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Studies on reverse genetic systems for avian influenza virus and the Borna disease virus

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Zusammenfassung

Aviäre Influenza A Viren (AIV), wie alle Influenza Viren, und das Virus der Bornaschen Krankheit (BDV) sind Negativstrang RNA-Viren, die ihr Genom im Kern der infizierten Zelle replizieren und transkribieren. Influenza A Viren infizieren Säuger und Vögel und AIV haben weltweit zu enormen Verlusten und Schäden in der Geflügelindustrie geführt. Außerdem stellen sie eine ständige Bedrohung der menschlichen Gesundheit dar. So wurde 1997 das aviäre Influenza A Virus vom Subtyp H5N1 direkt von Vögeln auf den Menschen übertragen und führte zum Tod von 6 von 18 infizierten Menschen in HongKong. BDV kann eine Vielzahl von Warmblütern infizieren und führt zu einer persistenten Infektion des zentralen Nervensystems des infizierten Tieres, welche eine neuropathologische Erkrankung auslösen kann.

Reverse Genetik-Systeme haben sich als nützliche Werkzeuge für die Analyse des viralen Replikationzyklus, der regulatorischen Funktion viraler Proteine und molekularer Pathogenitätsmechanismen erwiesen. Wissenschaftler haben sehr viel an reversen Genetik-Systemen für humane Influenza Viren gearbeitet, aber kaum für AIV. BDV ist immer noch eine mysteriöses Virus, zu dem es noch viele ungeklärte Fragen gibt. In dieser Studie habe ich versucht ein reverses Genetik-System für das aviäre Influenza Virus A/Goose/Guangdong/1/96 (H5N1) und BDV zu etablieren.

In dem ersten Teil meiner Arbeit wurden zur Erstellung eines reversen Genetik-System acht Pol I-Plasmide konstruiert, welche die kompletten cDNAs der acht Genomsegmente von A/Goose/Guangdong/1/96 (H5N1) enthalten. Da bei Arbeiten mit Influneza Viren vom Subtyp H5N1 die biologische Sicherheit berücksichtigt werden muß habe ich versucht Reassortante Viren zu generieren, welche als genetischen Hintergrund jeweils sieben Gensegmente des aviären Influenza Virus A/FPV/Rostock/34 (H7N1) (FPV, Laborstamm) und eins von A/Goose/Guangdong/1/96 (H5N1) enthalten. Hierdurch sollten meine neuen Plasmide funktionell getestet werden. Mindestens fünf Plasmide waren funktionsfähig, wie sich sowohl durch direkte (CAT-Analyse) als auch indirekte Untersuchungen (Erzeugung eines Reassortanten Virus) zeigen ließ.

Das Reassortante Virus GD1NSFPV, welches das NS-Segment von A/Goose/Guangdong/1/96 (H5N1) und die übrigen Segmente von FPV enthält, unterscheidet sich in seinen Vermehrungseigenschaften signifikant von dem Wild Typ FPV. Zur Untersuchung worin dieser Unterschied begründet liegt, habe ich die virale Induktion der Raf/MEK/ERK-Signalkaskade durch beide Viren analysiert, da diese zelluläre Kaskade für die Bildung infektiöser Viren wichtig ist. Da das NS1-Protein ein wichtiger Pathogenizitätsfaktor der viralen Replikation ist habe ich auch das NS1-Protein beider Viren näher untersucht. Die Ergebnisse zeigten keine großen Unterschiede in der Aktivierung der Raf/MEK/ERK-Kaskade, was darauf hindeutet, daß hierin kein wichtiger Grund für die unterschiedlichen Wachstumseigenschaften der beiden Viren liegen kann. Mit der Hilfe von zwei Proteindomänen übt das NS1-Protein seine proviralen Funktionen aus. Teilweise durch

Bindung freier RNA (dsRNA/mRNA), wodurch die Aktivierung zellulärer Verteidigungsmechanismen verhindert wird und die Translationseffizienz zellulärer mRNA zum Vorteil der Virusvermehrung reduziert wird. Die Aminosäuresequenz des NS1-Proteins von GD1NSFPV unterschiedet sich von der des FPV-NS1 speziell in der RNA-Bindungs- und in der Effekor-Domäne. Darüber hinaus resultieren dieses Unterschiede in einer Änderung der Hydrophilizität beider Proteine. Außerdem zeigte es sich, daß das reassortante GD1NSFPV Virus deutlich effizienter die zelluläre Interferonexpression unterdrückt, welche ein wichtiger initialer Schritt in der Etablierung der zellulären Immunität darstellt. Trotzdem muß der eigentliche Grund für die unterschiedlichen Vermehrungseigenschaften noch genauer bestimmt werden. Die Studien zur Pathogenität der Reassortante GD1NSFPV werden zukünftig durch Tierexperimente in China untersucht.

Im zweiten Teil meiner Arbeit habe ich, auf Grundlage neuer Daten (die Existenz eines „A"-Nukleotids am äußerten 3´-Ende der genomischen Einzelstrang-RNA von BDV und einem „U"-Nukleotid am 5´-Ende), neue Pol I-Expressionskonstrukte erstellt, die ein Reportergen (CAT) expremieren. Die Pol I-Transkripte beginnen mit einer „Hammerhead" Ribozymsequenz (HHR), die mit einem „A" startet, da die RNA-Polymerase I normalerweise nicht „U" als erstes Nukleotid einbaut. Ich konnte zeigen, daß die HHR-Versionen die Transkripte in cis schneiden und so neue 5´-Enden generieren, die mit dem genomischen „U" als erstes Nukleotid beginnen, und das die korrekten 3´-Enden der Transkripte durch ein Hepatitis Delta Virus-Ribozym (HDV) erzeugt werden, welches ebenfalls in cis schneidet. Darüber hinaus konnte ich zeigen, daß die korrekten Enden auch in vivo durch die beiden Ribozyme erzeugt werden, und das die BDV-Polymerase das antigenomische Mini-Transkript in einem BDV-abhängigen System repliziert und transkribiert. Weiterhin zeigen RPA-Ergebnisse, daß das putative Polyadenylierungssignal in der 3´-Nichtkodierenden-Region (NCR) wirklich von der viralen Polymerase genutzt wird. Zusätzlich konnte ich zeigen, daß das von dem Pol I-Expressionkonstrukt erzeugte Mini-Genom in einem plasmidbasierten reversen Genetik-System funktionell ist und dabei publizierte Daten bestätigen die zeigen, daß dieser Prozeß von einem empfindlichen Verhältnis zwischen BDV-Proteinen N und P abhängt.

Da AIV vom H5N1-Subtyp von großer Bedeutung für die Geflügelwirtschaft sind wird meine Arbeit helfen einen Grundlage für zukünftige Arbeiten über die Pathogenese dieser Viren zu legen und könnte zu neuen Antigenen für die Vakzinierung führen. Speziell die Analyse der Unterschiede in der Pathogenität zwischen der Reassortante GD1NSFPV und dem Wild Typ FPV werden helfen die besondere Rolle von NS1 für den viralen Replikationszyklus zu beleuchten.

Obwohl zwei reverse Genetik-Systeme während des Abschlusses meiner Arbeiten publiziert worden unterscheiden sich meine Mini-Genome in der spezifischen Nukleotidsequenz der cis-aktiven NCRs. Da diese von besonderer Bedeutung für die Replikation und Transkription des BDV-Genoms sind, war es wichtig zu zeigen, daß die vorhergesagten NCRs funktionell sind.

Meine Arbeit könnte somit die Basis für verbesserte reverse Genetik-Systeme für beide Viren (AIV und BDV) sein und helfen den Charakter dieser Viren besser zu verstehen.

Summary

Both, avian influenza virus (AIV) and the Borna disease virus (BDV), are negative-strand RNA viruses, that replicate and transcribe their genomes in the nucleus of infected cells. Influenza A viruses can infect mammals and all species of birds and AIV have caused enormous losses and heavy shocks to avian industries worldwide. These viruses are also potential threats to human health. Specifically, the avian H5N1 influenza virus was directly transmitted from birds to humans and led to the death of 6 of 18 infected people in Hongkong in 1997. BDV can infect a variety of warm-blooded animals and cause a persistent infection in the central nervous system of infected animals, which can lead to neuropathological disease.

Reverse genetic systems have proved that they are a very useful tools to analyze the viral life cycle, the regulatory function of viral proteins and molecular mechanisms of viral pathogenicity. Scientists have done a lot of research on reverse genetic systems for human influenza virus, but less on AIV. BDV is still a mysterious virus, about which there are many open questions that are not answered. In this study I tried to establish a reverse genetic system for the avian influenza virus A/Goose/Guangdong/1/96 (H5N1) and for BDV, respectively.

In the first part of my work, eight Pol I plasmids including the complete cDNAs of the eight segments of the A/Goose/Guangdong/1/96 (H5N1) have been constructed to establish a reverse genetic system for the A/Goose/Guangdong/1/96 (H5N1). As the biological safety has to be considered, I tried to rescue reassortant viruses using the genes of the strain A/FPV/Rostock/34 (H7N1) (FPV) as a genetic background with only one gene of A/Goose/Guangdong/1/96 (H5N1) in order to test that every plasmid with a complete cDNA of the strain A/Goose/Guangdong/1/96 (H5N1) virus is functional. At least five plasmids were proved to be functional either by a direct (CAT-assay) or indirect (generation of a reassortant virus) method.

The rescued reassortant GD1NSFPV virus, that carries the A/Goose/Guangdong/1/96 (H5N1) NS gene and the other 7 genes of FPV, differs in its growth characteristics significantly from the wild type virus FPV. In order to analyze why the reassortant GD1NSFPV is different from the wild type FPV, I investigated the induction of the Raf/MEK/ERK cascade with both viruses, as this cascade is important for the formation of infectious virus. Also I compared the NS1 protein of both viruses, as it is a major viral factor for the pathogenicity and replication efficiency of influenza A viruses. The results showed that there are no big differences in the activation Raf/MEK/ERK cascade between both viruses, which therefore can not be an important reason for the different growth characteristics of both viruses. With the help of two protein domains, the NS1 protein exerts its proviral functions in part by binding free RNA (dsRNA/mRNA) thereby preventing the activation of cellular defense mechanisms and reducing the translation efficiency of cellular mRNAs for the benefit of viral replication. The amino acids sequence of the NS1 protein of GD1NSFPV differs from that of FPV, specially in the RNA binding domain and in the effector domain. Moreover the differences in the RNA

binding domain and the effector domain of both NS1 proteins result in a difference of hydrophilicity of both proteins. Furthermore the reassortant GDNS1FPV virus more effectively prevented the cellular interferon expression, which is an important initial step to establish cellular/innate immunity. Nevertheless the key element that leads to the different growth characteristics of both viruses remains to be determined. The studies about the pathogenicity of the reassortant GD1NSFPV virus will be performed by animal experiments in future work in China.

In the second part, according to new data for the BDV genomic 5'- and 3'-ends (the existence of an additional "A" residue at the extreme 3'-end of the single-strand genomic RNA of BDV and a "U" residue probably to be found at the 5'-end), I have generated Pol I expression constructs expressing a reporter transcript (CAT gene). They start with a hammerhead ribozyme sequence beginning with an "A" residue, because the RNA polymerase I normally does not incorporate an "U" as the first residue. I could show that the hammerhead ribozyme cleaves the transcript in vitro in cis to generate a new 5'-end that starts with the genomic "U" residue, and that the correct 3'-end of the transcript is generated by a hepatitis delta virus (HDV) ribozyme that also cleaves the transcript in cis. Moreover I was able to show that the correct 3'- and 5'-ends of the mini-genome were also generated by the two ribozymes in vivo, and that the BDV polymerase can use the antigenomic mini-transcript for replication and transcription in a BDV-dependent system. Furthermore the RPA results indicate that the putative polyadenylation signal in the 3'-non coding region (NCR) is indeed used by the viral polymerase. I also showed that the mini-transcript generated by the Pol I expression construct is functional in a plasmid-based reverse genetic system, and I could confirm published results demonstrating that this process depends on the delicate ratio of the viral N and P proteins.

As AIVs of the H5N1 type are of major important for poultry production, my work will help to provide the basis for future work on the pathogenicity of these viruses and could lead to designed immunogens for vaccination. Specially an analysis of the differences in the pathogenicity between the reassortant GDNS1FPV and the wild type FPV will help to elucidate the specific role of the viral NS1 protein for the viral replication cycle.

Even though two reverse genetic systems for BDV have been published during the completion of my work on an according system, my mini-genome differs in the specific nucleotide sequence of the cis-active NCRs. As these are important for BDV-genome replication and transcription, it was important to show that the predicted NCR-sequences are functional. My work could therefore be a basis for improved reverse genetic systems for both viruses and might help us to understand the character of these viruses.

Introduction

1. Avian influenza and influenza A virus

1.1 Avian influenza

Avian influenza (AI) is an infectious disease caused by type A influenza virus. This disease can affect all species of birds and the symptoms of this disease can vary from a mild disease with little or no mortality to a highly fatal epidemic depending on the virus strains and host species. The highly pathogenic avian influenza (HPAI) has been classified as a " list A " disease by the O.I.E (Office International des Epizooties, the World Organization for Animal Health).

1.1.1 History

Avian influenza was firstly described in 1878 by Perroncito et al. as a serious disease of chicken in Italy, at that time this disease was called "fowl plague". The causative agent of this disease was isolated from a chicken in 1902, marking the first documented isolation of influenza virus (92). The first human influenza virus was isolated in 1933 (204). It was determined in 1955 that fowl plague virus is actually an influenza A virus (2). Only type A influenza viruses are known to cause natural infections of birds and this type also is the most common type leading to serious epidemics in humans, type B and C do not affect domestic animals. Influenza A viruses are divided into different subtypes based on the antigenic nature of their hemagglutinin (HA) and neuraminidase (NA) glycoprotein (116). Up to now 15 subtypes of HA (H1-H15) and 9 subtypes of NA (N1-N9) have been identified. But it is possible that all 15 HA and 9 NA subtypes of influenza A viruses can combine new viruses to infect avian species naturally.

1.1.2 Current situation

Since the first AI was reported in Italy in 1878, up to now many occurrences of AI were described in different areas of the USA (1983 - 1984, 1986, 1995), Mexico (1994-1995), England (1963, 1979, 1991), Germany (1979,1995 - 1996, 1998), Australia (1992, 1995, 1997), Ireland (1983 - 1984, 1997), Italy (1994, 1999), Iran (1998), China (1994), South Africa (1995), Pakistan (1994-1995, 1999), Hongkong (1997, 1999) (2). AI has caused enormous losses and heavy shocks to avian industries worldwide, and it still threats and destroys seriously the avian industries of the world. A low pathogenic avian influenza (LPAI) H7N2 has caused the infection of more than 4.7 million chickens and turkeys and about 4 million were destroyed in Virginia poultry farms in 2002 (148). An epidemic of HPAI H7N7 happened in the Netherlands, Belgium and Germany in 2003, it caused that more than 20 million chickens, expected to cost hundreds of millions of dollars, had to be slaughtered (1, 57). At the same time it also infected more than 80 people and one veterinarian died (57, 229).

Therefore, AI is a constant threat to the poultry industry, and avian influenza viruses are also potential threats to human health.

Aquatic birds are believed to be the natural host and reservoir of influenza A virus, they play a key role in spreading virus and leading to the infection of other birds and other species. Avian influenza viruses in wild aquatic birds are spread to susceptible birds through inhalation of viral particles in nasal and respiratory secretions and from contact with the feces of infected birds. Influenza A viruses infecting poultry can be divided into two groups on the basis of their pathogenicity: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The highly virulent viruses cause mortality in poultry as high as 100%. To date, all highly pathogenic isolates have been influenza A viruses of subtypes H5 and H7, although not all viruses of these subtypes can cause HPAI. Until the end of 20 century, 17 outbreaks of HPAI were reported in different countries and areas (2). Other AI viruses cause LPAI, the clinical signs are much less evident or absent and mortality is much lower. Sometimes environmental conditions or secondary infections may cause exacerbation of LPAI infection and result in more serious disease.

The HA glycoprotein on the surface of influenza A virus is synthesized as a precursor polypeptide (HA0) (30), which plays a central role in the pathogenicity of AIVs (207). In order to activate membrane fusion function and virus infectivity, the precursor HA0 must be cleaved into the disulfide linked subunits HA1 and HA2 (107, 120), so HA0 cleavage is regarded as a major determinant of pathogenicity. There are special molecular differences in the HA glycoprotein between HPAI and LPAI viruses. HA proteins of LPAI virus have a single arginine at the cleavage site (17). These viruses are limited to HA cleavage by extra cellular proteases and the viral replication is restricted in the host. HPAI viruses possess multiple basic amino acids at their HA cleavage sites and appear to be cleavable by an ubiquitous protease (17). These viruses are able to replicate in most tissues throughout the bird and cause death (181).

During the epidemic of avian influenza in the past time, the outbreaks of H5 and H7 HPAI have caused enormous losses to poultry industries worldwide. In 1983-1984 a epidemic of H5N2 HPAI occurred in Pennsylvania in United States (29) and resulted in the destruction of more than 17 million birds. It took more than 2 years to eradicate the disease by quarantine and slaughter, at a direct cost of more than $60 million and an indirect cost to the industry of more than $250 million. In 1994 another outbreak of H5N2 HPAI occurred in Mexico (94, 215), 18 million birds had to be slaughtered and it was eradicated from chickens by quarantine and use of inactivated vaccine, at the cost of more than $100 million. Avian influenza viruses (AIV) affect not only the poultry industries, but also are potential threats to the human health. Up to now three different subtypes of AIV have been detected in humans. In 1996, in England a H7N7 virus of avian origin was isolated from the eye of a woman with conjunctivitis who kept ducks (6). The most serious cases occurred in 1997 in Hongkong, 18 people were infected by a H5N1 virus of avian origin and 6 of them died (203). H9N2 viruses of avian origin were isolated from humans in China in 1999 (75, 78). Subsequently two

independent isolates of H9N2 virus of avian origin were obtained from two people who recovered from flu-like-illness in Hongkong (156). In February 2003, the same subtype H5N1, as in 1997 in Hongkong, was isolated from two members of one family in Hongkong. One of them died and other members of the same family were affected. Recently HPAI H7N7 broke out in Netherlands on March 2003 and spread to Belgium and Germany. It has caused the death of one veterinarian and 82 farmworkers and slaughtermen were affected by this avian flu virus (57, 229). Affected people have shown symptoms ranging from conjunctivitis to mild flu (57). It should be noticed that this virus can spread from human to human in a limited fashion, because the wife and child of one infected man were also infected, who had no direct contact with poultry (57).

Normally pigs play very important role as "mixing vessel" for the transmission of influenza virus between birds and humans (27). Since they are susceptible to infection with both avian and human influenza A virus, and are often involved in interspecies transmission, facilitated by regular close contact with human and birds. AIV HAs have receptor specificity for 2, 3-galactose sialic acid and human influenza virus for 2, 6-galactose sialic acid that are predominantly found in the according host, whereas pigs have both 2, 3- and 2, 6-galactose sialic acid linkages in the cell lining of the trachea. This can lead to modification of the receptor binding specifcities by mutation of avian influenza viruses from 2, 3 to 2, 6 linkage (98, 99) , that is the native linkage in humans (39). This provides a potential link from birds to human. But the avian influenza viruses can also overcome the species barrier and be transmitted to humans from birds directly without any intermediate (pig), for example H5N1 in Hongkong, H9N2 in China and H7N7 in Netherlands as mentioned above. Last not least reassortant viruses can form, that are able to replicate in humans but carry avian influenza HA proteins. This can lead to pandemic outbreaks.

1.1.3 Clinical symptoms

The main symptoms of HPAI in poultry are depression, loss of appetite, cessation of egg laying, nervous signs, swelling and blue discoloration of combs due to disturbance of blood circulation, ruffled feathers, severe respiratory signs and diarrhea. Sudden death can occur without any previous signs. Death occurs as early as 24 hours after the first signs of disease, and frequently with 48 hours, or can be delayed for as long as a week. The mortality rate can reach up to 100% depending on the species, age, the virus type involved and environmental factors. The clinical symptoms of LPAI consist of mild respiratory disease, depression and drop in egg production in laying birds.

1.2 Influenza A virus

1.2.1 Morphology and genome structure of influenza A virus

The virions of influenza A virus can exhibit a variety of shapes and sizes, ranging from spherical particles with a diameter about 100 nm to elongated filamentous forms of the virus. The virion is made up of a lipid envelope derived from the plasma memberane of host cells

(69) and nine viral structure proteins (Table 1 and Figure 1). The HA and NA glycoproteins are attached to the lipid envelope by short sequence of hydrophobic amino acids. The HA is responsible for binding of the virus to cell surface receptors (sialic acid) and for membrane fusion during virus entry into hosts cells. The function of the NA is to cleave sialic acid from the virus and host cells at the end of virus life cycle in order to allow mature virions to be released. Within the lipid envelope lies the integral membrane matrix protein M2, which is an ion channel that modulates the pH of intracellular compartments (35, 214). The viral matrix protein M1 is thought to play an important role in the process of assembly and budding. Perhaps M1 interacts with ribonucleoproteins (RNPs) (96, 139), the exact mechanism is still not known. The NS2 protein was originally designated as nonstructural protein. NS2 acts as nuclear transport factor (127, 149) and has been proved to be a minor component of virions (130, 133, 172). NS1 is an another nonstructural protein, which is a regulator of both mRNA splicing and translation and also modulates the interferon responses to viral infection (186, 236, 256). M1 and NS1 mRNAs are collinear transcripts, M2 and NS2 are coded by spliced transcript of their genome segments. An alternative reading frame of PB1 encodes PB1-F2 protein, which is transported into mitochondrion and can induce apoptosis. This protein is not essential in infected cells and not strongly conserved for some animal influenza viruses (31). The role of this protein in viral propagation is not known.

Table 1. Influenza A virus proteins and functions (stem A/PR/8/34, H1N1)
(modified from S. Ludwig *et al*., 1999)

Segment	***vRNA(nt)***	***Protein***	***AA***	***Functions***
1	2341	PB2	759	subunit of RDRP; "Cap-snatching"
2	2341	PB1 PB1-F2	757 87	catalytic subunit of RDRP not known
3	2233	PA	716	subunit of RDRP
4	1778	HA	566	surface-glycoprotein; receptor binding; membrane fusion
5	1565	NP	498	nucleoprotein; encapsidation of v/cRNA
6	1413	NA	454	surface-glycoprotein; neuraminidase; receptor disruption, virus releasing
7	1027	M1 M2	252 97	matrix protein; ion channel activity; protecting HA-conformation
8	890	NS1 NEP/NS2	230 121	post-transcription regulator; inhibition of (i) pre-mRMA splicing, (ii) polyadenylation (iii) PKR-activation nuclear export factor

Avian influenza virus (AIV) is a type A influenza virus belonging to the family *Orthomyxoviridae*. The family *Orthomyxoviridae* also includes influenza type B and C viruses. The type A, B, C is based upon the antigenic character of the viral M protein and the nucleoprotein (NP). Influenza A virus is a negative-sense RNA virus with a segment genome. The genome length is approximately 13.6 kb and the genome contains 8 segments encoding 11 proteins. In contrast to positive strand RNA viruses, the naked RNAs are not infectious. Only viral RNAs molecules encapsidated by the NP and the three subunits of the viral polymerase (PB1, PB2, PA) form RNPs, which are able to initiate viral replication and transcription (116).

A striking feature of influenza viruses is that the surface proteins are constantly changed due to mutations of the according genes. This is a result of the high mutation frequency of the RNA-dependent RNA-polymerases (RDRP). As the RDRP posses no proofreading function, the mutation frequency for influenza A virus genes for any single nucleotide appears to be about 10^{-3}-10^{-4} per replication compared to10^{-8} -10^{-11} for eukaryotic genes.

1.2.2 Propagation and genome replication of influenza A virus

The propagation cycle of the influenza A virus takes about eight hours. At the beginning the glycoprotein HA of influenza virions binds the cell surface receptor containing 2, 3- or 2, 6-galactose sialic acid linkages (adsorption), then endocytosis occurs (Figure 2). The release of the viral genome (uncoating) takes place in acid environment of the endocytotic vesicle. Firstly, low pH triggers conformational changes in the HA, which leads to exposure of lipophilic "fusion domain" of the HA2 that triggers fusion of the viral envelope and the vesicle membrane (penetration). Further the ion channel protein M2 is actived by the low pH to permit the flow of ions from the endosome to the virion interior, which leads to the reduction of the pH in the virions resulting in the dissocation of M1 from the RNPs (uncoating). All four proteins that are in the RNP (NP, PB1, PB2 and PA) contain nuclear localization signal (NLS) and the RNPs are actively transported into the cell nucleus through the nuclear pore complex (NPC) (Figure 2). In the late phase of propagation cycle newly formed RNPs are transported from the cell nucleus to the cytoplasm through the NPC (116). M1 and NEP/NS2 play an important role in exporting the RNPs and preventing the RNPs to be reimported into the nucleus (115, 116, 130).

After the RNPs are transported into the nucleus of the infected cell, the viral genome is transcribed and replicated by the RDRP. The RDRP of influenza A virus consists of three subunits: PB1, PB2 and PA. The NP encapsidates cRNAs and vRNAs, which is necessary for them to be recognized as templates for the viral polymerase. The vRNA with the four proteins together form the biologically active RNPs (116). Influenza v/cRNAs have short noncoding regions (NCRs) at their 5'-terminus (13 nt) and 3'-terminus (12 nt), which are highly conserved (116). It is known that the influenza vRNA promoter structure consists of the 5' - and 3'-terminal sequences of the RNA. The NCR sequences are partly complementary, thus base pairing can happen and short double stranded RNA (dsRNA) can be formed. The unique

conformation ("corkscrew" model) appears to be the core element in vRNA recognition by the viral polymerase (58). The cRNAs are synthesized on the basis of vRNAs as templates, then cRNAs act as templates for the synthesis of new vRNAs. Since the RDRP can not synthesize Cap structures (m7GpppNm), the PB2 subunit splices 5' Cap structure from the cellular mRNAs (Cap snatching), which serve as primers for the initiation of viral mRNA synthesis. (164, 165). Polyadenylation of the mRNAs occurs at a site 15 to 22 nucleotides before the 5´ end of the vRNA segment. Termination occurs apparently as a result of stuttering or reiterative copying of the stretch of U residues, thereby adding a poly(A) tail to the 3´ ends of the viral mRNAs (166, 178, 255).

NS1 is a multifunctional protein, which regulates the mRNA splicing and translation and plays a key role in the pathogenesis of influenza A virus (115, 130, 236). For example, functional cellular mRNAs are present in the cytoplasma, the synthesis of cellular proteins is practically switched off approximately after three hours of viral infection. At the same time viral proteins are efficiently translated. Although the details of "host cell shut off" are not completely understood, it is sure that the NS1 plays a special role. It inhibits splicing and polyadenlation of the cellular pre-mRNAs and keeps the cellular mRNAs in the nucleus (61, 128, 142). At the same time it binds to double stranded RNA (dsRNA) (80) and prevents the activation of the NF-κB pathway (236). It also blocks the dsRNA-dependent protein kinase PKR (15, 81, 129). PKR is activated by dsRNA, the activated PKR phosphorylates the α-subunit of the eukaryotic translation initiation fator elF2, resulting in the inhibition of viral mRNA translation, and assists in the production of the autocrine and paracrine acting IFN that initiates innate immunity and establishes an antiviral state (5, 64, 65). Thereby fighting the innate immune response of the cell, which is the first line of defense against pathogens.

NA, M2 and the HA precursor (HA0) are transported via the rER to the cell membrane in a signal recognition particle (SRP)-dependent manner (97). Posttranslational processing will take place upon the export, for example, the proteolytic cleavage of HA0 into two subunits HA1 and HA2, which are linked by a single disulphide bond. This cleavage is necessary for the virus to be infectious. Due to the newly generated N-terminus of HA_2, the fusion peptide is available to trigger the fusion of viral membrane and cellular membrane (116). Normally different cellular proteases are able to cleave the HA0, but it depends on the amino acid (AA) sequence at the HA0 cleavage site. The highly pathogenic H5 and H7 subtypes can be cleaved by ubiquitous proteases due to possessing the multiple basic AAs at HA0 cleavage sites. These viruses are able to replicate throughout the avian organism and damage vital organs and tissues which results in disease and death. The avian H5N1 virus, which in 1997 led to the death of 6 people of 18 infected humans, has the multiple basic AAs at HA0 cleavage sites (212). In contrast to the subtypes with a monobasic AA at the HA0 cleavage sites (H1, 2, 3, 4, 6, 8, 9, 10, 11, 12, 13, 14, 15), these viruses are limited to cleavage by host proteases and restricted to replication in the host.

The mature glycoprotein HA and NA and the nonglycosylated M2 are transferred to the cellular membrane as a trimer (HA) and/or as tetramers (NA, M2). In the nucleus M1 and NEP bind to the RNPs and are exported into the cytoplasma through the NPC. For the further maturation (assembly) the M1 retains the RNPs in the cytoplasm. Finally new mature viral particles pinche off from the plasma membrane (budding), but the mechanism of budding is not well understood. The virions enwrap themselves with the cellular membrane by budding, at the same time they also carry membrane components, for example proteins which contain the receptor determinant. As the ripe virions carry cellular membrane receptors, they can agglutinate with themselves and aggregate to a cluster. The enzymatic activity of the neuraminidase is needed to release the virions from the cell surface, because NA cleaves sialic acid from virus and cell surface glycoconjugates (receptors) (115, 116).

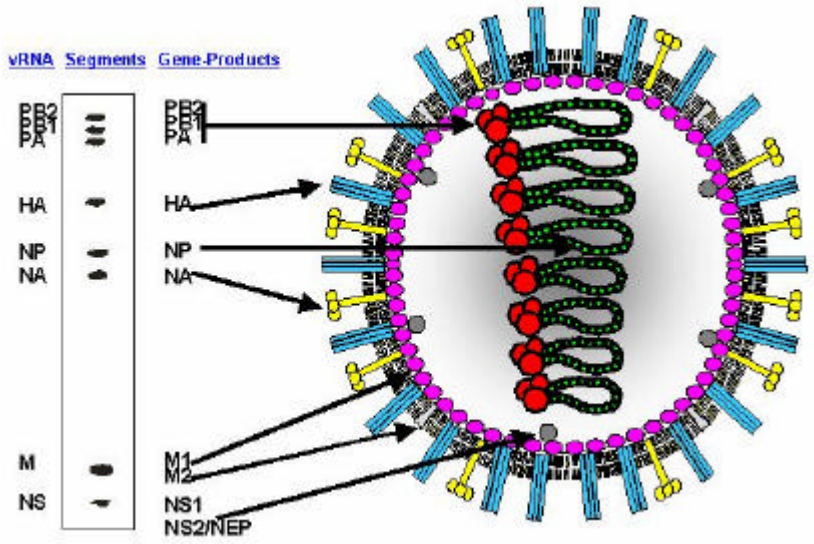


Figure 1: Schematic diagram of the influenza virion. Spherical Influenza A virus particle has a diameter of approximately 100 nm. The eight viral RNA segments were isolated in a urea polyacrylamide gel electrophoresis and detected by silver staining (left). The corresponding gene products and their accepted localization in the virion are right indicated. NS1 is not a component of mature virions (Adapted from S. Pleschka and S. Ludwig).

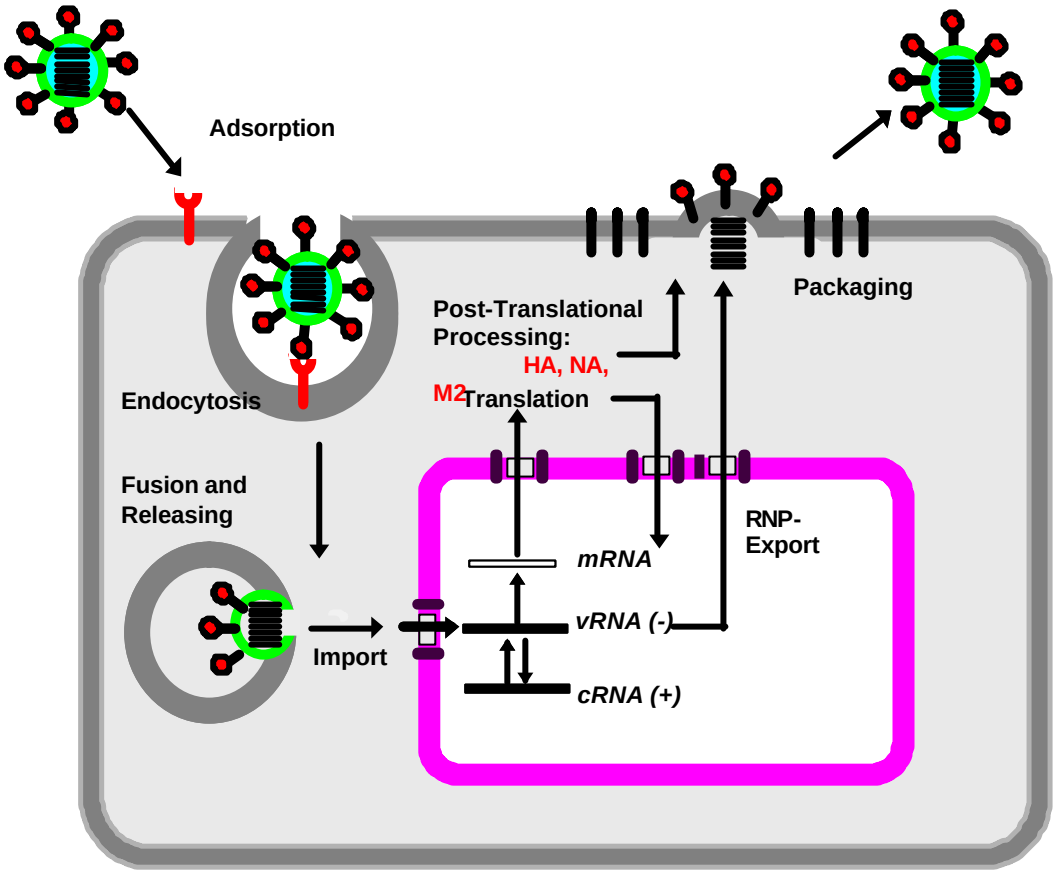


Figure 2: Propagation cycle of influenza virus. The virus binds to the cellular receptor determinant through the receptor-binding site in the HA molecules and enters the cell by receptor-mediated endocytosis. After the fusion between the viral membrane and the vesicle membrane the RNPs are released into the cytoplasma. The RNPs are then transported into the nucleus, where vRNA transcription and replication takes place. The viral mRNAs are transported into the cytoplasma and translated into viral proteins. The viral glycoproteins are transported to the cell surface through the exocytotic transport pathway. They are modified by various posttranslational processing. The replicative proteins move back into the nucleus and continue genome replication. In the late phase of the infection newly synthesized RNPs are exported from the nucleus and packed into the virions, which are finally released from the cell surface by budding (adapted form S. Pleschka).

1.2.3 The relation between human flu epidemics and AIV

Influenza type A, B, C viruses all can infect human beings, but only influenza type A viruses have caused catastrophic pandemics. The pandemic in 1918 to 1919 was particularly severe, between 20 to 40 million people in the world were killed. In the USA about 0.5% of the population died, half the population perished in Alaska and the Pacific islands (102).

Influenza A viruses are renowned for their capacity to cause epidemics on a nearly annual basis due to the continuously evolving nature of the surface glycoproteins, referred to as antigenic drift. This is due to the high error rate of the RDRP that lacks a proofreading function. Antigen shift describes that in unpredictable intervals viruses with completely different surface antigens are introduced into new hosts, as a result of genetic reassortment that can occur in mixed infection with different strains (243). The consequence of this reassortment is the appearance of a new virus subtypes containing a novel HA or NA that is immunologically distinct from those of the previous circulating strain (s). There is no immunprotection in the population, and therefore these viruses can cause global pandemics. Phylogenetic evidence suggests that an influenza virus (H1N1) containing eight gene segments from avian influenza virus was transmitted to human and pigs before 1918 and replaced the 1900 strain (H3N8) (242). This virus was probably carried from North America to Europe by American troops and caused the catastrophic spanish pandemic.

In 1957 an avian influenza virus from wild ducks provided three genes for the Asian influenza pandemic virus (H2N2) which kept five other genes from the circulating human strain (104). Similarly, the H3N2 virus that caused the "Hong Kong" pandemic of 1968 was a reassortant virus that derived its HA and PB1 genes from an avian influenza virus and the remaining gene segments from the circulating H2N2 virus (40, 104) (Figure 3).

