Expression of influenza virus non-structural protein 1 (NS1)

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To my grandmother

Abstract

Avian influenza poses a threat to many species including man, as shown by the current scenario in Southeast Asia. It appears that this particular type of influenza virus can spread to many species causing severe disease in these new species. For example, from 258 human cases 154 were fatal (WHO, 2006-11-29). Why some avian influenza virus have the ability to infect other species remains to be understood. One viral gene, coding for the non-structural protein 1 (NS1), appears to be an important factor for successfully transmission into a new host, by counter-acting the new host's immune system. The exact mechanism of action of NS1 is still unclear, but leads to down-regulation of various pathways of the immune defence. The aim of this study was to express NS1 proteins of influenza viruses originating from different hosts, including highly pathogenic avian influenza viruses, for use in investigations on the mechanism used by NS1 to interfere with the immune system of various hosts. In particular, studies on the interaction of NS1 with the RNA dependent protein kinase (PKR) were initiated, to determine differences in this interaction between high and low pathogenic influenza viruses from different hosts. By establishing tools and optimised assays, the work enables further studies on the role NS1 in immune evasion.

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BACKGROUND

History of influenza

Influenza is a contagious respiratory viral illness of global importance, caused by influenza virus. Influenza viruses have unique features among respiratory viruses, with the segmented genome and considerable antigenic diversity. They have been classified into three distinct types: A, B and C. Influenza A is responsible for occasional pandemics affecting millions of people worldwide, often associated with considerable morbidity and mortality. During the years 1918-1920, it caused one of the most destructive disease outbreaks in world history that become known as the Spanish flu pandemic. It resulted in the death of an estimated 50-100 million people (Johnson and Muller, 2002); thus, this influenza pandemic killed more people than the World War I. It is not known where the pandemic started but it has been suggested that it originated during the First World War, in the northern part of France (Oxford *et al*., 2005). Most parts of the world were affected. In Sweden about 35.000 people died from this disease. For unknown reasons the disease disappeared after this devastating outbreak. The disease occurred in the United States in 1924-25, and then again in 1929 in a much milder form. This virus continued to circulate in humans until 1957. It was then replaced by another human influenza virus, the Asian flu virus. Pandemic influenza A viruses emerged three times during the last century: in 1918 (H1N1 subtype), in 1957 (H2N2), and in 1968 (H3N2) (Cox and Subbarao, 2000; Webby and Webster 2003). Currently both H3N2 and H1N1, a late fifties variant that re-appeared in 1977, co-circulate in humans.

The recent circulation of highly pathogenic avian H5N1 viruses in Asia from 2003–to date has caused at least 258 human cases 154 were fatal as of November 29th 2006 (Peiris *et al.,* 2004, World Health Organization, WHO) and has raised concern about the development of a new pandemic like the Spanish Flu (Cox and Subbarao, 2000). How and when novel influenza viruses emerge as pandemic strains and their mechanism of pathogenesis is still not understood.

At the time of the great Spanish flu it was not known that the disease was caused by a virus. However, the causative agent of influenza in birds, the so-called fowl plague, was shown to be a 'virus' as early as 1901. It was not until 1955 that its relationship with other milder viruses isolated from birds, and with mammalian influenza A viruses (first isolated in the 1930s) was demonstrated (Schafer, 1955). In 1933 Smith, Andrewes and Laidlaw isolated the virus in ferrets (Smith, Andrewes and Laidlaw, 1933).

Meanwhile, Tumpey et al. (2005) reporting in Science show that the reconstructed 1918 Spanish influenza virus kills mice faster than any other influenza virus so far tested. So, there appears to be some viral factor that is especially potent.

Influenza A is classified into subtypes based on antigenic and genetic differences of the two viral surface proteins, HA and NA. To date, 16 HA and 9 NA subtypes have been detected in wild birds and poultry throughout the world (World Health

Organization 1980, Fouchier *et al.,* 2005). All 16 HA and 9 NA subtypes in the majority of possible combinations have been isolated from wild avian species (Alexander, 2000). The most recent type (H16) was isolated from black-headed gulls caught in Sweden in 1999 (Fouchier *et al.,* 2005).
Influenza A viruses infecting poultry can be divided into two groups. The very virulent viruses cause 'fowl plague', now termed highly pathogenic avian influenza (HPAI), in which mortality may be as high as 100%. There are also avian influenza viruses causing milder symptoms and these are referred to as low pathogenic avian influenza (LPAI). HPAI viruses are always of the H5 or H7 subtypes, however, there are LPAI viruses within the H5 and H7 subtypes as well. In wild birds throughout the world influenza viruses representing 16 HA and 9 NA antigenic subtypes have been detected in numerous combinations (Fouchier *et al.,* 2005). Low pathogenic influenza A virus strains may, after circulation in chickens or other domestic birds mutate into highly pathogenic influenza strains (Alexander *et al.,* 2000).

