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Generation of recombinant Influenza A derived Ribonucleoprotei n complexes

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Abstract

The influenza A derived ribonucleoprotei n (RNP) complexes consist of negative single stranded RNA covered with Nucleoprotein (NP). Complementary regions at the 5' and 3' termini can anneal to form a bul ged duplex structure that is essential for transcription and replication, which is achieved by a heterotrimeric polymerase complex, consisting of PA, PB1 and PB2. The polymerase is found to be tightly associated with vRNA 5' and 3' termini. Due to the fact that RNP complexes are the minimal infectious particle of influenza A virus, they are intended for vitro / in vivo gene delivery, as well as for generation of recombinant virus. Another application is the use of RNP complexes in vaccine design.

The goal of this study was to recombinantly express the 4 RNP proteins in an insect cell system, using baculovirus for gene delivery. We first established MOI / hpi combinations ideal for RNP formation in the nucleus and tried to visualize complexes in their native state, which was achieved by Blue Native page. We further attempted to isolate RNPs by velocity sedimentation and confirmed the presence of nucleoprotein in pellet fraction by western blot. To show functionality, insect cells were infected with baculovirus delivering RNP proteins together with baculovirus delivering negative single-stranded RNA coding for a GFP fusion protein. If polymerases work, negative sense RNA should be transcribed to mRNA, leading to GFP expression. For testing the activity of viral polymerases in an insect cell system, Sf9 cells were transfected with in vitro transcribed negative-strande d RNA and monitored for GFP expression. For further verifying in vitro transcribed RNA we infected CHO and MDCK cells with the influenza A strain PR/8/34 and transfected the cells with RNA. We finally observed 4 GFP expressing CHO cells leading to the assumption that in vitro transcribed RNA was correct.

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Abbreviations:

AD	Aqua dest.
ATB	Antibiotic mix
CHO	Cell line from Chinese hamster ovary
BALB/c	Albino strain of laboratory mice
BME	. - mercaptoethan ol
BN Page	Blue native page
BSA	Bovine serum albumine
BV	Budded virus
cDNA	Copy DNA
CIAP	Calf intestinal alkaline phosphatase
CPE	Cytopathological effect
cRNA	Copy RNA
dNTP	Deoxynucleotidet riphospate
dsRNA	Double-stranded RNA
EM	Electron microscopy
EtBr	Ethidiumbromide
FCS	Fetal calf serum
GFP	Green fluorescent protein
HA	Hemagglutinin
hpi	Hours post infection
hpt	Hours post transfection
LB	Luria Bertani
M1	Matrix protein 1
MDCK	Cell line from Madin Darby canine kidney
MOI	Multiplicity of infection
NA	Neuraminidase
NLS	Nuclear localization signal
NEP	Nuclear export protein
NES	Nuclear export signal
NP	Nucleoprotein
NTP	Nucleotidetriphp ophate
ODV	Occlusion derived virus
OPD	o - phenyl - diamine- dihydrochloride
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PR/8/34	Influenza A strain Puerto Rico / 8 / 34
PVDF	Polyvinylidene fluoride
RNPs	Ribonucleoprotei n complexes
RNP virus	Baculovirus expressing RNP proteins
RT	Room temperature
SDS	Sodium dodecyl sulfate
Sf9	Cell line from Spodoptera frugiperda
TAE	Tris - Acetate - EDTA
TPBS	PBS containing 0,01% Tween 20
vRNA	Viral RNA

1. Introduction:

1.1 The Influenza Virus:

1.1.1 General considerations:

Next to the common cold, influenza or "the flu" is perhaps the most familiar respiratory infection in the world (1).Influenza is caused by a virus that attacks mainly the upper respiratory tract – the nose, throat and bronchi and rarely also the lungs. The infection usually lasts for about a week and is characterized by sudden onset of high fever, headache, severe sickness and non-productive cough. Most people recover within one to two weeks without requiring any medical treatment. In the very young, the elderly and people suffering from medical conditions such as lung diseases, diabetes, cancer, kidney or heart problems, influenza poses a serious risk. In these people, the infection may lead to severe complications of underlying diseases, pneumonia and death.

Influenza rapidly spreads around the world in seasonal epidemics and imposes a considerable economic burden (2).

An influenza pandemic is a rare but recurrent event. Three pandemics have occurred in the previous century: "Spanish influenza" in 1918, "Asian influenza" in 1957 and "Hong Kong influenza" in 1968. The 1918 pandemic killed an estimated 40–50 million people worldwide. That pandemic, which was exceptional, is considered one of the deadliest disease events in human history. Subsequent pandemics were much milder, with an estimated 2 million deaths in 1957 and 1 million deaths in 1968.
A pandemic occurs when a new influenza virus emerges and starts spreading as easily as normal influenza – by coughing and sneezing. Because the virus is new, the human immune system will have no pre-existing immunity. This makes it likely that people who contract pandemic influenza will experience more serious disease than that caused by normal influenza (49).

1.1.2 Classification:

Three distinct types of influenza virus, dubbed A, B, and C, have been identified. Together these viruses, which are antigenically distinct from one another, comprise their own viral family, *Orthomyxoviridae* . Most cases of the flu, especially those that occur in epidemics or pandemics, are caused by the influenza A virus, which can affect a variety of animal species, but the B virus, which normally is only found in humans, is responsible for many localized outbreaks. The influenza C virus is morphologically and genetically different than the other two viruses and is generally nonsymptomatic, so is of little medical concern (1).

1.1.3 Morphology:

The structure of influenza virus is somewhat variable, but the virion particles are usually spherical or ovoid in shape and 80 to 120 nanometers in diameter. Sometimes filamentous forms of the virus occur as well, and are more common among some influenza strains than others (1).

Influenza A viruses have genomes comprising eight segments of RNA, varying in length between 890 and 2341 nucleotides, coding for 10 identified polypeptides. Nine of these polypeptides are incorporated into virions. The viral envelope is formed by three polypeptides: the *hemagglutinin (HA)* and *neuraminidase (NA)* glycoproteins, involved in cell entry and exit, respectively, and *M2*, a low abundance ion channel involved in uncoating and HA maturation. Underlying the membrane is the *matrix* or *M1* protein, the major structural component of the virion. M-protein is thought to act as an adaptor between the lipid envelope and the internal genome is probably the driving force behind virus budding (3). M1 and M2 protein are both coded by genome segment 7 and generated by differential splicing.

Inside the shell of M1 lie the RNPs: these comprise the genomic RNA segments in association with a trimeric RNA polymerase and stoichiometric quantities of *nucleoprotein (NP)* (4). The RNA polymerase consists of three subunits, *polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA)*. A detailed view on RNP complexes will be given in next chapter.

Also found in the virion are small quantities of *nuclear export protein (NEP) and non structural protein (NS1),* which are generated by alternative splicing of genome segment 8.

1.2 The influenza RNP complex - a more detailed view:

The influenza A ribonucleoprot ein complexes, which are composed of "-"sense single stranded RNA covered by nucleoprotein and the trimeric polymerase complex, as mentioned above, have a diameter of about 15 nm and a length between 50 and 100 nm depending on the RNA segment (5).

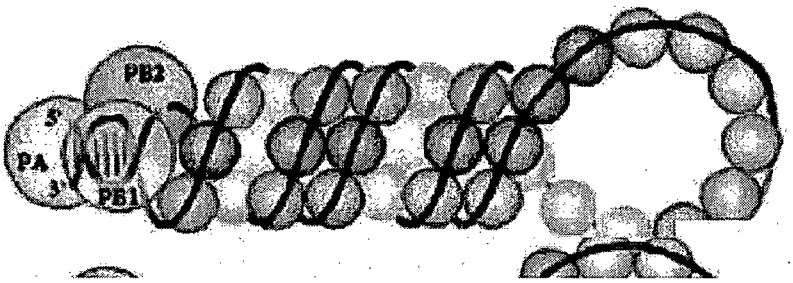

Figure 1.1 *Structural organisation of influenza virus RNPs. Blue spheres represent NP monomers with associated viral RNA molecule (black line). The single stranded vRNA is coiled into a hairpin structure with a short region of duplex (formed between the 5' and 3' ends) which forms the binding site for the heterotrimeric RNA-dependent RNA polymerase (from: Agustin Portela et al.).*

Each *vRNA* consists predominantly of coding sequences (in antisens e orientation), flanked at both ends by untrans lated regions (UTRs) that range from 19 to 58 bases long. Within these UTRs, the distal 12 and 13 noncoding bases that form the extreme 3' and 5' termini, respectively, of every segment are highly conserved among viral strains and among the eight segments themselves. These distal conserved sequences are partially complementary to each other and so can anneal to form a bulged duplex structure that is essential for transcription and replication of the segment (37).

The *infuenza virus promotor structure* consists of both termini of a vRNA molecule and may be separated into three parts: a proximal element (the first nine nucleotides from both ends of the molecule), followed by angular nucleotide, A10, which has no counterpart in the 3' sequence, and the distal promoter element, consisting on average of six RNA basepairs, i.e. positions 11-16 at the 5' end, which in most vRNAs are exactly complementary to positions 10 to 15 at the 3' end. Despite the overall sequence conservation, it has been shown repeatedly that compensatory double exchanges in the distal element, i.e. reconstitution by any other complementary RNA sequence, will regain the full level of promoter activity, while single nucleotide exchanges disrupting the structure abolish expression of an adjacent reporter gene (33).

Influenza virus NP is the major structural protein in RNPs and has multiple functions in the viral infectious cycle. It is a basic protein rich in arginine with a net positive charge pH 6.5 (6). Each copy of this protein of 56 kDa binds to about 24 nucleotides of viral RNA (5). In vitro RNA binding is nonspecifically, yet in vivo NP binds only to complete cRNA (plus polarity) and vRNA (minus polarity), forming cRNP and vRNP, respectively, and does not bind to viral mRNA (pluspolarity) possessing 5' cap and 3' poly(A) sequences (7). NP has nuclear localisation signals (21) for nuclear translocation and along with the polymerase complex plays a critical role in nuclear translocation of vRNP after uncoating of the infecting virus.

The existence of *NP-NP interactions* is important for maintaining the RNA template in an ordered conformation suitable for transcription by the polymerase and / or packaging into virions. EM visualization of RNPs reveals rod-like particles, often with loops at one end (Figure 1.1); these structures have been interpreted as strands of NP-RNA complexes forming a panhandle structure (23). The formation of these structures depends on a combination of RNA-termini, RNA-RNA, NP-RNA and NP-NP interactions (24).

The *influenza virus polymerase* is as mentioned before a heterotrimer with an aggregate molecular mass of 250 kDa (34) and is found to be tightly associated with 5' and 3' termini of the double stranded panhandle formed by RNA and nucleoprotein (Figure 1.1). No crystal structure is available for the influenza virus polymerase complex, but a low-resolution three-dimensio nal structure, determined by electron microscopy, showed that it is very compact, with no obvious boundaries between subunits (34). Protein-protein interactions between the individual polymerase subunits have been identified, suggesting that PB1 is the core of the polymerase complex. The N-terminal region of PB1 interacts with the C-terminal region of PA, while the C-terminal region of PB1 interacts with the N-terminal of PB2 subunit. No direct protein-protein interactions have been demonstrated between PB2 and PA (35, 36). All three polymerase subunits are required for transcription and replication activity both in vivo and in vitro (30, 38), although, controversially, Honda et al. reported that recombinant dimeric complexes of PB1-PB2 and PB1-PA have distinct transcriptase and replicase activities, respectively, in vitro (39). In recent years, it has been shown that NP interacts directly with PB1 and PB2 but not with PA, both in virus-infected cells and recombinant systems (40, 41). Consistent with this, an EM reconstruction of an RNP clearly shows two regions of contact between the polymerase complex and separate NP monomers (42).

The PB1 subunit contains the conserved motifs characteristic of RNA-dependent RNA polymerases (25, 26) and binds to the vRNA and cRNA promoters (27, 28). PB1 further has endonuclease activity required to snatch capped primers from host pre-mRNAs for viral RNA transcription (29). However, PB2 is responsible for recognition and binding the cap structure of host mRNAs (30, 31). The exact role of PA is less well understood, but it has a central role in both transcription and replication (32).

1.3 The influenza life Cycle:

As with all viruses, influenza virus needs to penetrate target cells to cause infection. So for enveloped viruses, the principal route of entry takes place by a combination of receptor binding and fusion. In the case of influenza virus, these events have generally been well characterized from a biochemical and biophysical perspective (8); however, many of the cell biological aspects of virus entry still remain unclear.

1.3.1 Viral Entry:

Entry of influenza virus starts with binding to sialic acid receptors present on cell surface (9). It has become established that the influenza A and B viruses can bind to sialic acid residues that are present on either glycoprotein or glycolipid (8). The specific conformation of the sialic acid linkage (. , 2-3 vs. . , 2-6) has also been established to control species tropism of the virus (10). The crystal structure of the viral hemagglutinin (HA) shows that it binds sialic acid substrates via surface pockets at the membrane-distal region of HA (8). *In vitro* assays, examining hemagglutination of red blood cells, have shown that certain gangliosides can act as influenza virus receptors (10).

After initial receptor binding, influenza virus particles are internalized in endosomes following the route used by other viruses and macromolecule s (11). Acidification of endosomes is achieved by the activity of the vacuolar proton ATPase (v[H1]ATPase). This enzyme pumps protons to the endosome interior at the expense of ATP hydrolysis (12), causing endosome acidification within a few minutes. Acidification induces conformational changes in several animal virus glycoproteins, including HA (13), so that hydrophobic regions of the proteins become more exposed, thereby increasing the tendency of the protein to interact with membranes (11, 14). The M2 ion channel further becomes activated due to the acid environment and is thought to permit a flow of ions into the viral interior leading to acidification. This causes M1 protein to dissociate from RNPs. The interaction of HA molecules with the endosomal membrane finally leads to fusion of the latter with the viral envelope, allowing release of the viral nucleocapsid into the cellular cytoplasm (15).