In 1997 the avian H5N1 influenza virus infected humans directly and resulted in the death of six people of 18 infected people (203). Recently HPAI H7N7 broke out in Netherland and led to death of one man from more than 80 infected people, even more alarming is the apparent human-to-human spread of the virus (57). All facts provided evidence for the role of avian influenza viruses in the emergence of human pathogenic strains (48).

As mentioned before pigs are thought to provide the "mixing vessel" for AIV and human influenza virus where genetic reassortment may occur. Early on it was speculated that the transmission of virus from pigs to humans resulted in the 1918 pandemic (87, 226). Therefore it should be given more concern where waterfowl, swines and humans have close contact, for example, in south China, that emphasizes the need for persistent influenza surveillance.

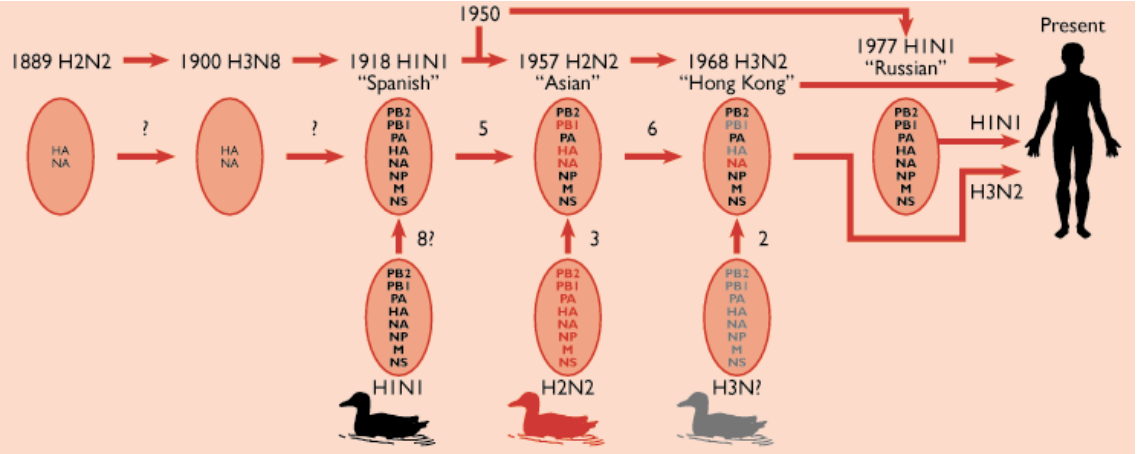


Figure 3: Postulated evolution of the influenza A viruses currently circulating in humans. Seroarcheology suggests that H2N2 and H3N8 influenza viruses circulated in humans in 1889 and 1900, respectively. Phylogenetic evidence suggests that an influenza virus possessing eight gene segments from avian influenza reservoirs was transmitted to humans and pigs before 1918 and replaced the 1900 strain. This virus was probably carried from North America to Europe by American troops and caused the catastrophic influenza pandemic of 1918. In 1957 the Asian pandemic virus acquired three genes (PB1, HA, and NA) from the avian influenza gene pool in wild ducks by genetic ressortment and kept five other genes from the circulating human strain. After the Asian strain appeared, the H1N1 strains disappeared from humans. In 1968 the Hong Kong pandemic virus acquired two genes (PB1 and HA) from the duck reservoir by reassortment and kept six genes from the virus circulating in humans. After the appearance of the Hong Kong strain, the H2N2 Asian strains were no longer detectable in humans. In 1977 the Russian H1N1 influenza virus that had circulated in humans in 1950 reappeared and spread in children and young adults. This virus probably escaped from a laboratory and has continued to cocirculate with the H3N2 influenza viruses in human population (adapted from Field Virology, 2001).

2. Borna disease

Borna disease (BD) is an infectious and fatal progressive Polioencephalitis that is caused by Borna disease virus (BDV). 200 years ago BD was documented in central Europe and primarily affected horses and sheep (140, 182). This name of the disease originated from the town of Borna in Saxonia, Germany, where a serious epidemic occurred between 1894 and 1896 (182). More sporadic occurrence of equine BD has been described in different areas of Germany, Switzerland (140), Liechtenstein (25) and in Austria (246). The mode of transmission is still unknown, but it is assumed to be through salival, nasal or conjunctival secretions (176). Rodents are suspected carriers of the virus, but the true host reservoir of BDV has not yet been identified (176, 206). BDV was firstly isolated in Giessen by Zwick et al. in 1927 (258).

2.1 Host range and clinical symptoms

Horses and sheep have been regarded as the main natural hosts of BDV. Other warm-blooded animals are also but rarely naturally infected, for example other Equidae, cattle, goats, rabbits and zoo animals (83, 175, 182). Seroepidemiological surveys suggest that BDV can infect humans and is associated with mental disorders (73, 125). Some reports indicated that BDV was isolated from humans and proved that the RNA of BDV can be detected from human samples (16). The published sequences of human strains show high homology with the sequences of strains used in the according laboratory. It is considerable whether these are contaminations of the laboratory strains or natural infections (162, 176, 206).

BDV causes a persistent infection in a variety of warm-blooded animals and different cell lines. The incubation period of natural BDV infection varies from 2 weeks to several months (174). Clinical symptoms in naturally and experimentally infected animals are not uniform, they depend on the host species and the virus strain. BDV infection in horses can result in acute or subacute symptoms with meningoencephalitis. Typical clinical signs of BD in horses involve simultaneous or consecutive changes in behavior, sensibility, mobility and in the function of autonomic nervous system (52).

2.2 BDV

BDV is an enveloped nonsegmented negative-strand RNA virus. BDV transcribes and replicates its genome in the nucleus of infected cells and uses the splicing machinery to regulate the expression of viral proteins (20, 41, 100, 194). According to its unique genetic and biologic features, BDV is the prototypic member of the family Bornaviridae, within the order Mononegavirales (50, 192). BDV is a highly neurotropic virus that persist in the central nervous system of infected animals (182). The virions of BDV are spherical with a diameter ranging from 70 to 130 nm (111). The length of the BDV genome is about 8900 nt, and can code for 10 possible proteins. At least six viral proteins can be found in the BDV-infected cell: the nucleoprotein (N), the phosphoprotein (P), a polypeptide of 10 kDa (p10/X), the matrix protein (M), the membrane glycoprotein (G) and the RNA-dependent RNA polymerase (L). Six open reading frames (ORFs) were identified in the BDV genome sequence (Figure 4). ORF I codes for N, ORF II for P, ORFx1 for P10/X, ORF III for M, ORF IV for G and ORF V for L. All these viral proteins have been detected with the help of specific antibodies (50, 192). RNA transcripts encoding BDV proteins are initiated at three transcriptional start sites (S1-S3) and terminated at four transcriptional termination sites (T1-T4) (Figure 4). RNA from the first transcription unit (S1 -T1) codes for the nucleoprotein N. Because there are two AUGs for initiation of the translation in the same ORF, two isoforms of the BDV N protein (p38 and p40) are found in BDV-infected cells (167). p38 is shorter than p40 about 13 amino acids (AA). A nuclear localization signal (NLS) for N protein has been characterized to the 13 N-terminal amino acid residues, with the motif 4KRR6 playing an essential role (109). Recently Perez et al. have proved that only p40 is strictly required for virus polymerase activity by a reverse genetic system (157).

RNA transcripts from the second transcriptional unit (S2 - T2) are bicistronic and code for the BDV protein p10/X (10 kDa) and the phosphoprotein P (24 kDa). The ORFx1 encoding the p10/X-protein overlaps N-terminal with ORF II, encoding P (Figure 4).

The translation of p10/X takes place most likely through leaky ribosome scanning. Regarding the genome localization of the p10/X-gene, the p10/X-protein is comparable with the C-proteins, which are found in representatives of the Mononegavirales replicating in the cytoplasm (e.g. Sendai, Measles, Vesicular Stomatitis virus (VSV)) (13, 70, 117, 118, 205). It could be demonstrated that the C-proteins of Sendai virus and VSV could have modulating effects on viral RNA synthesis in vitro and in vivo (23, 44, 91, 117, 220). p10/X colocalizes with N and P in the nucleus of the BDV-infected cells, that indicates an association of the p10/X-proteins with the viral RNP. Sequence analyses and functional investigations show that this protein contains a nuclear export signal in the N-terminal region (250), which could indicates a function as a genomic export factor.

BDV-P is phosphorylated predominantly by protein kinase C (PKC) and to a lesser extent by casein kinase II (200, 224). Viral phosphoproteins that serve as typically transcription factors are modulated in their functional activities through phosphorylation.

RNA transcripts originating from the third transcription start site (S3) can be terminated at the third termination site (T3) or the fourth (T4). Initiation of transcription at S3 predominantly results in the 2.8 kb RNA transcript. When transcription is terminated at T4, a 7.1 kb RNA is synthesized (Figure 4). In both cases, the RNA transcripts contain two introns (intron 1 and 2) (43, 194). These transcripts can code for M, G and L.

ORF III codes for the matrix protein, which is argued for that it exists as a nonglycosylated (16 kDa) or as a glycosylated (18 kDa) protein (108, 113, 209). Kraus et al. did not find any proof that matrix protein is a glycosylated protein. There are no consensus amino acid sequences (N-X-S/T) for putative attachment of N glycosidic carbohydrates within matrix protein (21, 108). The supposed N-glycosylation sites are N_{74}-I-Y and L-N_{88}-S-L-S (108). When the supposed N-glycosylation sites were introduced N_{74}-I-T_{76} and L-N_{88}-S-T_{90}-S, no carbohydrate attachment could be observed (113). This suggests that the matrix protein is a typical nonglycosylated protein associated at the inner surface of the viral membrane, as homologous proteins of other members of the order Mononegavirales (113). The matrix protein is thought to be a mediator between the nucleocapsid and the lipid-containing envelope, stabilizing the virus shape (114). Other groups suggests that matrix protein is a glycosylated protein that exists as a stable tetramer, with a relative molecular mass of 18 kDa (108, 209, 210). It can be recognized by neutralizing antibodies, which arise in the chronic phase of the disease of the experimentally infected rats (82, 208). It remains to be determined which form the matrix protein exists.

All S3-transcripts, which contain the intron 2, can code for the G (ORF IV) (Figure 4). In order to achieve an efficient translation of the G, intron 1 between the splice donor site 1 (SD1) and the splice acceptor site 1 (SA1) must be removed through splicing (193, 194). This only surface membrane glycoprotein of BDV is synthesized as a polypeptide with a molecular

mass of 57 kDa and N-glycosylated to a precursor glycoprotein of about 94 kDa (72, 175, 193). The precursor glycoprotein (gp94) is processed by the cellular protease furin into the C-terminal membrane-anchored subunit GP-C (gp43) and the identified N-terminal subunit GP-N (gp51) in BDV-infected cells (105), that is highly glycosylated. GP-N seems to be responsible for receptor binding as shown by pseudotype method of vesicular stomatitis virus containing a BDV GP/VSV-G chimeric glycoprotein (158). GP is obviously involved in the surface fusion between viral and cellular membrane after proteolytic activation of GP, which results in release of the viral genome into the cytoplasma (71, 173).

ORF V codes for an approximately 190 kDa protein (p190), the RDRP (L = RDRP), that can only be synthesized after splicing of intron 1 (SD1-SA1) and 2 (SD2-SA2) (192, 233). Translational initiation from the second AUG on the same ORF can generate a shorter form of the polymerase with approximately 180 kDa (p180) (192). Which form of the polymerase is actually active in infected cells, is at present not well-known. Within the coding region of the L-protein, a second alternative splice acceptor site (SA3) was recently described (42, 225). Alternative splicing of intron 2 gives rise to two new ORFs with coding capacities for a 8,9 kDa and a 165 kDa protein. Whether these two proteins are really formed in BDV infected cells and whether they have essential functions for the viral reproduction remains to be determined. Additionally to this "new" ORFs, a smaller one was recently described, which codes for a P'-protein with approximately 16 kDa (110). As p38/p40 (N-ORF) and p180/p190 (L-ORF), P' can be translated through a second start codon in the P-ORF. (Figure 4). Up to now it is also not known for P' whether it is necessary for the BDV reproduction. Another ORF (ORF VI) generated by splicing of intron 2 has been shown by Pleschka et al (192), but it remains to be investigated whether the ORF VI product is indeed synthesized during BDV infection.

There is still a lot of work to be done on functional studies of BDV proteins. A complexation between P and N could be proven by immunoprecipitation experiments with specific serums against P or N in infected cells. Two-hybrid analyses confirmed interactions between P and N (95, 201). Here it could be further proved that P is able to bind specifically not only N, but also p10/X. In contrast, N and p10/X can not form complexes in the two-hybrid system (201). However, the interaction of N with p10/X could also be shown by cotransfection studies in eucaryotic cells (134). The colocalization of N, P and p10/X in the nucleus of infected cells (201, 245, 250) could refer to p10/X as a component of the functional replication complexes.

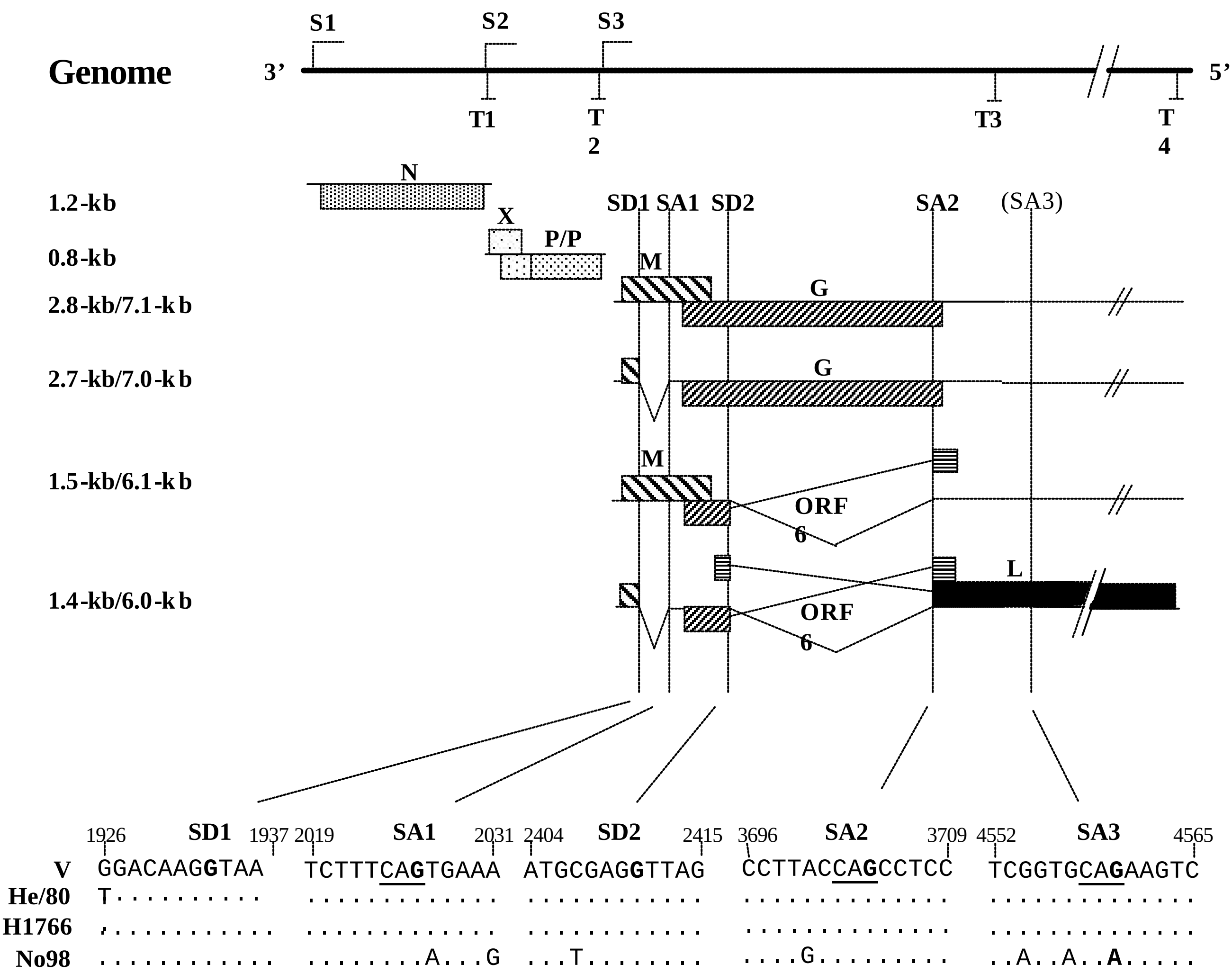


Figure 4: BDV genome organization

Consensus transcription map of four different BDV genomes: Position of transcription start sites (S1-S3) and termination sites (T1-T4) are indicated. Transcription orientation is marked by arrows. Locations and nucleotide sequences flanking splice donor (SD1,SD2) and acceptor sites (SA1-SA3) are given. ORFs are individually marked on the various viral transcripts. Letters N, X, P, P´, M, G and L indicate translation products that have been identified in infected cells by immunohistochemical methods. The putative products of ORF6 remains to be visualized. Due to the absence of a functional SA3 site in strain No/98, this consensus transcription map does not include SD2-SA3 spliced viral mRNAs and their predicted translation products (adapted from S. Pleschka *et al*., 2001).

It is accepted that per cell approximately 1-10 infectious virus particles are formed in 48 h, depending on the infected cell lines (26). Electron microscopy studies of negatively stained cell free infectious BDV particles have shown that the virions are spherical with the diameter approximately 100 nm (111, 257) and these virions will be set free after budding from the cell surface. In what extent the replication cycle of BDV is similar to influenza viruses, is not well-known. It is accepted that BDV is an enveloped, nuclear replicating, negative-strand

RNA virus with an fusion-active glycoprotein and that the virions are set free at the cell surface by budding (111, 257). But the life cycle of BDV and the regulation among the proteins are still waiting to be determined.

There are many open questions about BDV that are not answered yet, so the establishment of reverse genetic system for BDV is urgent and important. Reverse genetic systems for other viruses have proved that they are a very useful tool to analyze the viral life cycle, the regulatory function of viral proteins and molecular mechanisms of viral pathogenicity. Nevertheless the knowledge of the genetics of BDV is very limited and the development of a reverse genetic system for BDV is very tedious. In my study, I tried to establish a helper virus dependent reverse genetic system for BDV.

3. The progress of reverse genetic systems for negative-strand RNA viruses

The negative-strand RNA viruses are a large group of viruses that comprises some important human pathogens, including influenza, measles, mumps, rabies, respiratory syncytial, Ebola and Hanta viruses. In contrast to the positive strand RNA viruses, the genomic RNA of negative-strand RNA viruses is not infectious, and infectious virus particles must deliver their own RDRP into the infected cell to start the first round of virus specific mRNA synthesis. This means that the translation of their RNAs is possible by cellular ribosomes only after transcription of the viral genome by the RDRP. For genetic manipulation of the viral RNA-genome this has to be cloned as DNA from which it will then be expressed as RNA. This step from RNA to DNA to RNA explains the term "reverse genetics". The DNA derived RNA genome can only be replicated and transcribed by the viral RDRP.

3.1 Influenza virus

In 1989 the first system was developed for influenza viruses. In this approach the purified polymerase and nucleoprotein (NP) were mixed with in vitro synthesised vRNA to assemble the functional RNP complexes. The biologically active RNP was then transfected into the cells infected by influenza virus (132) (Figure 5). With the help of this reverse genetic system, it became possible to generate negative-strand RNA viruses containing the vRNA derived from cloned cDNAs. This means that these viruses can carry changed or additional genetic information (144). Site specifically mutated influenza viruses were firstly generated by Enami et al. (55). In this system, helper virus provides in trans the viral proteins required for amplification of the synthetic RNP complex. Different strategies can be used to select an reassortant virus containing a synthetic gene from helper viruses, for example antibody growth restriction (8, 56, 93, 183), temperature sensitivity (122, 251), host range restriction (55, 211), or drug resistance (28). Up to now selection systems have been established for six of the eight influenza A viral segments, these are PB2 (211), HA (56), NP (122), NA (55), M (28) and NS (56), and for the HA and NA segments of influenza B virus (8, 183). But the

application of this technology was limited and complex due to the demand for a strong selection system and technical expertise.

The second impetus for reverse genetics of influenza virus was the establishment of RNA polymerase I system for the intracellular synthesis of influenza virus RNA (146). RNA polymerase I is a cellular enzyme in nucleus that transcribes ribosomal RNA which lacks both 5' cap and 3' poly(A) tail. Transfection of cells with a plasmid containing cloned cDNA of influenza virus gene, flanked by RNA polymerase I promoter and terminator sequences, followed by influenza virus infection, resulted in the production of wild type and recombinant viruses carrying a reporter gene. Pleschka et al. successfully improved this system to generate clonal recombinant influenza virus in 1996 with synthetic NA (150) and HA. This system was also used to establish reverse genetics system for Thogoto viruses (six RNA segments, Orthomyxoviridae) (230, 241) which replicates in nucleus and other viruses, for example, Uukuniemi virus (3 RNA segments, Bunyaviridae) (59) which replicates in the cytoplasm. With both systems the RNP transfection method and RNA polymerase I system, transfectants must be selected from a vast background of helper virus, which requires a strong selection system and complicates the generation of growth defective viruses. Another drawback is that the efficiencies of virus generation are low.

Based on the system of Pleschka et al., a completely plasmid-based system was developed in 1999 by Neumann et al. and Fodor et al. (60, 145). This system is able to generate the influenza A virus entirely from cloned cDNA and can be used to introduce mutations into any gene segment. In this approach, the eight cDNAs were inserted between a human RNA polymerase I promoter and the mouse RNA polymerase I terminator or a hepatitis delta virus ribozyme respectively. Fodor et al. used the hepatitis delta virus ribozyme instead of the RNA polymerase I terminator in order to generate vRNA possessing the precise 3' end sequence. The expression of the four RNP-complex proteins was driven by the human cytomegalovirus promoter or the HMG-promoter respectively. Transfection of twelve plasmids into 10^6 293T cells led to virus recovery of more than 1×10^3 PFU of virus per ml of posttransfectional supernatant. The efficiency could be increased to 5×10^7 PFU after transfection of 17 plasmids (Figure 6). This highly efficient system was attributed to the high transfection efficiency of 293T cells, which led to a pool of cells that receive the full complement of constructs required to initiate virus replication.

An eight plasmid system, designated RNA polymerase I/II system, was developed by in 2000 (89). A cDNA encoding a viral segment was cloned in the negative-sense orientation between RNA polymerase I promoter and terminator sequence, and this cassette was in turn cloned in the positive-sense orientation between RNA polymerase II promoter (CMV) and a polyadenylation sequence. Transcription by RNA polymerase I yields negative-sense vRNA, whereas transcription by RNA polymerase II results in positive-sense mRNA synthesis. Thus the vRNA and mRNA are generated from the same template, circumventing the need for protein expression constructs (Figure 7). They also established the unidirectional RNA

polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids (90). In this approach, cotransfection of eight RNA pol I-pol II tandem promoter plasmids containing the cDNA of A/WSN/33 (H1N1) resulted in the generation of infectious influenza A virus, albeit with a lower yield than the bidirectional system. These two reverse genetics systems reduce the number of plasmids required for the recovery of influenza A viruses and allow the generation of reassortant viruses. Because both protein expression and vRNA synthesis are achieved from the same template, this system does not allow generation of virus like particles lacking or containing lethal mutations in one or more viral segments.

The RNA polymerase I system allows to introduce any desired mutation into the genome of influenza virus. This ability to manipulate the viral genome without technical limitation has profound implications on the study of the viral life cycle, functional regulation of viral proteins and molecular mechanisms of viral pathogenicity. Perhaps this system can also be used to create an live-attenuated influenza virus vaccine.

3.2 Nonsegmented negative-strand RNA-viruses (NNS viruses)

As the negative-strand RNA-viruses with segmented genome, the nonsegmented negative-strand RNA-viruses (NNS viruses) in the order Mononegavirales package their own RDRP together with the nonsegmented genome in virions. This way replication/transcription is initiated in the infected cell. The naked genome is not infectious and not able to begin the reproduction cycle. Most NNS viruses have been generated from antigenomic, positive-sense cRNA (177). At the beginning of the establishment of reverse genetic systems for NNS viruses, a synthetic Sendai virus-like RNA, containing the positive-sense coding region of the chloramphenicol acetyltransferase (CAT) gene replacing the coding region of the Sendai virus genome, was transcribed from a cDNA in vitro. When introduced into cells infected with Sendai virus, this RNA construct was transcribed, replicated, and packaged into infectious virions (152). Here a virus-like RNA, which codes for CAT reporter gene, could be assembled by the viral RDRP transcomplementation. Schnell et al. (196) established an approach to generate rabies virus from cloned cDNA for the first time for negative-stranded RNA viruses. Plasmids encoding the viral nucleocapsid protein (N) and the polymerase protein (L and P) under the control of T7 polymerase promoters were transfected into cells previously infected with recombinant vaccinia virus expressing the T7 polymerase protein. In addition to these plasmids, a plasmid encoding a full-length antigenomic viral RNA under the control of a T7 promoter and hepatitis delta ribozyme was also transfected into the cells. After transcription of RNAs from the T7 promoter and translation of the encoded proteins, nucleocapsid proteins assemble with the antigenomic RNAs and polymerase proteins, then replicate these RNPs to form RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and translation, infectious virus is assembled (Figure 8).

An effective DNA transfection system has also been developed for another Rhabdoviridae, vesicular stomatitis virus (VSV) (119, 247). Reverse genetic systems have also been

developed for paramyxoviruses. For example for measles virus, a cell line constitutively expressing T7 polymerase and the measles N and P proteins has been used for the rescue of infectious virus from full-length clones (171). Vaccinia virus based systems have allowed the rescue of respiratory syncytial viruses (RSV) (37) and of Sendai viruses (68, 103).

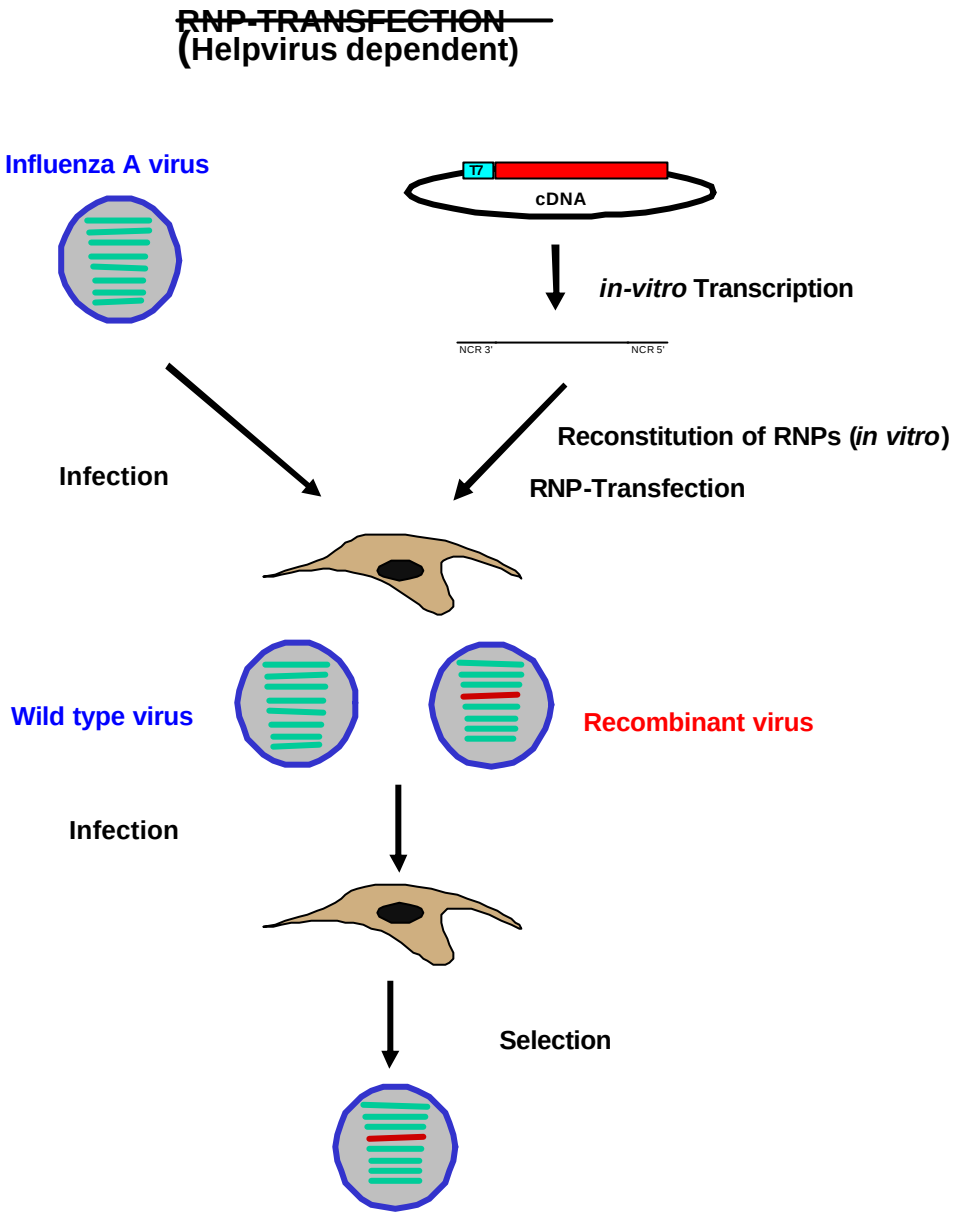


Figure 5. RNP transfection. For generating recombinant influenza virus, RNPs are reconstituted by in vitro purified viral polymerase subunits and NP and an in vitro T7-transcript. These reconstituted RNPs are transfected into cells, then these cells are infected by helper virus. A part of the newly formed virions contain the synthetic genomic segment. This method needs a selection system, that allows only the recombinant viruses to propagate.

Recently T7 polymerase system and Pol I system for BDV have been reported (157, 195), but up to now infectious BDV is not rescued by a reverse genetic system. BDV replicates its genome in the nucleus of the infected cell. Since the T7 polymerase works in the cytoplasm, it

might not be successful for the rescue of BDV by T7 polymerase system. A T7 generated minigenome that was replicated and transcribed by transiently expressed influenza virus proteins (RNPs) was not packaged into a helpervirus after infection of new cells with the supernatant of transfected cells. In contrast a PolI generated minigenome was (Pleschka, unpublished data). On the other hand BDV causes a very slow and persisting infection. This means that perhaps its genome replication requires a very exact and tuned system. In my study, I tried to establish a helper virus dependnet reverse genetic system for BDV analoge to the successful system for influenza viruses.

Synthesis of influenza virus entirely from cloned cDNAs

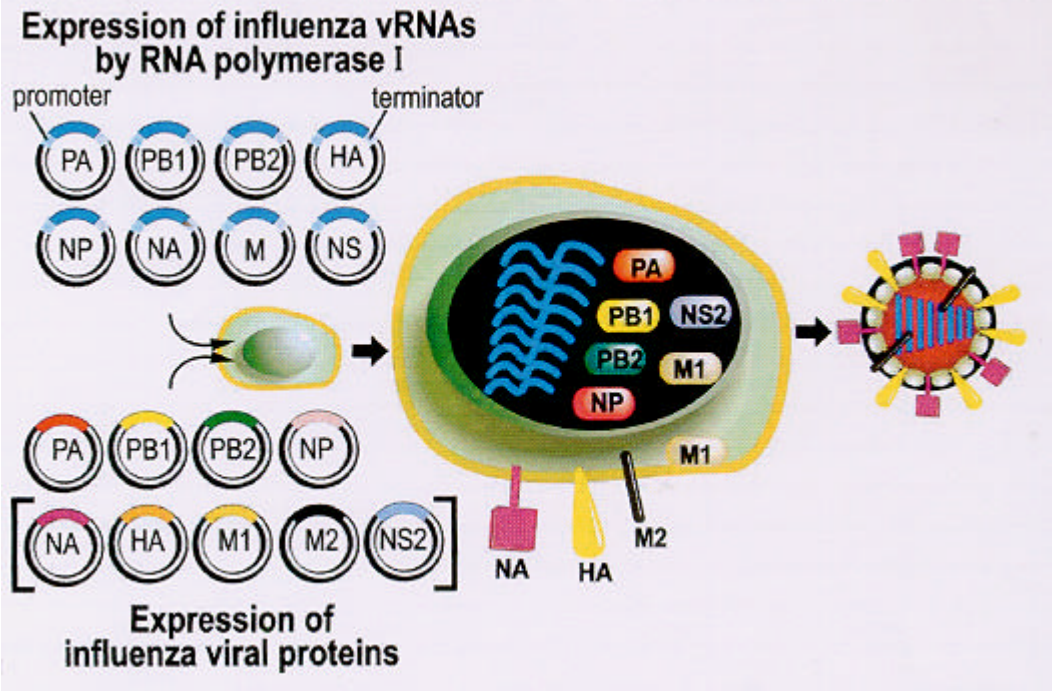


Figure 6: Synthesis of influenza virus entirely from cloned cDNAs. Plasmids containing the RNA polymerase I promoter, a cDNA for each of the eight viral RNA segments, and the RNA polymerase terminator are transfected into cells together with four plasmids for the expression of viral proteins required for vRNA replication and transcription (PB1, PB2, PA, and NP). This system (12 plasmids) yields more than 1 x10^7 infectious viruses per milliliter of supernatant from transfected cells. Cotransfection of cells with nine plasmids for expression of all viral structural proteins increases the efficiency of virus production and also permits production virus particles lacking genes or possessing genes containing some mutations. (adapted from Neumann, *et al*., 2001)

The RNA polymerase I/II system for the generation of influenza virus

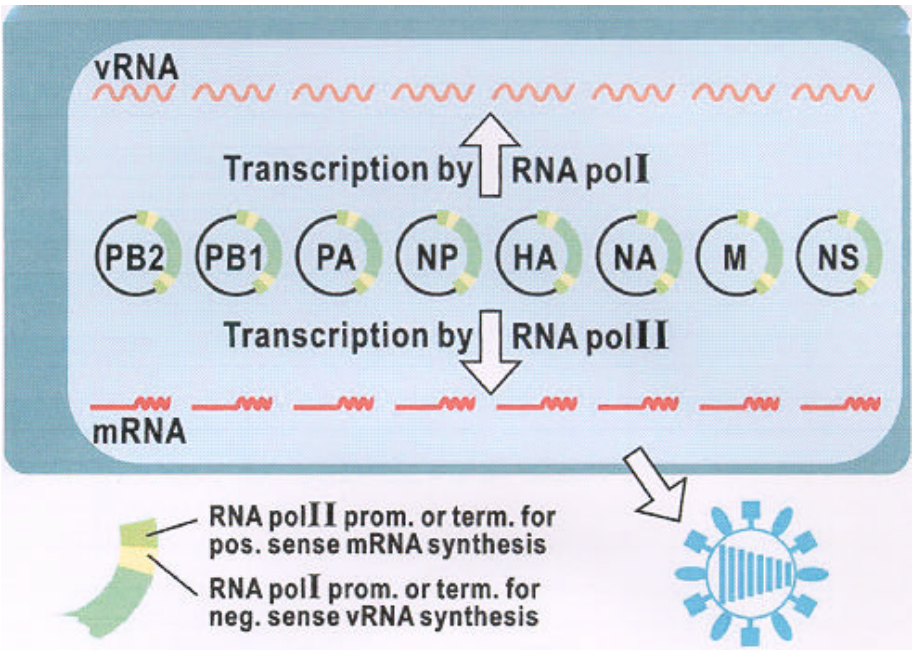


Figure 7. The RNA polymerase I/II system for the generation of influenza virus. Cells are transfected with plasmids that contain a cDNA encoding a viral RNA flanked by RNA polymerase I promoter and terminator sequence in a negative-sense orientation. This cassette is then inserted between RNA polymerase II promoter and terminator sequence in a positive-sense orientation. Transfection of these plasmids results in influenza vRNA sysnthesis by cellular RNA polymerase I and in mRNA sysnthesis by cellular RNA polymerase II. (adapted from Neumann, *et al.*, 2001)

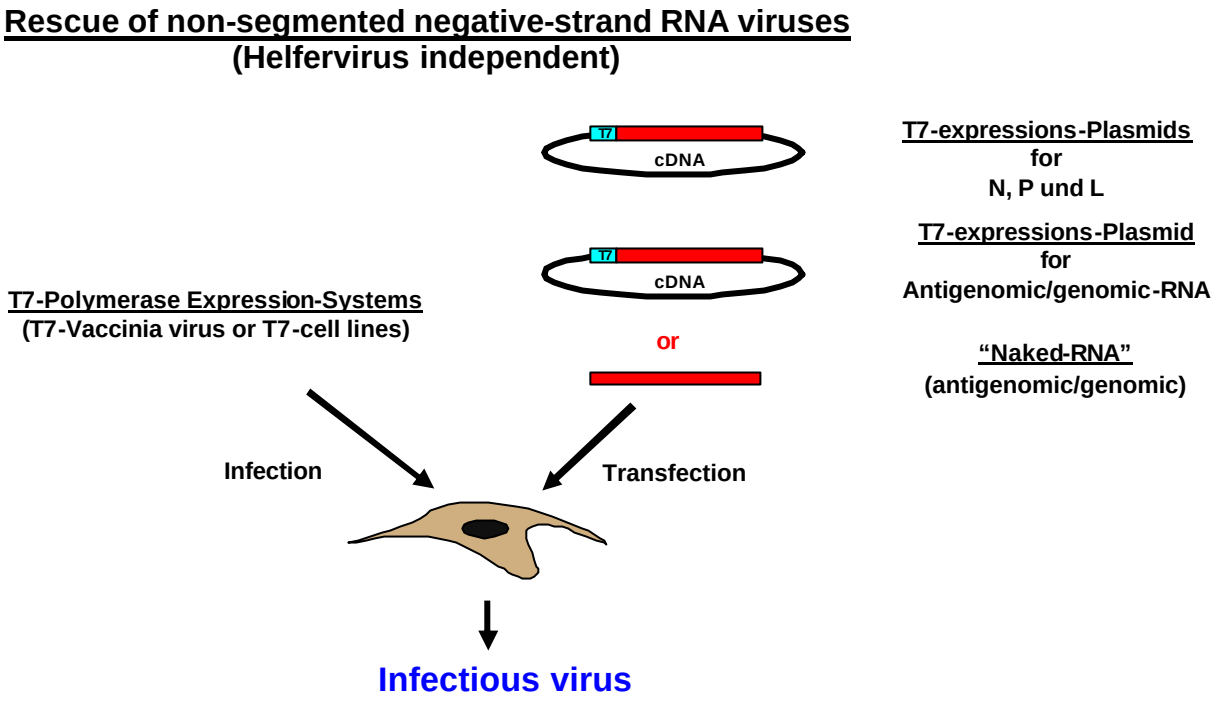


Figure 8. Reverse genetic systems for the rescue of infectious nonsegmented negative-strand RNA viruses from cDNA. Transcriptionally competent viral RNPs are made by cellular expression of the viral proteins N, P and L. This can be achieved by a variety of methods, including vaccinia virus-driven T7 expression and T7 complementing cell lines constitutively expressing T7 polymerase and viral proteins. The full-length viral RNA can be provided by transfecting plasmids expressing antigenomic or genomic RNA or by directly transfecting naked RNA (plus-sense or minus-sense). The intracellularly assembled RNPs are transcribed and replicated by the viral polymerae complex (N, P and L proteins) generating infectious viruses.

