Presentation of Recombinant Proteins in Modified Vaccinia Virus Ankara Extracellular Enveloped Virions

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1. SUMMARY

Modified vaccinia virus Ankara (MVA) is a highly attenuated vaccinia virus strain developed during the smallpox eradication campaign. Nowadays recombinant attenuated poxviruses gain importance as live carrier vaccines against different infectious diseases and in cancer therapy.

The aim of this work was to develop recombinant viral vectors, for presentation of a foreign antigen on the surface of extracellular enveloped particles (EEV). First, it was tested whether significant amounts of this viral form are produced by MVA in comparison to replication competent and widely used vaccinia virus strains. Using a number of independent approaches it could be shown that MVA infection in primary chicken embryo fibroblasts results in the production of enveloped viruses, but strikingly most of these were not released into the culture medium but remained attached to the plasma membrane. The results also showed that the replication competent vaccinia virus IHD-J is more efficient in trans-Golgi-network-wrapping and in releasing enveloped virions into the extracellular medium, while the WR strain is less efficient than MVA.

Two different strategies were followed to target the recombinant protein to the surface of extracellular enveloped viruses. Since it was shown that non-vaccinia virus proteins can be incorporated to some extent into the outer membrane, a native model type II membrane protein was used. To increase the chance of foreign protein incorporation a fusion protein was used which consisted of the transmembrane domain of a protein known to be specific for the outer membrane of extracellular enveloped virus and the extracellular domain of the foreign antigen which was used in its native form.
The data show that both proteins were incorporated into the extracellular enveloped virions produced in MVA infected chicken embryo fibroblasts, albeit with low efficiency. The transmembrane domain of the EEV specific protein was not sufficient to target the foreign protein specifically to the outer envelope.

2. INTRODUCTION

The *Poxviridae* comprise a large family of complex DNA viruses that replicate in the cytoplasm of vertebrate and invertebrate cells. The most notorious member, variola virus, caused smallpox and consequently had a profound impact on human mortality in historical times. Smallpox was eradicated in 1977, nearly two centuries after the introduction of prophylactic inoculations with cowpox and vaccinia virus. Vaccination with vaccinia virus contributed to present concepts of infectious diseases and immunity. Additionally, vaccinia virus was the first animal virus seen microscopically, grown in tissue culture, accurately titrated, physically purified, and chemically analyzed (Moss, 1996b).

Due to the ease of generating mutants, vaccinia virus has become a popular eukaryotic expression system. Since vaccinia virus can induce effective T cell immunity, against itself as well as to foreign proteins cloned into its genome, recombinant attenuated poxviruses are attractive candidates to be utilized in cancer therapy and vaccination programmes (Brochier et al., 1991; Hanke and McMichael, 2000) for review see (Moss, 1996a).

2.1 Historical background of vaccination

The first effective method of prevention of any infectious disease was applied to smallpox as early as the 10th century by the intranasal insufflation of dried smallpox scrab material or the inoculation of pustule fluid or scrab material into the skin, a practice called „inoculation“ or „variolation“. If the material used contained active virus, such operations produced smallpox. But for reasons that are not well understood, the disease induced in this manner was usually much milder than „natural“ smallpox, with case-fatality rates of 1-2% instead of 20-30%. A major disadvantage of variolation was that the virus was unchanged in virulence, and mild cases of inoculation smallpox could give rise to severe and fatal attacks of smallpox in contact persons (Fenner, 1988). In 1796 Jenner introduced cowpox virus, a related *Orthopoxvirus*, for preventing smallpox (Jenner, 1959). He challenged persons who had been injected with cowpox virus by inoculation with smallpox virus and showed that they were completely protected. The new procedure became known as vaccination (*vacca* = cow). By 1967 a Smallpox Eradication Unit was established at WHO headquarters, with the aim of global eradication within a decade. The WHO

program achieved this goal in October 1977, and the world was certified free of endemic smallpox in 1979 (WHO, 1980).

2.2 Taxonomic classification of poxviruses

The general properties of *Poxviridae* include a large (250-350 nm) complex brick-shaped virion containing enzymes that synthesize mRNA, a genome composed of a single linear double-stranded DNA molecule of 130-300 kilobase pairs (kbp) with a hairpin loop at each end, and a cytoplasmic site of replication. The *Poxviridae* are divided into two subfamilies, *Chordopoxviridae* and *Entomopoxviridae*, based on vertebrate and insect host range (for overview see table 1).

The similarity in restriction endonuclease maps of several *Orthopoxvirus* genomes (Esposito and Knight, 1985; Mackett and Archard, 1979) has been substantiated by more than 90% sequence identity of genes of vaccinia and variola viruses (Goebel et al., 1990; Massung et al., 1994; Shchelkunov et al., 1993). For at least the last 100 years vaccination against smallpox has been carried out with a distinct species of *Orthopoxvirus* now called vaccinia virus, which is different from cowpox virus, the agent that Jenner used. Subsequently, some preparations of vaccine came from pox lesions of horses and other lots were purportedly mixed with smallpox virus to increase their „potency“. Vaccinia virus has no natural host, although it has been isolated from buffalo in India (Dumbell and Richardson, 1993), and its origin has not been resolved (Baxby, 1981). The restriction endonuclease maps of the genomes of different strains of vaccinia virus from different parts of the world are very similar to each other and quite distinct from variola virus, cowpox virus, and other members of the poxvirus family that occur naturally (Esposito and Knight, 1985). It is not known whether vaccinia virus is the product of genetic recombination, a new species derived from cowpox or variola virus by prolonged serial passage, or the living representative of a now extinct virus (Baxby, 1981).
Despite the profound differences in pathogenicity of variola, vaccinia and cowpox viruses for humans, they are known to belong to the same Orthopoxvirus genus, accounting for their ability to cross protect.

Table 1: Family poxviridae (Moss, 1996b)

Subfamilies	Genera	Member viruses
Chordopoxviridae (vertebrate poxviruses)	*Orthopoxvirus*	Camelpox, cowpox, ectromelia, monkeypox, racoonpox, skunkpox, taterapox, Uasin Gishu, vaccinia, variola, volepox
	Parapoxvirus	Auzduk disease, chamois, contagious ecthyma, orf, pseudocowpox, parapox of deer, sealpox
	Avipoxvirus	Canarypox, fowlpox, juncopox , mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox, turkeypox
	Capripoxvirus	Goatpox, lumpy skin disease, sheeppox
	Leporipoxvirus	Hare fibroma, myxoma, rabbit fibroma, squirrel fibroma
	Suipoxvirus	Swinepox
	Molluscipoxvirus	Molluscum contagiosum
	Yatapoxvirus	Tanapox, Yaba monkey tumor
Entomopoxviridae (insect poxviruses)	*Entomopoxvirus A*	Melolontha melolontha
	Entomopoxvirus B	Amsacta moorei
	Entomopoxvirus C	Chironimus luridus

2.3 Modified vaccinia virus Ankara (MVA)

Although vaccination with vaccinia virus was a major achievement compared to variolation, cases of severe side effects occurred. Congenital or acquired immunodeficiency diseases, immunosuppression, and eczema were absolute contra-indications to vaccination (Fenner, 1988). During the course of the eradication campaign, several highly attenuated strains of vaccinia virus were developed to overcome these risks. MVA was derived from vaccinia virus strain Ankara that was

used in Turkey for vaccination against variola. The virus was attenuated by over 570 serial passages in chicken embryo fibroblast cells (CEF) (Mayr, 1975). The resulting MVA strain lost the capacity to productively infect mammalian cells and suffered six major deletions of DNA totaling 31,000 base pairs (bp). Compared to VV Copenhagen one third of the genes that encode in particular for host range and immune evasion factors are deleted or fragmented (Antoine et al., 1998). When tested in a variety of animal species, MVA was proven to be avirulent even in immunosuppressed animals (Mayr, 1978). Nevertheless, replication of viral DNA appeared normal and both early and late viral proteins were synthesized. This finding was surprising, since other vaccinia viruses with a restricted host range had lost this ability (Drillien et al., 1981; Drillien et al., 1978). Proteolytic processing of viral structural proteins was inhibited, for example in 4A and 4B, two major proteins of the virus core. Correct proteolytic processing of the core proteins is essential for virion morphogenesis, so only immature particles were detected by electron microscopy. In human cells, the assembly of infectious MVA particles is blocked at an early stage. Vaccinations of more than 125,000 humans with MVA showed no major cases of side effects.

Despite the inability of MVA to produce infectious progeny, recombinant viruses were able to synthesize high levels of a foreign protein in human cells, demonstrating the potential of MVA to serve as an exceptionally safe and highly efficient expression vector (Sutter and Moss, 1992).

2.4 Genome of vaccinia viruses

Poxviruses have linear double-stranded DNA genomes that vary from about 130 kbp in parapoxviruses to about 300 kbp in avipoxviruses. Inverted terminal repeats (ITRs), which are identical but oppositely oriented sequences at the two ends of the genome are present in all poxviruses examined. The length of the ITRs are variable even within a genus (Moss, 1996b).

The two strands of vaccinia virus DNA are connected by hairpin loops to form a covalently continuous polynucleotide chain (Baroudy et al., 1982; Geshelin and Berns, 1974). The genomes of several VV strains have been sequenced, including Copenhagen strain (Goebel et al., 1990) and MVA (Antoine et al., 1998). The genome of MVA is 178 kb in length, significantly smaller than that of the vaccinia Copenhagen genome, which is 192 kb. The 193 open reading frames (ORF) mapped in the MVA genome probably correspond to 177 genes, 25 of which are split and/or

have suffered mutations resulting in truncated proteins. Essential genes map within the highly conserved central region of the genome, whereas many of the genes that are dispensable for replication in tissue culture are closer to the ends. The apparent absence of introns, the short promotor sequences, and the relatively small sizes of many ORFs account for the packing of about 150-200 genes into the orthopoxvirus genome (Goebel et al., 1990). Both strands of the DNA are transcribed, yet extensive overlap of the ORFs is uncommon.

The convention adopted for naming the orthopoxvirus ORFs consists of using the Copenhagen *Hind*III restriction endonuclease DNA fragment letter from A to O, followed by the ORF number inside a specific *Hind*III fragment (from left to right), and L or R, depending on the direction of transcription of the ORF (Rosel and Moss, 1985).

Several different nomenclatures are in use for VV proteins. Initially they were named according to their apparent molecular weight (e.g. p21, p37 etc.), or their enzymatic activity (e.g. DNA polymerase). In recent years, however, as the proteins could be assigned to their corresponding genes in the viral genome, mostly the gene names are used. Purified VV virions contain more than 110 proteins that are usually synthesized late (Carrasco and Bravo, 1986; Essani and Dales, 1979; Jensen et al., 1996). Per definition, all proteins present in the virions could be called structural VV proteins. However, not all of these have necessarily a structural role, but rather are packaged because their enzymatic activities are needed for the process of early transcription upon infection of the cell (Gershon and Moss, 1990). The virion proteins are roughly divided into core and membrane proteins according to their solubility in non-ionic detergents under reducing conditions.

Many viral proteins, including some structural ones, modulate the course and pathogenesis of viral infection in the host animal and are as such not essential for replication in cultured cells. All VV proteins are roughly classified into early and late dependent on whether they are synthesized before or after DNA replication. Many viral enzymes are synthesized late, and are packaged into the virion to function during virus entry or early viral transcription. Other viral enzymes are synthesized early, and are required for DNA replication and late viral transcription.

2.5 Life cycle of vaccinia virus

The infection of a host cell by a virus can be divided into several steps (Levine, 1992):

1. Adsorption: Binding of the virus to receptors on the plasma membrane of the host cell.
2. Penetration: Entry of the virus into the host cell by penetrating the plasma membrane or endosome and delivery of the viral core into the cytoplasm.
3. Uncoating: Release of the viral genome into the host cell.
4. Synthesis of early viral proteins.
5. Replication of the viral genome.
6. Synthesis of late viral proteins.
7. Assembly: Posttranslational modification of viral and structural proteins and assembly of the viral particles.
8. Release of infectious particles (specific or by lysis of the host cell).

A schematic overview of the complex assembly process is depicted in Fig. 1 (Sanderson, 1999).

2.5.1 Adsorption and penetration

The entry pathway of poxviruses, including vaccinia virus, the prototype of this family, is not well characterized. Studies of poxviruses are complicated by the fact, that two structurally and antigenically distinct, infectious forms exist: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). Structurally, EEV consists of an IMV with an additional outer membrane containing proteins that are absent from IMV. The amount of EEV produced varies between different vaccinia virus strains and host cell types (Payne, 1980).