Influenza A viruses have been isolated from many species, including humans, pigs, horses, mink, marine mammals and wide range of domestic and wild birds (Murphy and Webster 1996, Webster *et al.,* 1992). Horses and swine are affected by influenza viruses, showing symptoms rather similar to humans. The virus comprise the subtypes H3N8 in equine and H1N1, H3N2 and H1N2 in swine. Equine influenza outbreaks are common also in Sweden but swine influenza appears only to cause milder symptoms in swine and is not commonly noted. The first isolation of high pathogenic influenza virus from feral birds was in 1961 from common terns (*Sterna hirundo*) in South Africa (Becker, 1996), but it was not until the mid-1970s that any systematic investigation of influenza in feral birds was undertaken. These revealed the enormous pools of influenza viruses now known to be present in the wild bird populations. Wild birds such as wild waterfowl, shorebirds and gulls are the main reservoirs of influenza viruses. All influenza virus strains infecting mammalian species originate from wild birds (Webster *et al.,* 1992).

Genome Structure and function

As mentioned earlier influenza is caused by influenza A virus. Influenza A viruses are negative sense, single-stranded RNA viruses, which together with influenza B, C and Thogotovirus, belong to the family *Orthomyxoviridae*. Influenza B can also cause similar symptoms as Influenza A, but generally in a milder form. Influenza C viruses infect mammals only, and generally do not cause disease. They are genetically distinct from A and B types. Of all influenza viruses, the leading cause of significant disease in both humans and animals is infection with influenza A. The following text only describes influenza type A virus. A model of the virus is shown in Figure1.

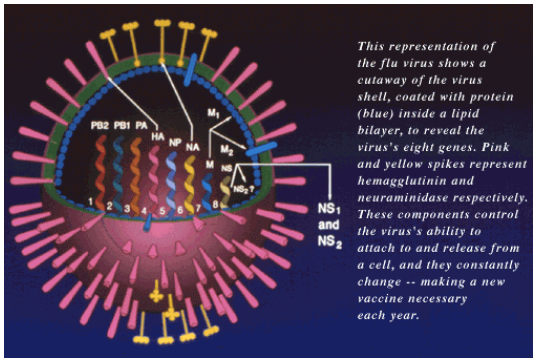

Figure 1. A model of influenza virus, showing the two types of surface proteins, haemagglutinin and neuraminidase, and the segmented genome. Source: www.psc.edu

The viral genome consists of eight segments of single stranded RNA of so-called negative polarity. Each of these segments encodes one polypeptide, except for PB1 segment, which encodes a protein called PB1-F2, and the two smallest ones that encode two proteins each. A total of 11 known proteins are encoded by the genome. The three largest segments (1, 2 and 3) encode polymerase proteins, PB1, PB2 and PA that are responsible for RNA synthesis. Segment numbers 4 and 6 encode surface proteins, hemagglutinin (HA) and neuraminidase (NA) that are involved in attachment, fusion between the viral envelope and cellular membrane, and release of virus particles. The 5th segment encodes a Nucleoprotein (NP) that protects the viral RNA, but has also other functions. The 7th segment encodes the M1 and M2 proteins. M1 is a matrix protein that covers the inner surface of the viral membrane. M2 forms an ion-channel that is important in the early steps of infection by lowering the pH and allows conformation changes if the HA, that then mediates the fusion process. The last segment encodes two proteins: non-structural protein 1 (NS1) and nuclear export protein (NEP), which are involved in various aspects in the process of taking over the host cell. The NS1 protein is not present in the virion but only expressed in infected cells.

Influenza A is one of infrequent viruses that have its genome in separate segments. This increases the potential for recombinants, so-called gene reassortants to form, and may contribute to the rapid development of new influenza strains in nature.

The virus life cycle

The life cycle of influenza virus is summarised in Figure 2. Attachment of influenza A virus requires sialic acid as receptor. However, strains vary in their affinities for different sialyloligosaccharides. For example, avian and equine virus strains prefer sialic acids attached to galactose with α-2,3 linkages (Connor *et al.,* 1994, Rogers and Paulson 1983), while human virus strains prefer the α-2,6 linkages. Hemagglutinin is the viral glycoprotein that binds to the cell receptor, sialic acid. The HA monomer is synthesized as a precursor that is glycosylated and cleaved into HA1 and HA2 subunits in order to successfully infect cells

(Steinhauer, 1999). Receptor-bound viruses are taken into the cell by endocytosis. In the low pH environment of the endosome, viral envelope fuses with the lipid bilayer of the vesicle, releasing the viral ribonucleoprotein (RNP) into the cell cytoplasm, from where it is transported into the nucleus. New viral proteins are translated from transcribed messenger RNA (mRNA), and the viral RNA is replicated from the negative stranded templates. The new viral RNA, is then encapsidated by the NP protein, and after acquiring the new matrix protein, is transported to sites at the cell surface where envelope HA and NA components are incorporated from the cell membrane. Progeny virions are formed and then released from the cell by budding.

