BIOLOGICAL CHARACTERIZATION OF TWO EVOLUTIONARILY RELATED PATHOGENS, EQUINE AND CANINE H3N8 INFLUENZA A VIRUSES

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BIOLOGICAL CHARACTERIZATION OF TWO EVOLUTIONARILY RELATED PATHOGENS, EQUINE AND CANINE INFLUENZA H3N8 VIRUSES

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Equine influenza H3N8 virus (EIV) jumped into dogs around the year 2000 and consequently caused the emergence of canine influenza H3N8 virus (CIV). This dog-specific virus has since been circulating primarily in the United States in animal shelters and places with high density dog populations. Host specificity and adaptation of influenza A viruses (IAVs) are not well understood, and so we compared the biological properties of EIV and CIV in order to further elucidate these properties. We used a variety of assays to characterize virus growth, infections in different host cells, receptor specificity, hemagglutinin (HA) cleavage, and infections in tracheal cultures. Despite numerous mutations between the genomes, we found minimal biological differences comparing EIV and CIV. Both viruses grew similarly in dog cells (MDCK) while they could not infect horse (EQKD) and human (A549) cells. Both viruses' receptor binding HA protein preferred α2-3 over α2-6 linked sialic acids, and there was also no difference comparing HA cleavage efficiency. Interestingly, infections in tracheal cultures showed CIV could not establish a productive infection in horse trachea compared to EIV. We also characterized an ancillary protein, PA-X, from both viruses by using reporter assays and RNA sequencing (RNA-seq). Reporter assays showed EIV and CIV PA-X had ribonuclease activity and suppressed β-galactosidase and GFP expression. Notably, EIV PA-X had significantly stronger activity compared to CIV PA-X. Using site directed mutagenesis we found this difference was due to a mutation at amino acid position 231 and the truncation at the C-

terminus. RNA-seq of cells transfected with plasmids encoding EIV and CIV PA-X revealed that they up-regulated the expression of many host genes compared to the controls. These altered genes were involved in various functions such as modulating the immune response, protein ubiquitination, ER-Golgi sorting and trafficking, and transcription. The RNA-seq analysis did not reveal any differences in gene expression comparing EIV and CIV PA-X samples however. Taken together our results showed EIV and CIV's biology was very similar despite many genetic differences between the two viruses, and this implies IAV host-switching and adaptation may be mediated by more subtle factors.

BIOGRAPHICAL SKETCH

Kurtis Feng started his undergraduate studies in 2007 at The Ohio State University and finished in 2011 earning a Bachelor of Science. He started his undergraduate research in Dr. Jianrong Li's laboratory in 2008 and received training in molecular virology. Kurtis worked in the Li laboratory for three years learning different experimental techniques and ways to improve his scientific writing. During this period he competed in several undergraduate research competitions with multiple first and second place finishes. Additionally, he secured undergraduate research grants based on his work on foodborne pathogens, human norovirus and hepatitis E virus. At the end of his undergraduate career he published a first author paper in *Applied and Environmental Microbiology* and co-authored a publication in the *Journal of Virology*. Kurtis started his graduate studies at Cornell University in 2011. He rotated through the laboratories of Dr. Joel Baines, Dr. Colin Parrish, and Dr. Toshi Kawate before joining Dr. Colin Parrish's laboratory in 2012 after earning the National Science Foundation graduate student research grant. Kurtis' research in the Parrish lab focused on studying equine and canine influenza H3N8 viruses. Of note, he presented a talk about these viruses at an influenza conference in Dublin, Ireland in 2013 and at the American Society for Virology meeting in Fort Collins, Colorado in 2014. He also traveled to Glasgow, Scotland in 2014 to observe and learn tracheal culturing techniques from collaborator Dr. Pablo Murcia. Kurtis published a first author paper in the *Journal of Virology* in 2015 characterizing the biological properties of equine and canine influenza viruses. He also published a first author paper in 2016 in *Virology* describing the functions of equine and canine influenza virus PA-X protein. He was also involved in other projects such as creating canine parvovirus (CPV) capsid mutants and engineering sialic acid binding probes for tissue staining.

Dedicated to my loving and patient parents, Frank and Amy

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CHAPTER I: INTRODUCTION

I.I VIRION AND GENOME ORGANIZATION

The influenza A virus (IAV) is a negative-sense segmented RNA virus that belongs to the family *Orthomyxoviridae*. The virus generates pleomorphic particles ranging from spherical ones (80-120 nm diameter) to filamentous particles that can reach 100 nm in width and 20 µm in length (1). Mutations in the virus' M1 matrix protein and the infected cell type determine the morphology of the particles (2, 3). Each particle is enveloped by a lipid membrane derived from host cells and two viral glycoproteins decorate this membrane, hemagglutinin (HA) and neuraminidase (NA), in addition to the M2 proton ion channel (4). Beneath the lipid membrane resides the viral capsid which is made from repeating units of M1 protein, and within this capsid contains the viral genome. The genome consists of eight negative-sense RNA segments wrapped around the viral nucleoprotein NP to form a complex called ribonucleoprotein (RNP). Each segment also associates with its own viral polymerase complex consisting of PA, PB1, and PB2 (5). The viral nuclear export protein (NEP) also resides within the capsid (6).

In addition to the nine structural proteins described above, the IAV genome also encodes three non-structural proteins: NS1, PB1-F2, and PA-X. The genome may also encode several other non-structural proteins but these are poorly characterized and some suggest they may just be "translational noise". These potential proteins include PB1-N40, PA-N155, PA-N182, M42, and NS3 (7).

I.II VIRAL LIFE CYCLE

The first step in the IAV's infection cycle is binding to the host's cellular receptor and this is initiated by the receptor binding protein HA. So far eighteen HA subtypes (H1-H18) have been identified based on serology and genetic divergence (8). The canonical receptor for HA is the terminal sialic acid in glycan chains present on the surface of host cells, however both H17 and H18 (from bat influenza viruses) do not seem to use sialic acid as the receptor (9). There are

approximately 300-400 HA homotrimeric spikes embedded in the virus' lipid envelope (10). Each HA spike consists of three major domains (11). First, the HA1 domain is the globular head at the distal end of the spike that contains the sialic acid binding pocket. Second, the HA2 domain is the stalk region of the spike and carries the HA fusion peptide. Lastly, the transmembrane domain makes up the proximal region of the spike and anchors the HA in the viral envelope. Upon binding to sialic acid the virus triggers receptor-mediated endocytosis and becomes internalized. Studies have shown IAV uses multiple routes for internalization such as clathrin and caveolin dependent pathways, and there is evidence that the virus can enter cells independent of both pathways as well (12, 13). Furthermore, there is also evidence of other methods of uptake such as macropinocytosis (14). Regardless of which method, the next step in IAV entry is navigating the host cell's endocytic system and subsequent release of the viral genome segments into the cytoplasm. The endocytic machinery is a complicated network with endosomes constantly being recycled and trafficked around the internalized particles. Rab proteins are essential for these processes and not surprisingly several have been implicated to be essential for influenza trafficking such as Rab5 and Rab7 (15). Virus particles are exposed to a two-step acidification process, once around pH = 6.0 in the early endosomes, and then a final step at pH = 5.0 in late endosomes (16). The acidic environment serves two purposes: first, the HA undergoes a conformational change exposing the hydrophobic fusion peptide which in turn inserts in to the opposing endosomal membrane. A second "foldback" rearrangement of the HA molecule pulls the viral membrane towards endosomal membrane leading to fusion. Secondly, the M2 ion channel in the viral envelope pumps protons into the viral capsid which causes the dissociation of the M1 matrix protein from the viral RNPs (16-19). The consequence of these events is the expulsion of viral RNPs into the cytoplasm.

Once released, the RNPs travel quickly and within 10 minutes enter the nucleus through nuclear pore complexes (NPCs) (20). The NP protein encodes several nuclear localization signals that allow importins, such as α1 and α5, to bind to and translocate the entire complex

across the nuclear membrane (21). Once inside the nucleus the viral RNA (vRNA) serves as the template for transcription and viral genome replication. Messenger RNA synthesis is initiated by PB2 binding to the 5' cap of host mRNA and PA cleaving the cap 10-13 nucleotides downstream of the structure through its N-terminal ribonuclease domain, and subsequently the cap is then used as a primer for viral transcription (22). Transcription proceeds through the active site of PB1 until a repeat of uridines is encountered and transcription can no longer proceed, due to the steric hindrance of the vRNA 5' end in proximity to the 3' end. This hindrance causes slippage and repeated copying of the Us which results in a poly(A) tail. The viral transcripts are then released from the polymerase complex and interact with host factors such as the splicing machinery (for alternatively spliced transcripts) and the NXF1/TAP complex for efficient nuclear export (23, 24), and once the viral transcripts are outside the nucleus they are translated by the host ribosomes. IAV transcription mainly occurs early on in the infection (2-6 hours post-infection) due to the switch to genome replication later in the infection. This switch has been attributed to several factors including running out of host mRNA cap donors, the expression of small viral RNAs that modulate the viral polymerase complex, and the nuclear import and accumulation of newly translated viral polymerase and NP proteins (22, 25). Replication of the viral genome involves two distinct steps: the generation of complimentary RNA (cRNA) and then using cRNA as the template to make vRNA. Since the cRNA does not contain a 5' cap or a 3' poly(A) tail the mechanism for making them is different compared to creating viral mRNA. Indeed, it has been proposed genome replication requires both a *cis*- and *trans*-acting polymerase complex. The first step is the release of the 3' end of the vRNA template from the *cis*-acting polymerase and this binds to the PB1 subunit of the *trans*-acting one, and elongation of cRNA begins when GTP binds to the active site as well. Next, the 3' end of the original vRNA template rebinds to the *cis*-acting polymerase while the 5' end of the newly synthesized cRNA binds to PB1 of the *trans*-acting polymerase. In order to finish elongating the cRNA, the 5' end of the vRNA template must be released from the *cis*-acting polymerase for the

trans-acting polymerase to bind to and read through the 5’ end. Lastly, the 5’ end of the vRNA template returns to its original position at the *cis*-acting polymerase while the newly created 3’ end of the cRNA is bound to the PB1 subunit of the *trans*-acting polymerase and NP is recruited to cover the newly synthesized strand forming cRNP. And thus new viral RNPs are created by going through this cycle again but cRNA is used as the template instead (22). After successful rounds of genome replication the last steps to the infection cycle are exporting viral components from the nucleus followed by assembly and release of newly synthesized particles.

Viral RNPs are exported out of the nucleus through several interactions with viral and host proteins. First, M1 and nuclear export protein (NEP) are imported into the nucleus and M1 specifically binds to RNPs and in turn NEP binds to M1. Next, the host exportin 1 (Crm1) recognizes the two nuclear export signals (NES) located in NEP and binds to the NEP-M1-RNP complex, and lastly, Crm1 is primed for export by binding to Ran-GTP (26). Once outside the nucleus, viral RNPs dissociate from the Crm1 complex through interaction with Human immunodeficiency virus Rev Binding protein (HRB) (27). The RNPs take advantage of recycling endosomes by binding to Rab11 present on the surfaces, and this allows them to traverse the cytoplasm through the microtubule network (26, 28, 29). The RNPs reach the apical area of the cellular membrane and here they dissociate from Rab11 (through GTP hydrolysis) and begin the packaging process. During this time HA, NA, and M2 membrane proteins have already traveled through the secretory pathway and have accumulated in large lipid rafts spanning the cellular membrane. Unique packaging signals residing at the terminal ends of the genome segments dictate specificity and efficiency to make sure all eight segments are brought together and packaged into virions (30-32). Indeed, electron tomography has shown the eight RNP segments are closely bundled together with segments having distinct lengths (33). During this packaging process, several viral proteins are involved in the maturation and subsequent budding of new virions. For example, M1 must interact with the cytoplasmic tails of HA, NA, and M2 in order to assemble the capsid as well as provide docking sites for the viral RNPs (34).

Additionally, many host factors have been shown to be necessary for budding including G-protein and kinase activity, ATPase activity, and the presence of actin filaments (35-37). The exact budding mechanism is not currently known but the overall picture has been visualized by electron microscopy. First, the cellular membrane protrudes out at concentrated regions of lipid rafts (heavily decorated by HA and NA), and this protrusion is caused by M1 assembly and the polarization of the RNP segments. Next, M2 is recruited to the periphery of the budding virion by interactions with M1 to mediate membrane scission. This occurs through the insertion of the M2 amphipathic helix into the cellular membrane, changing the plasma membrane's curvature and thus causing the virion to break off from the cell (26, 34). Additionally, NA modulates efficient release of virions and prevents aggregation by cleaving off sialic acids from the cellular membrane (38). These newly generated virions can now travel to other cells and start the replication cycle over again.

I.III INFLUENZA A VIRUS IN BIRDS

The natural reservoirs for most influenza viruses are birds. With the exception of bat influenza H17N10 and H18N11 viruses, all current circulating subtypes in animals can be traced back to having origins in wild birds (8). Indeed, the first IAV isolated from birds occurred in 1961 in South Africa and it was a H5N3 subtype. More thorough investigations of influenza in birds did not occur until the 1970s (39). Since then, IAVs have been isolated from over 100 species of birds spanning 15 orders – encompassing all major families (40). Interestingly, waterfowls (order Anseriformes) such as ducks and geese harbor the greatest number and diversity of IAVs and additionally these viruses are most widespread in them compared to other birds. For example, a survey of over 21,000 samples from all avian species showed 15% of waterfowl samples were infected with IAVs while the next highest isolation rates were 2.9% and 2.2% from orders Passeriformes (perching birds) and Charadriiformes (shorebirds) (41). Furthermore a 3-year study of ducks on lakes in Alberta, Canada revealed over 60% of the population were infected

by IAVs (42). Specific subtypes seem to prefer different bird species; H9 and H13 subtypes are found in Charadriiformes while H9 is rarely found in Anseriformes and H13 is never found in that order (43). There are two main groups of avian influenza viruses based on their pathogenicity in poultry: high pathogenicity avian influenza (HPAI) and low pathogenicity avian influenza (LPAI). LPAI viruses are the vast majority of viruses isolated from wild birds; they are asymptomatic in their hosts but sometimes can cause mild illness. In contrast, HPAI viruses (mainly H5 and H7 subtypes) can have up to a 100% mortality rate in chickens and are not maintained in wild bird populations (43). Indeed, HPAI viruses only emerge from LPAI viruses when specific point mutations accumulate in the genome and/or when gene reassortment events occur with other subtypes (40). Avian influenza viruses are also not stationary in their bird hosts; throughout history they have jumped and adapted to other animals, namely mammals such as humans, pigs, horses, dogs, seals, and minks (44).

I.IV INFLUENZA A VIRUS TRANSMISSION TO HUMANS

In modern society IAV is a major human pathogen causing several million cases of severe illnesses and up to half a million deaths around the globe annually (8). Major pandemics in which a virus from a different species is introduced into the human population and subsequently adapts and spreads from human to human are rare. In fact, there have only been four documented incidents in history so far. The largest and most devastating pandemic occurred nearly 100 years ago – the H1N1 influenza pandemic of 1918. Historians and scientists have estimated that up to a third of the world's population at the time was infected and between 50 and 100 million people died from bacterial co-infections (45, 46). The virus spread across the world in three simultaneous waves in Europe, Asia, and North America. A huge geopolitical event that contributed to the wide spread and the virulence of the virus was World War I (47, 48). Millions of soldiers from different countries were stationed in military camps and trenches, and the living conditions were cramped and unsanitary. Compound this with fear,

anxiety, exposure to the elements, and constant contact with wild and domestic animals, and thus it is not surprising to see why the virus spread rapidly throughout the world. The exact origins of the virus is currently unknown but sequence analysis suggests it most likely came directly from birds (49). Interestingly, the genome sequences from the 1918 virus do not resemble any other avian or mammalian influenza viruses due to a large number of silent nucleotide mutations. This characteristic suggests the virus may have jumped into the human population directly from an avian species and quickly adapted by picking up numerous mutations (45, 50). Additionally, the lack of influenza sequences predating 1917 has also made identifying the origins of the 1918 virus a difficult task.

The next pandemic in the 20th century occurred in 1957 and originated in Asia. The H2N2 virus emerged from the reassortment of the viral genome segments (antigenic shift); the HA, NA, and PB1 originated from an avian virus H2 subtype while the other segments came from the circulating H1N1 human virus (51). Children and the elderly were especially susceptible to the virus and death was usually caused by pneumonia. Interestingly though, unlike the 1918 H1N1 virus, the H2N2 virus caused pneumonia by itself without co-infections by bacteria (52). In total, around 2 million people succumbed to the virus worldwide and the virus displaced the H1N1 subtype in circulation (53, 54). In 1968 another pandemic broke out and its origins was Hong Kong and this was a result of another reassortment event; the virus had HA and PB1 segments from an avian H3 subtype while the other genome segments were derived from the circulating H2N2 human virus (54, 55). The virus spread worldwide and replaced the H2N2 virus resulting in two waves of severe influenza-related deaths; mortality rate was highest in North America between 1968-1969, while in Europe and Asia a majority (70%) of deaths occurred between 1969-1970. The observed delay in Europe and Asia has been attributed to antigenic drift (point mutations) in the NA gene (56). Overall though, the pandemic was quite mild compared to previous incarnations most likely due to pre-existing immunity against the human H2N2 virus. Interestingly, the human H1N1 virus was re-introduced into the human

population in 1977 most likely from a laboratory accident and the virus co-circulated with the human H3N2 virus globally until it was displaced during the 2009 pandemic (8).

The first pandemic in the 21st century started in April 2009 and initial cases came from Mexico and California. The H1N1 virus traveled rapidly supporting human to human transmission and by December 2009 there were 208 countries/territories that confirmed the virus was in humans (57). The virus emerged from a complex reassortment event in swine; it had segments from both classical (HA, NP, NS) and Eurasian (NA, M) swine influenza, avian influenza (PA, PB2), and human influenza (PB1) virus (58). The infection rate was higher in regions near the tropics such as Central and South America and Southeast Asia because influenza circulates year-round compared to temperate regions in which influenza is most prominent during colder months (58). Pre-existing medical conditions exacerbated infections and deaths were usually caused by bacterial co-infections leading to pneumonia (59). Interestingly, children and young and middle-aged adults were primarily affected by the virus. Indeed, older age groups were partially immune to the virus and this has been linked to the presence of cross-reactive antibodies based on hemagglutination inhibition assays (57). The mode of transmission was similar to seasonal influenza – contact with respiratory droplets from infected individuals. Additionally, many infected individuals had diarrhea and viral RNA was abundantly detected in the feces but live virus particles were not found and thus fecal transmission of the virus has not been proven (60). Overall, the pandemic was relatively mild (due to advancements in global infrastructure, influenza research, health care, disease surveillance and response, etc) and deemed to be over on August 2010 by the World Health Organization (WHO); in total there were 1.4 million reported cases and 25,000 deaths globally (58).

Although influenza pandemics have been rare throughout history, transmission of the virus from a non-human to a human host is not an unusual event. The difference between these “spillover” infections and pandemics is whether the virus gains the ability to efficiently transmit

among humans. Indeed, there are many subtypes of influenza viruses that have crossed the species barrier into humans at one point or another, including H5N1, H7N9, H9N2, H6N1, H7N7, H10N8, H7N2, and H7N3 (61-68). Several of these viruses gained access into the human population by jumping from domesticated animals such as poultry and swine. For example, the first reported incidence of an avian influenza virus in poultry transmitting to humans occurred in 1997 in Hong Kong (69). This outbreak of highly pathogenic avian H5N1 virus was curbed by removing many live poultry markets. However, the virus re-emerged in 2003 and since then it has spread to poultry in Europe, Asia, and Africa. Since January 6, 2015 over 690 cases of infected humans have been documented with a 70% fatality rate (70). Although a vast majority of these incidents have been direct transmission of the virus from poultry to humans, there was some evidence of limited transmission between humans as well (71, 72). If this virus adapts and possibly reassorts with circulating human viruses a large scale and deadly pandemic could arise as a result. The H7 subtypes are also known to jump into humans from poultry; documented cases include an outbreak in China in March 2013 (H7N9), an incident in Northern Italy in August 2013 (H7N7), and an outbreak in the Netherlands in 2003 (H7N7) (8). Since spring 2013 there have been over 400 reported cases of H7N9 in humans and researchers deduced multiple reassortment events between wild birds and poultry must have occurred for the creation of the virus. Indeed, the internal genes of the virus resembled H9N2 poultry viruses in China while the HA and NA genes were similar to duck and wild bird viruses in Southern China (70). Interestingly, human infections by H7 subtypes usually result in conjunctivitis which is rarely seen by other viral subtypes (73). Ever since the 2009 H1N1 pandemic, researchers have also taken a greater interest in studying the potential of swine viruses infecting humans. The pandemic H1N1 virus is currently enzootic in swine and has since been reassorting with other circulating swine viruses. For example, there have been over 340 cases of swine H3N2 (carries the M gene from the 2009 pandemic virus) infecting humans in over 10 US states since 2011 (74). This virus is antigenetically distinct from seasonal human

H3N2 viruses so there is potential for it to cause a pandemic due to a lack of pre-existing immunity. Furthermore, in 2012 there were a few cases of swine H1N2 (also carried the M gene from the pandemic virus) infecting humans in Minnesota after contact with pigs at a state fair (8).

In temperate regions such as the United States influenza usually circulates during the colder months, hence the terms “flu season” and “seasonal influenza”. Currently there are two subtypes of seasonal IAVs in the human population: H1N1 (derived from the 2009 pandemic virus) and H3N2 (originated from the 1968 pandemic). Interestingly, the H3N2 subtype is re-seeded across the world every year from variants that arise from East and Southeast Asia whereas H1N1 persists in local regions all over the world and does not move around the globe as much (75, 76).

I.V EMERGENCE OF CANINE INFLUENZA FROM HORSES AND BIRDS

The earliest documented record of horses suffering from an influenza-like illness was during the great epizootic of 1872 in Canada. The virus traveled quickly throughout North America and caused major disruptions in society because horses were heavily used for transportation. Indeed, when a major fire broke out in Boston a large portion of the city was burned to the ground because the human-pulled fire wagons could not respond fast enough (77). However, it was not until 84 years later (1956) in the Czech Republic that the first equine influenza virus (EIV) was isolated. The virus was a H7N7 subtype and was identified to be the cause of influenza outbreaks in horses throughout the world (77). An EIV H3N8 subtype was identified in 1963 in the United States from horses that traveled from South America to Florida, and it replaced the circulating H7N7 subtype in the 1970s. Interestingly, the H7N7 virus has not been isolated since then and is thus considered “extinct” (78). Like most influenza viruses, the origin of EIV H3N8 was probably an avian virus that jumped into horses in the past. The virus evolved to form two distinct lineages, American and European, and the American lineage has

been further divided into clades: South American, Kentucky, and Florida. The Florida clade became the most prevalent EIV and it was spread to Asia in 2007. The virus was then subsequently introduced to Australia from infected horses in Japan and it infected over 70,000 horses costing an estimated $400 million for the region (78). Interestingly, in 1989 a distinct H3N8 virus was isolated from an outbreak in horses in China – gene sequencing showed the virus closely resembled avian influenza viruses instead of traditional EIV which implied the outbreak was caused by direct transmission of avian influenza into horses. The virus had a high mortality rate (20%) in horses but did adapt in its new host and consequently went extinct (79).

Traditionally, horses were considered “dead ends” for EIV because the virus did not seem to transmit to new host species. This notion changed when racing greyhounds in Florida became sick in 2004 and sequencing results revealed EIV H3N8 was the causative agent (80). Further sequencing of archival samples revealed the virus most likely jumped into dogs and subsequently adapted to its new host around 2000 resulting in the establishment of canine influenza virus (CIV) H3N8. Within a year of the outbreak in Florida the virus spread throughout the United States to other race tracks and dog populations (80, 81). And since 2008, CIV has been maintained primarily in large animal shelters and rescue centers where there are high density dog populations with rapid turnover rates (82-84). The constant shuffling of dogs among shelters increases the chance of introducing the virus to naïve populations and thus allowing the virus to be steadily maintained. Indeed, despite the large number of household dogs (80 million) in the United States the virus is not maintained and transmitted in that population most likely due to the lack of continuous contact with infected dogs; most infections in small shelters and in the household dog population die out within a few days or weeks (82, 83). There have also been transmissions of EIV to dogs in other parts of the world including the United Kingdom and Australia but they were limited and the virus did not adapt (85, 86). Besides dogs, EIV H3N8 has also been reported to infect pigs in China and cats in laboratory inoculations (87, 88).