Materials and methods

1. Materials

1.1 Chemicals and reagents

Acetic acid	Roth, Germany
Acrylamide	Bio-Rad, USA
Acrylamide/bisacrylamide 30, 30%, 37.5:1	Roth, Germany
Acrylamide/bisacrylamide 40, 40%, 19:1	Roth, Germany
Agarose ultra pure	Roth, Germany
Ammonium persulfate (APS)	Serva, Germany
Ampicillin	ICN, USA
Agar high-gel strength	Serva, Germany
Boric acid	Roth, Germany
Bromophenol blue	Merck, Germany
Chloroform	Roth, Germany
Coomassie brilliant blue R 250	Merck, Germany
Dimethylsulfoxid (DMSO)	Sigma, USA
1,4-Diazabicyclo [2,2,2]octane (DABCO)	Merck, Germany
DEAE Dextran (Mw, 500,000)	Pharmacia Biotech, Denmark
Di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4)	Merck, Germany
Ethanol (absolute)	Roth, Germany
Ethidium bromide	Roche, Germany
Ethylenediamine tetraacetic acid (EDTA)	Fluka, USA
Extract of yeast powder	Merck, Germany
Formamide	Fluka, USA
Glycerol	Sigma, USA
Glycogen	Roche, Germany
Isoamyl alcohol	Roth, Germany
Isopropanol	Roth, Germany
Magnesium chloride ($MgCl_2$)	Merck, Germany
Mowiol 40 - 88	Aldrich, Germany
Methanol	Roth, Germany
N-2-hydroxyethylpiperazine (HEPES)	Sigma, USA
Oxoid Agar	Oxoid, England
Peptone from meat trypsin-digested	Merck, Germany
Potassium chloride (KCl)	Roth, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	Roth, Germany
Roti-phenol	Roth, Germany

Roti-phenol/ chloroform	Roth, Germany
Sodium acetic acid (NaAc)	Roth, Germany
Sodium chloride (NaCl)	Roth, Germany
Sodium dodecyl sulfate (SDS)	Merck, Germany
Sodium hydroxide (NaOH)	Merck, Germany
Sodium borohydride $NaBH_4$)	Roth, Germany
Sodium hydrogen carbonate ($NaHCO_3$)	Fluka, USA
Sodium citric acid	Roth, Germany
Silver nitrate	Merck, Germany
Tris base	Roth, Germany
N,N,N',N'-Tetramethyl-ethylene diamine (TEMED)	Serva, Germany
Urea	BRL, USA
ß-mercraptoethanol (MetOH)	Roth, Germany
Xylene cyanole	Sigma, USA

1.2 Enzymes and enzyme inhibitor

Klenow frament of DNA polymerase I	NEB, England
Restriction endonucleases	NEB, England
RQ1 RNase free DNase (1U/µl)	Promega, USA
Taq DNA polymerase (5U/µl)	Promega, USA
RNase inhibitor (RNasin, 40U/µl)	Promega, USA
T7 RNA Polymerase	Ambion, USA

1.3 Nucleotides and reaction buffer

DNA ladder mix	PEQLAB, Germany
10x restriction enzyme buffer 1, 2, 3 and 4	NEB, England
10x RQ1 DNase buffer	Promega, USA
10x Taq polymerase buffer	Promega, USA
Tris-HCl buffer for flash cat assay (pH 7.4)	Stratagene, Germany
Bio-Rad protein assay buffer	Bio-Rad, USA
Cell lysis buffer	Promega, USA

1.4 Kits

QIAGEN plasmid maxi kit	QIAGEN, Germany
QIAGEN plasmid midi kit	QIAGEN, Germany
QIAGEN RNA/DNA mini kit	QIAGEN, Germany
QIAprep spin miniprep kit	QIAGEN, Germany
TOPO TA cloning kit	Invitrogen, USA
Rapid DNA ligation kit	Roche, Germany
Rapid DNA ligation kit	NEB, England

Expand high fidelity PCR system	Roche, Germany
High pure PCR product purification kit	Roche, Germany
Flash CAT Non-Radioactive CAT Assay Kit	Stratagene, USA
MAXIscript™ (In vitro Transcription Kit)	Ambion, USA
RPAIII™ (Ribonuclease Protection Assay Kit)	Ambion, USA
BrightStar™ BioDetect™ (Nonistopic Detection Kit)	Ambion, USA

1.5 Materials for cell culture

Dulbecco's Modified Eagle's medium (DMEM)	Invitrogen, USA
Fetal calf serum (FCS)	PAN, Germany
Opti-MEM	Invitrogen, USA
100x penicillin-streptomycin solution	PAA, Austria
1x trypsin-EDTA (0.5 g/L trypsin, 0.2 g/L EDTA)	PAA, Austria
Tissue culture dish	Greiner, Germany
Tissue culture flask	NUNC, USA
Bovine Albumin	ICN, USA

1.6 E. coli strains, virus strains and cell lines

XL1-Blue	Stratagene, Germany
REB (rat embryo brain cells)	kindly provided by Dr. M. Flüß
BDV-H1766	kindly provided by PD Dr. J. A Richt

The following three cell lines were kindly provided by HDoz Dr. S. Pleschka:
293T (Human embryonic kidney cells)
Vero (African green monkey kidney cells)
MDCK (Madin-Darby canine kidney cells)

1.7 Plasmids

pcDNA3.1	Invitrogen, USA
pSV2 CAT	Stratagene, USA
pCAGGS T7	kindly provided by Dr. E. Muehlberger Philipps-Unversity Marburg, Marburg
pCITC Luci	kindly provided by Dr. N. Tautz Justus-Liebig -Universtiy, Giessen
p125-luci (IFN-ß promoter)	kindly provided by Prof. Dr. S Ludwig Heinrich Heine-University, Duesseldorf

The following plasmids were constructed by Wenjun Ma in Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science, China:
pCI-NP (A/Goose/Guangdong/1/96 (H5N1))
pCI-HA (A/Goose/Guangdong/1/96 (H5N1))

pCI-NA (A/Goose/Guangdong/1/96 (H5N1))
pCI-M (A/Goose/Guangdong/1/96 (H5N1))
pCI-NS (A/Goose/Guangdong/1/96 (H5N1))
The following plasmids were kindly provided by HDoz Dr. S. Pleschka:
pHMG-PA (A/WSN/33(H1N1))
pHMG-PB1 (A/WSN/33(H1N1))
pHMG-PB2(A/WSN/33(H1N1))
pHMG-NP(A/WSN/33(H1N1))
pPolI-CAT-RT(-)
pAM500 GFP
The following eight plasmids were kindly provided by Dr. R. Wagner Philipps-Unversity Marburg:
pPolI-PB1 (A/FPV/Rostock/34 (H7N1))
pPolI-PB2 (A/FPV/Rostock/34 (H7N1))
pPolI-PA (A/FPV/Rostock/34 (H7N1))
pPolI-NP (A/FPV/Rostock/34 (H7N1))
pPolI-HA (A/FPV/Rostock/34 (H7N1))
pPolI-NA (A/FPV/Rostock/34 (H7N1))
pPolI-M (A/FPV/Rostock/34 (H7N1))
pPolI-NS (A/FPV/Rostock/34 (H7N1))
The following three plasmids were kindly provided by Dr. M. Perez The Scripps Research Institue, Department of Neuropharmacology, La Jolla, USA:
pCAGGS-N (BDV)
pCAGGS-P (BDV)
pCAGGS-L (BDV)

1.8 Antisera and monoclonal antibodies

Biotin-SP-conjugated attinipure sheep anti-Mouse IgG	Dianova, Germany
Peroxidase-conjugated Streptavidin	Dianova, Germany
Furescein (FITC) conjugated goat anti-mouse IgG	Sigma, USA
Monoclonal Bo18/p40	kindly provided by PD Dr. J. A. Richt

1.9 DNA oligonucleotides

DNA oligonucleotides were ordered from MWG-Biotech, Germany and Bao biological technique company, China.

T7 clone Fw:	5'- GCG CCG TCT CTA ACA ACA AAC CAA TCA CCA TTC TTC TAA CAA AAT GAA CAC ACG CAA TGA ACA CGA TTA ACA TCG -3'
T7 clone Bw:	5'- CGC GTG GCC ATT ATT ACG CGA ACG CGA AGT CCG AC -3'

HH-Ribo Fw1:	5'- GCG CTC TAG AAC CAG AGG GCC CCG G -3'
HH-Ribo Bw1:	5'- GCG CAA GCT TCG TCT CTA ACG CAA CAG ACT CCT TAC GGA GTT TCG GCC TTT CGG CCT CAT CAG TGT TGC GTT AAC AAC TAA TAA CCC GGC GGC CC -3'
HH-Ribo Bw2:	5'- GCG CAA GCT TCG TCT CTA ACG CAA CAG ACT CCT TAC GGA GTT TCG GCC TTT CGG CCT CAT CAG TGT TGC GTT AAC TAA TAA CCC GGC GGC CC -3'
HH-Ribo Bw3:	5'- GCG CAA GCT TCG TCT CTA ACG CAA CAG ACT CCT TAC GGA GTT TCG GCC TTT CGG CCT CAT CAG TGT TGC GTT AAT AAC CCG GCG GCC C -3'
CAT29-52 Fw:	5'- CCG TTG ATA TAT CCC AAT CGC ATC -3'
CAT612-589 Bw:	5'- CAT TCT GCC GAC ATG GAA GCC ATC -3'
NS-NCR SapI Fw:	5'- GCG GGC TCT TCC ATT AGT AGA AAC AAG GGT G -3'
NS-NCR SapI Bw:	5'- GGG CGC TCT TCA GCC AGC AAA AGC AGG G -3'
Seq pBD Fw:	5'- GAC TCA CTA TAG GCT AG -3'
Seq pBD Bw:	5'- GTG TCC GTG TCG CGC G -3'
PA H5N1-Fw:	5'- CCA GCA AAA GCA GGT ACT GAT -3'
PA H5N1-Bw:	5'- TTA GTA GAA ACA AGG TAC TT -3'
PB1 H5N1-Fw:	5'-CCA GCA AAA GCA GGC AAA CAA -3'
PB1 H5N1-Bw:	5'- TTA GTA GAA ACA AGG CAT T -3'
PB2 H5N1-Fw:	5'- CCA GCA AAA GCA GGT CAA T -3'
PB2 H5N1-Bw:	5'- TTA GTA GAA ACA AGG TCG T -3'
NP H5N1-Fw:	5'- CGC GGG ATC CGC TCT TCT GCC AGC AAA AGC AGG GTA CCT AAT CAC TCA C -3'
NP H5N1-Bw:	5'- CGC GGG GCC CGC TCT TCC ATT AGT AGA AAC AAG GGT GGA ATT CTT TAA TTG TCG TAC TCC TC -3'
HA H5N1-Fw:	5'- GGC GAA GCT TGC TCT TCT GCC AGC AAA AGC AGG GGT ATA ATC TGT CAA AAT GGA GAG AAT AGT GCT TC -3'
HA H5N1-Bw:	5'- GGC GGG GCC CGC TCT TCC ATT AGT AGA AAC AAG GGT GTT TTT AAC TAC AAT CTG AAC TCA CAA ATT TAA ATG CAA ATT CTG CAT TG -3'
NA H5N1-Fw:	5'- GGC GGC TAG CGC TCT TCT GCC AGC AAA AGC AGG AGA TTA AAA TGA ATC CAA ATC AGA AGA TAA T -3'
NA H5N1-Bw:	5'- GGC GGG GCC CGC TCT TCC ATT AGT AGA AAC AAG GAG TTT TTT GAA CAA GCT TCT AGG CAA TG -3'
M H5N1-Fw:	5'- GGC GGG ATC CGC TCT TCT GCC AGC AAA AGC AGG TAC CTA TTG AAA AAT G -3'
M H5N1-Bw:	5'- GGC GGG GCC CGC TCT TCC ATT AGT AGA AAC AAG GTA GTT TTT TAC TC -3'

NS H5N1-Fw:	5'- GGC GAA GCT TGC TCT TCT GCC AGC AAA AGC AGG GTG ACA AAG AC -3'
NS H5N1-Bw:	5'- GGC GGG GCC CGC TCT TCC ATT AGT AGA AAC AAG GGT GTT TTT TAT -3'
Flu RT-primer:	5'- AGC AAA AGC AGG -3'
NS-RT Fw:	5'- CCA GCA AAAGCA GGG TGA CA -3'
NS-RT Bw:	5'- TTA GTA GAA ACA AGG GTG TT -3'

1.10 Other materials

Deoxynucleosidetriphosphate set (dNTP, 100mM)	Promega, USA
Eppendorf tube	Eppendorf, Germany
X-ray film	Kodak X-OMAT, USA
Syring (5ml, with 25G needle)	Braun, Germany
Falcon centrifuge tube	Falcon, Germany
Lipofectamine	Invitrogen, USA
Lipofectamine 2000	Invitrogen, USA
Plus Reagent	Invitrogen, USA
Microtiter plate (96 wells)	Greiner, Germany
TLC plate	Merck, Germany
SOC medium	Invitrogen, USA

2. Methods

2.1 DNA cloning and subcloning

2.1.1 Preparation of competent cells for eletroporation

Luri-Broth (LB) medium
0.5% (w/v) Extract of yeast powder
1% (w/v) Peptone from meat trypsin-digested
1% (w/v) NaCl
pH7.5, autoclaved

LB plate
1.5%(w/v) Agar high-gel strength in LB medium, autoclaved

Washing buffer
1mM HEPES pH 7.0

Competent cell stock solution
10% (v/v) glycerol
autoclaved

100 µl of Escherichia coli XL1-Blue was transferred into 10 ml LB medium. Cells were grown overnight at 37°C on the incubator shaker with moderate shaking (250 rpm). 10 ml culture medium was transferred into 1 L LB medium. Cells were grown for additional 3 - 4 h at 37°C until the OD_{595} reached 0.5-0.8. After cooling on ice for 30 min, cells were transferred into four sterile 250 ml centrifugation tubes and pelleted by centrifugation for 10 min at 10000 rpm, 4°C (Beckmann type 14). Then cells were sequentially resuspended in 500 ml of chilled 1mM HEPES (pH 7.0), pelleted again by centrifugation and resuspended in 250 ml of chilled 1mM HEPES (pH 7.0), pelleted by centrifugation again as above. The cells were resuspended in 50 ml chilled 10% glycerol in H_2O, centrifuged again as above and resuspended in 2.5 -3 ml 10% glycerol. Competent cells were aliquoted in 100 µl (per tube) on ice. The tube containing the competent cells were placed into nitrogen for freezing, then stocked immediately at -70°C.

2.1.2 Electroporation

Preparation
Chilled sterile electro cuvette (Bio-Rad, USA)
1 ml sterile SOC medium in an eppendorf tube
Thaw competent cells rapidly on ice

Transformation equipment setting
Ohm: 200
Capacitance: 25
Voltage: 2.5 KV
Time: time Const

10 ng of plasmid DNA or 20 µl of desalted ligation mixture was mixed in 100 µl of rapidly thawed competent cells by stirring gently with the pipette tip. Transformation mixture was added into the chilled cuvette, and cuvette was dried and placed into the device of the Gene Pulser (Bio-RAD, USA). Then two buttons were pushed at the same time until a signal was heard (the desired electroporation time is about 4 milliseconds). Immediately SOC medium was added into the cuvette and mixed with the cells. The cells and SOC medium were removed from the cuvette and given into the eppendorf tube, then incubated on the thermomixer comfort (eppendorf, USA) for 30 min at 37°C. If ligation mixture was transformed, the cells and SOC medium mixture was centrifuged shortly after incubation, then only 100 µl liquid was kept in the eppendorf tube, rest of supernatants were discarded. The cell pellet was resuspended in the 100 µl liquid as mentioned and spread onto a pre-warmed LB plate containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C. If the plasmid was transformed, 100 µl of cells and SOC medium mixture (after

incubation on thermomixer comfort) was spread onto pre-warmed LB plate containing 100 µg/ml ampicillin. The centrifugation and resuspension step is not needed.

2.1.3 Preparation of plasmid DNA

Solution I
50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)
autoclaved for 15 min at 10 lb/sq.

Solution II
0.2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

Plasmid DNA mini-preparation was normally done according to the following protocol: a single bacterial colony was transferred into 5 ml of LB medium containing 100 µg/µl ampicillin in a loosely capped 15 ml tube. The tube was incubated overnight at 37°C with vigorous shaking. 1.5 ml of culture was poured into a microfuge tube and centrifuged at 6000 rpm for 4 min at 4°C in a microfuge (Heraeus, Germany). The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 100 µl of solution I by vigorous vortexing, until the pellet was dispersed. Then 200 µl of prepared solution II was added, the contents were mixed by inverting the tube rapidly five times. 150 µl ice-cold solution III was subsequently added, the tube was vortexed gently for 10 seconds and stored on ice for 10 min. The tube was centrifuged at 13,000 rpm for 10 min at 4°C in a microfuge (Heraeus, Germany) and the supernatant was transferred to a new tube. The DNA was precipitated by adding 2 volumes of isopropanol at room temperature and the contents were mixed by vigorously vortexing. The tube was centrifuged at 13,000 rpm for 15 min at 4°C in a microfuge (Heraeus, Germany). After centrifugation the supernatant was removed by gentle aspiration, then the pellet was rinsed with the cooled 500 µl 70% ethanol and centrifuged for 2 min at 13,000 rpm. The supernatant was removed as above mentioned, the pellet of DNA was dried in the air for 10 min. Then the DNA was redissolved in 50 µl TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 µg/ml).

Plasmids DNA midi or maxi preparation was preformed using QIAprep plasmid midi or maxi kit. The preparations were done according to the manufacturer's protocol. The concentration of DNA was calculated by the measurement of the absorbance at two wavelengths, 260 nm and 280 nm, in a spectrophotometer (Pharmacia Sweden). Pure DNA should have an $OD_{260/280}$ ratio of approximately 1.8. The amount of DNA can be calculated by the following formula,

$\mu g/ml\ DNA = OD_{260} \times dilution\ factor \times 50$

2.1.4 Restriction endonuclease digestion

Digestion of DNA with restriction endonuclease was performed by pipetting DNA sample, 10x restriction buffer, restriction enzyme, and distilled and deionized water (ddH_2O) in a 1.5 ml eppendorf tube. The reaction mixture was incubated at the recommended temperature. Normally 1 or 5 U of restriction enzyme was used to digest 1 µg DNA. Restriction endonucleases were inactivated by heating 65°C for 15 min.

2.1.5 Filling recessed 3' termini by Klenow fragment of DNA polymerase I

After DNA digestion by restriction enzyme was inactivated by heating, 1 µl Klenow fragment of DNA polymerase I (5 U/µl) and 1 µl of 1 mM dNTPs were added into a 20 µl of digestion reaction. The mixture was incubated for 15 min at 25°C. Klenow fragment can be inactived by adding EDTA to a final concentration of 10 mM and heating at 75°C for 20 min.

2.1.6 Dephosphorylation

In order to prevent self-ligation of the linearized vector DNA during cloning, dephosphorylation of 5' termini of vector DNA was performed by using CIP (Alkaline Phosphatase, Calf intestinal). DNA sample was mixed with 1 µl CIP (1 U/µl) and 2 µl 10x CIP buffer or 10x restriction buffer (NEB) in a 20 µl of reaction. The mixture was incubated for 1 h at 37°C. DNA can be purified by gel purification, spin-column purification or phenol exaction. CIP can be inactived by heating treatment at 65°C for 20 min.

2.1.7 Phenolization and precipitation of DNA

Phenolization is normally employed to purify nucleic acid by removing proteins. The volume of reaction mixture containing DNA was adjusting to 200 µl with TE or ddH_2O. 1 vol of Roti-phenol/chloroform was added, and mixed by vortexing. After centrifuging at 13000 rpm, 4°C for 10 min, the upper aqueous phase was transferred to a new tube and precipitation with 1/10 vol 3 M NaAc (pH 5.2) and 2 vol of isopropanol. DNA pellet recovered by centrifugation was subsequently washed with 1 ml of 70% ethanol, air-dried , and redissolved in ddH_2O.

2.1.8 Agarose gel electrophoresis

20000x ethidium bromide stock solution
10 mg/ml ethidium bromide

6x loading buffer
0.025% (w/v) bromophenol blue
30% (v/v) glycerol

50x TAE

242 g	Tris base
57.1 ml	glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

0.8-2% agarose gels were routinely used for analyzing DNA. The agarose was melted in 1X TAE buffer using a microwave oven (Quelle, Germany), and cooled to about 55^{o}C. Before the gel was poured into a casting platform, ethidium bromide was added. DNA samples were mixed with appropriate amount of 6x loading buffer before loaded into the wells. Normally, the voltage of eletrophoresis was set to 10 V/cm (eletrophoresis apparatus, pharmacia Sweden). DNA was visualized by placing gel on a UV light source (Bachofer Germany), and picture was photographed directly by a photo system (MWG-BIOTECH Germany).

2.1.9 Preparation of DNA fragments

DNA bands were excised from gel and subsequently purified with a QIAquick gel extraction kit. The preparation was performed according to the manufacturer's instruction. After purification, 5-8 µl DNA was run on agarose gel for analyzing the purified DNA amount for ligation.

2.1.10 Ligation

Ligations were done with a rapid DNA ligation kit (Roche, Germany) or NEB rapid ligation kit. The appropriate amount of linearized vector DNA and purified DNA fragment were used. The exact operation was performed according to the manufacturer's instruction. The ligation products were purified with high pure PCR product purification kit according to the manufacturer's instruction, before they were used to electroporation.

2.2 Plasmids construction

2.2.1 Plasmid PCR

Plasmid DNA was diluted to 10 ng/µl, 2 µl were taken as template. Then the following reagents were added.

Templates (10 ng/µl)	2 µl
Forward primer (10 pmol/µl)	5 µl
Backward primer (10 pmol/µl)	5 µl
dNTPs mix (10 mM each)	2 µl
Taq DNA polymerase (5 U/µl, GIBCO)	0.5 µl
10x PCR buffer (GIBCO)	10 µl
25 mM $MgCl_2$	6 µl
ddH_2O	69.5 µl

This mixture was first denatured for 4 min at 94°C followed by cycles as shown below:

denaturation	2 min	94°C
annealing	30 s – 50 s	42°C – 60°C (according to primers)
extension	50 s – 2.5 min	72°C (about 30 s/500bp)
cycles	25 – 30	
final extension	7 min	72°C

cool to 4°C

PCR was performed on the PCR machine (GeneAmp, PCR system 2400, Perkin Elmer). After PCR, 10 µl of PCR product was loaded on a appropriate concentration agarose gel for analyzing.

2.2.2 RT-PCR

RNA was prepared from viruses or cells with the Qiagen RNA kits. If the RNA was extracted from the DNA transfected cells and RT-PCR was done for identification, it was necessary to digest DNA with DNaseI (Roche, Germany 10 U/µl) at 37°C for 30 min to delete the transfected DNA contamination. The appropriate amount of RNA was used as template for RT.

First - strand synthesis of cDNA

RNA	2-4 µg
RT-primer	3 µl (200 ng/µl)

DEPC H_2O was added to 20 µl, and reaction was incubated in PCR machine at 70°C for 10 min, immediately placed on the ice. Then the following components were added to the annealed primer/template-mix for reverse transcription (RT):

reverse transcriptase 5x reaction buffer (Invitrogen)	10 µl
10 mM dNTPs (10mM each, Invitrogen)	1 µl
RNasin ribonuclease inhibitor (40 U/µl, Promega)	1 µl
0.1 M DTT (Invitrogen)	5 µl
Superscript II $RNaseH^-$ (200 U/µl, Invitrogen)	0.5 µl
DEPC H_2O	12.5 µl

The reaction was incubated in PCR machine at 42°C for 90 min, then inactivated at 95°C for 5 min. 10 µl RT product was used as template for PCR (steps were the same as 2.1).

2.2.3 pPolI-HHR-CAT2.1#1, #2 and #3 and pPolI-HHR-CAT2.2#1, #2 and #3

The parent plasmid from which six new clones were made were pPOLI-BDVcCAT2.1SP#10 and pPOLI-BDVcCAT2.2SP#11, that contain human polymerase I promoter (252 bp), mouse polymerase I terminator (175 bp), BDV 5'- NCR and 3'- NCR, and reporter gene CAT. There is a hepatitis delta virus (HDV) ribozyme behind 5'-NCR, that cleaves the transcript in cis to generate the correct 3'-end. Plasmid PCR was done with pPOLI-BDVcCAT2.2SP#11 as template, (HH-Ribo Fw/ HH-Ribo Bw1, HH-Ribo Bw2 and HH-Ribo Bw3 as primers) PCR products were digested by XbaI and HindIII, then cloned into the XbaI and HindIII site of pcDNA3.1. New plasmids were named pcDNA3.1HHR#1, #2 and #3, and were sequenced to assure that the cloned sequences were correct.

These plasmids were digested by BsmBI (this site was designed in the three backwards primers), then the ends of the fragment were filled in by Klenow enzyme and digested by ApaI after filling in. The isolated DNA fragments were cloned into the ApaI and HpaI sites of pPOLI-BDVcCAT2.1SP#10 or pPOLI-BDVcCAT2.2SP#11 to generate pPolI-HHR-CAT2.1#1, #2 and #3 or pPolI-HHR-CAT2.2#1, #2 and #3, and the new plasmids were sequenced to determined that the sequences were correct.

The ribozyme assay (see results) was done to prove that both ribozymes (hammerhead ribozyme and HDV ribozme) were functional in vitro, and the three plasmids would then be used to transfect BDV infected Vero cells to prove that the BDV polymerase could recognize and use the primary RNA transcript (Pol I- transcript) for transcription and replication.

2.2.4 pcDNA3.1Ribo1p, pcDNA3.1Ribo1s-p, pcDNA3.1Ribo2 and pcDNA3.1Ribo3

In order to prove that the both ribozymes were functional in vitro, these four plasmids were constructed. The inserted fragments were prepared by PshAI digest of pPolI-HH-RCAT#1, #2 and #3 separately, and by PshAI and SmaI digest of pPolI-HHR-CAT#1. pcDNA3.1 was digested by EcoRV and dephosphorylated using CIP. The inserted fragments were cloned into the EcoRV site of pcDNA3.1 under the control of a T7 promoter (HHR ribozyme near T7 promoter) to generate pcDNA3.1Ribo1p, pcDNA3.1Ribo1s-p, pcDNA3.1Ribo2 and pcDNA3.1Ribo3. Then these new plasmids were digested by different restriction enzymes for identification.

2.2.5 pPCRII-TOPO-RPA

Plasmid pPCRII-TOPO-RPA was constructed for in vitro-transcription to generate a probe for a RNase protection assay (RPA). Firstly plasmid pPCRII-TOPO-T7 (see method 2.6) and pcDNA3.1Rib1 P (see method 2.4) were digested by HindIII and XbaI to generate the vector and the inserted fragment respectively, then the inserted fragment was cloned into the HindIII and XbaI sites of vector to generate the pPCRII-TOPO-RPA.

2.2.6 pPoll-HHR-T7

For the construction of this plasmid, plasmid PCR was done with pCAGGS T7 as the template (T7 clone Fw/ T7 clone Bw as primers) and the PCR product was cloned into pPCRII-TOPO vector to generate pPCRII-TOPO-T7. After identification by restriction enzyme digest and sequencing, pPCRII-TOPO-T7 was digested by BsmBI and filled in by Klenow enzyme, then digested by MscI. The inserted fragment was cloned into the HapI and MscI sites of pPOLI-BDVcCAT2.1SP#10 to generate pPOLI-BDVT7. Hammerhead ribozyme version 3 (HHR#3) was cut from pcDNA3.1HHR#3 by BsmBI and was also filled in by klenow enzyme, then digested by ApaI. The HHR#3 fragment was inserted into the HapI and ApaI sites of pPOLI-BDV-T7 to generate pPolI-HHR-T7.

2.2.7 pBD

pBD was kindly provided by Dr. Kanta Subbarao, influenza Branch, CDC. This plasmid includes the CMV promoter, human RNA polymerase I promoter, hepatitis delta virus (HDV) ribozyme and two SapI cloned sites.

2.2.8 pBD-PB1, -PB2, -PA

To construct these plasmids, reverse transcription was firstly performed with viral RNA as template (Flu RT-primer as primer), then PCR was done with PB1 H5N1-Fw/Bw, PB2 H5N1-Fw/Bw and PA H5N1-Fw/Bw as primers respectively. RT-PCR products were treated with T4 DNA polymerase (dTTP and dCTP), and pBD was digested by SapI and filled in by Klenow enzyme (dTTP and dATP). The treated PCR products were ligated with the linearized vector, and transformed into the E coli. cells. The positive plasmids were identified, and sequenced to ensure that the complete sequences were correct. pBD-PB1, pBD-PB2 or pBD-PA was transfected into 293T cells together with the according 3 plasmids of the helper plasmid set (pHMG-PB1, -PB2, -PA,-NP) and pPolI-CAT-RT(-). A CAT-assay was performed to prove that either one of the three plasmids (pBD-PB1, -PB2, -PA) is functional.

2.2.9 pBD-NP

To construct pBD-NP, a plasmid PCR was done with pCI-NP as template (NP H5N1-Fw/Bw), and the PCR product was digested by SapI, then inserted into the SapI site of the pBD to generate pBD-NP. pBD-NP was sequenced and transfected into 293T cells with pHMG-PB1, -PB2, -PA and pPolI-CAT-RT(-), then CAT assay was made to prove that it is functional.

2.2.10 pBD-HA, -NS

To construct pBD-HA, -NS, a plasmid PCR was done with pCI-HA and pCI-NS as template respectively (HA H5N1-Fw/Bw, NS H5N1-Fw/Bw), and the PCR products were digested by HindIII/ApaI, then inserted into the HindIII/ApaI sites of pcDNA3.1 to generate pcDNA3.1-HA and pcDNA3.1-NS. pcDNA3.1-HA and pcDNA3.1-NS were sequenced in order to ensure

that the complete sequences were correct, and they were digested by SapI, then inserted into the SapI site of pBD to generate pBD-HA and pBD-NS.

2.2.11 pBD-NA, -M

To construct pBD-NA, -M, a plasmid PCR was done with pCI-NA and pCI-M as template respectively (NA H5N1-Fw/Bw, M H5N1-Fw/Bw), and the PCR products were cloned into the pPCRII-Topo vector, then sequenced in order to ensure that the complete sequences were correct. They (pPCRII-Topo-NA and pPCRII-Topo-M) were digested by SapI, then inserted into the SapI site of pBD to generate pBD-NA and pBD-M.

2.3 Ribozyme assay

2.3.1 Plasmid linerization

10 µg of plasmids were digested by appropriate restriction enzyme. After being sure of complete digestion through analyzing by gel electrophoresis, plasmids were precipitated by adding these chemicals (1/10 volume 3 M NaAc pH5.2, 1-2 volume Isopropanol, 2 µl glycogen). After vortexing, this mixture was centrifuged for 15 min at 13000 rpm, 4°C. Then pellet was sequentially washed by 70% ethanol and centrifuged again for 5 min at 13000 rpm, 4°C, leaving the pellet as dry as possible. Then the pellet was resuspended in 11 µl of DEPC H_2O. 1 µl was used for measuring the DNA concentration.

2.3.2 T7 - transcription (in vitro)

1 µl of linearined DNA was taken as template. Then the following reagents were added.

DNA	1 µl (1 µg/µl)
10x transcription buffer (with DTT)	2 µl
ATP (10 mM)	1 µl
CTP (10 mM)	1 µl
GTP (10 mM)	1 µl
UTP (10 mM)	1 µl
RNasin (40U/µl)	1 µl
T7 RNA polymerase (15U/µl)	1µl
DEPC H_2O	11 µl

The mixture was mixed by pipetting, and incubated for 2 h at 37°C. Then adding 1 µl DNaseI (10U/µl) and mixing, the mixture was incubated at 37°C for 30 min. The mixture was precipitated as above mentioned (2.3.1), and washed once by 70% ethanol and dried. Finally the pellet was resuspended in 30 µl DEPC H_2O.

2.3.3 Ribozyme reaction

The following materials were added for ribozyme reaction, then the mixture was incubated at at 37°C for 30 min.

RNA	30 µl
25 mM $MgCl_2$	20 µl
RNasin (40U/µl)	1 µl

2.3.4 Running denaturing acrylamide gel

10x TBE

TBE is generally used at 1x final concentration for preparing gels and for gel running buffer. The following recipe is for 1 L of 10x TBE .

Tris base	109 g
Boric acid	55 g
0.5 M EDTA solution	40ml

then add ddH_2O to 1 L.

Denaturing acrylamide gel mix (5% acrylamide /8 M urea gel)

Urea (high quality)	7.2 g
10 x TBE	1.5 ml
40% acrylamide (acry: bis-acryl = 19:1)	1.9 ml

add ddH_2O to 15 ml. Then stirring at room temperature until the urea is completely dissolved, subsequently add:

10% ammonium persulfate	120 µl
TEMED	16 µl

Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour gel immediately.

Loading buffer

95% Formamide

0.025% xylene cyanol

0.025% bromophenol blue

18 mM EDTA, 0.025% SDS

Details of attaching gel to the running apparatus (Bio-Rad, USA) according to the manufacturer´s instructions. 1x TBE was used as the gel running buffer. Before loading the samples, the wells of urea-containing gels were rinsed. 2 µl sample with 8 µl loading buffer was incubated at 90°C for 3 min, then shortly centrifuged and loaded in the wells of gel. Gels should be run at about 20 V/cm gel length.