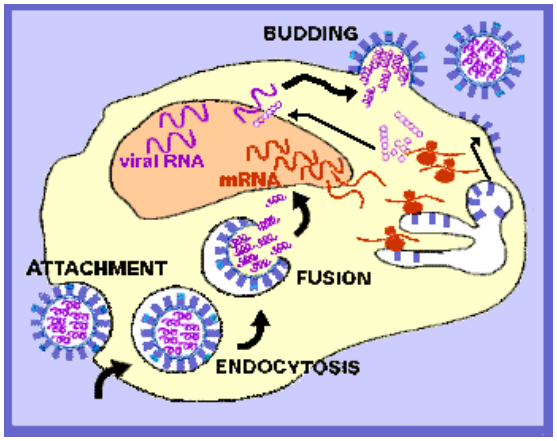

Figure 2. The infection cycle of influenza virus. Adapted from Stannard, 1995.

Influenza virus reservoir (wild birds)

Wild birds such as wild waterfowl, shorebirds and gulls are the main reservoirs of avian influenza viruses. Migratory waterfowl - most notably wild ducks - are the natural reservoir of avian influenza viruses, and these birds are also the most resistant to infection. They can carry the virus over great distances. In their natural host the viruses appear, in most cases, to be non–pathogenic. From this reservoir, virus can be transmitted to domestic birds or other hosts. All known influenza strains infecting humans and mammals originally circulated in the wild bird population (Ito and Kawaoka, 2000; Reid *et al.,* 1999). As everyone is aware of, the aggressive form of the “avian flu” has been detected also in Sweden, with a number of cases in wild birds such as tufted ducks, swans, owl and also a wild mink. To date about 65 cases have been proven positive for the highly pathogenic H5N1 virus.

Aquatic birds are the reservoir of avian influenza virus. In Sweden the swans and tufted ducks were the most affected by HPAI infection. (Photo: Bengt Ekberg and Erik Ågren)

Influenza in domestic birds

Domestic birds such as chickens, turkeys and ducks are highly susceptible to avian influenza virus. Direct or indirect contact of domestic flocks with wild migratory waterfowl has been implicated as a frequent cause of epidemics (Webster *et al.,* 1992). Millions of birds died from the disease or had to be culled in order to stop the outbreaks (Capua, 2001). The outbreaks in domestic birds have increased during the last years (Munster *et al.,* 2005). Live bird markets have also played an important role in the spread of epidemics. Also, water, where infected animals feed and live can have enormous quantities of viruses and act as source of infections.

Influenza A virus transmission to mammalian species

Influenza is not only a human disease as mentioned earlier. It is known to cause several of the most devastating diseases in other mammals as well. Influenza A viruses have been isolated from a variety of mammals, including pigs, horses, sea mammals, and mink (Webster *et al.,* 1992). Examples of bird to mammal transmission of influenza viruses have been reported (Callan *et al.,* 1995; Geraci *et*

al., 1982; Guo *et al.,* 1992; Hinshaw *et al.,* 1986; Hinshaw *et al*., 1984; Klingeborn *et al.*, 1985; Lvov *et al.,* 1978, Scholtissek *et al.,* 1983).

In 1979, an H1N1 avian virus was transmitted to pigs and is still circulating in this species (Pensaert *et al.,* 1981, Scholtissek *et al.,* 1983). In Southern China in 1996, an H1N1 virus was transmitted from birds to pigs (Guan *et al.,* 1996) but it does not appear to be circulating in these hosts at present. Avian virus strains have been found in pigs for example, a H4N6 strain in Canada (Karasin *et al.,* 2000). Viruses other than the H1, H3, N1, N2 and N7 subtypes have not been isolated from pigs.

Transmission of influenza virus to humans occurs occasionally and has often been fatal (Rota *et al.,* 1989). Beginning in 1979, in Europe, human H3N2 and avian H1N1 viruses co-circulated in pigs, and eventually re-assorted in this host (Castrucci *et al.,* 1993) generating a virus with its HA and NA genes from the human H3N2 virus and the remainder from the avian virus. In 1992, this reassortant virus was isolated from ill children in the Netherlands (Claas *et al.,* 1994). This incident support the theory that pigs serve as mixing vessels for the generation of avian-human reassortants, with the potential to cause human pandemics (Scholtissek *et al.,* 1988). In England, an avian influenza with an unusual subtype (H1N7) was isolated (Brown *et al.,* 1994). Two of its genes (NA and M) appear to be of equine origin, while the others seem to have originated in humans. Pigs are liable to variety of influenza viruses and should be monitored regularly.

