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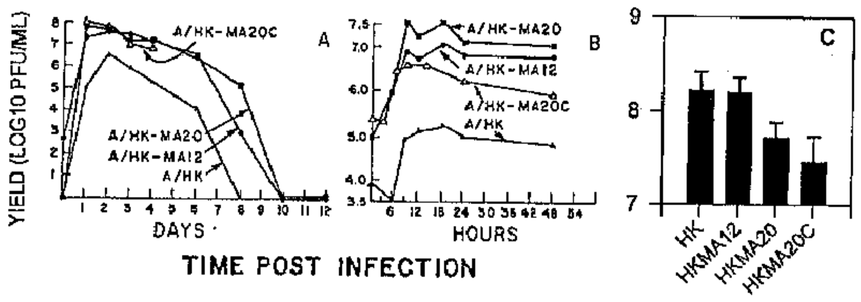
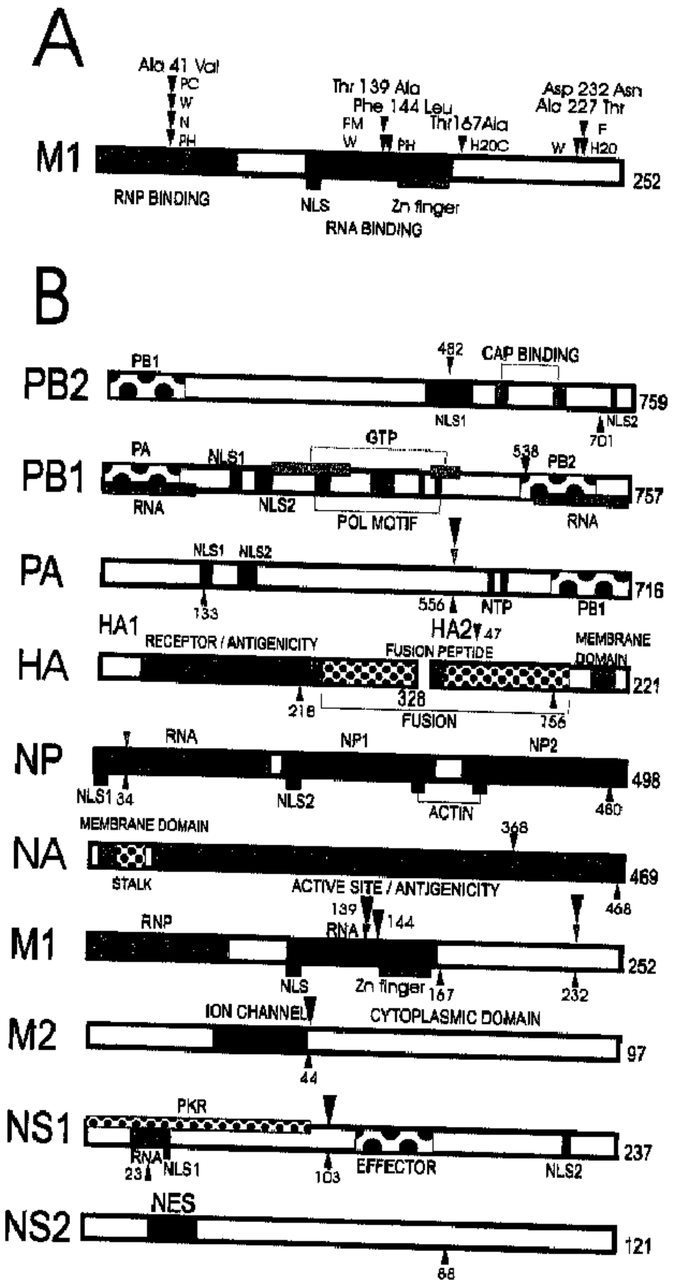
(54) Titre : PROCESSUS D'IDENTIFICATION DES MUTATIONS QUI CONTROLENT LA CAPACITE DES VIRUS DE SE MULTIPLIER ET DE CAUSER UNE MALADIE, ET L'APPLICATION DE CES MUTATIONS DANS LE DIAGNOSTIC VIRAL ET LA THERAPIE ANTIVIRALE

(54) Title: THE PROCESS OF IDENTIFICATION OF MUTATIONS THAT CONTROL THE ABILITY OF VIRUSES TO REPLICATE AND CAUSE DISEASE AND THE APPLICATION OF THESE MUTATIONS TO VIRAL DIAGNOSIS AND ANTIVIRAL THERAPY





(57) Abrégé/Abstract:
There is currently little understanding of the genetic basis for virulence or growth control in viruses. The invention provides methods of identifying mutations that control viral virulence and growth as well as the application of the detection of these

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(57) Abrégé(suite)/Abstract(continued):
mutations to diagnostic and therapeutic purposes. This patent identifies a process of selecting viral variants that accumulate biologically relevant mutations. The process involves the serial culturing of large populations of virus under conditions of restricted replication in this instance in the lung of mice but can include other types of tissues, that selects viruses with mutations that both increase replication and virulence. These mutations also involve regions of proteins that are in involved in controlling aspects of specific viral protein and RNA interactions. These regions define targets for antiviral drug intervention.

Abstract

There is currently little understanding of the genetic basis for virulence or growth control in viruses. The invention provides methods of identifying mutations that control viral virulence and growth as well as the application of the detection of these mutations to diagnostic and therapeutic purposes. This patent identifies a process of selecting viral variants that accumulate biologically relevant mutations. The process involves the serial culturing of large populations of virus under conditions of restricted replication in this instance in the lung of mice but can include other types of tissues, that selects viruses with mutations that both increase replication and virulence. These mutations also involve regions of proteins that are in involved in controlling aspects of specific viral protein and RNA interactions. These regions define targets for antiviral drug intervention.

This invention will be better understood by reference to the following examples, which illustrate but are not intended to limit the invention described herein.

Introduction:

Virulence is the measure of the ability of a pathogen to damage its host. Human influenza A virus infection typically causes tracheobronchitis with a low incidence of fatal pneumonia. In 1918 a virulent influenza A virus variant arose causing a devastating pandemic killing 50 million people (1). Although this virus was not isolated it must have possessed mutations that increased its virulence. The genomic sequence of 1918 viruses are being determined from archival tissues and while the sequence of the HA and NA genes are now available (2 Reid et al., 1999) we do not yet have the understanding of the molecular basis for virulence needed to interpret this information. The difficulty of discerning mutations that control virulence among the background of unselected mutations has been exemplified by sequence analysis of the highly virulent A/Hong Kong/156/97-like (H5N1) virus that recently infected humans directly from birds in Hong Kong (HK) (3). Even the most closely related avian isolate, A/Ck/HK/220/97 differs from this virus at 28 amino acids although both strains are equally virulent for chickens (4). Related H9N2 viruses that possess the same 6 internal protein genes have also infected people directly from birds in 1998-1999 indicating a continued threat to the human population by this lineage of viruses (1). There is thus a need to understand the genetic basis for virulence in influenza virus variants with the hope that specific mutations will be indicators and thus predictive of virulence.