The exact mechanism of how EIV adapted to infect dogs remains unknown. Although there are numerous differences in the CIV's genome compared to EIV's it has been challenging to identify the mutation(s) required for the host jump and subsequent adaptation of the virus. Some mutations in the CIV's genome are around the HA receptor binding site, but both EIV and CIV prefer the same host receptor type (89-91). Additionally, other characteristics comparing the two viruses such as *in vitro* growth and infections in different host cells also appear to be similar (89). Animal transmission studies showed EIV infected horses transmitted the virus to sentinel dogs, but CIV infected dogs did not transmit the virus to sentinel horses (92, 93). Indeed, there seems to be a host barrier for CIV to infect horses. Further evidence of this host barrier is poor infectivity of CIV in horses and ponies (experimental inoculation), in primary equine respiratory cells, and in horse tracheal cultures (89, 94, 95). Interestingly, CIV expressing both EIV HA and NA proteins was able to infect horse trachea better than the CIV wild type suggesting mutations in the viral glycoproteins may be important in overcoming the host barrier in horses (89). Another report showed dogs infected with EIV with a truncated NS1 gene (CIV always has the full-length variant) did not transmit the virus efficiently to other dogs suggesting NS1 may play a role in the adaptation of EIV in dogs (96).

A new CIV H3N2 subtype emerged in Korea in 2007 and sequencing results showed the gene segments were related to Eurasian and American avian influenza viruses (97). Though, details regarding how exactly the virus jumped into dogs and adapted are not presently known. The virus was most likely circulating in the Asian dog population for some time prior to its identification because it has been reported CIV H3N2 was in China since 2006 and in Korea since 2005 (98, 99). The virus seems to be expanding to other regions such as Thailand and in April 2015 the virus caused an outbreak in Chicago and since then thousands of dogs in the US have been reported to be infected by the virus (100, 101). Interestingly though, the virus appears to have a broader host range compared to CIV H3N8 because it has been isolated

from cats during an outbreak in Korea and it can also infect ferrets in experimental conditions with limited transmission (102-104).

I.VI VIRAL HOST TROPISM AND ADAPTATION

Influenza HA receptor recognition is a major factor with respect to determining host specificity. The link between the terminal sialic acid and the penultimate galactose in carbohydrate chains is important for receptor recognition. In general, avian influenza viruses prefer α2-3-linked sialic acids while human influenza viruses prefer α2-6-linked sialic acids (105). Indeed, avian influenza viruses reside primarily in the gut of birds due to the abundance of α2-3-linked sialic acids, whereas infections in humans are usually limited to the upper respiratory tract because of localized α2-6-linked sialic acids (8). Mutations in the receptor binding domain (RBD) can alter the specificity and consequently allow the virus to infect a different host. For example, mutations Q226L and G228S in the RBD of H2 and H3 subtypes change the receptor specificity preference from avian to human receptor (106). A similar change in receptor preference is observed in the H1 subtype with mutations E190D and D225G in the RBD (107). Furthermore, the D225G mutation in the 2009 H1N1 pandemic virus allowed it to bind to both α2-3- and α2-6-linked sialic acids which consequently increased its pathogenicity because α2-3-linked sialic acids are found in the lower respiratory tract of humans (108). However, not having these “signature mutations” in the RBD is not necessarily predictive of receptor preference. For instance, the ability for the avian H7N9 virus to bind human receptor is attributed to the Q226L mutation; however, not all isolates have that mutation which suggests other residues may play a role in HA binding to α2-6-linked sialic acids (109). In addition to mutations in the RBD, other changes in the HA can also alter receptor preference. For example, a report showed the loss of a glycosylation site in the avian H5N1 virus, along with several other mutations, caused the virus to have a greater affinity for α2-6-linked sialic acids (110). Another factor to consider is the HA cleavage site sequence; HA must be cleaved by specific cellular

proteases before virus is released from the cells, otherwise the HA will not be able to undergo the conformational change required for membrane fusion in the next round of replication. For instance, HPAI viruses have a polybasic cleavage site sequence which allows efficient cleavage by ubiquitous cellular proteases such as furin (111). On the other hand, human influenza viruses are cleaved by specific serine proteases, TMPRSS2 and HAT, found in the respiratory tract (112). Indeed, changes in the cleavage site could play a role in host specificity; CIV H3N8 has a threonine (T) at the P2 position (residue 328) of the cleavage site while EIV H3N8 always has an isoleucine (I), and this may be reflective of selective pressures from dog-specific proteases (89). Lastly, changes in the HA stalk region may increase HA stability which could facilitate transmission to new hosts based on ferret studies using H5N1, H7N1, and H9N2 viruses (113-115).

The influenza NA protein complements the HA because it is responsible for cleaving sialic acids on the cellular surface for efficient particle release after assembly. A precise balance of activity must be maintained between the two proteins for efficient infection and transmission (116). Thus, compensatory changes must be selected in the NA when the virus jumps to a new host and this is especially important if the receptor type is different. Although the receptor-destroying domain resides in the globular head, changes in the NA stalk length greatly changes enzymatic activity. For example, transmission of H5N1 and H7N7 viruses from wild birds to poultry frequently results in significant deletions in the stalk region, ranging from 19 to 35 amino acids depending on the subtype (117, 118). Although reports have shown deletions in the stalk region actually lowers the enzymatic activity which causes inefficient particle release, this characteristic may be important for counterbalancing the decreased HA binding affinity to the new host's receptor, and there is evidence the deletions enhance infection and replication of H7N1 in chickens (119, 120). Furthermore, it has been shown that a mammalian-adapted virus carrying the NA from a poultry-adapted virus could not transmit efficiently between ferrets, but this deficit was overcome by restoring the NA stalk length found typically in human-transmissible

viruses (121). Interestingly, when the H2N2 virus emerged in humans, causing the 1957 pandemic, the newly adapted N2 cleaved α2-6-linked sialic acids better than the avian N2; this further suggests NA is crucial in influenza virus host specificity and adaptation (122, 123).

The influenza polymerase complex is responsible for transcription and genome replication. Several key mutations have been identified in the PB1, PB2, and PA proteins that facilitate viral adaptation in new hosts. Specifically, PB2 appears to play the largest role compared to PB1 and PA. One of the most extensively studied mutations in PB2 resides in position 627; in almost all avian influenza viruses a glutamic acid (E) occupies that spot while changing it to a lysine (K) can immediately allow the virus to replicate in mammalian cells, increase its pathogenicity, and promote transmission in ferrets and guinea pigs (124-127). Indeed, many avian influenza viruses isolated from human samples had the PB2 E627K mutation, such as the H5N1 and H7N9 subtypes (128, 129). One reason why the lysine mutation is selected in mammalian hosts is temperature difference; influenza usually replicates in the gut of birds which is around 41°C while the human virus replicates in the upper respiratory tract which is around 33°C (130). It was demonstrated that viruses with 627K replicated better at 33°C than viruses with 627E, and this difference was mitigated as the temperature increased to 37°C (131, 132). Host factors have been identified that directly influences influenza polymerase activity based on their interactions with the PB2 627 residue such as the DEAD box RNA helicase 17, and thus are important when considering host specificity. Indeed, knocking-down that gene in human cells inhibited the human-adapted polymerase complex carrying PB2 627K but increased the activity of the avian-adapted polymerase complex with PB2 627E, although the precise mechanism is not currently known (133). For many years the exact step at which the avian polymerase is inhibited in human cells was not known, but a study in 2012 revealed that avian polymerase containing PB2 627E could initiate transcription but made defective cRNA which consequently limited genome replication (134). Although there is a lot of focus on investigating the link between PB2 627 and overcoming host barriers, the mutation is not

required for virus adaptation to new hosts. For example, the 2009 pandemic H1N1 virus did not have a lysine at position 627 and artificially changing it did not enhance virus replication in mice (135). Instead, it had mutations G590S and Q591R and these changes may serve a similar purpose as E627K because they confer a positive charge and reside on the same surface based on the partial crystal structure (136, 137). Other mutations in avian PB2 that enable adaptation and enhance polymerase activity in mammalian hosts include T271A, E158G, and D701N (130). Interestingly, the PB2 D701N mutation changes the virus' importin-α dependency; it switches the avian virus' dependency on importin-α3 to importin-α7 which in turn facilitates mammalian host adaptation (138).

Although influenza PA is less characterized compared to PB2 with respect to host specificity and adaptation, there is evidence that the protein is important for determining influenza host range. For example, restriction of an avian polymerase complex in human cells was overcome by replacing the avian PA with a human-origin one (139). In fact several mutations have been identified in the avian PA from the 2009 H1N1 pandemic virus that are involved in its adaptation to mammalian cells including T85I, G186S, and L336M (140). Many mutations in PA have also been identified that enhance its polymerase activity in human cells such as P400L, M423I, V476A, T552S, and V630E (139). Furthermore, serially passaging an avian H5N2 virus in mice increased its polymerase activity and replication in mice but not in chickens, and this was attributed to a host specific isoleucine (I) mutation at position 97 in PA (141). In 2012 a second protein was identified from influenza segment 3, deemed PA-X (142). Although it appears its main function is gene suppression via its endonuclease domain located at the N-terminus, it may be involved in host adaptation because certain viruses always carry a truncated form, such as canine influenza H3N8 and H3N2 and certain strains of swine influenza (143). Furthermore, a recent study showed the truncated form of PA-X may be important in the adaptation of influenza virus in pigs (144).

PB1 is currently the least characterized protein regarding virus adaptation. However, position 375 in PB1 has been determined to be important in defining host range. Most avian influenza viruses harbor an asparagine (N) at this position while human viruses have a serine (S) (50). In fact, the viruses that caused the pandemics in 1918, 1957, and 1968 all had the PB1 N375S mutation (145). This mutation seems to be dispensable because there is evidence of avian viruses carrying a serine and human viruses carrying an asparagine at position 375 (50). Unfortunately, whether mutations at this position affect polymerase activity or influence other aspects of viral replication in different host cells is currently not known.

A key process for facilitating viral adaptation and overcoming host barriers is countering the innate immune response. The interferon (IFN) response is one of the most potent antiviral defense system; expression of type I and type III IFNs (triggered by specific antigen pattern recognition receptors) result in the transcription of hundreds of genes – many of these inhibit viral replication and spread (130, 146). Influenza viruses encode several proteins that aim to combat host defense: NS1, PB2, PB1-F2, and NP (130). The NS1 protein is the main antagonist against the host immune response; it inhibits host gene expression by interfering with transcription and downstream processes such as mRNA processing and nuclear export (147). Furthermore, it inhibits the IFN pathway by blocking specific antigen pattern recognition receptors such as retinoic acid-inducible gene 1 (RIG-1) and it also inhibits the activation of transcription factors that are important in other aspects of the innate immune response such as the NF-κB complex (147). Phylogenetic studies have shown the NS1 sequence varies depending on the host which implies there are selective mutations necessary for adaptation, most likely for countering host-specific immune responses (148). There is empirical evidence that NS1's control of the IFN response varies depending on its origin; studies showed different avian NS1 proteins displayed different efficiencies with respect to inhibiting IFN-β production in human cells and the NS1 protein from the avian H7N9 virus that infected humans exhibited

greater IFN control in human cells compared to the NS1 protein from the H7 virus typically found in chickens (149-151).

Interestingly, PB2 has been shown to localize to the mitochondria to disrupt the mitochondrial antiviral signaling protein (MAVS) to inhibit IFN response (152, 153). The primary residue responsible for mitochondria localization is position 9; human virus has an asparagine (N) while avian influenza has an aspartic acid (D) which prevents PB2 from localizing to the mitochondria (154). A study showed that preventing the human PB2 from localizing to the mitochondria (by changing the key residue) caused a greater IFN response in mice and attenuated the virus (152). This suggests PB2 plays a role in viral adaptation by disrupting the host immune response in addition to regulating polymerase activity. Additionally, the PB1-F2 (a small non-structural protein generated from an alternative ORF in the PB1 gene) also localizes to the mitochondria in order to interact with MAVS to limit IFN induction (155, 156). Indeed, a report showed the mutation N66S increased the pathogenicity of the 1918 H1N1 pandemic virus and avian H5N1 virus in mice because the change enhanced IFN inhibition (157).

As stated previously, the induction of IFNs activates a multitude of genes that suppress viral replication. One of these genes encode for the MxA protein which is a major restriction factor for many types of RNA viruses; it binds to viral nucleocapsid protein and sequesters it (158). Influenza virus is restricted by MxA but the sensitivity depends on the virus; avian strains appear to be more sensitive to MxA than human viruses. Indeed, several influenza NP mutations in the 1918 and 2009 pandemic viruses were identified that allowed the viruses to evade human MxA. However, these mutations actually hindered virus growth and replication in mammalian MxA deficient cells which suggests that evading MxA is detrimental to overall viral fitness, and so there must be compensatory mutations in the viral genome that allow human viruses to overcome this (159).

I.VII DEVELOPMENT OF INFLUENZA VIRUS VACCINES

The first influenza A vaccines were created in the 1940s. The development of the vaccines involved growing the viruses in embryonated chicken eggs (method still used today), purifying the viruses, and then inactivating them using chemical means; these were called "whole-virus inactivated vaccines" (160). However, antigenic drift (accumulation of point mutations) in circulating viruses rendered these vaccines useless after one or two years and so the World Health Organization established a surveillance program in the 1950s to monitor relevant viruses for vaccine production (161). Most current vaccines aim to protect humans from infections by inducing antibodies against the HA head domain and also to some degree the NA protein as well (162). These are trivalent inactivated vaccines – meaning that they contain components from three inactivated viruses: two influenza A subyptes (H1N1 and H3N2) and one influenza B virus. The specific strains must be picked every year due to antigenic drift based on extensive surveillance and mathematical modeling of virus evolution (163). However, choosing the correct vaccine strains is still a difficult task and not picking the optimal ones leads to increased morbidity and mortality rates in the human population (164). An alternative approach to inactivated vaccines is the production of live attenuated influenza vaccines (LAIV); these contain an attenuated virus carrying the chosen NA and NA glycoproteins (165). The main advantage they offer compared to inactivated vaccines is they are introduced intranasally which stimulates a natural infection and subsequently elicit both the mucosal and systemic antibody and T cell responses. Indeed, inactivated vaccines usually elicit a weaker immune response in children and so LAIVs may be more appropriate for this age group (166, 167). Despite this advantage, they still suffer from the same problems as inactivated vaccines; antigenic drift in the HA head domain can cause the vaccine to lose effectiveness and production in eggs is expensive, laborious, time-consuming, and difficult to scale up. Furthermore, LAIVs have their own problem regarding incompatibility between the HA and NA glycoproteins and the attenuated virus backbone (165). Indeed, current vaccine approaches suffer from logistical constraints and are not well equipped in dealing with pandemics and rapidly mutating viral

strains. These issues call for a novel type of vaccine that will generate long-lasting immunity against all viral subtypes regardless of antigenic differences.

One of the most promising candidates for a universal influenza vaccine is the “stalk-based” vaccine. Indeed, the membrane proximal stalk region in the HA is conserved among most influenza viruses regardless of subtype, unlike the variable globular head domain (168). This region is highly conserved because it must undergo conformational changes that all influenza viruses need for membrane fusion and subsequent genome release into the cytoplasm. Efficient antibodies that target this region should completely abolish any productive infections. Since 1983, numerous laboratories have tried designing vaccine candidates using different portions of the HA stalk domain with various approaches (169-174). Currently there are several vaccine candidates in pre-clinical trials, and one promising contender is the “chimeric HA” construct. These constructs are designed by attaching the HA domains that are currently not associated with human adapted viruses (H4, H5, H6, H7, etc) to H1 and H3 stalk domains. It has been demonstrated that repeated immunizations with these chimeric HA constructs stimulated strong stalk-specific polyclonal antibody response which subsequently protected animals from H1 and H3 viral infections (175-178). Interestingly, it was shown that sequential infections with the 2009 H1N1 pandemic virus or vaccinations promoted the generation of stalk antibodies in the host (179, 180). Furthermore, infections with both H1N1 and H3N2 seasonal strains also boosted stalk neutralizing antibodies in humans and mice after successive infections (181). And thus chimeric HA vaccines are usually applied after an initial antibody “boost” by a low infectivity virus (168). Unfortunately, in general stalk antibodies have not reached the same level of protection compared to antibodies generated against the HA globular head domain even after repeated immunizations and boosters; there is evidence immunized animals showed clinical symptoms of varying degrees which suggest stalk antibodies may only confer partial immunity, especially against more pathogenic strains (176-178). However, new

developments and strategies are constantly being tested so a universal influenza vaccine based on stalk antibodies may not be too far off from the future (182).

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CHAPTER II

Equine and canine influenza H3N8 viruses show minimal biological differences despite phylogenetic divergence

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II.I PREFACE

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Author contributions are as follows:

Kurtis H. Feng – Lead author, conducted the vast majority of experiments and data analysis, wrote and edited the original manuscript for submission to Journal of Virology

Gaelle Gonzalez – Main contributor of the tracheal explant infections

Lingquan Deng – Main contributor of the glycan array binding experiments

Hai Yu – Minor contributor of the glycan array binding experiments

Victor L. Tse – Main contributor of the hemagglutinin cleavage assay

Lu Huang – Assisted in the flow cytometry experiments

Kai Huang – Assisted in virus stock sequencing

Brian R. Wasik – Assisted in the glycan array binding experiments, purification of proteins

Bin Zhou – Main contributor of the generation of influenza infectious plasmids

David E. Wentworth – Principal investigator of the laboratory responsible for creating the influenza infectious plasmids

Edward C. Holmes – Principal investigator of the laboratory responsible for creating the phylogenetic tree comparing equine and canine influenza virus, assisted manuscript revisions

Xi Chen – Principal investigator of the laboratory responsible for constructing the glycan array

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Pablo R. Murcia – Principal investigator of the laboratory responsible for the tracheal explant infections, assisted manuscript revisions

Colin R. Parrish – Corresponding author, principal investigator of the laboratory responsible for vast majority of conducted experiments, assisted manuscript revisions

II.II ABSTRACT

The A/H3N8 canine influenza virus (CIV) emerged from A/H3N8 equine influenza virus (EIV) around the year 2000 through the transfer of a single virus from horses to dogs. We defined and compared the biological properties of EIV and CIV by examining their genetic variation, infection and growth in different cell cultures, receptor specificity, hemagglutinin (HA) cleavage, and infection and growth in horse and dog tracheal explant cultures. Comparison of sequences of viruses from horses and dogs revealed mutations that may be linked to host adaptation and tropism. We prepared infectious clones of representative EIV and CIV strains that were similar to the consensus sequences of viruses from each host. The rescued viruses, including HA and NA double reassortants, exhibited similar long-term growth in MDCK cells. Different host cells showed varying levels of susceptibility to infection, but no differences in infectivity were seen when comparing viruses. All viruses preferred α2-3 over α2-6 linked sialic acids for infections, and glycan microarray analysis showed EIV and CIV HA-Fc fusion proteins bound only to α2-3 linked sialic acids. Cleavage assays showed EIV and CIV HA proteins required trypsin for efficient cleavage, and no differences in cleavage efficiency were seen. Inoculation of the viruses into tracheal explants revealed similar levels of infection and replication by each virus in dog trachea, although EIV was more infectious in horse trachea than CIV.

II.III INTRODUCTION

Influenza A viruses are maintained in aquatic birds as intestinal infections, occasionally transfer to and become established as respiratory infections in mammals including humans, and sometimes spread from one mammal to another (1, 2). Mammalian hosts that have been commonly seen to maintain avian-derived viruses include swine, horses, humans, mink, seals and recently, dogs (2-5). Host transfers between different birds, from birds to mammals, or between different mammalian hosts are relatively common, but mostly result in single infections or limited outbreaks. On rare occasions the host-transferred viruses go on to cause sustained epidemics or pandemics in their new hosts. Influenza viruses causing epidemics in new hosts often have mutations that appear to be specific to the new hosts in several gene segments, and in some cases these have been shown to control host adaptation (6-8). In many cases the transferred virus was observed to be a reassortant with segments from a number of different ancestors, or it soon reassorted with another influenza virus infecting that host (9, 10).

A number of different viral functions have been associated with host adaptation of influenza viruses. Specific sialic acid binding and/or cleavage are often key factors in host adaptation because sialic acids are primary influenza receptors, and mutations in the receptor interacting proteins, the hemagglutinin (HA) and neuraminidase (NA), often appear upon host transfer. Key traits include HA recognition of α2-3 or α2-6 linked sialic acids; avian viruses are generally specific for α2-3, and human viruses are generally specific for α2-6 linked receptors (11-13). There is often a coordination of the NA activity and specificity that correlates with HA binding, and the sialic acid linkages that are present in the host (14). Importantly, mutations in other gene segments are often seen, including PB2, PA, NP, M, and NS (15-18). In particular, polymerase subunits PB2 and PA control replication in different host cells and at different temperatures (19). Some mutations in the M gene segment have been associated with transmission (20), while NP mutations control the interactions with MxA, a host-derived antiviral molecule (21). Mutations in the NS1 gene control a variety of host-specific functions and innate

immune responses (22, 23). Despite the identification of these mutations, we lack a complete understanding of the factors that control specific virus-host range, particularly in nature, or of the host barriers that regulate the transfer of viruses to new hosts.

Here, we examined the host tropism associated with the transfer to and continuing replication of the A/H3N8 equine influenza virus (EIV) in dogs to create the phylogenetically distinct lineage of A/H3N8 canine influenza viruses (CIV) (Fig. 2.1) (3, 24-26). CIV was first identified in 2004 in Florida when it caused an outbreak in greyhounds in a training facility, and it was soon recognized to be closely related to EIV (3). Infected greyhounds carried the virus to different regions of the USA, and many other breeds of dogs have since been infected (24, 27, 28). CIV has continued to circulate in some regions of the USA, and for the past several years appears to have been primarily maintained in several hotspots where there are high-density and high turn-over dog populations, including animal shelters in New York City, Philadelphia, Colorado Springs, and Denver (26, 28, 29). Dogs appear to be a naturally receptive host of influenza virus because, in addition to the equine-origin A/H3N8 virus, an avian-origin A/H3N2 subtype has spread among dogs in Korea and China since 2006 (30, 31).

Examination of A/H3N8 isolates collected from dogs has shown that all segments contained CIV-specific mutations not seen in equine viruses, or only seen at very low frequencies (26, 28). However, it is not known how these CIV-specific mutations alter host range and tropism, or whether there has been ongoing selection of canine-adaptive mutations during the extended passage in dogs. To determine whether any of the CIV-specific mutations play a role in virus host-switching and adaptation, we examined protein sequences of all available CIV sequences deposited in GenBank (NCBI) in addition to sequences we determined from additional virus isolates. We also prepared reverse genetics plasmid sets of EIV and CIV and used these to derive viruses that we tested for host tropism and infectivity in cells. Additionally, we examined receptor specificity and HA cleavage, and lastly, we looked at growth and infectivity of these viruses in horse and dog tracheal explants.

II.IV MATERIALS AND METHODS

Cells and cell culture. All mammalian cells were grown at 37°C under 5% CO_2 and included Marbin-Darby canine kidney cells (MDCK), canine tumor fibroblasts (A72), Norden laboratory feline kidney cells (NLFK), human lung cancer cells (A549), human kidney embryonic kidney cells (HEK293T), ferret fibroblasts (Mpf), Chinese hamster ovary cells (CHO), and equine kidney cells (EQKD). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Insect cells (Sf9 and High Five) were grown in Grace's Insect Media supplemented with 10% FBS at 23°C. CHO cells express only α2-3 linked sialic acids (32). To generate CHO cells with varying levels of α2-6 linked sialic acids, cells were transfected with a plasmid expressing α2-6 sialyltransferase (33) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) and selected with hygromycin B (Life Technologies) at 250 μg/ml. Stably transfected cells were double stained to detect the levels of expression of α2-3 or α2-6 linked sialic acids. Biotinylated *Maackia amurensis* agglutinin type 1 (MAA1) (Vector Laboratories, Burlingame, CA) was used to detect α2-3 linked sialic acids and FITC conjugated *Sambucus nigra* agglutinin (SNA) (Vector Laboratories) was used to detect α2,6 linked sialic acids. Cells were incubated with biotinylated MAA1 for 1 h on ice, and then with FITC conjugated SNA and phycoerythrin (PE) conjugated streptavidin (Life Technologies). Cells were assayed by flow cytometry by following the commercial protocol using the Millipore Guava EasyCyte plus flow cytometer (EMD Millipore, Billerica, MA), and expression levels were analyzed by FlowJo software (TreeStar, Ashland, OR).

Viruses, plasmids, and virus rescue. A/equine/NY/61191/2003 and A/canine/NY/dog23/2009 were plaque purified and then passed in MDCK cells. The eight gene segments of each virus were cloned into modified pDZ plasmids (34). Co-cultures of HEK293T and MDCK cells (2:1 ratio) in 6 well plates were transfected with 300 ng of each of the 8 influenza plasmids using TransIT-LT1 (Mirus Bio, Madison, WI). At 24 h post-transfection,

medium was changed to DMEM with 0.3% BSA containing 1 µg/ml TPCK trypsin from bovine pancreas (Sigma-Aldrich, St Louis, MO). After 48 h, the supernatant was harvested and used to inoculate MDCK cells supplemented with 1 µg/ml trypsin in 6 well plates to grow P2 virus. The supernatant was harvested 72 h later and clarified by low speed centrifugation. Standard hemagglutination (HA) assays using 0.5% chicken erythrocytes (Lampire Biological Laboratories, Pipersville, PA) confirmed the presence of P2 virus (35). The virus was used to infect MDCK cells supplemented with 1 µg/ml trypsin in 75 cm^2 flasks to grow up working stocks of P3 virus. At 72 h post-infection, supernatant was harvested and clarified, and virus was frozen down at -80°C in 500 µl aliquots.