Transmission of an avian virus to horses was reported in a notable case of severe outbreak of respiratory disease in 1989, causing about 20% mortality (Guo et al., 1992). This new virus was named A/-Equine/Jilin/1/89 (H3N8), but later analyses demonstrated its avian origin. This virus disappeared for unknown reasons. Mink has been found to be naturally infected by avian Influenza A virus of the subtype H10N4 in Blekinge, Sweden (Klingeborn *et al.,* 1985). This was one of the first examples of a direct transmission of avian influenza into a mammalian species (Klingeborn *et al.,* 1985). Interestingly, during the outbreak of H5N1 in wild bird in early spring 2006 in Sweden, this virus has also been isolated from mink. There are also examples when influenza viruses were introduced from avian species to other mammals such as seals (Geraci *et al.,* 1982, Lang *at al.,* 1981) whales (Hinshaw *et al.,* 1984) tigers and leopards (Keawcharoen *et al.,* 2004) and in cats (Yinqst *et al.,* 2006).

Influenza A virus transmission to humans

There are only three known A subtypes of human influenza viruses (H1N1, H2N2, and H3N2). As mentioned earlier, both H3N2 and H1N1 are currently circulating among humans. It is proven that some genes of circulating human influenza A viruses came from birds originally. In fact, the current H3N2 carries 5 genes from the "Spanish flu" influenza virus, while the PB1, HA and NA have been replaced via avian influenza viruses. Influenza A (H5N1) virus – also called H5N1 virus is a subtype that occurs mainly in birds. H5N1 virus does not usually infect people, but infections with these viruses have occurred mostly resulting from direct or

close contact with H5N1 infected poultry or contaminated premises. Recent examples show a transmission of H5N1 virus directly from chicken to humans already in 1997, where 6 out of 18 infected people died (De Jong *et al.,* 1997; Class *et al.,* 1998; Subbarao *et al.,* 1998) and an H7N7 transmission associated with one fatal case (Foucier *et al.,* 2004). The current data as of 2006-11-29 shows a total of 258 confirmed cases with 154 deaths (www. who.int/en). The cases are from Azerbaijan (8/5), Cambodia (6/6), China (21/14), Djibouti (1/0), Egypt (15/7), Indonesia (74/56), Iraq (3/2), Thailand (25/17), Turkey (12/4), Viet Nam (93/42). The death rates are unusually high, about 50 per cent. It is not known whether milder forms of this disease occur.

Pathogenicity of avian influenza in other species

The common pathogenic feature for all HPAI has been the presence of a stretch of basic amino acids in the HA protein, rendering it to be easily cleaved by ubiquitous proteases. The cleaving of HA into two subunits is a requirement for virus infectivity. The situation in mammals cannot be explained by this cleavage ability. There are several examples, like the previously mentioned cases in mink some years ago, where the HA of this virus was not of the "easy cleavage kind" (Feldmann *et al.,* 1988). Another example is the HA of the Spanish flu that do not have this typical hallmark. Obviously, other factors are important in the introduction of influenza into new species and further spread in that species. The current H5N1 outbreak is, however, caused by a typical HPAI HA (Viseshakul *et al.,* 2004; Suarez *et al*., 1998). Thus, the HA may contribute to pathogenicity but is not the sole explanation.

In addition to the "infectivity issue" one major determinant for pathogenicity is the virus' ability to cope with the immune system of the new host. The H5N1 influenza virus that was transmitted to humans in 1997 is an example of this. The virus was highly virulent in humans, and unlike other human and avian viruses, was shown to be resistant to the innate immune system as exemplified by interferon (IFN) and tumor necrosis factor α (TNFα) (Seo *et al.,* 2002). This resistance was attributed to the NS1 protein of the virus. There are more substantial data pointing to a central role of the NS1 in pathogenesis of influenza virus infection (Basler *et al.,* 2001; Zejun *et al.,* 2006). The NS1 of avian influenza has evolved to be resistant to the avian IFN system and TNF-α defense, and opposite. The exact functions of NS1 are unclear at the moment. Several publications have shown that NS1 has many activities, including down regulation of IFN expression (Geiss *et al.,* 2002), binding to and preventing mRNA processing (Noah *et al*., 2003), RNA silencing suppressor (Otilia Delgado *et al.,* 2004; Bucher *et al.,* 2004; Li *et al.,* 2004). In all cases, the effects of NS1 lead to crippled host innate defense. A daunting scenario is that an avian influenza may acquire an extremely potent human, or animal NS1 protein and, thereby, gain the ability to bypass the innate immune defense and cause worldwide pandemics in humans or animals.