Vertebrate poxviruses were thought to enter cells by phagocytosis (viropexis) (Dales, 1964). When entry by fusion of the virus envelope with the host cell membrane was discovered for insect poxviruses (Granados, 1973), adsorption and penetration of vaccinia virus was re-examined. Studies indicated the occurrence of direct fusion between the envelope of the attached virus particles and the plasma membrane of the cell. Since also poxvirus particles closed within phagosomes were seen, those findings suggested, that vaccinia virus can enter cells by membrane fusion as well as by phagocytosis (Armstrong et al., 1973). In all of these experiments it was not distinguished between IMV and EEV. Payne and Norrby (Payne and Norrby, 1978)

examined the adsorption and penetration of IMVs and EEVs separately, and came to the conclusion, that intracellular and extracellular viruses enter the cell by different mechanisms. Kinetic studies showed, that EEV penetrated cells more rapidly than IMV (Domes, 1990; Payne and Norrby, 1978). The fusion of EEV with the plasma membrane of host cells presents an interesting topological problem. In fact, fusion of EEV would be expected to release IMV into the cytoplasm, facing the problem of removal of the IMV membrane and virus uncoating. Ichihashi (Ichihashi and Oie, 1996) proposed a model of EEV entry, assuming that EEV entry consists of binding to the cell, endocytosis, disruption of the EEV outer membrane within an acidified endosome, fusion of the exposed IMV with the endosomal membrane and release of the core into the cytoplasm. This model was strongly supported by Vanderplasschen et al. (Vanderplasschen et al., 1998a). The receptors responsible for virion entry have not been identified yet. A monoclonal antibody, called B2, which recognizes a trypsin-sensitive cell surface protein and inhibits the binding of IMV to cells was isolated (Chang et al., 1995). This antibody inhibits IMV binding sterically but the inhibition is incomplete, suggesting that there is more than one IMV receptor (Vanderplasschen et al., 1997). More recently it was shown, that binding of IMV activates a signaling cascade, linked to the formation of actin and ezrin containing protrusions at the plasma membrane, that seems to be essential for the entry of IMV. EEV appeared to enter the cell without the need for signaling and actin/membrane rearrangements (Locker et al., 2000). Electron micrographs suggested that the cores derived from both the IMV and EEV enter the cell at the plasma membrane where they discard all the surrounding membranes (Locker et al., 2000). In contrast to what would be expected of a simple fusion event, none of the IMV- and EEV-membrane proteins appear to be implanted into the plasma membrane since they could not be detected at the plasma membrane by immunoelectron microscopy. Instead, viral membrane fragments that were attached to the cell surface or in close proximity to the plasma membrane were decorated by antibodies to viral proteins (Locker et al., 2000; Pedersen et al., 2000). Because of those findings it was proposed that, rather than entering by membrane fusion, both viruses seem to undergo a „membrane shedding“ or „membrane unfolding“ at the plasma membrane, releasing the underlying core for delivery into the cytoplasm. This hypothesis leaves open how the core that is released at the plasma membrane, is subsequently translocated across that same membrane. While there is little consensus on the exact pathway, it is generally accepted that the first step of uncoating results in the delivery of VV cores, that contain the genome and associated viral enzymes into the cytoplasm

(Hollinshead et al., 1999; Locker et al., 2000; Moss, 1990; Pedersen et al., 2000; Vanderplasschen et al., 1998a).

2.5.2 Synthesis of VV-proteins and DNA replication

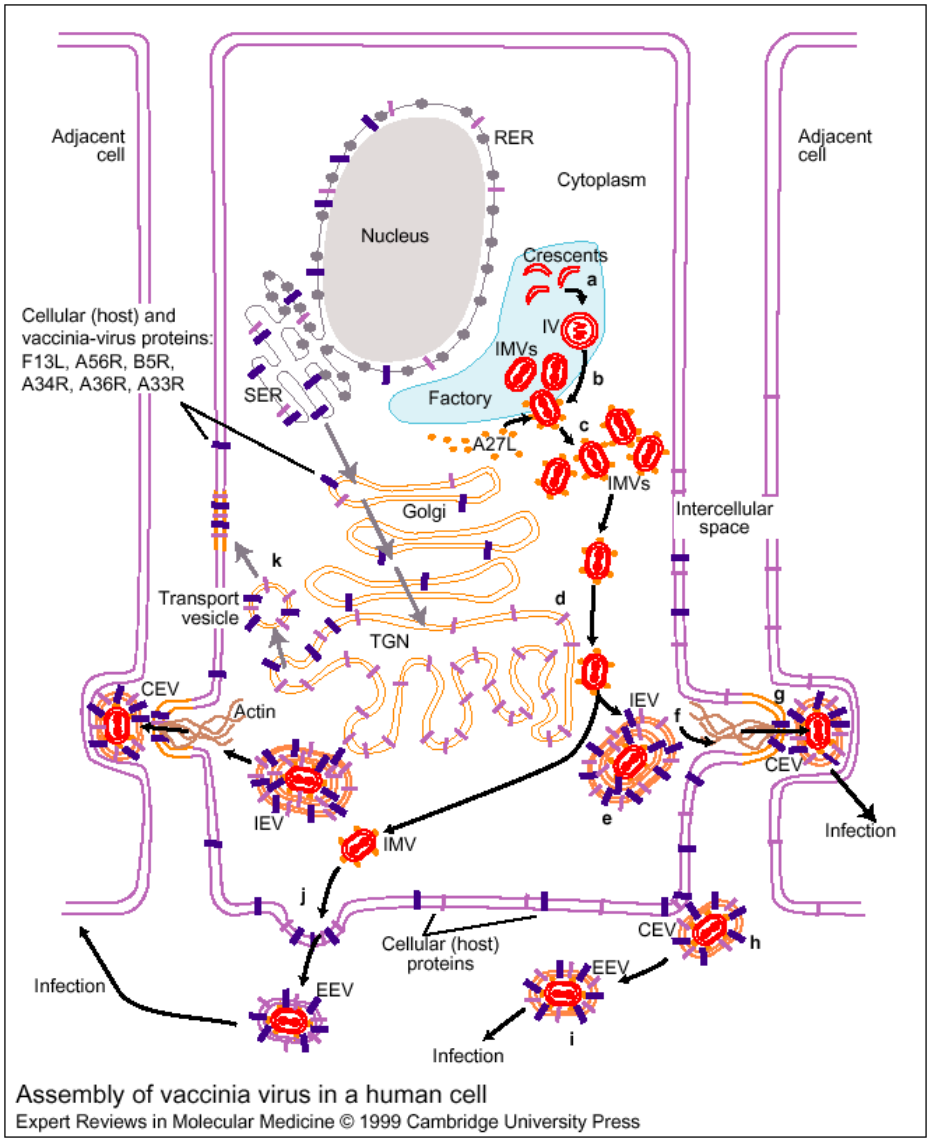
Besides DNA, also virus-encoded enzymes are present in the core, including a multisubunit DNA-dependent RNA polymerase, a transcription factor, capping and methylating enzymes, and a poly(A) polymerase, all of which form a complete apparatus for the transcription of mRNA. When the core enters the cytoplasm, approximately 100 genes (about half of the virus genes) are transcribed by the viral RNA polymerase. These early genes encode proteins involved in stimulation of the growth of neighboring cells, defense against host immune responses, replication of the viral genome, and transcription of the intermediate class of viral genes. During poxvirus replication, concatemeric intermediates that are resolved into unit-length genomes are formed. The replicated DNA molecules provide the templates for the successive expression of intermediate and late genes. Intermediate genes are less numerous and encode mostly for factors required for late transcription. During virus infection intermediate genes are only expressed once DNA replication has commenced, as late genes are expressed once the late transcription factors are available. They encode the structural proteins of the virus, enzymes that are to be packaged into virions for early transcription, and additional virulence factors. In general, late proteins are expressed at higher levels than early proteins due to their requirement in stoichiometric rather than catalytic amounts. Each temporal class of genes has its own characteristic promotor sequences that are recognized by specific viral proteins, providing the basis for a programmed cascade mechanism of gene regulation.

DNA replication has long been known to occur in distinct cytoplasmic sites, called the viral factories (Cairns, 1960). It was recently shown that these sites do not lie „free“ in the cytoplasm, but instead were found surrounded by ER membranes (Tolonen et al., 2001). This ER wrapping process correlated with DNA replication. About 3 hours post infection the entire replication site appeared to be ER enclosed, and this wrapping persisted throughout infection until the first evidence of virion assembly was seen. Interestingly, ER-wrapping coincided with a peak in viral DNA synthesis implying that wrapping may promote viral replication. ER wrapping may supply the replicating DNA a cytoplasmic scaffold as well as create a microenvironment that supports DNA synthesis (Tolonen et al., 2001).

2.5.3 Assembly

VV life cycle takes place entirely in the cytoplasm of the infected cell, where morphogenesis proceeds through a well-synchronized series of events. Assembly of VV starts at 5 to 6 h.p.i. with the formation of typical crescent-shaped membranes modified by viral membrane proteins. These crescents are perfectly spherical segments of membranes with a thickness of 8 nm and a diameter of 280 nm (Sodeik et al., 1994). According to the classical model of VV assembly, membrane crescents contain a single biological membrane bilayer that has no continuities to cellular membrane systems but ends openly in the cytosol (Dales and Mosbach, 1968; Hollinshead et al., 1999; Stern and Dales, 1974). It has been proposed that the crescents assemble de novo in the cytoplasm from cellular lipids and viral membrane proteins by a unique viral mechanism. The viral DNA is incorporated into the spherical immature virion (IV), and after several internal structural changes most likely induced by proteolytic cleavage of major core proteins, an intracellular mature virus (IMV) is formed. The core was thought to contain a proteanaceous core membrane enclosing the viral DNA, DNA associated proteins and viral enzymes. Lateral bodies are located on both sides of the core that therefore appears as a biconcave disk and has a dumb bell shape in cross sections. One outer membrane that contains both proteins and lipids, encloses core and lateral bodies, to build the mature IMV particle. In this model, IMV was postulated to be surrounded by a single lipid bilayer made de novo in the cell.

This model was challenged in 1993 by Sodeik et al. (Sodeik et al., 1993), who showed data to suggest that the crescent-shaped membranes are composed of two tightly apposed membranes derived from a cisterna of the smooth ER (Sodeik et al., 1993; Sodeik and Krijnse-Locker, 2002). It was subsequently shown that several viral membrane proteins are co-translationally inserted into the ER and retained in smooth ER membranes. Since these membrane proteins localize also to the crescent-shaped membranes, it was postulated that interactions between these ER-retained viral membrane proteins caused two lipid bilayers of a cellular ER cisterna to become so tightly apposed that they could easily be mistaken for one bilayer only. Furthermore these proteins were at least in part responsible for the formation of the characteristic crescent-shape of the viral membranes; reviewed in (Sodeik and Krijnse-Locker, 2002). These crescents engulf electron-dense material composed of viral core proteins and generate the completely spherical IV (Fig. 2A). Upon DNA uptake the spherical IV particles develop an internal core surrounding the DNA and mature into the infectious form of the virus, the intracellular mature virus (Fig. 2B).



Assembly of vaccinia virus in a human cell

Figure 1: Assembly of vaccinia virus in a human cell (Sanderson, 1999). SER = smooth endoplasmic reticulum; RER = rough endoplasmic reticulum. (a) Vaccinia virus morphogenesis starts in cytoplasmic factories with the formation of membrane crescents, which extend to form spherical immature virus (IV) particles. (b) IV particles are non-infectious until they undergo morphological condensation into brick-shaped IMV particles. (c) During infection, most IMV particles remain within the cell until they are released by cell lysis. However, some IMV particles become enveloped by membranes derived from either the trans-Golgi network (TGN) or tubular endosomes (d) to form intracellular enveloped virus (IEV) (e). (f) Proteins within the outer membrane of IEV particles induce the polarised, unidirectional polymerization of actin, which propels the particle towards the plasma membrane and assists the infection of neighboring cells. (g) During this process, the outer membrane of the IEV particle fuses with the plasma membrane of the cell, exposing an infectious particle on the cell surface and leaving proteins that induce actin polymerization within the plasma membrane. (h) If these particles remain attached to the cell they are called cell-associated enveloped virus (CEV); (I) however, if they are released from the cell they are called extracellular enveloped virus (EEV). (j) In addition to the classical mechanism of vaccinia virus assembly described above, EEV particles can also form via direct budding of IMV particles through the plasma membrane. This alternative mechanism of EEV formation might be facilitated by the transport of viral glycoproteins from the TGN to the plasma membrane (k).

The IMV is formed after the IV undergoes a major structural transformation, which coincides with the cleavage of several core proteins (Lee and Hruby, 1994; Moss and Rosenblum, 1973; Silver and Dales, 1982; Vanslyke et al., 1991). This cleavage presumably triggers the transition from the spherical IV to the brick-shaped IMV, and the generation of the morphologically and biochemically distinct core structure of the IMV. The IMV is roughly brick-shaped and measures around 300-310 x 240-245 x 110-130 nm^3. IMVs contain around 90% protein, 6% lipid, and 3% DNA, and weigh 5 x 10^{-15} g (Smadel, 1942; Zwartouw, 1964). In contrast to the classical model, the IMV is thought to contain two lipid bilayers that are very closely juxtaposed. Most recently it was proposed that the structure of the IMV is exceedingly more complex than presented above, and that the particle might be composed of one continuous folded membrane cisterna (Griffiths et al., 2001a; Griffiths et al., 2001b). One fold of the cisterna seems to enclose the genome and separates the genome from another (cytosolic) compartment. Moreover, the cisternae enclosing the DNA seem to be continuous with the membrane cisterna that surround the particles and seal the virion off at one side. The lateral bodies are proposed to be the sites where the outer membrane cisternae contact the viral core (Sodeik and Krijnse-Locker, 2002).