1.3.2 Nuclear translocation:

Most DNA viruses replicate in the nucleus of the infected cell (with some exceptions like pox viruses) where they use the cellular RNA polymerase II to make mRNAs that can be translated in the cytoplasm. Since RNA viruses cannot use cellular Pol II (because it is dependent on a DNA matrix) most RNA viruses replicate in the cytoplasm. Influenza virus is one of the very few RNA viruses to replicate in the nucleus. This is probably due to the fact that at least two of the genes (NS1/NEP and M1/M2) have full-length and spliced mRNAs leading to different proteins.

RNPs that have to enter the nucleus are carried in by importins (also called karyopherins). There are two types of importins, . and ., both of which are part of the importin . super-family. The proteins belonging to this family are characterized by a structure made up of a series of armadillo repeats. Importin . proteins recognize the NLS on the RNP protein. Once the cargo is bound by importin ., the complex is recognized and bound by importin . that subsequently binds to the fibrils of the nuclear core complex and is responsible for the actual translocation (22).

However, it is not known which NLS are used for this import. All individual protein components of the RNP have individual NLS and it is not know which of these signals in the assembled RNP are used for import. It is further not known whether the RNPs are transported as a group or whether each complex enters the nucleus independently through different pores (16).

1.3.3 Viral Replication:

In virus-infected cell nuclei, three modes of RNA synthesis occur: (i) transcription of the vRNA into mRNA which contains a cap structure at the 5' terminus and a poly(A) tail at the 3' terminus, (ii) the vRNA directed synthesis of a full-length cRNA without any modifications (the first step of replication), and (iii) the cRNA-directed synthesis of vRNA (the second step of replication) (17).

The molecular mechanism and regulation of transcription have been relatively elucidated by biochemical and genetic analyses. The PB2 subunit first binds specifically to the cap-1 structure at the 5' termini of nascent host cell mRNA

transcripts (18). Thereafter, the polymerase endonucleolytically cleaves the mRNAs to 10- to 13-mers (19). These short capped RNAs serve as primers for viral mRNA synthesis (20). The polymerase then transcribes viral RNAs (vRNAs) by elongation to nucleotides 17 to 22 from the 5' ends of the vRNAs. At this point it adds A residues to nascent RNA chains, leading to the formation of viral mRNA with both the cap-1 structure at the 5' terminus and the poly(A) tail at the 3' end. The mRNAs are transported to cytoplasm where translation takes place and are imported back into nucleus using their nuclear localisation signals.

The switch from mRNA transcription to production of cRNA depends on the level of free nucleoprotein; i.e. later in infection there is lots of NP in the nucleus, mRNA synthesis stops but cRNA synthesis continues.

1.3.4 Export of newly made RNPs:

After replication of the RNA and assembly of the new RNPs, these complexes have to leave the nucleus in order to be incorporated into new virus particles that bud at the cell membrane. For export of RNPs, both viral M1 and NEP proteins are needed (43). M1 is a two-domain protein. The N-terminal domain contains the NLS and the C-terminal domain has affinity for RNP (44). However, M1 does not have the necessary NES motif for nuclear export. This motif is found on the N-terminal domain of NEP. NEP can bind in vitro to an exportin (CRM1), which leads to the export of RNPs using an RanGTP dependent pathway (45). There are about 200 copies of NEP in a mature virus particle which could mean that some 20-30 M1-NEP complexes bind per RNP (46).

1.3.5 Formation and budding of new virus particle:

To be infectious, an influenza A virus particle must package at least one copy of each genome segment. *cis*-Acting signals necessary for packaging appear to reside within the 3' and 5' untranslated regions (UTRs) of each vRNA (48).

Influenza virus M1 proteins plays a critical role in the assembly and budding process of virions. Because of the presumed juxtaposition of the M1 protein between the viral envelope and the nucleocapsid (vRNP), M1 is proposed to interact with the cytoplasmic tail of transmembrane viral proteins on the outer side and the viral nucleocapsid (vRNP) on the inner side. These interactions are believed to trigger the budding process leading to the formation and release of virus particles. It is proposed that release of newly budded particles from the cell surface is triggered by neuraminidase (47).

One fundamental question in influenza virus genetics is whether the virus discriminates among its segments during the packaging process. While a variety of mechanisms could be envisioned, the issue is often framed in terms of two simple, hypothetical models that are intended to represent the extreme alternatives. Under the first model, called random packaging, each virion selectively incorporates viral segments (as opposed to other viral or cellular RNAs) but does not differentiate among them, so that the likelihood of acquiring the full complement of vRNAs is determined entirely by chance. The second model, by contrast, asserts that the virus targets each of the eight segments individually and independently, a phenomenon called specific packaging (48).

1.4 Vaccination strategies:

Influenza virus vaccines were first developed in the 1940s and consisted of partially purified preparations of influenza viruses grown in embryonated eggs. Because of substantial contamination by egg-derived components, these killed vaccines were highly pyrogenic and lacking in efficacy. A major breakthrough came with the development of the zonal ultracentrifugation in the 1960s. This technology, which originated from uses for military purposes, revolutionized the purification process and industrial production of many viruses for vaccines. To this day, it remains the basis for the manufacturing process of our influenza virus vaccines (61).

Currently two major types of influenza virus vaccines are available: The first type, which can further be divided into whole virus, split virus and virosome vaccines is composed of physically or chemically inactivated virus. The second type is a live attenuated vaccine.

1.4.1 Inactivated vaccines:

The first types of vaccines are *trivalent inactivated vaccines (TIV)*, which are the primary means of preventing influenza infection, due to the vast experience of their use worldwide. Viral strains used for production of TIVs are 6:2 reassortants with HA and NA of the recently circulating strain and the 6 backbone gene segments of influenza A virus Puerto Rico/8/34, which is a high yielding laboratory strain. The HA and NA antigens are derived from current H1N1 (hemagglutinin [HA] subtype 1; neuraminidase [NA] subtype 1) and H3N2 influenza A virus as well as an influenza B virus. Such reassortants are made by coinfecting eggs with both viruses and screening progeny for the desired 6:2 configuration. The virus is further harvested, chemically / physically inactivated and purified by ultracentrifugation (61, 62).

Whole viruse vaccines exhibit good levels of immunogenicity, but have greater reactogenicity too, in particular causing fever in children and, therefore, are not indicated for this age group. Split or subunit virus vaccines are formaldehyde-inactivated preparations, which, after purification, are chemically disrupted with a nonionic detergent (for example, Triton X-100). The difference refers to the degree of disintegration. These preparations show lower pyrogenicity than whole virus

vaccines, but lower immunogenicity too. Virosome vaccines are inactivated vaccines in which the HA and NA influenza virus surface antigens are incorporated into virosome (virus-like) particles that have an adjuvant role. This type of vaccine offers similar immunogenicity and safety to other vaccines (63).

In general, 1 dose for adults contains the equivalent of 45 . g HA (15 . g HA for each of the 3 antigenic components). This dose is approximately the amount of purified virus obtained from the allantoic fluid of 1 infected embryonated egg (61).

1.4.2 Live, attenuated vaccines:

The second major class of viral vaccines consists of live viruses. The only FDA licensed product against influenza is a cold-adapted attenuated vaccine. Influenza virus was passaged at 25°C in tissue culture (chicken kidney cells) and in embryonated eggs. This resulted in a cold-adapted, temperature-sensitive, and highly attenuated master strain (64). The annually updated vaccine strains are generated in the laboratory by reassortment with viruses more closely related to the currently circulating ones. The resulting vaccine strains (both A and B types) are 6:2 reassortants with the 6 nonsurface protein genes derived from the cold-adapted master strains and the HA and NA from circulating A and B viruses, reflecting the changing antigenicity. These cold-adapted influenza virus vaccines are easily administered by nasal spray. They induce local mucosal neutralizing immunity and cell mediated responses that may be longer lasting and more cross-protective than those elicited by chemically inactivated (killed) vaccine preparations (61).

1.4.3 Modern approaches:

As indicated, current FDA-licensed influenza vaccines are based on technologies developed in the 1960s and earlier. Through the breakthrough of reverse genetics techniques, infectious influenza viruses from plasmid DNAs transfected into tissue culture cells can now be rescued (65). This technology permits the construction of high-yield 6:2 seed viruses by mixing the 6 plasmid DNAs from a good-growing laboratory strain with the HA and NA DNAs obtained by cloning relevant genes from currently circulating viruses. Thus, within a 1- to 2-week period, the appropriate seed viruses could be generated for distribution to the manufacturers. The backbones of

the 6:2 recombinant viruses could be prepa red, tested, and distributed in advance. Similar approaches can be envisioned for the manufacturing of live, cold-adapted influenza virus vaccines. In this case, the backbone would consist of the 6 genes of the cold-adapted master strain. Again, the HA and NA of the currently circulating strains would be cloned and used for rescue in the plasmid-only reverse genetics system. Such an approach would have several advantages over the present manufacturing process (61).

Other promising approaches concern the use of replicati on-deficient preparations. For example, virus particles that lack the gene for the nuclear export protein will go through a single cycle of rep lication (without forming infectious particles) (66).

1.5 The influenza RNP complex - special features and applications:

Based on the work done by different groups, influenza RNP complexes are intended for their use in vaccine design and as adjuvant for increasing the immunogenicity of commercially available vaccines. This is due to the fact that in vertebrates, receptors of the innate immune system recognize pathogen-associate d molecular patterns in order to activate early defense mechanisms. Innate immune recognition plays an important role not only in clearing the majority of invading pathogens but also in priming and directing the adaptive immune response. The most prominent of the innate immune receptors belong to the Toll-like receptor (TLR) family. It has been shown that mammalian Toll-like receptor 3 recognizes double-stranded RNA (dsRNA), as present in influenza A RNP complexes, which acts as a molecular mimic associated with viral infection, and activates the NF-. B pathway. This leads to an activation of alpha / beta interferon (IFN-. / .), which enhances the primary antibody response. dsRNA also induces antiviral responses intracellularly, by directly activating RNA-dependent protein kinase (PKR), which causes an inhibition of cellular and viral protein synthesis via phosphorylation of eukaryotic translation initiation factor 2 . (eIF2 .) (69).

Ichinohe et al. have shown the mucosal adjuvant effect of synthetic dsRNA polyriboinosinic polyribocytidyl ic acid [poly (I:C)] against influenza virus by intranasal coadministration with inactivated hemagglutin in vaccine from PR8 (H1N1) in BALB/c

mice. This coadministration induced a high anti-HA immunoglobulin A (IgA) response in the nasal wash and IgG antibody response in the serum, while vaccination without poly (I:C) induced little response (70).

Transcriptionally active RNP complexes are the minimal infectious particle of influenza virus. This intends the use of their replicon for in vitro and in vivo gene delivery applications. The most commonly used technique has involved the assembly of purified RNP proteins with an in vitro transcribed RNA copy of the gene of interest, followed by transfection of target cells using cationic lipid formulations in vitro and possibly in vivo too. The heterotrimeric polymerase complex, once entered the nucleus of the target cell, transcribes negative-stranded RNA to mRNA, leading to transgene expression. This approach could possibly be used for gene therapy in the field of cancer treatment.

Another widely spread use of RNP complexes is the direct manipulation of influenza virus gene products and the creation of new recombinant virus not found in nature. The method is based on gene delivery technique, as described before, but carries an in-vitro transcribed RNA copy of a virus gene segment, followed by transfection into cells previously infected with a helper virus. Several transfection methods have been established, but recent work has shown that electroporation works best. Using a selection method, viruses containing the genetically engineered RNP (transfectant viruses) can be isolated. However, it is not clear that all of the viral proteins present in the RNP complexes are required since the helper virus is providing newly synthesized polymerase and NP proteins in the cells used for transfection. Shaw et al. have shown that influenza virion polymerase proteins are not necessary in the artificial RNP complexes used for the generation of influenza virus transfectants and that NP alone is sufficient. The role of NP may be 2-fold; protection of the RNA from degradation and transport to the cell nucleus where the helper virus supplies the necessary transcription and replication activities (71).

1.6 Baculovirus:

1.6.1 Classification:

Baculoviruses are large, double-stranded, circular DNA viruses which infect arthropods, mainly insects (50). Although baculoviruses infect over 600 species of insects, individual isolates normally show a limited host range and infect only closely related species (51). They were first studied in the context of their potential use as ecologically friendly biopesticides against pest caterpillars. The discovery that baculoviruses such as *Autographa californica multicapsid nucleopolyhedrov irus* (AcMNPV) could be developed as foreign protein expression systems in cultured insect cells quickened the pace of their characterization at the cellular and molecular levels. The cell lines most often used for these studies were derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* (Sf21) or a clonal derivative of this cell line (Sf9) (50).