Aims of this study

The hypothesis behind this study is that there are differences in the ability of NS1 proteins from influenza viruses isolated from different species to circumvent the host immune response. This could be a major factor in enabling the virus to change host specificity and cross the host species barrier. Thus, the overall aim of this study was to increase the understanding on NS1 and its role in the potential of avian influenza viruses to jump the host species barrier. In particular, the aim was: i) to express NS1 proteins from avian influenza viruses originating from different species, for further biochemical and biological studies; ii) to initiate studies on protein-protein interactions and on shut-down of IFN production.

Results and discussion

Expression of NS1 proteins from AIV originating from different species
A number of NS1 expression constructs were produced, comprising clones of high pathogenic (mink, Eurasian eagle-owl (eagle-owl), low pathogenic avian viruses, and other non-typed viruses of human origin. As shown in Figure 1, in the manuscript all the clones express the NS1 protein, evaluated by correct expected size (26 KDa). NS1 proteins from different AIV subtypes were expressed to high levels in the TNT system. The NS1 from mink and eagle-owl, the HPAI used in this study, were slightly shorter than the ones expressed from the remaining clones (LPAIV). This corresponds to a short, five amino acid deletions in the middle of the protein (Zohari et al., personal communication).
The hypothesis behind this study is that there are differences in the ability of NS1 proteins from avian influenza viruses isolated from different hosts to circumvent the host immune response. This could be a major factor in enabling influenza virus to change host specificity. In particular, we were interested to determine if influenza NS1 proteins originating from different hosts may differ in their ability to interact with the interferon induced and double-stranded RNA activated protein kinase (PKR). This protein has to be an essential component of the IFN-mediated cellular antiviral response (Li *et al.,* 2006). This antiviral effect is achieved through blocking of the viral protein synthesis by phosphorylation of the translation initiation factor eIF-2 (Samuel *et al.,* 1993) NS1 has previously been shown to inhibit this activity.To study this, NS1 proteins from AIV deriving from different host species were expressed in the TNT system for use as prey in GST pull-down assays. PKR proteins from different hosts were also expressed as fusion to GST and used as bait in the assays. The preliminary results of the GST pull-down assays could not be evaluated due to background signals, and the assay is under optimization to enable completion of the studies. Nevertheless, this work has produced tools for use in further studies on the functions of NS1. For example, by using the NS1 clones it was possible to obtain data that indicates that NS1 of LPAI viruses are better at inhibiting interferon production than HPAI virus (Örlen, H, poster presentation to USGBR). This unexpected result is interesting and is currently under more detailed investigation. It has been shown that NS1 of avian influenza A virus binds PKR and blocks its activation during virus infection (Shoudong et al., 2006). The completion of the

experiments from the present studies will be important to determine whether the established interaction is species specific, and if it differs between high and low pathogenic avian influenza viruses.

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Research report 1

Expression of the non-structural protein of avian influenza

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Key words: influenza virus, NS1, PKR, avian flu

Abstract

Avian influenza is a potential threat to many species including man. The viral gene encoding the NS1 protein is presumed to be an important factor for evasion of the innate immune system and, therefore, important for a successful transmission of the virus to a new host. The exact mechanism behind this function of NS1 is still unclear. Previous studies have shown that NS1 has different effects in the immune system, among them down-regulation of interferon (IFN) expression, inhibition of activation of the RNA dependent protein kinase (PKR). The aim of this study was to investigate whether these effects are species specific. NS1 proteins from avian influenza viruses originating from different hosts, as well as PKR proteins of horse, human and chicken origin were expressed. These were used to initiate studies on NS1-PKR interactions and on shutdown of IFN production. The completion of the experiments from these studies will be important to determine whether the interaction is species specific, and if it differs between high and low pathogenic avian influenza viruses.

Introduction

Influenza is caused by Influenza A virus, which together with Influenza B and C and Thogotovirus, belongs to the family *Ortomyxoviridae*. Of all influenza viruses, the leading cause of significant disease in both humans and animals is Influenza A infection. Influenza A viruses have the potential to cause devastating pandemics (Palese, 2004) and may cross species barriers as a whole and adapt to a new host (Ludwig *et al.,* 1995; Webster *et al.,* 1981).

The viral genome consists of eight segments of single-stranded RNA of negative polarity. Each of these segments encodes one polypeptide, except for PB1 segment, which encodes a protein called PB1-F2, and the two smallest ones that encode two proteins each. A total of 11 known proteins are encoded by the genome. The three largest segments (1, 2 and 3) encode polymerase proteins, PB1, PB2 and PA that are responsible for RNA synthesis. Segment number 4 and 6 encode surface proteins, hemagglutinin (HA) and neuraminidase (NA) that are involved in attachment, fusion between the viral envelope and cellular membrane, and release of virus particles. The 5th segment encodes a Nucleoprotein (NP) that protects the viral RNA, but has also other functions. The 7th segment encodes the M1 and M2 proteins. M1 is a matrix protein that covers the inner surface of the viral membrane. M2 forms an ion-channel that is important in the early steps of infection by lowering the pH and allows conformation changes if the HA that then mediates the fusion process. The last segment encodes two proteins; non-structural protein 1 (NS1) and nuclear export protein (NEP), which are involved in various aspects in the process of taking over the host cell. The NS1 protein is not present in the virion but only expressed in infected cells.