2.3.5 Silver staining

After separating the gel from the two glass plates of electrophoresis apparatus, the gel was firstly washed in solution I (25% ethanol, 10% acetic acid) for 5 min at room temperature. Subsequently washed in solution II (10% ethanol, 0.5% acetic acid) for 5 min, then the gel was stained in 1.8 g/L silver nitrate solution (1.8 g silver nitrate dissolved in 1 L H_2O) for 20 min. Meanwhile the reduction solution (3 g NaOH and 40 mg $NaBH_4$ dissolved in 100 ml H_2O) was prepared. When ready, 0.75 ml formaldehyde was added into the reduction solution. The gel was washed with ddH_2O for three times, then laid into the reduction solution until bands were seen about 2 - 5 min. Immediately the gel was placed in solution of 10% acetic acid, then the gel was dried and photographed.

2.4 Indirect Immunofluoresces Assay (IFA) and in situ immunhisto-chemical BDV-detection

2.4.1 IFA

100x Ca^{2+}/Mg^{2+} solution

1 g	$MgCl_2$
1.32 g	$CaCl_2$
100 ml	dH_2O

autoclaved, then filtered with the 0.2 µm filter column (SARSTEDT, Germany).

10 x PBS

8 0 g	NaCl
2 g	KCl
14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4

Dissolved in 800 ml of H_2O, then adjust the pH to 7.4 with HCl. Finally add H_2O to 1 L and autoclaved.

1x PBS^{++} buffer

500 ml	1x PBS (autoclaved)
5 ml	100x Ca^{2+}/Mg^{2+} solution

Cell fixing buffer (100 ml)

95 ml	PBS^{++}
4 ml	Formaldehyde (PFA)
1 ml	Triton X-100 (t-Octylphenoxypolyethoxyethanol)

Mowiol DABCO

2.4 g	Mowiol
6 g	Glycerol

6 ml dH_2O

Mixed by magnetic stirrer over night. Next day 12 ml 0.2 M Tris-HCl (pH 8.5) was added and incubated at 50°C for 30 min. The viscous mixture was centrifuged at 6000 rpm at room temperature for 15 min, and the supernatant was mixed with DABCO (final concentration 2.5%). The solution can be stored at -20°C for several months.

The confluent cells were trypsinized by trypsin-EDTA, and spilt and reseeded in the wells of 24 well plate containing sterile glass coverslip (12 mm). After 2-3 days incubation, the cells were confluent. The growth medium was removed from the culture vessel, and the cells were washed once with PBS^{++}, then the cells were fixed with 1 ml cell fixing buffer for 30 min at room temperature. After fixation, the cells were washed twice with PBS^{++}, subsequently 100 µl of the first antibody (Bo18 p40 mAB, 1:100 dilution with PBS^{++}/3% BSA) was added (the glass coverslip should be covered by the antibody solution) and incubated for 1 h at room temperature. After 1 h, the cells were washed once as above, then 100 µl the second antibody FITC conjugated goat anti mouse IgG (1:100 dilution with PBS^{++}/3% BSA) was added and incubated for 1 h at room temperature. Then the cells were washed twice as above, and once with dH_2O. The glass coverslip was fixed on the glass carrier with Mowiol, and hardened overnight. The analysis was done in next day with a fluorescence microscope.

2.4.2 In situ immunhistochemical BDV-detection

Substrate solution

One tablet H_2O_2 (Sigma, USA) and one tablet Diaminobenzidin tetrahydrochlorid (DAB) (3,3'-Diaminobenzidine Tablet Set, Sigma, USA) were dissolved in 15 ml 1x PBS.

The BDV infected and noninfected cells were grown confluent in the wells of a 24 well plate. The growth medium was removed from the culture vessel, and the cells were washed once with PBS^{++}, then fixed with 1 ml cell fixing buffer for 30 min at room temperature. After fixation, the cells were washed twice with PBS^{++}, subsequently 150 µl of the first antibody (Bo18 p40 mAB, 1:100 dilution with PBS^{++}/3% BSA) was added and incubated for 1 h at room temperature. After 1 h, the cells were washed once as above, then 150 µl the second antibody (biotin anti mouse, Dianova, Germany, 1:100 dilution with PBS^{++}/3% BSA) was added and incubated for 45 min at room temperature. Then the cells were washed once as above, then 200 µl Streptavidin/HR-Peroxidase-conjungated (Dianova, Germany 1:1000 dilution with PBS^{++}/3% BSA) was added and incubated for 30 min at room temperature. The cells were washed twice with PBS^{++}, 1 ml substrate solution was added for 10-20 min at room temperature. The reaction was stopped by washing with dH_2O twice. The plate was dried, and the analysis was done by a microscope.

2.5 Establishment of Vero cell line infected by BDV H1766

To generate new BDV infected Vero cells, I have tried to infect the new Vero cells with BDV directly, but this failed. Therefore I used BDV H1766 to infect primary REB cells (rat embryo brain cells that can be easily infected with BDV), and then co-culture the BDV infected REB cells together with the new Vero cells. After two passages, the REB cells were dead and the Vero cells were infected by BDV H1766. I proved that the new Vero cells were almost completely infected by BDV H1766 through in situ immunhistochemical BDV-detection (2.4.1) and indirect immuno-fluoresces assay (2.4.2).

2.6 DNA-transfection of eucaryotic cell cultures

Complete medium

450 ml	1x DMEM
50 ml	FCS
5 ml	Antibiotic (100x penicillin-streptomycin solution)

DMEM/BA/S/P medium (0.5 L)

492 ml	DMEM
3 ml	35% Bovine Albumin (ICN)
5 ml	Antibiotic (100x penicillin-streptomycin solution)

2.6.1 Transfection I; Normal Lipofectamine Reagent

Adherent cells: The cells in 35 mm dishes should be 70% - 80% confluent for transfection. DNA (5 µg) was diluted in 100 µl 1x DMEM (without serum and antibiotics) in a tube (5 ml polystyrene Round-Bottom Tube, Falcon), then 10 µl Plus Reagent was added into the mixture, mixed by pipetting and incubated at room temperature for 15 min. 7 µl Lipofectamine Reagent was diluted into 100 µl 1x DMEM (without serum and antibiotics) in a second tube (5 ml polystyrene Round-Bottom Tube, Falcon) and mixed by pipetting. The diluted Lipofectamine Reagent was added into the tube containing pre-complexed DNA and Plus Reagent, then mixed and incubated for 15 min at room temperature. While complexes were forming, the medium on the cells was replaced with 0.8 ml 1x DMEM (without serum and antibiotics). The DNA-Plus-Lipofectamine Reagent complexes (total 200 µl volume) were added each dishes containing fresh medium. The complexes were mixed into the medium gently and incubated at 37^{o}C at 5% CO_2 for 6 h. After 6 h, the medium containing the complexes was removed and replaced with the complete medium. The cells were continually incubated at 37^{o}C at 5% CO_2 for 24 - 48 h for assay. If this transfection was to rescue viruses, DMEM/BA/S/P medium was used to replace the medium containing DNA-Plus-Lipofectamine Reagent complexes.

2.6.2 Transfection II; Lipofectamine 2000

Suspension cells: 2-4 h prior to transfection, culture medium was refreshed with new medium without antibiotics (DMEM + 10% FCS). DNA (5 µg) was diluted in 50 µl of Opti-MEM I reduced serum medium and mixed gently in a tube (5 ml polystyrene Round-Bottom Tube, Falcon). Lipofectamine 2000 mixed gently before using, then 10 µl Lifectamine 2000 was diluted in 240 µl of Opti-MEM I in a second tube (5 ml polystyrene Round-Bottom Tube, Falcon), mixed gently and incubated not longer than 5 min at room temperature. The diluted Lipofectamine 2000 was added to the tube containing the diluted DNA, then mixed gently and incubated 20-30 min at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. During the complexes were formed, the confluent cells (75 cm^2 culture flask) were firstly washed with 1x PBS, then trypsinized by 5 ml 1x trypsin-EDTA. After digestion, 5 ml medium (DMEM + 10% FCS) was added into flask, the mixture was transferred into a 15 ml of Falcon centrifuge tube and centrifuged at 1000 rpm in a megafuge (Heraeus, Germany) for 5 min. The supernatant was removed by aspiration, then the cell pellet was resuspended by 10 ml medium (DMEM + 10% FCS) and reseeded into fresh 35 mm dishes (1 ml/dish). The DNA-Lipofectamine 2000 complexes (total volume 300 µl) were added into the new 35 mm dish containing cells and mixed gently. The cells were incubated at 37^oC at 5% CO_2 for 24 h, then the medium containing DNA-Lipofectamine 2000 complexes was replaced with the complete medium. The cells were continually incubated at 37^oC at 5% CO_2 for 24 - 48 h for assay. If this transfection was to rescue viruses, DMEM/BA/S/P medium was used to replace the medium containing DNA-Lipofectamine 2000 complexes.

2.7 Generation, amplification and purification of wild and reassortant avian influenza virus

293T cells (1×10^6) on 35 mm diameter dishes were transfected with 12 plasmids (5 µg DNA), with the use of Lipofectamine and plus reagent (Invitrogen, USA) according to the transfection I (method 2.6.1). For generating the wild type FPV, 1.2 µl (1µg/µl) of protein expression plasmids (pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP) were used for transfection and the ratio of pHMG-PB1 : pHMG-PB2 : pHMG-PA : pHMG-NP was 1:1:0.2:1 (these four plamids were mixed firstly according to this ratio). 3.8 µl (1µg/µl) of the RNA polymerase I plasmids were used for transfection. These RNA polymerase I plasmids are pPolI-PB1, pPolI-PB2, pPolI-PA, pPolI-NP, pPolI-HA, pPolI-NA, pPolI-M and pPolI-NS of the strain A/FPV/Rostock/34 (H7N1), and the ratio was pPolI-PB1 : pPolI-PB2 : pPolI-PA : pPolI-NP : pPolI-HA : pPolI-NA : pPolI-M : pPolI-NS =1:1:1:1:2:2:1:1 (these eight plasmids were mixed firstly according to this ratio).

To generate the reassortant virus (GD1NSFPV) of the strain A/FPV/Rostock/34 (H7N1) and A/Goose/Guangdong/1/96 (H5N1), only pPolI-NS of the strain A/FPV/Rostock/34 (H7N1) was substituted by pBD-NS. The amount of DNA for transfection and the ratio among plasmids was not changed. 48 h after transfection, the supernatants were harvested and used

to infect confluent MDCK on 6-cm-diameter dishes to amplify progeny viruses. Then plaque purification was done three times for purifying the viruses. Every times a single plaque (in agarose gel, method 2.9.1 plaque assay) was picked and mixed in 500 µl PBS/BA/P/S/Ca/Mg solution (see plaque assay recipie). Then the purified viruses were amplified and titrated on MDCK cells by plaque assay. The titrated viruses were stored in -70°C for the following experiments. For generating other reassortant viruses, for example GD1MFPV, GD1HAFPV, GD1NAFPV and GD1NPFPV and so on, only pPolI-M (pPolI -HA, -NA, -NP) was used to instead of the according plamid pPolI-M (pPolI-HA, -NA, -NP). The procedure, that is same protocol for generating the reassortant GD1NSFPV, was performed to rescue an reassortant virus.

2.8 Chloramphenicol Acetyltransferase (CAT)-Assay

2.8.1 Preparation of cell extracts

48 h after transfection (method 2.6.1) , cells were washed twice with 1x PBS, then scraped off with 1.5 ml of 1 x PBS and transferred into a microfuge tube. Cells were centrifuged at low speed (6000 rpm) in a microcentrifuge (Heraeus, Germany) at 4°C for 5 min and resuspended in 100 µl of 0.25 M Tris-HCl buffer (pH 7.4) for flash CAT assay (Stratagene, USA), then cell extracts were made by freezing (nitrogen) and thawing (37°C water bath) three times (5 min/times). The debris was pelleted by spinning for 10 min at 13000 rpm at 4°C in a microcentrifuge (Heraeus, Germany), then the supernatant was transferred to a fresh tube and the extracts were kept on ice.

2.8.2 Determination of protein amount

A 5 µl sample from the cell lysate was taken to measure the protein amount, other materials were added as followed.

5 µl	sample
200 µl	Bio-Rad protein assay buffer
800 µl	ddH_2O

The concentration of protein was calculated by the measurement of the absorbance at the wavelength 595 nm in a DU-70 spectrophotometer (Beckman). According to the indicated absorbance, the protein concentration of different samples could be calculated and compared.

2.8.3 CAT-Assay

For each reaction, mix a predetermined amount of protein with the following prechilled reaction components (Flash CAT Non-Radioactive CAT Assay Kit, Stratagene, USA) on ice.

65 µl of cell extracts plus reaction buffer (same amount protein)
25 µl of 4 mM Acetyl Coenzyme A (Acetyl CoA)
10 µl of BODIPY CAM substrate

The mixture was mixed briefly and incubated at 37°C for 14-16 h. The reaction was terminated by adding 500 µl ice-cold ethyl acetate and the samples were vortexed for 20 seconds. The samples were centrifuged at 13,000 rpm for 3 min, then about 450 µl of supernatant was transferred into a fresh tube and the ethyl acetate evaporated in a vacuum drier. The yellow residue was dissolved in 10 µl of ethyl acetate, then spotted onto TLC plates (20 x 20 cm Silica gel 60, MERCK, Germany) (1.5 cm from the bottom of the TLC plate and no less than 1 cm from each sample). To perform TLC, a closed TLC tank (containing a piece of Whatmann 3MM filter paper running the vertical height of the tank) was preequilibrated with an 87:13 mixture of chloroform-methanol to a depth of 1.0 cm. After samples had been applied and air-dried on the plate, chromatography was done until the solvent front was near the top of the plate. The TLC plates (were dried in air for several seconds under a hood, then analyzed under direct long-wavelength UV light (366 nm) and recorded by standard instant photography.

2.9 Plaque-Assay

MEM/BA (0.5 L)

100 ml	10x MEM (Gibco)
10 ml	Antibiotic (100x penicillin-streptomycin solution)
20 ml	7.5% $NaHCO_3$
6 ml	35% Bovine Albumin (BA) (ICN)
354 ml	ddH_2O (sterilized)

Viral dilution solution (PBS/BA/P/S/Ca/Mg)

20 ml	10x PBS
175 ml	dH_2O

autoclaved, then add the following solutions:

2 ml	Antibiotic (100x penicillin-streptomycin solution)
1.2 ml	35% Bovine Albumin (ICN)

mix completely, add:

2 ml	100x Ca^{2+}/Mg^{2+} solution

mix completely.

Agarose gel solution for plaque assay of influenza virus (50 ml)

8.5 ml	ddH_2O
25.0 ml	2x MEM/BA
0.5 ml	1% DEAE Dextran (Mw, 500,000 Pharmacia Biotech)
15.0 ml	2% Oxoid Agar
1.0 ml	5% $NaHCO_3$

2.9.1 Plaque-Assay

Virus was exactly diluted with viral dilution solution. The cells (MDCK) were seeded on 3.5 cm-diameter dishes and grown confluently. The cells were washed once with 1x PBS^{++}, then 100 µl viral solution was laid on the cells. The cells were incubated at room temperature for one hour, and shaken gently. At the same time 2% Oxoid agar was melted and incubated in the 42°C water bath. MEM/BA medium was also incubated in the 37°C water bath. After one hour, the viral solution was removed from the dishes, 2% Oxoid agar was mixed with MEM/BA (and the other solutions). 2 ml mixture was given to every plate. Until the agar became solid, the dishes were inversely laid in a 37°C, 5% CO_2 humidified incubator (Heraeus, Germany). After 2 or 3 days plaques were seen on the dishes.

2.9.2 Analysis of Plaque-Assay

Preparation of coomassie brilliant blue buffer (100 ml)

0.25 g	Coomassie brilliant blue R 250 (Merck)
90 ml	Methanol /H2O (1:1)
10 ml	acetic acid

filtered with normal filter paper.

The agar was taken away from the dishes with a spatula. Cells were stained with coomassie brilliant blue (1 ml/3.5 cm plate) at room temperature for 5 min, then the plates were washed gently with normal water. Plaques were calculated for different dilution and the total amount of plaques can be calculated by the following formula:

PFU/ml = amount of plaques × dilution factor × volume factor

volume factor: PFU is related to 1 ml. if a dish was infected with 100 µl viral dilution solution, the factor was 10.

2.10 Haemagglutination (HA)-Assay

2.10.1 Preparation of red blood cells (RBCs) from chicken blood

Red blood cells should be taken from SPF chickens. If SPF chickens are not available, blood may be taken from normal birds (chickens) that are shown to be free from antibodies to avian influenza. Firstly about 20 - 30 ml fresh chicken blood (free from antibodies to avian influenza) was transferred to a 50 ml sterilized Falcon centrifugation tube containing 10 ml of 3.7% sodium citric acid. The RBCs was washed with cooled PBS^{++} and centrifuged at 1500 rpm at 4°C for 10 min. The supernatant was carefully removed by aspiration, then RBCs pellet was washed with PBS++ and centrifuged. This was repeated twice as above mentioned. Finally the pellet of RBCs was diluted to 0.5% (v/v) with cooled PBS^{++} for haemagglutination assay.

2.10.2 HA-Assay

50 µl PBS^{++} was dispensed into each well of a plastic U-bottomed microtitre plate (96 wells), then 50 µl virus suspension (cell culture supernatant) was placed in the first well. Twofold dilutions of 50 µl volumes of the virus suspension were made across the plate, subsequently 50 µl of 0.5% chicken RBCs was dispensed to each well. The mixture of every well was mixed by tapping the plate gently and then the RBCs were allowed to settle for about 30 min at 4^oC.

2.10.3 Determination of HA-Units

HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The Haemagglutinating Units (HAU) are : 2^x, where X is the number of the last well without tear-shaped streaming.

2.11 Luciferase Activity-Assay

2.11.1 Preparation of passive lysis buffer

Passive lysis buffer was supplied as a 5x concentrate (Promega, USA). A sufficient quantity of the 1x working concentration was prepared by adding 1 volume of 5x passive lysis buffer to 4 volumes of distilled water and the mixture was mixed well.

2.11.2 Active lysis of cultured cells

The transfected cells with plasmids containing the luciferase reporter gene were incubated for the appropriate time, the growth medium was removed from the cultured cells by aspiration and a sufficient volume of 1x PBS was applied to rinse the bottom of the culture dishes. The dishes were swirled briefly to remove detached cells and residual growth medium. A minimal volume of 1x lysis reagent was dispensed into the culture dishes (200 µl/35 mm dish), and the culture dishes were rocked several times to ensure complete coverage of cells with lysis buffer. The attached cells were scraped from the dish, then the cells and all liquid were transferred to a microcentrifuge tube. The microcentrifuge tube was vortexed vigorously for 10-15 seconds, then centrifuged for 15 seconds at 12,000 g at room temperature or for 2 min at 4^oC. The supernatant was transferred to a new tube, and the cell lysates might be assayed directly or stored at -70^oC. The assay for measuring was performed according to the manufacturer's protocol of Microplate Luminometers machine (Molecular Devices, USA).

2.12 Western Blotting (Semi-dry)

2.12.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

0.5 M Tris pH 6.8 (100 ml)

6.05 g Tris-base

50 ml dH_2O

adjusted pH to 6.8 with HCl, filled to 100 ml with dH_2O.

1.5 M Tris pH 8.8 (100 ml)

18.15 g	Tris-base
50 ml	dH_2O

adjusted pH to 8.8 with HCl, filled to 100 ml with dH_2O.

5x SDS-PAGE buffer (5 L)

5 g	SDS
30 g	Tris-base
144 g	Glycine

dissolved in 5 L dH_2O.

Loading buffer (10 ml)

5 ml	8 M urea solution
2 ml	120 mM Tris (pH 7.6)
1 ml	20% (w/v) SDS
0.5 ml	Methanol
1.5 ml	Glycerin
0.05 g	Bromphenol blue

autoclaved.

Stacking gels:

30% Acrylamide	0.75 ml
0.5 M Tris pH 6.8	1.25 ml
10% SDS	50 µl
dH_2O	2.9 ml
10% APS	50 µl
TEMED	3 µl

Resolving gels (10%):

30% Acrylamide	3.3 ml
1.5 M Tris pH 8.8	2.5 ml
10% SDS	100 µl
dH_2O	4 ml
10% APS	50 µl
TEMED	5.5 µl

According to manufacturer's (Bio-Rad, USA) instruction the eletrophoresis apparatus was assembled, and the resolving gel was poured into the glass plate sandwich. After the resolving gel was polymerized, the stacking gel was poured on top of the resolving gel. The 150 µl of sample (firstly measure the protein concentration, see 2.8.2) with 75 µl loading buffer were incubated for 5 min at 95^{o}C and cooled on ice for 1 min, then shortly centrifuged and loaded

into wells of gel and Rainbow protein marker (Amersham, Germany) (2 µl Marker + 8 µl loading buffer) loaded as control. Gels should be run at about 20 V/cm gel length.

2.12.2 Transfer membrane in "Semi-dry" electroblotter

Transfer buffer (Semi-dry)

5.8 g	Tris-base
2.9 g	Glycine
0.17 g	SDS
200 ml	Methanol

dissolved in 1 L dH_2O.

A PVDF-Membrane (Roth, Germany) and six blotting papers (GB004 Scheicher & Schuell, Germany) were incubated in 100% Methanol for 2 min, and washed three times (5 min/times) in dH_2O, then equilibrated in the transfer buffer for 2-3 min. On the layer of 3 blotting papers the PVDF-Membrane was laid. The polyacrylamide gel and again 3 blotting papers were then placed on the PVDF-Membrane and made a "sandwich" (without any bubbles), and laid in "Semi-dry" Electroblotter (Scheicher & Schuell, Germany). The eletrophoresis power was set to 0.8 mA/cm^2 for transfer.

2.12.3 Antibody-incubation

10x TBS (Tris Buffer Saline)

24.2 g	Tris
80 g	NaCl

adjusted pH to 6.7 with HCl, filled to 1 L with dH_2O. 1x TBS buffer was used for washing membrane and diluting antibody.

1x TBST buffer

100 ml	10x TBS
900 ml	dH_2O
0.5 ml	Tween 20

Blocking buffer

5 g	nonfat dry milk
100 ml	1x TBST
1 ml	BSA

The PVDF-Membrane was washed for 5 min in 1x TBST buffer after the transfer, then the membrane was blocked in the blocking buffer overnight. Next day the membrane was washed in for 5 min in 1x TBST buffer, then incubated in the first antibody solution (p-ERK monoclonal antibody, 1:500 dilution, Santa Cruz) for 1 h at room temperature. After washing three times (5 min/once) in 1x TBST buffer, the membrane was incubated with the second

antibody solution (horse radish peroxidase conjugated anti mouse monoclonal antibody, 1:1000 dilution, Santa Cruz) for 1 h at room temperature.

2.12.4 ECL-reaction

As above washed three times (5 min/once) in 1x TBST buffer and washed once in 1x TBS again, the membrane was then incubated for 1 min in ECL (enhanced chemilu-minescence) solution (Amerham, Germany) between a glass plate and a clear plastic membrane. After 1 min, everything was laid into a photo cassette, then a film (Bio Max Film MR1, Kodak) was placed on top of the membrane and exposed for 1.5 - 5 min. The film was then developed (Protec, Processor-Technology, Germany).

In order to detect the ERK-2 protein, the attached antibody was striped from membrane by incubating with a 1:10 diluted strong stripping solution (Re-Blot Plus Western Blot Recycling Kit, Chemicon, Canada) for 30 min at room temperature. Then washed in 1x TBST buffer for 5 min, the membrane was incubated with the ERK-2 monoclonal antibody (1:1000 dilution, Santa Cruz) solution for 1 h at room temperature. Subsequently the membrane was washed three times in 1x TBST buffer and washed once in 1x TBS, then incubated for 1 min in ECL solution and proceeded as above.

2.13 RNase protection assay (RPA)

This assay was performed by using three kits from Ambion company: MAXIscript^{TM} kit (in vitro transcription); RPAIII^{TM} kit (ribonuclease protection assay); BrightStar^{TM} BioDetect^{TM} kit (Nonistopic detection kit).

2.13.1 Synthesis of the biotin labeled probe

Firstly, a pool of four nucleotides was prepared according the following procedure: four kinds of nucleotides were mixed and the final concentration of every nucleotide is 5 mmol/L. The ratio of unlabeled UTP and Biotin-16-UTP (Roche, Germany) is 2:1. (7.5 µl 100mmol/L ATP, 7.5 µl 100mmol/L CTP, 7.5 µl 100mmol/L GTP, 5 µl 100mmol/L UTP and 25 µl 10mmol/L Biotin-16-UTP dissolved in 97.5 µl of RNase-free H_2O).

In order to perform RPA, the biotin labeled experimental probe and the biotin labeled positive control probe were synthesized by T7 polymerase in vitro transcription. Firstly, the plasmid pPCRII-TOPO-RPA was linearized by BsmBI and purified as mentioned (method 2.3.1), the linearized pTRI-actin-Mouse plasmid was provided by MAXIscript^{TM} kit for generating the positive control probe, then T7-transcription in vitro was done and the following reagents were added.

DNA (1 µg/µl)	1 µl
10x transcription buffer (with DTT)	2 µl
BSA (1 mg/ml)	2 µl
dNTPs (10 mM)	2 µl
RNasin (40U/ul)	0.5 µl
(T7, T3, or SP6) Enzyme mix (T7 polymerase 15U/µl)	2µl
DEPC H_2O	10.5 µl

The mixture was mixed by pipetting, and incubated for 1 h at 37°C. Then adding 1 µl DNaseI (10U/µl) and mixing well, the mixture was incubated again at 37°C for 30 min. 1µl of 0.5 M EDTA was added to stop reaction, the probe was then purified by on a 6% denaturing polyacrylamide gel, from which the according bands were isolated.

2.13.2 Gel purification of probe

The RNA mixture was mixed with an equal volume of Gel Loading Buffer II (total 42 µl) and heated 3 min at 95^{o}C. 12 µl and 32 µl of the mixture (transcription mixture and the Gel Loading Buffer II) were loaded separately into two freshly-rinsed wells of a 6% denaturing polyacrylamide gel. This gel was run in the eletrophoresis apparatus (Bio-Rad, USA) for about 1 h at 120 volts until the bromophenol blue was approaching the bottom of the gel. Subsequently the gel was cut into two parts and one part including the 12 µl sample was stained by ethidium bromide. After staining, a thin plastic membrane was placed on the top of the gel and the band was visualized by UV light, then the position of the probe band, the position of the bromophenol blue and the scope of gel could be circled with Marker pen on the plastic membrane. When the plastic membrane was placed on the another part of the gel (not stained and including the 32 µl sample) according to the position of the bromophenol blue and the scope of gel, the probe area of the gel indicated by the circled region on the plastic membrane was excised with new scalpel, transferred to an RNase-free microfuge tube, and submerged in 350 µl of probe elution buffer (provide by the Ambion MAXIscript^{TM} in vitro transcription kit). This tube containing the gel piece was incubated at 37°C overnight, then the gel pieces were removed and the amount of biotin labeled probe was determined by the measurement of the absorbance at two wavelengths, 260 nm and 280 nm, in a spectrophotometer (Pharmacia Sweden). Pure RNA should have an $OD_{260/280}$ ratio of approximately 2.0. The amount of RNA can be calculated by the following formula,

$\mu g/ml\ RNA = OD_{260} \times dilution\ factor \times 40$

The labeled probe was stored at -20°C or -70°C.

2.13.3 RNA preparation and purification

5 µg pPolI-HHR-CAT2.1#3 was transfected into Vero and BDV infected Vero cells separately according to method 2.6.1. After three days of transfection, RNA was prepared

from the transfected cells with Qiagen RNeasy kit, then RNA was digested by RQ1 to remove the DNA contamination. The following reagents were added:

RNA (1µg/µl)	40 µl
RQ1 10x reaction buffer	20 µl
RQ1 enzyme (1U/µl)	80 µl
RNasin (40U/µl)	4 µl
DEPC H_2O	56 µl

After incubation for 1 h at 37°C, 20 µl of stop buffer (provided by Promega with RQ1 enzyme) was added and mixed, then incubated for 10 min at 65°C. Phenol/cholorform was used to purify the digested RNA sample, then direct PCR (control) was performed by using the digested RNA as template and specific CAT primers (CAT29-52 Fw/CAT612-589 Bw). When the PCR results were negative, the digested RNA could be used for RPA hybridization. If they were positive, the RNA sample has to be digested again by RQ1 as above, until the PCR results were negative.

2.13.4 Hybridization and RNase digestion of probe and sample RNA

This step was performed according to standard hybridization procedure of the instruction manual of RPAIII™ Kit of Ambion. 40 µg of digested RNA sample from Vero and BDV-Vero cells respectively was used for hybridization.

Tube	Sample RNA	Amount probe	RNase dilution
1	40 µg BDV-Vero	2400 ng	1:100
2	40 µg Vero	2400 ng	1:100
3	2 µg Yeast RNA	2400 ng	1:100
4	10 µg Mouse liver RNA	600 ng	1:100
5	2 µg Yeast RNA	2400 ng	No RNase

Briefly, sample RNA and labeled probe were mixed according to 600 pg probe per 10 µg total RNA. Two controls were set up for the experimental probe: 2 µl Yeast RNA was mixed with 2400 ng of the experimental probe (tube 3, 5). 5 M NH_4OAc supplied with the RPAIII™ kit was used to adjust the mixture (RNA sample and probe) concentration of NH_4OAc to 0.5 M, then 2.5 volumes of EtOH was added and mixed thoroughly for co-precipitating the probes and sample RNAs. The tubes were placed in -20°C for at least 15 min, then the RNAs were pelleted by centrifuging at 13000 rpm in a microcentrifuge for 15 min at 4°C. The supernatant was discarded and pellets were washed with 70% EtOH once, then pellets were dried for 5 min on the bench. Pellets were resuspended in 10 µl hybridization buffer provided by the RPAIII™ kit and the tubes were incubated for 3 - 4 min at 90 - 95°C. The tubes were then centrifuged briefly at low speed to collect the liquid at the bottom of the tube, then the tubes were incubated overnight at 42°C (16 -18h).

After hybridization, 150 µl of the diluted RNase mixture (the 6µl of RNaseA/T1 was added to 600 µl of RNase Digestion III buffer and mixed very well) was added to every tube except for tube #5 (only the 150 µl of the RNase Digestion III buffer without RNaseA/T1 was added). This "no" RNase control tube will serve as a control for probe integrity. The tubes were incubated for 30 min at 37^{o}C, then 225 µl RNase Inactivation/Precipitation III solution was added and vortexed, and these tubes were incubated for at least 15 min at -20^{o}C. Subsequently, these tubes were centrifuged at 13000 rpm in a microcentrifuge for 15 min at 4^{o}C. All supernatant was carefully removed from each tube, pellets were washed with 70% EtOH once, then pellets were dried in air for 5 min.

2.13.5 Separation and detection of protected fragments

After hybridization and RNase digestion, the precipitated sample was resuspended 4 µl RNase-free water. 4 µl gel loading buffer provied by the RPAIII^{TM} kit was added to every tube and every tube was incubated for 3 min at 90-95°C, then the samples were loaded into wells of a 6% denaturing polyacrylamide gel. Gel should be run at about 120 V constant voltage for 1 h, then electroblotting from polyacrylamide gel was performed according to the protocol provided by nonisotopic detection kit of Ambion. Briefly, a positively charged nylon (PCN) membrane (Ambion, Germany) and six blotting papers (GB004 Scheicher & Schuell, Germany) were wetted in 0.5x TBE. On the layer of 3 blotting papers the PCN membrane was laid. The polyacrylamide gel and again 3 blotting papers were then placed on the PCN membrane and made a "sandwich" (without any bubbles), and laid in "Semi-dry" Electroblotter (Scheicher & Schuell, Germany). The eletrophoresis power was set to 0.8 mA/cm^2 for transfer. Electroblotting was run for 1 h. Then UV crosslinking was done according the manufacturer's recommendation (UV Stratalinker 2000, Stratagene). That is, the membrane, which was removed from the sandwich, should be kept wet and exposed under the UV of 1.2 millijoules twice, then the protected bands were detected by using BrightStar^{TM} BioDetect^{TM} kit (Ambion USA).

In the detection procedure, all incubations should be done at room temperature with constant, gentle agitation in containers that allow free movement of the membrane and the membrane must remain wet throughout the procedure. The wash buffer, blocking buffer, Strep-Alkaline Phosphatase, the assay buffer and CDP-Star solution are all provided by BrightStar^{TM} BioDetect^{TM} kit.

After UV crosslinking, the membrane was washed with 1x wash buffer twice (5 min in 1 ml wash buffer/ cm^2 membrane/wash), then the membrane was incubated in blocking buffer twice (5 min in 0.5 ml blocking buffer/cm^2 membrane/incubation). Subsequently, the membrane was incubated in blocking buffer for 30 min (1 ml blocking buffer/cm^2 membrane), then the membrane was incubated in diluted Strep-Alkaline Phosphatase (Strep-AP) for 30 min (1 µl Strep-AP was added to 10 ml blocking buffer and mixed thoroughly). After incubation in diluted Strep-AP, the membrane was incubated in blocking buffer for 15

min (0.5 ml blocking buffer/cm^2 membrane), then washed in 1x wash buffer twice (15 min in 1 ml wash buffer/ cm^2 membrane/wash). The membrane was incubated in 1x assay buffer twice (2 min in 0.5 ml 1x assay buffer/cm^2 membrane/incubation), then incubated in 4 ml of CDP-Star solution for 5 min. Subsequently, the membrane was laid on a piece of filter paper to remove excess CDP-Star, without letting the membrane dry. The membrane was wraped in a single layer of plastic and laid into a photo cassette, then a film was placed on the membrane and exposed for 1 h at the beginning. The film was developed (Protec, Processor-Technology, Germany). An appropriate time was determined for exposures, until an optimal image was obtained.

Results

1 AIV

1.1 Testing the expression vector pBD

The expression vector pBD (kindly provided by Dr. Kanta Subbarao, influenza Branch, CDC, USA) includes the human cytomegalovirus (CMV) promoter for mRNA expression, the human RNA polymerase I promoter for v/cRNA expression, hepatitis delta virus (HDV) ribozyme and two SapI cloning sites (Figure 9). It was important for me to compare the ability of CMV promoter and human RNA polymerase I promoter of this vector expressing the reporter CAT gene, because this plasmid was to be used to clone the cDNAs of the eight RNA-segments of an avian influenza virus (A/Goose/Guangdong/1/96 (H5N1)) to establish a reverse genetic system. Therefore the reporter plasmid pBD-CAT was constructed which contains the CAT open reading frame in positive sense, flanked by the 3' and 5' noncoding regions of the NS-RNA segment of influenza virus A/Goose/Guangdong/1/96 (H5N1) (Figure 10). At the same time the CMV promoter was partially deleted to construct the plasmid pBD-CAT(-CMV) (Figure 10) to test the functionality of the Pol I promoter of pBD.

CAT-assays were performed to test the functionalities of the RNA Pol I and Pol II promoter. Although CAT protein was expressed by transfection of pBD-CAT(-CMV), the amount of CAT protein was almost equal to the amount of a 1/100 dilution of a sample from pBD-CAT transfected cells (Figure 11). This could be due to residual activity of the truncated CMV-promoter (Figure 10).

In order to test the functionality of the Pol I promoter, five plasmids were cotransfected into 293T cells. Four helper plasmids (pHMG- PB1, -PB2, -PA and -NP, which were kindly provided by HDoz Dr. S. Pleschka) were used to express the PB1, PB2, PA and NP proteins of influenza virus A/PR/8/34 under the control a mouse hydroxymethylglutary-coenzyme A reductase promoter (HMG). pPolI-CAT-RT(-), which was also kindly provided by HDoz Dr. S. Pleschka, contains CAT open reading in negative polarity flanked by the noncoding regions of the NS gene of influenza A/WSN/33 virus (161). The transfection of all five plasmids into 293T cells led to the expression of CAT protein, as shown in Fig. 12. This indicated that a negative-sense RNA synthesized from the pBD-CAT(-CMV) as from the pPolI-CAT-RT(-) was reconstituted intracellularly into functional RNPs with the polymerase subunits and the NP provided in trans from the helper plasmids. These RNAs were then transcribed by the reconstituted viral RNA polymerase into mRNA, which was translated into CAT protein. Transfection of only the pBD-CAT plasmid led to a strong CAT-activity due to the active Pol II (CMV) promoter (Figure 12). The result of the pBD-CAT(-CMV) with the helper plasmids tranfection demonstrated that the Pol I-transcript can only be replicated/transcribed by the viral RDRP. The CAT-assay results shown that both promoters were functional (Figure 11 and 12).