1.6.2 Life Cycle:

Infection of the host begins when insect larvae acquire the virus orally. Infection is first observed in the epithelial cells of the midgut, and this is followed in most cases by systemic infection. One hallmark of the baculovirus infection cycle is the production of two structurally and functionally distinct virion phenotypes. One virion phenotype, the *occlusion-derived virus (ODV)*, is found within the protective occlusion bodies. Once released from the occlusion body by the alkaline pH of the gut, the ODV initiates infection of the animal by infecting epithelial cells of the midgut. A second virion phenotype, the *budded virus (BV)*, is produced by budding from the surfaces of infected cells. The BV is initially produced from infected midgut epithelial cells and is believed to be essential for systemic infection, mediating movement of the virus from the midgut to other tissues and propagating the infection from cell to cell within the infected animal. BVs are highly infectious to tissues of the hemocoel and to cultured cells, whereas ODVs appear to be less infectious in cell culture or when injected into the hemocoel. The two virion phenotypes also differ in entry mechanisms (60).

1.6.3 Morphology:

Baculoviruses have large, rod-shaped virions that contain double-stranded, supercoiled DNA genomes ranging in size from 88 to over 160 kb depending on the viral strain. The most well-characterized baculovirus, the *Autographa californica* multinucleocapsid nuclear polyhydrosis virus (AcMNPV) has a genome of 134 kb and encodes approximately 150 genes (52).

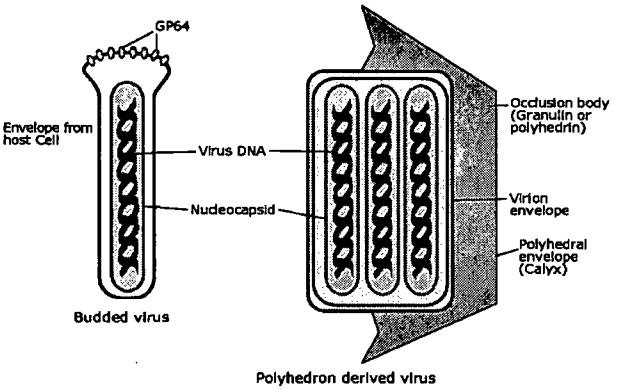

***Figure 1.2** Budded (BV) as well as occlusion derived (ODV) baculovirus phenotypes.*

Although nucleocapsids of the two phenotypes appear to be identical their envelopes are derived from different sources.

The baculoviral nucleocapsid has a size of 21 nm x 260 nm and is composed of at least one small putative DNA-binding protein, a capsid protein termed VP39, another putative capsid protein termed p87 (53) and the large viral genome. For efficient packaging, genomes must become highly condensed. Although histones neutralize the electrostatic repulsion of neighbouring DNA sequences in cellular DNA, they do not appear to be associated with DNA packaged within nucleocapsids (54). However, an arginine/serine-threonine-rich protein containing 54 amino acids (termed p6.9) has been shown to be present in virions. The high concentration of arginine, serine and threonine in these proteins is similar to that of protamines, which are a class of proteins present in many fish, avian and mammalian sperm nuclei and are involved in the production of highly condensed DNA (55).

BV acquires its envelope from the cell membrane and requires a glycoprotein, GP64, for being able to spread systemic infection. This protein, also known as GP67, is the major envelope protein, which forms structures called peplomers on one end of the budded virus particle. It is an extensively processed homotrimeric type I integral membrane glycoprotein and consists of 512 amino acids with four glycosylation sites at asparagine residues. It further contains oligomerization and fusion domains as well as hydrophobic transmembrane domains near the C-terminus (56).

However, within the *ODV* phenotype, nucleiocapsids are embedded in a polyhedron matrix. Polyhedrin is a protein of about 245 amino acids (29K) and is the major component of polyhedra. Polyhedrin has received considerable attention because it is hyperexpressed, constituting up to 18% or more of total cellular alkali-soluble protein late in infection. The polyhedrin gene is not essential for viral replication in cell culture, and is the most common locus for insertion of foreign genes in the baculovirus expression system (53).

1.6.4 Molecular biology of infection and replication:

Baculovirus has been shown to enter insect cells via adsorptive endocytosis, with the uptake to intracellular vesicles occurring between 10 and 20 min postinfection. The cellular receptor, however, has not been identified. The nucleocapsids are further released from endosomes, by an acid induced pathway, between 15 and 30 min postinfection with the help of the baculovirus membrane protein GP64 (57). After uncoating, the nucleocapsids induce the formation of actin filaments in the cytoplasm. This results from the activity of a viral protein known as actin rearrangement-inducing factor (Arif-1). Finally, nucleocapsids interact with the nuclear pore, enter the nucleus, mediated by actin filaments, and uncoat. DNA is now ready for transcription and replication (58).

Baculovirus genes are expressed in a transcriptional cascade in which each successive phase is dependent on the expression of genes during the previous phase. They can be divided into the general categories of early genes, which are transcribed by the host RNA polymerase II starting 0-6 hpi, and late genes which are transcribed by a virus-specific RNA polymerase, with a unique subunit composition.

This polymerase is resistant to .-amanitin (an RNA Pol II toxin) and initiates transcription from within a 5 bp late promoter element 6-24 hpi. Late gene expression is dependent on viral DNA replication and is not observed when DNA replication is inhibited. After the onset of DNA replication, most late genes are actively transcribed but the levels of expression decline at later times. However, expression of the very late polyhedrin gene, which encodes the major occlusion body protein, is initially delayed but subsequently reaches extremely high levels very late in the infection (approximately 24-72 hpi) (59).

1.6.5 Generation of recombinant baculovirus:

For baculoviruses to occlude large numbers of virions efficiently, massive amounts of polyhedrin must be produced during the infectious cycle. Although a hyperexpressed gene product, polyhedrin is not necessary for growth of the virus in cell culture. This has been exploited in the development of the baculovirus expression system, in which the polyhedrin gene is replaced by foreign genes under the control of the polyhedron promoter. The advantage of this system is that the recombinant baculovirus express high levels of eukaryotic gene products that are usually folded and processed in a manner similar to the native proteins (53).

2. Goals of this thesis:

The goal of this thesis was to generate recombinant baculovirus, expressing proteins coding for influenza A ribonucleoprot ein complexes. Assembly was monitored, the complexes were characterized and their functionality was tested.

Four distinct sections are present:

1. Cloning of transfer vectors containing the sequences coding for RNP proteins: Nucleoprotein (NP) and the trimeric polymerase complex (PA, PB1, PB2)

2. Generation of recombinant baculovirus , by cotransfection of transfer vectors containing the desired sequences and BaculoGold DNA, followed by plaque purification and rising of viral titers.

3. Generation and characterization of the RNP complexes.

4. Testing the ability of viral polymerases to transcribe "-"single-strand ed RNA in mRNA leading to reporter gene expression.

3. Materials and methods:

3.1 Cloning of baculoviral transfer vector:

3.1.1 Polymerase chain reaction:

For *preparative* PCRs KOD_{Hifi} DNA Polymerase (Novagen), a high fidelity thermostable polymerase was used. It generates blunt-ended PCR products with up to 6 kb in length. The enzyme's 3´→5´exonuclease-de pendent proofreading activity results in a low PCR mutation frequency.

For *screening* PCRs BIOTOOLS DNA Polymerase (Biotools) was used. Reactions were performed in a T3 Thermocycler PCR block. The mixtures for preparative and screening PCR are described in *table 3.1.*

Table 3.1 Mastermixes for screening and preparative PCR

	Screening PCR	Preparative PCR
Buffer	10 µL 10x Biotools Reaction Buffer	5 µL 10x KOD Hifi Buffer 1
DMSO	0 µL	1 µL
MgCL2	4 µL	2 µL
dNTPs	1 µL	5 µL
5' Primer	1 µL	4 µL
3' Primer	1 µL	4 µL
DNA polymerase	0,5 µL	0,4 µL
AD	82,5 µL - Volume of template	28,6 µL - Volume of template
Sum	100 µL	50 µL
Volume / Reaction	30 L	50 µL

PCR programs were based on manufacturers' recommendations:

1. Initial Denaturation step	95 – 98 °C	1 – 2 min.
2. Denaturation step	94 – 95 °C	15 – 30 sec.
3. Annealing step	56 °C	2 – 30 sec.
4. Elongation step	72 °C	20 sec. – 2 min.
5. Final Elongation step	72 °C	5 min.

Steps 2 - 4 were repeated 25 - 30 times. The exact conditions depend on melting temperature of the primers, length of the desired PCR fragment and the polymerase used.

3.1.2 Agarose gel electrophoresis:

Agarose gel electrophoresis is used for separating and analyzing DNA molecules of different size. This is achieved by the moving of negatively charged molecules in the agarose matrix containing an electric field. Shorter molecules will run further than longer ones. The nucleic acids are visualized in the gel by addition of the fluorescent dye Ethidiumbromide. It intercalates between the bases of DNA and gives strong fluorescence when exposed to UV light.

For our purposes we used 1% agarose gels regarding to *table 3.3* and run them in TAE buffer containing ETBr (*table 3.5*). Analytical gels were run at 130V and preparative gels at 90V (constant voltage in both cases).

Table 3.2 Loading Buffer

6x BX buffer	
Bromphenolblue	0,25 %
Xylene cyanol	0,25 %
Glycerin	30 %
AD	69,5 %

Table 3.3 Gel formulation

1% Agarose gel	
Agarose	4 g
TAE	8 mL
EtBr	20 µL
AD	392 mL

Table 3.4 TAE Buffer

50x TAE buffer	
Tris base	242 g
Acetic acid	57,1 g
EDTA 0,5 M	100 mL
with AD up to 1000 mL	

Table 3.5 Running Buffer

Running Buffer	
TAE	200 mL
EtBr	300 µL
with AD up to 10 000 mL	

3.1.3 Preparation of transfer vector and inserts:

As transfer vector we used pBacPAK 8 (clontech) which was linearized with 20 units of XhoI and 10 units of NotI in buffer 3 for 2,5 h at 37°C (enzymes, buffer - NewEngland BioLabs). Preventing religation, the vector was dephosphorylated with 2 units of Calf Intestinal Alkaline Phospatase. Cleaning was achieved by applicating the vector to a preparative gel, followed by an extraction using "Wizard® SV Gel and PCR Clean-Up System" (Promega).

Templates for amplification were provided by Theresa Schinko. The desired inserts (NP, PA, PB1, PB2) were PCR amplified using KOD Hifi DNA polymerase and primers carrying restriction sites for XhoI and NotI (NP, PA, PB1, PB2 – XhoI - back; NP, PA, PB1, PB2 – Not I - for). Inserts were further digested with XhoI and NotI and cleaning was preformed using "Wizard® SV Gel and PCR Clean-Up System" (Promega).

3.1.4 Ligation:

Ligation was carried out using 200ng of vector and 273ng of NP, 391ng of PA, 415ng of PB1 and 415ng of PB2. The components, including 400 units of T4 DNA ligase and T4 ligase buffer, were mixed on ice and ligated on 16°C over night.

3.1.5 Ethanol precipitation:

Ethanol precipitation is used for getting rid of small DNA fragments as well as salts and T4 DNA ligase.

For precipitating DNA 2,5 volumes of 96% ethanol and 0,1 volumes of 3M ammonium acetate were added to the ligation. After an incubation of 15 min at 4°C the tube was centrifuged at 16 000g for 15 min. The supernatant was discarded and the precipitated DNA was further washed with 2,5 volumes of 70% ethanol and again centrifuged for 15 min at 16 000g. The pellet was dissolved in 8 µL AD and was now ready for transformation.

3.1.6 Preparation of electrocompetent *E.coli* DH10B cells:

20 mL of LB-media *(table3.6)* were inoculated with one colony of DH10B cells and incubated over night at 37°C while shaking. The next day 400 mL of LB-media in a 2000 mL flask were inoculated with 4 mL of the overnight culture and grown at 37°C to an OD_{600} of 0,6. Cells were centrifuged at 4000 rpm for 8 min and washed 4 times with 500 mL 1mM HEPES at 4°C (centrifugation is performed as described previously). After last centrifugation cellular pellet was resuspended in 3 mL of 10% glycerin and aliquoted to 50 µL portions in eppendorf tubes. These were subsequently frozen in liquid nitrogen and stored at -80°C.

Table 3.6 LB-media for cultivation of E.coli cells

LB-media	
Peptone from Caseine	10 g
NaCl	10 g
Yeast Extract	5 g
with AD up to 1000 mL	

Set pH to 7,0 using 5M NaOH

3.1.7 Transformation:

Transformation was performed by electropo ration which is a standard method for transforming E.coli cells. For our experiments we used a" BioRAD MicroPulser™, (BioRAD).

50 µL aliquots of DH10B cells were thawed on ice, mixed with the ethanol precipitated ligations and immediately trans ferred to an electroporation cuvette. The parameters on the device were set to 2,5 kV, 1000 . and 25 µF. A time constant between 4,5 and 5 ms indicated that electroporatio ns worked.

Subsequently after the pulse, the cells were added to 950 µL of SOC-media (*table 3.7*) and incubated at 37°C for 1 h while shaking. This step is necessary for chilling the cells until they were plated out on LB-agar plates (*table 3.8*) containing ampicillin (100 µg / mL). The antibiotic pre ssure is used for selecting positive clones.

Table 3.7 SOC-media for chilling of cells

SOC-media	
Bacto - Trypton	20 g/L
Yeast Extract	5 g/L
NaCl	0,6 g/L
KCL	0,2 g/L
MgCL2	1 g/L
MgSO4	1,2 g/L
Glucose	3,6 g/L
AD	

Table 3.8 LB-Agar

LB-Agar	
Peptone from Caseine	10 g/L
NaCl	10 g/L
Yeast Extract	5 g/L
Agar	15 g/L
AD	

3.1.8 Screening for positive clones:

16 colonies from each plate (pBacPAK8 containing sequences for NP, PA, PB1, PB2) were picked and screened for positive clones using Biotools DNA polymerase. 4 positives clones were further cultivated over night in 5 mL of LB-media containing ampicillin and plasmid-DNA was extracted using "Wizard® *Plus* SV Minipreps DNA Purification System" (Promega). A restriction digest with XhoI and NotI was performed for confirming positive clones.