There are no clinical isolates of human influenza (excepting the atypical A/HK/156/97 H5N1) that are known to differ in virulence (5), therefore necessitating the analysis of influenza infection in animals. A/HK/156/97 is not only virulent for chickens but also for mice without prior adaptation (6) indicating a shared genetic basis for disease production among species. Influenza virus is partially host restricted where virus from one host does not transmit or cause disease in other hosts unless adapted by cycles of infection. Serial passage of human influenza virus in mice results in the selection of highly virulent variants that have acquired mutations in multiple genes since analyses of genetic reassortants that possess mixtures of genes from virulent and avirulent strains have identified various groupings of genes in the control of virulence, that in aggregate implicate all 8 genome segments (7). These data have lead to the untested assumption that virulence cannot be genetically predicted because there are too

many degrees of freedom in the control of virulence. To begin to address this, a complete sequence comparison was done between the A/FM/1/47 (H1N1) parental strain (FM) and its mouse-adapted variant, FM-MA, that had increased $10^{4.6}$ fold in virulence on the basis of LD_{50}. This identified single amino acid substitutions in 5 of its 10 genes. Reintroduction of each of these mutations into the parental FM strain not only confirmed their roles in increasing virulence but also in replicative fitness for the mouse (8). These findings were compelling since they show a clear relationship between replicative fitness and the ability to damage the host. It was also surprising that the mouse-adapted variant did not possess unselected mutations that typically accumulate in clinical isolates. This however, would be predicted from studies of viral adaptation in cell culture where genetic variation is a function of virus population size (9). Serial passage of large populations of virus under novel conditions allows competition among all possible mutants with the selection of optimal genotypes. In contrast, the transfer of small populations, typical of normal disease transmission, leads to the fixation of unselected mutations due to stochastic effects. This process termed Muller's ratchet results in the accumulation of deleterious mutants (9).

The primary feature of organisms with adaptive mutations is that they increase in prevalence in the population due to improved fitness. A strong indicator of adaptive change at the molecular level is convergent evolution characterized by the repeated and independent occurrence of the same mutations, termed parallel evolution, or mutation at the same sites but with different amino acids, termed directional evolution (10). This is the standard criterion for identifying mutations responsible for drug and inhibitor resistance providing evidence for convergent evolution in many organisms including influenza virus (11).

Since the parental A/FM/1/47 strain had been subjected to mouse passage immediately on isolation in 1947 (12) it carries pre-existing mouse adaptive mutations detectable on analysis by genetic reassortment (13,14). In the present study, the prototype H3N2 clinical isolate, A/HK/1/68, without a prior history of mouse passage, was used to generate virulent variants by serial mouse-lung passage. The 2 objectives of this analysis were to identify the location and nature of the mutations that control virulence and secondly to assess the predictive value of these sites for other virulent viruses. Genomic sequencing of a highly virulent mouse-adapted variant identified 11 mutations that were selected on mouse adaptation. Sequencing of clonal variants showed that most of these mutations were positively

selected in the population and affected specific functional regions of individual genes that define functional themes for regulating virulence. Parallel mutations were shown to have occurred in the M1 gene of independently derived mouse-adapted variants. Furthermore 6 mutations were exclusively or predominantly associated with virulent viruses from avian and mammalian sources, suggesting a common role for these mutations in the control of virulence. Experimental evolution recapitulated natural evolution of virulence such that the Hong Kong H5N1 viruses possessed 4 mutations in common with mouse-adapted strains suggesting their instrumental operation in the unusual virulence of this group of viruses.

Methods

Viruses

A/Phillippines/2/82 (H3N2) and the prototype human H3N2 isolate, A/HK1/68 were obtained from the Laboratory Centre for Disease Control, Health Canada, Ottawa. Viruses were clonally purified by 2 plaque isolations in MDCK cell monolayer followed by stock preparation in the allantoic cavity of 9 day old chicken embryos. Virus was passaged in mouse lungs by cycles of intranasal infection under halothane anaesthesia for groups of 3 mice for 3 days as described previously (13). Mouse-adapted viruses were clonally isolated and titrated by plaque assays on MDCK cell monolayer as described (13)

Nucleotide Sequencing

Viral RNA was purified by phenol extraction from stock virus as previously described (14). Each genome segment was amplified by RT-PCR and purified on Sepahcryl S400 spin-columns (Pharmacia) before direct, dideoxy terminated cycle sequencing using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase (Perkin Elmer, Mississauga) and an Applied Biosystems automated sequencer, Model ABI 373. Genome segment specific primers for RT-PCR were complementary to the first 16 nucleotides of each segment in combination with a primer complementary to the 12 nucleotides at the 3' end of vRNA and all possessed a 5' terminal adapter sequence of CCGC. Sequencing primers (sequences available on request) were complementary to related H3N2 viruses; Genbank accession no. J02135-40, and X59240 respectively.

SDS PAGE

MDCK cells were infected, [^{35}S]Met-labeled and analyzed as described previously (13). Labeled virus was purified by adsorption to guinea pig erythrocytes. Trypsin was used at 1 ug/ml for 15 minutes at 37° C to cleave hemagglutinin. Tunicamycin was used at 10 ug/ml from 5.5 h post infection onward with pulse-labeling at 6.5 h pi. for 1.5 h. Immunoprecipitations were performed as described previously using A/HK/1/68 specific rabbit immune serum (15).

Virulence assay

Virulence was measured as the lethal dose in CD1 strain, Swiss Webster, mice. The median lethal dose (LD_{50}) in pfu was measured by intranasal infection of 5 groups of 5 mice each with serial 10 fold dilutions of virus as described previously (13). Survival was monitored for 10 days.

Software for Database analysis

To find all known mutations for a specific position in a protein, a search program was developed using PERL in conjunction with a local setup of the BLAST program (Basic Local Alignment Search Tool, (17)) and the Influenza Sequence Database downloaded from the Los Alamos National Laboratory (current Jan. 2001) (http://www.flu.lanl.gov/). The PERL script was accessed via a web interface and parsed the output, flat query-anchored alignments from BLAST, for sequences differing in the user selected position of the query protein sequence. The PERL script is available from the authors and the BLAST program from NCBI (ftp://ncbi.nlm.nih.gov/blast/).

Statistical analysis

LD_{50} values were determined using the Karber method (13). The significance of differences in virulence and growth values was determined using the Z statistic for a standard normal distribution. The probability of multiple independent mutations in the same clonal isolate was predicted by the Poisson distribution using the observed mutation frequency.

Growth in MDCK cells, chicken allantoic cavity and mouse-lung

MDCK cells were infected at an moi of 5 pfu/cell and incubated at 34°C in the presence of 1 ug/ml trypsin (14). Supernatants were collected over a 48 hr period and plaque assays were performed in duplicate for each sample. Groups of CD-1 mice were infected intranasally with $5x10^3$ pfu of each virus. Over a 10 day period lungs were removed from groups of 3 mice, pooled and sonicated for quantification by plaque assay on MDCK monolayers as described above. The yield in chicken egg

allantoic cavity was determined by infecting 9-10 day old chicken embryos with 10^4 pfu and incubation at 48 hr at 34°C. Pools of 3 eggs each (2-6 pools/virus) were titrated by plaque assay on MDCK cells.

Results

Mouse-adapted variants of the human isolate, A/HK1/68, (HK) were produced by serial lung to lung passage beginning with inoculation of 10^6 pfu of virus per mouse. Subsequent passages involved the inoculation of 50 uL of 1/10 diluted lung extract into groups of mice and since the lung extracts contained 10^{6-7}pfu/mL at each passage (data not shown) this represented an inoculum of 10^{4-5}pfu/mouse. Virulence was assayed by LD_{50} after 12 and 20 passages for both the total population of virus obtained directly from titrated lungs extracts without further culturing as well as 6 clonal isolates from each of these passage levels obtained by plaque purification of 10^7 fold diluted lung extracts in MDCK cells (Table 1). The parental virus was totally avirulent for mice, $LD_{50} > 10^{7.7}$ pfu, however after 12 passages the LD_{50} of the population of virus in lung extracts had decreased $>10^4$ fold and after 20 passages $>10^5$ fold, indicating that these virus populations were in the process of acquiring mutations that profoundly affect virulence. Several HK mouse adapted (HKMA) clonal isolates from each passage level had similar virulence to their respective populations and were thus representative of these populations, however some were less virulent indicating some genetic heterogeneity within these populations. HKMA20C was the most virulent virus clone $LD_{50} = 10^{2.5}$ pfu, being similar to the passage 20 population (Table 1).