Virus titration. Virus stocks were quantified by tissue culture infectious dose 50 ($TCID_{50}$), HA assays, and genome copies (RT-qPCR). $TCID_{50}$ was performed using 96 well plates seeded with MDCK cells. Briefly, virus stocks were 10-fold serially diluted in DMEM and 50 µl volumes were inoculated into each well across 8 rows. After 48 h incubation, cells were fixed with 4% paraformaldehyde (PFA) for 10 min. Cells were then washed with phosphate-buffered saline (PBS) and mouse IgG anti-nucleoprotein (NP) antibody (Creative Diagnostics, Shirley, NY) was added to each well in permeabilization buffer (PBS with 0.5% saponin). After 1 h incubation, cells were washed with PBS and then incubated for 1 h with goat IgG anti-mouse Alexa Fluor 488 conjugated antibody (Life Technologies) in permeabilization buffer. Cells were washed with PBS and viewed by a Nikon TE300 fluorescent microscope. Each well was scored as positive for infection as long as there was a single infected cell, and $TCID_{50}$ was calculated using the Reed and Muench method. Standard HA assays were performed using 0.5% chicken erythrocytes as mentioned above. Virus genome copies were calculated by RT-qPCR targeting the influenza M gene segment (36). First, viral RNA was extracted using QIAmp Viral RNA mini kit (Qiagen, Venlo, Netherlands). Next, the viral RNA, two outside primers specific for influenza M, and a TaqMan probe were used to set up standard reaction cocktails using the QuantiTect Probe PCR kit (Qiagen) supplemented with ImProm-II reverse transcriptase (Promega,

Madison, WI). Samples were exposed to a reverse transcription step and subsequent 40 cycle amplification step in an AB StepOnePlus RT-PCR machine (Applied Biosciences, Foster City, CA), and genome copies per µl were calculated based on the C_T values of a standard curve generated using the influenza M gene plasmid.

Phylogenetic analysis. A total of 400 representative HA sequences of EIV and CIV sequences were download from GenBank. A minimum sequence length comprising at least the HA1 domain was set, and identical sequences were excluded. The sequences were easily aligned using the MAFFT method available in Geneious (37), resulting in a total alignment length of 1710 bp. The phylogenetic relationships among these sequences were then determined using the maximum likelihood (ML) approach available in the PhyML program (38). This analysis utilized the generalized time reversible (GTR+) model of nucleotide substitution and a combination of subtree pruning and regrafting (SPR) and nearest-neighbor interchange (NNI) branch-swapping. The robustness of individual nodes on the phylogeny was assessed using Shimodaira-Hasegawa (SH)-like branch supports.

Virus sequencing. Virus RNA was extracted using QIAmp Viral RNA mini kit. The cDNA was synthesized using AMV reverse transcriptase (Promega) and universal primer Uni12 (5′-AGCAAAAGCAGG-3′). Eight gene segments (HA, NA, NP, M, NS, PA, PB1, PB2) were amplified by PCR with EIV and CIV gene specific primers. The PCR products were purified using E.Z.N.A. Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA). Purified DNA was sequenced using an Applied Biosystems 3730xl DNA Analyzer (Life Technologies) at Cornell University Institute of Biotechnology, and full-length genes were assembled using Lasergene software (DNASTAR, Madison, WI).

Virus sequence analysis. Consensus protein sequences of EIV and CIV were generated and compared representing differences in host and time of sampling. In this context a “consensus” sequence is used to define the most common amino acid in all available EIVs and CIVs (i.e. epidemiological scale) and not simply those from a single host. All available EIV and

CIV sequences in GenBank were used in addition to four newly sequenced CIVs from Pennsylvania (2009), Virginia (2010), and New York (2011 and 2013). Sequence alignments were performed using MEGA (Arizona State University, Phoenix, AZ), and Clustal Omega (EMBL-EBI, Cambridge, UK) to find the most common amino acid at each position. Three groups of consensus EIV and CIV protein sequences (HA, NA, M1, NP, NS1, PA, PB1, PB2) were generated. The first group represented EIV isolates sampled close to the ancestor of the CIVs, starting from 1990 to 2011; the second group represented CIV isolates sampled soon after the emergence in dogs (between 2003 and 2007); while the third group represented CIV isolates sampled after the virus had been circulating in dogs for at least 8 years, from 2008 to 2013. Both EIV and CIV plasmid sets were compared with their respective consensus sequences to ensure they were good representatives of EIV and CIV. The accession numbers of the newly sequenced CIVs can be obtained from the data set KM359803-KM359864.

Virus growth curves. MDCK cells were seeded in 12 well plates. Upon reaching confluency, cells were washed with DMEM and incubated with virus diluted in DMEM containing 0.3% BSA and 1 µg/ml trypsin at a multiplicity of infection (MOI) = 0.0006 based on $TCID_{50}$ for 1h at 37°C. Cells were then washed with and replenished with fresh DMEM containing 0.3% BSA and 1 µg/ml trypsin. Supernatants were harvested from each well every 24 h for 5 days and stored at -80°C. $TCID_{50}$ and genome copies for all time points were determined as described above.

Virus infection of cell lines derived from different hosts. Different host cells were seeded in 48 well plates. Upon reaching confluency, cells were washed and incubated with virus diluted in DMEM containing 0.3% BSA and 0.5 µg/ml trypsin at MOI = 0.05 based on $TCID_{50}$ for 1h at 37°C. Cells were then washed and replenished with fresh DMEM containing 0.3% BSA and 0.5 µg/ml trypsin. Cells were fixed with 4% PFA for 10 min at 24 h post-infection and stained for NP expression as described above and DAPI (Life Technologies) stained for 5 min following the commercial protocol. Stained cells were visualized by fluorescence microscopy.

Infections of different host cells were also quantified by flow cytometry. Briefly, cells were grown in 48 well plates and upon reaching confluency, inoculated with virus as described above at MOI = 0.1 based on $TCID_{50}$. Cells were harvested and fixed with 4% PFA for 10 min at 24 and 48 h post-infection. Cells were stained for NP and quantified by flow cytometry as described above, and results were analyzed by FlowJo.

Lectin staining. Cells were grown in 48 well plates, and upon reaching confluency, they were collected and fixed using 4% PFA. Cells were incubated with either FITC conjugated MAA1 or FITC conjugated SNA to detect α2-3 or α2-6 linked sialic acids, respectively. After 1 h incubation cells were washed with PBS with 1% BSA. Cells were assayed by flow cytometry as described above, and results were analyzed using FlowJo. In addition to flow cytometry, fluorescence microscopy was also used to look at lectin stained cells.

Construction and purification of HA-Fc fusion proteins. The EIV and CIV HA ectodomains (the same sequences as the reverse genetics plasmids) were fused to human IgG1 Fc at the C terminus followed by a hexa-histidine-tag (39, 40). The baculovirus gp64 secretion peptide was fused to the constructs at the N terminus. The genes were synthesized by GeneScript (Piscataway, NJ) and cloned into pFastBac-1 (Life Technologies) to generate recombinant bacmids following the commercial protocol. Recombinant baculoviruses were recovered by bacmid transfection into Sf9 insect cells using Cellfectin II (Life Technologies). Viruses were then used to infect suspension High Five cells and two days post-infection the proteins were purified by binding to a HiTrap ProteinG HP 5 ml column (GE Healthcare Life Sciences, Piscataway, NJ) and eluted with 0.1M Citrate, pH 3.0 (pH neutralization to 7.8 with 1M Tris, pH 9.0) using ÄKTA FPLC system (GE Healthcare Life Sciences). The HA-Fc containing fractions were dialyzed in PBS and concentrated using 30kD Amicon Ultra-15 centrifugal filter tubes (EMD Millipore). The proteins were stored at -80°C in aliquots. Concentration was measured using Beer-Lambert Law calculation based on A280 reading.

Testing virus receptor specificity. CHO and CHO cells expressing α2-6 linked sialic acids (6H4 cells) were grown in 48 well plates. When confluent, cells were inoculated with viruses as described above at MOI = 1 based on $TCID_{50}$. After 24 h post-infection, cells were collected and fixed by 4% PFA. Cells were stained for NP and quantified by flow cytometry as described above, and results were analyzed using FlowJo. Purified EIV and CIV HA-Fc proteins were used in glycan binding microarrays. The microarrays were fabricated using epoxide-derivatized glass slides and the high-throughput protein binding screening was carried out as previously described (41, 42). Briefly, freshly printed glycan microarray slides were blocked by ethanolamine, washed and dried, and then fitted in a multi-well microarray hybridization cassette (ArrayIt, CA) to divide into subarrays. The subarrays were blocked with ovalbumin (1% w/v) in PBS (pH 7.4) for 1 h at RT in a humid chamber with gentle shaking. Subsequently, the diluted HA-Fc samples were added to the subarrays and incubated for 2 h at room temperature with gentling shaking, and lastly the slides were extensively washed. Fluorescently labeled antibody (Cy3-labeled goat anti-human IgG, Jackson ImmunoResearch Laboratories) was then applied and incubated for 1 h. Following final washes and drying, the developed glycan microarray slides were scanned with a Genepix 4000B microarray scanner (Molecular Devices Corp., Union City, CA). Data analysis was done using the Genepix Pro 7.0 analysis software (Molecular Devices Corp., Union City, CA).

HA cleavage assays. HEK293T cells were seeded in 24 well plates coated with poly-D-lysine. Upon reaching 70% confluency, 500 ng of each respective HA plasmid were transfected using Lipofectamine 2000 (Life Technologies) following the manufacturer's protocol. After 18 h post-transfection, cells were washed with PBS and incubated with trypsin at 3 µg/ml for 15 min. Cells were kept at 4°C and surface biotinylated using sulfo-NHS-SS-biotin (Thermo Scientific) at 250 µg/ml following the manufacturer's protocol for 30 min. Excess biotin was quenched using 50 mM glycine for 10 min. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (EMD Millipore) with complete protease inhibitor cocktail tablets (Roche, Nutley, NJ) for 10 min.

Lysed cells were high speed centrifuged for 20 min at 4°C. Supernatant was collected and incubated with 50% suspension of streptavidin agarose beads (Thermo Scientific) for 18 h while rotating at 4°C. Beads were then washed with RIPA buffer and resuspended in 2× Laemmli sample buffer containing 10% beta-mercaptoethanol for Western blotting (43). HA bands were detected using goat IgG polyclonal anti-H3 HA antibody (BEI Resources, Manassas, VA) followed by rabbit IgG anti-goat antibody conjugated to horseradish peroxidase (Thermo Scientific). Western blot images were taken using a FujiFilm LAS – 3000 imaging system. The pixel density of HA bands were measured by Image J (National Institutes of Health, Bethesda, MD), and relative cleavage efficiencies were calculated based on the formula: $(HA_1/HA_0 + HA_1) \times 100\%$ (43).

Infection of horse and dog tracheal explants. Tracheal explant cultures were acquired, prepared, maintained, and tested for viability as described previously (44-46). Viruses were used to infect explants by inoculating 400 $TCID_{50}$ units of each virus directly on the epithelium layer. Virus growth was assayed by plaque assays in MDCK cells every 24 h as described previously (44). Explant sections were used for hematoxylin and eosin staining, and also for virus antigen NP staining on days 1, 3, and 5 post-infection (44). Due to the difficulty of obtaining fresh horse trachea from healthy animals, only one experimental replicate was done using the horse tracheal explants.

Statistics. Statistical significance was measured by the Student's t-test using GraphPad Prism when appropriate.

II.V RESULTS

EIV and CIV genetic analysis. Three sets of epidemiological scale consensus protein sequences were generated: EIV (1990 – 2011), early CIV (CIV 2003 – 2007), and more recent CIV (2008 – 2013) (Fig. 2.2). Sequence alignments revealed consensus amino acid mutations in the eight major proteins. Some mutations were only seen between EIV and early CIV

sequences, while others only appeared between the early CIV and more recent CIV sequences. HA exhibited the greatest number of mutations, while M1 showed the least. In addition, a phylogenetic analysis of 400 EIV and CIV HA sequences clearly showed the CIV sequences formed a single monophyletic group distinct from EIV (Fig. 2.1).

Our analysis revealed substitutions at putative HA antigenic sites (residues 54, 75, 83, 92, 159, 216), receptor binding pocket (residues 222, 223), and sites that may influence HA cleavage (residues 328, 483) (47-50). Although there were no NA mutations in the active site, changes at position 149 may affect sialidase activity because the 150 loop can incorporate sialic acid derivatives to inhibit NA enzymatic activity (51). Mutations at the N terminus of NP (residues 27, 52) were located in RNA and PB2 binding domains, and mutations at the C terminus (residues 359, 375, 498) may influence NP polymerization and binding to host actin (52). The M1 138 mutation was in a domain that is responsible for polymerization and binding to NP (53). Mutations in NS1 could potentially change a number of interactions with host proteins including poly(A)-binding proteins I and II, importin-α, nucleolin, and translation initiation factors (54). Structural insights into the polymerase proteins have revealed functional domains, and our analysis revealed mutations in those regions. For example, mutations in PB2 (residues 374, 389) were located in the host RNA cap binding domain, C terminus mutations in PB1 (residues 687, 754) may affect binding to PB2, and mutations in PA (residues 327, 348, 388, 400, 444, 675) may alter interactions with PB1 (55). The mutation at position 27 in PA was located in the endonuclease domain and with the recent discovery of PA-X, the mutation may affect PA-X specific host gene suppression (56-59). Additionally, position 400 in PA and 292 in PB2 may be host determinants and play roles in virus adaptation (60, 61).

Rescued viruses and virus growth. An EIV sampled close to the ancestral sequence of CIV (A/equine/NY/61191/2003) and a CIV sampled from 9 years after the virus transferred into dogs (A/canine/NY/dog23/2009) were chosen as representatives of these viruses based on comparing their sequences to the consensus, and were rescued by reverse genetics.

Additionally, two reassortant viruses were recovered: EIV with CIV HA and NA and the reciprocal virus, CIV with EIV HA and NA. All viruses reached a high infectious titer after minimal passages in MDCK cells and were able to hemagglutinate chicken erythrocytes (Table 2.1). The HA and NA genes of the virus stocks were sequenced and there were no mutations comparing the sequences to the reverse genetics plasmids. Five-day growth curves for the viruses were conducted in MDCK cells, and there was no significant difference ($p>0.05$) in yields of infectious particles (Fig. 2.3A) or RNA copies (Fig. 2.3B). Infectious titers peaked for all viruses between 48 and 72 h and then steadily declined. RNA copies reached their peaks at similar timepoints and then plateaued.

EIV and CIV infections in different host cells. Viruses were used to inoculate cells (MOI = 0.05) from several hosts: MDCK (dog), A72 (dog), NLFK (cat), Mpf (ferret), A549 (human), and EQKD (horse). Cells were stained for virus NP at 24 h post-infection and visually the infectivity looked similar comparing the viruses, however, different host cells exhibited varying levels of susceptibility to infection (Fig. 2.4). MDCK, A72, and NLFK cells were all heavily infected. Mpf cells were moderately infected, and A549 and EQKD cells were poorly infected. To further analyze infectivity in different host cells, viruses were used to inoculate four host cells (two that were permissive to infection, MDCK and A72 cells, and two that were least permissive to infection, A549 and EQKD cells) (MOI = 0.1) and the percentage of infected cells was quantified by flow cytometry at 24 and 48 h post-infection (Fig. 2.5A). There was no significant difference ($p>0.05$) in infectivity comparing the viruses. At 24 h post infection, around 40% of MDCK and A72 cells were infected by all viruses. In contrast, about 15% of A549 and EQKD cells stained positive for infection. At 48 h post infection, the percentage of MDCK and A72 infected cells rose to around 70 – 80%, while the percentage increase of infected A549 and EQKD cells was much more modest, around 20% (Fig. 2.5A). To ensure A549 and EQKD cells can be infected by influenza, the laboratory adapted human virus A/Puerto Rico/8/1934 H1N1 (PR8) was used to infect A549 and EQKD cells (MOI = 0.05) and its infectivity was compared to

EIV and CIV at 24 h post-infection (Fig. 2.5B). PR8 infected high proportions of A549 and EQKD cells in contrast to EIV and CIV.

EIV and CIV receptor specificity. To determine if the difference in infectivity comparing various host cells was due to virus receptor availability and/or differences, cells were lectin stained and visualized by microscopy and fluorescence was measured by flow cytometry (Fig. 2.6). MDCK, A72, A549, and EQKD cells all stained positive for both α2-3 and α2-6 linked sialic acids, while NLFK and Mpf cells were stained predominately for α2-3 linked sialic acids (Fig. 2.6A and 2.6B). Overall, there was a higher relative concentration of α2-3 linked sialic acids compared to α2-6 linked sialic acids in all cell lines (Fig. 2.6C). CHO cells expressed only α2-3 linked sialic acids while CHO cells stably transfected with α2-6 sialyltransferase (6H4) expressed both α2-3 and α2-6 linked sialic acids (Fig. 2.7A). Viruses were used to inoculate these cells (MOI = 1) and infectivity was assayed by flow cytometry 24 h post-infection. There was no significant difference ($p>0.05$) in infectivity among the viruses, but all viruses showed around a 50% reduction in their ability to infect 6H4 cells compared to CHO cells (Fig. 2.7B). To further analyze EIV and CIV receptor specificity, HA-Fc fusion proteins were generated and used to bind different glycans on microarrays. The binding specificity for the EIV and CIV HA-Fc proteins were similar to each other; both bound to α2-3 linked sialic acids and neither bound to any α2-6 linked sialic acids in the array (Fig. 2.7C).

EIV and CIV HA cleavage efficiency. HA cleavage assays showed that both wild type proteins and CIV HA mutants (T328I and T483N) were efficiently cleaved by trypsin under the conditions of this trial (Fig. 2.8A). There was an extra band detected of around 38 kDa, and this was most likely degraded HA product detected by the polyclonal antibody. CIV HA T483N had a lower percentage of cleavage compared to EIV and CIV HA T328I (Fig. 2.8B). However, the difference was minor (<20%), and overall there was no significant difference ($p>0.05$) in cleavage efficiency among the four proteins when tested for trypsin cleavage. All four HA proteins had minimal cleavage (<10%) without trypsin.

EIV and CIV infections of dog and horse tracheal explants. Inoculation of dog tracheal explants with EIV, CIV, and the reassortants showed that all of the viruses infected explants to similar levels. Histology showed canine tracheal explants that were infected with wild type and reassortant viruses progressively had their epithelium layer thinned out (Fig. 2.9A), and this was most noticeable on day 5 compared to the control. Additionally, the number of ciliated cells gradually decreased in infected explants, and completely disappeared on day 5 for all viruses, and these changes in the tracheal architecture were due to the presence of virus as confirmed by antigen detection (Fig. 2.9B). Virus was detected in the epithelium and did not infect the basal cells to a large degree. Growth for all viruses was similar, reaching maximum titers between 48 and 72 h post-infection (Fig. 2.9C). Interestingly, at 24 h post-infection, the wild type viruses' titers were around 2 logs lower ($p<0.05$) compared to the reassortant viruses' titers.

Inoculation of horse tracheal explants with the same viruses showed EIV reached several logs higher in titer compared to CIV on each day post-infection. CIV with EIV HA and NA grew better compared to CIV, although its growth was still poor compared to the two EIVs. Conversely, EIV with CIV HA and NA reached lower titers (1-2 log difference) compared to EIV, but it still grew to higher titers than the two CIVs (Fig. 2.10C). Histology showed the epithelium layer of the equine tracheal cultures did not show an obvious decrease in thickness compared to the control, and the number of ciliated cells also was not greatly reduced on day 5 (Fig. 2.10A). Indeed, the ciliated cells only decreased in number when virus was detected in the epithelium (Fig. 2.10B), and overall virus detection was not as consistent compared to the infected dog tracheal explants. CIV was not detected in the horse trachea on days 1, 3, and 5 post-infection, and CIV with EIV HA and NA was not detected on day 1 post-infection (Fig. 2.10B and 2.10C). Interestingly, EIV with CIV HA and NA was only detected after screening several sections by NP staining on day 5 but growth was observed on each day post-infection.

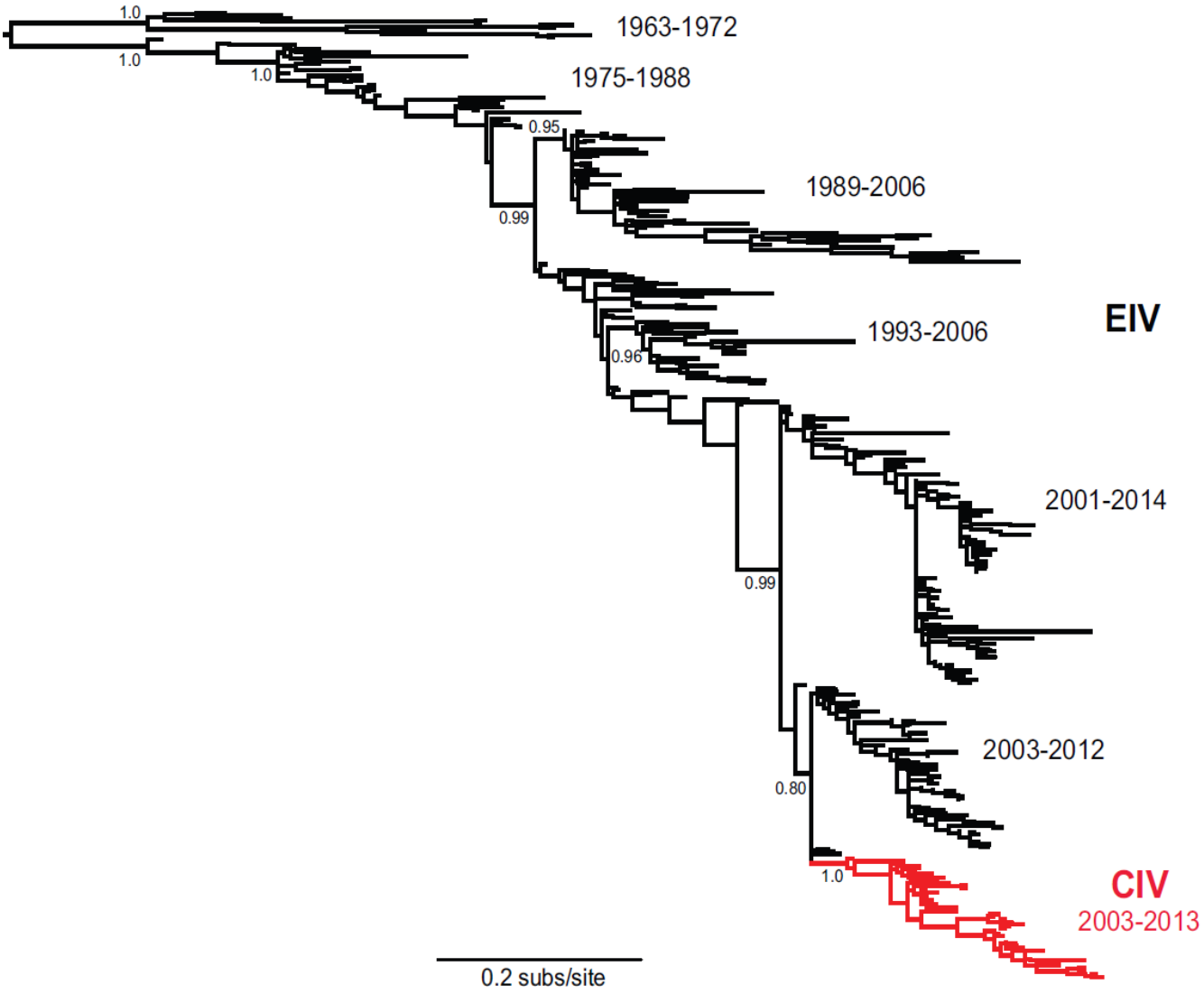

FIG 2.1. Maximum likelihood phylogenetic tree of 400 EIV and CIV HA sequences, with the latter shown in red. All tip labels were removed for clarity of representation. SH-like branch supports are shown for key nodes, as are time ranges of sampling for the main clusters of viral sequences. The CIV sequences clearly form a single monophyletic group, indicative of a single viral emergence event in dogs.