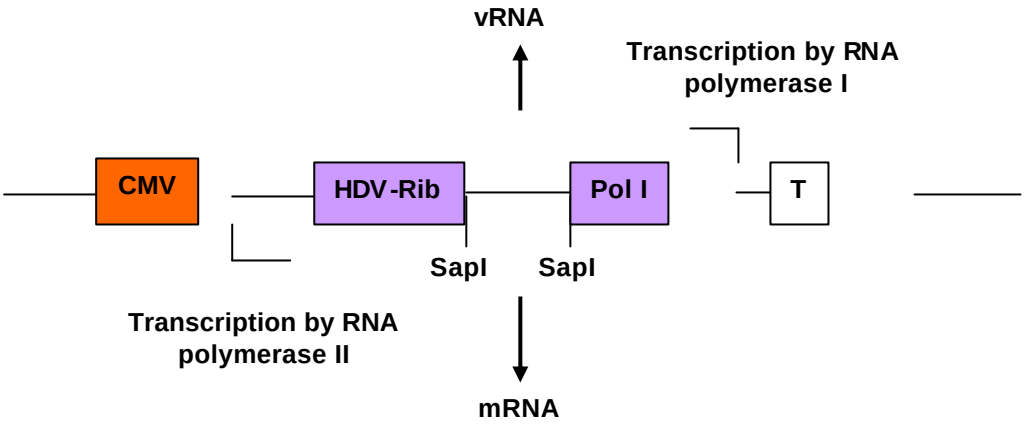


Figure 9: Schematic diagram of the vector pBD. The cDNA of each of the eight influenza segments was inserted into the SapI sites. The Pol I transcription unit (Pol I promoter and HDV-Rib) is flanked by the Pol II promoter (CMV) of the human cytomegalovirus and a polyadenylation signal (T) of the gene encoding the bovine growth hormone. After transfection, negative-sense vRNA is synthesized by cellular RNA polymerase I from the Pol I promoter. Transcription by Pol II (CMV) yields mRNAs.

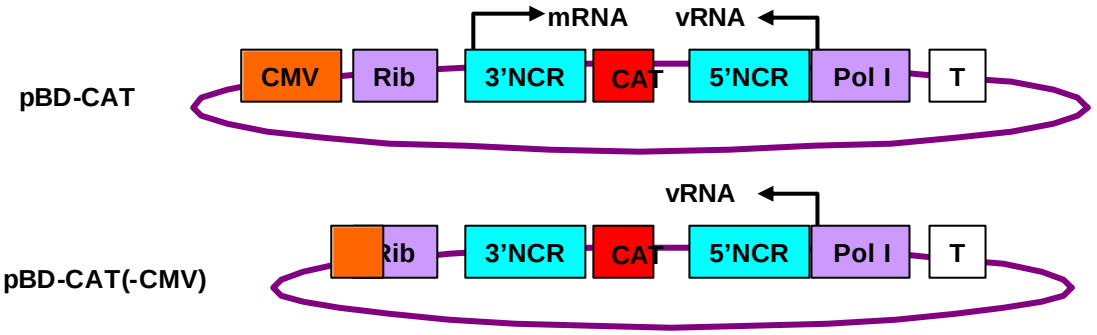


Figure 10: Schematic diagram of the pBD-CAT and pBD-CAT(-CMV). The reporter gene CAT, flanked by the 3' and 5' noncoding regions of the NS-RNA segment of influenza virus A/Goose/Guangdong/1/96 (H5N1), was inserted between the Pol I promoter and HDV-ribozyme. After tranfection of pBD-CAT, two types of molecules are synthesized (mRNA from Pol II promoter and vRNA from Pol I promoter) as indicated. Pol II (CMV) promoter was deleted (but not completely as indicated) in order to test the functionality of Pol I promoter by CAT-assay.

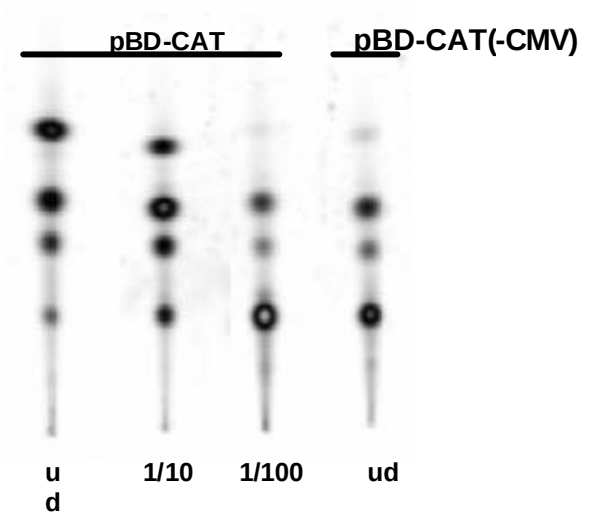


Figure 11: CAT expression in 293T cells transfected with pBD-CAT or pBD-CAT(-CMV). Approximately 10^6 adherent 293T cells were transfected with 5 µg pBD-CAT or pBD-CAT(-CMV) using plus reagent and lipofectamine reagent (Invitrogen) according to the manufacturer's instruction. Cell extracts were prepared and 50 µl samples were used for CAT-assay. pBD-CAT transfected 293T cells, cell extracts were diluted as indicated (ud; 1/10; 1/100) for CAT-assay. pBD-CAT(-CMV) transfected into 293T cells and cell extract were used undiluted for CAT-assay. "ud" means undiluted.

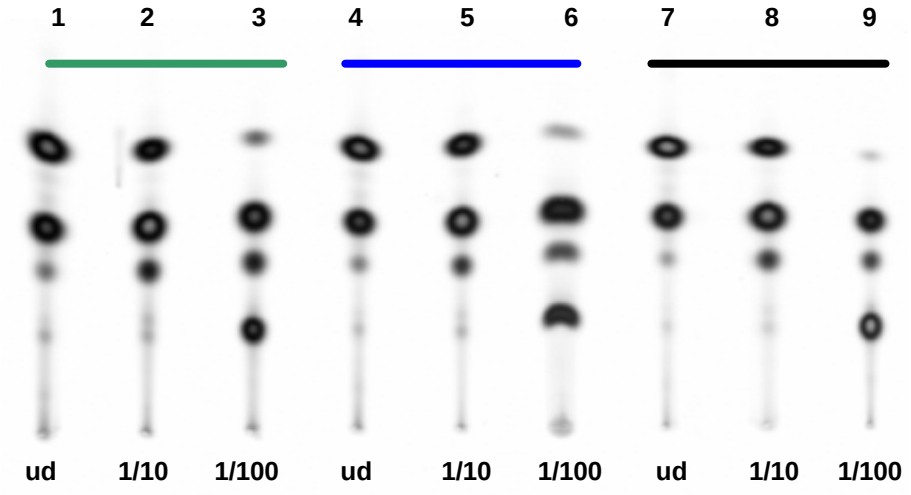


Figure 12: CAT expression in 293T cells tranfected with different plasmids combinations. Lane 1, 2, 3: 1.4 µg of pPolI-CAT-RT(-), cotransfected into 293T cells on 35 mm diameter dishes together with 3.6 µg of a DNA mixture of pHMG- PB1, -PB2, -PA and -NP as positive control (the ratio of PB1: PB2: PA: NP always was 1:1:1:2). Lane 4, 5, 6: 1.4 µg of pBD-CAT(-CMV) were cotransfected into 293T cells together with 3.6 µg of a DNA mixture of pHMG- PB1, -PB2, -PA and -NP. Lane 7, 8, 9: 5 µg of pBD-CAT transfected into 293T cells. Cell extracts of every transfection was diluted as indicated (ud; 1/10; 1/100) for CAT-assay.

2.1 Construction of pBD-PB1, -PB2, -PA, -NP, -HA, -NA, -M, -NS

In order to establish a reverse genetic system of an avian influenza virus (A/Goose/Guangdong/1/96 (H5N1)), I used the POLI-POLII system. Therefore I have cloned the complete cDNAs of the eight segments of the strain A/Goose/Guangdong/1/96 (H5N1) into the expression vector pBD according to the method and materials. The newly constructed plasmids are pBD-PB1, -PB2, -PA, -NP, -HA, -NA, -M, -NS. Every plasmid was identified by restriction enzymes digest (Figure 13) and was sequenced. The sequence results showed that only one nucleotide (G549A) of the NS gene was different compared to the original sequence of NS gene (the original sequence refers to sequences of the eight segments of the strain A/Goose/Guangdong/1/96 (H5N1) that were sequenced by National Avian Influenza center, Harbin Veterinary Research Institute, CAAS, China) and the genebank A/Goose/Guangdong/1/96 (H5N1) NS sequence (AF144307), and led to an NS1 protein with an AA difference Glu175Lys. There were two nucleotide (G592A, A690G) differences in the M gene compared to the original M sequence and A/Goose/Guangdong/1/96 (H5N1) M sequences from the genebank (AF144306). This resulted in AA changes Met189IIe and His222Arg in the M1 protein. Only one nucleotide (G51A) of the NA gene was different compared to the original sequence of the NA gene and A/Goose/Guangdong/1/96 (H5N1) NA sequence from the genebank (AF144304), and led to the AA difference Gly11Arg. There was no difference in the complete HA gene. The polymerase genes (PB1, PB2 and PA) and NP gene were not completely sequenced, as a functional assay was performed to prove that they are correct (Result 1.3).

1.3 Testing the cloned polymerase genes and NP gene

Unlike positive-strand RNA viruses, the negative-sense viral RNAs of influenza viruses are not infectious. Only vRNA molecules encapsidated with the NP and three polymerase proteins (PB1, PB2, PA) to form RNPs are able to initiate a viral replication and transcription cycle. So pBD-PB1 (pBD-PB2, -PA or -NP) can be cotransfected into 293T cells in combination with the functional helper plasmids (pHMG-PB2, pHMG-PA and pHMG-NP, pPolI-CAT-RT(-)) (161) respectively, then CAT-assays were done to test the functionality of the cloned polymerase genes and the NP gene. For example 1.4 µg pPolI-CAT-RT(-) was cotransfected into 293T cells on 35 mm diameter dishes together with 3.6 µg of the four helper plasmids (pBD-PB1 (avian influenza), pHMG-PB2, pHMG-PA and pHMG-NP (human influenza: A/PR/8/34), the ratio of the four plasmids is pBD-PB1: pHMG-PB2: pHMG-PA: pHMG-NP = 1:1:1:2) using lipofectamine and Plus Reagent on adherent cells. Furthermore three transfections were done to test functionality of the other avian influenza virus three genes (Figure 14). As positive control a transfection (pHMG-PB1, pHMG-PB2, pHMG-PA, pHMG-NP and 1.4 µg pPolI-CAT-RT(-)) was done, as a negative control only pPolI-CAT-RT(-) was transfected. 48 h after transfection, samples were prepared and CAT-assays were performed. The results showed that the new plasmids expressing the avian

influenza virus polymerase protein and the NP protein are functional (Figure 14). Then the four newly constructed plasmids together with pPolI-CAT-RT(-) were cotransfected into 293T cells and a CAT-assay was performed in order to compare the CAT expression by the newly constructed plamids with the helper plasmids expressing the genes of the human influenza virus as a positive control. The negative control were done as described above. These results showed that the CAT amount expressed by the POLI-POLII system was higher compared to system that uses separated promoter: the POLI for vRNA and the PolII for the PB1, PB2, PA and NP proteins (Figure 15).

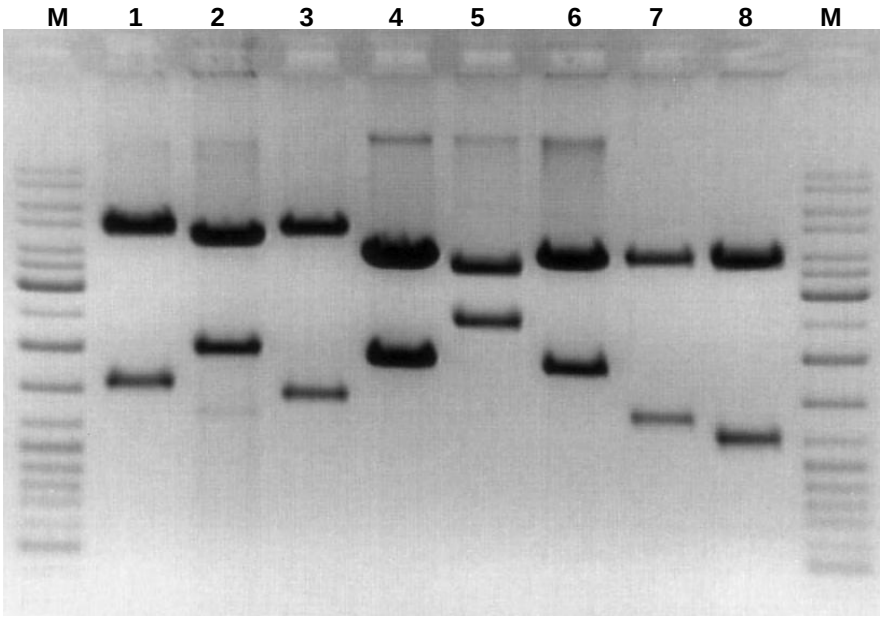


Figure 13: Characterization of pBD-PB1, -PB2, -PA, -NP, -HA, -NA, -M, -NS by restriction enzyme digest. Different restriction enzymes were used to digest the constructed plasmids for identification. Lane 1: pBD-PB1 was digested by EcoR I, (1603 nt and 5086 nt); Lane 2: pBD-PB2 by BamH I, (2006 nt and 4683 nt); Lane 3: pBD-PA by BamH I, (1506 nt and 5074 nt); Lane 4: pBD-NP by XbaI (1907 nt and 4006 nt); Lane 5: pBD-HA by NdeI (2466 nt and 3661 nt); Lane 6: pBD-NA by HindIII (1851 nt and 3955 nt); Lane 7: pBD-M by XbaI (1369 nt and 4006 nt); Lane 8: pBD-NS by XbaI (1232 nt and 4006 nt). All digest showed the expected bands.

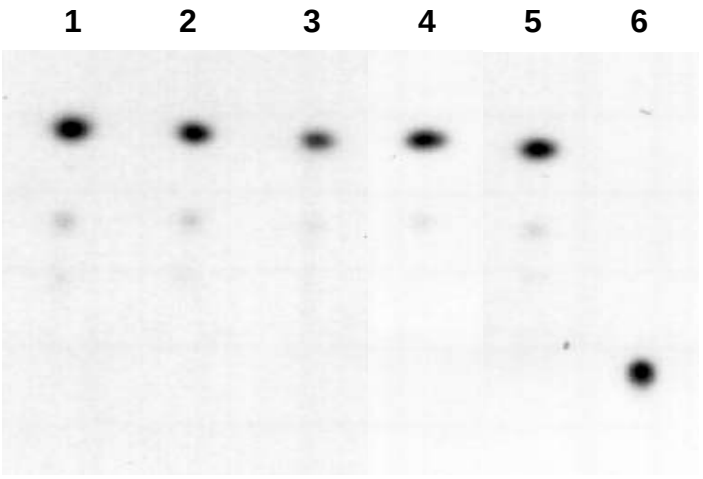


Figure 14: Testing the functionality of the cloned polymerase genes and the NP gene by CAT-assay. Different combinations of plasmids were made to transfect 293T cells. Independent of the expression vectors used (pHMG or pBD), the ratio of PB1: PB2: PA: NP always was 1:1:1:2. These four plamids were mixed according to the ratio. 5 µg DNA in total was used for every transfection. 3.6 µg of DNA mixture of the PB1, PB2, PA and NP plasmids was cotransfected into 293T cells on 3.5 mm diameter dishes together with 1.4 µg of pPolI-CAT-RT(-). 1. pHMG-PA, -PB1, -PB2, -NP + pPolI-CAT-RT(-); 2. pBD-PA, pHMG-PB1,-PB2,-NP + pPolI-CAT-RT(-); 3. pBD-PB1, pHMG-PA,-PB2,-NP + pPolI-CAT-RT(-); 4. pBD-PB2, pHMG-PA,-PB1,-NP + pPolI-CAT-RT(-); 5. pBD-NP, pHMG-PB1,-PB2, -PA + pPolI-CAT-RT(-); 6. pPolI-CAT-RT(-).

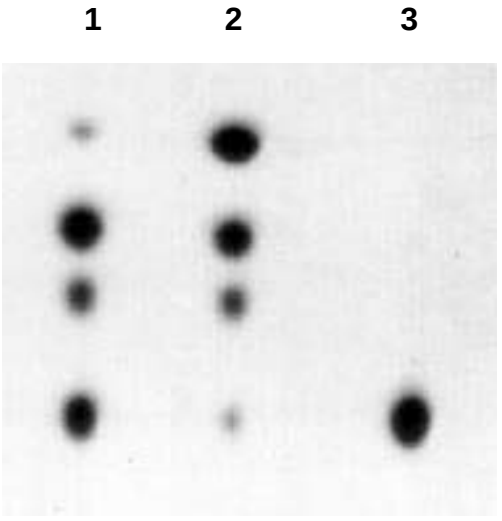


Figure 15: Comparison of CAT expression in 293T cells transfected with pPolI-CAT-RT(-) and pHMG expression vectors of the influenza virus A/PR/8/34 genes (PB1, PB2, PA and NP), and pBD expression vectors of the avian influenza virus A/Goose/Guangdong/1/96 (H5N1) genes (PB1, PB2, PA and NP). The ratio of PB1: PB2: PA: NP is 1:1:1:2. 1. pHMG-PB1, -PB2, -PA, -NP + pPolI-CAT-RT(-); 2. pBD-PB1,-PB2, -PA, -NP + pPolI-CAT-RT(-); 3. pPolI-CAT-RT(-).

1.4 Generation of reassortant avian influenza virus

In order to prove that the eight plasmids encoding the genes of the avian influenza strain A/Goose/Guangdong/1/96 (H5N1) are functional, I tried to rescue reassortant viruses using the genetic background of the avian influenza strain A/FPV/Rostock/34 (H7N1) (this strain is also a pathogenic avian influenza virus that is used regularly in the laboratory) with one gene of A/Goose/Guangdong/1/96 (H5N1). Because the plasmids including the genes of the strain A/FPV/Rostock/34 (H7N1) (provided by R. Wagner, Marburg) are controlled only by the RNA polymerase I promoter, the helper plasmids (pHMG-PA, -PB1, -PB2, -NP) must be used to express the polymerase proteins and NP to generate the biological active RNPs (Figure 16). To obtain reassortant viruses, 12 plasmids were transfected into confluent 293T cells (Figure 16). I have substituted the PB1, PB2, PA, NP, HA, NA, M and NS gene of A/FPV/Rostock/34 (H7N1) by the according genes of the A/Goose/Guangdong/1/96 (H5N1) respectively to generate a reassortant virus, but only the wild-type A/FPV/Rostock/34 (FPV) virus and a reassortant virus (GD1NSFPV) which carries the A/Goose/Guangdong/1/96 (H5N1) NS gene and the other 7 genes of FPV were successfully rescued. The rescued viruses were then purified by three plaque passages on MDCK cells.

1.5 Identification of reassortant virus by RT-PCR

The reassortant virus obtained in the rescue experiment was analyzed by RT-PCR of the viral RNA to confirm that the NS-segment is from A/Goose/Guangdong/1/96 (H5N1). The isolated RNAs from the supernatants of cultured cells infected by wild-type and reassortant virus were treated by DNaseI to remove residual DNA and purified by phenol/chloroform extraction. Half of the RNA was used as a template for RT reaction with Flu RT-primer for the RT of influenza viral RNA, the other half was used as a template for direct PCR (control) with a pair of NS-specific primers (NS-RT Fw/NS-RT Bw). PCR products were subjected to restriction analysis with endonucleases and analyzed by agarose gel electrophoresis. With the RNA of wild-type and reassortant virus, the RT-PCRs yielded products of about 890 nucleotides. The RT-PCR product of the NS-RNA of the reassortant virus was sensitive to HaeIII (after digestion, about 523 nt and 367nt bands should be generated), whereas no cleavage occurred when the RT-PCR product of wild-type NS-segment of FPV virus digested (Figure 17).

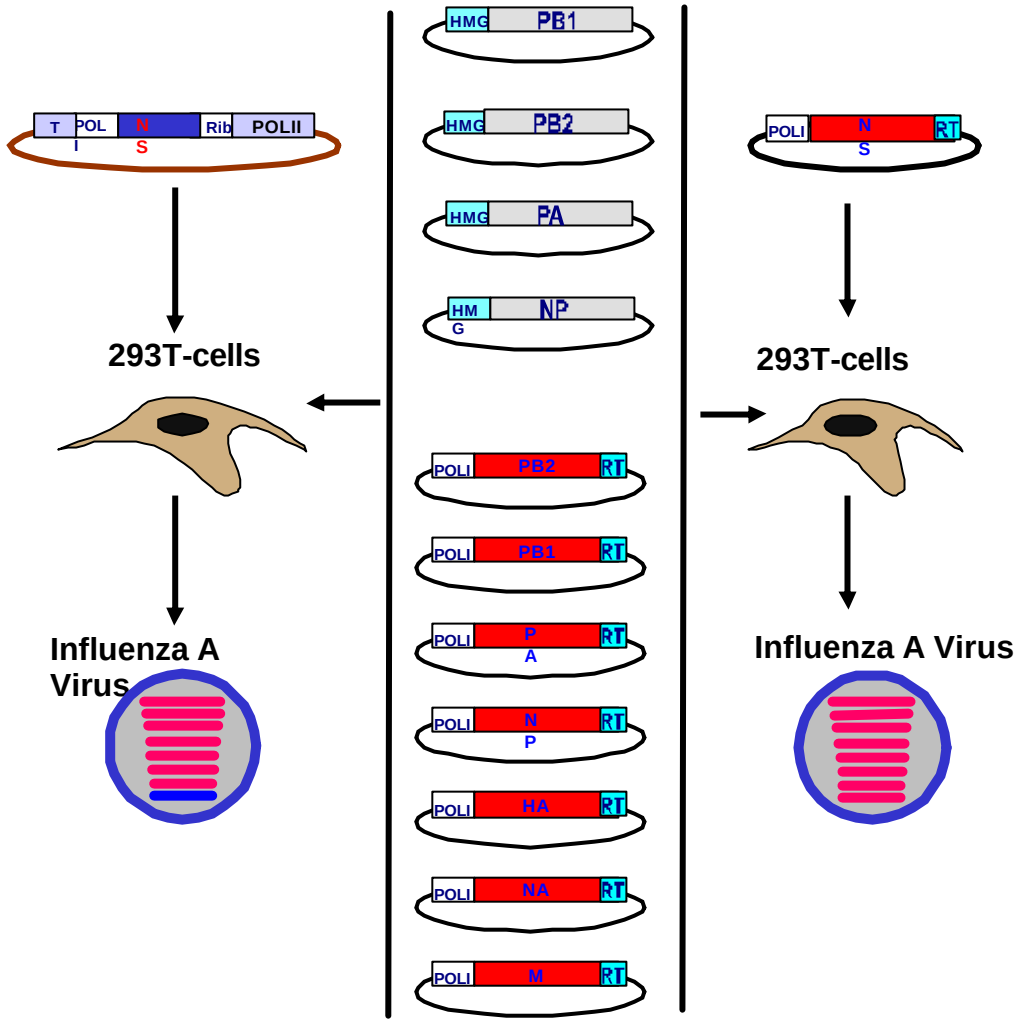


Figure 16: Reverse genetics method for the generation of wild and reassortant avian influenza viruses. Plasmids containing the RNA plymerase I promoter, a cDNA for each of the eight viral RNA segments of the strain A/FPV/Rostock/34 (H7N1), and RNA polymerase I terminator are transfected into 293T cells together with plasmids expressing PA, PB1, PB2 and NP proteins (pHMG-PA, -PB1, -PB2, -NP). To generate the reassortant virus, the NS gene of the strain A/FPV/Rostock/34 (H7N1) was substituted by the NS gene of the strain A/Goose/Guangdong/1/96 (H5N1).

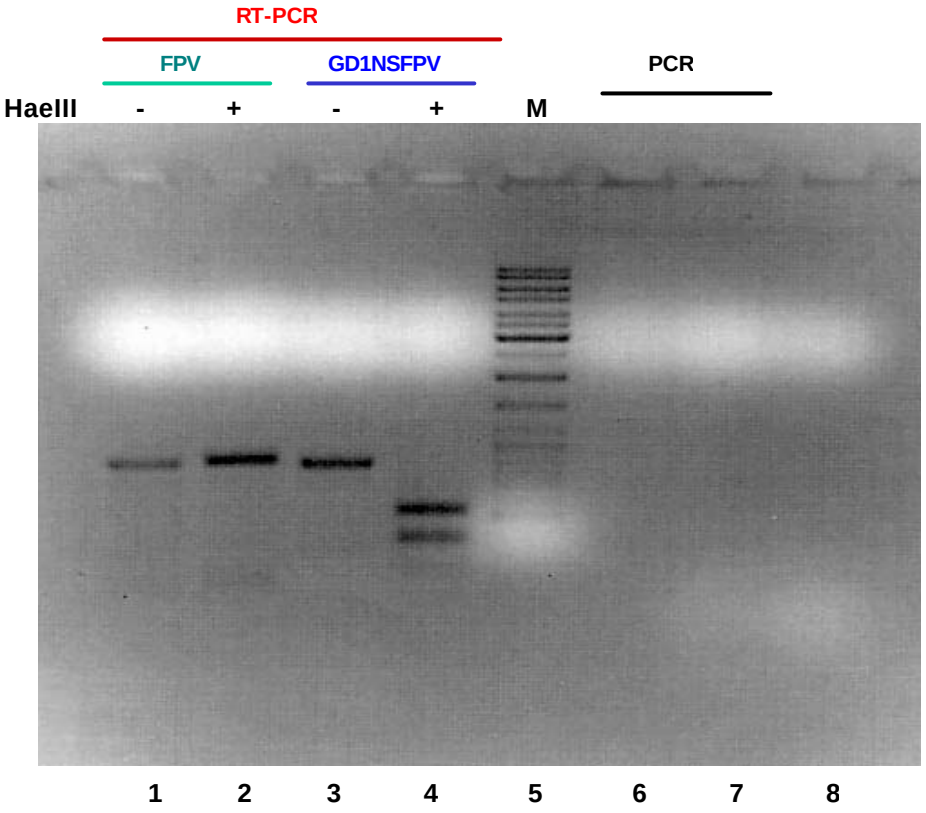


Figure 17: RT-PCR of RNA isolated from wild-type FPV and reassortant virus (GD1NSFPV). RT-PCR products were incubated with HaeIII as indicated and separated on an agarose gel. Direct PCR was done with the RNA as templates or ddH_2O was used as RT-PCR control. Lane 6: the RNA of wild-type FPV; Lane 7: the RNA of GD1NSFPV; Lane 8: RT-PCR control.

1.6 Growth of reassortant virus in cell culture

It was of great interest to see how both viruses (wild-type FPV and reassortant avian influenza virus GD1NSFPV) replicate in cells. Therefore MDCK cells were infected at an MOI of 0.001 by wild-type FPV and reassortant GD1NSFPV viruses respectively to allow multicycle replication, supernatants were monitored for HA titers and plaque assay at different time points. The HA titers showed that the reassortant GD1NSFPV growed apparently faster than the wild type FPV (Figure18). The studies on the growth characterics were also performed by plaque assay on MDCK monolayers. Results obtained by this approach indicated that the number of plaque- forming units (PFU) formed by the reassortant GD1NSFPV was more than 40 fold higher than by the wild-type FPV (Figure 19). Moreover, the plaques formed by the reassortant GD1NSFPV were larger than those formed by the wild-type FPV (Figure 20).

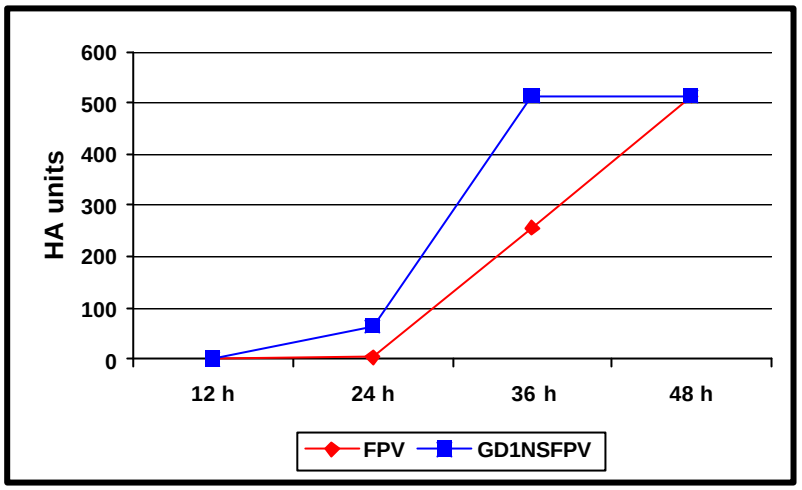


Figure 18: Growth curves of wild-type FPV and reassortant GD1NSFPV. MDCK monolayers were infected at an MOI of 0.001 with wild-type FPV and reassortant GD1NSFPV respectively, and supernatants were monitored for HA titers at the time points indicated.

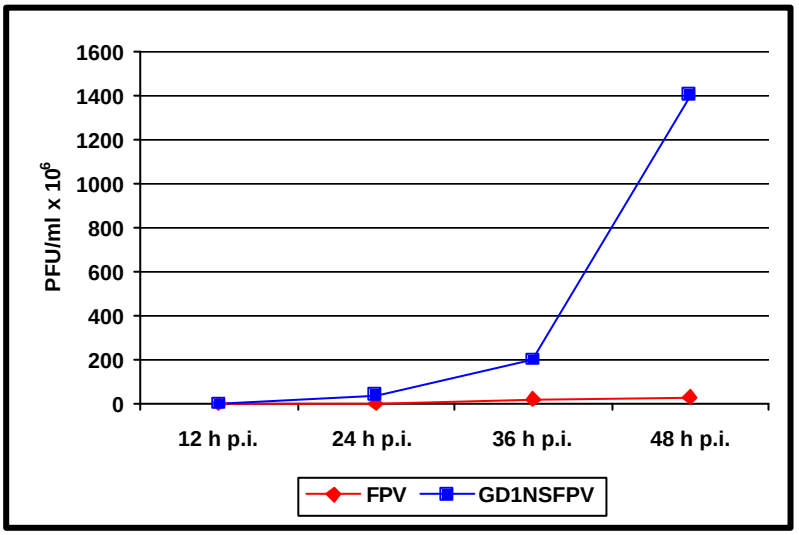
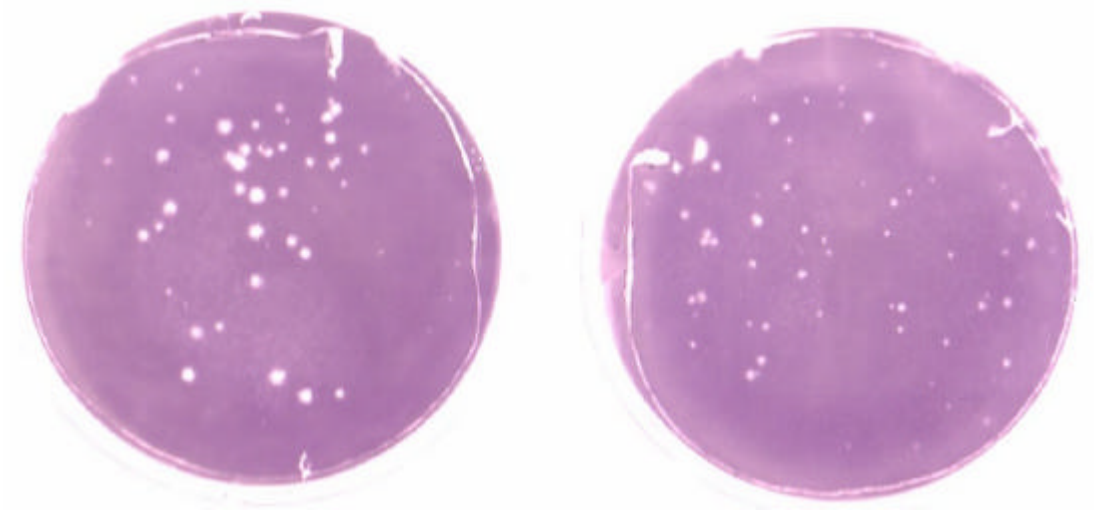


Figure 19: Growth curves of the wild-type FPV and the reassortant GD1NSFPV. MDCK monolayers were infected at an MOI of 0.001 with wild-type FPV and reassortant GD1NSFPV, and the supernatants were monitored for plaque assay (PFU) at the time points indicated.



GD1NSFPV **FPV**

Figure 20: Comparison of plaques formed by wild-type FPV and reassortant GD1NSFPV. Plaques formed by the reassortant GD1NSFPV virus were larger than those formed by the wild-type FPV as indicated.

1.7 Interferon-ß (IFN-ß) induction in cells infected with wild-type FPV and reassortant GD1NSFPV

IFN synthesis is one of the early responses of the host against viral infection. Previous studies shown that an influenza virus with a deletion in the NS1 gene, delNS1 virus, can only replicate efficiently in an IFN-deficient system, suggesting that the NS1 protein of influenza virus inhibits the IFN system of the host (65, 218). As the reassortant virus that differs from the wild-type FPV only by the NS-segment grows to significantly higher titer and forms bigger plaques, I investigated the IFN-ß responses through the induction of the IFN-ß reporter plasmid in response to infection by both viruses. The results showed that the induction of the IFN promoter dependent reporter (luciferase gene is controlled by IFN-ß promoter) by the wild-type FPV was much higher (about three folds) than by the reassortant GD1NSFPV (Figure 21).

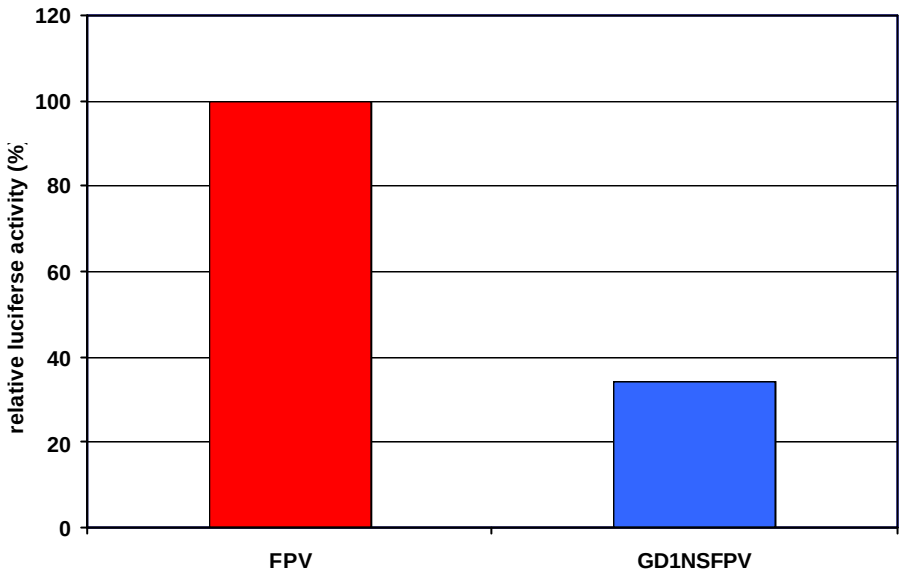


Figure 21: The induction of the IFN dependent reporter by the wild-type FPV and reassortant GD1NSFPV virus. MDCK cells were transfected with 100 ng of the IFN-ß promoter luciferase reporter plamids pIFN-Luci. After 24 h, cells were mock infected or infected with the wild-type FPV or the reassortant GD1NSFPV virus at an MOI of 1. Cell extracts were prepared at 4 h postinfection in cell lysis buffer (Promega) and assayed for luciferase activity. For a comparison mock values were subtracted, and enzyme activity induced by wild-type FPV was set to 100%. Average values determined in four independent experiments are shown.

1.8 Investigation of the Raf/MEK/ERK cascade activation between the wild type FPV and reassortant GD1NSFPV virus.

The Raf/MEK/ERK cascade is the prototype of mitogen-activated protein kinase (MAPK) cascade and has an important role in cell growth, differentiation and survival (24, 46, 179). Growth factor-induced signals are transmitted by consecutive phosphorylation from the serine/threonine kinase Raf via the dual-specificity kinase MEK (MAPK kinase/ERK kinase) to ERK. Active ERK subsequently translocates to the nucleus to phosphorylate a variety of substrates and mediates changes in gene expression (179, 227). A variety of DNA and RNA viruses induce signaling via MAPK pathways in infected host cells, suggesting that these kinase cascade may play a functional role in virus replication (14, 22, 180). Pleschka et al. (163) have proved that this cascade is also activated by influenza virus, and signaling through the mitogen cascade seems to be essential for RNP export from the nucleus. As the reassortant GD1NSFPV differs from the wild type FPV in growth titer, I investigated whether this cascade is differently induced by the infection of wild type or reassortant influenza virus. The

results showed that both viruses induce MAPK-signaling, but there were no big difference in the activation Raf/MEK/ERK cascade by both viruses (Figure 22).