One of each construct was chosen for sequencing.

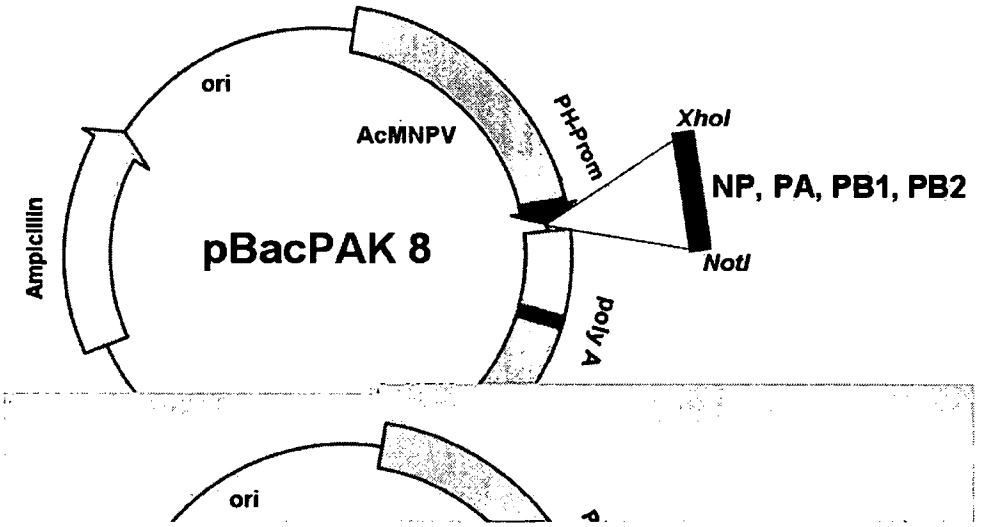

Figure 3.1 Map of transfer vectors.

3.1.9 Sequencing:

Inserts were PCR amplified using KOD Hifi DNA polymerase and vector primers (5' 24-ph-back, 3' 1660-for, 5' NP-744-back, 5' PA-731-back, 5' PB1-716-back, 5' PB2-752-back) . The sequencing itself was done by IBL, Vienna, Austria.

3.2 Generation of recombinant Baculovirus:

3.2.1 Insect cell strain and growth media:

For generation of recombinant baculovirus and the following baculoviral experiments the insect cell line Sf9 was used which was originally derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* . Sf9 cells are highly susceptible to infection with baculovirus and can be used with all baculovirus expression vectors. Sf9 cells were cultivated on IP-L 41 media (*table 3.9*), containing 3% FCS at 27°C.

Table 3.9 IP-L 41 media for Sf9 cells

IP-L 41 media	
Media Power	795 g
Sucrose	297 g
NaCl	56 g
$CaCl_2$	10 g
$NaHCO_3$	7 g
NaOH 2M	150 ml
Yeast Extract	72 g
Lipid stock	50 mL
Pluronic F68 10% v/v	500 mL
AD	to 20,5 kg

Set pH to 6,2 with NaOH 2M

3.2.2 Co-Transfection:

The Co-Transfection was performed with the baculovirus transfer vectors coding for NP, PA, PB1, PB2 and BacloGold DNA (BD Bioscience Pharmingen) using Cellfectin™ transfection reagent (Invitrogen).

Sf9 cells were grown to confluence and passaged the day before transfection. Cells were harvested and counted. For each transfection 1×10^5 cells / cm^2 were seeded in a 12-well plate and incubated at 27°C until they attached to the surface. Cells were washed twice with IP-L 41 media to get rid of the FCS and further incubated with 200 µL of serum free media.

In the meantime transfection mixes were prepared: Mixture A containing 30 ng of BaculoGold DNA and 300 ng of transfer vector up to 15 µL with IP-L 41 in Sarstedt tubes. Mixture B containing 7 µL of Cellfectin up to 15 µL with IP-L 41 in Sarstedt tubes. The mixtures were combined, mixed by pipetting and incubated for 30 min at RT. The solutions were applied to the cells and incubated for 5h at 27°C. For not

stressing the cells too much, media was replaced with 1,5 mL IP-L 41 containing 10% FCS and 1x antibiotic mix. After 5 - 6 days transfection supernatants were harvested.

3.2.3 Plaque Assay:

The Plaque Assay was used for plaque purification and titer determination. Sf9 cells were passaged the day before and 2×10^6 cells were seeded in a 40 mm cell culture dish. After attachment to the surface, the cells were washed twice with IP-L 41. For both uses 1:10 serial dilutions of virus stocks were performed in IP-L 41 and 500 µL of the dilutions were applied to the cells. After incubation with occasional shaking for 1 h at RT the supernatant was removed and cells were overlaid with IP-L 41 containing 1% low melt agarose, 10 % FCS and 1x antibiotic mix. The dishes with solid agar overlay were put into a bag with high humidity and incubated for 5 days at 27°C. The plaques were visualized with MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5 - diphenyltetrazol ium bromide, Sigma Aldrich) and either counted for titer determination or picked for further amplification.

The picked plaques were transferred into 12-well plates containing 1×10^5 Sf9 cells / cm^2 in IP-L 41 (3 % FCS, 1x ATB) and incubated for 5 days at 27°C. The generated seed stocks were further amplified till they reached titers of about 10^8 pfu / mL, which was determined by plaque assay.

3.3 Generation and characterization of RNP complexes:

3.3.1 Generation of RNP complexes by infection:

For generation of RNP complexes Sf9 cells were passaged the day before and seeded into T175 roux flasks with a density of 1 x 10^5 cells / cm^2 (IP-L 41, 3 % FCS, 1x ATB). The cells were then infected with working stocks of baculovirus expressing RNP proteins NP, PA, PB1 and PB2.

Different MOIs were tested for determining the best values of protein expression. Titers of viral stocks and resulting volumes for infection are shown in *table 3.10*.

Table 3.10 Viral Titers and volumes for infection

MOI		1	10	50	
surface of flask [cm2]	175	1,75E+07	1,75E+08	8,75E+08	
total cell number	1,75E+07				
NP [pfu / mL]	8,01E+08	21,85	218,5	1092,4	µL
PA [pfu / mL]	7,89E+08	22,18	221,8		µL
PB1 [pfu / mL]	7,79E+08	22,46	224,6	1123,2	µL
PB2 [pfu / mL]	7,44E+08	23,52	235,2		µL

3.3.2 Generation of cellular extracts:

For not destroying RNP complexes a very gentle method of generating cellular extracts was necessary. Two different methods were available:

A. The infected cells were harvested and washed twice with PBS buffer (*table 3.13*). For lysing the cellular pellet was resuspended in 1 mL of T/C buffer (0,1 M citric acid, 2 % Triton X 100) and incubated while shaking for 20 h at 27°C. Nuclei were collected by centrifugation at 2500 g for 10 min. The supernatant constituting the cytoplasm was removed and the nucleic pellet was washed with and resuspended in PBS buffer. For breaking the nuclear membrane, glass beads were added and the solution was vigorously vortexed for 10 times 1 min, with incubation on ice in between. Glass beads were removed and the nucleic and cytoplasmatic extracts were immediately used for further experiments or stored at -20°C.

B. The infected cells were harvested and washed twice with PBS buffer. The pellet was resuspended in 1 mL of buffer A (*table 3.11*) and incubated on ice for 15 min which leaded to a swelling of the cells. Lysing was performed by addition of 62,5 µL 10 % NP40 (v/v). Nuclei were immediately spun down at 16 000 g for 30 sec at 4°C and the supernatant contributing the cytoplasm was removed. The pellet was washed twice with PBS buffer to get rid of cytoplasmatic proteins and resupended in 200 – 1000 µL of buffer B (*table 3.12*), depending on the desired protein concentration. Nuclei were lysed for 20 min, while shaking, in an ice bath and nuclear debris was spun down for 15 min at 16 000 g at 4°C. Nucleic and cytoplasmatic extracts were immediately used for further experiments or stored at -20°C.

Table 3.11 Buffer A

Buffer A	
1 M Na.HEPES pH 7,9	500 µL
2,5 M KCL	200 µL
0,5 M EDTA pH 8,0	10 µL
0,2 M EGTA pH 8,0	25 µL
AD	49 mL

Table 3.12 Buffer B

Buffer B	
1 M Na.HEPES pH 7,9	300 µL
2,5 M KCL	2,4 mL
0,5 M EDTA pH 8,0	60 µL
0,2 M EGTA pH 8,	150 µL
50 % Glyerol	3 mL
AD	9 mL

DTT and PMSF were added to 1 mM final and 0,5 mM final respectively, immediately before use.

Table 3.13 PBS Buffer

10x PBS Buffer	
NaCl	80 g
KCl	2 g
Na_2HPO_4	14,4 g
KH_2PO_4	2,4 g
with AD up to 1000 mL	

Set pH to 6,5 using HCL

The harvesting of the infected cells and preparation of cellular extracts was performed at different hpis.

3.3.3 SDS – Page:

Sodium dodecyl sulphate polyacrylamide gel glectrophoresis (SDS-Page) is a standard method for protein analytics. In our case it was used for several purposes, like determination of best method for preparing cellular extracts.

Samples were mixed 1:2 with loading buffer containing SDS and . -mercaptoethanol according to *table 3.14*. SDS is an anionic detergent which denatures proteins and applies a negative charge to each protein in proportion to its mass. BME acts as reducing agent which further denatures the proteins by reducin g disulfide linkages, thus overcoming some forms of higher order protein folding.
Samples were heated up to 100°C in a wate r bath for 10 min and applied on a gel or stored at -20°C.

Table 3.14 SDS loading buffer

2x SDS loading dye	
SDS	1 g
Glycerin	2 mL
Bromphenolblue 0,1 %	2 mL
Tris 1M pH 6,8	1,25 mL
with AD up to 10 mL	

add BME 1:10 immediately before use

For preparing a gel, the gel-cassette was built up regarding to manufacturers instructions and rinsed with AD. A 10 % separating gel mix was prepared according to *table 3.15*, immediately poured into the cassette and covered with 500 µL isopropanol for getting a sharp boarder.

Table 3.15 Separating gel mix

10 % Separating gel	
AD	7,5 ml
Separating gel buffer *(table 3.16)*	3,75 mL
Acrylamide / Bisacrylamide 37,5:1	3,75 mL
SDS 20 %	75 µL
TEMED	20 µL
APS 20 %	50 µL

Table 3.16 Separating gel buffer

Separating gel buffer	
Tris Base	18,2 g
AD	upt to 500 mL
pH was set to 8,8 using HCL	

After polymerization of the separating gel, the isopropanol was removed and a 5 % stacking gel mix was prepared according to *table 3.17*, based on stacking gel premix (*table 3.18*) and stacking gel buffer (*table 3.19*).

Table 3.17 Stacking gel mix

Stacking gel	
Stacking gel PREmix	4 mL
TEMED	8 µL
APS 20 %	17 µL

Table 3.18 Stacking gel PREmix

5 % Stacking gel PREmix	
AD	24,2 ml
Stacking gel buffer	10 mL
Acrylamide / Bisacrylamide 37,5:1	5 mL
SDS 20 %	400 µL

Table 3.19 Stacking gel buffer

Stacking gel buffer	
Tris Base	30,5 g
AD	upt to 500 mL
pH was set to 6,8 using HCL	

The solution was immediately poured into the chamber and 15 slot combs were added. After polymerization, the gel-glass sandwich was removed form the pouring cassette and transferred to the electrophoresis device. Combs were removed and the device was filled up with 1x Laemmli buffer according to *table 3.20.*

Table 3.20 Laemmli Buffer

5x Laemmli Buffer	
Tris Base	15,1 g
Glycine	94 g
SDS 10 %	50 mL
AD	up tp 1000 mL

Samples and 5 µL of "Page Ruler Prestained Protein ladder" (Fermentas) were applied on the gel. The run was performed at 200 V, constant voltage, for 80 min.

3.3.4 Western-Blot:

The gels from SDS-Page were blotted on a PVDF membrane (8,5 x 7,5 cm) after activation in methanol, for being able to visualize NP protein. Gel, membrane and filter papers were incubated in Semi-Dry Transfer Buffer according to *table 3.21.* The components were assembled in a "Transplot SD Semi Dry Transfer Cell" (BioRAD) and blotting was performed at 170 mA for 50 min.

Table 3.21 Transfer Buffer

Semi-Dry Transfer Buffer	
Tris Base	3,03 g
Glycine	14,4 g
Methanol	200 mL
AD	up to 1000 mL

After blotting, the membrane was incubated in TPBS (PBS buffer, 0,01 % Tween 20) containing 3 % milk powder, for blocking unspecific binding sites and washed three times with TPBS. The membrane was further incubated for 1 h with the primary antibody "MAB anti-NP influenza A"(1:10000 in TPBS containing 1 % milk powder), which was kindly provided by Andrej Egorov. For getting rid of unbound antibody the membrane was again washed with TPBS. As secondary antibody "Anti-Mouse IgG Alkaline Phophatase Conjugate" (Sigma Aldrich) (1:1000 in TPBS containing 1 % milk powder) was applied to the membrane. After a final washing step, detection was carried out using CDP-Star reagent (NewEngland Biolabs) according to manufacturers' recommendation. Luminescence was detected in a Lumilmager™ (Boehringer Mannheim).