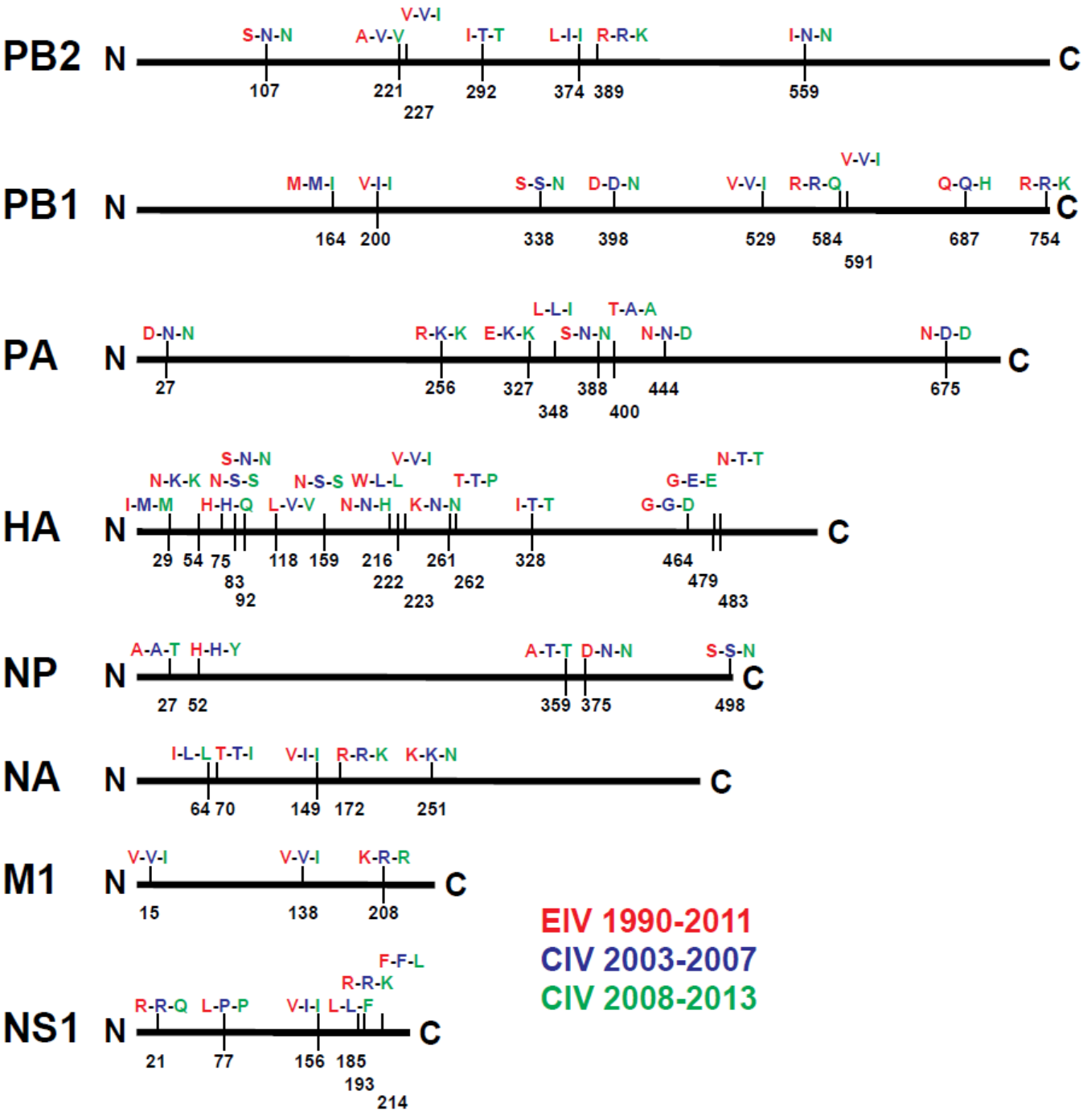
PB2
N
C
S-N-N
A-V-V
V-V-I
I-T-T
L-I-I
R-R-K
I-N-N
107
221
227
292
374
389
559
PB1
M-M-I
V-I-I
S-S-N
D-D-N
V-V-I
R-R-Q
V-V-I
Q-Q-H
R-R-K
164
200
338
398
529
584
591
687
754
PA
D-N-N
R-K-K
E-K-K
S-N-N
L-L-I
T-A-A
N-N-D
N-D-D
27
256
327
348
388
400
444
675
HA
S-N-N
N-K-K
N-S-S
N-S-S
V-V-I
W-L-L
T-T-P
N-T-T
G-E-E
I-M-M
H-H-Q
L-V-V
N-N-H
K-N-N
I-T-T
G-G-D
29
54
75
83
92
118
159
216
222
223
261
262
328
464
479
483
NP
A-A-T
H-H-Y
A-T-T
D-N-N
S-S-N
27
52
359
375
498
NA
I-L-L
T-T-I
V-I-I
R-R-K
K-K-N
64
70
149
172
251
M1
V-V-I
V-V-I
K-R-R
15
138
208
NS1
F-F-L
R-R-K
R-R-Q
L-P-P
V-I-I
L-L-F
21
77
156
185
193
214
EIV 1990-2011
CIV 2003-2007
CIV 2008-2013

FIG 2.2. Comparison of EIV and CIV epidemiological scale consensus protein sequences. The consensus sequences of eight major influenza proteins were generated for EIV 1990-2011, for early CIV 2003-2007, and for more recent CIV 2008-2013. The three consensus sequences were aligned and differences were indicated by vertical bars along each protein. Each vertical bar indicates the amino acid position (H3 and N2 numbering) and specific amino acids differences for EIV (red), early CIV (blue), and more recent CIV (green) sequences. A full vertical bar indicates a change comparing EIV to early CIV and no change comparing early CIV to more recent CIV. A half vertical bar indicates no change comparing EIV to early CIV but a change comparing early CIV to more recent CIV. Additionally, EIV NS1 can be truncated by 11 amino acids at the C terminus while CIV NS1 is never truncated except for the first reported CIV sequence in 2003 (A/canine/Florida/242/2003 (H3N8)).

TABLE 2.1. Reverse genetics rescue EIV, CIV, and reassortant viruses. A representative EIV (A/equine/NY61191/2003) and CIV (A/canine/NY/dog23/2009) were recovered from 8 plasmid reverse genetics along with HA and NA double reassortant viruses. Virus rescue was confirmed by $TCID_{50}$, virus genome copies, and HA assays using chicken erythrocytes.

Virus	Log $TCID_{50}$ / ml	Log genome copies / µl	HA units
EIV	**6.82**	**7.99**	**64**
CIV	**6.63**	**7.28**	**32**
EIV / CIV HA+NA	**6.40**	**7.11**	**32**
CIV / EIV HA+NA	**6.47**	**7.26**	**32**

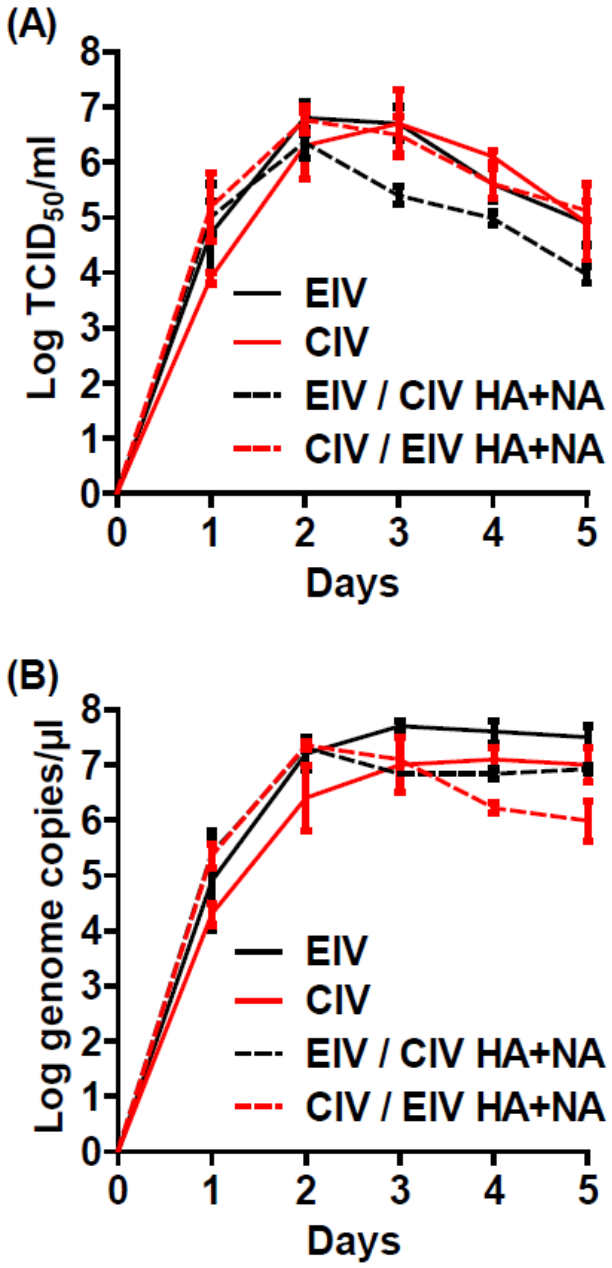

FIG 2.3. EIV, CIV, and reassortant virus growth curves in MDCK cells. Viruses were used to inoculate MDCK cells at MOI = 0.0006 in the presence of 1 µg/ml trypsin. Infectious titer (A) and genome copies per µl (B) were determined every 24 h post-infection. Error bars represent the standard deviation of three independent experiments.

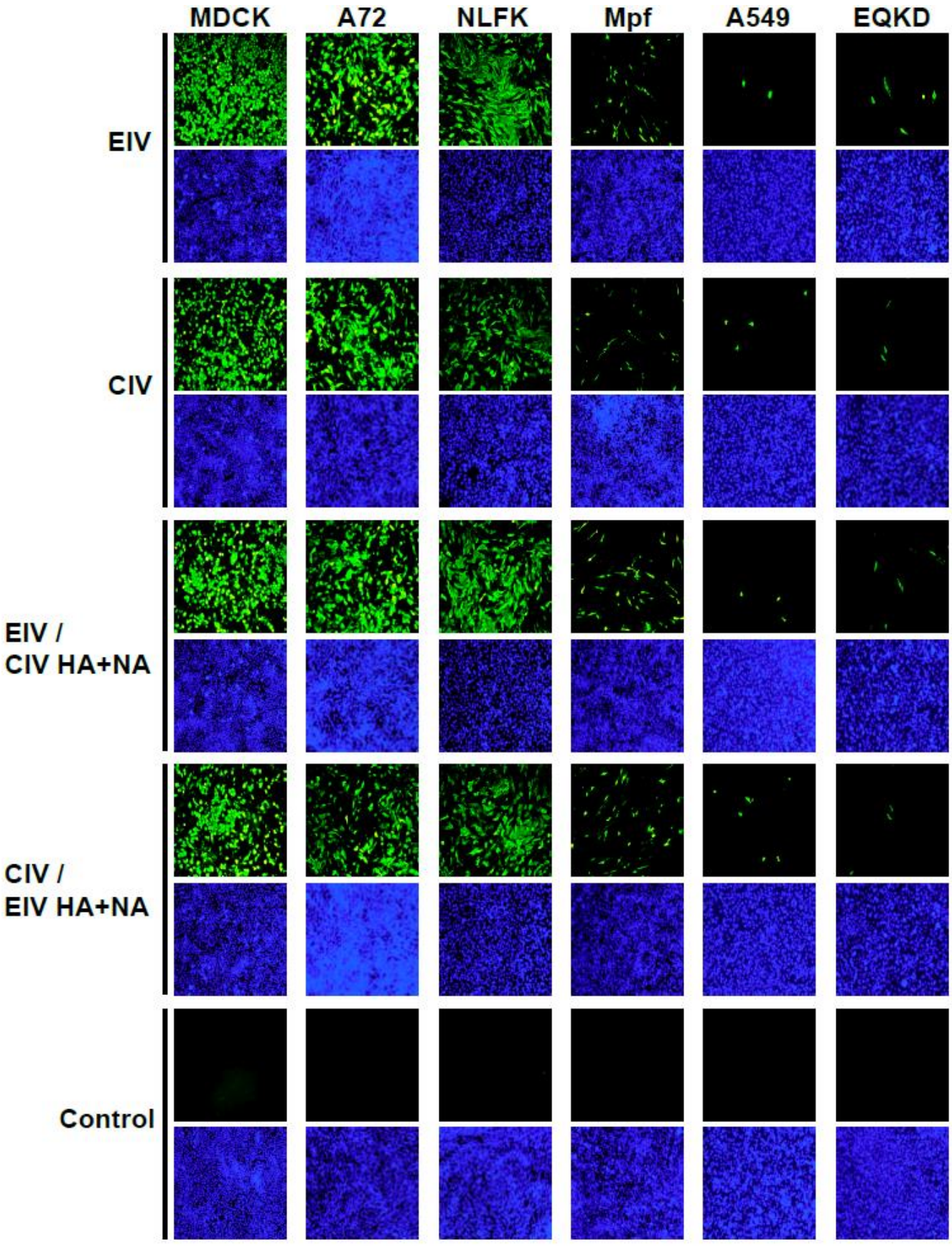
MDCK
A72
NLFK
Mpf
A549
EQKD
EIV
CIV
EIV /
CIV HA+NA
CIV /
EIV HA+NA
Control

FIG 2.4. Immunofluorescence images of EIV, CIV, and reassortant virus infections in different host cells. Viruses were used to inoculate different cells at MOI = 0.05 in the presence of 0.5 µg/ml trypsin. Anti-NP staining was used to detect virus at 24 h post-infection and DAPI was used for nuclear staining. Images were taken at 100× magnification using a fluorescent microscope as overlays. The overlays were kept as separate panels for clarity.

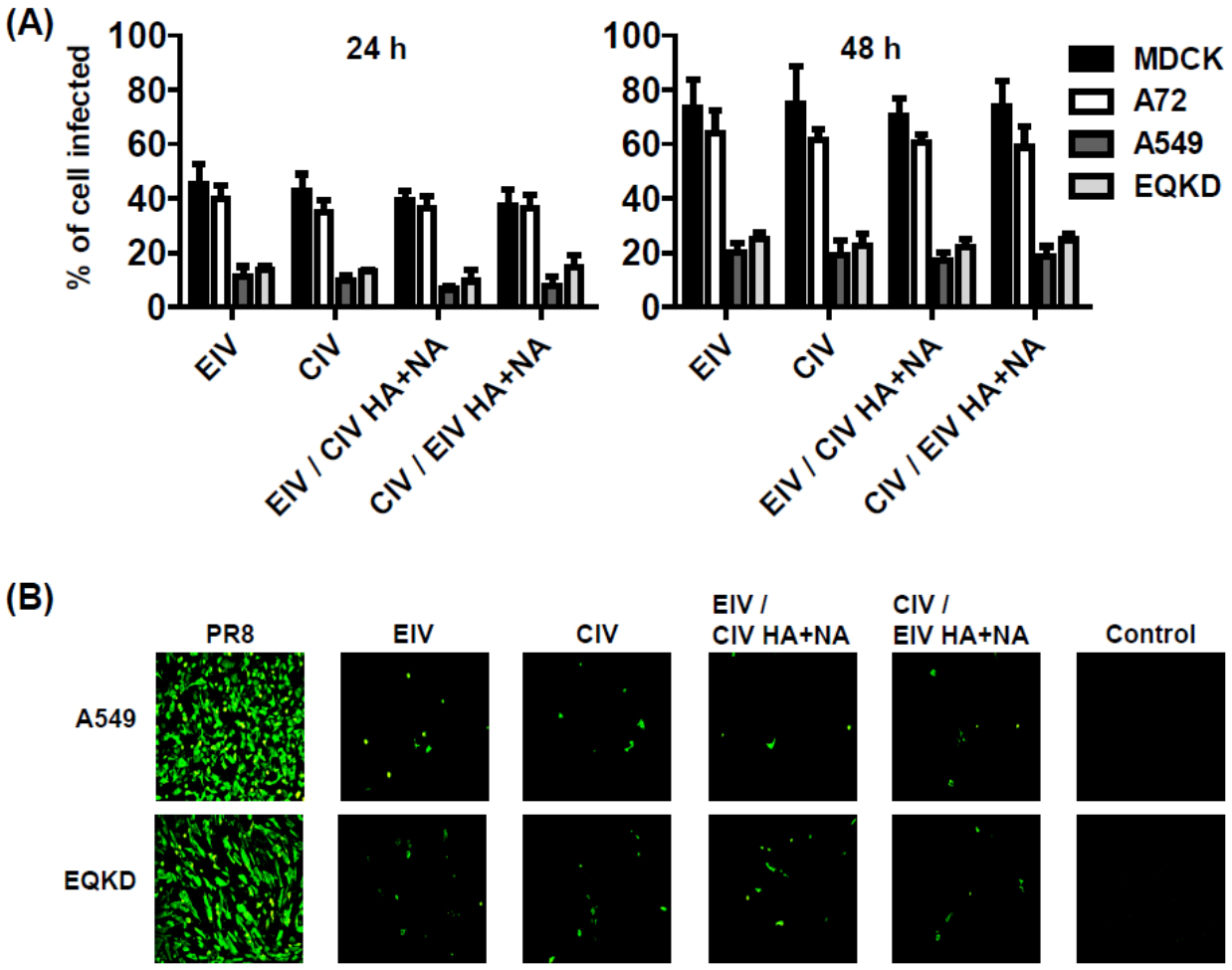

FIG 2.5. Quantification of EIV, CIV, and reassortant virus infections in different host cells, and PR8 infections of cells least permissive to EIV and CIV. Viruses were used to inoculate different host cells at MOI = 0.1 in the presence of 0.5 µg/ml trypsin and quantified by flow cytometry at 24 h and 48 h post-infection (A). Similarly, PR8, EIV, CIV, and reassortant viruses were used to inoculate A549 and EQKD cells at MOI = 0.05 in the in the presence of 0.5 µg/ml trypsin and visualized by fluorescence microscopy (anti-NP staining) 24 post-infection (B). Error bars represent the standard deviation of three independent experiments.

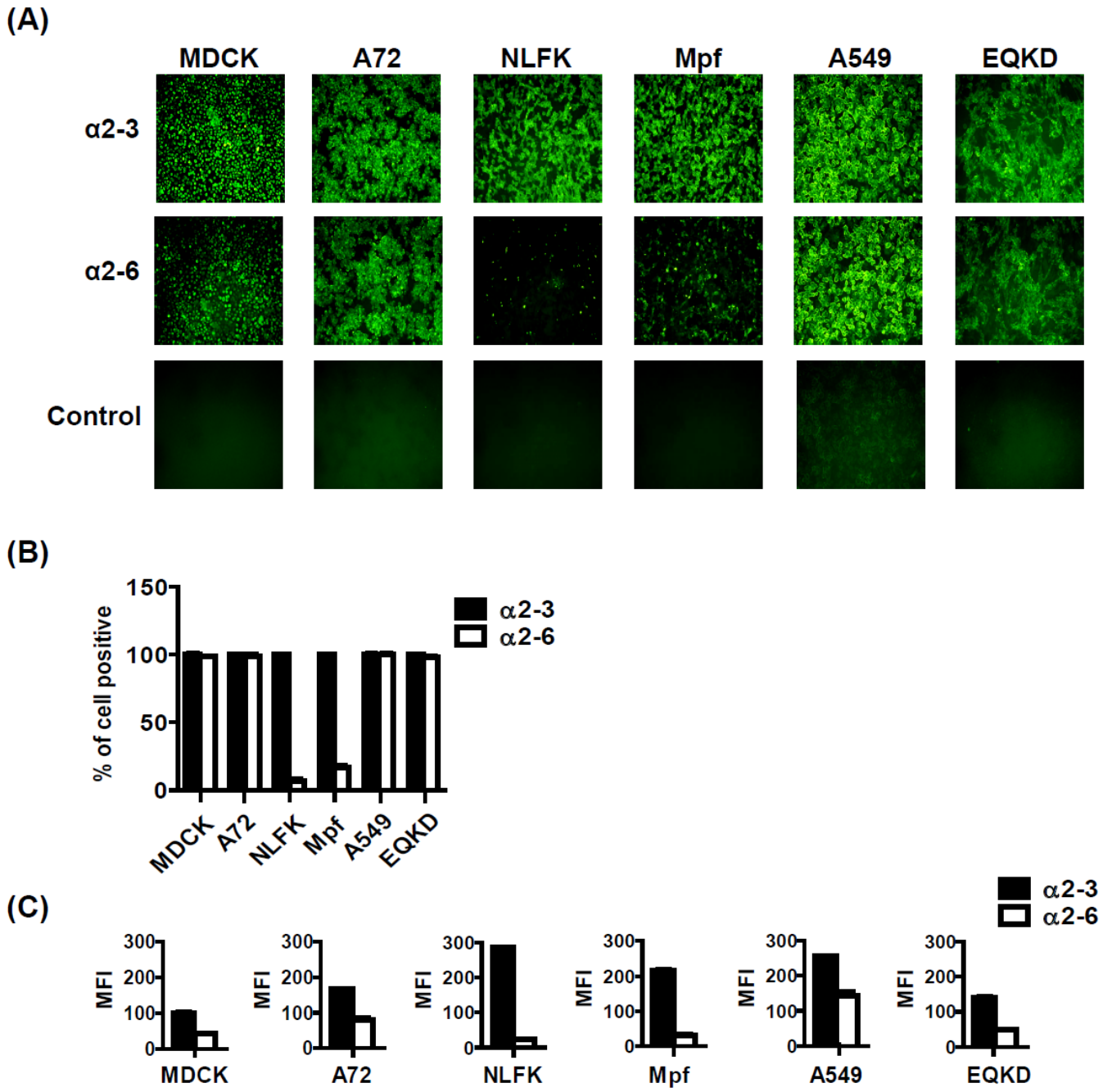

FIG 2.6. Lectin staining of different host cells. Different host cells were lectin stained using FITC conjugated MAAI to detect α2-3 linked sialic acids and FITC conjugated SNA to detect α2-6 linked sialic acids and visualized by fluorescent microscopy (A) and assayed by flow cytometry (B). Relative abundance of the sialic acids was measured by flow cytometry as well (C). Error bars represent the standard deviation of three independent experiments.

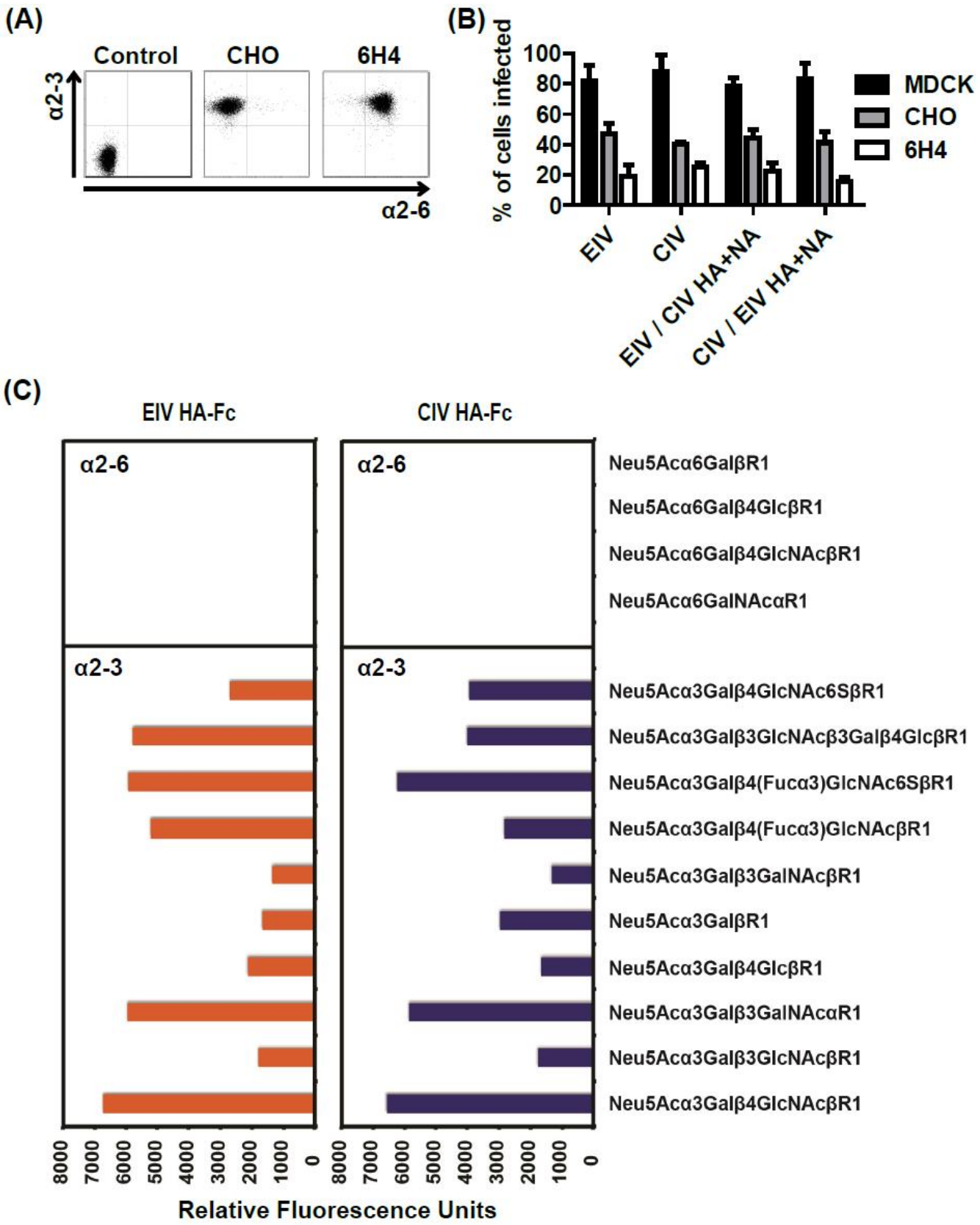
(A)
Control
CHO
6H4
α2-3
α2-6
(B)
% of cells infected
100
80
60
40
20
0
MDCK
CHO
6H4
EIV
CIV
EIV / CIV HA+NA
CIV / EIV HA+NA
(C)
EIV HA-Fc
CIV HA-Fc
α2-6
α2-3
Neu5Acα6GalβR1
Neu5Acα6Galβ4GlcβR1
Neu5Acα6Galβ4GlcNAcβR1
Neu5Acα6GalNAcαR1
Neu5Acα3Galβ4GlcNAc6SβR1
Neu5Acα3Galβ3GlcNAcβ3Galβ4GlcβR1
Neu5Acα3Galβ4(Fucα3)GlcNAc6SβR1
Neu5Acα3Galβ4(Fucα3)GlcNAcβR1
Neu5Acα3Galβ3GalNAcβR1
Neu5Acα3GalβR1
Neu5Acα3Galβ4GlcβR1
Neu5Acα3Galβ3GalNAcαR1
Neu5Acα3Galβ3GlcNAcβR1
Neu5Acα3Galβ4GlcNAcβR1
8000
7000
6000
5000
4000
3000
2000
1000
0
Relative Fluorescence Units

FIG 2.7. EIV and CIV receptor specificity. Lectin staining showed CHO cells only expressed α2-3 linked sialic acids while 6H4 cells expressed both α2-3 and α2-6 linked sialic acids (A). EIV, CIV, and reassortant viruses were used to inoculate MDCK (control cells), CHO, and 6H4 cells at MOI = 1 in in the presence of 0.5 µg/ml trypsin. After 24 h post-infection cells were stained for anti-NP and quantified by flow cytometry (B). EIV and CIV HA-Fc fusion proteins were used to bind α2-3 and α2-6 linked sialic acids on glycan microarrays (C). Error bars represent the standard deviation of three independent experiments.

