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The effect of avian influenza virus NS1 allele on virus replication and innate gene expression in avian cells

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ABSTRACT

The NS1 gene encoded by Type A influenza virus circulates as two alleles, the A and B allele. The immunomodulatory properties of the NS1 A allele have been thoroughly examined; however, comparisons of allele function have been predominantly made in mammalian systems. Here we show that counter to the current understanding of allele function in mammals, the two alleles similarly regulate elements of the type I interferon (IFN) signaling pathway, including the interferon-inducible genes *Mx* and 2′–5′ oligoadenylate synthase (2′–5′ OAS), and IL-6, which share the same induction pathway as the interferons in embryo fibroblasts from chickens, turkeys or ducks. Replication of two reassortant viruses demonstrated that the B allele virus replicates more and to higher titers than the A allele virus in duck cells; however, the A allele virus replicates more in the cells from chickens and turkeys. Finally, chimeric constructs were used to identify a region of the NS1 gene that conferred the statistically significant differences in expression and replication observed between the alleles.

1. Introduction

Avian Influenza Virus (AIV) is a Type A Influenza Virus, and infects a wide variety of avian and mammalian hosts including humans (Cardona et al., 2009). The pathogenesis of AIV infection in these species is the subject of many studies and much is known about the AIV replication cycle, the functions of the proteins it encodes, and virus–host interactions. The nonstructural 1 (NS1) protein has been demonstrated to have a role in evading the host immune system and in augmenting viral replication. These functions have been identified using mammalian cells or animal models primarily with human strains of influenza A viruses. In contrast, the role of NS1 in avian systems has been only rarely studied. An understanding of the avian immune response to AIV infection is an area of critical need because of their roles as viral reservoirs, and spillover hosts such as chickens and turkeys, which can serve to amplify and expand influenza in epidemics and in which, highly pathogenic viruses can emerge.

Infections with AIV can be economically significant in domestic chickens, turkeys, and ducks. An outbreak of highly pathogenic H5N1 in 2003–2005 in Vietnam alone is estimated to have cost between 76 and 450 million US dollars (McLeod and Guerne-Bleich, 2006). The interaction between avian hosts and AIV is complicated; not all strains behave in the same way within a single avian species. Conversely, not all avian species behave similarly in response to a single AIV. In a study comparing infections with 12 low pathogenicity AIV (LPAIV) isolates in chickens, turkeys, and ducks, turkeys displayed the highest morbidity, followed by chickens then ducks which demonstrates variation in disease manifestation between species in response to single isolates. Within each species, there was marked variation in levels of virus shed that was dependent on the virus strain (Spackman et al., 2010). Furthermore, in a study that compared the gene expression of chicken embryo fibroblasts infected with two different isolates of highly pathogenic H5N1, the authors observed considerable variation in host gene expression as well as virus growth (Sarmento et al., 2008). The strain dependent responses in these studies may

Abbreviations: AIV, avian influenza virus; NS1, non-structural 1; vRNA, viral ribonucleic acid; CEF, chicken embryo fibroblasts; TEF, turkey embryo fibroblasts; DEF, duck embryo fibroblasts; nt, nucleotide.

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be due in part to NS1 and its role in modulating the host's immune responses.

The NS1 protein is composed of multiple domains that interact with several host proteins resulting in the regulation of viral RNA (vRNA) synthesis, enhanced viral protein synthesis, increased viral replication, and evasion of the host's immune response. The regulation of vRNA synthesis is likely due to the effector domain (ED) as carboxy terminal truncations have been shown to result in decreased vRNA levels (Falcon et al., 2004), confirming the findings of earlier studies (Wolstenholme et al., 1980). Viral protein synthesis is regulated via interactions with eukaryotic translation initiation factor 4GI (eIF4GI) and polyadenosine binding protein II (PABPII) which preferentially recruit the cellular translation complex to viral mRNA (Aragon et al., 2000; Burgui et al., 2003). NS1 has two major strategies to confound the host immune system: binding RNA and interacting with host cellular proteins. RNA binding is thought to prevent the activation of host cellular sensors such as protein kinase R (PKR), retinoic-acid inducible gene I (RIG-I), and 2′–5′ OAS which recognize aberrant ribonucleotide structures such as dsRNA and viral ssRNA (Talon et al., 2000; Min and Krug, 2006; Pichlmair et al., 2006; Silverman, 2007). NS1 interaction with host proteins is complicated, as NS1 may prevent the activation of the interferon (IFN) pathways at times either pre-transcription or post-transcription of IFN genes, thus preventing cellular activation of an antiviral state, Pre-transcriptional inhibition is carried out by NS1 inactivation of PKR and RIG-I (via its interaction with TRIM25), thereby effectively preventing the activation of the signaling cascade leading to IFN transcription (Min et al., 2007; Gack et al., 2009). Post-transcriptional inhibition is accomplished by preventing nuclear export of cellular mRNA by binding the cleavage and polyadenylation specificity factor (CPSF30) and the poly(a)-binding protein II (Nemeroff et al., 1998; Chen et al., 1999; Twu et al., 2006).