3.3.5 Silver staining:

Following electrophoresis and western blotting, the gel was stained, allowing visualization of the separated proteins. After staining, different proteins will appear as distinct bands within the gel.

Table 3.22 Fixation solution

Fixation solution	
EtOH	50 %
HAc	10 %
with AD up to 100 %	

Table 3.23 Incubation solution

Incubation solution	
$Na_2S_2O_3$	1 g
NaAc	34 g
EtOH	150 mL
with AD up to 500 mL	

Table 3.24 Silver nitrate

Silver nitrate	
$AgNO_3$	0,5 g
with AD up to 500 mL	

Table 3.25 Developing solution

Developing solution	
$NaCO_3$	12,5 g
with AD up to 500 mL	

Table 3.26 Stop solution

Stop solution	
NaEDTA	7,8 g
with AD up to 500 mL	

Therefore, the gel was removed form the device and incubated in fixation solution for at least 60 min. A 15 min incubation step in incubation solution, containing 62,5 µL glutaraldehylde / 25 mL, followed. After 15 min of washing with AD the gel was incubated in $AgNO_3$ solution, containing 5 µL formaldehyde / 25 mL. Excess $AgNO_3$ was washed away and the embedded silver was visualized by incubation in developing solution,containing 5 µL formaldehyde / 25 mL. A final washing step and incubation in stop solution completed the staining procedure. Different solutions were prepared according to *tables* 3.22 – 26.

3.3.6 Enzyme Linked Immunosorbent Assay:

The ELISA results will help us determining the best MOI / hpi combination for high production rates of RNP proteins and generation of RNP complexes.

For this purpose the wells of a 96-well plate were covered with 170 µL of coating buffer (0,1 N $NaHCO_3$-buffer pH 9,6-9,8) each. Cytoplasmatic and nucleic extracts (170 µL) were applied to wells in the first row and serial 1:2 dilutions were performed. 75 µL of the diluted samples were applied to a fresh 96- well coating plate, shaked for 1 h at RT and incubated over night at 4°C. This incubation step is necessary for adsorption of proteins to the surface of the wells. The plate was three times washed with TPBS and incubated with TPBS 1% BSA for blocking unspecific binding sites. The plate was again washed with TPBS and primary antibody was applied: "Monoclonal Antibody to Influenza A Nucleoprotein" (Acris Antibodies) 1:200 in TPBS 1 % BSA. The plate was incubated, while shaking, for 60 min and unbound antibody was washed away. As secondary antibody "Anit-Mouse IgG Peroxidase conjugate" (Sigma Aldrich) 1:1000 in TPBS, 1 % BSA, was used. A detection buffer was prepared containing 10 mL Peroxidase coloring buffer (Na_2HPO_4/citric acid buffer, pH5), 100 µL OPD (o – phenyl – diamine- dihydrochloride) and 10 µL Peroxidase. 100 µL each were applied to the washed and empty wells. OPD is cut by the enzyme and a color reaction becomes visible. The reaction was stopped by addition of 100 µL H_2SO_4 and the plate was analyzed using "Sunrise 12-channel microplate absorbance reader for 96-well plates" (Tecan).

3.3.7 Blue native page:

In standard SDS-PAGE, the charge-shift molecule is SDS. The SDS denatures proteins and binds to them conferring a net negative charge, allowing the proteins to migrate in one direction towards the anode. In BN PAGE, developed by Schägger et al., the Coomassie® G-250 binds to proteins and confers a net negative charge while maintaining the proteins in their native state without any protein denaturation. The G-250 is present in the cathode buffer to provide a continuous flow of Coomassie® G-250 into the gel, and is added to samples containing non-ionic detergent prior to loading the samples onto the gel. The gels do not contain any G-250.
The binding of Coomassie® G-250 to proteins has an advantage that was of great benefit for our work: RNP proteins have basic isoelectric points (pI) which results in a net positive charge in normal laemmli gel systems. In BN-page proteins are covered with a net negative charge, allowing them to migrate towards the anode.

We based our Blue Native page experiment on a commercially available 4-20 % Novex® Tris – Glycine gel (Invitrogen). Electrophoresis was preformed according to manufacturers' recommendation. The cytoplasmatic and nucleic extracts form *chapter 3.3.2 (B)* were mixed 1:2 with Tris-Glycine Native Sample buffer, incubated at RT for 15 min and applied on the gel. Buffers were prepared according to *table 3.27 /3.28.*

Table 3.27 Native gel sample buffer

2x Tris-Glycine Native Sample buffer	
0,5M Tris HCl, pH 8,6	400 µL
Glycerol	200 µL
0,1 % (w/v) Coomassie G-250)	50 µL
AD	350 µL

Table 3.28 Native gel running buffer

10xTris-Glycine Native Running buffer	
Tris Base	5,8 g
Glycine	28,8 g
AD	up to 200 mL

Right before starting the run, 200 µL Coomassie® G-250 were added to the cathode buffer. The run was performed at 125 V, constant voltage, for 4 h.

After finishing the run, the gel was incubated in 0,1 % SDS for 15 min for giving the proteins enough negative charge for blotting.

A PVDF membrane (Roth) was cut 8,5 x 7,5 cm and activated by incubation in methanol. The blotting experiment for Novex gels was performed regarding to manufacturers recommendation. Blotting was accomplished at 25 V, constant voltage, for 90 min using 1x Tris-Glycine Transfer buffer containing 20 % methanol according to *table 3.29*.

Table 3.29 Transfer Buffer + MetOH

1x Transfer buffer 20 % MetOH	
25x Transfer buffer	100 mL
Methanol	20 mL
AD	380 mL

Table 3.30 Native Transfer Buffer

25x Tris-Glycine Transfer buffer	
Tris Base	1,82 g
Glycine	9 g
AD	up to 50 mL

The blocking and detection was performed as described in *chapter 3.3.4.*

3.3.8 Ultracentrifugation:

Ultracentrifugation is a useful method for pelleting large macromolecular complexes. So it seemed to be the ideal method for separating RNP complexes form other nuclear proteins. Therefore, pH 7 - 4 buffered, glycerol step gradients (10 – 40 %) were prepared in centrifugation buckets by freezing the different phases at – 80°C, before applying a new gradient step. The nuclear extracts form *chapter 3.3.2 (B)* were applied on the gradient and centrifugation was preformed in a SW41 Ti rotor at 35 000 rpm for 3 h using a Beckman L8-80M ultracentrifuge.

Different fractions were collected and the pellet was resuspended in 200 µL of PBS buffer. Fractions and dissolved pellet were subsequently mixed 1:2 with SDS-Page loading dye (*table 3.14*) and denatured by boiling for 10 min. Samples were loaded on a SDS gel and blotted as described in chapters *3.3.3 SDS-Page* and *3.3.4 Western Blot*. As primary antibody we used "MAB anti-NP influenza A" (1:10 000) and as secondary antibody "Anti Mouse IgG Alkaline Phosphatase Conjugate" (1:1000). Detection was carried out by CDP-Star.

3.4 Functionality testing:

3.4.1 Infection:

Sf9 cells were passaged the day before and seeded in a T175 roux flask with a density of 1 x 10^5 cells / cm^2. The medium was supplement ed with 1x antibiotic mix for avoiding bacterial contaminations. Infections were performed with baculovirus expressing RNP-proteins (NP, PA, PB1, PB2) with an MOI of 10 and with baculovirus delivering "-" sense single-stranded RNA (MOI 10) which were kindly provided by Theresa Schinko (*figure 3.2*). The cells were checked for GFP expression 24, 48 and 72 h post infection using a UV microscope.

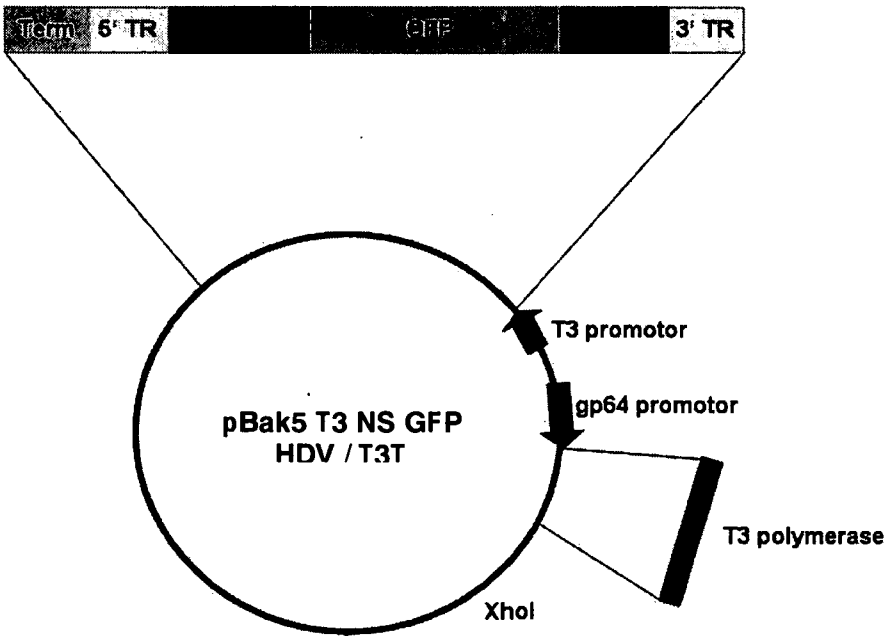

Figure 3.2 Map of transfer vectors of RNA deliveri ng baculovirus: pBak5 T3 NS GFP HDV, carrying a HDV ribozyme, and pBak5 T3 NS GFP T3T, carrying the T3 terminator, for the termination of T3 polymerase.

3.4.2 In vitro transcription:

For being able to confirm the functionality of influenza A polymerases PA, PB1 and PB2 an in vitro transcribed RNA, coding for GFP, was generated. The transcription was preformed using "AmpliScribe™ T3 High Yield Transcription Kit" (EPICENTRE Biotechnologies) according to manufacturers ' recommendation . As templates for transcription we used pBak5 T3 NS GFP HDV and pBak5 T3 NS GFP T3T vectors (*figure 3.2*) linearized with XhoI, which only differ in their termination sequence for T3 polymerase. For really being sure to get correctly terminated RNAs we decided to use a PCR fragment carrying the T3 promoter and the desired sequence as well. This was produced by KOD Hifi DNA polymerase according to *chapter 3.1.1 Polymerase Chain Reaction* using Bak5 T3 NS GFP HDV vector as template and T3-Prom-GFP-back (carrying the T3 promotor sequence) and NS1gfpNep-back primers. The in vitro transcription mix was prepared according to *table 3.31*.

Table 3.31 Reaction mix for in vitro transcription

AmpliScribe T3 Transcription Mix	
Linearized template DNA carrying T3 promoter	1 µg
10x Ampli Scribe T3 Reaction Buffer	2 µL
100 mM ATP	1,5 µL
100 mM CTP	1,5 µL
100 mM GTP	1,5 µL
100 mM UTP	1,5 µL
100 mM DTT	2 µL
AmpliScribe T3 Enzyme Solution	2 µL
Rnase-Free water	up to 20 µL

The solution was incubated at 37°C for 2 h and DNA was removed by addition of 1 µL (1 MBU) of RNase-Free DNase to the standard 20 µL AmpliScribe transcription reaction. RNA transcripts were purified by ammonium acetate precipitation. This method selectively precipitates RNA while leaving much of the DNA, protein and un-incorporated NTPs in the supernatant.

1 volume of 5 M ammonium acetate was added to the solution and incubated for 15 min on ice. The RNA was pelleted by centrifugation at 12 000 g for 15 min at 4°C and further washed with 70 % ethanol. The pellet was resuspended in RNase-Free water and stored at -80°C.

3.4.3 Transfection of infected Sf9 cells:

Sf9 cells were passaged the day before and seeded in a 6-well plate with a density of 1 x 10^5 cells / cm^2. The cells were infected with baculovirus delivering RNP-proteins with MOI 1, 2 and 10. The transfection was carried out 4 h and 24 h post infection using FlyFectin™ (OZBiosciences). Two mixtures were prepared: Mixture A containing 8 µL FlyFectin™ and 22 µl IP-L 41 and Mixture B containing 4 µg of in vitro transcribed RNA up to 30 µL with IP-L 41. We used RNA generated form templates carrying the T3 and HDV terminator as well as RNA generated from PCR product, just to test the effects of different terminations. Mixture A and B were combined, mixed by pipetting up and down and incubated for 20 min at RT. In the meantime cells were washed twice and finally covered with 1 mL IP-L 41. The transfection mixture was filled up to 500 µL with serum free media and applied to the cells. After an incubation of 4 h, media was replaced by IP-L 41 containing 3 % FCS and 1x antibiotic mix. Cells were checked for GFP expression 24 and 48 h after transfection.

3.4.4 Infection and transfection of MDCK cells:

For verifying the in vitro transcribed RNA, MDCK cells, which were kindly provided by Helga Fekete, were infected with an influenza A virus and transfected with RNA. Therefore, MDCK were seeded in a 6-well plate to nearly 40 % confluence the day before infection and transfection. Cells were washed twice with PBS buffer and infected with 200 µl of influenza A strain PuertoRico/8/34 for 30 min, while gentle shaking. Virus supernatant was filled up to 2 mL with DMEM / HAM's F12 (1:1, Biochrom). 4 hpi cells were washed twice with PBS buffer and covered with 2 mL fresh media. Two transfection mixtures were prepared according to manufacturers' recommendation: Mixture A containing 4 µg of in vitro transcribed RNA / 4 µg of positive control plasmid (pAcGFP, which was kindly provided by Simon Itig) diluted to 100 µL media and mixture B containing 16 µL DreamFect Gold (OZBioschiences) diluted to 100 µL with media. Both solutions were combined, mixed by pipetting and incubated for 20 min. The transfection mixtures were applied to the cells and further incubated at 27°C. Cells were checked for GFP expression 24 and 48 h post transfection.