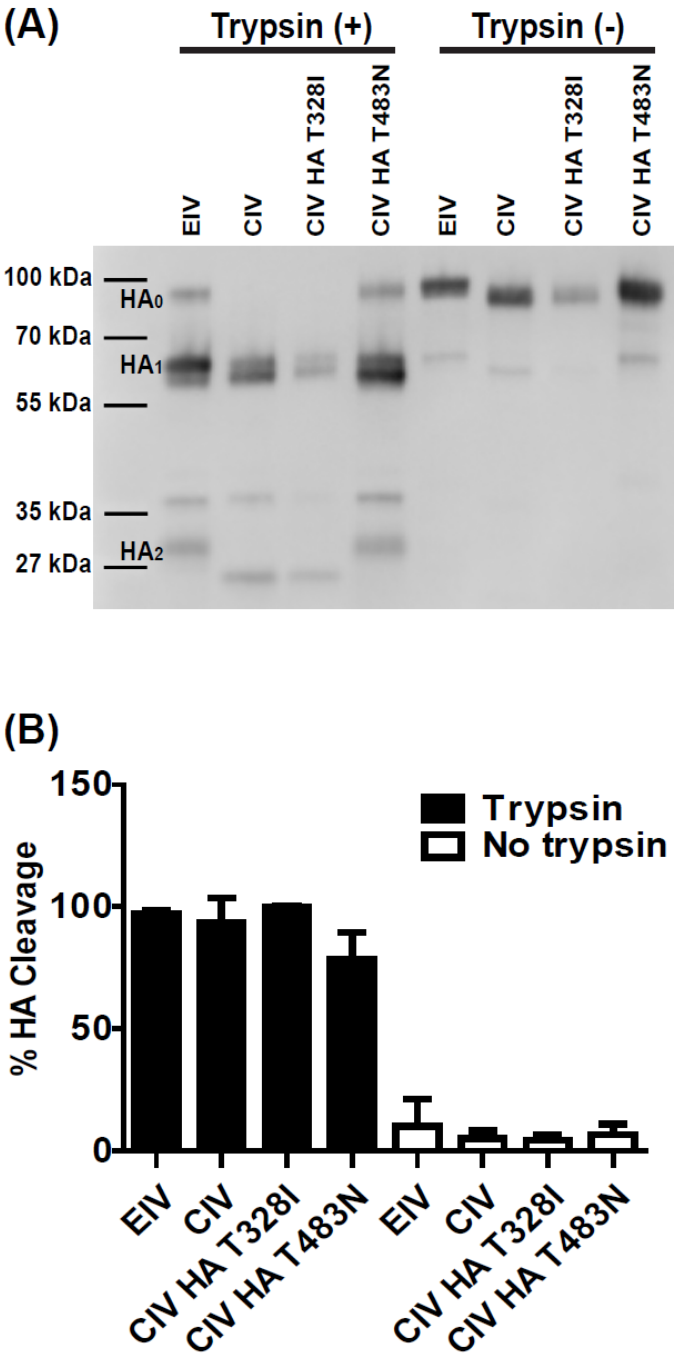

FIG 2.8. EIV and CIV HA cleavage assays. Both wild type HA proteins, CIV HA T328I, and CIV HA T483N were surface expressed and biotinylated. Western blot was used to look at HA_0 cleavage into HA_1 and HA_2 (A). Cleavage efficiency was determined by band density of the Western blots and following the formula: $(HA_1/HA_0 + HA_1) \times 100\%$ (B). Error bars represent the standard deviation of three independent experiments.

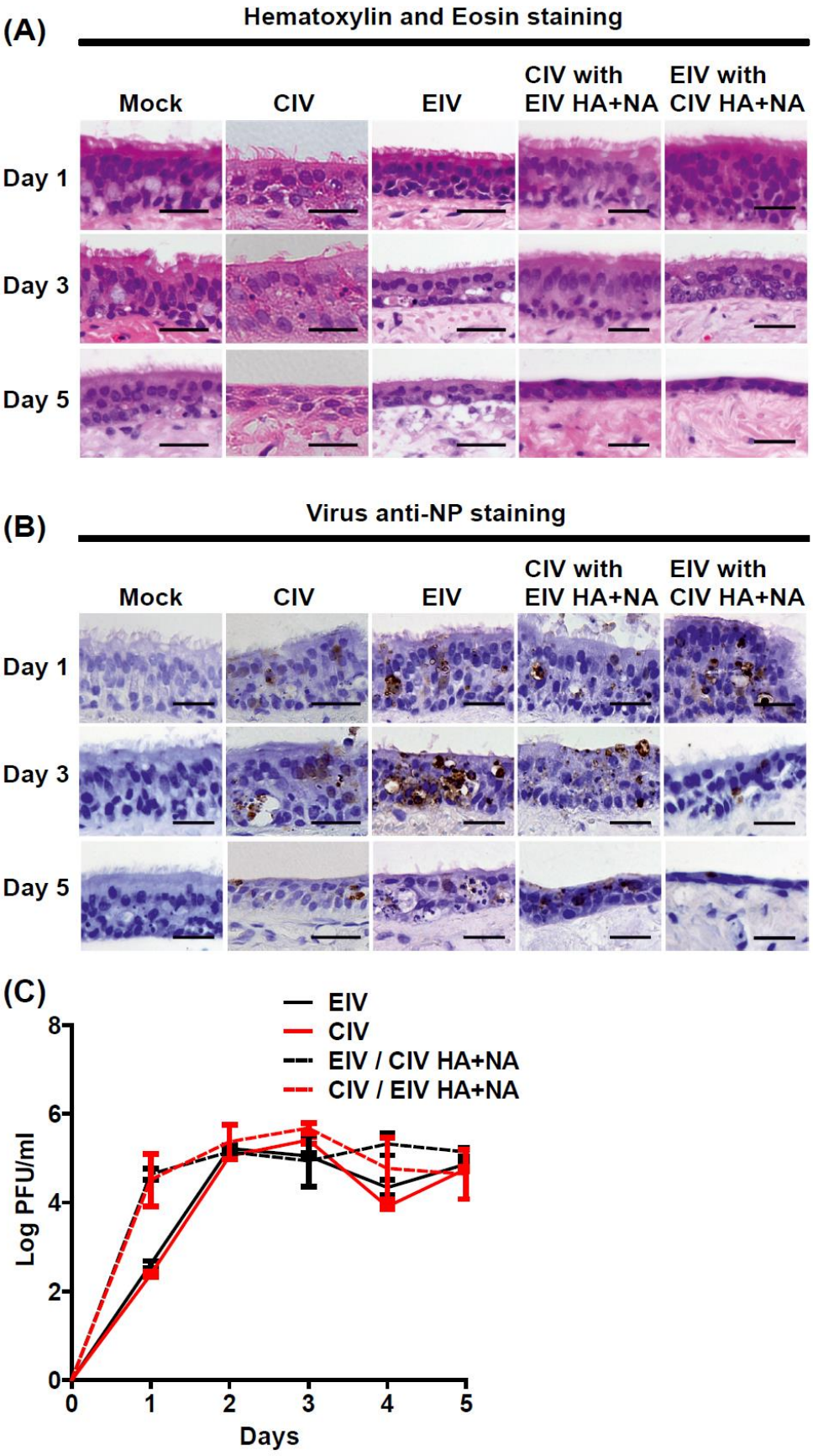
(A)
Hematoxylin and Eosin staining
Mock
CIV
EIV
CIV with EIV HA+NA
EIV with CIV HA+NA
Day 1
Day 3
Day 5
(B)
Virus anti-NP staining
Mock
CIV
EIV
CIV with EIV HA+NA
EIV with CIV HA+NA
Day 1
Day 3
Day 5
(C)
EIV
CIV
EIV / CIV HA+NA
CIV / EIV HA+NA
Log PFU/ml
0
2
4
6
8
0
1
2
3
4
5
Days

FIG 2.9. EIV, CIV, and reassortant virus infections of dog tracheal explants. Viruses (400 $TCID_{50}$ units) were used to inoculate dog tracheal explants, and tissues were collected for hematoxylin and eosin staining (A) and for anti-NP staining (B) at days 1, 3, and 5 post-infection. Virus growth was assayed every 24 h for 5 days by plaque assays in MDCK cells (C). Error bars represent the standard deviation of three independent experiments. Black scale bars represent 50 µm.

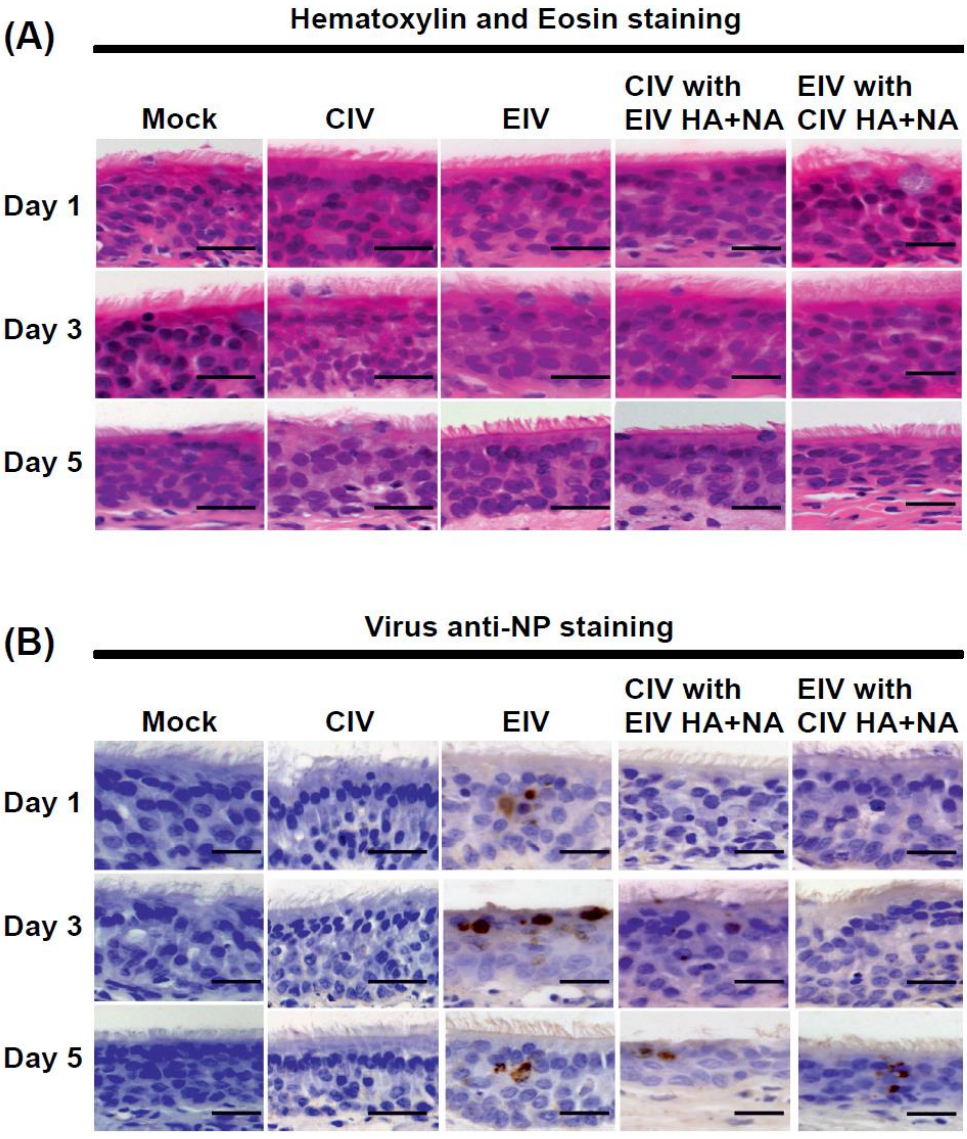
(A)
Hematoxylin and Eosin staining
Mock
CIV
EIV
CIV with EIV HA+NA
EIV with CIV HA+NA
Day 1
Day 3
Day 5
(B)
Virus anti-NP staining
Mock
CIV
EIV
CIV with EIV HA+NA
EIV with CIV HA+NA
Day 1
Day 3
Day 5
(C)
EIV
CIV
EIV / CIV HA+NA
CIV / EIV HA+NA
Log PFU/ml
10
8
6
4
2
0
0
1
2
3
4
5
Days

FIG 2.10. EIV, CIV, and reassortant virus infections of horse trachea explants. Viruses (400 $TCID_{50}$ units) were used to inoculate dog tracheal explants, and tissues were collected for hematoxylin and eosin staining (A) and for anti-NP staining (B) at days 1, 3, and 5 post-infection. Virus growth was assayed every 24 h for 5 days by plaque assays in MDCK cells (C). Black scale bars represent 50 µm.

II.VI DISCUSSION

Genetic differences in the EIV and CIV HA showed subtle differences in receptor specificity and cleavage efficiency. There are five "signature" mutations that distinguish CIV HA from EIV HA: N54K, N83S, W222L, I328T, and N483T (Fig. 2.2) (25, 62). The W222L mutation is located in the sialic acid receptor binding pocket, and studies have shown that HA mutations in the 220 loop can alter binding to α2-3- and α2-6 linked sialic acids (35, 49, 63). There is also evidence that the W222L mutation in CIV H3N2 allowed the virus to infect dogs more efficiently (64), such that the species jump and subsequent adaptation of EIV H3N8 in dogs may have been mediated by changes in receptor recognition. However, no infectivity differences were seen between the viruses (or the reassortants) when those were inoculated into CHO or into 6H4 cells. However, all viruses exhibited a 50% reduction in their ability to infect 6H4 cells relative to CHO cells (Fig. 2.7B). This suggests the viruses did not differ in their recognition of α2-3 and α2-6 linked sialic acids, and in fact may prefer α2-3 linked sialic acids for infections. Furthermore, there were no significant differences in infectivity among viruses based on the infection of various host cells, which further suggest minimal differences in receptor specificity (Fig. 2.4 and 2.5A). Furthermore, a recent study showed EIV and CIV bound strongly to α2-3 and not α2-6 linked sialic acids using sialic acid glycopolymers (62), consistent with our conclusion.

Binding of EIV and CIV HA-Fc proteins to glycan microarrays showed support for the CHO and 6H4 cells infection results. Indeed, both HA-Fc proteins bound strongly to α2-3 linked sialic acids in a similar pattern and neither protein bound to any α2-6 linked sialic acids (Fig. 2.7C). This preference towards binding to α2-3 (classical avian receptors) over α2-6 (classical human receptors) linked sialic acids suggests a potential human host restriction barrier for both viruses. Interestingly, there were differences in the relative binding preference to the various α2-3 linked glycans. For example, EIV HA-Fc showed higher binding to Neu5Acα3Galβ3GlcNAcβ3Galβ4GlcβR1 and Neu5Acα3Galβ4(Fucα3)GlcNAcβR1, while CIV

HA-Fc showed higher binding to Neu5Acα3Galβ4GlcNAc6SβR1 and Neu5Acα3GalβR1. These findings suggest there are subtle differences between EIV and CIV HA's ability to recognize specific sialic acids. Indeed, a recent study revealed the atomic structures of EIV and CIV HA to be nearly identical and both proteins preferred binding to α2-3 over α2-6 linked sialic acids, but there were subtle differences; CIV HA bound better to sulfated sialic acids compared to EIV HA (65). Small differences such as these may be important for understanding virus host adaptation and tropism.

Previous research has suggested that the signature HA I328T mutation, which is at the P2 position of the cleavage site, may influence influenza HA cleavage (25, 62). However, we mutated the CIV HA to the EIV background and it did not influence protein cleavage efficiency with and without trypsin compared to the wild type under the conditions of our assay (Fig. 2.8B). We also tested whether the signature HA N483T mutation, a glycosylation site that is close to the cleavage site in the HA structure, might influence cleavage by sterically hindering protease activity due to the presence of glycans. Changing the site in CIV to the EIV codon did not dramatically influence HA cleavage efficiency with and without trypsin (Fig. 2.8B). Overall, both EIV and CIV HA required trypsin activation and there were no major differences in efficiency of cleavage comparing the wild type with the mutant proteins. Different hosts have been shown to express different proteases that can cleave HA (66), and it is therefore possible that there are dog specific proteases that have selected the I328T and N483T mutations in CIV HA which allow for better replication, and testing dog specific proteases might provide further insight into any differences in cleavage efficiency.

Other genetic differences between EIV and CIV may play roles in host adaptation and tropism. Although many mutations documented here reside in known functional domains (Fig. 2.2), it is still unclear whether the specific mutations actually affect influenza host adaptation and replication, or contribute to any host-specific functions. Interestingly, most (8 of 9) of the mutations in PB1 occurred between early and more recent CIV sequences (Fig. 2.2),

whereas in the other proteins most mutations occurred between EIV and the earliest (2003 or 2004) CIV isolates (PB2, PA, HA), or there was no difference between EIV and the two CIV groups (NP, NA, M1, NS1) (Fig. 2.2). Whether mutations that occurred between early and more recent CIV isolates indicate adaptive changes to its canine host remain to be elucidated. Of the PB2 mutations, one (I292T) has been suggested to facilitate human H1N1 and H3N2 adaptation (60). Consequently, its possible role in dog adaptation needs to be considered. One mutation in PA (T400A) has also been reported to distinguish between human and avian influenza viruses. Human viruses almost always have a leucine at this site while avian viruses show either serine or proline (61), such that it may represent another host range determinant in CIV. In addition, we found EIV NS1 can be naturally truncated by 11 amino acids at the C terminus while CIV NS1 is never truncated, except for the first CIV isolate. A previous study showed the 2009 human pandemic H1N1 virus also had an 11 amino acid truncation which resulted in inefficient suppression of host genes, and extension of NS1 to full length restored its binding to host poly(A)-binding protein II which increased NS1 host gene suppression activity (67). As mentioned above, the mutation at the N terminus of PA could affect PA-X host gene suppression. Furthermore, both CIVs (H3N8 and H3N2) have a 20 amino acid truncation at the C terminus of PA-X implying that the truncation may be a host determinant in dogs (57). In fact, a comparison of human full length and truncated PA-X revealed a difference in gene suppression activity (58). Taken together, these genetic differences between EIV and CIV may be a consequence of dog-specific host pressures, including those generated by the innate and adaptive immune responses.

EIV and CIV did not show infectivity differences in various host cells. Despite the relatively high number of mutations observed in the virus genomes, both wild type and reassortant viruses grew similarly in dog (MDCK) cells, which are generally considered the most susceptible cells for influenza viruses (Fig. 2.3). Other mammalian cells tested showed differences in susceptibility to infection (Fig. 2.4). The reassortant viruses appeared similar to

the wild types, which was not surprising since the wild type viruses did not show any infectivity differences. Interestingly, the viruses infected human (A549) and horse (EQKD) cells poorly (Fig. 2.4, 2.5A). Previous results have indicated there is a host barrier for respiratory infection of CIV in horses (36, 62, 68, 69), so a low infectivity for CIV in EQKD cells may reflect a host difference that is also seen in kidney cells. However, EIV also infected EQKD cells poorly, so that the block in infection may be due to biological differences between horse kidney and airway respiratory cells. The poor infection of CIV in A549 cells was interesting because there has been no reported cases of CIV (or EIV) naturally transmitting to humans, including people who were regularly exposed to CIV infected dogs (70); whether there is a correlation between the poor infectivity in A549 cells and virus transmission to humans is unknown. Interestingly, the highly laboratory adapted PR8 strain was able to infect both A549 and EQKD cells to high levels compared to the horse and dog viruses (Fig. 2.5B). This suggests both cell types were permissive to influenza infection, and the limited infection from EIV and CIV was attributable to those specific viruses' biology. The poor infectivity in these cells could not be explained by sialic acid linkages because both A549 and EQKD cells expressed high levels of both α2-3 and α2-6 linked sialic acids (Fig. 2.6), similar to cells that were permissive to infection. Indeed, the infectivity differences between host cells was likely not due to differences in sialic acid binding because both EIV and CIV HA bound to sialic acid similarly as described above. The viruses were able to infect cat (NLFK) and ferret (Mpf) cells, although the infection in ferret cells was poorer (Fig. 2.4). Both cells stained positive predominately for α2-3 linked sialic acids (Fig. 2.6), and so this suggests the infectivity difference between NLFK and Mpf cells was likely not due to receptor differences. These results were interesting because ferrets are susceptible to influenza and are used as a model for human virus infection and transmission (71), and there is already evidence that CIV H3N2 can infect cats (72, 73). Additionally, a previous study showed that EIV replicated in the upper respiratory tract in live ferrets but was restricted in the lungs (74). There are no extended analysis or reports of CIV H3N8 infections in ferrets, but seroconversion has

been observed after inoculating ferrets (25). Interestingly, there is evidence of limited CIV H3N2 infection and transmission between ferrets in laboratory settings, but the virus could not transmit from dogs to ferrets (75, 76). Taken together, there is potential for EIV and CIV to infect other mammalian hosts and subsequently adapt.

EIV and CIV infection of tracheal explants revealed a host-specific barrier. To simulate the natural environment in which EIV and CIV cause infections, dog and horse tracheal explant cultures were prepared and used for virus infections. Overall, the results showed the viruses could infect dog trachea, and there were no major differences among the viruses with respect to damaging the epithelium layer or the location of virus antigens in the tracheal explants (Fig. 2.9). The reassortant viruses reached a greater titer (around 2 logs) relative to the wild types at 24 h post-infection, and this suggests the protein mismatching altered viral biology (Fig. 2.9C). However, the change was not dramatic overall because growth was very similar when comparing the viruses after 24 h post-infection; reaching peak titer between 48 and 72 h post-infection, similar to growth in MDCK cells (Fig. 2.3). In horse trachea all viruses caused some level of infection, but the physical damage of the epithelium was less pronounced compared to the infections of the dog trachea, which may be due to the horse trachea being more structurally robust (Fig. 2.10). Interestingly, EIV grew much better compared to CIV which further provides evidence of a host-specific barrier (Fig. 2.10C). Replacing the CIV HA and NA with EIV HA and NA allowed the virus to grow slightly better and stabilized (detected virus on days 3 and 5 post-infection) the virus compared to the wild type CIV. Furthermore, replacing the EIV HA and NA with CIV HA and NA attenuated virus growth by around 1-2 logs across 5 days compared to the wild type EIV. These findings suggest the difference in growth between EIV and CIV in horse trachea may be attributed to the mutations in the glycoproteins. Overall, the reassortant viruses grew similarly with respect to their wild type counterparts, and the experiment was carried out in one experimental replicate, and thus further experimentation using horse trachea and reassortant viruses is needed for validation. Also, the apparent

attenuation of growth of CIV and EIV with CIV glycoproteins in horse trachea may not have been caused by differences in α2-3 and α2-6 linked sialic acids compared to dog trachea as past studies have shown the existence of both in dog and horse tracheal tissues (77, 78). However, the distribution was shown to be different. While horse trachea epithelial cells stained positive for both sialic acids, dog trachea epithelial cells showed stronger staining for α2-3 linked sialic acids. Interestingly the lamina propria of dog trachea stained positive for both. However, it is important to note that sialic acid distribution, variety, and presence can vary depending on individual animals and their age (79).