NS1 exists as two alleles circulating in specific host ranges (von Hoyningen-Huene and Scholtissek, 1983; Treanor et al., 1989) both of which have the functional domains described although functionality has only been characterized in A alleles of the gene. The A allele is found in humans, non-human mammals, and avian species, while the B allele has been detected exclusively in avian species with very few exceptions (Ludwig et al., 1991; Guo et al., 1992; Zohari et al., 2008). The sequences are relatively conserved within alleles, which have 93–100% amino acid identity, but can be as much as 31% divergent between alleles. This difference at the amino acid level may impart a difference in function. Early studies showed that replication in the rhesus macaque resulted in lower titers for a B allele virus compared with an A allele virus (Treanor et al., 1989). More recently, IFN levels were compared in NS1 vector transfected mink and human lung cells which showed that the A allele suppressed IFN more efficiently and that this effect was localized to the RNA binding domain (RBD) of the A allele (Munir et al., 2011b). Moreover, the authors examined inhibition of NF-κB, the signaling system by which IFN is induced, by similar methods and found that the A allele was more potent at inhibiting NF-κB in these cell types (Munir et al., 2012). These findings suggest that the A allele, which is more broadly distributed in mammals is more efficient at evading mammalian host defense mechanisms than is the B allele under the conditions employed in these studies. However, the functional differences between the alleles in avian hosts are still unknown. It was the purpose of our study to examine the response to infection with an LPAIV H9N2 bearing either the A or B allele of NS1 in the cells of three important avian hosts and identify differences in immune gene expression and viral titers. We further sought to identify regions of the respective alleles responsible for differences in these outcomes by using chimeric NS1 alleles.

Table 1
Age of avian embryonating eggs at time of fibroblast preparation.

Avian egg	Days of embyonation
Chicken	11
Turkey	14.5
Duck	13

2. Materials and methods

2.1. Embryo cell isolation and cell culture

Avian embryo fibroblasts were derived from specific-pathogen-free chicken (Charles River, Wilmington, MA), commercial turkey (Willmar Poultry Co, Willmar, MN), and commercial Pekin duck (Maple Leaf Farms, Leesburg, IN) eggs as follows. Briefly, after embryonating for the number of days indicated in Table 1, six eggs of each avian species were chilled at 4 °C then disinfected by spraying their surface with 70% ethanol in water. Embryo fibroblasts were prepared according to standard methods (Martin et al., 1971). Cells were resuspended in 10% DMSO and 20% FCS in DMEM then aliquoted in 1 mL volumes at approximately 5×10^7 cells/mL. Cell aliquots were frozen in liquid nitrogen until further use.

Chicken embryo fibroblasts (CEF), turkey embryo fibroblasts (TEF), and duck embryofibroblasts (DEF) were thawed, washed three times in sterile PBS, and resuspended in DMEM supplemented with 10% FCS, 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. $1–2 \times 10^6$ cells were seeded per well on 12-well plates (Corning, Corning, NY) and incubated at 37 °C and 5% CO_2. When the cells reached confluency (2–4 days), a single well of each CEF, DEF, or TEF culture was trypsinized and quantified by hemocytometer.

2.2. Viruses and infection

Reassortant NS1 A allele and B allele viruses were generated by reverse genetics using A/guinea fowl/Hong Kong/WF10/99 (H9N2) WF10 plasmids (Song et al., 2007) as a recipient virus. The NS genes of low-pathogenicity avian influenza viruses A/duck/WA/663/97 (H11N9, A Genbank Accession no. KC833454) and A/greater white fronted goose/California/HKWF446/2007 (H10N7, B allele Genbank Accession no. CY034169) were cloned, transfected with the remaining seven WF10 cloned genes, and recovered from mixed 293T and MDCK culture as described previously (Hoffmann et al., 2002). The reassortant viruses are referred to from this point on as WF10A (A allele reassortant) and WF10B (B allele reassortant). Culture supernatants were centrifuged at 10,000 RPM for 5 min then 200 μL of supernatant were inoculated into ten-day-old embryonating chicken eggs (Charles River, Wilmington, MA). After 72 h, allantoic fluid was harvested from eggs, and diluted 1:10 before inoculation into eggs for a second passage. Second pass viral allantoic fluid was titered by plaque assay as described below.

Chimeric constructs were created by designing primers at two positions in the NS1 gene where A/duck/WA/663/97 and A/greater white fronted goose/California/HKWF446/2007 are identical. We posited that selecting fusion positions at regions of identical sequence would have a lesser negative impact on the chimeric gene function due to preservation of conserved sequence in the final construct. The primer sequences designed for these purposes can be found in Table 2. These primers were paired with NS1 forward and reverse primers (Bm-NS-1 and Bm-NS-890R respectively (Hoffmann et al., 2002)) to generate 5′ and 3′ segments of the A allele and B allele sequences. These segments were then paired using the 5′ end of the A allele and the 3′ allele of the B allele and vice versa and used as cDNA templates in a final PCR reaction using Bm-NS-1 and Bm-NS-890R for the generation of chimeric NS1