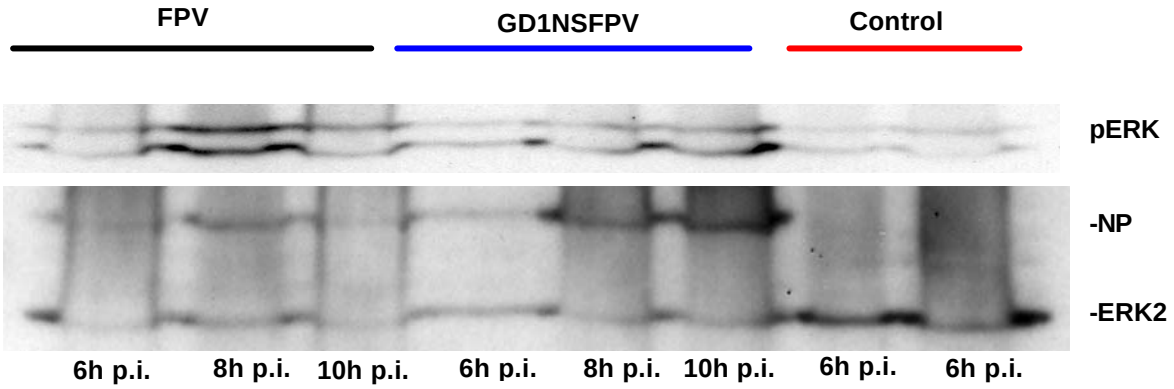


Figure 22: FPV and GD1NSFPV induce activation of ERK in MDCK cells. MDCK cells were mock treated (Control), or infected with FPV or GD1NSFPV at an MOI of 5 and incubated for 6, 8, or 10 h as indicated. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were blotted onto polyvinylidene difluoride membrane. The membrane was incubated with the first antibody (pERK monoclonal antibody for the detection of activated ERK) and a second antibody (horse radish peroxidase conjugated anti mouse monoclonal antibody). ECL was detected by a ECL^{TM} Western blotting detection reagents kit (Amerham, Germany). ERK2 Western blots using an anti ERK2-antibody were analyzed to confirm equal loading of the kinases. The ECL results were visualized on X-ray films. NP proteins was detected to control virus replication.

1.9 Comparison of NS gene nucleotide and amino acid sequence of the strain A/FPV/Rostock/34 (H7N1) and A/Goose/Guangdong/1/96 (H5N1)

The reassortant GD1NSFPV virus differs from the wild type FPV virus only in the NS-segment, but the former grows to significantly higher titer and forms bigger plaques than the later. So I compared the NS gene nucleotide acid and amino acid (AA) sequences of the strain A/FPV/Rostock/34 (H7N1) with the strain A/Goose/Guangdong/1/96 (H5N1). The length of NS-segment of both viruses is 890 nt and encodes the NS1 and NS2 proteins due to splicing of the mRNA. The NS1 protein encoded by both viruses is 231 amino acids long, and the NS2 protein encoded by both viruses is 122 amino acids long. NS1 is designated as nonstructural because it is synthesized in infected cells, but is not incorporated into virions (116). The NS1 proteins of influenza A possesses two important functional domains: an RNA-binding domain near the amino-terminal end (amino acids 1-73) and an effector domain in the carboxyl half the molecule (amino acids 74-231) (147, 168, 169) furthermore the binding domains are conserved to different extents among influenza A and B viruses (234). The binding domain specifically binds to the RNA poly(A), U6 snRNA and double-stranded (ds) RNA, and the activation of PKR kinase is blocked by NS protein binding to dsRNA (see discussion). The effector domain of the NS1 protein is required for the inhibition of the nuclear export of the poly(A)-containing cellular mRNA and the inhibition of pre-mRNA splicing (128, 234) by

binding and inhibiting the function of two cellular proteins. These two proteins, that are required for the 3'-end processing of the cellular pre-mRNAs, are the 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) and the poly(A)-binding protein II (PABII) (32, 123, 142). The binding sites for the CPSF and PABII are near the carboxy end of the NS1 protein: the CPSF binding site is centered around amino acid 186 and the PABII binding site is located in the 223-237 amino acid region (123). Another study demonstrated that NS1 protein has a latent nuclear export signal (NES) that is located at the amino end of the effector domain (amino acids 134-147). This NES is inhibited by the adjacent 14 AA of the effector domain (amino acids 148-161) (124). In addition, the NS1 protein contains two nuclear localization signals (NLS) (Figure 23) (76), it has been proved that NLS2 seems to be dispensable for the function of the NS1 protein in regulating the nuclear export of viral mRNA (168). The NS2 protein, which contains a nuclear export signal near the amino-terminal end (amino acids 11-23) (149), mediates the nuclear export of viral RNPs.

The NS1 gene of the strain A/FPV/Rostock/34 (H7N1) is only 69.7% identical to the NS1 of the strain A/Goose/Guangdong/1/96 (H5N1) at the nucleotide level, and only 69.3% at the AA level. In the RNA-binding domain (amino acids 1-73) of the NS1 protein of A/FPV/Rostock/34 (H7N1), there are seven continuous AA differences to NS1 protein of the strain of A/Goose/Guangdong/1/96 (H5N1). The differences are $_{21}$RFADQEM$_{28}$ to $_{21}$LLSMRDM$_{28}$, and result in a change of hydrophilicity (Figure 23 and 24). There are eight AA different in the position 134 to 161 of the effector domain of NS1 protein between the strain A/FPV/Rostock/34 (H7N1) and the strain A/Goose/Guangdong/1/96 (H5N1), which also leads to a change of hydrophilicity (Figure 23 and 24). The conserved L at the position 146 in the effector domain of NS1 protein of FPV, which is crucial for the function of both NS1 and HIV-1 Rev protein (137, 168) (HIV-1 Rev protein contains two functional domains (135) and facilitate the nucleocytoplasmic transport of unspliced and partially spliced viral mRNA (79, 136)), is changed to S in the according position of GDNS1FPV. The binding site for the CPSF (amino acid 184-188) among the two NS1 proteins of both viruses is completely conserved, but the binding site for the PABII between two NS1 proteins of both viruses is different (Figure 23). The NS2 gene of the strain A/FPV/Rostock/34 (H7N1) was 81.4% identical to the NS2 of the strain A/Goose/Guangdong/1/96 (H5N1) at the nucleotide level, and 82% at the amino acid level. Two amino acids (M14Q, G22E) were different in the NES of NS2 protein of the stain A/FPV/Rostock/34 (H7N1) compared to the NS2 protein of the strain A/Goose/Guangdong/1/96 (H5N1) (Figure 25).

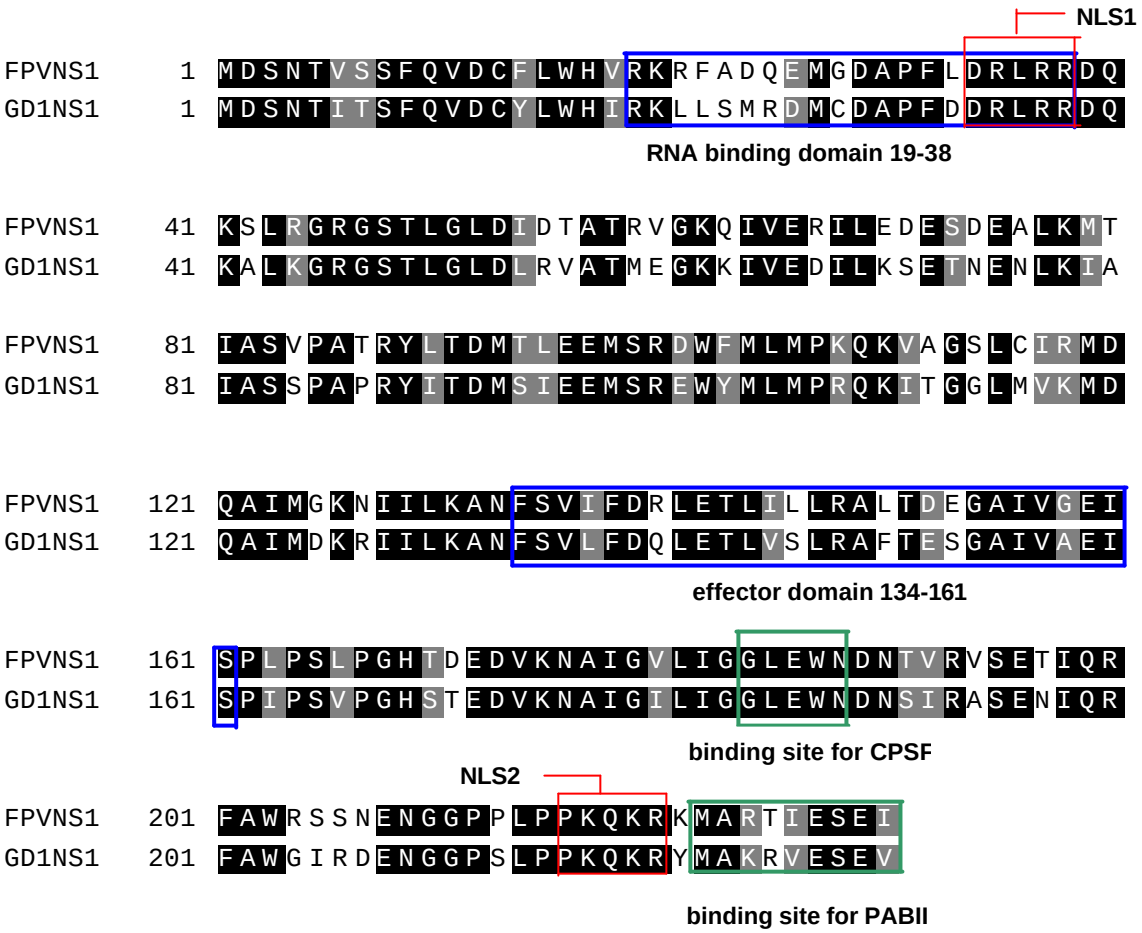


Figure 23: Comparison of the NS1 protein of the strain A/FPV/Rostock/34 (H7N1) (FPVNS1) and A/Goose/Guangdong/1/96 (H5N1) (GD1NS1). Identical amino acids are marked by black color, and the position 19-38 of RNA-binding domain and the position 134-161 of effector domain are outlined in blue rectangles. The two nuclear location signals are indicated by red rectangles. The binding sites for the CPSF and for the PABII are outlined in green rectangles.

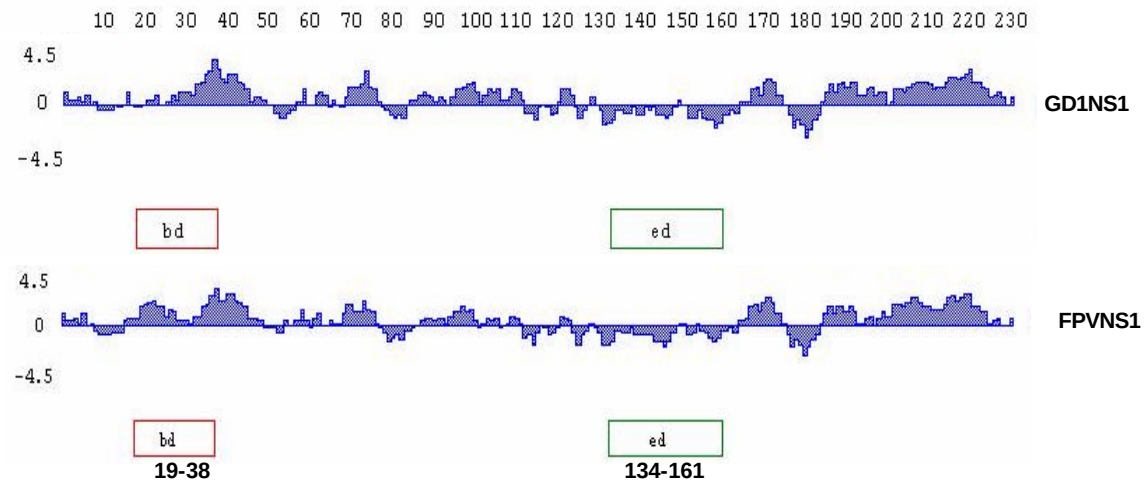


Figure 24: Comparison of hydrophilicity and hydrophobicity of the NS1 protein of the strain A/FPV/Rostock/34 (H7N1) and A/Goose/Guangdong/1/96 (H5N1). This analysis was done by the Protean of DNASTAR software (DNASTAR, Inc. USA). This method predicts regional hydropathy of proteins from their amino acid sequences, using the approach of Kyte and Doolittle (1982). Hydropathy values are assigned for all amino acids and are then averaged. The average is plotted as shown. The value of nine residues is averaged in this analysis. If the value is more than 0, it means hydrophilicity. If the value is less than 0, it means hydrophobicity. The position 19-38 in the RNA-binding domain (bd) and the position 134-161 in the effector domain (ed) are shown. The hydrophilicity is apparent different in the position 19-38 in the RNA-binding domains and also little different in the position 134-161 in the effector domains of both NS1 proteins.

```
GD1NS2     1 MDSNTITSFQDILQRMSKMQLESSSVDLNGMITQFERLKI
FPVNS2     1 MDSNTVSSFQDILMRMSKMQLGSSSEDLNGMITQFESLKL

                                NES

GD1NS2    41 YRDSLGESVMRMGDLHSLQNRNATWRNELSQKFEEIRWLI
FPVNS2    41 YRDSLGEAVMRMGDLHSLQSRNGKWREQLSQKFEEIRWLI

GD1NS2    81 AECRNILTKTENSFEQITFLQALQLLEVESEIRTFSFQL
FPVNS2    81 EEVRHRLKITENSFEQITFMQALQLPEVEQEIRTFSFQL

GD1NS2   121 I
FPVNS2   121 I
```

Figure 25: Comparison of the NS2 protein of the strain A/FPV/Rostock/34 (H7N1) (FPVNS2) and A/Goose/Guangdong/1/96 (H5N1) (GD1NS2). Identical amino acids are marked by black color, and the nuclear export signals (NES) is outlined with a blue rectangle.

2. BDV

2.1 Plasmids construction

Pleschka et al. proved the existence of an additional "A" residue at the extreme 3' end of the single-strand genomic RNA of BDV (162) and that a terminal "U" residue is probably found at the 5' end. The result is very important because correct structures of the genome ends are most critical parameters for the development of reverse genetics techniques that permits the genetic manipulation of BDV. To generate a primary RNA transcript in vivo that can be replicated by the viral polymerase, I used the Pol I-system, because RNA polymerase I is an abundant nuclear enzyme which transcribes ribosomal RNA, that doesn't contain 5'-cap or 3'-poly(A) structures. Moreover RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences. Therefore Pol I-transcripts resemble viral genomic RNAs and can be replicated by viral polymerases (146). As the Pol I normally does not readly incorporate an "U" as the first residue (normally A or G as first residue; I, Grummt personal communication), I have generated new Pol I expression constructs (using the pPOLI-BDVcCAT2.1SP#10 and pPOLI-BDVcCAT2.2SP#11 as the parental plasmids constructed by S. Pleschka) that express a transcript, that start with a hammerhead ribozyme (HHR) sequence (suggested by M. Schwemmle and cooperation with our laboratory) beginning with an "A" residue (Figure 26). The hammerhead ribozyme should cleave the transcript in cis to generate a new 5'-end that starts with the genomic "U" residue. The correct 3'-end of the transcript is assured by a hepatitis delta virus (HDV) ribozyme that also cleaves the transcript in cis generating the correct 3'-end of the transcript (Figure 27). This technique is used to generate Pol I-transcripts with correct 3'-ends in the Pol I-system for reverse genetics of influenza (60, 161). I used three versions of the HHR and developed three different constructs including the three HHR versions, because I could not estimate which version would be functional. I used the parental plasmids: (1) pPOLI-BDVcCAT2.1SP#10 and (2) pPOLI-BDVcCAT2.2SP#11. They contain the human RNA polymerase I promoter and terminator, HDV ribozyme and a CAT reporter gene in positive orientation. Only three nucleotides were changed in the anti-genomic BDV 3'NCR of the (2) pPOLI-BDVcCAT2.2SP#11 compared to the (1) pPOLI-BDVcCAT2.1SP#10. I have constructed six new plasmids (Figure 27) with a HHR expressing a CAT reporter gene RNA (in positive orientation) flanked by the BDV-NCR. They were controlled by restriction enzyme digest (Figure 28) and sequenced to assure the correct sequences. The sequence results showed that the inserted HHR sequences were exact. In total 6 kinds of constructs (pPolIHHRCAT2.1#1, #2, #3; pPolIHHRCAT2.2#1, #2, #3) for the expression of the CAT gene from a Pol I-transcript, that should resemble a BDV-like genomic RNA, were successfully constructed.

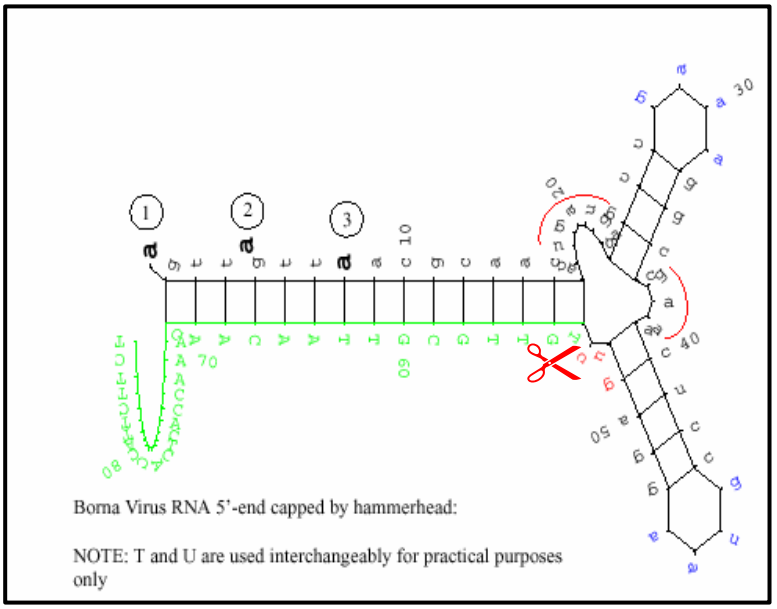


Figure 26: Hammerhead Ribozyme. The Pol I-transcript should begin with a 5' "A" of the hammerhead ribozyme. Bold "a" 1, 2 & 3 are three alternative 5' ends. Red "guc" is the cleavage site of the hammerhead sequence. Green is the native Borna virus 5' end sequence (in DNA code) which should be released by hammerhead after self cleavage.

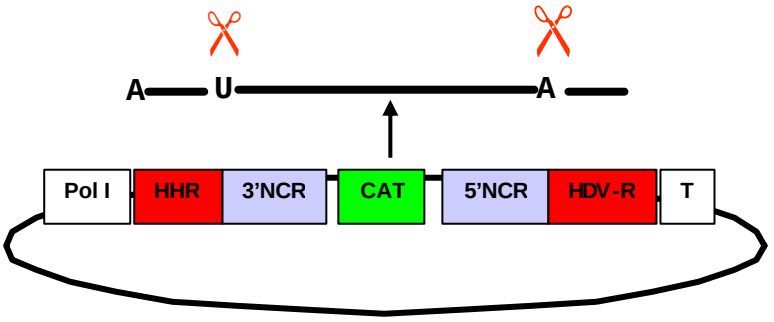


Figure 27: Schematic diagram of the RNA polymerase I transcription plasmid with HHR and HDV-Ribozyme. The transcription by RNA polymerase I will begin with the first "A" of hammerhead sequence. The hammerhead ribozyme should cleave the transcript in cis to generate a new 5'-end that starts with the genomic "U" residue. The correct 3'-end of the transcript is assured by a HDV ribozyme that also cleaves the transcript in cis generating the correct 3'-end of the transcript. There are two nucleotide changes (C38A, G44C) in anti-genomic BDV 3' NCR of the vector pPOLI-BDVcCAT2.2SP#11 compared to the pPOLI-BDVcCAT2.1SP#10, the other parts of two vectors are completely the same. There are three versions of HHR, the length of them is 53 nt, 50 nt and 46 nt respectively. So the plasmids were named pPolIHHRCAT2.1#1, #2, #3; pPolIHHRCAT2.2#1, #2, #3.

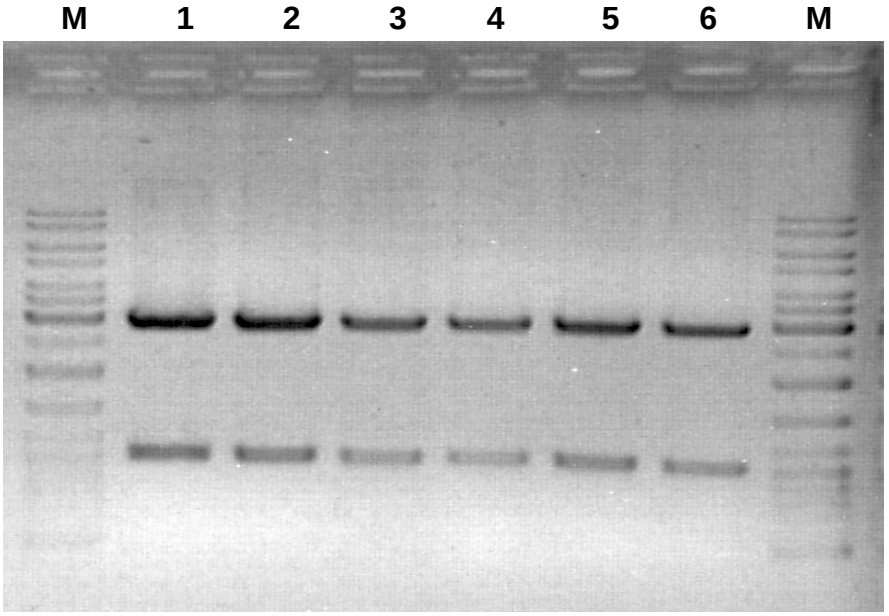


Figure 28: Restriction analysis of the new constructed plsmids. The new constructed plasmids was identified by restriction enzyme digest (PshAI). All digests show the expected bands. Lane 1: pPolIHHRCAT2.1#1 (1055 nt and 2887 nt); Lane 2: pPolIHHRCAT2.1#2 (1052 nt and 2887 nt); Lane 3: pPolIHHRCAT2.1#3 (1048 nt and 2887 nt); Lane 4: pPolIHHRCAT2.2#1 (1055 nt and 2887 nt); Lane 5: pPolIHHRCAT2.2#2 (1052 nt and 2887 nt); Lane 6: pPolIHHRCAT2.2#1 (1048 nt and 2887 nt).

2.2 Ribozyme assay

2.2.1 Plasmid construction

In order to prove that both ribozymes of the new constructs are functional in vitro, four plasmids (pcDNA3.1Ribo1s-p, pcDNA3.1Ribo1p, pcDNA3.1Ribo2 and pcDNA3.1Ribo3) were constructed for a ribozyme assay (Figure 29). These four plasmids were identified by different restriction enzymes digest, the results obtained by this approach showed that these four plasmids were successfully constructed (Figure 30).

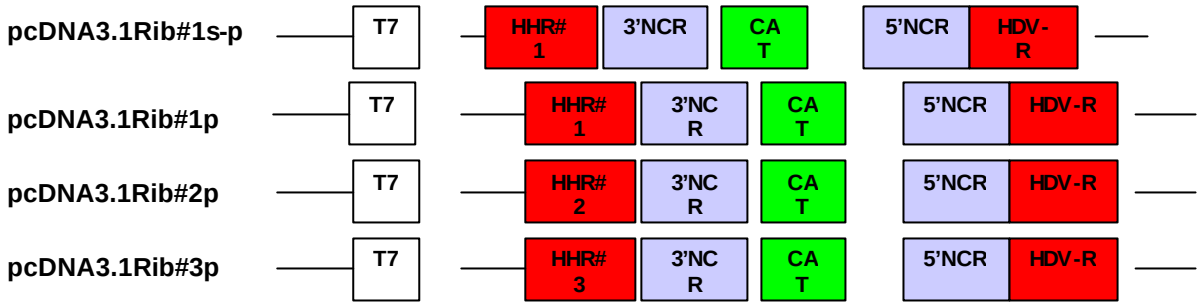


Figure 29: Schematic diagram of four different constructions for a ribozyme assay. These constructs differ only in the length of different HHR version except for pcDNA3.1Ribo1s-p. The part between the T7 promoter and HHR#1 is a little shorter in pcDNA3.1Ribo1s-p than in the other three constructs (pcDNA3.1Ribo1p, pcDNA3.1Ribo2 and pcDNA3.1Ribo3). HHR#1, HHR#2 and HHR#1 are 53 nt, 50 nt and 46 nt respectively.

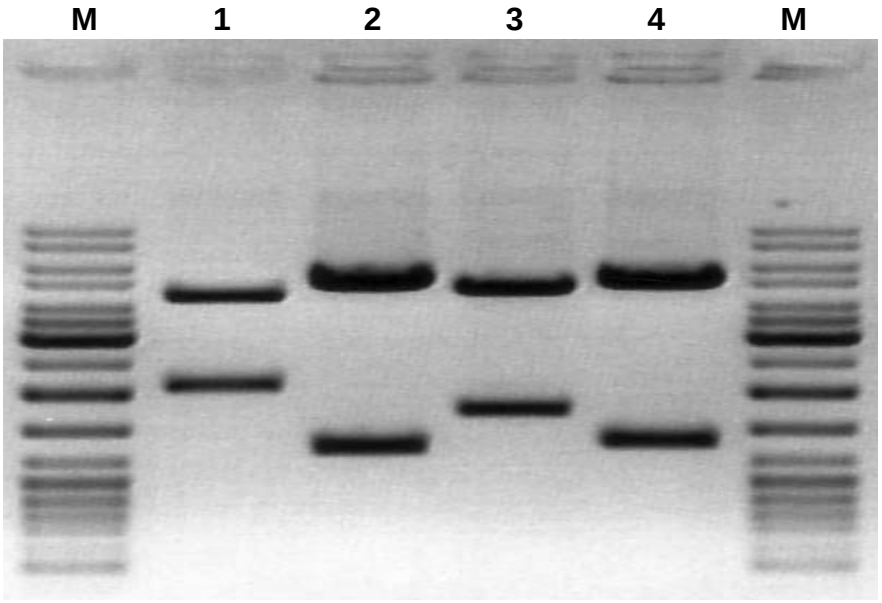


Figure 30: Plasmids identification by different restriction enzymes. All digest show the expected bands. Lane 1: pcDNA3.1Ribo1p digested by SmaI (2108 nt and 4379 nt); Lane 2: pcDNA3.1Ribo1s-p digested by NdeI (1335 nt and 5091 nt); Lane 3: pcDNA3.1Ribo2 digested by MscI (1737 nt and 4747 nt); Lane 4: pcDNA3.1Ribo3 digested by NdeI (1393 nt and 5091nt).

2.2.2 Ribozyme assay

Briefly, the four plamids were linearized by restriction enzyme digest (XbaI), then T7-transcriptions were performed in vitro. The RNAs obtained by T7-transcription were assayed for ribozyme reaction with the appropriate concentration of $MgCl_2$ at 37^oC for 30 min. Subsequently, the RNA was analyzed by the denaturing acrylamide gel eletrophoresis, then

the gel was stained with a silver nitrate solution. The results showed that both ribozymes cleave the primary transcript and generate the expected bands. Approximately 100 nt bands were generated by HDV ribozyme cleavage in all four constructs (pcDNA3.1Ribo1s-p, pcDNA3.1Ribo1p, pcDNA3.1Ribo2 and pcDNA3.1Ribo3) and 187 nt (pcDNA3.1Ribo1s-p), 248 nt (pcDNA3.1Ribo1p), 245 nt (pcDNA3.1Ribo2) and 241 nt (pcDNA3.1Ribo3) bands were obtained by HHR cleavage respectively (Figure 31). Other bands were generated (Figure 32), when ribozymes did not cleave completely. This results indicated that three versions HHR and HDV ribozymes were functional in vitro.

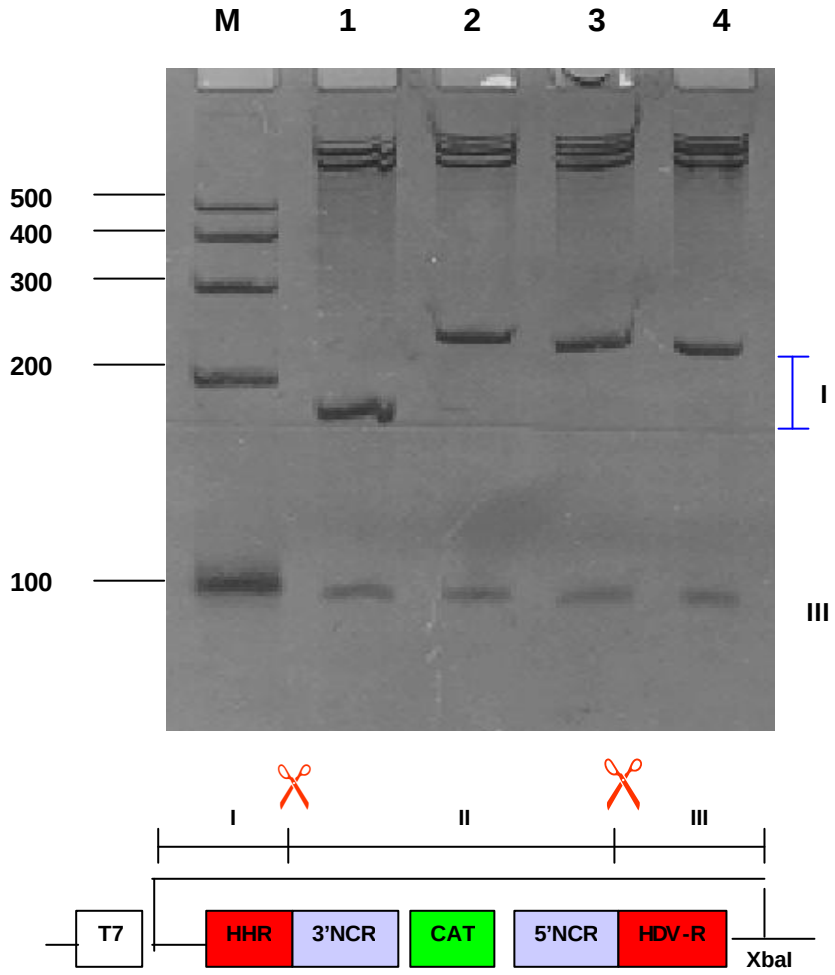


Figure 31: Self-cleavage analysis of the hammerhead ribozyme and the hepatitis delta virus ribozyme in vitro. Reaction products were separated on 5% PAGE, denaturing 8 M urea gels. Lane 1: pcDNA3.1Ribo1s-p; Lane 2: pcDNA3.1Ribo1p; Lane 3: pcDNA3.1Ribo2; Lane 4: pcDNA3.1Ribo3.

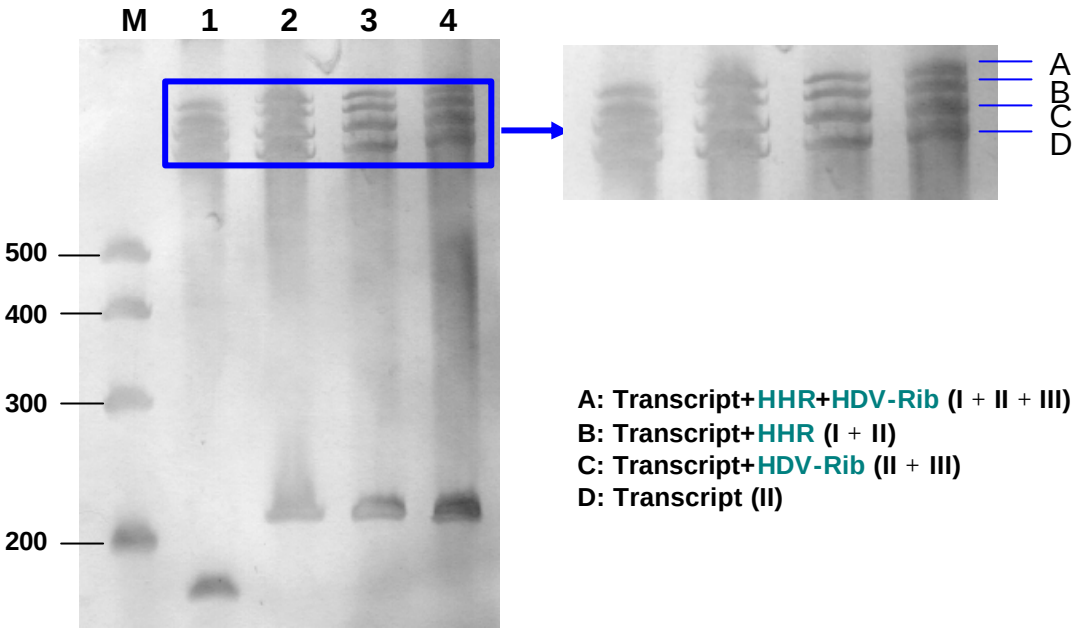


Figure 32: Self-cleavage analysis of the hammer head ribozyme and the hepatitis delta virus ribozyme in vitro. Reaction products were separated on 2.8% PAGE, denaturing 8 M urea gels. Lane 1: pcDNA3.1Ribo1s-p; Lane 2: pcDNA3.1Ribo1p; Lane 3: pcDNA3.1Ribo2; Lane 4: pcDNA3.1Ribo3.

2.3 Establishment of a Vero cell line infected by BDV H1766

In order to prove that my constructed "mini-genome" Pol I-transcripts for BDV were functional, I performed transfections (Pol I plasmids transfected into Vero cells and into BDV infected Vero cells) and RT-PCR was used to prove that BDV polymerase can recognize and use the primary RNA transcript (PolI-transcript) for replication and transcription (Figure 33). The RT-PCR results obtained in the first experiments showed that the transcription results of Vero cells and BDV infected Vero cells were the same (about 590 nt band) (Figure 34).

The results indicated that the control Vero cells were contaminated with BDV. This was confirmed by IFA (Figure 35). I had to use new Vero cells as a control that were not contaminated by BDV. The new Vero cells I received proved not to be contaminated by BDV in IFA (Figure 36), but the new Vero cells and the BDV infected Vero cells were from different laboratories and were different in growth speed. It was very difficult to control the amount of cells in order to control the condition for transfection for both cell lines. So I had to establish new Vero cells infected by BDV (strain H1766). I tried to use BDV H1766 to infect Vero cells directly, but this approach failed. I had to use BDV H1766 to infect the primary rat embryo brain (REB) cells, and then co-culture the BDV infected REB cells together with the new Vero cells (Figure 37). After two passages, the REB cells were dead and the Vero cells were infected by BDV H1766. I have proved that the new Vero cells were almost completely infected by BDV H1766 through in situ immunhistochemical BDV-detection (Figure 38) and IFA (Figure 39).