Overall these results showed that despite six years of continuous evolution in dogs that separated the two viruses tested here, and the accumulation of mutations in all of the genomic segments such that EIV and CIV are clearly phylogenetically distinct (Fig. 2.1), there appeared to be minimal biological differences between them. We showed that the viruses infected various host cells and with no infectivity differences among the viruses, although different host cells exhibited varying degrees of permissiveness. We also showed that the viruses preferred α2-3 over α2-6 linked sialic acid receptors, and there may be subtle differences in receptor recognition. Virus inoculation in tracheal explants revealed limited CIV infectivity in horse trachea, and the restriction factors may reside in the receptor binding proteins. Notably, although EIV has circulated in many parts of the world since it emerged in 1963, CIV has not spread in a sustained fashion beyond North America, where it has been maintained mainly in large, high turnover, animal shelters. Given the scarce phenotypic differences between EIV and CIV, the relatively limited spread of CIV among the domestic dog population may reflect a lack of epidemiological contacts rather than constraints on viral fitness. This suggests that inter-species transmission and adaptation of influenza virus in this case is mediated by subtle factors.

II.VII ACKNOWLEDGMENTS

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CHAPTER III

Comparing the functions of equine and canine influenza H3N8 virus PA-X proteins: suppression of reporter gene expression and modulation of global host gene expression

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III.I PREFACE

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Author contributions are as follows:

Kurtis H. Feng – Lead author, conducted the vast majority of experiments and data analysis, wrote and edited the original manuscript for submission to Virology

Miao Sun – Main contributor of the RNA sequencing analysis

Sho Iketani – Assisted in PA-X plasmid construction and mutagenesis

Edward C. Holmes – Principal investigator, assisted manuscript revisions

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III.II ABSTRACT

The influenza PA-X protein is translated from the PA open reading frame from frameshifting and suppresses cellular gene expression due to its ribonuclease activity. We further defined the functional roles of PA-X by comparing PA-X proteins from two related viruses – equine influenza (EIV) and canine influenza (CIV) H3N8 – that differ in a C-terminal truncation and internal mutations. *In vitro* reporter gene assays revealed that both proteins were able to suppress gene expression. Interestingly, EIV PA-X demonstrated ~50% greater activity compared to CIV PA-X, and we identified the mutations that caused this difference. We used RNA-seq to evaluate the effects of PA-X on host gene expression after transfection into cultured cells. There were no significant differences in this property between EIV and CIV PA-X proteins, but expression of either resulted in the up-regulation of genes when compared to controls, most notably immunity-related proteins, trafficking proteins, and transcription factors.

III.III INTRODUCTION

The segmented influenza A virus (IAV) genome encodes 9 structural proteins and 3 non-structural proteins, although it potentially encodes several other proteins (1). One non-structural protein, termed PA-X, was first identified in 2012 and is derived from the PA segment. It shares the first 191 amino acids with the PA protein, but has a unique C-terminal region derived from a +1 frameshift during translation (2, 3). Two natural variants of PA-X exist; the more common form has a length of 252 amino acids, while a C-terminally truncated 232 amino acid variant is found in H3N8 and H3N2 canine influenza viruses, the human H1N1/09 pandemic virus, and in some subtypes of swine influenza virus (3). Interestingly, it was recently shown that the PA-X C-terminal truncation in swine influenza may play a role in viral adaptation in pigs (4).

Several studies characterizing the infections of mutant viruses that expressed varying levels of PA-X showed that it modulated the host immune response, virus pathogenicity, and virus growth both *in vitro* and *in vivo* (5-9). Interestingly, the biological impact of the protein was dependent on the virus subtype and the host. For example, no difference in virus replication was observed comparing the wild type 1918 H1N1 and PA-X deficient virus infections in mice (2), while PA-X deficient HPAIV H5N1 reached higher titers in cell cultures and in mice compared to the wild type (6). Furthermore, it was shown that wild type 2009 human H1N1 virus replicated better in human respiratory cells compared to a mutant that expressed lower levels of PA-X (8). In addition to influencing virus replication, PA-X suppresses the host immune response by degrading host transcripts by its ribonuclease activity. For instance, loss of PA-X resulted in a stronger inflammatory response in mice, chickens, and ducks (2, 6, 7), an increased expression of IFN-β in mice (8), as well as greater virulence in the animal models. The ribonuclease activity of PA-X was demonstrated by its ability to suppress reporter gene expression and confirmed by incubating RNA substrates with purified PA-X protein (10). More recently it was shown that this ribonuclease activity was specific for host transcripts generated from Pol II while ignoring products from Pol I and Pol III (11). This ribonuclease domain has been attributed to the N-

terminal region, although several recent publications have shown that the C-terminal region may also be important in regulating ribonuclease activity (10, 12, 13). Interestingly, PA-X has also been shown to modulate other virus-host interactions such as preventing stress granule formation (and thereby preventing translational arrest), increasing the accumulation of poly(A)-binding proteins within the nucleus, and exhibiting anti-apoptotic activity (5, 6, 14).

The equine influenza (EIV) and canine influenza (CIV) H3N8 PA-X proteins differ in both length and sequence. We therefore sought to examine these proteins for functional differences by comparing gene suppression ability using reporter gene assays and by evaluating the details of their influence on host gene expression through RNA sequencing (RNA-seq). CIV emerged from EIV around 2000, and since then it has fixed a unique set of mutations in all its genes, including PA-X (15). By evaluating the biology of the two PA-X proteins we sought to further define PA-X function and to gain a better understanding of virus evolution and adaptation.

III.IV MATERIALS AND METHODS

Cells and cell culture. HEK293 human cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2.

PA-X protein sequence analysis. EIV and CIV PA-X protein sequences were compared using sequences of virus isolates deposited in GenBank (NCBI). In this context a "consensus" sequence is used to define the most common amino acid at each position for each EIV and CIV when comparing multiple protein sequences. We utilized EIV isolates sampled close to the ancestor CIV, starting from 1990 to 2013, and CIV isolates sampled soon after the emergence in dogs, from 2004 to 2013. Sequence alignments and the generation of consensus sequences were performed as described previously (15).

Plasmids and cloning. EIV and CIV PA-X genes were cloned into a mammalian expression plasmid, pcDNA3.1(-), and also into a T7 promoter plasmid, pT7CFE1-Chis (Thermo Fisher Scientific) as described previously (2). The EIV NP gene was also cloned into

pcDNA3.1(-) as a control. The genes were derived from strains A/equine/NY/61191/2003 and A/canine/NY/dog23/2009 (15), and the sequences were checked to ensure they matched the consensus. A mammalian plasmid expressing green fluorescent protein (GFP) under the control of the CMV promoter (pCAGGS-GFP) was kindly provided by Dr. Luis Martinez-Sobrido, and a mammalian plasmid expressing β-galactosidase (pSV-β-Galactosidase) was purchased from Promega.

Site-directed mutagenesis. Custom primers were constructed and used in Phusion (New England Biolabs) PCR mutagenesis following the commercial protocol. All mutagenesis of EIV and CIV PA-X were conducted in the pcDNA3.1(-) background. Single mutations in the EIV PA-X background included: D27N, D108A, S231F, and a 20 amino acid C-terminal truncation. Single mutations in the CIV PA-X background included: N27D, D108A, F231S, and a 20 amino acid C-terminal elongation. Combinations of mutations were made in the CIV PA-X background as well: N27D F231S, N27D with the C-terminal elongation, F231S with the C-terminal elongation, and N27D F231S with the C-terminal elongation. Based on the sequences, EIV PA-X wild type and CIV PA-X N27D F231S with the C-terminal elongation were identical. Similarly, EIV PA-X with the C-terminal truncation and CIV PA-X N27D F231S also had the same sequences. Lastly, EIV PA-X D27N and EIV PA-X S231F had the same sequences as CIV PA-X F231S with the C-terminal elongation and CIV PA-X N27D with the C-terminal elongation, respectively.

***In vitro* translation (IVT).** Both EIV and CIV PA-X were translated by *in vitro* translation using the 1-Step Human Coupled IVT Kit (Thermo Fisher Scientific). After translation samples were denatured and separated on a 10% SDS-PAGE gel by electrophoresis. The proteins were transferred onto a nitrocellulose membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). After transfer, the membrane was blocked in 5% nonfat dried milk overnight on a shaker at 4°C. The next day the membrane was incubated with an affinity purified rabbit IgG anti-PA-X peptide antibody (Pacific Immunology) for 1 h on a shaker. This antibody was custom produced

using the PA-X peptide sequence VSPREAKRQLKKDLKSQG (2). Next, the membrane was washed thoroughly using PBST and incubated with a goat IgG anti-rabbit HRP conjugated antibody (Jackson ImmunoResearch Laboratories, Inc) for 1 h on a shaker. The membrane was washed thoroughly using PBST and incubated with SuperSignal (Thermo Fisher Scientific) chemiluminescent substrate for 3 min. Protein bands were visualized using the ChemiDoc MP System (Bio-Rad).

β-galactosidase reporter assay. HEK293 cells were seeded in 24 well plates. Upon reaching 90% confluency cells were co-transfected with 400 ng of β-galactosidase and 4 – 400 ng of effector plasmid. The total concentration of DNA was 800 ng for each sample; empty pcDNA3.1(-) was used to standardize the concentration when necessary. Transfection cocktails were prepared in 100 µl of OPTI-MEM (Thermo Fisher Scientific) mixed with 3 µl of TransIT – 293T transfection reagent (Mirus Bio LLC) following the commercial protocol. After 48 h post-transfection cells were harvested and centrifuged at max speed for 5 min. The supernatant was decanted for each sample and cell pellets were lysed using 100 µl radioimmune precipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with protease inhibitors (Roche). Cell lysates were then centrifuged at max speed for 15 min to pellet down cell debris. The supernatant was collected and each sample was incubated with 100 µl of ortho-Nitrophenyl-β-galactoside (ONPG) substrate (Thermo Fisher Scientific) in Nunc MaxiSorp flat-bottom 96 well plates (eBioscience). A Tecan microplate reader was used to measure absorbance at 415 nm.

GFP reporter assay. HEK293 cells were seeded in 24 well plates. Upon reaching 90% confluency cells were co-transfected with 400 ng of GFP and 400 ng of effector plasmid. The transfection cocktails were prepared as described above. After 48 h post-transfection cells were viewed by a Nikon TE300 fluorescent microscope. Next, cells were resuspended in 0.5% BSA and assayed by flow cytometry following the commercial protocol using the Millipore Guava EasyCyte plus flow cytometer. The mean fluorescent intensity (MFI) was calculated using FlowJo (TreeStar) software.

RNA sequencing (RNA-seq). HEK293 cells were seeded in 24 well plates. Upon reaching 90% confluency the cells were transfected with 400 ng of effector plasmid. The transfection cocktails were prepared as described above. After 48 h cells were harvested and total RNA was extracted from each sample using an RNeasy Mini Kit (Qiagen). On-column DNase digestion was performed to remove genomic DNA contamination. Purified RNA samples were analyzed for quality at the Cornell University Institute of Biotechnology, and library construction and subsequent sequencing of RNA samples were performed by the Cornell University RNA Sequencing Core (RSC).

RNA-seq data analysis. RNA-seq reads were mapped to the human genome (hg19) using STAR aligner (version 2.4.2). FeatureCounts (version 1.5.0) was used to count the raw number of reads covering each gene, using the GTF file downloaded from the UCSC table browser (hg19 refGene). We then called differentially expressed genes from the EIV and CIV PA-X transfected samples against all other control samples (cells transfected with the pcDNA3.1 empty vector, or expressing GFP or NP) using the exact test in the edgeR software package, applying a FDR cutoff of 0.05, and a fold-change cutoff of at least 2. The resulting list of genes was plotted in a heatmap using the gplots software package.

III.V RESULTS

Genetic analysis and the expression of EIV and CIV PA-X. Aligning the EIV and CIV PA-X consensus sequences revealed three differences (Fig. 3.1A). Two of the differences were point mutations at amino acid positions 27 and 231. Specifically, EIV PA-X had an Asp and Ser at those positions, while CIV PA-X possessed an Asn and Phe, respectively. The third difference was that CIV PA-X had a 20 amino acid truncation at the C-terminus compared to the full-length variant of EIV PA-X. Expression of the PA-X genes by IVT yielded protein bands around 20 kDa; the CIV PA-X band ran slightly faster than the EIV PA-X band most likely due to its truncation (Fig. 3.1B). We used IVT because other methods of detection were not able to

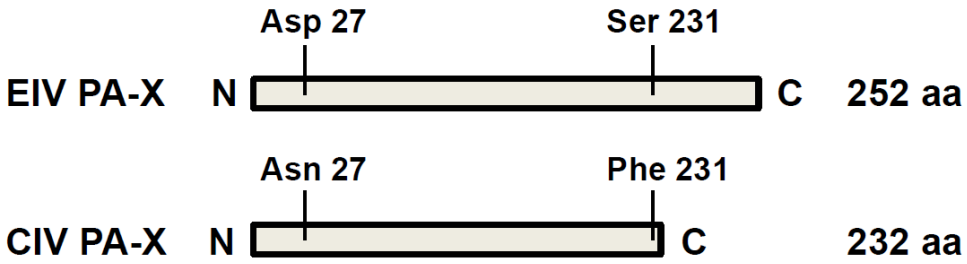
detect the protein (data not shown), in accordance with previous reports (2). Another study suggested that PA-X is difficult to detect due to strong self-suppression (13). Co-expression of β-galactosidase and PA-X in cell culture showed that both EIV and CIV PA-X were able to suppress gene expression in a dose dependent manner (Fig. 3.1C). At 400 ng there was no significant difference in β-galactosidase activity ($p>0.05$) comparing EIV and CIV PA-X transfected cells; however, as less PA-X DNA was used for transfections a consistent trend appeared: EIV PA-X had significantly ($p<0.05$) stronger suppression ability compared to CIV PA-X. The difference between the two grew larger as the concentration of DNA decreased from 40 – 4 ng (Fig. 3.1C). This difference was confirmed by flow cytometry (Fig. 3.2A) and microscopy (Fig. 3.2B) based on co-expression of PA-X and GFP. D108A mutants were tested as controls to ensure the ribonuclease domain resided at the N-terminus as previously described (2). Both mutants showed no gene suppression and thus, along with the protein bands shown by IVT (Fig. 3.1B), confirmed both EIV and CIV PA-X were properly expressed.

EIV and CIV PA-X site-directed mutagenesis. EIV and CIV PA-X mutants were created to identify the PA-X sequences associated with the difference in gene suppression activity. EIV PA-X D27N did not change its phenotype to resemble CIV PA-X. However, both S231F and its 20 amino acid C-terminal truncation mutants showed weaker suppression of GFP, similar to that seen for CIV PA-X based on flow cytometry (Fig. 3.3A) and microscopy (Fig. 3.3B). Next, reciprocal mutations were made in the CIV PA-X background: N27D, F231S, and a 20 amino acid C-terminal elongation mutant to match EIV PA-X. Interestingly, none of the three mutants resulted in a stronger GFP gene suppression phenotype compared to EIV PA-X based on flow cytometry (Fig. 3.4A) and microscopy (Fig. 3.4B). This suggested a combination of mutations must be required in the CIV PA-X background to change its phenotype to match EIV PA-X's. All possible combinations were created and results showed two mutants had phenotypes like EIV PA-X's based on flow cytometry (Fig. 3.5A) and microscopy (Fig. 3.5B):

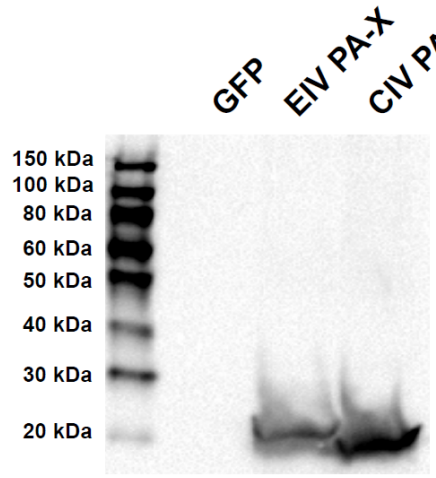
one had all three EIV PA-X mutations and the other construct carried two out of three mutations, F231S and the elongated C-terminus.

EIV and CIV PA-X's effects on host gene expression. RNA-seq analysis identified genes that were differentially expressed ($p<0.001$) comparing the controls (pcDNA3.1, GFP, NP) to the EIV and CIV PA-X samples, and this was organized into a heatmap (Fig. 3.6). Each gene was further analyzed using the NCBI database and notable/related genes were extracted from the heatmap and tabulated (Table 3.1). These genes showed higher levels compared to the controls and encoded proteins related to the immune response, ER-golgi trafficking, transcription. All down-regulated genes relative to the controls were not included in the table because of inconsistent levels of expression and variation within the same sample category, and because several of these gene products have not been characterized (Fig. 3.6). When comparing the EIV and CIV PA-X samples alone, there were no statistically significant differences ($p>0.05$) in detected gene expression.

(A)


Asp 27
Ser 231
EIV PA-X
N
C
252 aa
Asn 27
Phe 231
CIV PA-X
N
C
232 aa

(B)


GFP
EIV PA-X
CIV PA-X
150 kDa
100 kDa
80 kDa
60 kDa
50 kDa
40 kDa
30 kDa
20 kDa

(C)

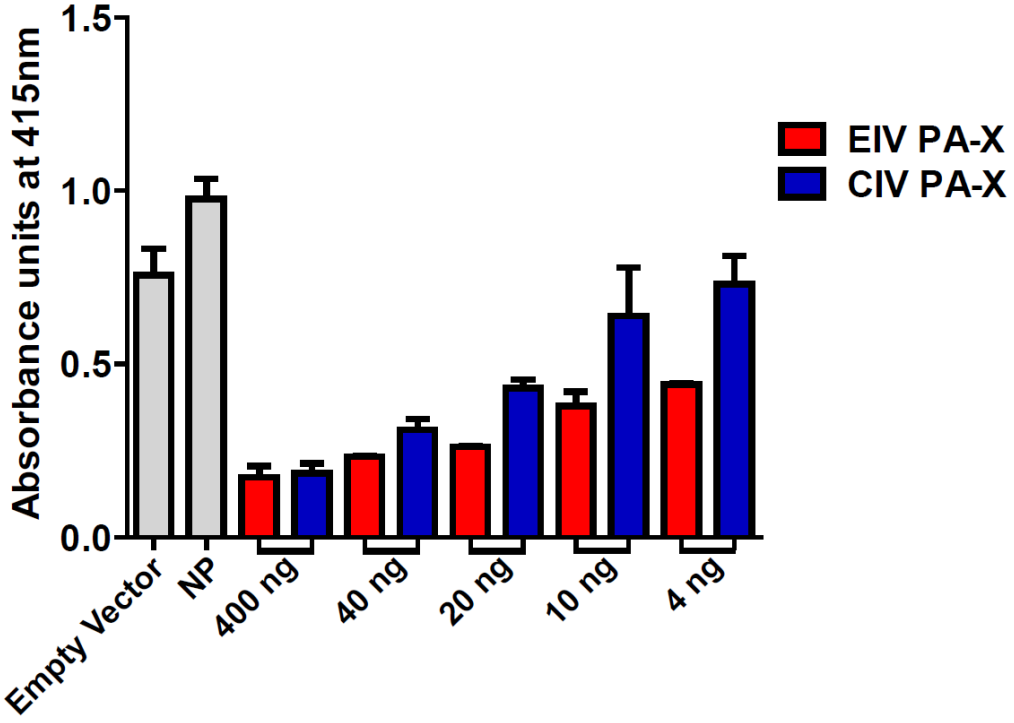

Absorbance units at 415nm
1.5
1.0
0.5
0.0
EIV PA-X
CIV PA-X
Empty Vector
NP
400 ng
40 ng
20 ng
10 ng
4 ng

FIG 3.1. Genetic comparison and expression of EIV and CIV PA-X in cell culture. (A) Comparison of EIV and CIV PA-X consensus protein sequences revealed three differences, two point mutations and one deletion. (B) Expression of PA-X was detected after IVT by Western blotting using a rabbit anti-PA-X peptide antibody. (C) EIV and CIV PA-X gene suppression ability was assayed by β-galactosidase reporter assay in a dose-dependent manner. Error bars represented the standard deviation of three independent experiments.

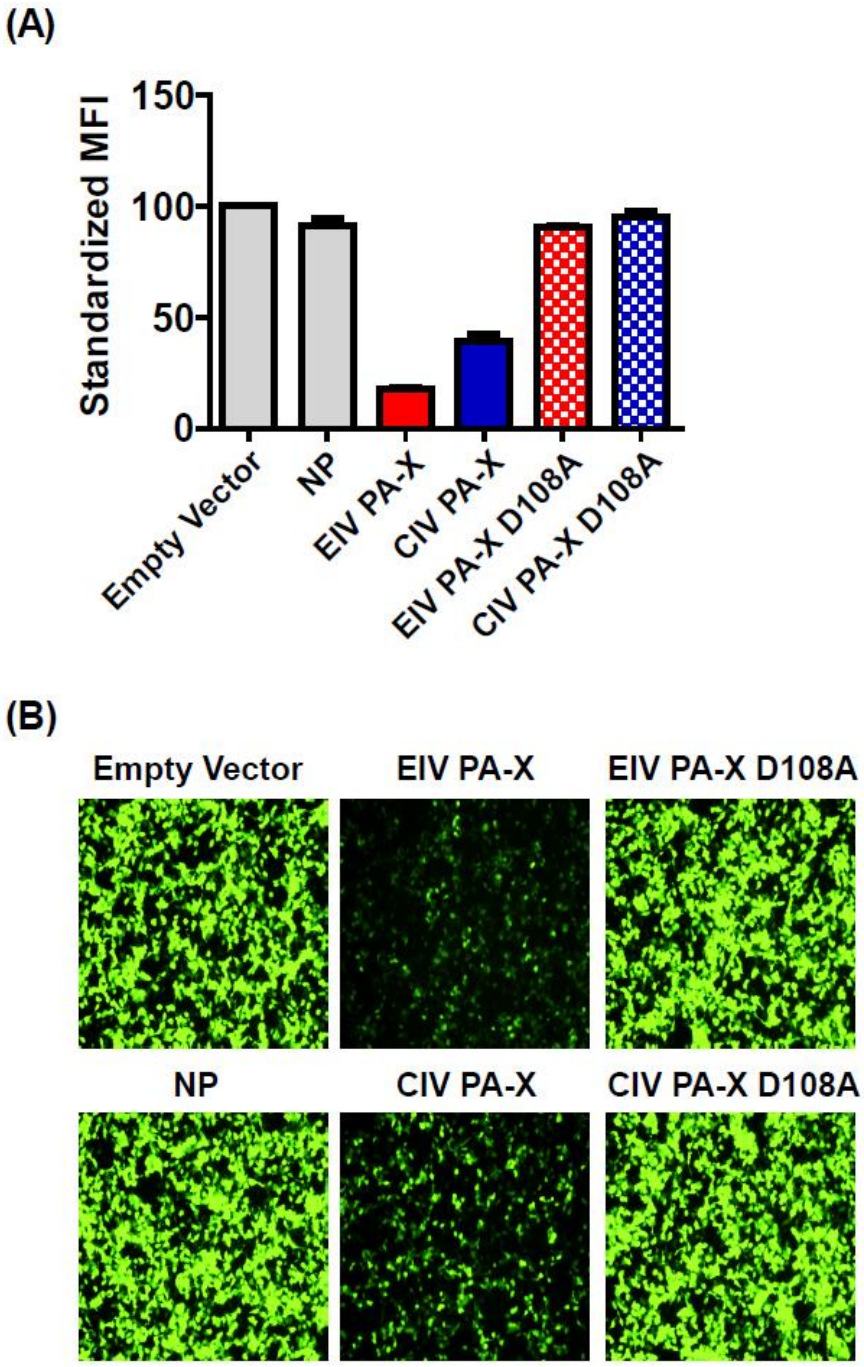


FIG 3.2. EIV and CIV PA-X GFP reporter assay. Wild types and PA-X D108A mutants were assayed for their ability to suppress GFP expression in cell culture. Results were shown by comparing (A) fluorescent intensity and (B) by microscopy. In (A) the fluorescent intensity was standardized to the empty vector transfected control cells, and error bars represented the standard deviation of three independent experiments.