Sequence analysis

To identify the mutations that are responsible for the increased ability to cause fatal lung infection we initially sequenced the genome of the most virulent clonal isolate, HKMA20C, as well as the HK parent for comparison. A total of 11 amino acid substitutions involving 8 of the 10 viral proteins (Table 2) and 4 silent substitutions were detected, that produced 2 coding changes in each of PA, HA, and NP, as well as single amino acid substitutions in PB2, NA, M1, M2 and NS and no mutations in PB1 and NS2. The noncoding changes (data not shown) included an insertion of an extra A in the poly-A coding region of the NA gene (Table 2).

Although it is clear that these mutations as a group must account for the difference in biology of the HKMA20C variant it is not clear that they are all instrumental in adaptation to increased virulence. Since adaptive changes increase replicative fitness, viruses that possess these changes will be present at a greater frequency in the virus population than their rate of formation predicted from the

mutation frequency. To detect mutations that were positively selected on mouse-adaptation we tested for the null hypothesis that the mutations in individual virus clones were randomly and thus independently generated. Given the observed frequency of coding mutations in HKMA20C of 2.2 x 10^{-3}/aa, the probability of having 2 viruses with the same mutation due to independent mutational events is predicted by chance to be P = 5 x 10^{-6}/n-1 where n is the number of isolates tested. The occurrence of the same mutations in more than one isolate is thus significant evidence of positive selection rather than the occurrence of independent mutational events. The HKMA12 and HKMA20 variants were subjected to sequence analysis to determine if they shared mutations in common with HKMA20C. Genome segments 4-8 were completely sequenced as well as the mutant loci detected in the PB2 and PA genes. The HKMA12 clone possessed 5 mutations in common with both of the passage 20 clones and HKMA20 possessed 6 mutations in common with HKMA20C indicating positive selection of 6 mutations. The low probability of pairs of viruses with 5 or 6 mutations in common is also a clear indication of positive selection as this is extremely unlikely to occur by chance (Poisson distribution P $\leq$ 1 x 10^{-13} and P $\leq$ 1.4 x 10^{-17})

Since 2 more mutations were detected in HKMA20, (M1-Asp232Asn and NS1-Val23Ala), segment 7 and 8 (M1, M2, NS1 and NS2 genes) were sequenced for HKMA-12A, -12B and all the remaining HKMA20-series of clones listed in Table 1. This analysis identified a further mutation, NS2 Lys88Arg, and demonstrated positive selection for 4 of 6 mutations in the M and NS genes (Table 3). All of these mutations were associated with a greater duration of adaptation since they were found in passage 20 clones and not in passage 12 clones.

Protein changes

The HA2 Thr156Asn substitution is expected to result in the loss of a glycosylation site that would be detectable by increased mobility on SDS PAGE. Electrophoresis of infected MDCK cell proteins showed that the uncleaved form of HA protein possessed higher electrophoretic mobility than HK for 3 of 6 of passage 12 clones and 6 of 6 passage 20 clones (data not shown). The change in electrophoretic mobility of HA0 was due to decreased glycosylation for all clones except HKMA12A, since the unglycosylated forms of all of the other variant HA0 proteins resulting from tunicamycin treatment were indistinguishable in electrophoretic mobility from the parental virus (data not shown). The mutations affecting glycosylation were confirmed to map to the HA2 subunit by trypsin cleavage

of purified virions since this subunit was shifted to higher relative electrophoretic mobility corresponding to a 5.5 kD decrease in apparent size relative to the HA2 of the HK parent (data not shown).

On SDS PAGE analysis it was observed that the NP proteins of HKMA20B, -C and -D possessed increased mobility that corresponded to a 1 kD decrease in apparent size (data not shown). This correlated with the presence of the mutation at aa 480 of HKMA20C relative to HKMA20 and HKMA12. The occurrence of this mutation in 3 of 6 passage 20 clones indicated positive selection.

Growth in Different hosts.

The relative ability of HK and the HKMA-12, -20 and -20C clones to replicate was assessed in mouse lung, MDCK cells and chicken allantoic cavity. All of the mouse-adapted clones grew faster and to higher titre than HK in mouse lung (Fig. 1A). Comparison of the yield 1 day post-infection as well as the maximum yields of infectious virus indicated that the HKMA20C virus had the greatest increase in replicative fitness relative to HK in the mouse which was consistent with its relative virulence (Table 4). The increases in fitness 1 day post infection were dramatic, demonstrating the replicative basis for selection of mouse-adapted variants. All of the mouse adapted clones tested also had an increased ability to replicate in MDCK cells (Fig 2), however both of the passage 20 mouse adapted clones yielded less infectious virus in chicken allantoic cavity than the HK parent or the less passaged HKMA12 clone ($P \leq 0.05$) (Fig 1C). Thus mouse adaptation selected variants with increased replicative fitness in the mouse but some of the mutations conferring these properties were host specific.

Evidence of parallel evolution

Although there is only fragmentary analysis of the mutations in genes of mouse-adapted variants of human influenza virus, the M1 genes of A/PC/2/73-MA (H3N2) and the neurotropic H1N1 variants WSN, and NWS have been determined both before and after mouse-passage (16). In addition to the FM and HK mouse-adapted variants, we have also sequenced the M1 gene of a clonal variant of A/Phillippines/2/82 (H3N2) obtained by 12 serial passages alternating between mouse lung and egg (unpublished data). Comparison of the mutations selected on mouse-adaptation in these viruses demonstrates impressive evidence of parallel evolution with 3 of 6 sites being repeatedly mutated to the same amino acid (Fig 2); the Ala227Thr change in FM presumably was selected during its first cycle

of mouse-adaptation. The pattern of amino acid substitution was also not random with 4 of 6 sites occurring in pairs, 5 amino acids apart, at 139-144 and 227-232.

To discover instances of parallel evolution between MA variants and other influenza strains in the Influenza Sequence Database (www.flu.lanl.gov), we produced software that sorts the output from the BLAST alignment (17) to detect variation at specific amino acid locations. Comparisons showed that 14 of 18 mutation sites mutated in mouse-adapted variants (Tables 2 and 3, Fig. 2), had mutated independently in naturally virulent or laboratory passaged isolates of influenza virus (bold in Table 5). Six sites of mutation were exclusively or predominantly associated with virulent viruses, primarily involving parallel changes and firmly supporting their roles in virulence (red arrows in Fig2, Table 5). This is a minimal assessment since the virulence properties of many viruses are undetermined or unpublished.

Discussion

Adaptation is believed to be the driving force in evolution where organisms are selected in nature due to increased fitness conferred by beneficial mutations. This paper extends the analysis of the mutational basis for virulence using mouse-adaptation since this system provides the identification of mutations that generate evolutionarily relevant phenotypic variation. The pattern of mutations associated with HK-MA variants was consistent with the assembly of mutations into genomes by reassortment followed by competition among viruses with different mutations on the same genome segment.

Positive Selection

A total of 14 coding and one insertion mutation were observed on mouse adaptation of HK. The positive selection of 11 of 14 mutations indicates that they confer a replicative advantage, however there is also reason to believe that the remaining 3 mutations observed in individual clones from the passage 20 population were also adaptive. The Glu133Gly mutation in the PA protein occurred in the middle of the first nuclear localizations signal at aa 124-139 (11), and may affects this activity. The M1-Thr167Ala mutation was independently selected in 1972 to become fixed in the H3N2 lineage (Table 5) indicating that this mutation is normally selected in human passage. The NS2-LysArg88 mutation was found in several virulent viruses including the virulent Hong Kong H5N1 lineage of viruses suggesting that this is an adaptive mutation that plays a role in modulating virulence (Table5). Thus all of the mutations detected on mouse-passage of HK virus were deemed to be instrumental in adaptation to increased virulence since they were either being positively selected, affected functionally important regions of proteins (discussed below) and/or were found to have occurred independently in other virulent or passaged influenza viruses (Table 5, Fig 2). From previous genetic analysis of the A/FM/1/47-MA variant, all the HKMA mutations would be expected to be adaptive (8,14).