Influenza A is classified in subtypes, based on antigenic and genetic differences in the two viral surface proteins, HA and NA. Sixteen different HA subtypes (H1-

H16) and nine NA (N1-N9) subtypes have been identified. From avian species all 16 HA and 9 NA subtypes have been isolated in the majority of possible combinations (Alexander, 2000). The most recent subtype (H16) was isolated from black-headed gulls caught in Sweden and the Netherlands in 1999 (Fouchier, 2005).

Influenza A viruses have been isolated from many species, including humans, pigs, horses, mink, marine mammals and wide range of domestic and wild birds (Fouchier *et al.,* 2005; Webster *et al.,* 1992). Wild birds, predominantly ducks, geese and shorebirds form the reservoir of influenza A viruses in nature. The viruses do not cause disease in the reservoir, however when in contact with susceptible hosts, infection is transmitted and may result in devastating disease.

To be able to successfully transmit into the new host avian influenza has to cope with the immune system of the new host. Several studies have shown that a viral gene, coding for the NS1 protein is involved in different roles including down-regulation of IFN expression (Geiss *et al.,* 2002), binding to and preventing mRNA processing (Noah *et al,* 2003), RNA silencing suppressor (Otilia-Delgado *et al.,* 2004; Bucher *et al.,* 2004). All the described effects of NS1 lead to crippled host innate defence, suggesting an important role of NS1 in immune evasion. Therefore, understanding the functions of the NS1 protein is needed in order to elucidate the mechanism of immune evasion and to determine the factors behind jumping of host species barrier.

Materials and Methods

Viruses
Avian influenza viruses, representing both HPAI and LPAI subtypes and originating from different host species (wild birds, mink, eagle-owl, chicken and human) were used in this study. The viruses were obtained from NVI, either as alantoic fluid preparations, that were used as source of RNA, or as extracted RNA (HPAI and other non-typed viruses of human origin). The samples included: H7N7, H4N6, H12N5, H6N8, H5N1, among others.

RNA-extraction
RNA was extracted with TRIzol LS Reagent (Invitrogen,USA), according to the manufacturer's instructions. Briefly, 250µl of samples were mixed with 750 µl of TRIzol and incubated for 5 minutes at room temperature. Afterwards, 200µl of chloroform was added, and the tubes were shaken vigorously for 15 sec and then centrifuged at 13 000 rpm (Sigma, Germany) for 15 minutes. The RNA was collected from the supernatant and precipitated overnight with 500µl of isopropanol at -20°C. The precipitated RNA was centrifuged at 13 000 rpm for 20 minutes at 4°C and then washed with cold 75% ethanol. The resulting pellets were air dried, re-suspended in 30µl of DMPC water and stored at -20°C for further use.

Synthesis of cDNA
A mix of 5µl RNA, 2µl of pdN6 (1:10) and 3µl of DMPC water was denatured at 65°C for 10 minutes and then chilled on ice. The reverse transcription mix

comprised 5µl of 5×first standard buffer (Invitrogen), 2µl of DTT, 2.5µl of mM dNTP mix (Amersham Biosciences) 24U of RNA guard (Amersham Biosciences) and 200U of Moloney murine leukaemia virus reverse transcriptase. Transcription was performed at 37°C for 90 min followed by the inactivation of enzyme at 95°C for 5 min.

PCR for amplification of the NS1 gene of AIV
The reaction mixture of 50µl, comprised 30µl of sterile distilled water; 5 µl of 10×PCR buffer; 1µl of 10mM dNTP mix; 2µl of each 10µM primer; 3µl of 25 mM MgCl; 1U of Taq DNA (Ampli Taq, Perkin-Elmer); and 5 µl of cDNA. Thermocyling profile included initial denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 94°C for 45sec; annealing at 56°C for 60 sec; and extension at 72°C for 90 sec and a final extension at 72°C for 7 min. These were amplified on a PTC-200 Peltier Termal Cycler machine.

RT-PCR for amplification of PKR from different cell types (chicken, human, horse)
Extraction of RNA was done using the Trizol reagent, as described before, from lysates of a B cell line, HEK 293T, and equine dermal cells, as source of chicken, human and equine PKR, respectively. Synthesis of cDNA was also performed as described before, except that a reverse primer specific for each PKR type was used. The reaction mixture for the PCR contained 33.5µl of sterile distilled water, 5 µl of 10×PCR buffer, 1µl of 10mM dNTP mix, 2µl of each 10µM primer, 4µl of 25 mM MgCl; 2U of Taq DNA polymerase (Ampli Taq, Perkin-Elmer); and 2.5 µl of cDNA. The thermocyling profile included initial denaturation at 95°C for 2min, 95°C for 30sec; annealing at 59°C for 30 sec; 72°C for 1.30min-30 cycles, 72°C for 7 min final extension and 4°C hold.