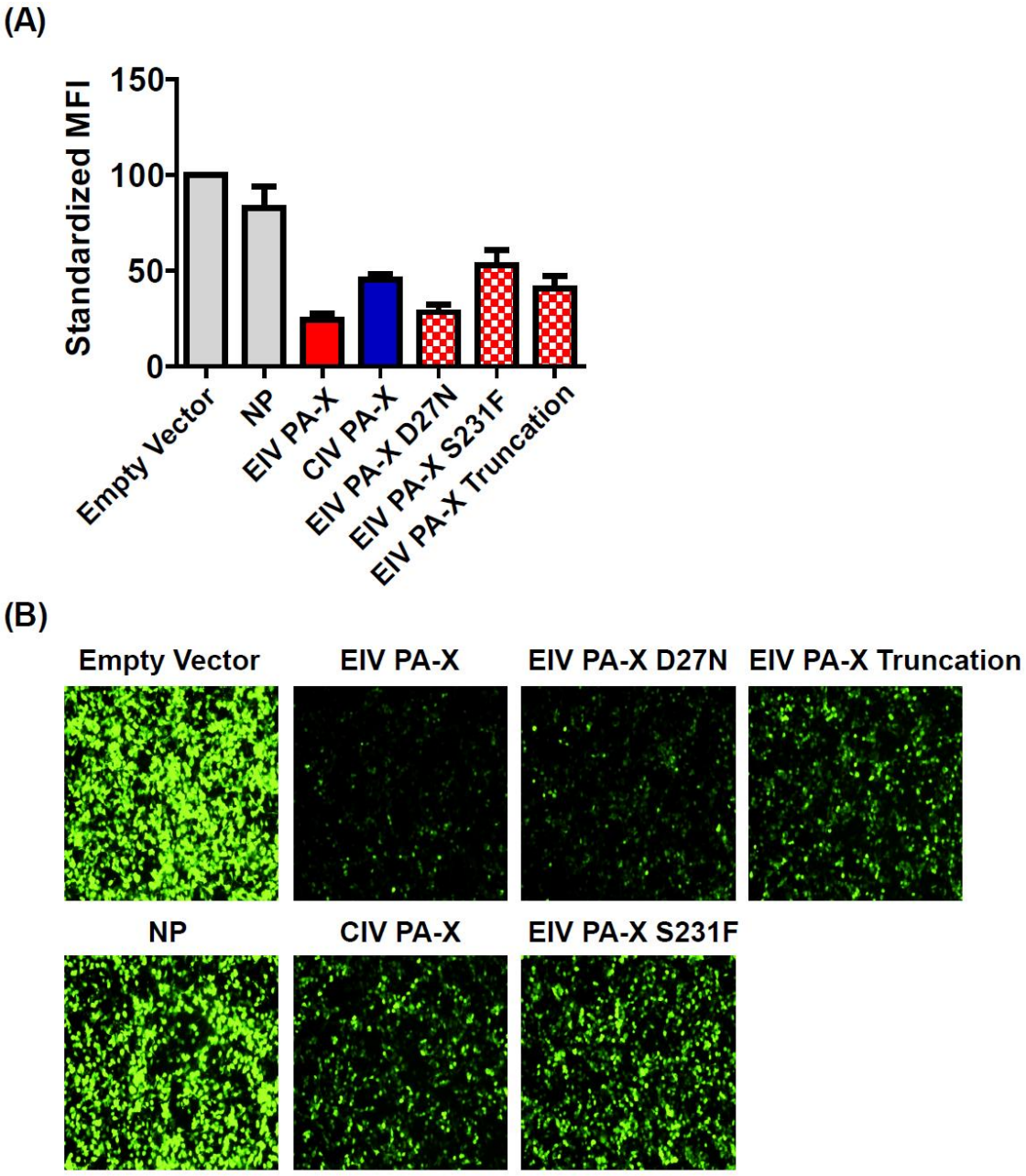


FIG 3.3. EIV PA-X mutants GFP reporter assay. EIV PA-X mutants were made based on the differences in CIV PA-X. The mutants were assayed for their ability to suppress GFP expression in cell culture. Results were shown by comparing (A) fluorescent intensity and (B) by microscopy (B). In (A) the fluorescent intensity was standardized to the empty vector transfected control cells, and error bars represented the standard deviation of three independent experiments.

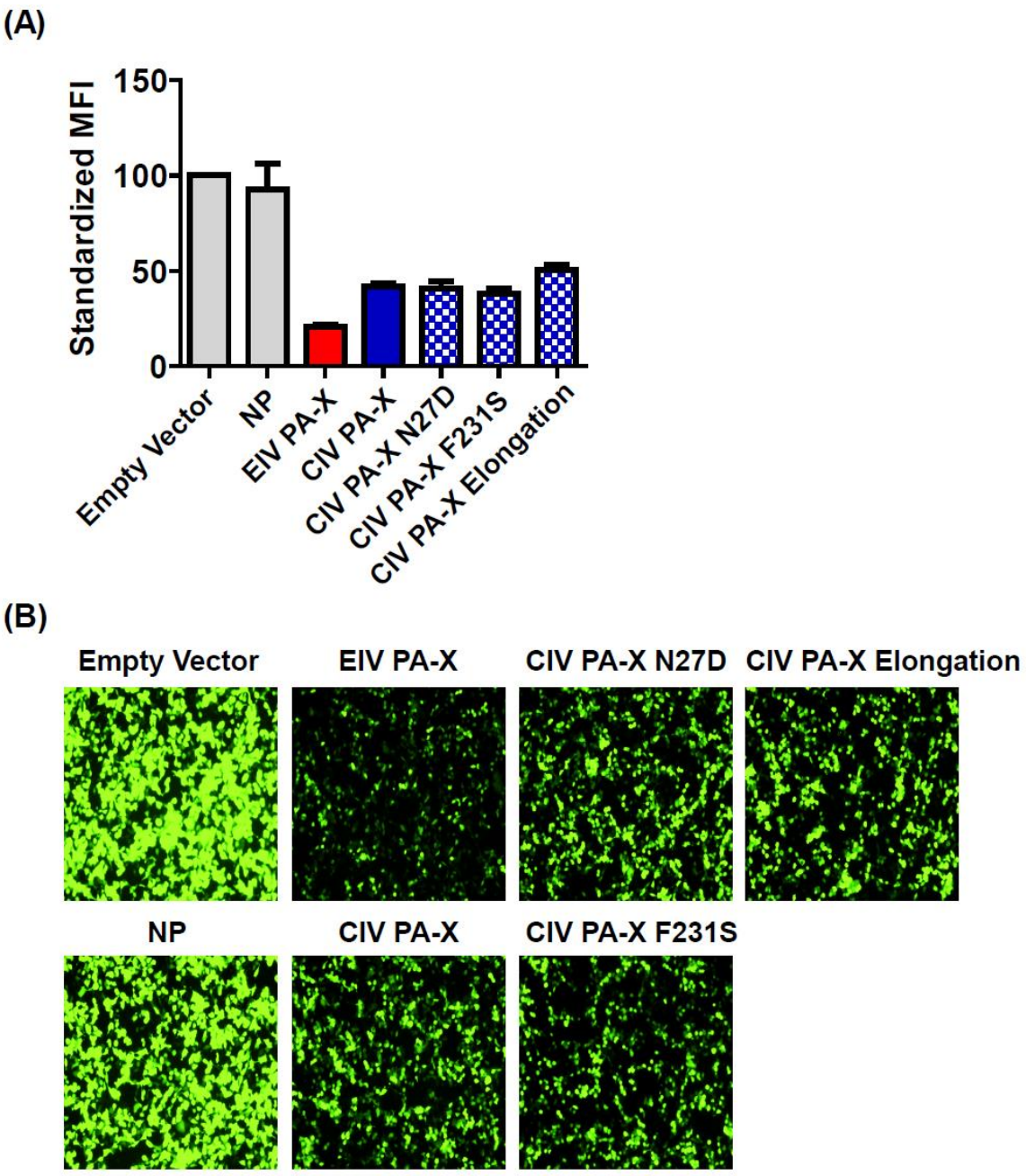


FIG 3.4. CIV PA-X mutants GFP reporter assay. Reciprocal CIV PA-X mutants were made based on the EIV PA-X mutants. The mutants were assayed for their ability to suppress GFP expression in cell culture. Results were shown by comparing (A) fluorescent intensity and (B) by microscopy. In (A) the fluorescent intensity was standardized to the empty vector transfected control cells, and error bars represented the standard deviation of three independent experiments.

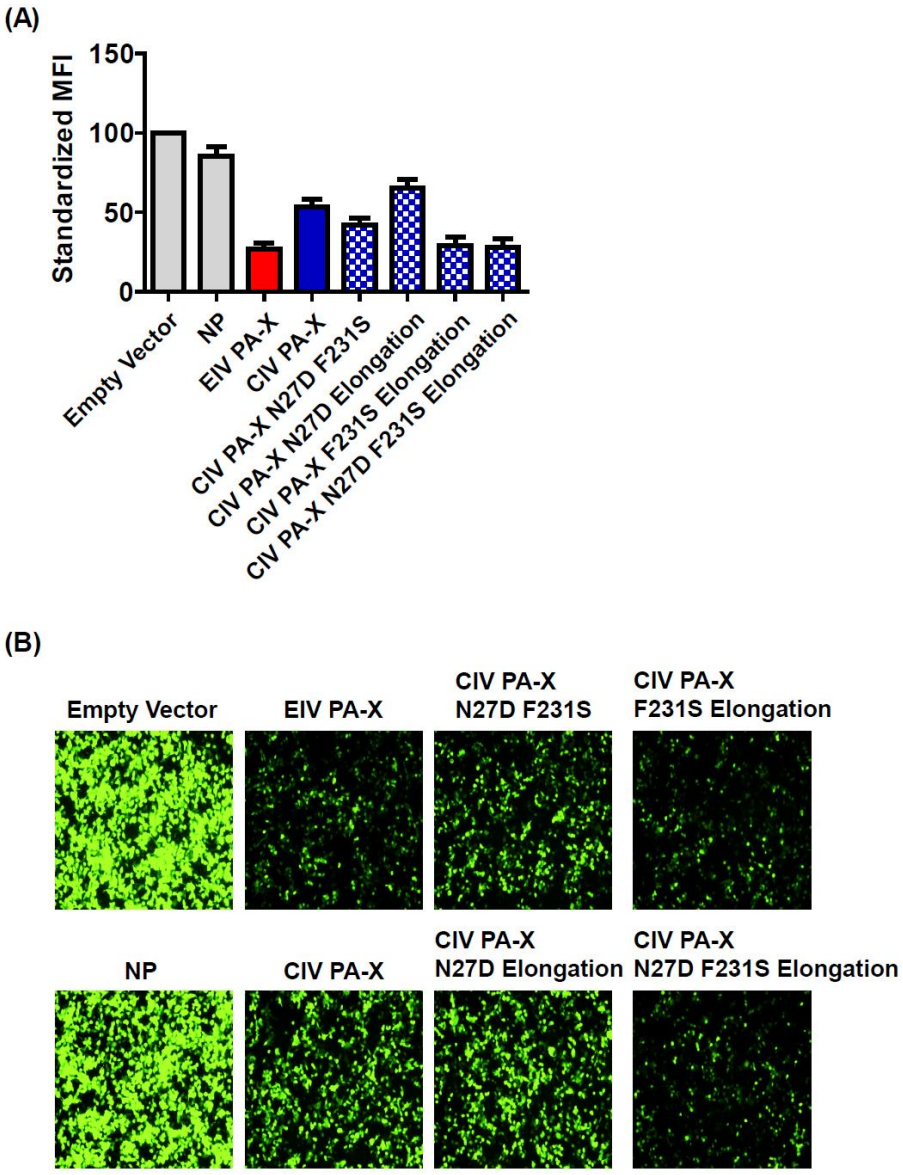


FIG 3.5. Multiple CIV PA-X mutants GFP reporter assay. All possible CIV PA-X mutant combinations were made to identify the key changes that resulted in the gene suppression phenotype difference comparing EIV and CIV PA-X. The mutants were assayed for their ability to suppress GFP expression in cell culture. Results were shown by comparing (A) fluorescent intensity and (B) by microscopy (B). In (A) the fluorescent intensity was standardized to the empty vector transfected control cells, and error bars represented the standard deviation of three independent experiments.

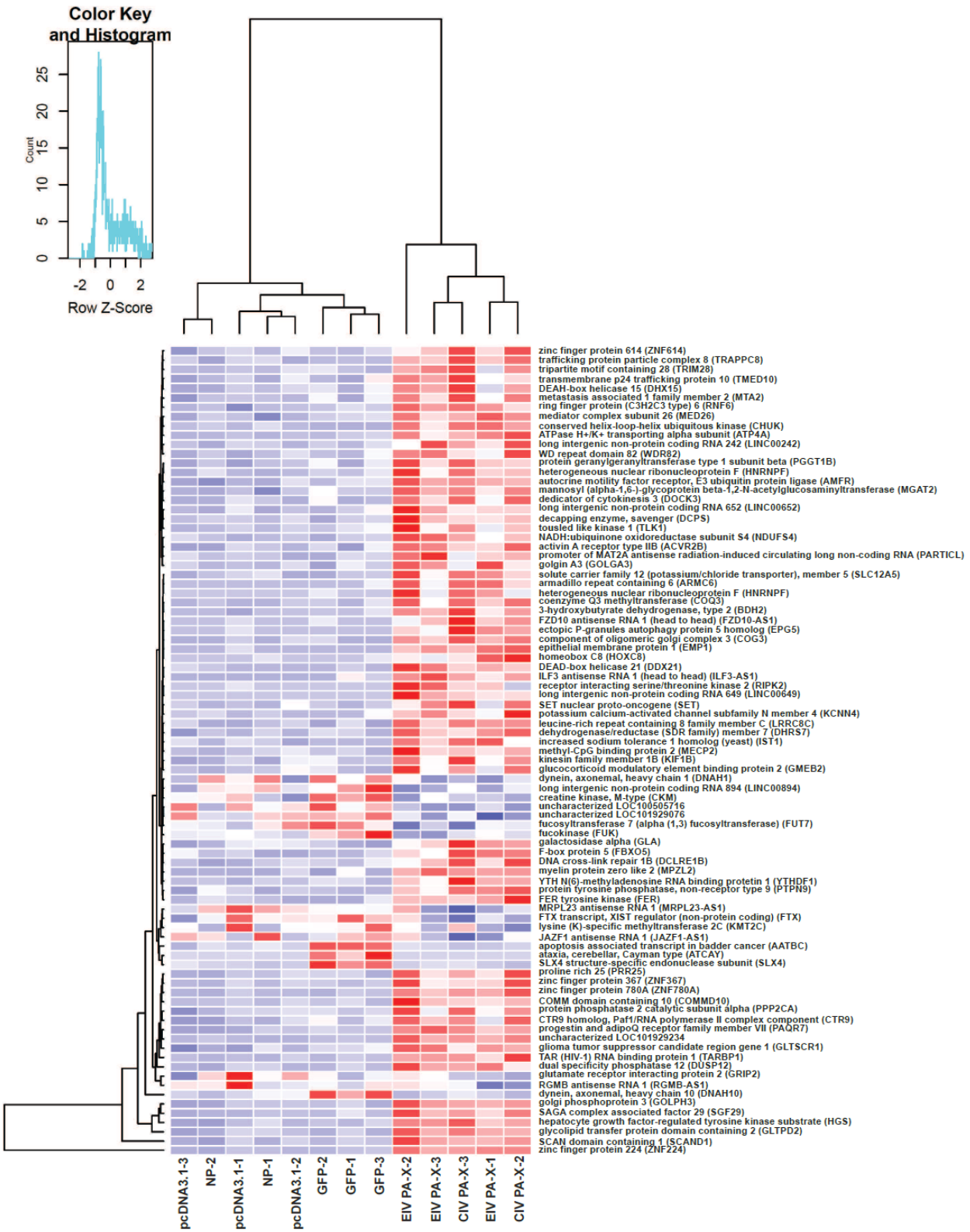

Color Key
and Histogram
Count
0
5
10
15
20
25
-2
0
2
Row Z-Score
zinc finger protein 614 (ZNF614)
trafficking protein particle complex 8 (TRAPPC8)
tripartite motif containing 28 (TRIM28)
transmembrane p24 trafficking protein 10 (TMED10)
DEAH-box helicase 15 (DHX15)
metastasis associated 1 family member 2 (MTA2)
ring finger protein (C3H2C3 type) 6 (RNF6)
mediator complex subunit 26 (MED26)
conserved helix-loop-helix ubiquitous kinase (CHUK)
ATPase H+/K+ transporting alpha subunit (ATP4A)
long intergenic non-protein coding RNA 242 (LINC00242)
WD repeat domain 82 (WDR82)
protein geranylgeranyltransferase type 1 subunit beta (PGGT1B)
heterogeneous nuclear ribonucleoprotein F (HNRNPF)
autocrine motility factor receptor, E3 ubiquitin protein ligase (AMFR)
mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (MGAT2)
dedicator of cytokinesis 3 (DOCK3)
long intergenic non-protein coding RNA 652 (LINC00652)
decapping enzyme, savenger (DCPS)
tousled like kinase 1 (TLK1)
NADH:ubiquinone oxidoreductase subunit S4 (NDUFS4)
activin A receptor type IIB (ACVR2B)
promoter of MAT2A antisense radiation-induced circulating long non-coding RNA (PARTICL)
golgin A3 (GOLGA3)
solute carrier family 12 (potassium/chloride transporter), member 5 (SLC12A5)
armadillo repeat containing 6 (ARMC6)
heterogeneous nuclear ribonucleoprotein F (HNRNPF)
coenzyme Q3 methyltransferase (COQ3)
3-hydroxybutyrate dehydrogenase, type 2 (BDH2)
FZD10 antisense RNA 1 (head to head) (FZD10-AS1)
ectopic P-granules autophagy protein 5 homolog (EPG5)
component of oligomeric golgi complex 3 (COG3)
epithelial membrane protein 1 (EMP1)
homeobox C8 (HOXC8)
DEAD-box helicase 21 (DDX21)
ILF3 antisense RNA 1 (head to head) (ILF3-AS1)
receptor interacting serine/threonine kinase 2 (RIPK2)
long intergenic non-protein coding RNA 649 (LINC00649)
SET nuclear proto-oncogene (SET)
potassium calcium-activated channel subfamily N member 4 (KCNN4)
leucine-rich repeat containing 8 family member C (LRRC8C)
dehydrogenase/reductase (SDR family) member 7 (DHRS7)
increased sodium tolerance 1 homolog (yeast) (IST1)
methyl-CpG binding protein 2 (MECP2)
kinesin family member 1B (KIF1B)
glucocorticoid modulatory element binding protein 2 (GMEB2)
dynein, axonemal, heavy chain 1 (DNAH1)
long intergenic non-protein coding RNA 894 (LINC00894)
creatine kinase, M-type (CKM)
uncharacterized LOC100505716
uncharacterized LOC101929076
fucosyltransferase 7 (alpha (1,3) fucosyltransferase) (FUT7)
fucokinase (FUK)
galactosidase alpha (GLA)
F-box protein 5 (FBXO5)
DNA cross-link repair 1B (DCLRE1B)
myelin protein zero like 2 (MPZL2)
YTH N(6)-methyladenosine RNA binding protein 1 (YTHDF1)
protein tyrosine phosphatase, non-receptor type 9 (PTPN9)
FER tyrosine kinase (FER)
MRPL23 antisense RNA 1 (MRPL23-AS1)
FTX transcript, XIST regulator (non-protein coding) (FTX)
lysine (K)-specific methyltransferase 2C (KMT2C)
JAZF1 antisense RNA 1 (JAZF1-AS1)
apoptosis associated transcript in badder cancer (AATBC)
ataxia, cerebellar, Cayman type (ATCAY)
SLX4 structure-specific endonuclease subunit (SLX4)
proline rich 25 (PRR25)
zinc finger protein 367 (ZNF367)
zinc finger protein 780A (ZNF780A)
COMM domain containing 10 (COMMD10)
protein phosphatase 2 catalytic subunit alpha (PPP2CA)
CTR9 homolog, Paf1/RNA polymerase II complex component (CTR9)
progestin and adipoQ receptor family member VII (PAQR7)
uncharacterized LOC101929234
glioma tumor suppressor candidate region gene 1 (GLTSCR1)
TAR (HIV-1) RNA binding protein 1 (TARBP1)
dual specificity phosphatase 12 (DUSP12)
glutamate receptor interacting protein 2 (GRIP2)
RGMB antisense RNA 1 (RGMB-AS1)
dynein, axonemal, heavy chain 10 (DNAH10)
golgi phosphoprotein 3 (GOLPH3)
SAGA complex associated factor 29 (SGF29)
hepatocyte growth factor-regulated tyrosine kinase substrate (HGS)
glycolipid transfer protein domain containing 2 (GLTPD2)
SCAN domain containing 1 (SCAND1)
zinc finger protein 224 (ZNF224)
pcDNA3.1-3
NP-2
pcDNA3.1-1
NP-1
pcDNA3.1-2
GFP-2
GFP-1
GFP-3
EIV PA-X-2
EIV PA-X-3
CIV PA-X-3
EIV PA-X-1
CIV PA-X-2

FIG 3.6. Heatmap generated after stringent statistical analysis showing differentially expressed genes comparing EIV and CIV PA-X samples to controls. Controls (pcDNA, NP, GFP) and PA-X samples (EIV and CIV) were labeled at the bottom of the heatmap while gene names (full and abbreviated) were labeled on the right side. The heatmap shows three independent replicates for each sample except for one NP and one CIV sample which represent extreme outliers. Blue shading indicated down-regulation of gene expression while red shading indicated up-regulation of gene expression.

Table 3.1. Genes that showed significant differential expression from the RNA-seq analysis heatmap comparing PA-X samples to the controls were individually analyzed and tabulated. Genes (abbreviated) are displayed in the first column followed by a brief description in the second column. All genes shown here were up-regulated in expression relative to the controls with RNAseq cut-offs of 0.05 FDR and > 2 fold change.

Gene	Key Words / Functions
AMFR	E3 ubiquitin ligase, ubiquitination pathway, takes part in ER-associated protein degradation (ERAD)
ATP4A	ion channel, hydrogen / potassium exchange
CHUK	kinase, phosphorylates NF-κB inhibitory proteins for ubiquitination and subsequent degradation, promotes NF-κB response
COG3	regulates ER-golgi transport, defines golgi morphology, protein glycosylation
COMMD10	modulates activity of E3 ubiquitin ligase, may down-regulate NF-κB response
COQ3	methyltransferase, functions in mitochondria, takes part in the electron transport chain (ETC)
CTR9	binds to RNA polymerase II, takes part in histone methylation, regulates transcription
DCLRE1B	DNA interstrand cross-link repair, promotes transcription
DCPS	takes part in mRNA degradation pathway, up-regulated during virus infections
DDX21	putative RNA helicase, may regulate transcription
DHX15	putative pre-mRNA splicing function, may regulate gene expression
DUSP12	phosphatase, negatively regulates mitogen-activated protein (MAP) kinase superfamily
FER	kinase, plays role in activating NF-κB response
GOLGA3	regulates golgi transport
GOLPH3	regulates golgi transport, vesicle budding, takes part in secretion pathway
HGS	endosomal sorting, sorts ubiquitinated proteins for degradation
HNRNPF*	associates with pre-mRNA, splicing and transport, plays role in mRNA maturation
HOXC8	transcription factor for cell development
IST1	vesicle budding, interacts with endosomal sorting complexes required for transport (ESCRT)
KCNN4	ion channel, calcium activated, promotes potassium influx into cells
MECP2	suppresses transcription by binding to methylated promoters
MED26	subunit of cofactor required for SP1 activation (CRSP) transcription factor
MGAT2	functions in golgi, converts N-glycans for protein glycosylation
MTA2	takes part in chromatin remodeling to facilitate transcription
NDUFS4	oxidoreductase, functions in mitochondria, takes part in the electron transport chain (ETC)
PPP2CA	phosphatase, negative control of cell growth
PTPN9	phosphatase, functions in golgi, takes part in transferring hydrophobic ligands
RIPK2	kinase, ubiquitination pathway, plays role in activating NF-κB response
RNF6	E3 ubiquitin ligase, ubiquitination pathway, may bind DNA to regulate transcription
SCAND1	binds to and regulates transcription factor myeloid zinc factor 1B
SET	prevents histone acetylation, inhibits transcription
SGF29	promotes transcription through association with histone acetyltransferases
SLC12A5	ion channel, lowers intracellular chloride concentration
TMED10	cis-golgi network, ER, takes part in early secretory pathway, cargo loading
TRAPPC8	may be involved in ER to golgi trafficking early on
TRIM28	represses transcription, binds to E3 ubiquitin ligase (MDM2), inhibits apoptosis by marking p53 for degradation
WDR82	promotes transcription by histone H3 methylation
ZNF367	promotes transcription
ZNF614	may be involved in transcription regulation
ZNF780A	may be involved in transcription regulation
	*Two variants of HNRNPF were identified

III.VI DISCUSSION

Genetic differences between EIV and CIV PA-X resulted in a phenotypic difference comparing gene suppression. We have previously shown that the numerous genetic changes between EIV and CIV did not result in easily detected significant differences in comparisons of virus growth and infectivity in different host cells, HA cleavage, or receptor specificity (15). In contrast, we show here that genetic differences between the PA-X proteins of the two viruses resulted in a biological difference, such that EIV PA-X had a greater ($p<0.05$) gene suppression ability compared with CIV PA-X based on β-galactosidase and GFP reporter assays (Fig. 3.1 – 3.5), although it is not clear how this difference would influence the evolution and adaptation of EIV to dogs. We used HEK293 cells for these reporter assays because dog (MDCK and A72) and horse cells (EQKD) could not be efficiently transfected after various attempts (data not shown). Furthermore, previous studies that analyzed PA-X's reporter gene suppression ability also used HEK293 cells for both human- and avian-origin PA-X proteins (12, 16).