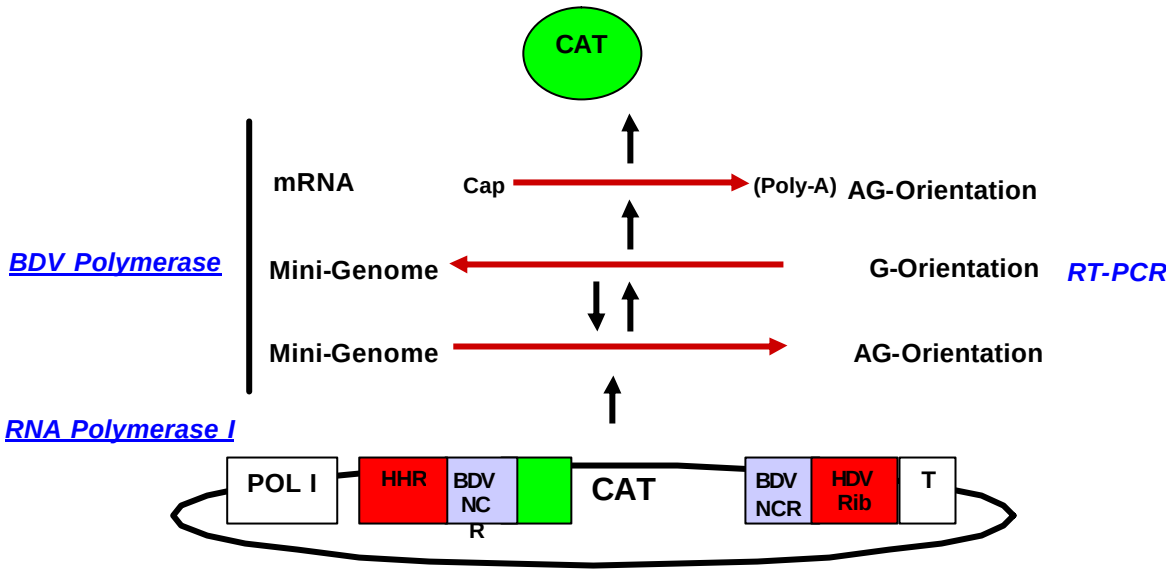


Figure 33: Mechanism of RT-PCR detection of BDV-specific replication intermediates from transfected BDV-infected Vero cells. Primary transcription (antigenomic orientation) will be done by cellular RNA polymerase I, then the primary transcript will be used by BDV polymerase and replicated and transcribed. The genomic orientated transcript can only be generated when BDV polymerase uses the antigenomic primary transcript to initiate replication and transcription. So RT-PCR can be used to detect BDV-specific replication.

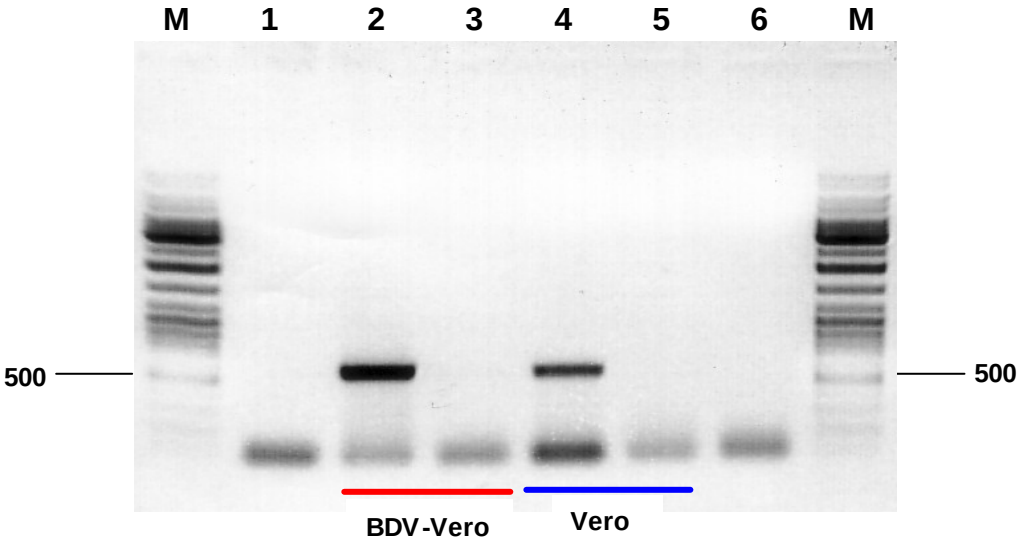


Figure 34: RT-PCR detection of BDV-specific replication intermediates from transfected Vero cells (BDV-infected and non-infected). pPolI-HHR-CAT2.1#1 was transfected into the BDV-infected and non-infected vero cells, then RNA was extracted from the cells. RNA was digested by DNaseI, then half of digested RNA was used for RT with the specific primer (CAT29-52) and subsequent PCR with a pair of CAT-specific primers (CAT29-52 Fw, CAT612-589 Bw), another part was used for direct PCR. About 590 nt bands should be obtained. PCR products were analyzed on gels. Lane 2, 4: RT-PCR; Lane 3, 5: PCR. Lane 1: RT-PCR control. Lane 5: PCR control.

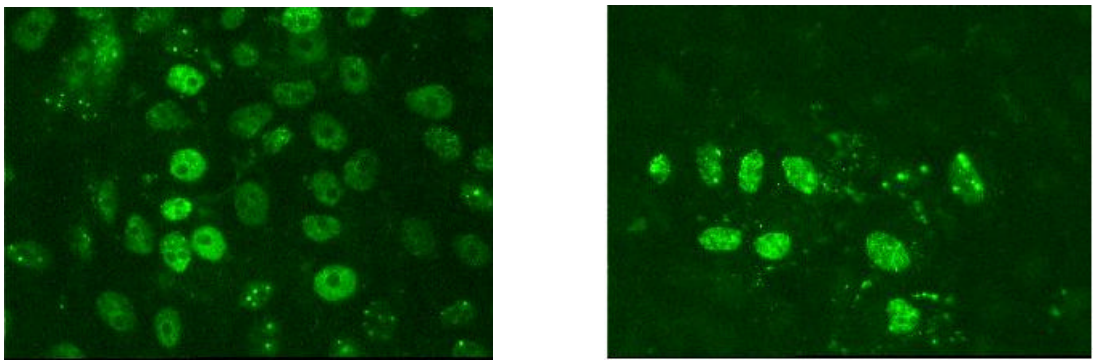


Figure 35: Detection BDV by indirect immunofluoresces assay. BDV-Vero and Vero cells were stained with a mouse monoclonal antibody against BDV nucleoprotein (Bo18, 1:100 dilution). Goat anti-mouse IgG fluorescein isothiocyanate (1:100 dilution) was used as secondary antibody. The typical nuclear staining showed (green) in BDV-Vero cells (x40). Part of vero cells were specifically stained in the nucleus. This indicated that Vero cells were contaminated by BDV.

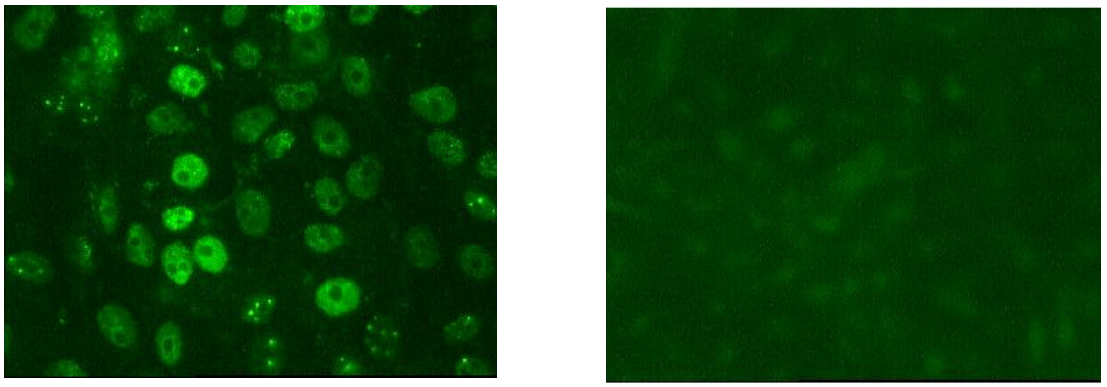


Figure 36: Testing new Vero cells by IFA. Marburg-vero and BDV-Vero cells were stained with a mouse monoclonal antibody against BDV nucleoprotein Bo18 (1:100 dilution) and goat anti-mouse IgG fluorescein isothiocyanate (1:100 dilution). The nucleus of Marburg-Vero cells were not stained compared to the BDV-Vero cells (x40).

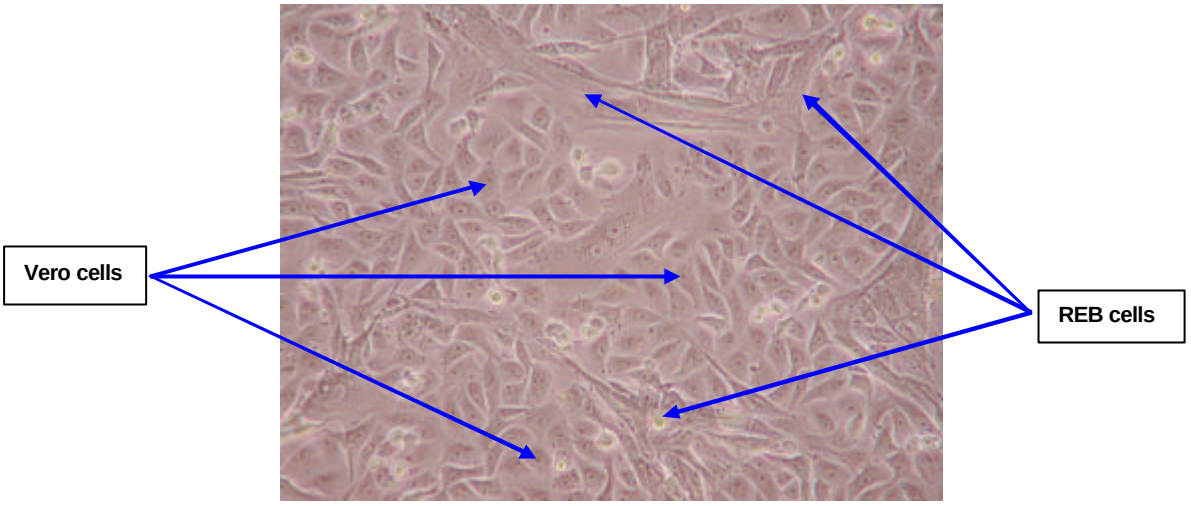


Figure 37: Co-cultivation of BDV H1766 infected REB cells with the Marburg-Vero cells. It is very difficult to infect the passaged cell lines (Vero cell) directly by BDV. BDV infection spreads by contact between infected cells and noninfected cells. Primary REB cells can be easily infected with BDV, therefore BDV infected primary REB cells were cocultivated with Vero cells. After two passages, the infected REB cells (arrow indicated) were dead.

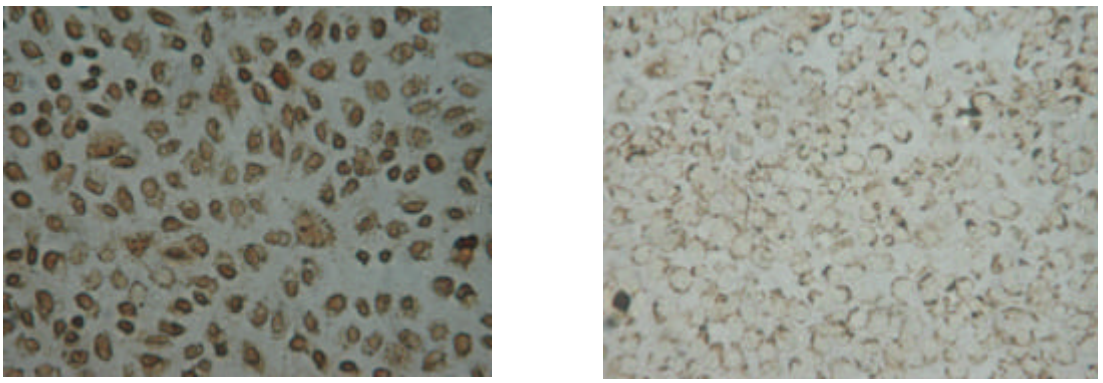


Figure 38: In situ immunhistochemical BDV-detection. Vero and BDV-Vero cells were stained with a mouse monoclonal antibody against BDV nucleoprotein (Bo18, 1:100 dilution) and biotin anti-mouse IgG (1:100 dilution). Subsequently the cells were incubated with Streptavidin/HR-Peroxidase-conjungated (1:1000 dilution), then incubated with 1 ml the substrate solution. The nucleus of BDV-Vero cells was stained brown, but not in Vero cells.

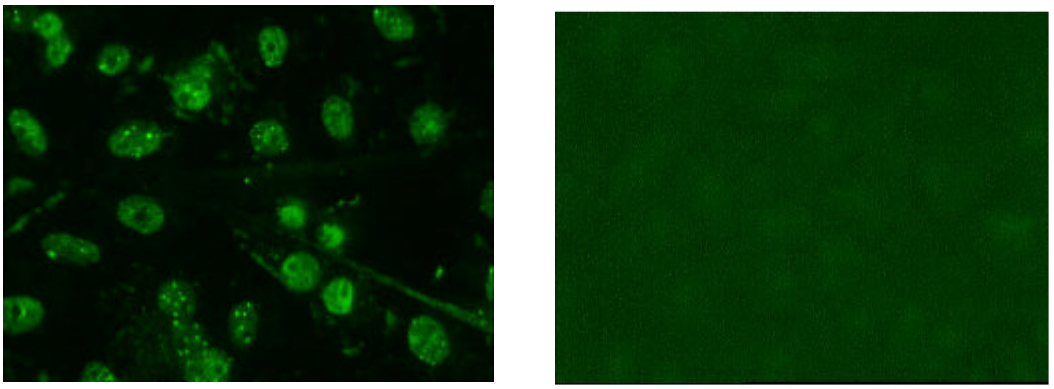


Figure 39: IFA of BDV H1766 infected Vero cells. BDV-Vero and Vero cells were stained with a mouse monoclonal antibody against BDV nucleoprotein (Bo18, 1:100 dilution). Goat anti-mouse IgG fluorescein isothiocyanate (1:100 dilution) was used as secondary antibody. The typical nuclear staining was seen (green) in BDV-Vero cells compared to the control Vero cells.

2.4 RT-PCR detection BDV-specific replication intermediates from transfected Vero cells (BDV-infected and non-infected)

pPolI-HHR-CAT2.1#3 was transfected into BDV-infected and non-infected Vero cells, then RNA was prepared from cells. 4 µg RNA was treated with RQ1 (RNase-free DNase I), 1/3 of the total digested RNA was used to perform PCR with a pair of CAT-specific primers to test for plasmid contamination. As the PCR result was negative (no 590 nt band), which means that no plasmid DNA was left that could lead to false positive RT-PCR result, 1/3 of the total digested RNA was used to perform RT-PCR and direct PCR respectively. RT-PCR result shown that a specific band (about 590 nt) was only obtained from the RNA sample of BDV-infected Vero cells compared to the control (Figure 40). The RT-PCR product was digested by EcoRI and the expected bands (about 185 nt and 400nt) were obtained (Figure 41) and proved that the RT-PCR product is specific and correct. This results suggested that the correct 3'- and 5'-ends of the minigenome were generated by splicing of the two ribozymes in vivo and the BDV polymerase can use the antigenomic mini-transcript for replication.

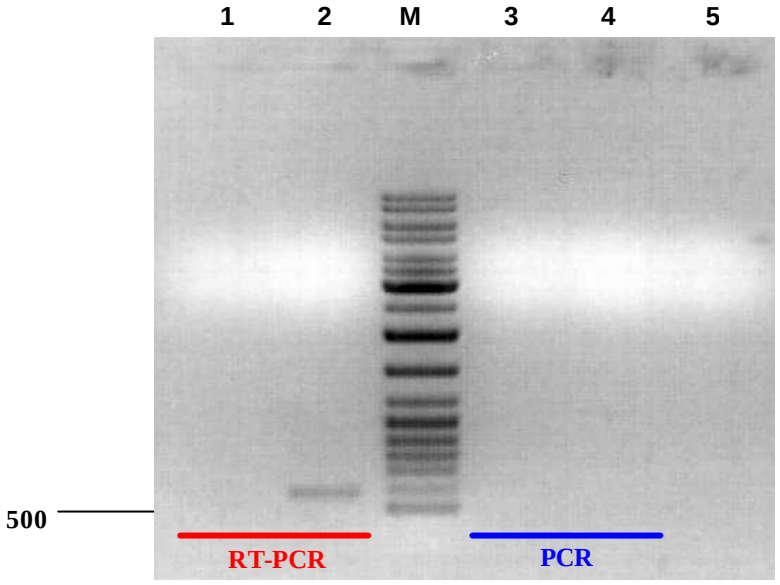


Figure 40: RT-PCR detection of BDV-specific replication intermediates from transfected Vero cells (BDV-infected and non-infected). pPolI-HHR-CAT2.1#3 was transfected into the BDV-infected and non-infected vero cells, then RNA was extracted from cells. RNA was digested by RQI, then RT-PCR and direct PCR was performed (specific primer (CAT29-52) for RT) with a pair of CAT-specific primers (CAT29-52Fw, CAT612-589Bw). A 590 nt band should be obtained. PCR products were analyzed on a 1% gel. Lane 1, 3: RNA from Vero cells; Lane 2, 4: RNA from BDV infected Vero cells. Lane 5: RT-PCR control.

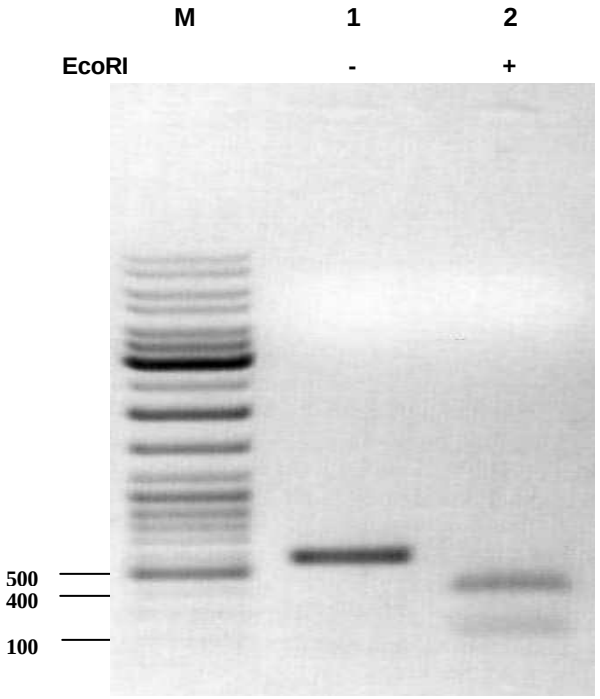


Figure 41: Analysis of RT-PCR product by restriction enzyme. RT-PCR products were incubated with EcoRI as indicated and separated on an agarose gel. About 185 nt and 400nt band should be obtained after digestion.

2.5 RPA detection BDV-specific transcription products from transfected Vero cells (BDV-infected and non-infected)

pPCRII-TOPO-RPA, which contains a T7 promoter, the HDV-Ribozyme, the 5'NCR and part of CAT gene, was used to generate a probe of genomic orientation and 441 bp in length. It will hybridize with the mRNA and the antigenomic RNA of the PolI-primary transcript generated by the BDV polymerase (Figure 42). 311 bp and a 249 bp long double-stranded RNA should be generated by the hybridization of RNA sample of BDV-infected Vero with the specific probe that is protected against RNase A/T1 digestion. The RPA result showed that about 311 bp and a 250 bp bands were protected by hybridization of the RNA sample from BDV-infected Vero by the specific probe (Figure 43). This result indicated that BDV polymerase can recognize and use the mini-genome for transcription and that the two ribozymes perform self-cleavage in vivo.

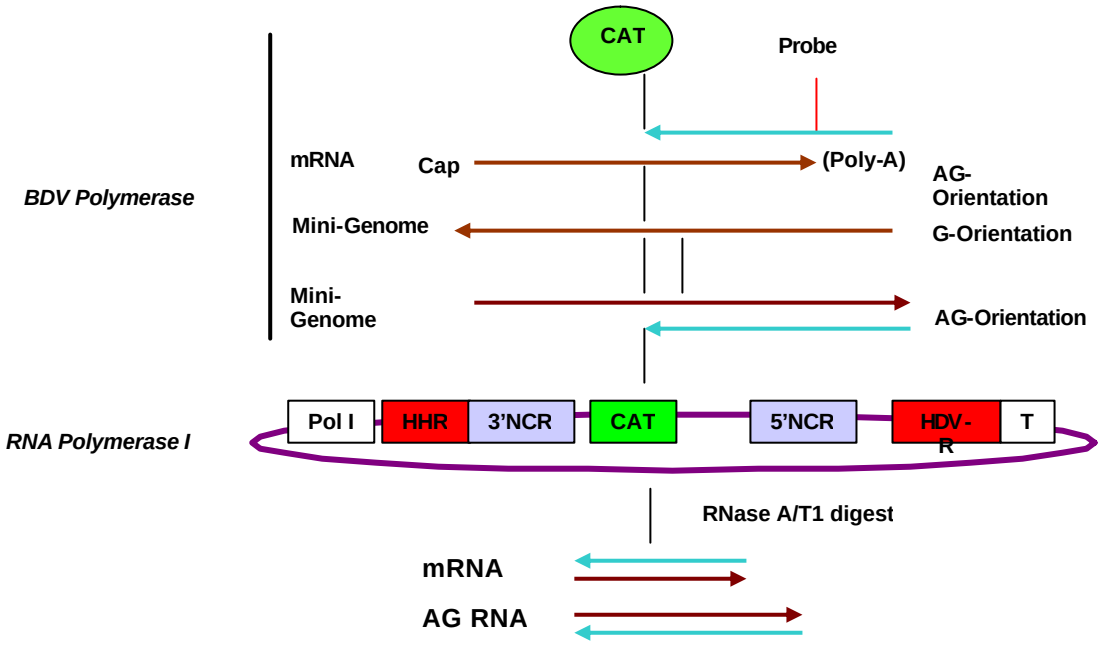


Figure 42: Scheme of RPA detection of BDV-specific transcription products from transfected BDV-infected Vero cells. Primary transcription (antigenomic orientation) through the cellular RNA polymerase I, then the primary transcript can be used by BDV polymerase and replicated and transcribed. The genomic orientation transcript can only be generated when BDV polymerase uses the antigenomic primary transcript to initiate the replication and transcription. mRNA is generated with 5'-Cap and 3'-Poly(A) by BDV polymerase. When a genetic orientation specific RNA probe is synthesized and will hybridize with the mRNA and the primary transcriptional antigenomic orientation RNA, two different length of the double stranded RNAs will be protected after RNaseA/T1 digestion due to the 3'-end difference among two antigenomic orientated RNAs (3'-end of mRNA with Poly(A) that can not hybridize with the probe). So RPA can be used to detect BDV-specific transcription.

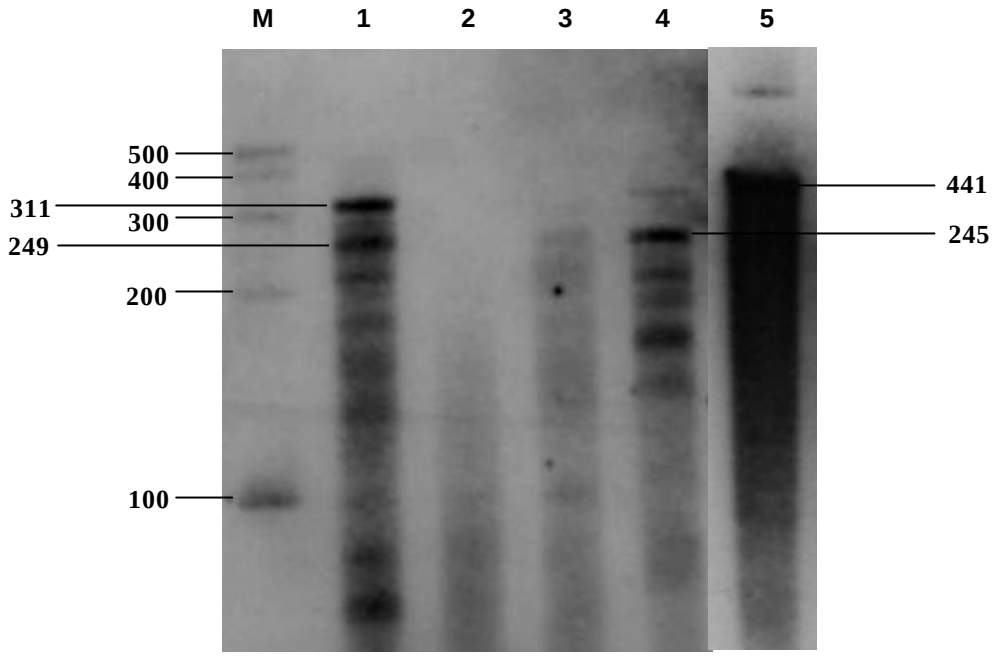


Figure 43: Analysis of BDV-specific transcription from transfected Vero cells (BDV-infected and non-infected) by RPA. RNA was hybridized with the according probes, then digested by the RnaseA/T1 and separated on 6% denaturing polyacrylamide gel. Electroblotting from polyacrylamide gel was performed. The protected bands were detected using BrightStar™ BioDetect™ kit (Ambion USA) according to the standard protocol. Lane 1: RNA sample from the plasmid transfected BDV infected Vero cells; Lane 2: RNA sample from the plasmid transfected Vero cells; Lane 3 and 5: yeast RNA; Lane 4: mouse liver RNA (positive control provided by kit and hybridized with the positive probe; 245 nt RNA band was protected). Except for Lane 5 (RPA-probe), others were digested by RNaseA/T1.

2.6 Luciferase activity assay

pPolI-HHR-T7 (Figure 44) was transfected together with pCITC Luci (expressing luciferase reporter gene under the control T7 promoter) into Vero cells and BDV-infected cells separately. Two additional combinations of transfections (pCAGGS T7+ pCITC Luci; pcDNA3.1+ pCITC Luci) were performed as positive and negative control. If the BDV polymerase can use the "mini-genome" transcript generated by pPolI-HHR-T7 for replication and transcription, T7 polymerase will be expressed and will recognize the T7 promoter in the pCITC Luci resulting in the expression of luciferase. The luciferse activity assays showed that the activity of luciferase was induced by pPolI-HHR-T7 compared to the control (Figure 45). This result indicates that the "mini-genome" transcript generated by pPolI-HHR-T7 can be recognized by the BDV polymerase for replication and transcription, and proves that the 3'- and 5'-ends that I used are functional.

Figure 44. Schematic diagram of the pPol I-HHR-T7. The T7 polymerase gene and very short part of CAT gene (179 nt), flanked by the 3' and 5' noncoding regions of the BDV, was inserted between the Pol I promoter, HHR ribozyme and HDV-ribozyme, Pol I terminitor.

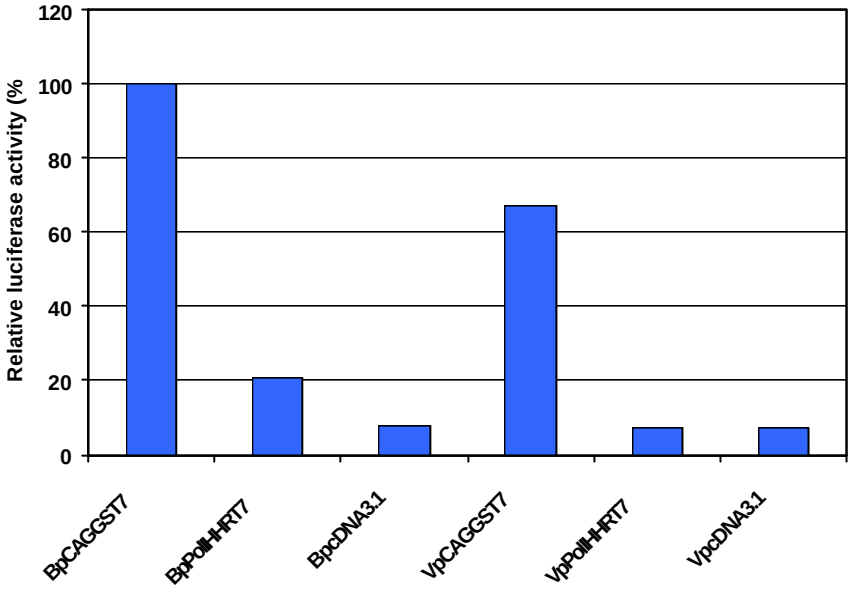


Figure 45: The induction of the luciferase by the different combinations of transfections. Different plasmid combinations were transfected into Vero cells and BDV-infected Vero cells with a total of 5 µg DNA. Cell extracts were prepared at 48 h posttransfection in cell lysis buffer (Promega) and assayed for luciferase activity. For a comparison enzyme activity induced by the transfection of pCAGGS T7 and pCITC Luci in BDV-infected Vero cells was set to 100%. Average values determined in three independent experiments are shown. BpCAGGST7, BpPolI-HHR-T7 and BpcDNA3.1 mean that pCAGGS T7+ pCITC Luci, pPolI-HHR-T7+ pCITC Luci and pcDNA3.1+ pCITC Luci were transfected into BDV-infected Vero cells respectively. VpCAGGST7, VpPolIHHRT7 and VpcDNA3.1 mean that pCAGGS T7+ pCITC Luci, pPolI-HHR-T7+ pCITC Luci and pcDNA3.1+ pCITC Luci were transfected into Vero cells respectively.

2.7 Luciferase acitivity assay and CAT assay by a plasmid-based reverse genetic system

I could show that the Pol I expression construct is functional in the BDV-dependent system (BDV-infected Vero cells). In order to prove whether this construct is also functional in a plasmid-based reverse genetic system, pPol I-HHR-CAT2.1#3 and pPolI-HHR-T7 with different plasmids (pCAGGS-L, pCAGGS-N and pCAGGS-P or pCITC Luci) for the expression of BDV-N, -P and –L were transfected into 293T cells respectively according to the method 2.6.2, then luciferase activity assay and CAT assay were performed. The results of these two assays showed that the activities of reporter genes (luciferase and CAT) were detected, and that the mini-genome expression depended on a delicate balance of the N- and P- expression plasmid as reported (157) (Figure 45 and 46). An N:P ratio of 5:1 resulted in a very low activity of luciferase (Figure 45), when the N:P ratio was 12.5:1, a higher activity of luciferase and CAT was detected, although the CAT activity is not very strong (Figure 45 and 46). These results indicated that this Pol I expression construct is also functional in the plamid-based reverse genetic system.

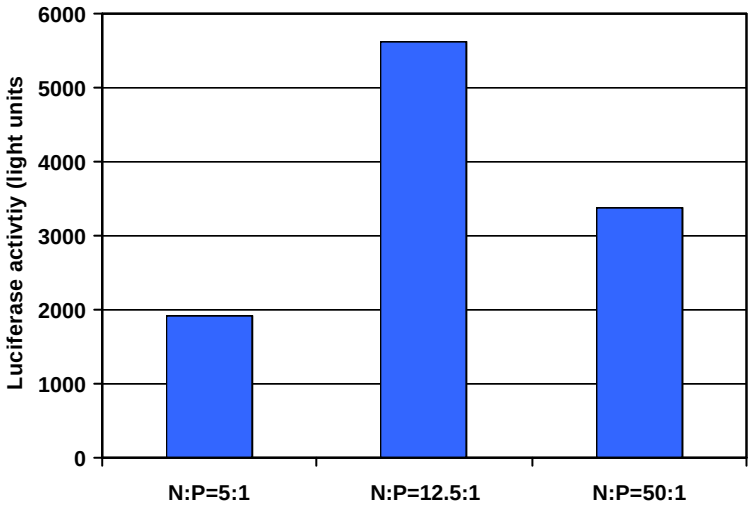


Figure 45: The induction of the luciferase by the different combinations of transfections. 293T cells were transfected using lipofectamine 2000 with different plasmid combinations. At 60 h after tranfection, cell extracts were prepared in cell lysis buffer (Promega) and assayed for luciferase activity. For a comparison negative control values were subtracted. N:P=5:1 transfection combination is: pPolI-HHR-T7 (1µg), pCITC Luci (1µg), pCAGGS-N (1µg), pCAGGS-P (0.2µg) and pCAGGS-L (1µg); N:P=12.5:1 transfection combination is: pPolI-HHR-T7 (1µg), pCITC Luci (1µg), pCAGGS-N (1µg), pCAGGS-P (0.08µg) and pCAGGS-L (1µg); N:P=50:1 transfection combination is: pPolI-HHR-T7 (1µg), pCITC Luci (1µg), pCAGGS-N (1µg), pCAGGS-P (0.02µg) and pCAGGS-L (1µg); Negative control transfection is: pCAGGS (2µg), pPolI-HHR-T7 (1µg) and pCITC Luci (1µg).

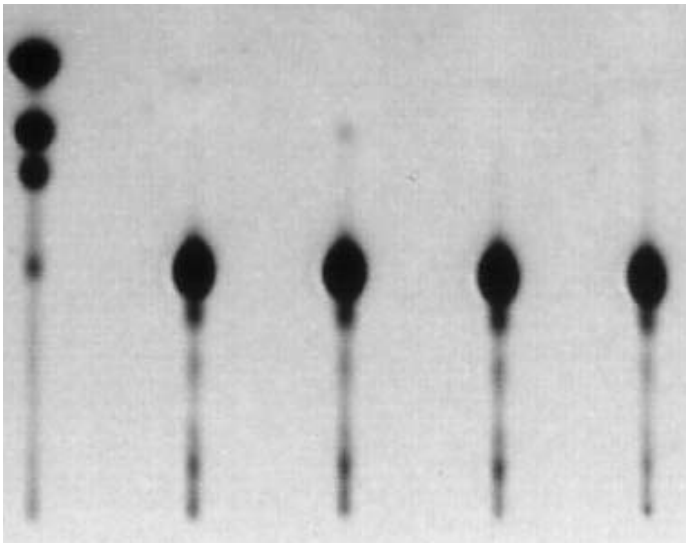


Figure 46: CAT expression in 293T cells transfected with pPol I-HHR-CAT2.1#3 and the combinations of the pCAGGS-N, -P and -L. 293T cells were transfected, as described in figure 45. 60 h after tranfection, cell extracts were prepared and assayed for CAT activity. pSV2CAT (3µg) transfection as a positive control, pCAGGS (2µg) and pPol I-HHR-CAT2.1#3 (1µg) transfection as a negative control. N:P=5 transfection combination is: pPolI-HHR-CAT2.1#3 (1µg), pCAGGS-N (1µg), pCAGGS-P (0.2µg) and pCAGGS-L (1µg); N:P=12.5:1 transfection combination is: pPolI-HHR-CAT2.1#3 (1µg), pCAGGS-N (1µg), pCAGGS-P (0.08µg) and pCAGGS-L (1µg); N:P=50:1 transfection combination is: pPolI-HHR-CAT2.1#3 (1µg), pCAGGS-N (1µg), pCAGGS-P (0.02µg) and pCAGGS-L (1µg).

Discussion

1. AIV

1.1 Establishing a reverse genetic system for avian influenza virus (AIV) A/Goose/Guangdong/1/96 (H5N1)

Since the first reverse genetic system (helper virus-dependent system) was developed for influenza virus (55, 132) that allowed to genetically manipulate RNA segments of influenza virus, up to now new systems to rescue influenza infectious viruses entirely from cloned cDNAs have been established (60, 89, 145). The ability to generate infectious RNA viruses from cloned cDNAs has contributed greatly to our biological understanding of this pathogens and to improved methods of disease control (150). Scientists have done a lot of research on human influenza virus, but less on AIV. I chose AIV A/Goose/Guangdong/1/96 (H5N1) as a research target, because this virus shows high homology to the AIV of the outbreak in HongKong in 1997. The new and efficient system to generate recombinant influenza viruses from cDNA should be useful for mutagenesis studies and the production of vaccines. This study could be beneficial to seek the accurate reasons why this AIV was transmitted directly to humans. In this study I employed the Pol I-Pol II system to rescue the AIV A/Goose/Guangdong/1/96 (H5N1). In this plasmid-based expression system, viral cDNA is inserted between the RNA polymerase I (Pol I) promoter and HDV ribozyme sequences. This entire Pol I transcription unit is flanked by an RNA polymerase II (Pol II) CMV promoter and terminator site. The orientation of the two transcription units allows the synthesis of negative-sense viral RNA and positive-sense mRNA from one viral cDNA template. I have constructed eight plasmids (pBD-PB1, -PB2, -PA, -NP, -HA, -NA, -M, -NS) including the complete cDNAs of the eight segments of the strain A/Goose/Guangdong/1/96 (H5N1). These constructed plasmids were identified by restriction enzymes digestion (Figure 13) and were sequenced. At least five plasmids (pBD-PB1, -PB2, -PA, -NP and pBD-NS) were proved to be functional by direct (CAT-assay) and indirect (generation of a reassortant virus) method. The eight plasmids should be used directly to rescue the AIV A/Goose/Guangdong/1/96 (H5N1). Although the strain of A/Goose/Guangdong/1/96 (H5N1) is different from the H5N1 which broke out in Hongkong, the biological safety has to be considered to prevent any human infection, therefore I tried to rescue the reassortant virus using the genes of the strain A/FPV/Rostock/34 (H7N1) as a genetic background with only one gene of A/Goose/Guangdong/1/96 (H5N1), in order to test that every plasmid including the complete cDNAs of the strain avian A/Goose/Guangdong/1/96 (H5N1) virus is functional. The study to generate the infectious avian influenza virus from cloned cDNAs of the strain A/Goose/Guangdong/1/96 (H5N1) will be done in the National Avian Influenza Reference Laboratory of the Ministry of Agriculture, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science (CAAS), China.