Parallel evolution associated with virulence

Comparison of mutations in the highly conserved M1 gene of mouse adapted strains indicated strong evidence for parallel evolution where 3 sites were repeatedly mutated to the same amino acid. The pattern of amino acid variation in the M1 gene involves regions of protein and RNA interaction that may be important in regulating virulence (Fig 2). Comparative analysis for common variation with these and other HK-MA mutations identified 14 of 18 mutations that had occurred in other virulent viruses. The most significant occurrences of parallel evolution were seen for 6 sites that were exclusively or

predominantly associated with mouse-adapted or naturally virulent viruses (PA-556, M1-139, M1-144, M1-232,; M2-44 and NS1-103) strongly supporting their roles in virulence (Fig2 and Table 5). This indicates that adaptation to increased virulence in the mouse is predictive of mutational events that occur independently in other virulent viruses. The occurrence of multiple sites of convergent mutation in individual viruses is highly significant of a shared genetic basis for virulence. Three sites were in common with the FM parental strain and probably represent mutations selected on mouse-adaptation in 1947 (Table 5).

With respect to the highly virulent A/HK/156/97 human strain it is compelling that this virus possessed 4 amino acid mutations, PB2-Asp701Asn, M1-Phe144Leu, NS1-Phe103Leu and NS2-LysArg88, in parallel with HK and A/Philippines/2/82 (H3N2) mouse-adapted variants and suggests that they operate in this virus to mediate its extreme virulence. The PB2-Asp701Asn mutation is located between the second nuclear localization signal (NLS2) at 736-739 (11) and the cap binding motif at 634-650 (11) both of which could be modified in activity. The M1-144 mutation is adjacent to a zinc finger motif (aa 148-162) in an RNA binding domain (aa 135-165) that could modulate interactions with the ribonucelocapsid during replication (11). The FM-MA M1-Ala139Thr mutation in this region has been confirmed to increase virulence and replication (8,14). The NS1 gene is required for counteracting several antiviral effects of interferon (31) and so the Phe103Leu mutation may be affecting this function since mice lacking an interferon response can develop a systemic infection with neurovirulent influenza virus (31) that is similar to A/HK/156/97 infection of wild type mice (6). The remaining mutation, NS2 Lys88Arg, may be affecting the function of this protein in nuclear export of viral nucleocapsids (11). Sequence analysis of A/HK/156/97 clones that are attenuated in virulence for mice was associated with reversion of the PB2 Asp701Asn mutation as well as 2 mutations, NS1-Asp101Asn and HA1-Pro211Thr, that are adjacent to the NS1-103 and HA1-218 observed on mouse adaptation (32); further supporting the relevance of these mutations as determinants of virulence.

Convergence with Human Lineages of Virus

Several MA mutations were convergent with mutations that have been selected in humans. The NS1 Val23Ala, mutation is convergent with most influenza viruses including human H1N1 and H2N2 strains (Tab 5). The M1 Thr167Ala mutation is convergent with the human H3N2 lineage, that acquired this mutation in 1972 (Tab 5). The M1 Ala227Thr mutation is convergent with the same lineage since

1993. Thus experimental evolution recapitulates the selective processes of natural evolution of influenza viruses. Furthermore many of the natural influenza isolates that possessed mutations in parallel with mouse-adapted strains had recently been adapted to a new host often in conjunction with reassortment as seen for the avian to animal transfer (e.g. A/Equine/Jilin/1/89 (28)) and human to swine transfer (data not shown). This further suggests that the natural adaptive processes accompanying host switching of influenza viruses are in part common with experimental adaptation.

Mouse adapted variants possess mutations in specific functional regions

It is apparent that increased virulence is due to mutations that increase the ability of influenza virus to exploit the host environment. Given the location of mutations selected on mouse-adaptation (Table 5) they appear to implicate the central processes of viral replication and implicate sites of interaction with host and viral components. Specific types of functional regions that are repeatedly mutated include RNA binding sites as seen for NS1-23, M1-139 and 144; nuclear localization signals of polymerase subunits, PB2-482 in FM-MA,(8) and PA-133; and mutations at subunit interfaces of HA, HA1-218 in HKMA variants and HA2-47 in FM-MA (14). The NP-480 mutation is adjacent to amino acid 479 that affects NP oligomerization (11) and the PB2 binding site of the PB1 polymerase subunit is mutated in FM-MA (8,11).

The Thr156Asn mutation of HA2 results in the loss of a glycosylation site adjacent to the cleavage site of HA0 and may be relevant to cleavage activation since there is detectable cleavage of HA without trypsin for the HKMA12, 20, and 20C strains produced in MDCK cells (data not shown). This is analogous to the loss of a glycosylation site near the cleavage site of the HA of A/CK/Pennsylvania/1370/83 (H5N2) that resulted in increased cleavability and high virulence (33). The other HA mutation, Gly218W of HA1, occurs at the trimer interface adjacent to the receptor binding site. Mutations at this site in monoclonal antibody escape mutants have been shown not only to affect HA structure such that antibody binding is maintained without neutralization but concomitantly results in alteration in receptor specificity and the pH threshold for membrane fusion (34). Consistent with this, the HKMA12 -20 and 20C variants were altered in pH of fusion, resistance to β-inhibitor (mouse serum lectin), and receptor binding (data not shown). A common feature of other mouse-adapted variants is mutations in HA associated with resistance to inhibitors and in some instances altered pH of fusion as seen for FM-MA (8,14,16).

Host-restrictive mutation

The nature of the polymerase and NP proteins have been implicated in host restriction (11). The HKMA20C variant has become host restricted in its growth characteristics possibly due to the PA-133 mutation in NLS1. The FM-MA variant has the same growth properties due to a combination of mutations in the PB2 (NLS1) and PB1 (PB2 binding site) subunits (8). The PA-556 mutation may also be relevant to host-specificity since it was only found in mouse-adapted strains (Table 5). The NP gene has evolved into host specific lineages (11) where Asp34 is found in all human clinical isolates and Gly34 in all other animal and avian isolates with exceptions that include mouse-adapted variants (Table 5). The Gly34 substitution was also detected in A/Ohio/103/83-like viruses obtained from a chronically infected immunocompromised child (21) indicating that this substitution is also selected during persistent human infection.

In conclusion adaptation is due to selection of host dependent as well as host-independent mutations that result in heightened exploitation of the host seen clinically as increased disease. Furthermore the process of experimental adaptation in the mouse appears to recapitulate natural evolution and is predictive of mutational events in other influenza viruses including the virulent H5N1Hong Kong and probably the 1918 pandemic strain. The means of achieving optimal competitive advantage appears to be controlled primarily by a discrete number of mutations in specific regions of the influenza virus genome that are, at least in part, predictable. Future studies will address the mechanism of action of adaptive mutations which are expected to explain growth control and identify novel targets for antiviral therapy. The adaptive approach to analyzing virulence should also have general applicability to other viruses such as HIV.

Abbreviation footnote: MA, mouse adapted; PB1, PB2 and PA, RNA polymerase subunits; HA, hemagglutinin; NP, nucleoprotein, NA, neuraminidase; M1 matrix; NS1, nonstructural protein ; HK, Hong Kong; Ck, chicken; Dk, duck, pfu, plaque forming unit.

Data deposition footnote: Genbank, accession no. AF348170-AF348206, AF348912-3

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Figure Captions

Fig. 1 Yield of HK and HKMA variants in different hosts. (A) Mouse lung; each point represents the average of 3 mice; values could not be obtained for HKMA20C after 4 days due to lethality. (B) Yield in MDCK cells.(C) The yield of infectious virus in chicken allantoic cavity; values are the average plus standard deviation of 2 to 6 pools of 3 eggs.