The results showed the mutation S231F and the C-terminal truncation in EIV PA-X changed its gene suppression phenotype to resemble CIV PA-X's, however; the reciprocal mutations in CIV PA-X (F231S and elongating the C-terminus) did not convert its phenotype to resemble EIV PA-X's (Fig. 3.3 – 3.4). Interestingly, a complete conversion to EIV PA-X's level of GFP suppression required both mutations in the CIV PA-X backbone (Fig. 3.5). This indicated that both position 231 and the C-terminal elongated tail defined EIV PA-X's stronger phenotype, thereby providing evidence that amino acids near the truncation site of PA-X, such as position 231, can influence function. These results are consistent with previously published reports that showed the 252 amino acid long (full-length) PA-X protein from different IAV subtypes had greater ribonuclease activity compared to the 232 amino acids truncated variant (10, 12). Taken together this indicates that the C-terminus most likely interacts with or regulates the N-terminal ribonuclease domain (Fig. 3.2). Understanding the mechanism clearly requires additional studies, such as solving the structures of the PA-X in full-length and truncated variants.

Expression of EIV and CIV PA-X alone resulted in significant up-regulated expression of host genes. Several previous reports have examined the effects of PA-X on host response by comparing wild type and mutant virus infections, and shown that PA-X was important for modulating various immune responses (2, 6-8, 12, 14). To further elucidate PA-X's effects on the host, we transfected plasmids expressing only PA-X into cell cultures and used RNA-seq to analyze the transcripts. While artificial, this allowed us to study PA-X's influence on the cell, as there would not be other expressed viral proteins that might change PA-X's interactions with host gene expression (Fig. 3.6). Notably, many genes were up-regulated (Table 3.1), a number of which were related to specific aspects of the innate immunity response. For example, we found genes related to the modulation of the NF-κB transcription factor: a well described response that leads to the transcription of several anti-viral cytokines, such as IFN-β (17). It has been previously noted that productive IAV infections required the activation of NF-κB (18, 19), and influenza HA and vRNA have been shown to trigger the pathway while influenza NS1 inhibited it (20-22). Our results suggest that influenza PA-X may also be directly involved in up-regulating genes involved in both activating (CHUK, FER, RIPK2 – all kinases) and repressing (COMMD10) the pathway, and thus playing an important role in virus replication. Another immunity related gene (TRIM28) that was up-regulated by EIV and CIV PA-X modulated apoptosis, a well described antiviral response (23-25). TRIM28 interacts with ubiquitin E3 ligase, MDM2, to drive ubiquitination and subsequent degradation of tumor suppressor protein p53, resulting in inhibition of apoptosis (26, 27). Our finding was consistent with studies that described PA-X (from H1N1 and H5N1 subtypes) as having anti-apoptotic activity (6, 7). Lastly, we also found two phosphatases (DUSP12, PPP2CA) that negatively regulated the MAP kinase cascades; these pathways have been shown to drive expression of antiviral cytokines such as TNF-α which causes an inflammatory response in cells (28). This finding further suggests that PA-X is able to function as a negative controller of the immune response.

Interestingly, our results revealed the up-regulation of two E3 ubiquitin ligases (AMFR, RNF6). Ubiquitination can either promote or restrict influenza virus infections. For example, while E3 ubiquitin ligase TRIM32 restricts influenza by marking PB1 for proteasomal degradation (29), there is also evidence that NEDD4 facilitates infections by accelerating the turnover rate of the antiviral factor IFITM3 (30). Additionally, disrupting the ubiquitination pathway by treating cells with proteasome inhibitors results in a decrease in infection (31). Thus, our results suggest that PA-X may control the expression of some E3 ubiquitin ligases to potentially modulate infections.

It was previously suggested that PA-X might be accumulated in the late phase of a virus infection due to the inefficiency of frame-shifting during translation and hence might exert more influence during this period (9). Our over-expression of PA-X in cells (no frame-shifting) may therefore have simulated a late phase infection environment and caused up-regulation of genes that reflect this time window. For example, several genes involved in vesicle transport and budding (COG3, GOLGA3, GOLPH3, IST1, TMED10, TRAPPC8) and related to protein post-translational modification in the Golgi (COG3, MGAT2, PTPN9) were up-regulated. The up-regulation of these genes makes sense in the context of a late virus infection phase – when viral components need to go through the secretory pathway (HA, NA, M2) and traverse the endocytic system via recycling endosomes (viral RNPs) in order to congregate at the cellular membrane in preparation for assembly and egress (32-36).

The RNA-seq results revealed that many genes involved with transcription and/or protein expression were up-regulated, although these were more generic as opposed to targeting a specific pathway or transcription factor. These genes can be sorted into two categories: (1) genes that, when expressed, produced proteins that modulated transcription and (2) those that regulated gene expression after transcription. The first category included genes related to histone modification (CTR9, SET, SGF29, WDR82), DNA template binding (DCLRE1B, MECP2, MTA2), and other putative transcription factors (HOXC8, ZNF367, ZNF614, ZNF780A). The

second category included genes associated with pre-mRNA splicing and maturation (DHX15, HNRNPF – two variants) and an mRNA de-capping enzyme, DCPS. Of note, viruses that carry their own de-capping enzymes, such as influenza and vaccinia viruses, use them to facilitate virus gene expression while inhibiting host translation (37, 38). Consequently, PA-X's ability to up-regulate a host de-capping enzyme may provide an additional method for IAV to inhibit host translation.

More in-depth studies will be required in the future to fully understand the mechanisms underlying these apparent changes in host gene expression, particularly whether PA-X interacts directly with the described genes or through binding partners. Additionally, it will be important to use RNA-seq to analyze the consequences of live virus infections, such as wild type and PA-X negative viruses, and compare their gene expression profiles with transfected PA-X's. Comparing these profiles would help to reveal a clearer picture of the effects of influenza PA-X on host gene expression. Although the RNA-seq analysis did not reveal any differences in gene expression when comparing EIV and CIV PA-X samples, this was not surprising given that we previously observed that CIV and EIV exhibited few phenotypic differences in assays that considered virus growth in cell culture, infections in different host cells, receptor (sialic acid) binding with purified HAs, and HA cleavage efficiency (15). For the future it may be possible to use RNA-seq to compare EIV and CIV PA-X effects when expressed in dog and horse cells. As stated previously, we have tried transfections using dog (MDCK and A72) and horse (EQKD) cells but the efficiencies were very poor and inconsistent based on GFP reporter; we would like to further explore this comparison at a later date.

III.VII ACKNOWLEDEMENTS

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CHAPTER IV: CONCLUSION AND FUTURE PERSPECTIVE

IV.I FINDING A DIFFERENTIATING HORSE CELL LINE

Based on our viral infections using horse and dog tracheal cultures and other studies using animal models, there is clearly a host barrier in horses that blocks CIV infections (1-3). Identifying exactly what viral genes and/or mutations that prevent CIV from infecting horses would further define influenza host tropism and adaptation. However, it is not practical to test infections using animals and/or tracheal cultures because of high cost, intensive labor, availability, and other limiting factors. Extensive experiments using these *in vivo* and *ex vivo* methods should be reserved for viral constructs that hold the most promise in revealing the genes and/or mutations necessary for overcoming the host barrier in horses. Thus, the most practical approach this problem is by testing different viral infections using a horse cell line that is permissive to EIV but not CIV. In our study we tested equine kidney cells (EQKD) but they were non-permissive to both EIV and CIV. There are currently two horse-derived cells available commercially from ATCC, E. Derm (NBL-6) and an uncharacterized horse cell line. One or both of these cells may restrict CIV but not EIV – similar to the animal and tracheal infections.

IV.II GENERATING APPROPRIATE VIRAL CONSTRUCTS

If an appropriate horse cell line is found, several new viral mutants should be constructed in order to identify the genes and/or mutations that block CIV from infecting those cells. In our previous study, we constructed viruses carrying single HA mutations at and near the cleavage site in addition to HA and NA double reassortant viruses (1). New mutants should include HA and NA single reassortant viruses and polymerase reassortant viruses, in particular swapping the PB2 segment. Since the receptor-interacting proteins and the polymerase complex have been extensively described to be critical in determining influenza host range (see Chapter I.X), focusing on these reassortant viruses first would provide the greatest chance of identifying a gene segment(s) responsible for blocking CIV infections in horse cells. Once a

specific gene(s) is identified individual mutations need to be made in order to further narrow down the amino acid residues that play a role in determining host range.

IV.III *IN VIVO* EXPERIMENTS TO CONFIRM HOST RANGE DETERMINANTS

Infecting horses with different CIV mutants would be the definitive method in determining the residues critical for overcoming the host barrier. The constructs that should be tested are the ones that can infect horse cells. Horse tracheal cultures can also be infected as an alternative – in a similar method described in our previous work (1). Another aspect that can be analyzed is CIV transmission from horse to horse. Indeed, if a specific CIV mutant(s) can infect horses, perhaps they can also facilitate transmission. However because EIV can infect dogs, dog tracheal cultures, and dog cells, determining the changes in EIV that are specific for dog adaptation is a challenge. One possible method would be passaging EIV in dogs (and alternatively, dog cells) and monitor for changes in the viral genome and seeing if those genetic changes increase viral growth. And even if the mutations do not affect viral growth, they may still be indicative of adaptive changes if they match previously described mutations (1).

IV.IV FURTHER RNA-SEQ EXPERIMENTS USING VIRAL INFECTIONS

Our RNA-seq experiments using transfected EIV and CIV PA-X genes showed many host genes were significantly up-regulated. However, these changes may not be reflective of PA-X's effects in viral infections. In order to gain a clearer picture of PA-X function, RNA-seq experiments should be conducted for wild type and PA-X negative viruses. Only by comparing the results from plasmid expression of PA-X to infections can we gain a better understanding of PA-X function with respect to modulating host genes. Comparing the results between wild type and negative PA-X viruses could also reveal differences between EIV and CIV, such that their respective PA-X genes could modulate the expression of different host genes. Using dog and horse cells instead of human cells may also reveal host-specific differences.

IV.V MODIFIED SIALIC ACIDS AND THE HOST RANGE OF EIV AND CIV

As stated previously, influenza viruses bind to sialic acids present on host cells to gain entry (see Chapter I.X). Additionally, viruses from different hosts will bind to sialic acids with specific linkages, such as α2-3 and α2-6. From our previous study and other reports (1, 4, 5), it appears EIV and CIV both bind to α2-3 linked sialic acids despite numerous mutations in their HAs. This binding may explain why both viruses grew similarly in MDCK cells and had similar infection phenotypes in various host cells based on our results (see Chapter II.X). However, there seems to be some differences comparing sialic acid binding between EIV and CIV such that the EIV HA binds to sulfated sialic acids better than CIV (5). Indeed, sialic acids are often naturally modified in different positions in its 9 carbon backbone such as hydroxylation, methylation, sulfenylation, and phosphorylation (6). These modifications create a wide diversity of sialic acids in their hosts and viruses have adapted, accordingly. For example, influenza C virus and bovine coronaviruses specifically bind to 9-O-acetylated sialic acids while mouse hepatitis C virus uses 4-O-acetylated sialic acids to gain entry (7, 8). Although there is currently no evidence that influenza A viruses are affected by modified sialic acids, it is worth investigating when it appears EIV and CIV HAs have different binding affinities to sulfated sialic acids. Indeed, modified sialic acids could be a host range determinant for EIV and CIV – the viruses may both prefer α2-3 linked sialic acids but they could vary in binding to specific modifications present in dogs and horses. Indeed, horse tissues may express significant amounts of 4-O-acetylated sialic acids because horse serum contains α2-macroglobulin that is rich in 4-O-acetylated sialic acids (9). These glycoproteins actually neutralize human H2 and H3 influenza subtypes because once the virus binds to the sialic acid, its NA is not able to cleave off the sialic acid and so the virus remains tethered (9). Additional evidence that horses may express 4-O-acetylated sialic acids in various tissues is that esterases specific for 4-O-acetyl groups have been found in horse liver (6). And so perhaps the reason why CIV is not able to infect horses is because it is inhibited by the presence of 4-O-acetylated sialic acids.

IV.VI EXPRESSION OF HEMAGGLUTININ ESTERASE FUSION PROTEINS

In order to determine if modified sialic acids such as 4-O- and 9-O-acetylated sialic acids affect EIV and CIV binding, we needed proteins that can detect them in cell culture and in tissue samples. There are currently no commercial antibodies or lectins that can recognize these modified sialic acids, and so we constructed hemagglutinin esterase (HE) fusion proteins based on a previous study (8). These proteins were derived from the lectin binding domain of mouse hepatitis virus S strain (MHV-S) HE and bovine coronavirus Mebus strain (BCoV-Mebus) HE. These HEs were fused to human Fc and recombinant baculovirus expressing these constructs were rescued in Sf9 insect cells. High Five suspension insect cells were used to scale up the infections and two days post-infection proteins were bound to a HiTrap ProteinG HP column and purified using FPLC as described previously in our report (1). Four fusion proteins were constructed: MHV-HE-Fc inactive, MHV-HE-Fc active, BCoV-HE-Fc inactive, and BCoV-He-Fc active. The inactive fusion proteins bound to either 4-O- (MHV) or 9-O-acetylated sialic acids (BCoV) and remained bound to them. The active forms bound to the same acetylated sialic acids, respectively, but they released themselves after cleaving off the acetyl group. And thus, the inactive forms can be used as “probes” to detect 4-O- and 9-O-acetylated sialic acids while the active forms can remove the specific acetyl modifications. In order to confirm their specificities, solid phase binding assays were conducted using horse serum glycoproteins (4-O-acetylated sialic acids) and bovine submaxillary mucin (9-O-acetyalted sialic acids). Results showed that both inactive HE-Fcs were highly specific for their respective target with minimal background and cross-reactivity (Fig. 4.1A). Additionally, the active HE-Fcs specifically cleaved off their respective acetyl groups – the inactive HE-Fcs were not able to bind to their substrates after pre-treatment with the active enzymes (Fig. 4.1B). The inactive HE-Fcs were also able to hemagglutinate erythrocytes from different animal species as well, showing horse erythrocytes expressed 4-O-acetylated sialic acids and chicken erythrocytes expressed 9-O-acetylated sialic acids. Cat and dog erythrocytes expressed neither (Fig. 4.2).

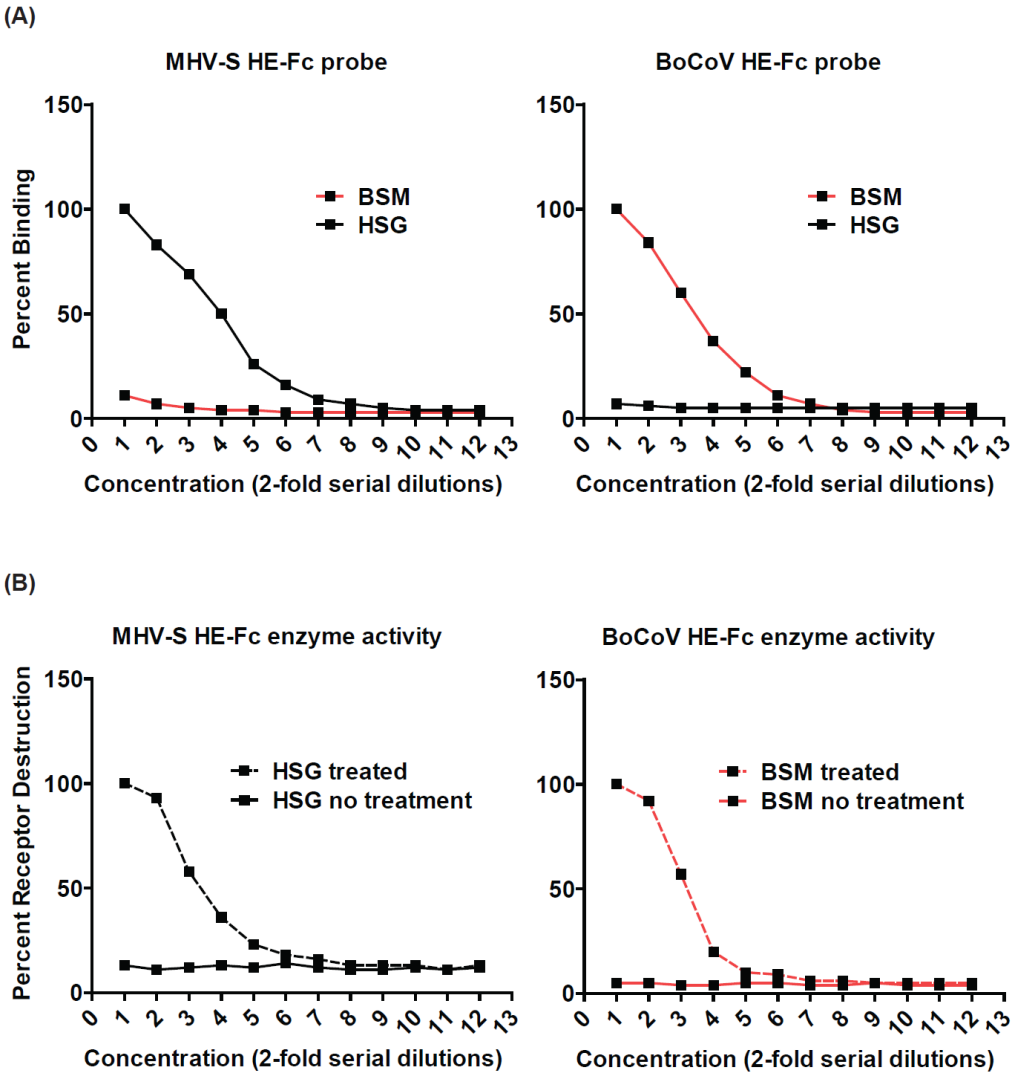

FIG 4.1. HE-Fc probe specificity and enzymatic activity. (A) MHV-S and BoCoV HE-Fc were used to bind BSM (9-O-acetylated sialic acids) and HSG (4-O-acetylated sialic acids) in solid phase binding assays to test specificity. The initial concentration of each probe was 100 ug/ml and was serially diluted 2-fold. Absorbance was measured at 450 nm after incubation with an anti-human IgG HRP conjugate and was standardized to the highest value. (B) HSG and BSM were treated with 2-fold serially diluted HE-Fc enzymes (100 ng/ml starting concentration) for 2 h at 37°C. Samples were then incubated with either MHV-S or BoCoV HE-Fc probes and absorbance was measured at 450 nm and standardized based on the lowest absorbance value (highest percent receptor destruction).

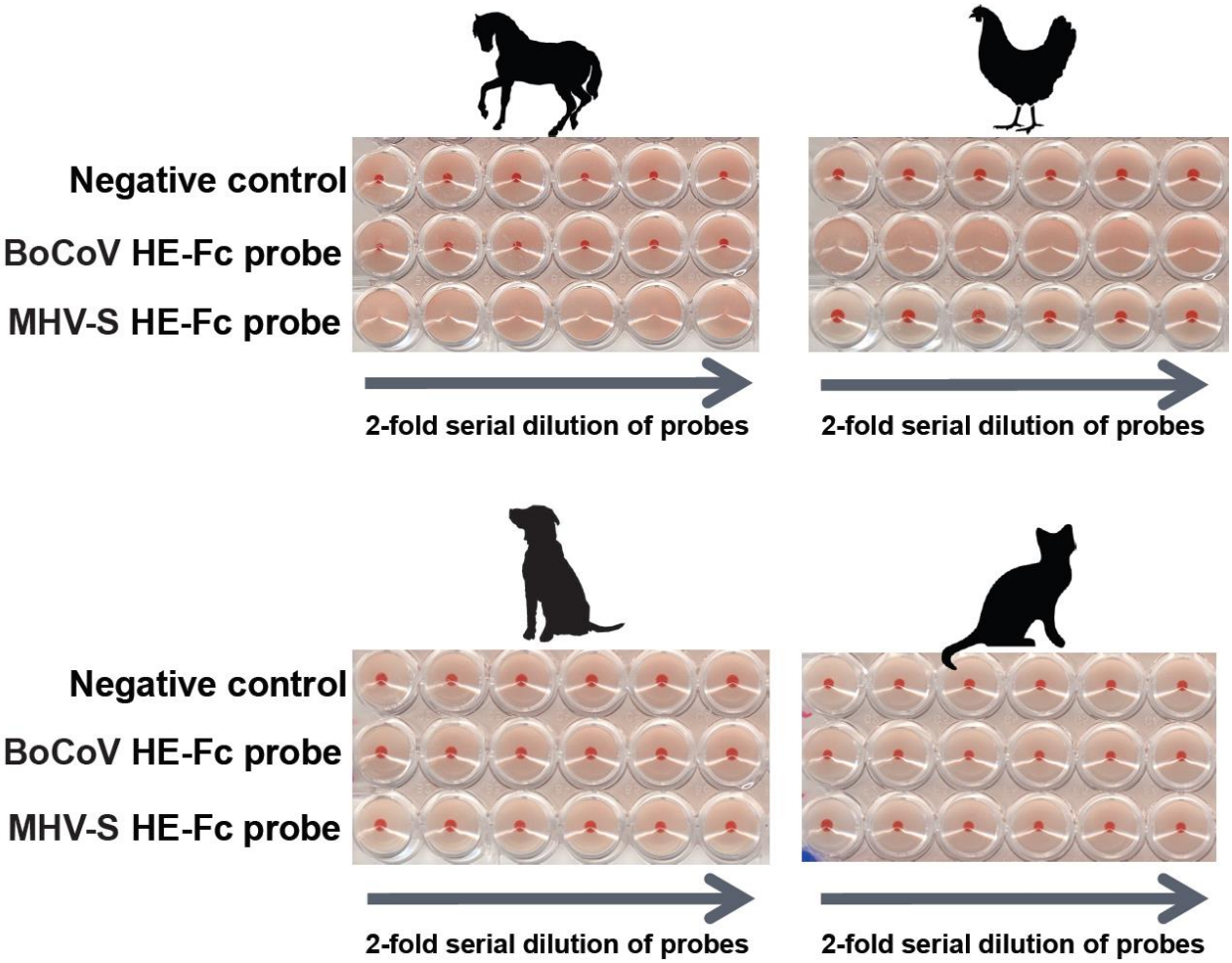

FIG 4.2. HE-Fc probe HA assays using erythrocytes from different animals. HA assays were performed using fresh erythrocytes prepared in PBS. Each well contained 50 ul of erythrocyte solution mixed with 50 ul of either MHV-S or BoCoV HE-Fc. The starting concentration of each probe was 10 ug and was serially diluted by 2-fold. Assays were read after 2 h incubation at room temperature.

IV.VII USING HEMAGGLUTININ ESTERASE FUSION PROTEINS

EIV and CIV may differ in their ability to bind to 4-O- and 9-O-acetylated sialic acids and this difference may be a host range determinant. For example, using horse cells that are permissive to EIV but not CIV, we can use these probes to determine if that block is due to the presence of these modified sialic acids. There is a high possibility that horse cells express 4-O-aceylated sialic acids because both horse serum glycoproteins and horse erythrocytes do. We can use the inactive 4-O HE-Fc to see if the cultured cells do as well and if so, we can use the active 4-O HE-Fc to cleave off those acetyl groups. Next, we can infect these cells with EIV and CIV and compare the results to cells that have not been treated with the 4-O HE-Fc enzyme. A change in infectivity, such that CIV is able to infect the horse cells, would suggest the presence of 4-O-acetylated sialic acids block CIV infection. This type of assay can be repeated with the 9-O HE-Fc proteins – as well as using different types of host cells. In order to get a clearer picture of the distribution of these modified sialic acids in horses and dogs and whether or not they affect viral binding, immunohistochemistry should be used to assay tracheal tissue – because the normal location of these viral infections is in the respiratory tract. Similarly to cell cultures, we can use the HE-Fc proteins to probe for the modified sialic acids and to cleave off the specific 4-O and 9-O acetyl groups. We have already constructed EIV and CIV HA-Fc fusion proteins (1) so we can use them to bind the tracheal tissue before and after HE-Fc enzyme treatment. For instance, if the CIV HA-Fc is able to bind to horse tracheal tissue more efficiently after cleaving off the acetyl groups, it would provide further evidence that the host barrier that prevents CIV from infecting horses is the presence of modified sialic acids. A more definitive method would be treating live tracheal cultures with the HE-Fc enzymes and seeing if CIV is able to infect horse tracheal explants. One major hurdle in these experiments is that since sialic acids are recycled and refreshed in live cells, making sure the acetyl groups stay cleaved off poses a considerable challenge. One way of solving this issue would be treating the cells with different concentrations of HE-Fc enzymes and then probing the cells at various time intervals to

see when the modified sialic acids recover. The time between when the modified sialic acids are cleaved and when they recover would be the optimal time frame for the infection experiments.

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