1.2 Generation the reassorant avian influenza viruses

In order to prove that the eight plasmids (including the genes of the AIV A/Goose/Guangdong/1/96 (H5N1)) are functional, I tried to rescue reassortant viruses using the genetic background of the avian influenza strain A/FPV/Rostock/34 (H7N1) with one gene of A/Goose/Guangdong/1/96(H5N1). I have substituted the PB1, PB2, PA, NP, HA, NA, M and NS gene of A/FPV/Rostock/34 (H7N1) by the according genes of the A/Goose/Guangdong/1/96 (H5N1) respectively to generate a reassortant virus, but only the wild-type A/FPV/Rostock/34 (FPV) virus and a reassortant virus (GD1NSFPV) which carries the A/Goose/Guangdong/1/96 (H5N1) NS gene and the other 7 genes of FPV were successfully rescued.

It is known that transmission of influenza A viruses is restricted by host range, this means that a specific influenza A virus can not replicate efficiently in any hosts and cell lines, normally only in the specific host cells. Although influenza A viruses are occasionally transmitted from one animal species to another, for example the AIV (A/Hongkong/156/97(H5N1)) infected human beings directly in Hongkong (203). Normally avian influenza viruses replicate poorly in humans (11), while human viruses do not replicate efficiently in birds (86, 141, 244). But the two influenza virus strains (A/FPV/Rostock/34 (H7N1) and A/Goose/Guangdong/1/96 (H5N1)) were isolated from birds, therefore there should be no species barriers between them. Studies have shown that the ion channel activity of the influenza virus M2 protein stabilizes the native form of fowl plaque virus (FPV) hemagglutinin during intracellular transport (34, 213, 216). These results indicated cooperation between the HA and M2 proteins, which is very important for the generation a new reassortant influenza virus between FPV and other strains. In early studies of the rescue of temperature-sensitive reassortant virus, the HA gene of the A/FPV/Rostock/34 (H7N1) did not segregate with the human matrix (M) gene (199). The sequence results showed that there are only two AA differences in M1 protein of A/Goose/Guangdong/1/96 (H5N1) compared to the known AA sequences of M1. Maybe the M1/M2 protein of the A/Goose/Guangdong/1/96 (H5N1) can not cooperate with the HA protein of A/FPV/Rostock/34 (H7N1), it is perhaps the reason why I did not rescue a ressortant virus containing the A/Goose/Guangdong/1/96 (H5N1) M gene and the other 7 genes of FPV.

I was also unable to generate ressortant viruses possessing only the H5N1 virus HA or NA in the background of the FPV genes in my study. Studies have suggested that a balance of HA and NA activities is important for efficient growth of influenza virus (4, 77, 84, 184, 231). The lack of balanced activities between HA and NA from both viruses might well explain my failure to generate the desired reassortant viruses. A reassortant virus possessing the HA and the NA genes or the HA and the M genes from the H5N1 virus can be perhaps produced, I will try to do this in future work.

The NP protein is thought to be the major determinant of host range and species specificity (198). The rescue experiments of temperature sensitive (ts) mutants of the A/FPV/Rostock/34 (H7N1) by Scholtissek and his colleagues provide the direct evidence for the active

participation of NP in determining host range. FPV reassortants with a human influenza viral NP were no longer capable of forming plaques on the chicken embryo cells and had lost their pathogenicity for chickens (197). The NP gene of FPV could be replaced by that of the different avian influenza strains through double infection, moreover the rescue frequency of the FPV-NP mutants is very high (198). This indicated that the NP gene from the avian influenza strains should be able to replace the NP gene of FPV. The NP gene of the strain A/FPV/Rostock/34 (H7N1) is 90.2% identical to the NP of the strain A/Goose/Guangdong/1/96 (H5N1) at the nucleotide level, and 98% at the amino acid level. Why a reassortant virus containing the NP gene of the A/Goose/Guangdong/1/96 (H5N1) was not rescued in my study, it is not clear. Maybe the reasons are caused by different methods (double infection or reverse genetic based on the plasmids), or the NP gene of the A/Goose/Guangdong/1/96 (H5N1) does not cooperate with the other genes of the FPV. Why a reassortant virus containing the PB1 or PB2 or PA gene of the A/Goose/Guangdong/1/96 (H5N1) was not rescued in this my study, remains unclear.

1.3 Activation of Raf/MEK/ERK cascade by both the wild type FPV and reassortant GD1NSFPV viruses

Scientists have done a lot of research about DNA viruses inducing signaling through MAPK pathways in infected host cells, therefore much is known about how DNA viruses interact with host cell (14, 22, 180). Although interaction of RNA viruses with the Raf/MEK/ERK signaling pathway has been reported, little information is know compared to the DNA viruses. For example, Planz et al. proved that the signaling through MEK is essential for BDV spread, but the molecular mechnism of how BDV interacts with the cellular Raf/MEK/ERK signaling pathway remians to be elucidated (160).

This cascade is also activated by influenza virus, and signaling through the mitogen cascade seems to be essential for RNP export from the nucleus and accordingly for virus titer (163). The question was whether there are differences in the activation of the mitogentic Raf/MEK/ERK cascade between both the wild type FPV and reassortant GD1NSFPV, because the reassortant GD1NSFPV grows to higher titer compared to the wild type FPV. The results showed that both viruses induce MAPK-signaling, but there are no big differences in the activation of the Raf/MEK/ERK cascade induced by both viruses. According to this result, it can be speculated that the activation of the Raf/MEK/ERK cascade is not a determinanting factor which results in the different titer of both viruses.

1.4 Character of the reassortant GD1NSFPV and the role of the NS gene for viral replication

The reassortant GD1NSFPV virus that differs from the wild-type FPV only by the NS-segment grows to significantly higher titers and forms bigger plaques than the wild-type FPV. Furthermore the induction of the IFN promoter dependent reporter by the wild-type FPV was much higher (about three folds) than by the reassortant GD1NSFPV (luciferase gene is

controlled by IFN-ß promoter) (Figure 21). The titer formed by the reassortant GD1NSFPV virus is comparable to another FPV-type influenza virus (H7N7), which is used in the laboratory and grows to the same high titers. It can be speculated that the pathogenicity of the GD1NSFPV is highly enhanced compared to the wild-type FPV (H7N1). The differences between the wild-type FPV (H7N1) and the reassortant GD1NSFPV are subjected to the NS gene.

The NS gene of influenza A virus encodes both the NS1 and the NS2 (NEP) protein. NS1 is a nonstructural protein and is expressed to high levels in virus-infected cells. The NS1 protein has been shown to have many functionalities, such as inhibition of the host mRNA polyadenylation (142) and the pre-mRNA splicing (61, 128, 249), inhibition of the nuclear export of polyadenylated host mRNA (32, 170), stimulation of the translation of the viral mRNA (3, 49, 53, 54), and modulation of viral RNA transcription and replication (138, 202). Another important function is to bind to dsRNA (80). Some studies suggested that binding of dsRNA by the NS1 protein prevents the activation of the double-stranded RNA activated protein kinase (PKR) (15, 81, 129, 219). PKR, the 68 kDa double-strand-RNA dependent kinase, is activated by dsRNA or by interaction with specific proteins (45, 154, 155, 159, 248) and is highly upregulated in response to the virus-induced cytokine IFN (187). Activated PKR phosphorylates the α-subunit of the eukaryotic translation initiation fator elF2, resulting in the inhibition of protein synthesis and hence virus replication (185). These functions of the NS1 protein are very useful for efficient viral replication and the "shut off" of the host cell protein expression.

Garcia-Sastre et al. generated an infectious recombinant influenza A/PR/8/34 virus lacking the NS1 gene (delNS1). They deleted the NS1 open reading frame except for the first 10 amino acids, which are shared with the viral NS2 protein (65). This virus only replicates efficiently in interferon-deficient systems, this findings supports the suggestion that the NS1 protein is a virally encoded inhibitor of the IFN-mediated antiviral responses, such as the activation of PKR. In my study the induction of the IFN-promoter dependent reporter gene by the wild-type FPV was much higher than by the reassortant GD1NSFPV. This results suggests that the NS1 protein of the GD1NSFPV virus is more efficient to counteract IFN-ß and interferon-mediated cellular response to virus-infection than that of the wild-type FPV.

The IFN system is a major component of the host innate immune response to viral infections. IFN is synthesized in response to the viral infection due to the activation of several transcription factors, including IFN regulatory factor (IRF3 and IRF7), AP1 and NF-κB (101, 188, 189, 223, 239, 240, 253). Since dsRNA also induces the activation of these factors, it is assumed that viral dsRNA intermediates generated during viral replication are at least partially responsible for the induction of interferon secretion. Activation of the synthesis of IFN during the viral infection results in the transcriptional activation of many host genes (about 250) involved in antiviral defense mechanisms. Because of the importance of IFN-α/β in antiviral host responses, many viruses have evolved different strategies to subvert the IFN

system. For example, multiple IFN-antagonist actives have been showed in vaccinia virus, adenovirus and hepatitis c virus (12, 18, 47, 62, 63, 106, 121, 221, 222). Several negative-strand RNA viruses have been reported to encode inhibitors of the IFN signaling pathway, such as the V proteins of the paramyxoviruses (SV5 and PIV2) (51, 254), the VP35 protein of the Ebola virus (10), the NS1 and NS2 proteins of bovine respiratory syncytial virus (190), the C proteins of Sendai virus (66, 67, 74, 112), the P proteins of the Borna disease virus (228), the V proteins of Newcastle disease virus and the V, W, and C protein of the Nipah virus (153). For influenza virus, the NS1 protein, which has RNA-binding activity (80), prevents production of IFN by inhibiting the activation of the transcription factors IRF3 and AP1 and NF-κB and blocking the activation of the IFN induced antiviral protein PKR and the (2'-5') oligoadenylate synthetase (OAS) (15, 65, 131, 217, 236).

Pervious studies have proved that the NS1 protein of influenza A virus possesses two important functional domains: an RNA-binding domain near the amino-terminal end (amino acids 1-73) and an effector domain in the carboxyl half the molecule (amino acids 74-231) (168, 169). Furthermore the RNA-binding domain of the NS1 protein is conserved among influenza A and B viruses (234). This suggests that this domain, which specifically binds to the RNA poly(A), U6 snRNA and double-stranded (ds) RNA, is important for the replication of these two types of influenza viruses. In contrast, the NS1 protein of the influenza B virus lacks an effector domain that functions like that of the NS1 protein of influenza A virus. The effector domain of NS1 protein of influenza A virus is required for the inhibition of the nuclear export of poly(A)-containing cellular mRNA and the inhibition of pre-mRNA splicing (234) by binding and inhibiting the function of two cellular proteins. These two proteins are required for the 3'-end processing of the cellular pre-mRNAs: the 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) and the poly(A)-binding protein II (PABII) (32, 123, 142). CPSF factor binds to the AAUAAA poly(A) signal in cellular pre-mRNA and is required for cleavage at the 3' site at which poly(A) addition occurs (7, 36). When the CPSF factor is inhibited, cellular pre-mRNA are not cleaved. The PABII protein is required for the processive elongation of poly(A) chains catalyzed by the cellular poly(A) polymerase (7, 232). When the effector domain of NS1 protein targets the PABII protein, the elongation of the 3'-short poly(A)of the cleaved pre-mRNAs is blocked as a consequence of this interaction. Thus the NS1 protein effectively blocks the 3'-end processing of cellular pre-mRNAs in influenza virus infected cells (32). The polyadenylation of viral mRNAs is not affected, as these RNAs receive their poly(A) tail by "stuttering" of the RDRP at the specific poly(A) signal in the viral RNA.

The RNA binding/dimerization domain in the NS1 protein of influenza A virus (amino acids 1-73) exhibits a dimeric six-helical folding (33, 126). Wang et al. have proved that the dimeric structure of the NS1 protein of influenza A virus is essential for RNA binding, because any alanine replacement (D12, R19, D29, R35, and R46 are required for the maintenance of the dimer structure) that cause disruption of the dimer results in the loss of

RNA-binding activity (235) Interestingly, R38 was found to be the only AA which is absolutely required for RNA binding but not for protein dimerization. This finding indicated that R38 probably interacts directly with the RNA target. The lysine at the position 41 in helix 2 makes an additional strong contribution to the affinity of RNA binding (235).

The binding domain and effector domain of NS1 protein are important for the character of influenza virus, so I compared the amino acids sequences of the NS1 protein between the wild-type FPV and GD1NSFPV. The results showed that the NS1 protein of the strain A/FPV/Rostock/34 (H7N1) was only 69.3% identical to the NS1 of the strain A/Goose/Guangdong/1/96 (H5N1). Specifically, the continuous difference (FPV $_{21}$RFADQEMG$_{28}$; GD1NSFPV $_{21}$LLSMRDMC$_{28}$) in the RNA-binding domain of NS1 protein of both viruses resulted in the change of hydrophilicity of NS1 protein (Figure 24). Maybe this change leads to a structural change of the NS1 protein, which influences the function of NS1 protein and results in the difference of both viruses. R38 and K41 in RNA-binding domains of both NS1 protein, which have been proved to be very important for the RNA binding activity (235), are conserved. The position R44 in the RNA-binding domain of NS1 protein of FPV is K44 in the NS1 protein of the GD1NSFPV, R44 and R37 are not required for RNA binding (235). The other differences among the RNA-binding domain of the NS1 protein of both viruses might be responsible for the RNA binding activity, because only 67.1% AA are identical in the RNA-binding domain of the NS1 protein of both viruses.

The NS1 effector domain contains a 10 AA (amino acids 138-147) sequence which is similar to a consensus sequence found in the effector domains of HIV-1 Rev proteins (168). Particularly, the two Ls which are located at positions 7 and 9 of this sequence are crucial for the function of both the NS1 and HIV-1 Rev protein (137, 168). HIV-1 Rev protein interacts with host nuclear targets to regulate the nuclear export of cellular mRNA and viral mRNA. There are eight AA differences in the position 134 to 161 in the effector domain of NS1 protein of the strain A/FPV/Rostock/34 (H7N1) compared to the NS1 protein of the strain A/Goose/Guangdong/1/96 (H5N1), and this also results in a change of hydrophilicity (Figure 23 and 24). The L at the position 9 of the conserved 10 AA sequence in the effector domain of NS1 protein of FPV (L146 in the effector domain) is changed to S in the according position of GDNS1FPV. It is not known what effect is caused by this change for the NS1 protein function of GDNS1FPV compared to that of FPV.

The binding sites for the cellular proteins CPSF and PAB II have been identified and are near the carboxy end of the NS1 protein: the CPSF binding site is centered around amino acid 186 and the PAB II binding site is located in the 223-237 amino acid region (123). A recombinant influenza A/Udorn/72 virus has been generated encoding an NS1 protein that has an altered amino acid sequence centered around amino acid 186 ($_{184}$GLEWN$_{188}$→$_{184}$RFLRY$_{188}$), and as a result it does not bind the 30 kDa subunit of CPSF (147). This recombinant virus is highly attenuated, suggesting this binding site in the NS1 protein is required for efficient virus replication (147). Comparing the binding site of the 30 kDa CPSF in the NS1 protein of

influenza A/Udorn /72 virus with that of FPV and GDNS1FPV, I found that the binding sites of the 30 kDa CPSF of the three influenza viruses are completely conserved ($_{184}$GLEWN$_{188}$), suggesting that this binding site is crucial for efficient viral replication. The binding site for PAB II protein in the NS1 protein of both FPV and GDNS1FPV viruses is different (FPV $_{223}$MARTIESE$_{230}$; GDNS1FPV $_{223}$MARTIESE$_{230}$), it is not known whether these differences in the NS1 protein of both viruses effects their abilities to bind to PABII resulting in different growth.

The NS1 protein would be able to sequester cellular pre-mRNAs and mRNAs in the nucleus due to the ability to inhibit pre-mRNA splicing and the nuclear export of the poly(A)-containing mRNA. Consequently, more of cellular capped RNAs would be accessible to the viral cap-dependent endonuclease (PB2) in the nucleus for the production of the capped RNA primers that are needed for viral mRNA synthesis (115). The change in hydrophilicity might be important for the structure and abilities of the RNA binding domain and the effector domain, and could result in a functional change of the NS1 protein. Thus, the NS1 protein of GDNS1FPV might be more efficient to inhibit the cellular pre-mRNA splicing and the nuclear export of the poly(A)-containing mRNA than that of FPV virus, and leads to enhanced replication.

The influenza virus NS2 protein mediates the nuclear export of the viral RNPs and has been proposed to act as an adapter molecule between the nuclear export machinery and the viral RNP complex (149). Nuclear export is controlled by export receptors (for example the highly conserved chromosome region maintenance 1 protein (CRM1)) that mediates the nuclear export of nuclear export signal (NES) containing substrates. A functional Leucine-rich NES was mapped in the N-terminal region of the NS2 protein (Figure 25), because it interacts with nucleoprorins in the yeast two-hybrid system, and can functionally replace the effector domain of the human immunodefciency virus type 1 Rev protein (149) that is presumed to interact with host nuclear protein to regulate the nuclear export of viral pre-mRNAs (135). Neumann et al. demonstrated that NS2 interacts with the cellular export factor hCRM1, but the NS2-NES is not required for the interaction with hCRM1 (143). But replacement of the putative NS2-NES by alanine (M16A, M19A, L21A) resulted in abolishing the nuclear export of NP and no virus could be rescued in a plasmid transfection system (143), suggesting that the NES function is crucial for viral replication. In addition, the C terminus of NS2 has been shown to interact with M1 both in vitro and in vivo (237, 252). According to these observations, it has been proposed that NS2 mediates the export of vRNPs by acting as an adapter protein between the nuclear export machinery and the M1-vRNP complex (143, 149, 151). A recent report shows that the structure of NS2 is characterized by a high degree of plasticity, suggesting that NS2 may indeed exist in multiple conformations in vivo (127) to mediate the nuclear export of the viral RNPs (238).

The NS2 gene of the strain A/FPV/Rostock/34 (H7N1) is only 81.4% identical to the NS2 of the strain A/Goose/Guangdong/1/96 (H5N1) at the nucleotide level, and 82% at the amino acid level. Two amino acids (M14Q, G22E) were different in the NES of NS2 protein (amino

acids 11-24) of the strain A/FPV/Rostock/34 (H7N1) compared to the NS2 protein of the strain A/Goose/Guangdong/1/96 (H5N1). The amino acids M16, M19, L21 in the NES of the NS2 protein, which are crucial for viral replication and for NP nucleocytoplasmic transport, were not changed in both viruses. The "conserved" M at the position 14 in the NES of the NS2 protein of FPV was changed to Q in the NES of the strain A/Goose/Guangdong/1/96 (H5N1), it is not known whether this change affects the function of the NES. There are many other differences between both NS2 proteins except for the NES. It remains to be determined whether they affect the structure or the functionality of NS2 protein, and whether the NS2 protein of the GDNS1FPV mediates much more efficiently the nuclear export of the viral RNPs than that of FPV, resulting in higher titer.

In general, the reassortant GDNS1FPV grows to significantly higher titers and forms larger plaques than the wild-type FPV, differing only in the NS-segment. Additionally, this reassortant virus inhibits more efficiently the IFN activation than the wild type. It can be speculated that the NS gene must contribute to the enhanced pathogenicity of the reassortant virus compared to the wild-type FPV. Animal (chicken) experiments will be done to investigate the pathogenic difference of both viruses in China.

2. BDV

2.1 Reverse genetics systems for negative-strand RNA virus

Negative-strand RNA viruses are different from positive-strand RNA viruses, because the genomic RNA of negative-strand RNA viruses is not infectious, and infectious virus particle must deliver their own RNA-dependent RNA polymerase (RDRP) into the infected cell to initiate the first round of virus specific mRNA synthesis. Thus generating negative-strand RNA viruses from cDNAs poses a greater challenge, specially for the segmented, negative-stranded RNA viruses, as one must produce a separate vRNA for each gene segment. 1990, a breakthrough in reverse genetics technology in the negative-stranded RNA field was achieved, when Palese and colleagues established the first system for modifying negative-stranded RNA viruses (influenza virus) by reconstituting the biological activity RNPs in vitro (55, 132) Nevertheless this system has not been successful in the rescue of nonsegmented, negative-stranded (NNS) RNA viruses, as reproducible reconstitution of infectious RNPs containing full length genomes, all of which are greater than 11 kb in length, could not been achieved (177).

An effective DNA transfection systems have been developed for cytoplasmatic replicating viruses like Rabies virus by Schnell et al in 1994 (196). Plasmids encoding the viral nucleocapsid protein (N) and the polymerase proteins (L and P) under the control of T7 polymerase promoters were transfected into cells previously infected with recombinant vaccinia virus expressing the T7 polymerase protein. In addition to these plasmids, a plasmid encoding a full-length antigenomic viral RNA under the control of a T7 promoter and a hepatitis delta ribozyme was co-transfected into the cells. After transcription of RNAs from

the T7 promoters and translation of the encoded proteins, nucleocapsid proteins assemble with the antigenomic RNAs and polymerase proteins, then replicate these RNPs to form new RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and translation, infectious virus was assembled. Subsequently, Vesicular stomatitis virus (VSV) (119, 247), Measles virus (171), Respiratory syncytial virus (RSV) (37) Sendai virus (68, 103), Bunyamwera virus (19), Rinderpest (9) Human parainfluenza virus type 3 (HPIV-3) (88) and Simian virus 5 (SV5) (85), which are all cytoplasmatic replicating viruses, were successfully recovered from the cDNAs by almost the same strategy.

Following the approach outlined by Schnell et al (196), Bridgen & Elliott firstly generated a segmented negative-stranded RNA virus (Bunyamwera virus belongs to family Bunyaviridae) in 1996 (19), demonstrating the feasibility of artificially producing more than one vRNA.

In 1996, a plasmid-based system for expression of influenza RNPs was reported (161). Here an improved RNA polymerase I system was used for the intracellular synthesis of influenza virus RNA as described by Neumann et al (146). In 1999, a completely plasmid-based system was developed to generate an infectious influenza particle from cDNAs by Neumann et al. and Fodor et al. (60, 145). Thogoto virus, another member of the order *Orthomyxoviridae* with six segments of negative-sense RNA, was generated from plasmids by T7 RNA polymerase system (230).

These reverse genetics systems allow to manipulate the viral genome of negative-stranded RNA viruses without technical limitation. It has profound implications on the study of the viral life cycle, functional regulation of viral proteins and molecular mechanisms of viral pathogenicity. Finally reverse genetics has opened the way to develop live attenuated virus vaccines and vaccine vectors.

2.2 Reverse genetic system for BDV

BDV is a nonsegmented negative-stranded (NNS) RNA virus that replicates it's about 8900 nt genome in the nucleus of infected cells as influenza viruses. In contrast to other NNS viruses, up to now reverse genetics system for BDV developed very slowly. There are many different reasons that hindered the establishment of a reverse genetic system for BDV. The genetic information about BDV was very limited, and the complete genome sequence of only two closely related BDV strains, named He/80 and V, were available until 2001 (21, 43). The genomes of these two strains show 95% sequence identity, and the L protein of the He/80 was reported to lack 24 amino acids at the C terminus, and the last 3 nt at the 5'- end differed completely from that of the strain V. Since genome ends are of critical importance for efficient replication and transcription of negative-strand RNA viruses (38), correct 5'- and 3'-ends of BDV are very crucial for developing a reverse genetic system for BDV. In 2001, Pleschka et al. (162) determined the complete nucleotide sequences of two other strains (H1766 and No/98) of BDV and compared them to the reference strains V and He/80. They showed conservation of coding and terminal sequences in these four different isolates of BDV. Specially they found an additional A residue at the extreme 3' end of the single-

stranded RNA genome in all four BDV strains. If the 3'-end of the BDV genome is indeed occupied by an A residue, the BDV polymerase has to initiate replication with UTP. Compared to the polymerases of other viruses from the order Mononegavirales, they all seem to initiate transcription by employing either ATP or GTP (38). This constellation of BDV would therefore represent a unique case among nonsegmented negative-stranded RNA viruses.

Additionally, the polymerase of BDV seems to have a very low activity. Also BDV causes a very slow and persisting infection. This means that perhaps its genome replication requires a very exact and tuned system.

A T7 polymerase system was reported by Schneider et al. (195) to express the reporter CAT gene by reconstituting the BDV polymerase complex in vivo. In this approach, co-expression in BSR-T7 cells of BDV-L, N and P (expression L, N and p plasmids were controlled by T7 promoter) together with a negative-sense mini-genome RNA containing a CAT gene was sufficient to induce L-dependent synthesis of CAT protein. Because this virus replicates its genome in the nucleus of infected cell as influenza virus, the T7 polymerase, that works in the cytoplasm, might not be successful to rescue BDV by a T7 polymerase system. A T7 generated minigenome that was replicated and transcribed by transiently expressed influenza virus proteins (RNPs) was not packaged into a helpervirus after infection of the new cells with the supernatant of the transfected cells. In contrast a Pol I generated minigenome was. (Pleschka, unpublished data).

Recently, a Pol I reverse genetics system for BDV was developed (157). A BDV RNA mini-genome was synthesized intracellularly from a plasmid driven by RNA polymerase I. Co-transfection with plasmids expressing the BDV-L, N and P under the control of RNA polymerase II allowed BDV mini-genome replication and expression. It is noted that both systems used an artifical 5'NCR beginning with the "G" residue and that in this Pol I system a 3'NCR encompassing the transcription initiation signal of the BDV N protein was used. In my study, I used the original BDV NCR (5' NCR is complementary with 3' NCR) for my Pol I mini-genome construct, and HHR and HDV ribozymes were used to generate a correct 5'- and 3'-end respectively. I tried to establish a reverse genetic system for BDV analogue to the successful system for influenza viruses.

2.3 New construct for generating a "mini-transcript" of BDV

Pleschka et al. proved the existence of an additional "U" residue at the extreme 5' end of the single-strand anti-genomic RNA of BDV (162). Also other sequence specificities in the noncoding regions that could be important for replication and transcription. Furthermore a consensus sequence of the polymerase gene was established. The results are very important because correct structures of the genome ends and of the polymerase gene are most critical parameters for the development of reverse genetics techniques that permits the genetic manipulation of BDV. To generate a primary RNA transcript in vivo that can be replicated by the viral polymerase, I used the Pol I-system, because RNA polymerase I is an abundant

nuclear enzyme which transcribes ribosomal RNA, that doesn't contain 5'-cap or 3'-poly(A) structures. Moreover RNA polymerase I initiates and terminates transcription at defined promoter and terminator sequences. Therefore Pol I-transcripts resemble viral genomic RNAs and can be replicated by viral polymerases (146). As the Pol I normally does not incorporate an "U" as the first residue (normally A or G as first residue; I, Grummt personal communication), I have generated new Pol I expression constructs that express a transcript, that starts with a hammerhead ribozyme (HHR) sequence beginning with an "A" residue. The hammerhead ribozyme should cleave the transcript in cis to generate a new 5'-end that starts with the genomic "U" residue. The correct 3'-end of the transcript is assured by a HDV ribozyme that also cleaves the transcript in cis generating the correct 3'-end of the transcript. This technique is used to generate Pol I-transcripts with correct 3'-ends in the Pol I-system for reverse genetics of influenza (60, 161).

I constructed new plasmids (pPolI-HHR-CAT2.1#1, #2, #3) with HHR expressing a CAT reporter gene RNA and tried to analyze whether these constructs can be recognized and transcribed by BDV polymerase. The ribozyme assay result showed that the three versions of HHR together with HDV ribozymes are functional and generate the correct 5'- and 3'-end in vitro. So pPolI-HHR-CAT2.1#3 (including HHR version #3) was used to transfect Vero and BDV-infected Vero cells for in vivo assays (RT-PCR and RPA assay). After transcription, the HHR sequence is folded in its secondary structure (see Figure 26) where part of it anneals to the newly generated 5' end sequence before the HHR cleaves itself off. As the remaining HHR sequence will leave the newly generated 5' end, it seems to be easy when the stretch of annealed HHR is short. I choose the HHR version #3 with the shorter stretch of homology to the new 5' end, this way the correct 5' end should be accessible for the BDV-RDRP. The RT-PCR results demonstrated that an replication intermediate of genomic orientation was generated from pPolI-HHR-CAT2.1#3 primary transcript (antigenomic orientation). This "mini-genome" can be recognized and replicated by the BDV polymerase, because the replication intermediate (shown by RT-PCR) can only be generated when the BDV polymerase uses the primary transcript to initiate replication. Furthermore the primary transcript and the according mRNA was detected in a RPA assay. These results indicate that primary transcript can be replicated and transcribed, and that the BDV 5'- and 3'- end NCR sequences that I have used are functional.

On the other hand the transcription termination sites of BDV was proposed to be the poly(U) sites of the BDV genome (21, 43), Schneeman et al. (191) confirmed that there are four transcription termination sites of BDV genome and each termination site consists of 6 or 7 U residues as known in other viruses of Mononegavirales. It seems likely that polyadenylation for BDV also occurs by polymerase stuttering on these U residues like for influenza virus. The RPA results showed that a short band (249 nt) “mRNA” was detected, confirming that the polyadenylation signal is indeed used in the trancription termination of the BDV genome, because the probe can not hybrized completely with the mRNA resulting in partial digest by

RNAase. In contrast the probe hybridizes completely with the primary transcript resulting in a longer fully protected probe (311 nt).

To generate an amplified and highly sensitive reporter system, a Pol I-plasmid expressing the T7 polymerase gene flanked by the BDV 3' and 5' NCR and the ribozymes sequences was constructed. This plasmid was transfected together with plasmids expressing luciferase reporter gene under the control of the T7 promoter into Vero cells and BDV infected Vero cells respectively, to prove that this "mini-genome" can be recognized and transcribed by the BDV polymerase. The luciferase assay results indicated that the polymerase of BDV can indeed use the "mini-transcript" generated by pPolI-HHR-T7 for replication and transcription, although the relative luciferase activity is not very high.

In the plasmid-based reverse genetic system, the Pol I expression construct has also shown to be functional by CAT and luciferse activity assays. These results furthermore prove that the functional 5'-NCR and 3'NCR can be generated by the Pol I expression construct of the reported system, although the activity of CAT and luciferase is not as strong compared to the data reported. Maybe the following reasons can present a good explanation. Firstly, this is the first experiment, a strong activity of the reporter gene should be detected, if the experimental condition is further optimized. Secondly, different NCR ends were used in my Pol I construct compared to the two reported systems. G of 5'NCR and A of 3'NCR were used in the mini-genome constructs, this means that the last nucleotide is not homologous and overhanging ends will be formed, which is different from the orignal ends in my construct according the expected sequences. The reported Pol I and T7 systems can work very well in vitro, maybe infectious BDV can be rescued by the reported Pol I system, but it remians to be determined whether it can represent the true viral condition in vivo. Moreover, different virus strains and cell lines were used in the reported Pol I system compared to my system. The He/80 strain was used to determine the 5'- and 3'-NCR ends in the reported Pol I system, but my construct is according to the strain BDV H1766.

In general, the new construct for generating a "mini-genome" of BDV is functional not only in the helper virus dependent system, but also in the plasmid-based reverse genetics system. This study might be the basis for establishing a reverse genetic system for the rescue of BDV in the future, that will play an role in understanding the character of the BDV.

3 Perspectives

3.1 AIV

Five Pol I expression plasmids for a reverse genetic system have been proved to be functional, and the sequences of other three genes should be correct, because there are very little differences compared to the sequences from the genebank and the original sequences. I will therefore try to rescue an infectious avian influenza virus A/Goose/Guangdong/1/96 (H5N1)

using these eight plasmids under safe conditions (P3 laboratory) in China, and establish a reverse genetic system for this strain. Some mutation studies will be performed by this reverse genetic system, in order to learn more about the molecular biology of this virus.

The HA is very important for the pathogenicity of influenza A viruses. But the reassortant GDNS1FPV differs from the wild type FPV only by the NS-segment. I will compare the pathogenicity of these three viruses (wild type FPV; reassortant GDNS1FPV and A/Goose/Guangdong/1/96 (H5N1)) by tissue cultures and animal experiments. These experiments will be very useful to learn more about pathogenicity of avian influenza viruses. Moreover, as the reassortant GDNS1FPV inhibits more efficiently the IFN activation than the wild type FPV, a series of mutation experiments will be done to investigate which domain is responsible for this function. This will benefit the development of the live attenuated influenza vaccine in the future.

In nature, it is possible that both highly pathogenic avian influenza viruses can infect the same host and lead to a new reassortant virus that might be much more pathogenic than the parental viruses. Therefore it is necessary to try to rescue the other ressortant viruses by different plasmid combinations as mentioned in the discussion, in order to study how the highly pathogenic H5 and H7 avian influenza viruses recombine in nature.

3.2 BDV

Compared to the reported two reverse genetic systems (T7 and Pol I), I used the original BDV NCR for the mini-genome construct in my system. Moreover I have shown that this Pol I construct is functional not only in the helper virus dependent system but also in the plasmid-based reverse genetic system. Artificial NCR ends were created (G of 5'NCR and A of 3'NCR) in the reported two systems, this means that overhanging ends will be formed. This is until now not the case for other NNS viruses and it can not be estimated what effect such ends would have for viral replication. Maybe the infectious BDV can be rescued by the reported Pol I system, but it remains to be investigated that it represents the true condition of BDV in vivo (animals).

The optimal condition for my Pol I system will be investigated, in order to study the 5'NCR and 3' NCR functions by mutations in the future and to rescue infectious BDV by this system. I hope my system will be useful to understand more molecular biology of BDV.

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Abbreviations

AA	amino acids
APS	Ammonium persulfate
AI	Avian influenza
AIV	Avian influenza virus
BD	Borna disease
BDV	Borna disease virus
bp	base pairs
BSA	bovine serum albumin
^{o}C	centigrade
cm	centimeter
CAT	chloramphenicol acetyltransferase
CMV	cytomegalovirus
CPSF	cleavage and polyadenylation specificity factor
CTP	cytidine triphosphate
ddH2O	deionized distilled water
DMEM	Dulbecco's Modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
eIF2	eukaryotic translation initiation factor 2
ERK	extracellular signal regulated kinase
et al.	*et alii* (=and others)
FCS	fetal calf serum
FPV	fowl plague virus
g	gram
GFP	green fluorescent protein
GTP	guanosine triphosphate
GP	glycoprotein, BDV
h	hour(s)
HA	hemagglutinin
HEPES	N-2-hydroxyethylpiperazine
HDV	Hepatitis delta virus
His	histamine
HPAIV	highly pathogenic avian influenza virus
IFA	indirect immunofluoresces assay
IFN	interferon
IRF3	IFN regulatory factor 3
kb	kilobasepairs
kDa	kilodalton
L	RNA-dependent RNA.polymerase, BDV
LPAIV	lowly pathogenic avian influenza virus
M	molar
M	matrixprotein, BDV
M1	matrixprotein, Influenza
M2	ion channel protein, Influenza
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase/ERK kinase

mg	milligram
min	minute(s)
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
N	nucleoprotein, BDV
NA	neuraminidase
NCR	noncoding region(s)
NEP/NS2	nuclear export factor
NES	nuclear export signal
NLS	nuclear location signal
NP	nucleoprotein
NPC	nuclear pore complex
NS1	nonstructural protein
ng	nanogram
nt	nucleotide(s)
NTP	ribonucleoside triphosphate
OD	optical density
P	phosphoprotein, BDV
p10/x	10 kilo Dalton protein, BDV
PA	subunit of RDRP, influenza
PABII	poly(A)-binding protein II
PAGE	polyacrylamide gel eletrophoresis
PB1	subunit of RDRP, influenza
PB2	subunit of RDRP, influenza
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit(s)
PKR	dsRNA activated protein kinase
pmol	picomolar
PolyA	polyadenylic acid
Pol I	RNA polymerase I
RDRP	RNA-dependent RNA-polymerase
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
RNPs	ribonucleoproteins
RPA	RNase protection assay
RT	reverse transcription
s	second(s)
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris-hydroxymethylaminomethane
ts	temperature sensitive
Tween 20	polyoxyethylenesorbiten monolaurate
U	unit(s)
U	uridine
UTP	uridine triphosphate
UV	ultraviolet
V	volt

vol	volume
vRNA	viral RNA
v/v	volume percentage
w/v	weight percentage
µg	microgram
µl	microliter
µM	micromolar

Erklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig durchgeführt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Gießen, den 26. 09. 2003

Wenjun Ma