initiation consists in the addition of a GTP to the 3'-OH of the first initiating ATP and is followed by the next base (Fig. 8). Viral mRNA synthesis is *primer-dependent* and the primer is obtained from the host mRNAs by the cap-snatching method using the PB2 subunit. The primer is base-paired with the U at the 3' end of the viral RNA. The Y residue in the YG template-binding pair is suggested to be involved in base stacking as well as hydrogen bonding to the ribose moiety at the 3'- growing end and the G provides the necessary kink for the effective positioning with the template. The catalytic site amino acid R^{486} is positioned near the 3'- growing end for proton abstraction. The invariant K^{481} is shown to be involved in the NTP selection.

(The epsilon group of K^{481} could form the necessary hydrogen bonds with the substrate in the ternary complex). The catalytic Mg^{2+} is shown coordinating with the catalytic D^{445} and D^{446}, D^{305} shielding the β- and γ- phosphates of the incoming NTP and exposing the α-phosphate to the electrophilic- nucleophilic attack at the polymerizing site (Figs. 8 and 9).

- **Step 2.** Proton abstraction by the active site amino acid K^{265} (R^{486} in transcription site) with the simultaneous electrophilic-nucleophilic attack at the polymerase site. Formation of phosphodiester bond between the 3'-Oxyanion and the α-phosphate of the NTP.
- **Step 3.** Proton transfer from the active site amino acid K^{265} (R^{486} in transcription site) to the pyrophosphate and formation of inorganic pyrophosphate.
- **Step 4.** Restoration of the active site and translocation of the enzyme to the next nucleotide (Figs. 8 and 9).

5. Conclusions

The human influenza viral RNA polymerase is a heterotrimeric enzyme and made up of three different subunits, viz. two basic protein subunits (PB1 and PB2) and an acidic protein subunit (PA). The PB1 polymerase subunits of the viruses contain the active sites for nucleotide polymerization and perform the synthesis of both the mRNAs and gRNAs. The two typical invariant catalytic metal-binding motifs, viz. –GDN and –SDD- reported in (-) strand RNA viruses are also found in the PB1 polymerase subunits. Furthermore, MSA analysis shows two possible catalytic regions in the polymerase PB1 catalytic subunits from all the three human influenza viruses. The two possible catalytic regions suggest that the polymerase may follow a dual mode, i.e., it could use one for mRNA synthesis (transcription) and the other for viral gRNA synthesis (replication) depending upon its association to the cap-snatching PB2 (for mRNA synthesis, transcription) or its association with the proofreading PA subunit for gRNA synthesis (replication). It could use the same metal-binding motifs for both the mRNA and gRNA syntheses. Thus, all the three human influenza viruses exhibit common and distinct features among them. These data throw more light on the functioning of the key enzyme and may help in designing structure-based antiviral drugs targeting the PB1 subunit's active sites for the treatment of flu in the future**.**

Compliance with ethical standards

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