Fig 2 Location of mutations selected on mouse-adaption on the functional genetic map of Influenza A virus. Panel A The mutations observed on mouse passage in M1 are shown with arrows for WSN (W), NWS (N), A/PC/2/73-MA (PC), FM (F), FM-MA (FM), HKMA-20 and -20C (H20, H20C), and A/Philippines/2/82-MA (PH) (11). Panel B. The location of mutations in the genetic map (11) seen in FM-MA that have been confirmed to control virulence are shown with black downward arrows. Mutations observed in mouse adapted A/HK/1/68 clones are shown with upward arrows. Sites that are consistently associated with virulence are marked with red arrows. The location of mutant sites that are consistently associated with virulence are marked with large grey downward arrows and the pre-existing common mutation in FM virus is indicated in small gray downward arrows;

Table 1 Virulence of mouse-adapted isolates of A/HK/1/68

A/HK/1/68 Viruses passaged in mouse lung	LD50 (pfu)	Change in LD50 (LOG_{10} decrease)
Unpassaged A/HK/1/68 parent Clone HK	$>10^{7.7}$	NA
Passage 12 population	$10^{3.7}$	>4.0
Passage 12 clone HKMA12	$10^{4.0}$	>3.7
Passage 12 clone HKMA12A	$10^{3.9}$	>3.8
Passage 12 clone HKMA12B	$10^{3.6}$	>4.1
Passage 12 clone HKMA12C	$10^{4.6}$	>3.1
Passage 12 clone HKMA12D	$>10^{4.7}$	ND
Passage 12 clone HKMA12E	$>10^{4.9}$	ND
Passage 20 population	$10^{2.7}$*	>5.0
Passage 20 clone HKMA20	$10^{4.2}$	>3.5
Passage 20 clone HKMA20A	$10^{4.0}$	>3.7
Passage 20 clone HKMA20B	$10^{3.0}$*	>4.7
Passage 20 clone HKMA20C	$10^{2.6\pm0.3}$	>5.1
Passage 20 clone HKMA20D	$10^{3.5}$	>4.2
Passage 20 clone HKMA20E	$10^{3.6}$	>4.1

*, indicates viruses that are not significantly different in virulence relative to HKMA20C, (Z value, $P \leq 0.05$); NA, not applicable; ND not done

Table 2 The nature and location and predicted amino acid changes (in bold) for HK and HKMA viruses; (nucleotides in parentheses)

	PB2	PA		HA1	HA2	NP		NA		M1	M2	NS1
	701 (2128)	133 (422)	556 (1691)	218 (729)	156 (1531)	34 (145)	480 (1483)	468 (1422)	(1457)†	167 (524)	44 (155)	103 (333)
HK	Asp (G)	Glu (A)	Gln (A)	Gly (G)	Thr (C)	Asp (G)	Asp (G)	Pro (C)	-	Thr (A)	Asp (G)	Phe (T)
HK-MA20C	**Asn** (A)	**Gly** (G)	**Arg** (G)	**Trp** (T)	**Asn** (A)	**Asn** (A)	**Asn** (A)	**His** (A)	(+Ade)	**Ala** (G)	**Asn** (A)	**Leu** (C)
HK-MA12	**Asn** (A)*	Glu (A)*	Gln (A)*	**Trp** (T)	**Asn** (A)	**Asn** (A)	Asp (G)	**His** (A)	-	Thr (A)	Asp (G)	Phe (T)
HK-MA20	**Asn** (A)*	Glu (A)*	**Arg** (G)*	**Trp** (T)	**Asn** (A)	**Asn** (A)	Asp (G)	**His** (A)	-	Thr (A)	Asp (G)	Phe (T)

* partially sequenced; † indicates insertion of non-coding nucleotide or none (-)

Table 3 Mutations in the M1, M2, NS1, and NS2 genes for clones of mouse-adapted A/HK/1/68

Virus	Amino acid variation (nucleotide position)					
	M1		M2	NS1		NS2
	167 (524)	232 (617)	44 (155)	23 (94)	103 (333)	88 (289)
HK	Thr (A)	Asp (G)	Asp (G)	Val (T)	Phe (T)	Lys (A)
HK-MA12	Thr (A)	Asp (G)	Asp (G)	Val (T)	Phe (T)	Lys (A)
HK-MA12A	Thr (A)	Asp (G)	Asp (G)	Val (T)	Phe (T)	Lys (A)
HK-MA12B	Thr (A)	Asp (G)	Asp (G)	Val (T)	Phe (T)	Lys (A)
HK-MA20	Thr (A)	**Asn (A)**	Asp (G)	**Ala (C)**	Phe (T)	Lys (A)
HK-MA20A	Thr (A)	**Asn (A)**	Asp (G)	**Ala (C)**	Phe (T)	Lys (A)
HK-MA20B	Thr (A)	Asp (G)	**Asn (A)**	Va l(T)	**Leu (C)**	**Arg (G)**
HK-MA20C	**Ala (G)**	Asp (G)	**Asn (A)**	Val (T)	**Leu (C)**	Lys (A)
HK-MA20D	Thr (A)	Asp (G)	Asp (G)	Val (T)	**Leu (C)**	Lys (A)
HK-MA20E	Thr (A)	Asp (G)	**Asn (A)**	Va l(T)	Phe (T)	Lys (A)

Table 4 Relative ability of HK and HKMA variants to replicate in mouse-lung.

virus	Yield in mouse lung 1 day post infection		Maximum Yield in mouse lung	
	Titre (pfu/ml)	Relative fitness	Titre (pfu/ml)	Relative fitness
HK	1.1e5	1	4.8e6	1
HK MA12	2.0e7	180	3.6e7	7.5
HK MA20	5.6e7	510	5.6e7	12
HK MA20C	1.3e8	1,180	1.3e8	27

Table 5 The occurrence of mutations in other influenza strains at sites in common with mouse-adapted variants (**naturally virulent (NV) and serially passaged (SP) shown in bold**) (n=number compared) (page 1 of 3)

PB2-Asp701Asn	**Asn701:A/HK/156/97 H5N1 (NV,32)**; All equine (n=3) (H3N8, H7N7); A/Sw/Germany/2/81 H1N1; (n=121)
PA-Glu133Gly	conserved (n=98)
PA-Gln556Arg (n=98)	**His556: A/WSN/33 H1N1 (SP,16) Pro556: A/FM/1/47 H1N1 (SP,12);**
HA1-Gly218Trp	**Arg218: A/Mink/Sweden/84 H10N4 (NV,18)**; A/CK/Germany/N/49 H10N7; A/SW/England/690421/95 H1N2; A/Umea/2/99 H3N2; **Asn218: A/Dk/England/1/56 H11N6 (NV,19)**; Ser218:A/Grey Teal/Australia/2/79 H4N4; Glu218:A/Ck/Jena/1816/87 H7N7; Thr218:A/SW/Scotland/410440/94 H1N2; Ala218: A/Brazil/18/96 H3N2 (n=1324)
HA2-Thr156Asn	Ile156: ts1/1/FPV/Rostock/34 H7N1 (n=1324)
NP-Asp34Asn	Asn34: A/Sw/HK/6/76 H3N2; A/Victoria/3/75 H3N2 (transfectant virus); Gly34:H1N1 swine viruses in humans (data not shown); A/HK/427/98 H1N1; A/HK/470/97 H1N1; **A/PR/8/34*H1N1 (SP,16); A/ WS/33 H1N1 (SP,16); A/FM/1/47 H1N1 (SP,12); A/AA/6/60-*ca* H2N2 (SP,20); A/Ohio/101/83-like H1N1 (persistent infection,21); Ser34: A/Ck/FPV/Rostock/34 H7N1 (SP,22)**; A/China/2000 (unknown subtype); A/SW/Cambridge/35 (H1N1);: A/SW/Cambridge/39 (H1N1); A/Hickox/40 (H1N1); A/Dk/Ukraine/2/60 (H11N8); (n=233)