3.4.5 Infection and magnetofection of MDCK cells:

For increasing transfection efficiency, another method called Magnetofection™ (OZBiosciences) was used. It exploits magnetic force exerted upon gene vectors associated with magnetic particles to drive the vectors towards, possibly even into, the target cells. In this manner, the complete applied vector dose gets concentrated on the cells within a few minutes so that 100% of the cells get in contact with a significant vector dose (67).

Therefore, MDCK cells were seeded to 70 % confluence the day before in a 6-well plate and infected with the influenza A strain PuertoRico/8/34. Magnetofection was carried out using DreamFect Gold and CombiMag (OZBiosciences) which is a magnetic particle preparation designed to be combined with any commercially available transfection reagent such as cationic polymers and lipids. CombiMag has been used successfully with plasmid DNA, antisense oligonucleotides , mRNA and siRNAs (67). Transfection reagent – RNA / DNA complexes were prepared as described in *chapter 3.4.4 Infection and Transfection of MDCK cells,* and immediately mixed with 8 µL of CombiMag particle solution by pipetting up and down several times. After an incubation of 20 min, the magnetofection mix was applied to the cells and plates were incubated for 15 min on a magnetic plate. Supernatants were finally replaced by 2 mL of fresh media and cells were checked for GFP expression 24, 48 h post transfection.

3.4.6 RNA-Isolation:

To examine the transfection efficiency of RNA transfected cells, RNA was isolated using Trizol LS (Invitrogen).

Transfected cells form chapter 3.4.5 *Infection and Magnetofection of MDCK cells* were washed twice with PBS buffer and excess RNA was removed by RNase treatment. Cells were therefore incubated for 1h at 37°C with a 1:100 dilution of RNase A and again washed with PBS buffer. For replacing cells, they were treated with 200 µL of trypsin (1 mg/mL) and transferred to a Sarstedt tube. Cells were washed with PBS buffer and resuspended in 250 µL of PBS. Addition of 750 µL Trizol LS to each sample followed by 5 min incubation at RT lysed the cells. 200 µL chloroform were further added and samples were incubated for 15 min at RT. For separating phases, tubes were centrifuged at 12 000 g at 4°C. Around 700 µL of the upper (aqueous) phase were removed and subsequently mixed with 500 µL

isopropanol. For precipitating RNA, tubes were incubated at RT and centrifuged for 10 min at 12 000 g and 4°C. The RNA pellet was washed with 70 % ethanol and further proceeded to DNase digestion. This step was necessary to remove excess DNA, which would falsify reverse transcription.

A mix containing 2 µL DNaseI (Fermentas), 5 µL 10x DNaseI buffer (Fermentas) and 43 µL RNase-Free water was prepared, in which the RNA pellet was dissolved. After an incubation of 60 min at 37°C 5 µL of 25 mM EDTA were added and the digest was heated up to 65°C for 10 min. This step leads to an inactivation of DNaseI. EDTA stabilizes RNA at high temperatures.

3.4.7 Reverse Transcription:

Reverse transcription leads to formation of first strand cDNA from isolated RNA. We based our experiments on SuperScript ™ III Rverese Transcriptase Kit (Invitrogen).
8 µL of DNase digested RNA were mixed with 1 µL of 10 mM dNTP mix and 1 µL of NS1gfpNEP-back primer. The solution was incubated for 5 min at 65°C and further chilled on ice for primer annealing → annealing mix. A cDNA synthesis mix was prepared according to *table 3.32*.

Table 3.32 Mix for cDNA syntesis

cDNA synthesis mix	
10x Reverse Transcription Buffer	2 µL
25 mM MgCl2	4 µL
0,1 M DTT	2 µL
RNaseOUT	1 µL
SuperScript III RT	0,2 µL
AD	0,8 µL

The cDNA synthesis mix and the annealing mix were combined and incubated for 50 min at 50°C. Inactivation was performed by heating up to 85°C for 10 min followed by chilling on ice. To get rid of RNA, 1 µL RNase H was added and incubated at 37°C for 20 min.
For synthesizing the second DNA strand, a PCR, using Biotools DNA polymerase according to *chapter 3.1.1 Polymerase chain Reaction,* was performed. As primers NS1gfpNEP-for and NS1gfpNEP-back were used.

3.4.8 Nucleofection of CHO cells:

For getting better transfecti on efficiencies another experi ment was designed. Due to the fact that transfection efficiencies in MDCK cells are quite low, we decided to transfect CHO cells which normally show much higher rates, although virus replication is suboptimal.

CHO-K1 cells, kindly provided by Simon Itig, were counted and 5×10^5 cells were centrifuged at 100 g for 8 min. The supernatant was removed and cells were resuspended in Nucleofector Solution (amaxa) containing 82 µL Cell Line Nucleofector Solution V, 18 µL Supplement and 22 µg of in vitro transcribed RNA / 6 µg of positive control (pAcGFP). The nucleofection mix was transferred to an electroporation cuvette and a pulse was applied using program U-24 (Nucleofector ™ I, amaxa). Cells were immediately transferred in a prewarmed 6 well plate containing 2 mL of CHO media, supplemented with 10 % FCS, and incubated for 4 h at 37°C. The high amount of FCS caused the cells to settle down and stick to the surface of the well.

For infection, cells were washed carefully with PBS and infected with the influenza A strain PuertoRico/8/34 for 30 min. Virus supernatant was removed and cells were further incubated in CHO media containing 3 % FCS at 37°C. Cells were checked for GFP expression 24 and 48 h after infection.

4. Results:

4.1 Generation of recombinant Baculovirus:

After cloning and transformation of E.coli DH10B, cells were striked out on LB agar plates containing ampicillin. The antibiotic pressure led to a selection of positive clones, because only those carrying the desired plasmid (pBacPAK 8 – NP / PA / PB1 / PB2) were resistant to ampicillin. Around 20 colonies each were observed and 16 were picked for PCR screening. From positive clones plasmid DNA was isolated and a restriction digest, using different enzyme combinations, was performed for confirming positive clones.

One of each construct was chosen for sequencing. This showed that there are no mutations, compared to template sequences. But after comparison with original PuertoRico/8/34 sequences derived from NCBI database, we indeed saw that there are point mutations. Checking amino acids showed that mutations are silent and do almost not affect protein sequence.

After confirming the correct sequence, constructs were co-transfected in Sf9 cells for generating recombinant baculovirus. 5 days after transfection almost no CPE was observable. For purifying virus clones, a plaque assay was performed and 4 plaques were picked each construct. The presence of well separated plaques showed that co-transfection worked and recombinant virus was produced. Viral clones were further amplified for getting high titers, which were determined by another plaque assay. We finally got titers for our working stocks in the range of $7 - 8 \times 10^8$ pfu / mL, which was a good value for further infection experiments (detailed results are shown in *table 3.10*).

4.2 Generation of cellular extracts from infected Sf9 cells:

Sf9 cells were infected with working stocks of baculovirus coding for RNP-proteins. 40 h post infection cells were harvested and cytoplasmatic / nucleic extracts were prepared using two different methods. Extracts were immediately applied on a SDS-gel and blotted on a PVDF membrane. For visualizing total protein a silver stain was performed.

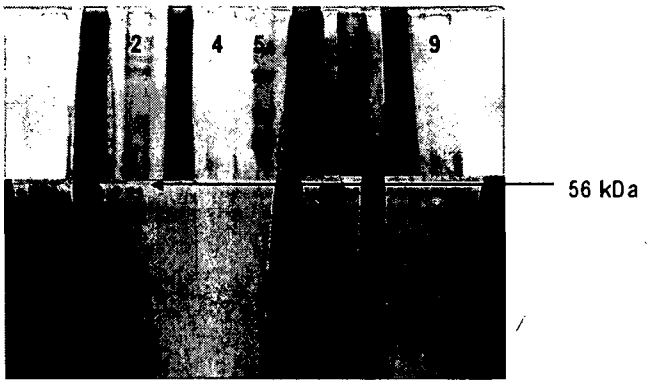

***Figure** 4.1 Silver stain of cytoplasmatic and nuclear extracts. Lanes 1 and 2 represent the cytoplasmatic and nuclear extracts prepared by method B regarding to chapter 3.3.2 Generation of cellular extracts. Lanes 3 and 4 represent extracts prepared by method A. Lane 5 contains PageRuler pre Stained Protein Ladder as standard. Lanes 6-9 represent the same samples as 1-4, but in different amounts loaded on the gel.*

Figure 4.1 shows the silver stain of cellular extracts. It can clearly be seen that preparation of cytoplasmatic extracts (lanes 1,3) worked fine using both methods. A lot of protein is visible along the whole spectrum of size.

Preparation of nucleic extracts using method A (lane 4) did not give the desired result. Almost no protein is visible, indicating that the breaking of nuclear membrane, using glass beads, was not ideal for preparation of nucleic extracts. However, nucleic extracts produced by method B (lane 2) worked fine, because a lot of protein is visible in the stain. At a height of 56 kDa a strong band is visible. For verifying this

band to be influenza A nucleoprotein a western blot was performed using "Avian Flu NP antibody" as primary antibody and "Anti Mouse IgG Alkaline Phosphatase Conjugate" as secondary. Detection was carried out by CDP-Star. The blot did not show any binding of antibody, because of wrong antibody formulation.

However, for further experiments method B was used for preparing cellular extracts.

4.4 Influence of MOI and hpi on NP expression and localization:

For testing the influence of MOI and hpi on the expression and localization of NP protein, an ELISA was performed. Sf9 cells were infected with different MOIs and cellular extracts were prepared at different times after infection. ELISA was carried out using "Monoclonal Antibody to Influenza A Nucleoprotein" as primary and "Anti Mouse IgG Peroxidase Conjugate" as secondary antibody.

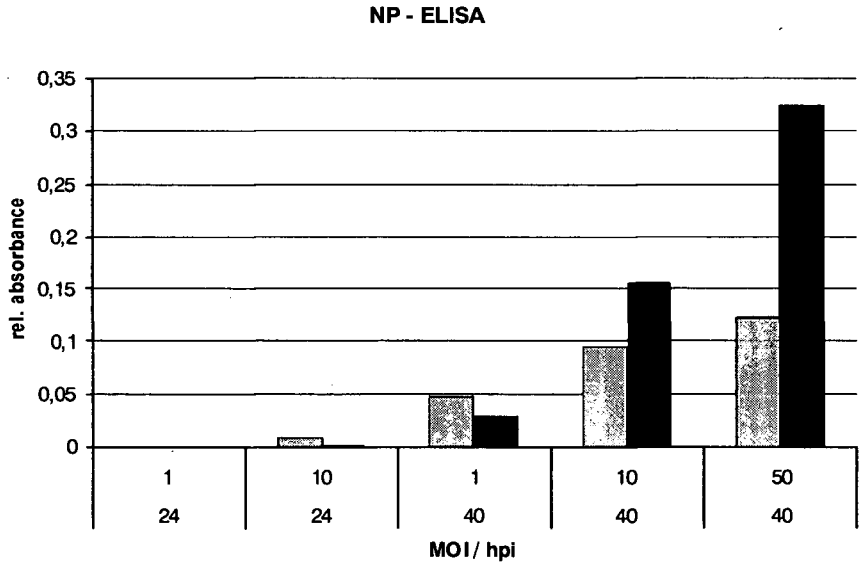

Figure 4.2 ELISA of cytoplasmatic and nuclear extracts prepared from infected Sf9 cells 24 and 40 h post infection. Cells were infected with virus delivering RNP proteins at different MOIs. As primary antibody "Monoclonal Antibody to Influenza A Nucleoprotein" and as secondary "Anti-Mouse IgG Peroxidase Conjugate" were used.

Figure 4.2 shows that expression of nucleoprote in becomes slightly active 24 hours post infection. This goes side by side with results described in literature. Polyhedrin promoter becomes active 24 hpi and shows full activity 48 hpi. The extremely low absorption values belong to a poor binding of primary antibody.

However, 40 hpi an increase in protein expression, using different MOIs, was detectable.

MOI plays an important role because at MOI 1 more NP is located in the cytoplasm. Raising the MOI to 10 or even 50 causes a significant increase of NP in the nucleus. So, for not overburdening cells, we decided to use an MOI of 10 for further experiments, because protein production is much higher compared to MOI 1 and NP localization is more comfortable for RNP formation.

4.5 Blue Native Page:

For being able to visualize a RNP complex, a blue native page was performed. RNP complexes can be formed without the presence of RNA due to direct interactions between RNP proteins, as described in *chapter 1.3.* The size is at least 300 kDa resulting from its components: NP ~ 56 kDa; PA, PB1, PB2 ~ 80 kDa.

For performing the Blue Native Page, Sf9 cells were on the one hand infected with baculovirus coding for RNP proteins and on the other hand just with baculovirus coding for NP. If a complex is formed, composed of all its components, the size should be much lager compared to complexes just formed by NP.

Infected cells were harvested 40 hpi and nuclear extracts were prepared. The BN page was performed according to Schägger et. al and immediately blotted on a PVDF membrane. The gel was finally silver stained.