2.5.4 Release

IMVs provide the majority of infectious progeny of VV and remain inside the infected cell until cell-lysis but can also bud off under some conditions (Tsutsui, 1983). A fraction of the IMVs becomes further enveloped by a double membrane derived from the early tubular endosomes (Tooze et al., 1993) or trans-Golgi network (Schmelz et al., 1994) (Fig. 2C). Envelopment of IMV must involve the specific interaction between one or more IMV surface proteins and the cytosolic domain of one or more EEV proteins, which become enriched in the wrapping membranes. The efficiency and degree of this wrapping process depends on the combination of virus strain and cell type used. Once envelopment is complete, the virus particles are surrounded by two additional membranes compared to the IMV and are called intracellular enveloped virus (IEV). For a while it was thought that the IEVs polymerize actin-tails with which they are actively moved through the cytoplasm towards the plasma membrane, with which they will then fuse (Fig. 2D) to release the extracellular enveloped virus (EEV) into the extracellular environment (Cudmore et al., 1995). Recently, however, it was shown that IEVs move in a kinesin-dependent fashion on microtubules towards the plasma membrane. Upon fusion of the outer-most

membrane of the IEV with the plasma membrane actin tails are made at this cellular membrane. The purpose of these tails appears to be the formation of long plasma membrane-derived filopodia, with an EEV attached on their tip (called CEV, see below), a process that may aid in virus cell-to-cell spread (Hollinshead et al., 2001; Moss and Ward, 2001; Rietdorf et al., 2001; Ward and Moss, 2001). These data are consistent with other results showing that the EEV is important for virus dissemination both *in vitro* and *in vivo* (Appleyard et al., 1971; Blasco and Moss, 1991; Boulter and Appleyard, 1973; Payne, 1980; Payne and Kristensson, 1985).

EEVs contain an additional envelope compared to IMV and are actively released from the infected cell. With most strains of VV a substantial fraction of the EEV remains attached to the cell surface and is termed cell-associated enveloped virus (CEV) (Blasco and Moss, 1992), constituting the viral form that is attached to the actin-tail containing filopodia. The EEV protein required for both microtubule- and actin-dependent motility is thought to be the gene product of A36R. This protein may indirectly bind to the so-called TPR domain of conventional kinesins' light chain (Rietdorf et al., 2001), thus mediating the observed microtubule-dependent movement. At the plasma membrane the same protein then may become tyrosine phosphorylated by cellular scr-kinases, a prerequisite for the formation of actin tails (Frischknecht et al., 1999a). With the formation of the IEV/EEV VV has thus evolved a very efficient and rapid mechanism for virus egress and cell-to-cell spread.

2.6 Infectious forms of vaccinia virus

As mentioned above, five morphologically different viral forms exist: immature virus (IV), intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV), and extracellular enveloped virus (EEV). IMV and CEV/EEV are the infectious forms of the virus, that stay inside the infected cell until lysis (IMV) or are actively released (CEV and EEV) and enter the cells by different mechanisms.

The properties of the additional envelope present on CEV/EEV are responsible for these differences.

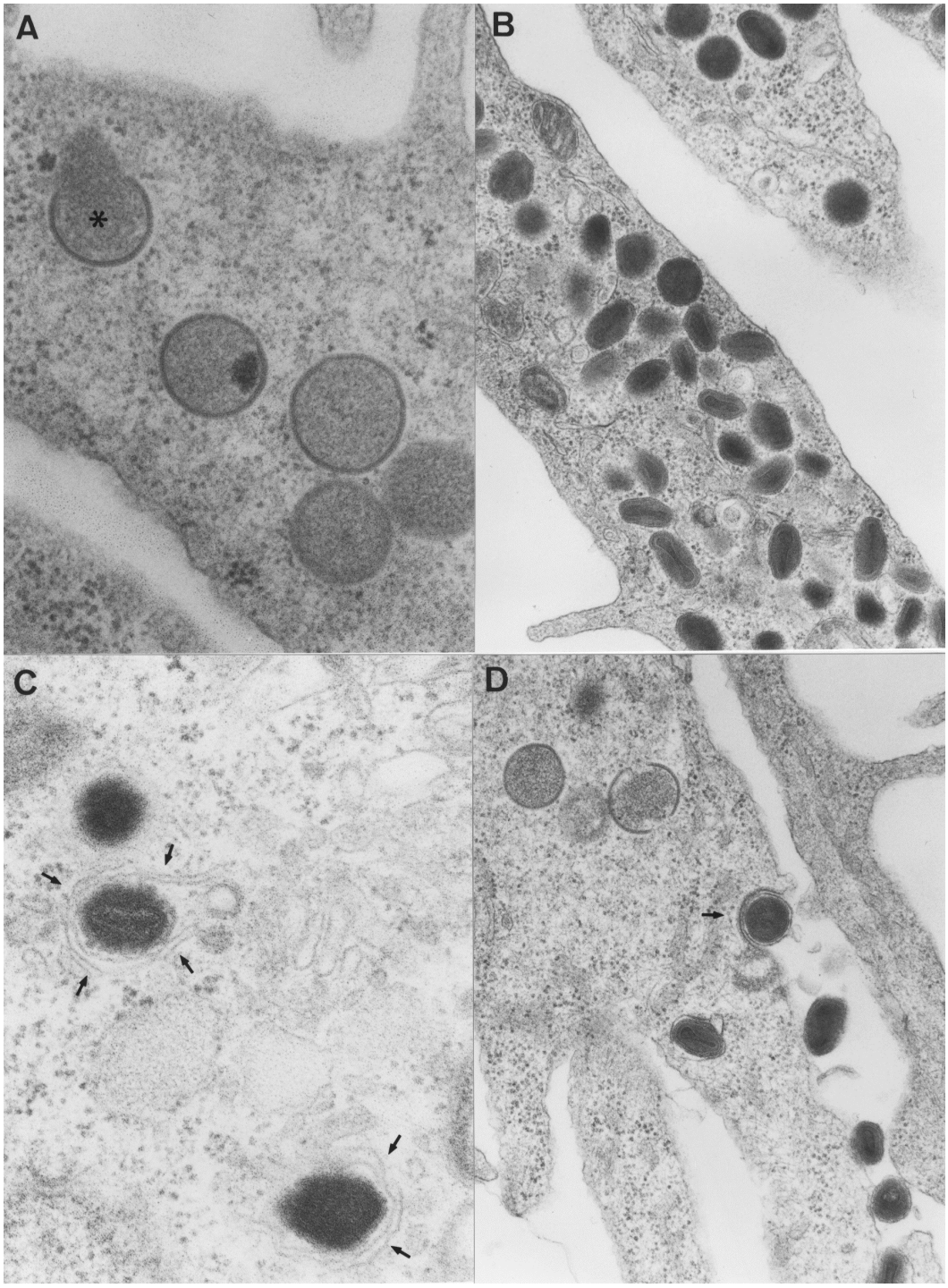


Figure 2: EM pictures of vaccinia virus virions at different stages of assembly. (A) Spherical IVs, IV uptaking DNA (*). (B) IMVs accumulating inside the cell. (C) TGN-wrapping of IMVs (arrows pointing to the wrapping membranes) (D) IEV fusing with the plasma membrane (arrow), CEV attached to the cell surface.

2.6.1 EEV specific proteins

At least 10 proteins which are absent from the IMV are associated with the outer envelope (Payne, 1978; Payne, 1979) and five of which have been identified, which endow EEV with different biological and immunological properties.

These are: A56R, encoding the virus haemagglutinin (HA), a heavily glycosylated protein of 86 kDa (Payne and Norrby, 1976; Shida, 1986); F13L, encoding a 37 kDa protein, p37 (Hirt et al., 1986); B5R, encoding a 42 kDa glycoprotein, gp42 (Engelstad et al., 1992; Isaacs et al., 1992); A33R, encoding a 23-28 kDa glycoprotein, gp23-28 (Roper et al., 1996); and A34R, encoding a triplet of glycoproteins of 22-24 kDa, gp22-24 (Duncan and Smith, 1992). In addition, the A36R, encoding a 45-50 kDa protein, p45-50 (Parkinson and Smith, 1994) is present in IEV but not IMV, CEV or EEV particles (van Eijl et al., 2000). Lastly, the F12L protein was identified recently as a 65 kDa protein (Zhang et al., 2000), which, like the A36R protein, is present only in IEV particles (van Eijl et al., 2000).

None of the EEV- and IEV-specific proteins are required for the formation of IMV, but they have different roles thereafter. In the absence of F13L (Blasco et al., 1991) or B5R (Engelstad and Smith, 1993; Wolffe et al., 1993), the wrapping of IMV by TGN membranes to form IEVs, is reduced or abolished and therefore subsequent stages of morphogenesis are inhibited. Deletion or repression of A33R (Roper et al., 1998), A34R (Duncan and Smith, 1992; McIntosh and Smith, 1996; Sanderson et al., 1998) or A36R (Parkinson and Smith, 1994; Sanderson et al., 1998; Wolffe et al., 1998) enables IEV, CEV and EEV to be formed, but cell-to-cell virus spread is reduced and these mutants have a small plaque phenotype (Zhang et al., 2000).

Enhanced levels of EEV are released when gene A33R (Roper et al., 1998) or A34R (McIntosh and Smith, 1996) is deleted, but without A34R the EEV has a reduced infectivity. Enhanced levels of EEV are also formed when the A34R (Blasco et al., 1993) or B5R proteins (Herrera et al., 1998; Mathew et al., 1998; Mathew et al., 2001) are mutated.

The loss of the A36R (Parkinson and Smith, 1994) or F12L (Zhang et al., 2000) genes caused a reduction in EEV formation. For all theses mutants the production of intracellular actin tails is reduced or abolished, providing a direct correlation between actin tail formation and virus cell-to-cell spread. Recently it was shown that A36R is required for both microtubule-mediated IEV movement and actin tail formation at the plasma membrane (Rietdorf et al., 2001).

The only IEV or EEV-specific protein not required for EEV formation is A56R (Ichihashi and Dales, 1971; Sanderson et al., 1998).

Table 2: Properties of vaccinia virus mutants lacking specific EEV proteins (Smith and Vanderplasschen, 1998)

Mutant	Plaque size	IMV	IEV	EEV	Actin tails	Virulence
vΔA33R	tiny	2-3 x ↓	Incomplete wrapping	2-4 x ↑	no, only short+slender	?
vΔA34R	tiny	normal	few	19-24 x ↑ infectivity 5x ↓	no	attenuated
vΔA36R	small	normal	normal	3 x ↓	no	attenuated
vΔA56R	normal	normal	normal	normal	normal	virulent
vΔB5R	small	normal	few	5 x ↓	few	attenuated
vΔF12L	tiny	2 x ↓	normal	7 x ↓	few	attenuated
vΔF13L	tiny	normal	few	5 x ↓	few	attenuated

2.6.2 Properties of the CEV/EEV envelope

The presence of different proteins on the surface of IMV and EEV give these virions different biological and immunological properties that are adapted to their different roles in VV pathogenesis. As mentioned before, EEV and IMV bind to distinct cellular receptors and penetrate cells by different mechanisms. Moreover, EEV, in contrast to IMV, is resistant to antibody neutralization (Ichihashi, 1996; Vanderplasschen et al., 1997). A major difference between IMV and EEV is also that IMV excludes host proteins from its surface whereas EEV does not. Vanderplasschen et al. (Vanderplasschen et al., 1998b) suggested that the incorporation of host complement control proteins into the envelope of EEV makes these particles resistant to complement.

2.7 Modern vaccines

Due to modern safety requirements vaccination procedures carried out like „in the old days“ are no longer an option. Safety and effectiveness are prerequisites for vaccine development. Since MVA has shown to be an exceptionally safe live vaccine and has the potential to serve as an expression vector for foreign proteins, this highly attenuated vaccinia virus strain is a candidate to be used as a non-virulent vector for heterologous antigens. Understanding of the vertebrate immune system helps to generate vaccines with increased efficiency in modulating the immune responses.

Invading organisms or components of those have to be evaluated for their non-self origin by innate immunity. Recognition as such leads to activation of host defense. The innate immune system therefore uses germline-encoded proteins that are expressed constitutively or are induced rapidly within hours following infection. Macrophages attack invading microorganisms upon detection, and further activate immune responses by releasing proinflammatory mediators.

Vertebrates have developed an acquired immunity that provides a higher degree of structural specificity. Acquired immunity is provided by B and T lymphocytes each of which express distinct cellular or soluble antigen receptors that have arisen by somatic gene rearrangement and are selected for foreign antigen reactivity within days.