NP-Asp480Asn	conserved ; (n=233)
NA-Pro468His	**Ala468: A/CK/Beijing/1/94-+related strains H9N2 (NV,23,30)**; Ser468: A/USSR/1/77 H1N1; (n=284)
M1-Ala41Val	**Val41: A/Ck/FPV/Weybridge/34 H7N7 (NV,22); A/CK/FPV/Ros/34 H7N7 (NV,22); A/PR/8/34 H1N1 (SP,16)**; A/Taiwan/1769/96 H1N1; A/Sw/Iowa/15/30 H1N1; (n=226)
M1-Thr139Ala	conserved: (n=226)
M1-Phe144Leu	**Leu144: A/Ck/Brescia/1902 N7N7 (NV,22); A/Ck/FPV/Dobson/27 H7N7 (NV,22); A/Ck/FPV/Weybridg/34 H7N7 (NV,22); A/HK/156/97+ related strains H5N1 (NV,24); A/Env/HK/437-4/99 +related strains H5N1 (NV,25); A/CK/Beijing/1/94+ related strains H9N2 (NV,23,30); A/Teal/HK/W312/97 H6N1 (NV,26)**; A/AA/6/60 (H2N2); (n=226)
M1-Thr167Ala	Ala167: All post-1972 human H3N2 strains; A/Denver/57 H1N1; **A/Ck/FPV/Rostock/34 H7N1 (NV,22)**; A/Quail/HK/AF157/92 H9N2; A/SW/North Carolina/35922/98 H3N2; A/SW/Ontario/41848/97 H3N2; A/Ck/Pennsylvania/11767-1/97 H7N2; A/CK/New York/8030-2/96 H7N2; Ile167: A/Mallard/New York/6750/78 H2N2; A/Leningrad/134/57 +derivative H2N2; (n=226)
M1-Ala227Thr	Thr227: A/Hebei/12/93 + related human H3N2 strains; **A/Ck/Beijing/1/94 H9N2 (NV,23); A/Dk/HK/Y280/97 H9N2 (NV,23)**; A/Sw/Ontario/41848/97 H3N2; A/Sw/England/191973//92 H7N1; A/Eq/Prague/1/56 H7N7 A/HK/427/97 H1N1; A/HK/470/97 H1N1; (n=226)

M1-Asp232Asn	**Asn232: A/FM/1/47 H1N1 (SP,12); A/Goose/Guangdong/1/96 H5N1 (NV,27); A/Env/HK/437-4/99 + related strains H5N1 (NV,25);** (n=226)
M2-Asp44Asn	**Asn44: A/CK/FPV/Rostock/34 H7N1 (NV,22); Tyr44: A/CK/Penn/1370/83 H5N2 (NV,22);** (n=226)
NS1-Val23Ala	Ala23: all human H1N1 + H2N2 except A/Leningrad/134/57-like; Val23: all human H3N2; Ala/Ser23: all other non-human viruses; (n=257)
NS1-Phe103Leu	**Leu103: A/HK/156/97 + related strains H5N1 (NV,24); A/Env/H K/437-4/99 + related strains H5N1 (NV,25); A/CK/Beijing/1/94 +related strains (H9N2) (NV,23,30); A/Teal/HK/W312/97 + related strains, H6N1 (NV,26); Ser103: A/PR/8/34* H1N1 (SP,16);** (n=257)
NS2-Lys88Arg	**Arg88: A/HK/156/97+ related strain H5N1 (NV,24); A/Quail/HK/G1/97, H9N2 (NV,23); A/Ck/HK/G9/97. H9N2 (NV,23);** A/HK/1073/99-like H9N2; A/Turkey/Colorado/13356/91 H7N3; A/Shorebird/Delaware/9/96 H9N2; **Thr88: A/Equine/Jilin/1/89 H3N8 (NV, 28); A/Env/HK/437-4/99 + related strains H5N1 (NV,25); A/Goose/Quangdong/1/96 H5N1 (NV,27); A/Ck/Queretero/7653-20/95-like H5N2 (NV,29);** 38 other avian stains (not shown) ; (n=257)

(*) Including viruses containing A/PR/8/34 genes (data not shown).

(End of Table 5)

Statement of Claims

1 A method of identifying mutations in influenza A viruses that control replication and/or virulence by comparing the genome sequence of a reference virus to a virus obtained by repeated, sequential culturing of this virus population. Thus this process embodies the repeated and sequential passage of virus populations where a portion of the virus population, that possesses a sizable fraction of the total genetic variation that is possible at each nucleotide position in the virus genome and resulting from a prior infection is inoculated into uninfected cells or animals to produce subsequent viral populations. This is followed by recovery and comparative sequence analysis of the resulting variant viruses.

2 The method or use of claim 1 where the virus is a member of the Orthomyxoviridae of human or animal origin.

3 The method or use of claim 1 where the virus is passaged in animals including rodents and domestic animals, as well as tissues or cells of same grown in vitro.

4 The method or use of claim 1 wherein the virus is not a member of the Orthomyxoviridae but is of human, animal, plant, or bacterial origin.

5 The application of means of detection of mutations in influenza A virus including those at amino acid positions 481 and 701 of the PB2 gene; 538 of the PB1 gene; 133 and 556 of the PA gene; 218 of the HA1 gene (H2 numbering); 47 and 156 of the HA2 gene (H2 numbering); 368 and 468 of the NA gene (N2 numbering); 34 and 480 of the NP gene; 41, 139, 144, 167, 227, and 232 of the M1 gene; 44 of the M2 gene; 23 and 103 of the NS1 gene; and 88 of the NS2 gene, as well as mutations identified by the method or use of claim 1, including any means

that is dependent on protein or nucleic acid sequence, either directly or indirectly, for the purpose of diagnostic detection or biological characterization of viral strains

6 The application of mutations in influenza A virus at amino acid positions 481 and 701 of the PB2 gene; 538 of the PB1 gene; 133 and 556 of the PA gene; 218 of the HA1 gene (H2 numbering); 47 and 156 of the HA2 gene (H2 numbering); 368 and 468 of the NA gene (N2 numbering); 34 and 480 of the NP gene; 41, 139, 144, 167, 227, and 232 of the M1 gene; 44 of the M2 gene; 23 and 103 of the NS1 gene; and 88 of the NS2 gene, as well as mutations identified by the method or use of claim 1 to the production of viruses with increased ability to replicate by introduction of any of said mutations into viruses using natural or molecular methods.