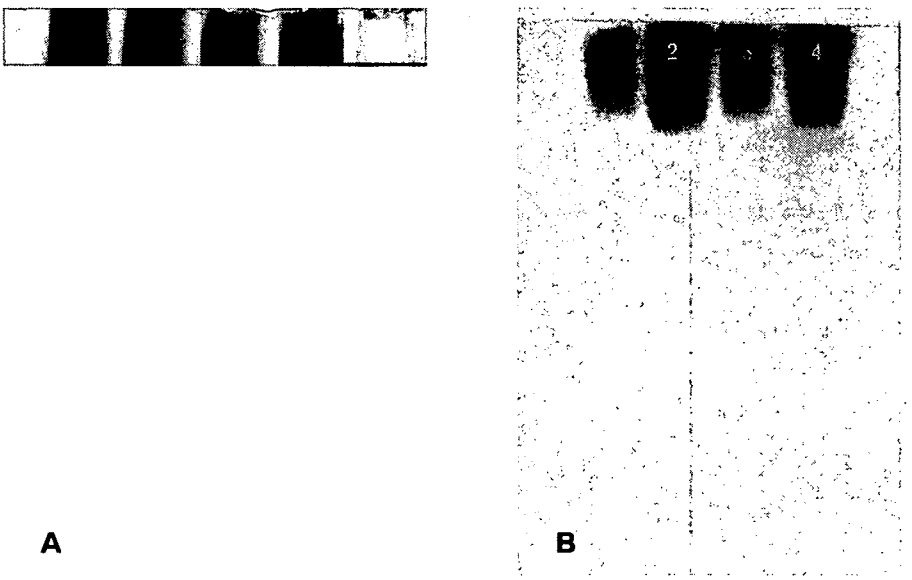

Figure 4.3 [A] *Silver stain of Blue Native Page. Cytoplasmatic and nuclear extracts from cells infected with RNP virus (lanes 1, 3) show equal amounts of protein. Extracts of cells just infected with NP virus too (lanes 2, 4). For determining the size of bands Page Ruler Pre Stained Protein Ladder was loaded (lane 5).*

Figure 4.3 [B] *Western blot of the gel. As primary antibody "MAB anti-NP influenza A" and as secondary "Anti-Mouse IgG Alkaline Phosphatase Conjugate" were used. Detection was carried out by CDP-Star.*

Looking at silver stain (*Figure 4.3 [A]*), cytoplasmatic extracts of RNP / NP infected cells show that total amount of protein and separation patterns are the same (lanes 1,2). However, western blot (*Figure 4.3 [B]*) gives a different result. The cytoplasmatic extract from NP infected cells (lane 2) shows a smear in the upper region of the blot, which indicates that influenza A nucleoprotein forms oligomers of different size, even in the absence of RNA. The cytoplasmatic extract from RNP infected cells (lane 1) gives almost no detection of nucleoprotein . A reason could be that RNP complexes are too large for being separated in a polyacrylamide gel, due to their size of at least 300 kDa.

Nuclear extracts from NP as well as RNP infected cells (lanes 3, 4 respectively) show the same results. There are equal amounts of total protein detectable in silver stain, but western blot gives a signal with NP infected cells and almost none from RNP infected cells.

Blue Native Page demonstrated that RNP complexes are indeed formed in nucleus as well as in cytoplasm.

4.6 Ultracentrifugation:

For purifying RNP complexes, nuclear extracts from RNP infected cells were centrifuged over a pH 7 – 4 buffered glycerol step gradient (10 – 40 %). Undergoing pH 5,5 RNP complexes should precipitate and only be found in the pellet whereas other proteins should stay in the fractions above.

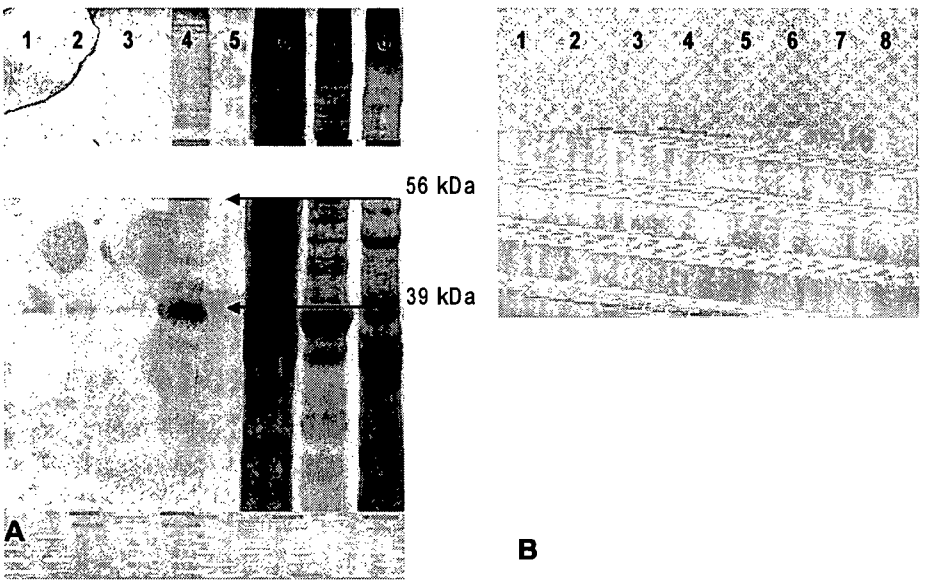

Figure 4.5 [A] *Silver stain of ultracentrifu ged nuclear extracts from RNP infected cells (MOI 10, 40 hpi). Lanes 1-3 contain different fractions of centrifuged supernatant, whereas resuspended pellet is visible in lane 4. Lane 6 contains a positive control for western blot (infected sf9 cells, directly mixed with loading buffer and boiled). Negative controls for ultracentrifug ation (not centrifuged cytoplasmatic and nuclear extracts) are visible in lanes 8, 7 respectively.*

Figure 4.5 [B] *shows western blot of the gel. As primary antibody "MAB anti-NP influenza A" and as secondary "Anti-Mouse IgG Alkaline Phosphatase Conjugate" were used. Detection was carried out by CDP-Star.*

Figure 4.5 shows silver stain [A] and western blot [B] of various fractions as well as resuspended pellet. Looking at the pellet fraction in silver stain (lane 4), a distinct band with a size of 56 kDa is visible and background is much lower compared to negative control (lane 7; nucle ar extracts before ultracent rifugation). Western blot, using "MAB anti-NP influenza A" as primary and "Anti Mouse IgG Alkaline

Phosphatase Conjugate" as secondary antibody verifies this band to be influenza A nucleoprotein. So pelleting of RNP complexes was successful, although purity is not very high, because a lot of other proteins were pelleted too. Especially one with a size of 39 kDa was found in large amounts in the pellet fraction. We think this protein to be baculovirus major coat protein p39, which has a size of 39 kDa. p39 forms, due to its function as coat protein, large macromolecular structures which were pelleted together with RNP complexes.

For verifying the successful pelleting of RNP complexes, nuclear extracts from Sf9 cells infected only with NP virus and non infected Sf9 cells were prepared.

Ultracentrifugation was preformed the same way as described above and fractions as well as resupended pellet were immediately analyzed by SDS page.

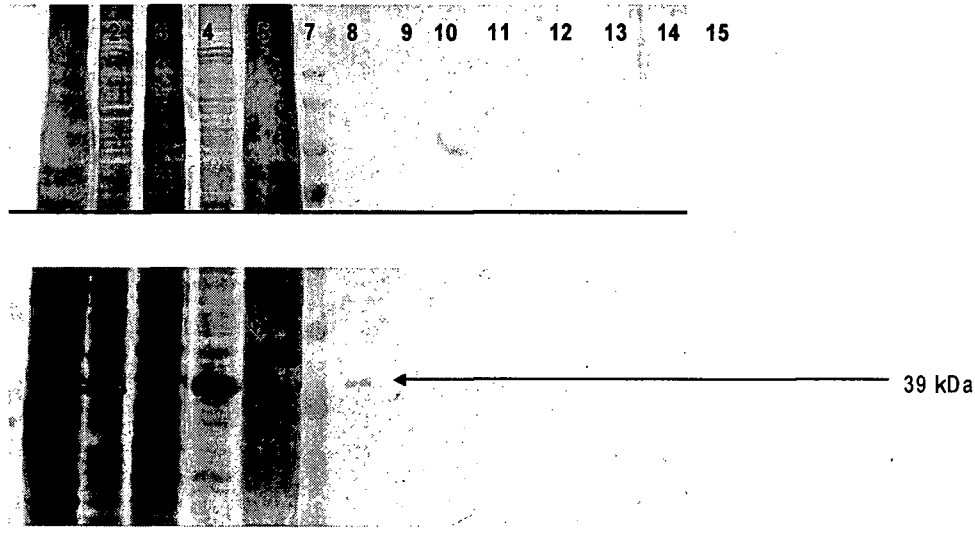

***Figure 4.6** Silver stain of different fractions after ultracentrifugation of negative control samples. Extracts were derived from non infected Sf9 cells or cells just infected with NP virus (MOI 10, 40 hpi). Lanes 1, 3 contain cytoplasmatic extracts of none / infected cells before centrifugation. Lanes 2 and 4 show nuclear extracts. Centrifugation of nuclear extracts from NP infected cells does not give any pelleting (lanes 8 -11). The same result is observable after centrifugation of non infected cells (lanes 12 -15)*

Looking at centrifugatio n resu lts of NP infected cells (*figure 4.6*, lanes 8 - 11), no pelleting of protein is visible in silver stain. Just a very weak band in pellet fraction (lane 8) at a size of 39 kDa, is detectable. This confers to p39 coat protein. From none infected cells (lanes 12 -15), no pelleting was observable. This demonstrates that pelleting of RNP complexes using ultracentrifugat ion indeed worked.

4.7 Infection of Sf9 cells with virus coding for RNP proteins and "–"sense single stranded RNA:

The goal was to show functionality of recombinantly expressed influenza A RNP complexes in Sf9 cells. Cells were therefore infected with baculovirus expressing RNP proteins together with virus delivering "–"sense single stranded RNA (different MOIs were used). If polymerases are functional, "-"sense RNA is be transcribed to mRNA leading to expression of a reporter gene. The "–"stranded RNA codes for a fusion protein of influenza A NS noncoding regions and GFP. Cells were checked 24, 48 and 72 hours post infection. Starting 24 hpi a CPE was detectable, but there was no GFP expression. Checking cells at la ter time points did not give any positive result.

4.8 Infection and Transfection of Sf9 cells with in vitro transcribed RNA:

Another way for testing the functionality of influenza polymeras es in a Sf9 system was to infect cells with baculovirus coding for RNP proteins and transfecting cells at different hpis with different amounts of in vitro transcribed RNA. As positive control for transfection, a pBacPAK8 vector with GFP under control of the polyhedrin promoter was used. Transfection was carried out using Cellfectin ™. Cells were checked for GFP expression 24, 48 and 72 hours post transfection, but no green fluorescence was detectable, not even in positive control.

4.9 Infection and Transfection of MDCK cells:

To examine the correctness of in vitro transcribed RNA, MDCK cells were infected with influenza A strain PuertoRico/8/34 and transfected with in vitro transcribed RNA using DreamFect Gold. To enhance transfection efficiency Magnetofection was performed. As control for transfection, pAcGFP vector was used.

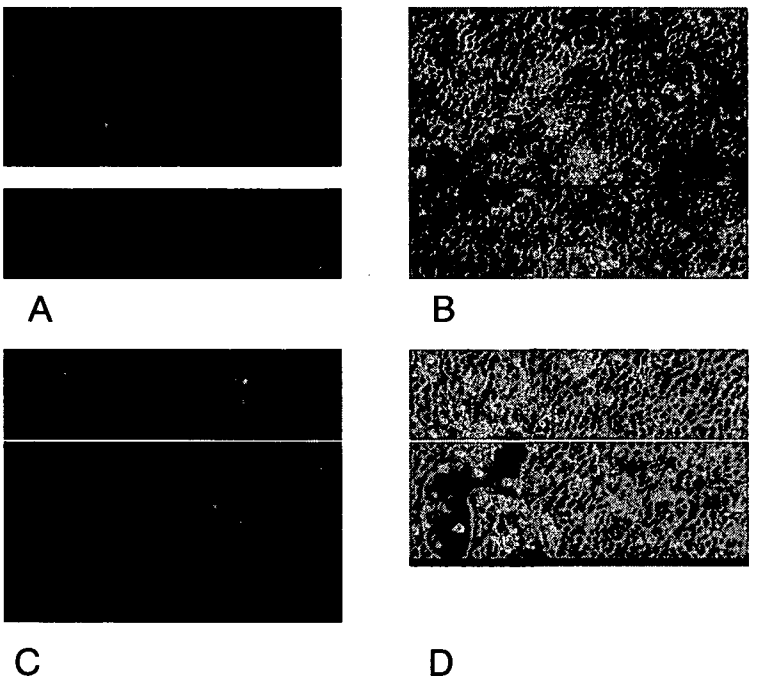

Figure 4.7 *Results of pAcGFP transfected MDCK cells. Transfection using DremFect Gold gave a positive result 24 hours post transfection (A, B). Using Magnetofection, efficiency was improved 4-5 fold (C, D).*

Cells transfected with positive control using DreamFect Gold showed GFP expression 24 hours post transfection (*Figure 4, 7 A*). Compared to total cell number (*Figure 4.7 B*) we came to an efficiency of 5 - 10 %, which corresponds to manufacturers' data. Transfection efficiency was greatly improved to 40 % positive transfected cells using Magnetofection ™ (*Figure 4.7 C*)

However, transfection of in vitro transcribed RNA (generated from templates carrying the HDV and T3 terminator, as well as PCR generated templates) did not give any positive results using DreamFect Gold, nor using Magnetofection ™.