The humoral immune response is driven by B lymphocytes which present highly variable immunoglobulins as antigen receptors on their surface. After contact with an antigen naive B cells differentiate into plasma cells which secrete high amounts of specific antibody, and into memory cells which allow a fast antibody release after a renewed antigenic contact. Antibodies, which bind with a high affinity to toxins, viruses or bacteria are able to neutralize those pathogens. Mostly, antibodies are covering the pathogens and bind to the Fc-receptor on phagocytes, mediating their uptake and degradation. Intracellular pathogens like viruses can be eliminated by another mechanism. Degradation products are presented on the cell surface via MHC class I molecules, this counts also for viral proteins after infection. Cytotoxic T cells (CTL) recognize peptides presented by MHC class I molecules, which are present on the surface on all nucleated cells. When the peptide presented is recognized as non-self the infected cell will be killed. The demand for an efficient vaccine should be the activation of both systems, B and T cell immunity.

2.8 Aim of the work

MVA is increasingly becoming an important candidate to be used as an attenuated live vaccine against various diseases in humans. Most studies on VV particles have been carried out with the replication competent vaccinia virus strains IHD-J and WR. In this work the ability of MVA to produce enveloped viral forms was one focus of investigation. Those enveloped virions are of special interest, since their outer envelopes are able to incorporate foreign and cellular proteins.

It is known that MVA, like other VV strains, induces effective T cell immunity against recombinant proteins even though ist replication is blocked in mammalian cells.

Presentation of the foreign antigen on the surface of the viral particle could lead to induction of B cell immunity and therefore modulates the type of immune response.
The aim of this work was the presentation of heterologous antigens on the outer surface of MVA virions. For reason mentioned above, extracellular enveloped virus was chosen as the viral particle of integration. The model antigen used in this work was the hemagglutinin of canine distemper virus (CDV.H), a type II transmembrane glycoprotein. Recombinant viruses containing the wildtype CDV.H or chimeric proteins consisting of the transmembrane domain of known EEV specific proteins and the extracellular domain of CDV.H should be produced and presentation of the foreign protein on the surface of EEV characterized.

3. MATERIALS

3.1 Abbreviations

AB/AM	Antibiotic-antimycotic solution
Amp	Ampicillin
APS	Ammonium peroxidisulfate
AraC	Arabinosylcytosine
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CEV	Cell associated virus
CPE	Cytopathic effect
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
EEV	Extracellular enveloped virus
EGTA	Egtazic acid
EM	Electron Micoscropy
ER	Endoplasmatic reticulum
EtBr	Ethidium bromide
FCS	Fetal calf serum
GFP	Green fluorescent protein
HEPES	N-[2-Hydroyxlethyl]piperazine-N'-[2-ethanesulfonic acid]
h.p.i.	hours post infection
IMCBH	N1-Isonicotinoyl-N2-3-Methyl-4-Chlor-Benzoyl-Hydrazin
IEV	Intracellular enveloped virus
IMV	Intracellular mature virus
IV	Immature virus
LB	Luria-Bertani bacterial medium
m.o.i.	Multiplicity of infection
MOPS	4-Morpholine-propanesulfonic acid
OD	Optical density
PFA	Paraformaldehyde
p.f.u.	Plaque forming unit
PIPES	1,4-Piperazinediethanesulfonic acid
rpm	rounds per minute
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-Tetramethylethylendiamine
TGN	trans Golgi network
TNE	Tris-NaCl-EDTA buffer
VV	Vaccinia virus

wt Wild type

3.2 Buffers and solutions

All the solutions used for cell culture were sterilized (autoclaved or filtered with 0.2 μm filters).

Ampicillin stock

100 mg/ml ampicillin

Ampuwa H_2O

stored at –20°C

Cellular lysis buffer for Western Blot 5x

250m; Tris/HCl pH 6.8

10% SDS

7.5% Glycerin

12.5% 2-Mercaptoethanol

0.5% Bromophenol blue

DNA loading buffer 6x

0.25% bromophenol blue

40% (w/v) sucrose in water

Fixing solution 1 for EM

8% PFA

0.2M PHEM

0.2% glutaraldehyde

Fixing solution 2 for EM

8% PFA

0.1M PHEM

Glutaraldehyde 25% (EM grade)

25% glutaraldehyde

dissolved in A.dest.

Paraformaldehyde 16%

16% PFA

dissolved in A.dest. with 0.16% Na_2CO_3 at 80°C

stored at –20°C

PBG buffer (**P**BS-**B**SA-**G**elatine)

1% FSG (fish skin gelatine)

0.8% BSA in 20 mM glycine

dissolved in PBS

stored at –20°C

PHEM buffer 0.4M pH6.9 (**P**IPES-**H**EPES-**E**GTA-**M**gCl_2)

240 mM PIPES

100mM HEPES

8mM $MgCl_2$

40mM EGTA

adjusted pH to 6.9 with KOH

stored at –20°C

Phosphate buffered saline (PBS) pH 7.4

140mM NaCl

5.4mM KCl

9.7mM $Na_2HPO_4x2H_2O$

2mM KH_2PO_4

Aqua bidest.

Protein loading buffer 5x

250mM Tris pH 6.8

10% SDS

7.5% Glycerine

0.5% bromophenol blue

12.5% 2-Mercaptoethanol

SDS-electrophoresis running buffer 10x

0.25M Tris Base

0.192M Glycine

1% SDS

SDS-electrophoresis transfer buffer 10x

0.25% Tris Base

0.192M Glycine

SDS-electrophoresis washing buffer 5x pH 7.5

0.25M Tris Base

0.75M NaCl

0.25 vol.% Tween 20

adjust to pH 7.5 with HCl

SDS-protein running gel 10%

A.dest.	4.0 ml
30% acrylamide mix	3.3 ml
1.5M Tris pH 8.8	2.5 ml
10& SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.004 ml

SDS-protein stacking gel 5%

A.dest.	3.4 ml
30% acrylamide mix	0.83 ml
1M Tris pH 6.8	0.63 ml
10% SDS	0.05 ml

10% APS	0.05 ml
TEMED	0.005 ml

Stripping buffer for WB

100mM 2-Mercaptoethanol

2% SDS

62.5mM Tris/HCl pH 6.7

TAE buffer 50x pH 8.0

2M Tris-acetate

0.5M NaCl

50mM EDTA

TE buffer

10mM Tris/HCl

1mM EDTA

TMN buffer (**T**ris-**M**gCl_2-**N**aCl)

0.01M Tris pH 7.5

1.5mM $MgCl_2$

10mM NaCl

TNE buffer pH 7.4

40mM Tris/HCl

1mM EDTA

150mM NaCl

3.3 Media

RPMI 1640 Medium with Glutamax-1	GibcoBRL, Karlsruhe
MEM (Minimal Essential Medium)	GibcoBRL, Karlsruhe
Dulbecco's MEM with Glutamax-1, Sodium Pyrovate 4500 mg/l glucose, pyridoxine	GibcoBRL, Karlsruhe
OptiMem	GibcoBRL, Karlsruhe
Lactalbumin Hydrolysate 10% Solution	GibcoBRL, Karlsruhe
BMS (Basalmedium Supplement)	Biochrom KG, Berlin
Trypsin-EDTA (1x) in HBSS w/o CA&MG W/EDTA 4N1	GibcoBRL, Karlsruhe
Antibiotics-Antimycotics prepared with 10000 U/ml penicillin G sodium 10000 μg/ml streptomycin sulfate 25 μg/ml amphotericin B in 0.85% saline	GibcoBRL, Karlsruhe
Fetal calf serum (FCS)	GibcoBRL, Karlsruhe

3.4 Antibodies

rabbit serum α-VV	Sigma, Taufkirchen
rabbit serum α-CDV.H	provided by Ricardo Witteck, Lausanne
rabbit serum α-B5R	provided by Ricardo Witteck, Lausanne
MAb α-B5R	provided by Gerhard Hiller (Schmelz et al., 1994)
MAb α-A17L	directed against the N-terminus of A17L
Goat anti rabbit HRP	Dianova, Hamburg
Goat anti rat FITC	Dianova, Hamburg
Phalloidin/rhodamine	Sigma, Taufkirchen

3.5 Cells

CEF	primary chicken embryo fibroblasts
BHK –21	baby hamster kidney (ATCC CCL-10)
RK-13	rabbit kidney (ATCC CCL-37)
BSC-40	african green monkey kidney (provided by Stewart Shuman, New York)

3.6 Viruses

Vaccinia virus strain MVA (cloned isolate F6) (Mayr, 1975)

Virus from the 582nd CEF passage was used for this study

MVA-ssP-K1L-gfp (Staib et al., 2000)

WR

IHD-J

3.7 Oligonucleotides

(used as primers)

NIH-GS83:	5‘-GAATGCACATACATAAGTACCGGCATCTCTAGCAGT-3‘
IIIfl1b:	5‘-CACCAGCGTCTACATGACGAGCTTCCGAGTTCC-3‘
II 0011b-3‘:	5‘-CGTCGTTTAAACATATGACTAGGGACC-3‘
II 0011b-5‘:	5‘-CGTTGTATACACATATTGAGTTGTCTG-3‘

4. METHODS

4.1 Bacterial techniques

4.1.1 Transformation of bacteria with plasmid DNA

Two different protocols were used for bacterial transformation. Electroporation was performed to transform electrocompetent DH10B cells with newly made plasmids immediately after ligation, and heat shock protocol for transformation of HB101 cells with purified plasmid DNA.

4.1.1.1 Preparation of electrocompetent cells

A single bacterial colony was inoculated in 10 ml of LB medium in a small glass tube and incubated O/N at 37°C shaking. The next morning the O/N culture was inoculated in 200 ml LB medium in a 1 l flask, and the cells were grown until an OD_{600} of 0.5 was reached (about 2 h shaking at 180 rpm). The cells were cooled on ice, transferred into Beckmann centrifuge tubes, and centrifuged for 15 min at 4200xg in a precooled GS-3 rotor at 4°C. The cell pellets were washed first in 200 ml ice-cold and sterile Ampuwa water, then in 100 ml Ampuwa water and finally resuspended in 10 ml cold 10% glycerol (filtered). Cells were spun down for 10 min at 4200xg, resuspended in 600 µl of cold 10% glycerol, aliquoted (50 µl/sterile Eppendorf tube), frozen and stored at –80°C.

4.1.1.2 Electrotransformation

For electroporation DH10B cells and the mixture samples obtained after the ligation reaction were thawed on ice. 5 µl of the ligation mixture (out of 20 µl) were added to 25 µl of DH10B cells and 10 µl A. dest. and mixed. The cells and the DNA were incubated on ice for some minutes and introduced to an ice-cold electroporation cuvette. The cells were shocked by 1 pulse of 1.8 kV in 3-4 msec. Immediately after the pulse 1 ml of RT SOC medium was added and the suspension was transferred into a glass tube, then incubated at 37°C for 60-90 min shaking. 100 µl of suspension was plated on prewarmed LB/Amp plates (100 mg/ml ampicillin) and incubated at 37°C O/N.

4.1.1.3 Heat shock transformation

HB101 cells were thawed on ice, and aliquots of 100 µl for each transformation were transferred into prechilled 15 ml Falcon 2059 polypropylene tubes. 1 ng of transforming plasmid DNA was added to the cells, mixed gently and incubated on ice for 30 min. The cells were heat shocked for 45 sec. at 42°C in a water bath. The cells were immediately transferred

on ice and incubated for 2 min. 1 ml of RT SOC medium was added to each tube. The tubes were incubated at 37°C for 1 h by shaking at 260 rpm. 200 µl of the cell suspension was spread on prewarmed LB/Amp plates (100 mg/ml ampicillin) and incubated O/N at 37°C.

4.1.2 Plasmid DNA isolation from E.coli

4.1.2.1 Qiagen maxi preparation

The plasmids used in the transfection experiments and the generation of recombinant MVA were prepared with the Qiagen maxiprep kit, according to the manufacturer's instructions. Qiagen plasmid purification protocols are based on a modified alkaline lysis procedure, followed by adsorption of plasmid DNA to Qiagen anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer, concentrated and desalted by isopropanol precipitation.

Briefly, plasmids were prepared from E.coli DH10B or HB101 bacterial cultures grown in the presence of the selective antibiotic ampicillin. A single colony was inoculated into 3 ml of standard LB medium in the presence of ampicillin (100 µg/ml), and grown O/N at 37°C with vigorous shaking (260 rpm). The miniculture was diluted 1:500 into a larger volume of selective medium and grown to saturation (O/N) at 37°C with gently shaking (100 rpm).

The bacterial cells were harvested and centrifuged at 4200xg for 10 min at RT in a Beckman Avanti J-25 Centrifuge using a JA-10 rotor. The pellet was resuspended in 10 ml ice-cold buffer P1 containing RNaseA (1 mg/10 ml) and transferred into appropriate tubes for the JA 25.50 rotor. 10 ml of buffer P2 were added, mixed gently, and the mixture was incubated at RT for less than 5 min. 10 ml of ice-cold buffer P3 was added, mixed gently, and the mixture incubated on ice for 20 min. The suspension was centrifuged at 25000xg for 30 min at 4°C. The supernatant was transferred into a new tube and the centrifugation was repeated under the same conditions. The Qiagen column was equilibrated with 10 ml buffer QBT. Subsequently, the supernatant from the cell lysate was applied to the column. Plasmid DNA bound to Qiagen anion-exchange resin was washed twice with 30 ml buffer QC and eluted with 15 ml buffer QF, and collected into another centrifugation tube. The DNA was precipitated with 0.7 vol of isopropanol, incubated 5 min at RT and centrifuged at 25000xg for 30 min at 4°C. The DNA pellet was washed with 70% ethanol, centrifuged for 10 min at 25000xg, dried and dissolved in 300-500 µl 1xTE buffer. The DNA was stored at –20°C.