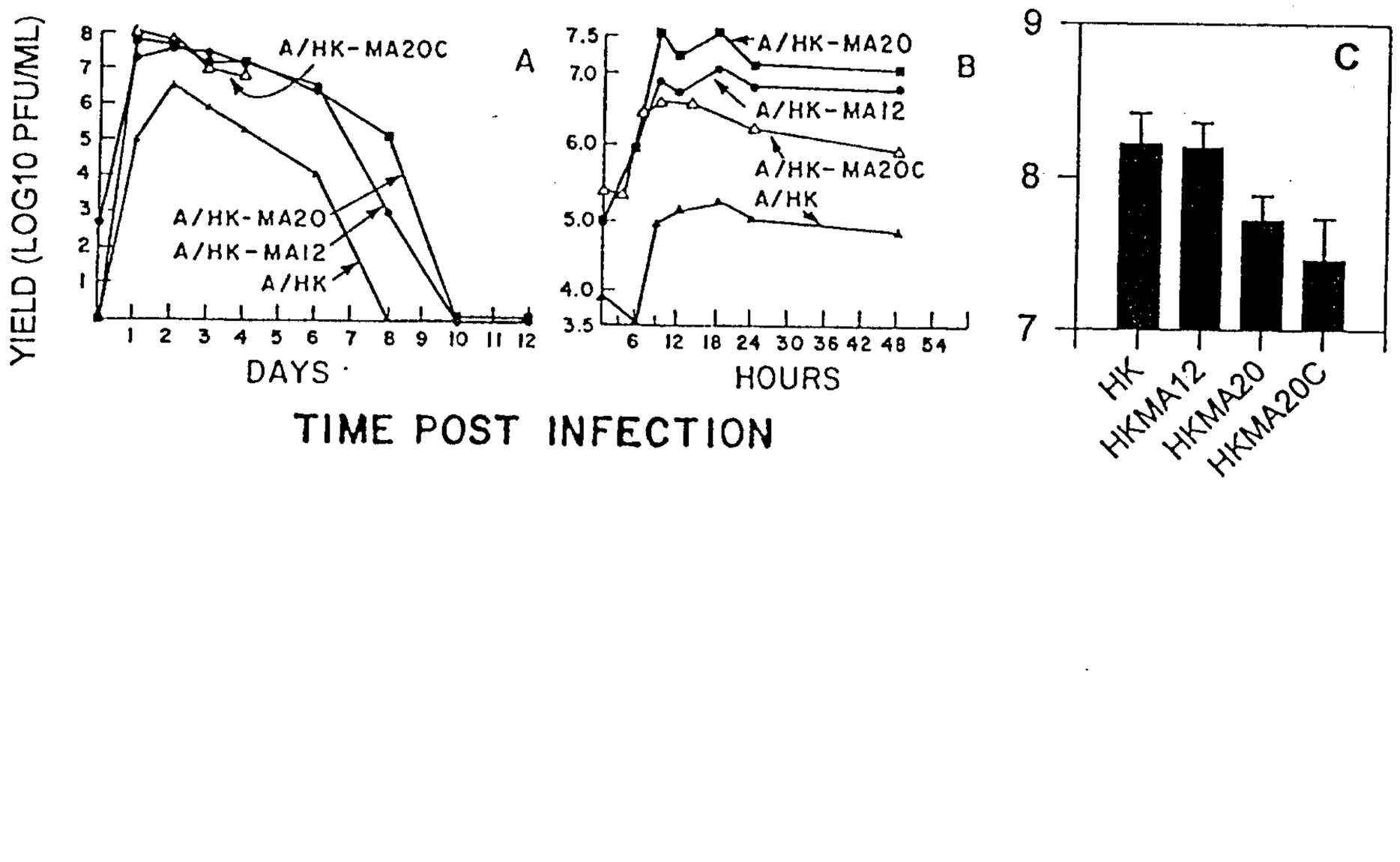
7 The application of means of detection of mutations detected by the method or use of claim 1 wherein the virus is not an Orthomyxovirus but is isolated from an animal, plant, or bacteria, by any means that measures protein or nucleic acid sequence, either directly or indirectly, for the purpose of diagnosis or biological characterization or modulating growth and biology of viral strains.

8 The introduction of mutations identified by the method or use of claim 1 or mutations in influenza A virus at amino acid positions 481 and 701 of the PB2 gene; 538 of the PB1 gene; 133 and 556 of the PA gene; 218 of the HA1 gene (H2 numbering); 47 and 156 of the HA2 gene (H2 numbering); 368 and 468 of the NA gene (N2 numbering); 34 and 480 of the NP gene; 41, 139, 144, 167, 227, and 232 of the M1 gene; 44 of the M2 gene; 23 and 103 of the NS1 gene; and 88 of the NS2 gene, into viruses to increase or decrease their ability to cause disease including the production of live attenuated vaccines.

9 The application of mutations identified by the method or use of claim 1 or mutations in influenza A virus at amino acid positions 481 and 701 of the PB2 gene; 538 of the PB1 gene; 133 and 556 of the PA gene; 218 of the HA1 gene (H2 numbering); 47 and 156 of the HA2 gene (H2 numbering); 368 and 468 of the NA gene (N2 numbering); 34 and 480 of the NP gene; 41, 139, 144, 167, 227, and 232 of the M1 gene; 44 of the M2 gene; 23 and 103 of the NS1 gene; and 88 of the NS2 gene, to the production of a therapeutic viruses.

10 The development and application of therapeutic and prophylactic drugs as well as gene products for the treatment of viral infection that affect molecular interaction defined by or involved with mutations discovered by the method or use of claim 1 or mutations in influenza A virus at amino acid positions 481 and 701 of the PB2 gene; 538 of the PB1 gene; 133 and 556 of the PA gene; 218 of the HA1 gene (H2 numbering); 47 and 156 of the HA2 gene (H2 numbering); 368 and 468 of the NA gene (N2 numbering); 34 and 480 of the NP gene; 41, 139, 144, 167, 227, and 232 of the M1 gene; 44 of the M2 gene; 23 and 103 of the NS1 gene; and 88 of the NS2 gene.

FIGURE 1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HOURS
C
HK
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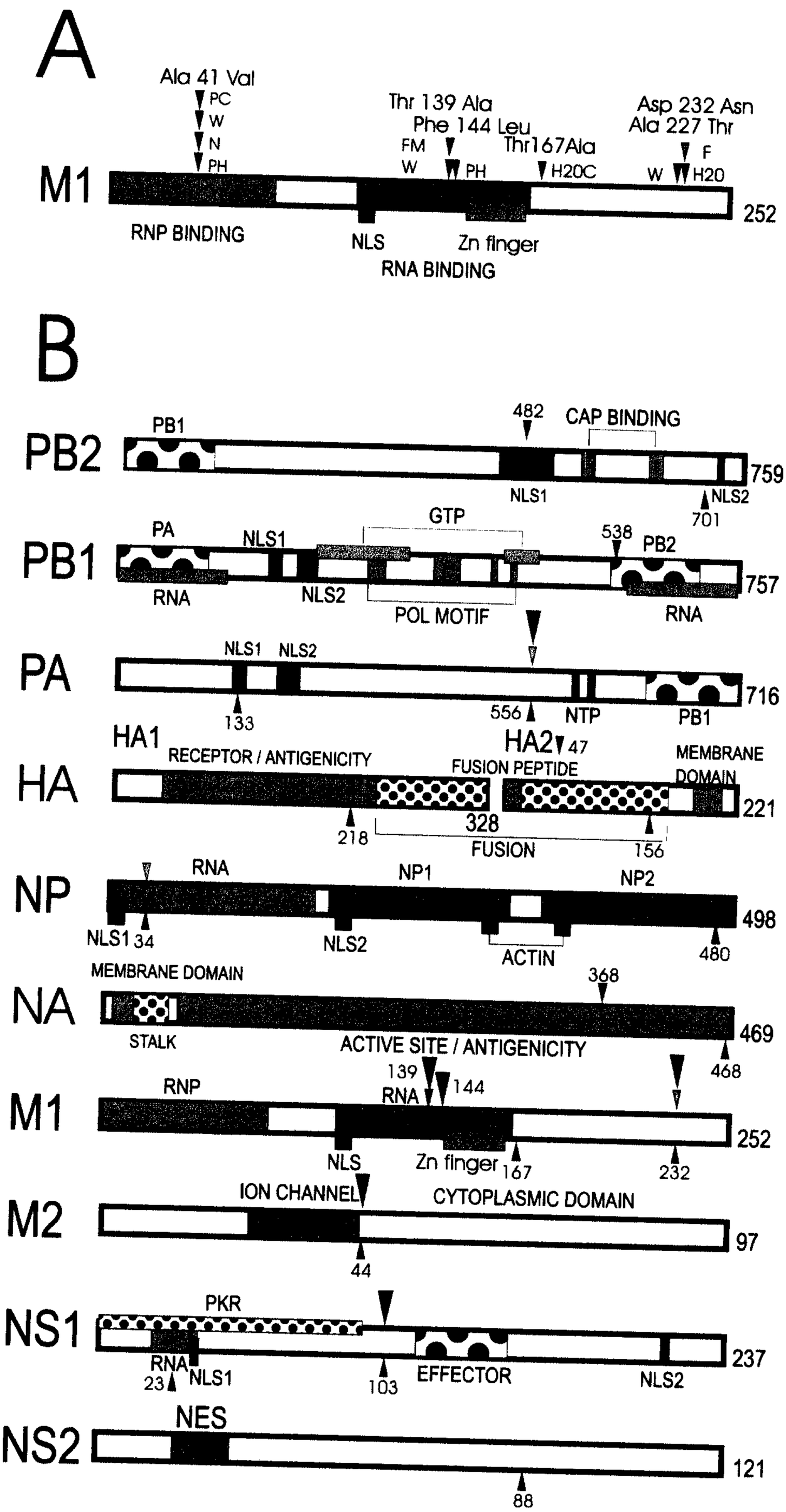
HKMA20
HKMA20C
TIME POST INFECTION