All the primers used in the RT-PCRs are listed in Table 1

Table 1. Primers used in RT-PCR amplifications

Name	**Sequence (5'-3')**	**Purpose**
NS1-AllFORECO	CCGAATTCAGCAAAAGCAGGGTGACAAAG	NS1PCR forward
NS1ALLBACKXBA	CCTCTAGAAATAGAAACAAGGGTGTTTAT	NS1PCR reverse
NS1 FKPN	ATTCGGTACCAGCAAAAGCAGGGTGACAAAG	NS1PCR forward
NS1RXBA	TACCTCTAGAAGTAGAAACAAGGGTGTTTTTTAT	NS1PCR reverse
CHPKF	CGTGGATCCATGGACCGAGAGTGCATG	Chicken PKR PCR forward
CHPKR	TACCTCGAGTTAGTGACTGTAAGC	RT, Chicken PCR, reverse
HUPKF	CGTGGATCCATGTCCCAGAGCAGG	Human PKR PCR forward
HUPKR	TACCTCGAGTTACTTTCTTTCTGCTAT	PT, human PKR PCR, reverse
For horse were used human's primers		

The PCR products (5µl) were run on 1% an agarose gel stained with ethidium bromide and visualized under UV light. They were then purified using the SV Wizard DNA Purification System (Promega).

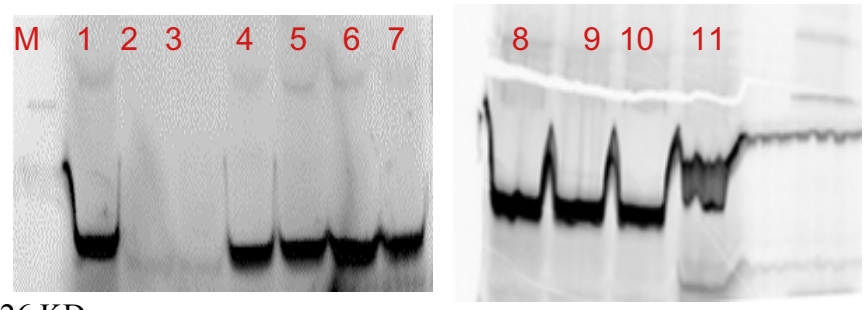
Cloning of NS1 DNA in pcDNA 3.1+ and of PKR DNA in pGEX-5T
The NS1 PCR products and the plasmid pCDNA-3.1+ were cleaved with *EcoRI* and *XbaI*-restriction enzymes (or *KpnI-XbaI* for NS1 DNA that had an internal *EcoRI* cleavage site). The PKR PCR products and the plasmid PGEX-5T were cleaved with *BamHI* and *XhoI* restriction enzymes. All DNA cleavages were performed following a standard protocol. The digested DNAs were ligated to the corresponding vector, using T4 DNA ligase and overnight incubation at 16°C. The ligation reactions were used to transform competent DH5α cells, following a standard procedure for chemical transformation. Recombinant clones were screened by colony PCR with the same primers used generate the respective PCR products. Three positive bacteria colonies selected from each transformation were used for purification of plasmid mini-preps, using the SV Wizard Plus SV Minipreps DNA Purification System (Promega). The mini-preps were then cleaved by restriction enzymes to check for presence and correct orientation of the inserts.

Protein expression
The confirmed NS1 clones were translated *in vitro* in the coupled transcription/translation system (TNT) from Promega, following the manufacturer's instructions, and the option of labeling translation products with ^{35}S methionine. The GST-PKR proteins were expressed in *E coli* BL21 cells. Optimization of expression conditions was performed by evaluating protein production at different time-points, at different temperatures and with different concentration of IPTG as inducer.

GST pull-down analysis
The assays have been described earlier (Berg et al., 1998). In brief, the TNT expressed NS1 and GST-PKR were mixed in a small volume in phosphate buffer. The proteins were incubated in room temperature for 30 minutes. Then GST-sepharose beads were added and incubated in a larger volume for three hours. The bound proteins were then retrieved on the beads, washed and analyzed on SDS-PAGE

Results

Expression of NS1 proteins from AIV originating from different hosts
A number of NS1 expression constructs were produced, comprising clones of high pathogenic (mink, eagle-owl), low pathogenic avian viruses, and other non-typed viruses of human origin. As shown in Figure 1, all the clones express the NS1 protein, evaluated by correct expected size (26 KDa). NS1 proteins from different AIV subtypes were expressed to high levels in the TNT system. The NS1 from mink and eagle-owl, the HPAI used in this study, were slightly shorter than the ones expressed from the remaining clones (LPAIV). This corresponds to a short, five amino acid deletions in the middle of the protein (Zohari et al., personal communication).