4.1.2.2 Qiagen mini preparation

This procedure, which isolates rapidly plasmid DNA from bacteria using the alkaline lysis method followed by ethanol precipitation, was used during the cloning process to isolate small amounts of DNA from a large number of colonies. The cells are disrupted by treatment with detergents and osmotic shock, followed by the alkaline denaturation of the nucleic acids. The

renaturation of the nucleic acids occurs at low pH. Chromosomal E.coli DNA renatures intermolecularly, leading to a high molecular network structure, whereas the plasmid DNA because of the topological form of its single DNA strands renatures intramolecularly. Proteins are denatured by SDS. Cellular debris, denatured proteins and chromosomal DNA are separated from plasmid DNA and RNA by centrifugation.

Briefly, each single colony was inoculated into 3 ml of LB medium (plus 100 µg/ml ampicillin) in glass tubes and grown O/N at 37°C with vigorous shaking (260 rpm). The next day 1.5 ml of the miniculture were transferred into Eppendorf tubes, and the bacterial cells were pelleted at 4000xg for 5 min in a benchtop centrifuge. The pellet was resuspended in 100 µl of ice-cold buffer P1 containing RNaseA and incubated at RT for 5 min. 200 µl of buffer P2 was added, mixed gently, and the mixture was incubated at RT for 2-3 min. 150 µl of ice-cold buffer P3 was added and mixed gently. The mix was incubated on ice for 5-10 min and then centrifuged at 10000xg for 10 min. The supernatant was transferred in a new Eppendorf tube containing 1 ml ice-cold EtOH and 10 µl 3M NaAc. The suspension was vortexed, incubated on ice for 20 min, and centrifuged for 20 min at 10000xg and 4°C. The pellet was washed with 300 µl of 70% EtOH, spun down at 10000xg for 3 min, air dried for about 30 min and dissolved in 50 µl destilled water. The DNA was stored at –20°C.

4.2 DNA techniques

4.2.1 In vitro modification and recombination of DNA

4.2.1.1 Preparation of vectors and inserts by digestion with restriction enzymes

The fragmentation of DNA with restriction enzymes is a common method to obtain DNA targets to generate new plasmids by in vitro recombination. Restriction endonucleases are enzymes, which recognize specific dsDNA-sequences and cleave both strands. Three types of restriction endonucleases are known. Type II restriction enzymes are most valuable for gene manipulation and cleave the duplex at specific target sites. Those are generally between 4 and 8 bp of length and very specific. After the cleavage 5‘-phosphorylated and 3‘-hydroxylated ends are originated. In principle each restriction enzyme needs its individual reaction conditions for hydrolysis, which are given by the manufacturer. If two or more of the needed restriction endonucleases require the same conditions, the DNA can be fragmented with a mixture of these enzymes. The typical reaction was performed in 20-50 µl total volume, using 2-10 µg DNA and 5-10 U restriction enzyme in the appropriate conditions (buffers and temperature) for 2-3 hours. The enzyme was inactivated by heating to 65°C for 10 min.

4.2.1.2 Enzymatic in vitro amplification of DNA by Taq-polymerase

The polymerase chain reaction (PCR) allows to amplify a selected DNA sequence in vitro. The synthesis starts at two primers flanking the DNA sequence of interest. The PCR is carried out in thermocyclers and consists generally of 3 steps. Each parameter has to be optimized according to the individual template-primer combination.

The initial denaturation of dsDNA at 94°C follows the sequence specific annealing of the primers at 45-65°C. Finally the thermostable DNA polymerase (temperature optimum at 72-74°C) starts the synthesis of new DNA at the 3'-OH ends of both primers that are hybridized to the DNA template. This cycle is repeated 20-35 times. A final incubation at 72°C for 2-7 min allows to finish the synthesis of incomplete DNA amplificates.

4.2.1.3 Dephosphorylation of 5' ends of DNA fragments with alkaline phosphatase

To avoid religations of linearized vectors, the 5'-phosphate of the DNA is removed to create free 5'-hydroxylated ends. For this 10-50 pmol DNA were incubated for 1 h at 37°C in the presence of 1 U alkaline phosphatase in the appropriate dephosphorylation buffer. The enzyme was inactivated by incubation at 65°C for 1 h after adding 1/10 volume 50 mM EDTA.

4.2.1.4 Creation of blunt ends by T4 DNA-polymerase or Klenow enzyme

To ligate DNA fragments lacking compatible ends the sticky ends need to be blunted. Klenow enzyme catalyzes the polymerization of DNA at the short hydroxylated ends in the presence of dNTPs. Therefore, 5'-overhangs of ssDNA can be filled in to create double stranded blunt ends. The typical reaction was performed for 30 min at 30°C in 1x PCR buffer in a total volume of 100 µl using 1U Klenow enzyme and 0.1 pmol dNTPs. The enzyme was inactivated at 65°C for 30 min.

T4 DNA-polymerase has a very active single strand specific 3'-5'-exonuclease activity in the absence of dNTPs, removing therefore the 3'-overhangs of ssDNA. The reaction was performed in 1xT4 reaction buffer in a total volume of 50 µl for 30 min at 37°C.

4.2.1.5 Ligation of DNA fragments by T4 DNA-ligase

The DNA-ligase of bacteriophage T4 catalyzes the phosphodiester binding between neighboring 3'-hydroxylated and 5'-phosphorylated ends of dsDNA. 1U of this enzyme is defined as the amount necessary to catalyze the conversion of 1nmol 32Pi in ATP at 37°C in 20 min (Weiss-Unit).

In this work all ligations were performed in 1xT4 ligation buffer using 1 µl T4 DNA-ligase (Life Technologies) in a total volume of 15 µl. Generally 50-200 ng of vector DNA were incubated with insert DNA (molar ratio of vector to insert 1:2-5) at 14°C overnight. The sample volume was filled up with A.dest. to 50 µl, and the T4-DNA ligase was inactivated at 70°C for 10 min.

For electroporation the DNA was precipitated with 500 µl butanol, air-dried and dissolved in 20 µl A.dest.

4.2.2 Purification of nucleic acids

4.2.2.1 Removal of proteins by phenol/chlorophorm extraction

Nucleic acid solutions containing a high amount of proteins or enzymes, which are difficult to inactivate (e.g. RNaseA) need to be purified by phenol extraction.

The DNA solution was mixed with the same volume of phenol/chlorophorm (1:1) and centrifuged for 2 min at maximum speed in a benchtop centrifuge. The upper water phase containing the DNA was removed carefully and re-extracted if necessary (for instance if a visible interphase of denatured proteins was present). For further use of the DNA the phenol has to be removed by extraction with chlorophorm/isoamylethanol (24:1, same volume). Subsequently, the DNA was precipitated and eluted in an appropriate amount of 1xTE buffer.

4.2.2.2 Concentration of nucleic acids by ethanol precipitation

Solutions with a low concentration of nucleic acids can be concentrated by ethanol precipitation. The DNA solution was mixed with 1/10 volume of 3M NaAc and 2.5-3 volumes of ice-cold ethanol and incubated on ice for 20 min. The nucleic acids then precipitate as sodium salts. The samples were centrifuged in a precooled benchtop centrifuge for 15 min at 12000xg and 4°C. The pellet was washed with 70% ethanol and air dried for 30 min or in a „speed-vac“ concentrator for 1-2 min. The DNA was eluted in an appropriate volume of 1xTE buffer or water.

4.2.2.3 Photometric estimation of purity and concentration of nucleic acids

According to the law of Lambert-Beer the concentration of a solution is directly proportional to its extinction or absorption.

To determine quality and yield, the DNA was measured in an UV spectrophotometer (Pharmacia Biotech) at 260 nm (absorption maximum of nucleic acids) and 280 nm (absorption maximum of proteins), normally diluted 1:50 in destilled water. The nucleic acid concentration was calculated by the spectrophotometric conversions:

dsDNA	1 OD_{260} = 50 µg/ml
ssDNA	1 OD_{260} = 40 µg/ml
ss oligonucleotides	1 OD_{260} = 30 µg/ml

In addition the ratio A_{260}/A_{280} was measured, which should be 1.7-1.9 for protein free solutions of nucleic acids.

4.2.2.4 Isolation of DNA fragments from agarose gels (silica adsorption)

The DNA band was excised from the agarose gel with a clean, sharp scalpel. The gel slice was minimized in size by removing excess agarose and weighed. 3 volumes of buffer QX1 were added to 1 volume of gel for DNA fragments of 100 bp to 4 kb. For DNA fragments > 4 kb 2 volumes of water were added additionally. The silica matrix was resuspended by vortexing for 30 sec and added according to the DNA amount:

< 2 μg DNA	10 μl of Qiaex II
2-10 μg DNA	30 μl of Qiaex II
each additional 10 μg DNA	add additional 30 μl of Qiaex II

This mixture was incubated at 50°C for 10 min with occasionally mixing to keep the silica matrix in suspension. This allows the release of DNA from the melting agarose and adsorption to the silica matrix. Since the adsorption is only efficient at pH < 7.5, those conditions need to be checked and if necessary adjusted by adding of 10 μl 3M sodium acetate, pH 5.5, followed by an incubation at RT for further 10 min. The sample was centrifuged for 30 sec and the supernatant removed carefully with a pipet. The pellet containing the DNA was washed once with 500 μl buffer QX1 to remove residual agarose contaminations and twice with 500 μl buffer PE to remove residual salt contaminations. The pellet was air-dried for 30 min until it became white. The DNA was eluted in 20 μl water by vortexing the pellet and incubation according to the size of the DNA fragments:

DNA fragments < 4 kb	RT for 5 min
DNA fragments 4-10 kb	50°C for 5 min
DNA fragments > 10 kb	50°C for 50 min

The sample was centrifuged for 30 sec at 10000xg in a benchtop centrifuge and the supernatant, containing the purified DNA, was pipeted into a clean Eppendorf tube and stored at –20°C.

4.2.2.5 Purification of PCR products

After the PCR procedure, surplus nucleotides, primers and enzymes have to be separated from the PCR product. This is possible by using a commercial available kit (e.g. Qiagen PCR purification kit) or by loading the sample to an agarose gel and recovering the DNA as described above.

4.2.3 Analysis of plasmid DNA

4.2.3.1 Restriction analysis

Fragmentation of DNA by restriction enzymes can be used to check the insertion and orientation of a foreign gene in the plasmid vector after cloning. Preferably one or two enzymes were chosen, cutting once in the insert and once in the vector, leading to DNA fragments easily distinguishable in size.

Analysis of plasmid DNA obtained after mini or maxi preparation was carried out in a total volume of 10-20 µl, using 2.5-5 U enzyme in the appropriate buffer. After incubation for 1 h the sample was loaded to an agarose gel for further analysis.

4.2.3.2 Analysis by horizontal agarose gel-electrophoresis

Agarose, which is extracted from seaweed, is a linear polymer, that forms a matrix upon hardening after boiled up in a desired buffer. When an electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates towards the anode. The rate of migration is determined by the molecular size of the DNA, the agarose concentration, the conformation of the DNA, the applied voltage and the composition of the electrophoresis buffer. The paracristalline mesh system of agarose gels allows the separation of high molecular DNA. The density is dependent on the agarose concentration, varying between 0.5 and 2.5%. For resolution of linear dsDNA fragments of 0.5-10 kb an agarose concentration of 1% is recommended.

The agarose was dissolved in 1xTAE buffer by boiling. After cooling down to 50-60°C, ethidium bromide was added to a final concentration of 0.5-1 µg/ml and thoroughly mixed. The solution was poured into a gel chamber, a comb was introduced to create the slots, and the gel left to polymerize. After the gel was completely set it was mounted into the electrophoresis tank and covered with 1xTAE buffer. DNA samples mixed with DNA gel-loading buffer were introduced into the slots of the submerged gel. A voltage of 5-10 V/cm was applied for the requisite time.

Ethidum bromide intercalates with the DNA and can be detected by ultraviolet light (250-360 nm). The minimum amount of DNA that can be detected by photography of ethidium-bromide-stained gels is about 20 ng in a 0.5 cm wide band.

4. 3 Analysis of proteins

4.3.1 Discontinous denaturating PAA-gel electrophoresis (SDS-PAGE)

Proteins are composed of different amino acids and do not have a constant ratio of mass and charge. By heating up the proteins, the three dimensional structure unfolds and the highly ionic detergent SDS can bind, making all proteins irrespective of their amino acid composition negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. Modifications to the polypeptide backbone, such as N- or O-linked glycosylation, however, have a significant impact on the apparent molecular weight. Thus, the apparent molecular weight of glycosylated proteins is not a true reflection of the mass of the polypeptide chain.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by a bifunctional agent. The effective range of separation depends on the concentration of

polyacrylamide used to cast the gel and on the amount of cross-linking. Cross-links formed from bisacrylamide add rigidity and tensile strength to the gel and form pores through which the SDS-polypeptide complexes must pass. The SDS-polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes.