Fugure 2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PA
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PB2
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NLS2
POL MOTIF
RNA
PA
NLS1
NLS2
716
133
556
NTP
PB1
HA
HA1
RECEPTOR / ANTIGENICITY
HA2
47
FUSION PEPTIDE
MEMBRANE DOMAIN
221
218
328
FUSION
156
NP
RNA
NP1
NP2
498
NLS1
34
NLS2
ACTIN
480
NA
MEMBRANE DOMAIN
368
469
STALK
ACTIVE SITE / ANTIGENICITY
468
M1
RNP
139
144
RNA
252
NLS
Zn finger
167
232
M2
ION CHANNEL
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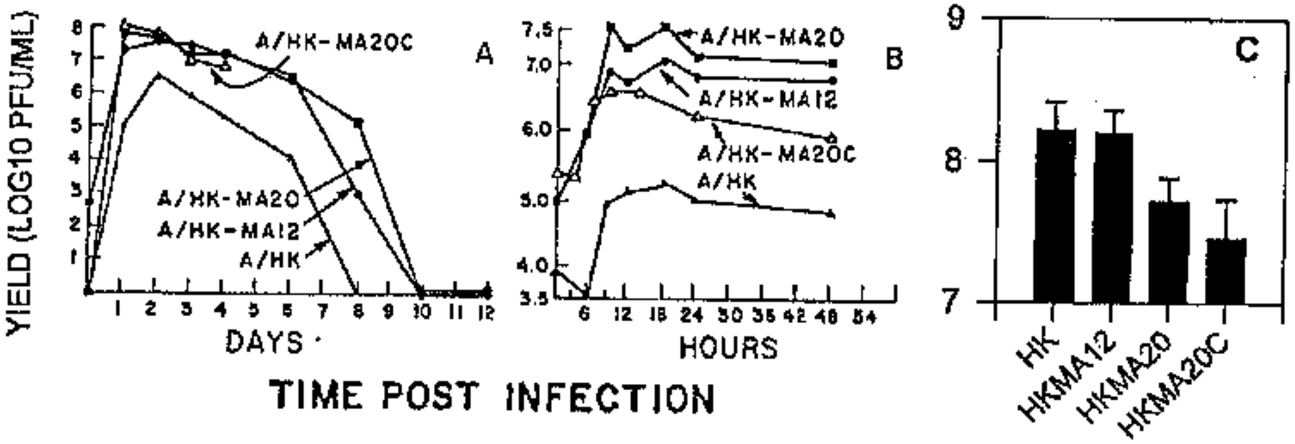
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A/HK-MA20
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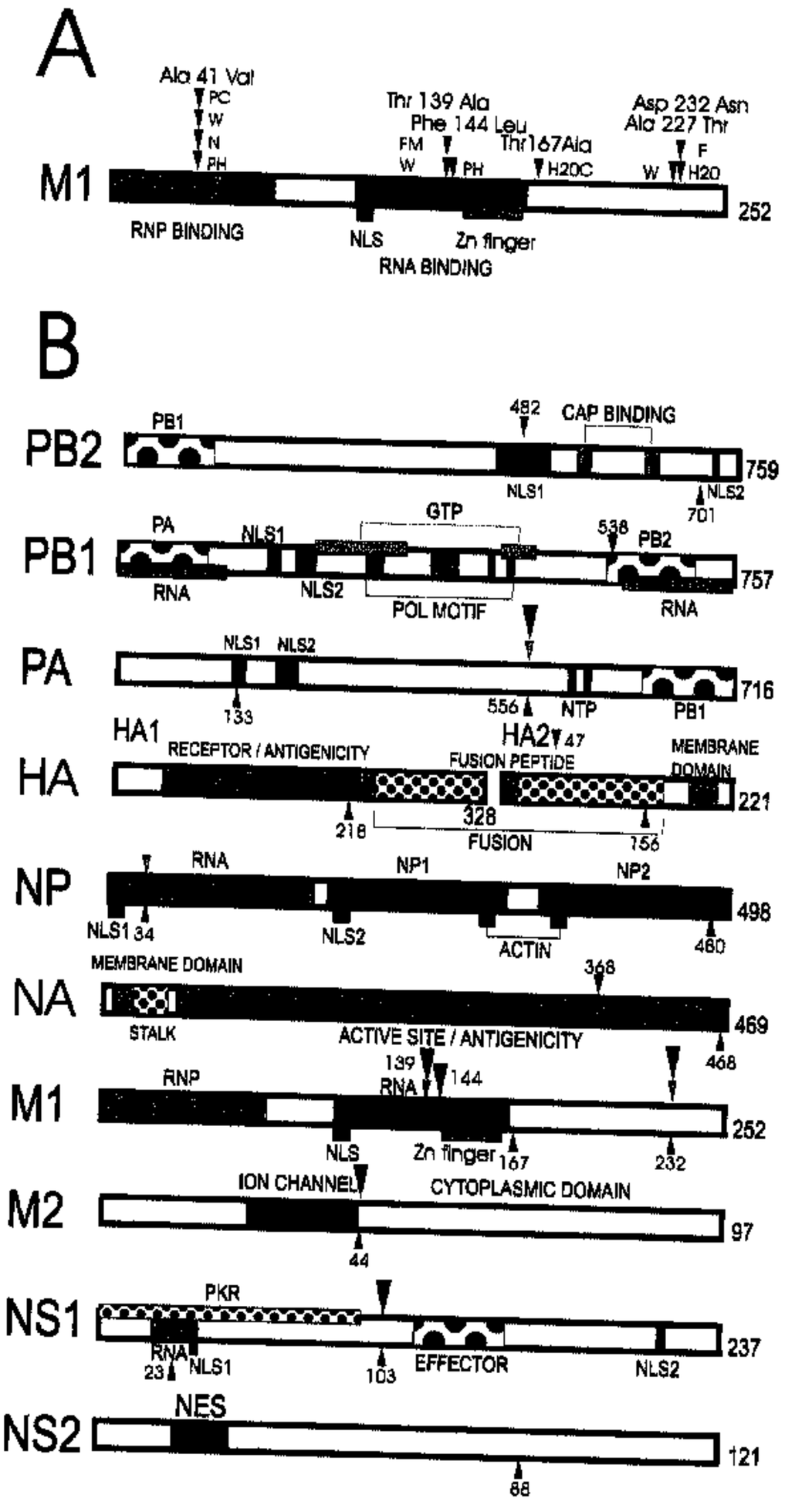
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CAP BINDING
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701
PB1
PA
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GTP
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