4. 10 RNA isolation and Reveres Transcription:

RNA was isolated from cells infected with influenza A strain PuertoRico/8/34 and transfected with in vitro transcribed RNA using Magnetofection ™. For getting rid of excess RNA in supernatant, cells were treated with RNase A. cDNA was produced and screened for the presence of NS1gfpNEP fusion gene.

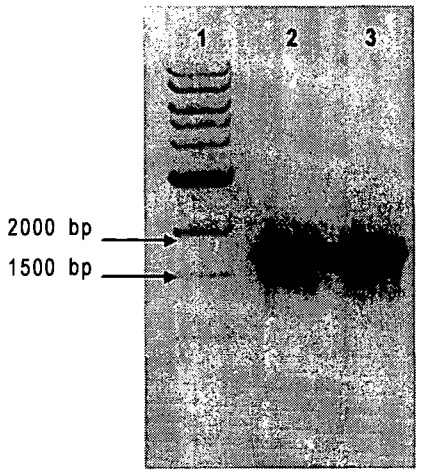

Figure 4.8 *Results of RNA isolation and in vitro transcription experiments. Lane 1 shows 1kb ladder for being able to determine the length of PCR fragment. Lanes 2 and 3 show the results of Reverse Transcription using different amounts of cDNA as template for final PCR (1 / 2 µL). Desired fragments with a length of 1700 bp are visible.*

After completing of second strand by Biotools PCR, bands were visible in a height of 1700 bp (*Figure 4.8*) which suits to our desired insert. This indicates that transfection of in vitro transcribed RNA indeed worked.

4.11Nucleofection and infection of CHO cells:

As final experiment we decided to transfect CHO cells with in vitro transcribed RNA and infect them with influenza A PuertoRico /8/34. Nucleofection of CHO cells is known to generate high transfection efficiencies. Cells were transfected with different amounts of RNA / pAcGFP as positive control. For attaching cells to the surface of the wells, they were incubated with 10 % FCS and infected with virus 4 hours post transfection.

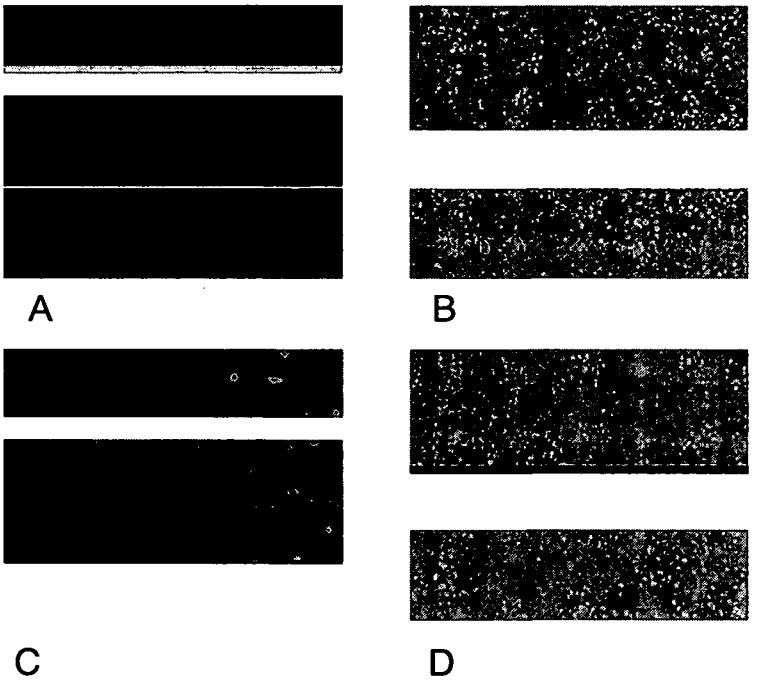

***Figure** 4.9 CHO cells transfected with 20 µg in vitro transcribed RNA / 4 µg pAcGFP as control using nucleofection. Cells were infected with influenza PR/8/34 4 hours post transfection. Transfection with in vitro transcribed RNA, monitored 24 hours post transfection, showed a positive result. 4 GFP expressing clones were visible (A). Transfection efficiency in CHO cells using nucleofection was around 90 % (B).*

Nucleofection of 20 µg in vitro transcribed RNA, followed by infection showed 4 GFP expressing cells monitored 24 hours post transfected (*Figure 4.9 A*). Positive cells were transfected with RNA generated from template carrying the HDV terminator. This indicates that RNA is correct, but efficiency is very low.

5. Discussion:

The goal of this thesis was to recombinant ly express influenza A RNP proteins in an insect cell system, using baculovirus for gene delivery. We further wanted to show that these proteins built a functional RNP complex, which can be used for in vitro and / or in vivo gene delivery as well as fo r the production of recombinant influenza virus. Another possible application is the use of RNP complexes, due to the presence of double stranded RNA, as an adjuvant for increasing the immunogenicity of inactivated influenza vaccine, or even as basis for novel vaccine design.

5.1 Generation and characterization of RNP complexes:

Subsequently after infection, baculoviral DNA moves to the nucleus where replication takes place. RNP proteins, under control of the late polyhedrin promoter, get expressed starting 16 – 24 hpi . Due to their nuclear localization signals, proteins move back to the nucleus where they accumulate and RNP complexes are built up.

For being able to check infected Sf9 cells for RNP production, the preparation of nuclear extracts was indispensable. Due to the fact that RNP complexes must not be denatured for analyzing them in their native states, we decided to use 2 very gentle methods and compared their efficiencies. The results are shown *figure 4.1.* Breaking of cellular membrane worked well using both methods, whereas preparing nuclear extracts was only successful using method B.

The next step was the establishment of MOI / hpi combinations ideal for RNP formation in the nucleus, which was monito red by performing an ELISA of cellular extracts. The results are shown in *figure 4.2* . It can clearly be seen that MOI as well as hpi play a critical role for yielding high protein expression and nuclear localization. Transgene expression gives detectable yields 24 hpi, but significantly increases 40 hpi. The higher the MOI, the higher the amount of NP and the more NP is located in the nucleus. The nuclear enrichment of RNP proteins is due to missing of M1 and NEP protein, which causes newly, built complexes to leave the nucleus. So, infecting Sf9 cells with an MOI of 10 and preparing cellular extracts 40 hpi seemed to be best for further experiments.

For visualizing RNP complexes, a Blue Native Page was performed, whose results are shown in *figure 4.3 [A] and [B].* Although total amount of protein seems to be the same in extracts of NP and RNP infected cells *[A]*, Western Blot *[B]* from the former shows NP located at various sizes on top of the blot. This confirms the presence of NP-NP interactions and the formation of NP oligomers in vitro. RNP extracts, however, show significantly lower amounts of NP on top of the blot, leading to the assumption that built RNP complexes are too large for being visualized in their native state in a gel. It further assumes that the trimeric polymerase complexes directly bind to NP oligomers and that complexes are formed even in the absence of viral RNA. NP, that is detected anyway, could be derived from denaturing processes. Finally, the results verify the assumption that RNP complexes are indeed formed.

Based on former results and work done by Olga P. Zoueva et al, we tried to separate RNP complexes using ultracentrifugation. Results are shown in *figure 4.5 [A], [B]* and in *figure 4.6.* Pelleting of RNP complexes seemd to be successful due to the presence of a distinct band of 56 kDa, constituting NP, which was confirmed by western blot. The missing of antibodies against influenza A polymerase proteins, hindered us to show the presence of PA, PB1 and PB2 in the pellet fraction. The affirmation that pelleting worked anyway was achieved by centrifuging extracts from just NP or non infected cells. Results from the former show that molecular weigh of NP oligomers is too low for pelleting, because no bands were visible. Just a very weak one at 39 kDa, present in RNP preparations as well, was detectable. Due to the fact that this band is missing in extracts of non infected cells, we suggested it to be the baculoviral major capsid protein VP39.

Data, obtained in former experiments show that recombinantly expressed RNP complexes can be pelleted by ultracentrifugation over a pH 7-4 buffered glycerol step gradient, but purity is not very high as indicated in *figure 4.5 [A] lane 4*. A lot of other protein is present in pellet fraction, which may get carried along with RNP and / or VP39 complexes.
So, ultracentrifugation is not the most sufficient method for purifying recombinantly expressed RNP proteins from Sf9 cell nuclei. Another method would be to generated tagged RNP proteins and isolate them using affinity chromatography.

5.2 Functionality testing:

To examine the functionality of influenza A RNP complexes in an insect cell system, Sf9 cells were infected with baculovirus delivering RNP proteins together with baculovirus delivering negative sense single stranded RNA of a NS1-GFP-NEP fusion protein. A map is shown in *figure 3.2*.
Viral polymerase now should recognize the negative stranded RNA and mRNA transcription, leading to GFP expression, should take place. Monitoring the cells for GFP expression, however, did not give any positive result. So, there are two possible reasons for this experiment not to work. Either the polymerases are not active in the insect cell system, which could be due to the low temperature of 27°C, or baculovirus failed delivering negative single stranded RNA in a correct order.

Transfection experiments in Sf9 cells, using in vitro transcribed RNA, did not give any GFP expression as well. So, the question if polymerases are inactive, or if there's something wrong with our RNA construct, still remained unclear.

To verify the in vitro transcribed RNA, we performed further transfection experiments, but decided to switch from an insect cell to a mammalian cell system and provide polymerase proteins by infectious influenza A virus. MDCK cells and influenza A PuertoRico/8/3 4 seemed to be the best choice. Due to the fact that polymerases are now derived from infectious virus, activity is negligible. If RNA is correct, GFP expression should have been detectable, but checking the cells at different hpts did not give any positive result.

Looking at transfection efficiencies of positive controls showed that efficiency is very low. So a further reason for the experiment not to work was that cells acquire too little RNA for yielding detectable amounts of GFP.

To overcome this problem we adopted another transfection method called Magnetofection, which is supposed to give high transfection efficiencies and low cytotoxicity. Comparing the results from positive controls using any transfection reagent, like in experiments before, and Magnetofection showed a 5 times higher number of positively transfected cells.

To examine whether the RNA actually enters the cell, whole RNA was isolated from MDCK cells, transfected by Magnetofecti on, and transcribed into cDNA. Due to the fact that RNA forms complexes with transfe ction reagent that might stick to outer surface of the cells, a false positive result was possible. A RNase treatment of the cellular supernatant, leading to a removal of possibly contaminat ing RNA, seemed to be adequate. The result is shown in *figure 4.8.* Bands are visible in a height of 1700 bp indicating that our desired fragm ent (NS1-GFP-NEP) indeed entered the cells. A quantification of in vitro transcribed RNA / cell was not possible by using this method.

Due to the fact that MDCK cells transfected by Magnetofectio n actually acquire some RNA, we suggested the amount to be too low for detectable GFP expression.

To overcome this difficulty we decided to use CHO K1 cells, which are known to be susceptible to influenza virus infection as well, for our final transfection experiment. Results are shown in *figure 4.9.* The presence of 4 GFP expressing cells confirms that negative stranded RNA is indeed transcribed to mRNA leading to reporter gene expression. One thing remains unclear. Why is the efficiency, although we transfected high amounts of RNA, that low.

Sequences at the 3' and 5' termini of vira l RNA play a critical role in promoter activity. Ramon Flick et al. showed the requirement for a double-strande d structure in the promoter-distal element at some stage during the propagation cycle. Several experiments that introduced either an A-C mismatch or a G-U RNA base-pair into either of the central positions of the short RNA double strand resulted in inactive versus nearly fully active promoter variant s (33). So if there are mutations in our RNA 5' and 3' termini, this could lead to a reduced promotor activity, explaining our low transfection efficiencies. The sequencin g of the RNA construct, which was done by Theresa Schinko, showed a failure of the T3 terminator to terminate transcription at the 5' end. The sequencing of RNA generated from the template carrying the HDV ribozyme was not successful at all.

6. Conclusion:

The use of baculoviral gene delivery system for the in vitro assembly of recombinant influenza A derived RNP complexes seemed to be a feasible method, because baculovirus is a very strong gene expression system with high yields expected, even when coexpressing several products. The great advantage over the isolation of influenza virion derived RNP proteins is the aspect of yield and the fact that RNP proteins can be tagged which eases the purification procedure drastically. It is further possible to coexpress negative-stranded RNA which will enable us to assemble functional RNP complexes in an insect cell system, overcoming the need for in vitro RNA transcription.

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9. Appendix:

9.1 Primers for cloning and screening:

PA – XhoI - back:

5' – GAT GAT CTC GAG ATG GAA GAT TTT GTG CGA CAA TG – 3'

PA – NotI - for:

5' – GAT GAT GCG GCC GCC TAA CTC AAT GCA TGT GTA AG – 3'

NP – XhoI - back:

5' – GAT GAT CTC GAG ATG GCG TCC CAA GGC ACC – 3'

NP –NotI - for:

5' – GAT GAT GCG GCC GCT TAA TTG TCG TAC TCC TC – 3'

PB1 – XhoI - back:

5' – GAT GAT CTC GAG ATG GAT GTC AAT CCG ACC TTA C – 3'

PB1 – NotI - for:

5' – GAT GAT GCG GCC GCC TAT TTT TGC CGT CTG AG – 3'

PB2 – XhoI - back:

5' – GAT GAT CTC GAG ATG GAA AGA ATA AAA GAA CTA AG – 3'

PB2 – NotI - for:

3' – GAT GAT GCG GCC GCC TAA TTG ATG GCC ATC CG – 3'