To obtain sharp protein bands, the gel is composed of a stacking gel (5%, pH 6.8) and a resolving gel (6-15%, pH 8.8). The chloride ions in the sample and in the stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecules. Between the leading and the trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient which sweeps the polypeptides from the sample and deposits them on the surface of the resolving gel. There, the higher pH of the resolving gel favors the ionization of glycine, and the resulting glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size by sieving.

The samples were prepared by heating them in 1x SDS gel-loading buffer to 95°C for 10 min to denature the proteins and a brief sonication to shear the DNA. The gel was placed in a vertical position into the electrophoresis tank and the samples loaded into the wells. A voltage of 8 V/cm was applied to the gel. After the dye front had moved into the resolving gel, the voltage was increased to 15 V/cm, and the gel ran until the bromophenol blue front reached the bottom of the resolving gel.

4.3.2 Identification of proteins by immunoblotting (Western blotting)

Western blotting is a useful method for the identification and quantitation of specific proteins in complex mixtures of proteins that are not radiolabeled. Because electrophoretic separation of proteins is almost always carried out under denaturing conditions, any problems of solubilization, aggregation and coprecipitation of the target protein with adventitious proteins are eliminated. Antibodies react specifically with antigenic epitopes displayed by the target protein attached to the solid support.

After separating the proteins by SDS-polyacrylamide gel electrophoresis according to their molecular weight, they were transferred to a nitrocellulose membrane (BioRad) using a Semi-dry Electroblotter (Sartorius). In the electric field the negative charged proteins migrate towards the anode, leaving the gel and meeting the PVDF-membrane, where they hold on via hydrophobic and electrostatic interactions.

Whatman 3MM paper and the nitrocelulose membrane were cut to the size of the resolving gel. Whatman paper, membrane and PAA resolving gel were soaked in 1x transfer buffer and

sandwiched in the blotting apparatus as follows:

+ anode +

3 MM Whatman paper

PVDF-membrane

PAA-resolving gel

3MM Whatman paper

- cathode -

The components have to fit tightly to each other, without introducing bubbles. The transfer occurred in 1 h 30 min at 150 mA, maximum 20 V (mA = cm^2 of membrane x 0.8).

The immobilized proteins on the membrane can be detected by immunological methods. First the membrane was blocked in 1x washing buffer (see 3.2) containing 1% BSA for 1 h at RT or at 4°C O/N. The primary antibody was diluted in blocking buffer and incubated with the membrane for 1.5 h at RT with continuous agitation. The membrane was washed 3x with washing buffer, then the secondary antibody (horseradish peroxidase conjugated), directed against the Fc-part of the primary antibody, was incubated with the membrane in blocking buffer for 45 min. After washing the membrane 3x with washing buffer Lumi-Light Western Blotting Substrate (Roche) was added and incubated for 5 min, rocking.

The chemiluminescence reaction results in the emission of photons (wavelength 425 nm), which are detectable by X-ray films. The exposure time to the film is dependent of the signal intensity. 10-50 pg antigen are detectable with this method.

4.3.3 Stripping of the Western Blot

Substrate and antibodies can be stripped off the membrane, and the blot can be reused for further Western blot analysis.

The membrane was incubated for 1 h at 50°C in stripping buffer (see 3.2) and washed 2x for 10 min in PBS.

The membrane is now ready to start with a new immunoblotting procedure, beginning with blocking procedure as described above.

3.3.4 Quantification of proteins

The determination of total protein can be carried out in a UV spectrophotometer, measuring the absorbance at 260 and 280 nm. The concentration of proteins (C) can be calculated as follows:

$C\ (mg/ml) = 1.55\ A_{280} - 0.76\ A_{260}$

Another common method is the determination using the bicinchoninic acid (BCA) kit from Pierce according to the manufacturer's instructions. This system combines the biuret reaction (proteins reducing Cu^{2+} in an alkaline medium to produce Cu^{+}) with the highly selective detection reagent BCA. BCA in the form of its water-soluble solution salt, is a sensitive, stable and highly specific reagent for the cuprous ion and forms a complex BCA-Cu^{+}-BCA, exhibiting a strong absorbance at 562 nm. Dilutions in TMN buffer (for samples obtained after CsCl

gradient purification) for a series of 8 calibration standards using a BSA stock solution of 2 mg/ml were prepared, sufficient for a working range of 20-2000 µg/ml. The reaction was carried out in microtiter plates. 25 µl of each standard, TMN buffer (blank) or samples were pipeted into the appropriate microwell plate wells. 200 µl of the working reagent (50 parts of reagent A mixed with 1 part of reagent B) were added to each well, and the plate was incubated at 37°C for 30 min. The absorbance was measured using a microelisa plate reader (BioRad) at 570 nm. A standard curve was prepared by plotting the absorbance values obtained on the x-axis against the specific standard protein concentrations on the y-axis, performing linear regression analysis with Microsoft Excel 5.0. Using the standard curve, the protein concentration for each unknown protein sample was determined.

4.4 Cell culture techniques

4.4.1 Host cells for in vitro cultivation of MVA

MVA grows efficiently in avian cells and baby hamster kidney cells (BHK21). After introducing the Vaccinia Virus specific K1L gene into the genome of MVA as a selection marker, this recombinant MVA is able to multiply in rabbit kidney cells (RK13).

4.4.1.1 Preparation of chicken embryo fibroblasts (CEF)

Since MVA was generated on primary chicken embryo fibroblasts, those cells were used mostly in this work. CEFs were prepared weekly using 20 chicken embryos (9-11 days old):
The upper part of the fertilized eggs was cleaned with 70% ethanol and opened with a sterile scissor. The embryos were drawn out with a sterile forceps, decapitated and transferred to a dish containing sterile PBS. Legs and inner organs were removed. The embryos were collected into a 20 ml syringe without a needle and squeezed into an Erlenmeyer flask containing 200 ml PBS, where the cell clumps sedimented. After 1 min the supernatant was discarded, and the cells washed again with 200 ml PBS. Cell aggregates were disturbed by quick magnetic stirring for 25 min in the presence of 50 ml Trypsin. The suspension was filtered carefully through 2 layers of sterile mesh (avoiding to pour cell clumps on the mesh). For the remaining cell clumps in the Erlenmeyer flask this step was repeated once or twice, using 25 ml Trypsin each. The filtrate was filled up with PBS to 300 ml, distributed into six 50 ml Falcon tubes and centrifuged for 7 min at 500xg and 4°C in a Heraeus swing out centrifuge. The supernatant was discarded and the pellet resuspended well in 10 ml PBS with a 10 ml pipet. The suspension was filled up with PBS to 50 ml total volume per Falcon tube and centrifuged as before. This washing step was repeated twice to remove the Trypsin. The pellets were pooled and resuspended in 10 ml medium (MEM containing 10% Lactalbumin, 5% BMS and 1% Antibiotics/Antimycotics). 50 ml medium was added and the cell suspension was filtered again through 2 layers of mesh to remove remaining cell clumps. Those cells were then introduced into 2 l medium and distributed into T225 flasks, using 40-50 ml per flask. The cells were incubated at 37°C and 5% CO_2 and should be confluent within 3-4 days.

It is possible to obtain at least a first passage of very good cell quality by splitting them 1:5 - 1:10. Primary CEFs with a subconfluent monolayer can „rest“ at 30°C for one week and then be used for the first passage, still having a very nice cell quality. Generally CEFs were prepared freshly.

4.4.1.2 Cell culture techniques for BHK21 and RK13 cells

BHK21 and RK13 are adherent cells. The growth by cell division is inhibited by contact with neighboring cells, leading to a confluent monolayer.

The cells were maintained under standard cell culture conditions using RPMI1640. The media were supplemented with 1% antibiotic-antimycotic solution and 10% fetal calf serum (FCS, inactivated for 30 min at 56°C) for RK13 cells and 7% FCS for BHK21 cells respectively. The cells were kept in culture for serial passages (maximum 50), and were expanded 1:5 – 1:10 twice weekly by trypsinization. The old medium was removed from the cells and the monolayer washed with PBS. Trypsin-EDTA solution was introduced according to the growth area and the number of cells (e.g. 5 ml for a T185 cell culture flask, $\approx 2x10^7$ cells) and incubated for 1-2 min for BHK21 and 10-15 min at 37°C for RK13 respectively, until the cells detached from the bottom of the flask. The cells were resuspended in media and distributed into new cell culture flasks in the appropriate amount of media (e.g. 25-30 ml for T185).

For long time storage in liquid nitrogen (-196°C) the media of a T25 flask was removed, the cells washed with PBS and the confluent monolayer treated with 2-3 ml Trypsin/EDTA as described above. The cells were transferred into a 15 ml Falcon tube and pelleted for 5 min at 500xg. The supernatant was removed and the pellet resuspended in 1.5 ml FCS. RPMI1640 with the recommended amount of FCS and 20% DMSO was added and mixed gently. The cells were aliquoted into cryovials (1 ml each vial), incubated for 10 min on ice, stored for 1-2 days at –80°C and then transferred into liquid nitrogen for long term storage.

To take the frozen cells in culture, the cryovials were warmed up to 37°C as quick as possible. Immediately after the freezing medium was thawed, the cells were introduced into 10 ml prewarmed RPMI1640 containing the recommended amount of FCS, and pelleted at 500xg for 5 min. The cell pellet was resuspended in medium, transferred to a T25 cell culture flask and incubated at 37°C and 5% CO_2.

4.4.2 Amplification of Vaccinia Virus

The amplification of MVA to generate working stocks (crude stocks) can be carried out on CEF and BHK21.

Ten T185 cell culture flasks with subconfluent monolayers of CEF or BHK21 cells were infected with a m.o.i. of 0.1-1 p.f.u. in 10 ml medium per flask with occasional shaking. After 1 h 15 ml medium per flask were added. The cells were kept in the incubator until lysis (36 h for m.o.i. of 1, 2-3 days for m.o.i. of 0.1). During the infection the cells round up and finally

detach. The cells were harvested by scraping them off from the bottom of the flask and transferred into centrifuge tubes. After a centrifugation for 15 min at 4°C and 3500xg, the pellet was resuspended in 1 ml medium without supplements per flask, obtaining 10 ml virus suspension in total. The virions inside the cells were released by disrupting the cells. This was done by 3 cycles of freeze-thawing using dry ice and a 37°C water bath, vortexing vigorously after the thawing. Those crude stocks were stored at –80°C and sonicated briefly before use.
Virus stocks of WR and IHD-J, respectively were generated by infection of HeLa cells with a m.o.i. of 0.1. Intracellular virus was isolated and semi-purified at 3 days post infection as described elsewhere (Pedersen et al., 2000).

4.4.3 Determination of infectivity

4.4.3.1 Titration of MVA ($TCID_{50}$)

To determine the titer of a virus suspension subconfluent monolayers of BHK21 or CEF cells in 96-well plates were infected with different dilutions. To avoid aggregations of virus particles, the suspension was sonicated 3 times for 1 min in ice water, followed by vigorous vortexing. The homogenous virus suspension was diluted in $\log_{10}$ steps, usually beginning with 10^{-2}. 100 µl virus dilution was added to 100 µl medium on top of the cells into 8 wells per dilution. The cells were incubated for 5 days at 37°C and 5% CO_2. The titer was calculated by the number of wells showing a cytopathic effect (CPE) of the cells according to the method of Spearman & Kaerber:
First the $\log_{10}$ of the dilution where 50% of the wells show an infection was determined:

$\log_{10} = x - d/2 + (d\Sigma r/n)$

x = highest dilution with all 8 wells showing a CPE
d = $\log_{10}$ of the dilution factor (d = 1 for 10 fold dilutions)
r = number of positive wells per dilution
n = total number of wells per dilution (here: n = 8)
The reverse value resulting of this formula is the virus titer referred to the infectious dose per unit volume, in this case $TCID_{50}$/0.1 ml, because the cells were infected with 100 µl virus dilution per well. The virus titer, given in $TCID_{50}$/ml can be calculated.

4.4.3.2 Titration of MVA by immunostaining

MVA was plaque titrated on CEFs grown in 6-well plates infected with 10 fold serial dilutions of the virus. Cells were fixed at 24 h.p.i. with a 1:1 mixture of methanol:acetone, and the virus detected with a rabbit serum against Vaccinia Virus (Sigma) in a dilution of 1:500 – 1:1000. The procedure is described in detail in 4.5.4.2. With this procedure the plaque forming unit/ml was calculated.