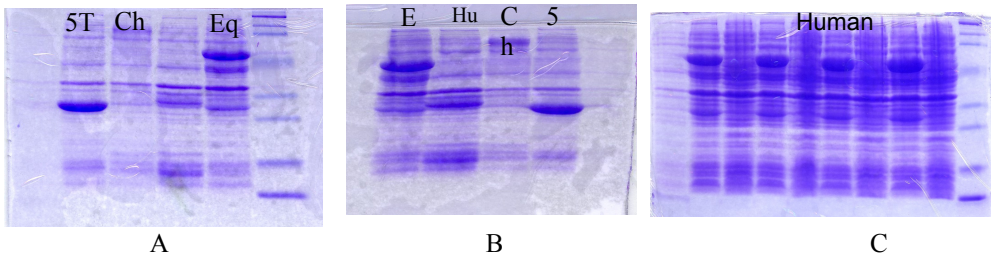
26 KDa <26 KDa

Figure 1. Protein expression from NS1 constructs. NS1 genes of different AIV were cloned in pCDNA 3.1+ and translated *in vitro* using the TNT system.

M: marker; lanes 1 and 11:NS1 control plasmid; lane 2:TNT reaction control, no plasmid; lane 3:pGFP-C3 plasmid; lane 4:pNS1-5, lane 5:pNS1-4; lane 6:pNS1-3; lane 7:pNS1-1; lane 8:pNS1-mink; lane 9:pNS1- eagle-owl; lane 10:pNS1-mink.

Expression of GST-PKR fusion proteins

The PKR genes from chicken, human and horse were cloned as fusions to GST in pGEX-5T plasmid. They were expressed in *E. coli* and subsequently tested for expression level, solubility and possibility to be purified on GST-beads. As shown in Figure 2, fusion proteins of the expected size were expressed. However, the level of expression and solubility was different between the chicken, human and horse PKR. While high expression levels were obtained for human PKR, protein production was lower from horse constructs, and poor from the chicken, on repeated experiments at different expression conditions. Nevertheless, the solubility of the human and the horse PKR protein was good and suitable for use in GST- pull-down experiments.



A B C

Figure 2. Expression of GST- PKR fusion proteins. PKR genes were cloned as fusions to GST and expressed in BL 21 cells. The human PKR gel (Panel C) shows a time course evaluation of protein production, with and without inducers.

GST pull down to determine interaction between NS1 and PKR
An initial GST-pull-down experiment was done using GST-PKR protein bound to beads and then the various TNT expressed NS1 protein were added to the beads. The proteins were incubated and their interaction assessed after washing the beads thoroughly and analyzing the protein by scanning SDS-PAGE gels for radiolabel signal. Plain GST protein was used as a negative control. A signal for NS1 could be detected in the gels. However, there was a background signal in the negative control, which prompted need to optimize the assay to reduce the non-specific binding. Dilution of the prey and bait proteins was used in the optimization.

Discussion

The hypothesis behind this study is that there are differences in the ability of NS1 proteins from avian influenza viruses isolated from different hosts to circumvent the host immune response. This could be a major factor in enabling influenza virus to change host specificity. In particular, we were interested to determine if influenza NS1 proteins originating from different hosts may differ in their ability to interact with the interferon induced and double-stranded RNA activated protein kinase (PKR). To study this, NS1 proteins from AIV deriving from different host species were cloned into an eukaryotic expression vector. This strategy will most likely render the proteins in an active form. To verify the expression of the various NS1 genes we utilized the TNT expression system. These TNT- produced proteins were then used as prey in GST pull-down assays. PKR proteins from different hosts were also expressed as fusion to GST and used as bait in the assays. The preliminary results of the GST pull-down assays could not be evaluated due to background signals, and the assay is under optimization to enable completion of the studies. Nevertheless, this work has produced tools for use in further studies on the functions of NS1. For example, by using the NS1 clones it was possible to obtain data that indicates that NS1 of LPAI viruses are better at inhibiting interferon production than HPAI virus (Örlen, H, poster presentation to USGBR). This unexpected, but most interesting finding is currently investigated in great detail. It has been shown that NS1 of avian influenza A virus binds PKR and blocks its activation during virus infection (Shoudong et al., 2006). The completion of the experiments from the present studies will be important to determine whether the established interaction is species specific, and if it differs between high and low pathogenic avian influenza viruses.
Furthermore, we have made reagents that will be used for further studies of RNA interference and many other aspects of NS1 function.

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