4.4.3.3 Titration of WR and IHD-J by cristal violet staining

WR and IHD-J were plaque titrated on BSC-40 cells by infecting monolayers with 10 fold serial dilutions of the virus. Cells were fixed and plaques visualized 24 h.p.i. with a mixture of 0.2% crystal violet and 3% formaldehyde in PBS. Living cells incorporate the crystal violet and obtain a dark blue color, whereas dead cells do not. With the number of white plaques on a blue monolayer the plaque forming unit/ml is calculated.

4.4.4 Plasmid-DNA transfection of infected adherent cells

Different methods and kits for transfection of DNA into eukaryotic cells are commercially available. In this work LipofectAmine Plus Reagent (Life Technologies) was used.

4.4.4.1 Stable transfection for generation of recombinant MVA

The generation of recombinant vaccinia viruses requires a stable insertion of the foreign DNA into the virus genome. Infection of the cells with a low m.o.i. of virus and transfection with a surplus of plasmid DNA increases the possibility to generate recombinants. The plasmids used in this work were constructed to allow homologous recombination of the gene of interest into the MVA genome. Those genes are subjected to transcriptional control of vaccinia virus specific promotors.

CEF or BHK21 cells were grown in 6 well plates to 80% confluence. The medium was removed and replaced by a virus dilution with a moi of 0.05-0.1 p.f.u. in 800 µl serum-free OptiMem per well. The cells were incubated for 1.5 h at 37°C and 5% CO_2 with occasional shaking. Meanwhile the transfection reagents were prepared:

Mix A: 15 µg Plasmid DNA were pre-complexed with 15 µl Plus reagent in 200 µl serum-free OptiMem for 15 min at RT.

Mix B: 15 µl LipofectAmin reagent were mixed with 200 µl serum-free OptiMem.

Mix A and B were combined, mixed and incubated for 15 min at RT. DNA-LipofectAmin complexes are now forming.

The infection-medium was removed from the cells, and the monolayers washed twice with serum-free prewarmed OptiMem.

The transfection mix was added to each well containing 555 µl OptiMem, reaching a total volume of 1 ml per 6 well. The complexes were mixed gently into the medium and incubated at 37°C and 5% CO_2 over night. The next day, the transfection mix was replaced by 2 ml complete medium and incubated for another day. The cells were harvested by scraping them off with a rubber policeman in 1-1.5 ml medium and frozen at –80°C.

4.4.4.2 Transient transfection

Detection of protein expression and protein localization in the cell requires the infection of every cell to provide enzymes for gene transcription.

4.4.4.2.1 Transient transfection for protein detection in western blot analysis

Infection and transfection of the cells were performed as described above, but the cells were infected with a moi of 10 p.f.u. The cells were scraped off in 0.5-1 ml medium, transferred into a 1.5 ml Eppendorf tube and spun down for 2 min at 8000xg in a benchtop centrifuge. The pellet was resuspended in 300 µl 2x sample buffer for SDS-PAGE gel electrophoresis and stored at –20°C.

4.4.4.2.2 Transient transfection for protein localization in electron microscopic studies

CEF cells grown in petri dishes (∅ 6 cm) to 80% confluency were infected with a m.o.i. of 10 p.f.u. in 2 ml MEM without supplements for 1.5 h.

2.5 µg DNA was diluted in 250 µl MEM and 8 µl Plus reagent, mixed and incubated for 15 min at RT.

225 µl MEM and 12 µl LipofectAmin reagent were mixed, added to the pre-complexed DNA, mixed again and incubated for another 15 min at RT.

The infection-medium was removed from the cells and the monolayers washed twice with MEM. 2 ml MEM were introduced to the petri dish, the transfection mix (500 µl) was added, mixed gently by shaking and incubated at 37°C and 5% CO_2. After 3 h the transfection-medium was replaced by MEM containing 5% FCS and 1% antibiotics/antimycotics. 40 hours post transfection the cells were fixed for electron microscopy analysis (see 4.6.2)

4.5 Generation and characterization of recombinant MVA

High rates of recombination occur within poxvirus-infected cells. The vaccinia virus genome recombines on average once in 1000 replication cycles. Recombination does not require late gene products and can also occur between virus-derived genomic DNA and transfected plasmid DNA. The transfected plasmids contain an insertion cassette of the gene of interest under control of a vaccinia virus specific promotor and the vaccinia virus K1L gene as a selection marker. This cassette is flanked upstream and downstream by sequences homologous to sequences of the MVA genome. The homologous sequences are chosen in a way to allow site directed recombination into regions where deletions had occurred naturally in the MVA genome.

The K1L gene is expressed early in infection and is required for replication of vaccinia virus in rabbit kidney cells (RK13). Viral genomes rapidly eliminate direct repeats with the formation of intra- and intermolecular recombination products. The K1L gene is flanked by lacZ-repeats, allowing its deletion by intramolecular recombination.

4.5.1 Generation of rMVA by homologous recombination

4.5.1.1 Growth selection of recombinant MVA on RK13 cell monolayers

Since MVA wt is unable to replicate efficiently in RK13 cells, this cell-line is used for growth-selection of recombinant MVA. In contrast to the usually plaque formation as a result of virus

infection, RK13 cells show a different phenotype when infected with MVA containing the K1L gene. The infected cell and the neighboring cells lose their growth inhibition achieved by cell-cell contact and move towards the infected cell in the center, building a three-dimensional aggregate.

The virus suspension from the transfection (see 4.4.4.1) was freeze-thawed 3 times, sonicated in iced water for 1 min using a cup sonicator (Sonoplus DH 200, Bandelin, Germany), plated on confluent RK13 cells in 6 well plates in dilutions from 10^{-1} to 10^{-4}, and incubated at 37°C and 5% CO_2 for 72 hours. Three days after infection aggregations (foci) of RK13 cells infected with recombinant MVA were picked in a 20 µl volume by aspiration with an air-displacement pipette (Gilson, Villiers-le-Bel, France) and transferred to microcentrifuge tubes containing 500 µl medium, and processed by freeze-thawing and sonication for further infections of RK13 cell monolayers. In this first round it was recommended to pick 10-20 different foci (viral clones). Not all foci contain recombinant viruses, also transient expression of the K1L gene in remaining plasmids allows wt MVA to grow on RK13 cells. In the second passage such viruses show a negative growth. Cloning of recombinant MVA by passaging on RK13 cells was continued until PCR analysis of viral DNA extracted from RK13 cells confirmed to absence of wild type sequence (see 4.5.3.2). Screening for synthesis of target protein by immunostaining of fixed cell monolayers (see 4.5.4.2) or Western blot analysis of cell lysates (see 4.5.4.1) was carried out.

4.5.1.2 Deletion of the marker gene K1L

After elimination of parental MVA ten-fold serial dilutions of the recombinant viruses were used for infection of semi-confluent BHK21 or CEF cells grown in 6-well tissue-culture plates. BHK21 and CEF cells show a strong cytopathic effect resulting in the lysis of the infected cells, finally leading to the typical plaque formation („holes“ in the cell monolayer). Those plaques were picked as described above. Absence of selective pressure allows the elimination of the K1L-gene by intramolecular recombination. Recombinant MVA were passaged until PCR analysis confirmed the absence of the K1L gene. K1L-free recombinant viruses should regain their growth deficiency on RK13, which was tested in parallel by infecting RK13 cell monolayers.

Cloning of recombinant MVA can also be carried out in 96-well plates. Wells containing one single CPE were harvested in total, followed by the procedure described above. In view of the fact that MVA produces a lot of extracellular enveloped viruses (EEV), which might contaminate the selected clones, this method is advantageous for generating recombinants and deleting the K1L-gene by avoiding EEV-spread using physical barriers.

Alternatively EEV-spread can be blocked by an agarose overlay. 2 h.p.i. the inoculum was removed and cultures supplemented with a 1:1 mixture of 2x medium (MEM, 2% FCS) and 2% Low-Melting-Point Agarose (Gibco-BRL). In this case, the CPEs were picked by aspiration using an inserted tip of a sterile cotton-plugged Pasteur pipette. Cells were scraped and

aspirated together with an agarose plug by squeezing a rubber bulb on the Pasteur pipette and the material was transferred to a tube containing 0.5 ml medium.
Recombinant viruses were amplified upon infection of BHK21/CEF cell monolayers (see 4.4.2.1).

4.5.2 Analysis of recombinant MVA-DNA

Recombinant MVA has to meet the demands of insertion of the foreign gene into the virus genome, expression of the foreign protein and elimination of parental wt MVA and the host range gene K1L.
In this work PCR analysis was used to check the elimination of wt MVA and K1L and the insertion of the foreign gene.

4.5.2.1 Isolation of MVA-DNA

Recombinant viruses were grown at a high m.o.i. in 6-well tissue culture plates until strong cytopathic effects of the cell monolayer were visible (usually the 10^{-1} dilution of a passage was sufficient). The cells were scraped off in 0.5-1 ml medium, transferred into a microcentrifuge tube and freeze-thawed three times to release the virions from the infected cells. Cellular debris were removed by centrifugation for 5 min at 320xg in a benchtop centrifuge. 350 µl supernatant containing the virus was introduced to a new Eppendorf tube, mixed with 50 µl 10x TEN buffer, 5 µl proteinase K (10 mg/ml in 1mM $CaCl_2$), and 50 µl 10% SDS and incubated for 2-3 hours at 56°C.
The DNA was purified by phenol/chlorophorm extraction (see 4.2.2.1), precipitated with ethanol (see 4.2.2.2) and eluted in 50 µl water or 1xTE buffer.

4.5.2.2 PCR analysis of recombinant MVA-DNA

MVA wt and recombinant DNA differ in size at the insertion-site of the foreign gene. Primers NIHGS83 and IIIfl1B are chosen complementary to the flanking sequences. Three PCR products can be distinguished (a schematic overview is depicted in Fig. 22C):

- wt DNA, amplification of the naturally deletion site: ≈ 600 bp for deletion III
- recombinant MVA including the K1L gene: ≈ 600 bp wt DNA + 1100 bp K1L gene + foreign gene
- recombinant MVA: ≈ 600 bp wt DNA + foreign gene

MVA wt DNA and the transfected plasmids were used as controls. The reaction was carried out in 0.2 ml PCR tubes in a total volume of 100 µl, using PCR Master (Roche)

MVA-DNA	10 µl (out of 50 µl ≈ 200-500 ng)
PCR Mastermix	50 µl (1x PCR buffer, 2.5 U Taq polymerase in Brij 35, 0.005% (v/v), dNTP each 0.2mM, 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$)

primers	5 µl each (10 pmol/µl)
H_2O	30 µl

DNA was denatured at 94°C for 2 min and went through 30 cycles of:

DNA-denaturation	94°C, 1 min
primer-annealing	55°C, 1 min
elongation	72°C, 4 min

The reaction was stopped by an incubation of 7 min at 72°C, and cooled down to 4°C. 10-20 µl of the reaction sample were analyzed by gel electrophoresis (see 4.2.3.2). Since the PCR-fragment for recombinant MVA still including the K1L gene might be quite big and therefore difficult to amplify, the absence of the K1L gene was confirmed by testing the growth ability on RK13 cells.

4.5.3 Detection of recombinant protein in MVA infected cells

4.5.3.1 Protein detection by Western blot analysis

Immunoblotting was performed as described (see 4.3.2). Polyclonal rabbit serum anti-CDV.H was used at a dilution of 1:5000, anti-rabbit peroxidase in a dilution of 1:10000.

4.5.3.2 Protein detection by immunostaining

Proteins can also be detected in fixed and permeabilized monolayers of virus infected cells using antibodies and horseradish peroxidase conjugates. The visualization occurs by adding o-dianisidine the substrate for horseradish peroxidase. The procedure is shown for cells grown in 6-well plates.

Medium was removed and the cell monolayers fixed and permeabelized with a 1:1 mixture of acetone:methanol (1 ml per well) for 2-10 min at RT. The fix was removed and the monolayers dried for some minutes. 2 ml PBS-2% FCS were added for blocking (1 h at RT or O/N at 4°C) to minimize unspecific binding of the antibodies. The first antibody was diluted in blocking buffer and incubated on the cells (2 ml/well) for 1-3 h at RT, by gentle rocking. 1^{st} antibody was removed and the cells washed three times with PBS to remove unbound antibodies. 2^{nd} antibody (horseradish peroxidase-conjugated, raised against the Fc-part of the 1^{st} antibody) was diluted in blocking buffer, introduced to the cells (2ml/well) and incubated for 1-2 h at RT, rocking gently. Meanwhile a saturated solution of dianisidine (Sigma No. 09143) in 500 µl absolute ethanol was prepared, vortexed thoroughly and stored in the dark for at least 30 min. Undissolved dianisidine was pelleted briefly, 200 µl saturated dianisidine solution was mixed with 9.8 ml PBS and filtrated (0.2 µm). 20 µl H_2O_2 (>30%) were added and the substrate introduced to the cells (0.5-1 ml per well) after washing them 3 x with PBS. Stained foci are visible within 5-20 min. The staining reaction was stopped by washing with water or PBS.

4.5.4 Multiple step growth curve

To investigate the ability of virus to productively grow in target cell monolayers this highly sensitive method was established.

The number of target cells grown in one well of a 6-well plate was determined and virus material homogenized by sonication was diluted in suitable medium to allow infection of target cells at a m.o.i. of 0.05 infectious units (IU)/cell using a 1 ml volume per well. Growth medium was removed, 1 ml virus suspension per well was added and adsorbed for 1 h at 37°C. The inoculum was removed and the cell monolayers washed carefully three times with medium (2 ml/well) to remove free virus. 1.5 ml growth medium including 2% FCS and 1% AB/AM were added per well and incubated at 37°C and 5% CO_2.

At 0, 12, 24, 48, 72 hours after infection supernatant and cell lysate of the infected cultures were harvested separately. The supernatant (containing EEV) was transfered to an Eppendorf tube, 1.5 ml growth medium was added to the cell monolayer, cells (containing all cell associated viruses) were scraped off and transfered with the medium into a new Eppendorf tube. Aliquots were frozen and stored at –80°C.

To determine the amount of infectious virus present at the various times after infection, the cell lysates were freeze-thawed (3x) and sonicated, the supernatant was used fresh directly after harvesting and titrated as described (see 4.4.3).

Alternatively, this method can be carried out in 12-well plates, using 0.5 ml inoculum and 1 ml growth medium.

4.5.5 Virus purification on CsCl gradients

This procedure allows to separate different enveloped virus particles according to their density. Enveloped viruses such as EEV and CEV sediment at a buoyant density of about 1.24 g/ml, whereas a density of 1.27 to 1.28 g/ml is characteristic for IMV.

4.5.5.1 Separation and concentration of virus fractions

Confluent CEFs grown in 7 cell culture flasks T225 were infected at an m.o.i. of 0.1 or 10 p.f.u. in 35 ml medium/flask and harvested at 50 h.p.i. or 40 h.p.i. respectively.

Extracellular fraction:

Medium was harvested, the cell monolayers washed with 10 ml PBS/flask, and the PBS pooled with the medium in a 50 ml Falcon tube. The supernatant was clarified by centrifugation at 1000xg and 4°C for 30 min. The clarification was repeated until the suspension was very clear. The supernatant was introduced in Beckmann ultraclear centrifuge tubes and pelleted for 1 h at 40000xg and 4°C in a Beckman Optima LE-80K Ultracentrifuge using a SW 28 rotor. Pellets were pooled and resuspended in 7 ml TMN buffer (see 3.2).

Cell associated fraction:

5 ml Trypsin/EDTA were introduced into each flask and incubated on the monolayers until cell detachment. During this procedure CEVs detach from the cells. Cells and trypsin were pooled

and introduced into a 50 ml Falcon tube containing 10 ml FCS to stop the trypsin reaction. The sample was clarified twice at 1000xg for 30 min. The supernatant (CEV fraction) was pelleted and resuspended in TMN as described above.

Intracellular fraction:

The remaining pellet after clarification of the cell associated fraction was resuspended in 45 ml PBS, freeze-thawed and sonicated 3x to disrupt the cells and release the intracellular viruses. Clarification and concentration was performed as described above.

For using these fractions directly in Western blot analysis 5 T225 flasks were infected, the pellets were resuspended in 600 µl TMN buffer and stored at –80°C.

4.5.5.2 Sucrose cushion

4 ml of sterile 25% (w/w) sucrose in TMN buffer in a 12 ml ultraclear centrifuge tube (Beckmann) were carefully overlaid with 7 ml virus suspension. The virus fractions were centrifuged for 2 h at 160000xg and 4°C in a SW 41 rotor through the sucrose cushion. The pellets were resuspended thoroughly in 0.5 ml TMN buffer and sonicated briefly before being layered over a preformed CsCl gradient.

4.5.5.3 Cesium chloride gradient (Payne and Norrby, 1976)

The gradients were prepared one day before the centrifugation. CsCl solutions in TMN of different concentrations (ρ = 1.3, ρ = 1.25, ρ = 1.2) were made, the concentration adjusted by measuring the viscosity with a refractometer:

$\rho = 10.2402\ \eta - 12.6483$

ρ = concentration

η = density

CsCl gradients were formed in 12 ml ultra clear centrifuge tubes (Beckmann) using 2.5 ml of 1.3 g/ml, 3.75 ml of 1.25 g/ml and 5 ml of 1.2 g/ml and equilibrated over night at 4°C.

Virus suspensions were loaded on the gradient and centrifuged for 1 h at 18°C and 180000xg in a Beckmann Ultracentrifuge using a SW 41 rotor. Fractions ($\approx$ 0.5 ml) were collected from the bottom of the tube. The absorbance at 280 nm and the density of each fraction were measured. Fractions corresponding to viral peaks were used for further analysis.

4.6 Electron microscopic analysis

EM analysis allows a direct observation of infected cells or single virus particles. Studies were carried out with a Philips 400T electron microscope, using magnifications of 8000 to 17000. In this work two different methods were used.

4.6.1 Immunolabeling and negative staining of viral particles

Purified virus particles obtained after CsCl density centrifugation are already characterized due to their envelopment by the buoyant density of their sedimentation. The following procedure allows to investigate the state of the particles and the associated proteins.

FormVar-coated copper grids (1% FormVar in chlorophorm) were carbonated and made hydrophilic by submitting them to a glow discharge. Labeling was performed by floating the grids on solutions placed as droplets onto Parafilm sheets (Griffiths et al., 1983). Grids (300 mesh) were incubated on 20 µl virus suspension from CsCl gradient fractions corresponding to viral peaks (as measured by OD_{260}) for 10 min, washed in PBS and blocked for 5 min in 20 µl PBG (see 3.3). Antibodies were diluted in PBG as recommended, and the grids were incubated on top of a drop of antibody solution for 15-20 min. After washing 6 times in PBS grids were incubated for 15 min with anti rabbit protein A gold (10 nm), diluted in PBG according to the manufacturers instruction. Grids were washed 6 x in PBS and 6 x in $dddH_2O$, negative stained and protected against collapse during air drying in a 4:1 mixture of 2% methyl- cellulose and 3% uranyl acetate for 10 min on ice. Excess fluid was removed from the grids by touching them with a filter paper, and the specimen were air dried while held in wire loops.

4.6.2 Electron microscopic analysis of infected cells

In this work EM analysis of infected cells was used to quantify the different forms of MVA virions (IV, IMV, IEV, CEV and EEV) at various time points of infection, using different multiplicities of infection.

4.6.2.1 Infection and drug treatment

CEFs grown in petri dishes (∅ 6 cm) were infected with MVA at an m.o.i. of 10 or 0.1 p.f.u. in 2 ml serum free MEM for 1 h. Monolayers were washed twice with MEM, then incubated with 5 ml MEM (5% FCS) at 37°C and 5% CO_2 until fixation.

In the case of drug treatment 10 µg/ml IMCBH (provided by Aventis Pharma, Frankfurt, stock solution: 10 mg/ml) was added to the growth medium immediately after the infection and 500 ng/ml Brefeldin A (SIGMA, stock solution: 5 mg/ml in 75% ethanol) 14 h.p.i. respectively. The drugs remained in the medium until fixation. IMCBH specifically inhibits release of EEV by blocking the formation of IEV (Payne and Kristenson, 1979). The target of IMCBH is a 37 kDa EEV protein, the gene product of F13L. This protein is essential for EEV formation (Blasco and Moss, 1991). Brefeldin A causes the ER to fuse with the Golgi complex. Consequently, membrane traffic is blocked and newly synthesized proteins can not leave the fused ER/Golgi. The drug has been shown to block EEV formation most likely because EEV membrane proteins are unable to reach the TGN.

4.6.2.2 Fixation

Cells were fixed at various time points after infection. To the medium an equal volume of fixing solution 1 (see 3.2)(leading to a final concentration of 4% PFA and 0.1% glutaraldehyde) was added, and cells were fixed for at least 2 h at RT. Most of the fixing solution was removed, until about 1 ml was left. Cells were scraped from the dish in the fix, transferred into a 1.5 ml Eppendorf tube and pelleted at top speed for 2-5 min in a benchtop centrifuge. The fix was removed and replaced by 0.5 ml fixing solution 2 without disturbing the pellet.

Those fixed samples were stored at 4°C.

4.6.2.3 Epon embedding

Epon embedding requires cells fixed in 1% glutaraldehyde, the cell pellets were therefore fixed once more with 1% glutaraldehyde in 200mM HEPES buffer pH 7.4 for 1 h at RT. The fix was removed and the pellets washed 3 x with HEPES buffer for 5-10 min each, then 3 x with water since HEPES is disturbing the osmium. Contrast is achieved by incubating the cell pellet with a mixture of 1% osmiumtetroxide (OsO_4) and 1.5% potassium ferrocyanide ($K_3Fe(CN)_6$, light sensitive) in H_2O for 20 min at RT in the dark. The cells were washed 3 x with water, followed by 3 x washes with HEPES. Cells were slowly dehydrated, beginning with two incubations in 50% EtOH for 10-15 min each, followed by an incubation in a saturated uranylacetate solution (> 5%) in 70% EtOH over night. The next day the cells were washed 3 x in 90% EtOH for 2 min, then 3 x in 100% dried EtOH for 10 min each. Slowly the ethanol was replaced by Epon, starting with a 1:1 mixture of Epon:100% dried EtOH. After an incubation period of 30 min this mixture was replaced by 75% Epon and 25% dried EtOH, and incubated for 2 h. Finally the cell pellet was incubated in pure Epon over night. Fresh Epon was poured into an embedding mould and a tiny piece of the pellet (max. 1 mm) was introduced to the outer side. The sample was incubated for several hours and polymerized over night in the oven at 60°C.

4.6.2.4 Sample preparation

Epon embedded samples were trimmed to the appropriate size and pyramidal shape. Fine sections of a thickness of about 70 nm were obtained by hand-driven or automatic cutting with a Reichert ultracut microtome (Leica) using a diamond knife (Drukker). The cut sections floated on the surface of $dddH_2O$ at the back of the knife. Finally, they were transferred to grids (hexagonal, 100 mesh, treated as described before) by dipping those into the water and fishing out the floating sections carefully to avoid overlapping.

Contrast was achieved by incubating the grids in a closed glass petri dish for 25 sec in drops of Reynold's Lead Citrate in the presence of sodiumhydroxide pellets. Sodiumhydroxide binds CO_2 of the atmosphere, avoiding the precipitation of Pb. Grids were washed in $dddH_2O$ and air dried.

4.7 Immunofluorescence

CEF cells grown on 11 mm coverslips for 24 hours were infected at an moi of 5 of MVA. At 16 hours p.i. the cells were washed with PBS and fixed for 10 min at RT with 3% PFA in PHGM buffer. The fix was washed away with PBS and the coverslip was incubated cell side down on a drop of 30 µl rat anti-B5R in PBS supplemented with 5% FCS and 10mM Glycine for 20-30 min at RT. Surplus of the first antibody was removed by washing the coverslip 3-4 times over 10-15 min in PBS with 10mM Glycine. The infected cells were introduced to the second antibody (anti-rat FITC) for 20-30 min in the dark. This first labeling was applied for staining CEVs on the cell surface.

Subsequently, the cells were permeabilized with 0.1% Triton-X-100 in PBS for 10 min at RT. After washing the cells 3-4 times as described above actin was stained with phalloidin-rhodamine in PBS (5% FCS and 10mM Glycine) for 10-15 min. After a final washing step the cells were embedded in 5 µl phenydiamine/moviol.

All records were done using the EMBL Advanced Light Microscopy facility. The resulting digital images were edited using the Adobe 4.0 software package.

5. RESULTS

As outlined in the introduction, the clinical safety and immunological properties of MVA makes this gene expression vector an important candidate for the generation of recombinant live vaccines against different diseases. Improvement of the viral vector can furthermore be achieved by the presentation of recombinant proteins on the surface of the vaccinia virus particles. This would allow the induction of both humoral and cellular immune response against the foreign antigen.

The extracellular enveloped form of vaccinia viruses was chosen as the particle of integration, since these virions can easily be obtained from the supernatant of infected cells without necessity of further purification. At the onset of this work little was known about the production of EEV by this highly modified vaccinia virus strain. Therefore, one focus of this work was to study the ability of MVA to produce the enveloped infectious form of vaccinia viruses in more detail.

Different laboratories work with slightly different MVA strains. Throughout this study MVA passage 582, clone F6, described and characterized by Mayr (Mayr, 1975) and Meyer (Meyer et al., 1991), referred here as wtMVA or MVA, was used.

5.1 Production of enveloped virus by MVA

The amount of EEV produced varies significantly depending on the vaccinia virus strain and cell line used (Payne, 1979; Payne, 1980). The Western Reserve (WR) and International Health Department-J (IHD-J) strain of vaccinia virus are derivatives of the New York City Board of Health strain, and produce similar amounts of CEV but differ in the extent of EEV formation (Blasco and Moss, 1992). VV strains which produce large amounts of EEV, such as IHD-J, produce comet-shaped plaques if incubated under liquid overlay, instead of sharply defined round ones as described for WR (Appleyard et al., 1971). This comet-shaped plaque phenotype represents unidirectional spread of virus from the primary plaque (comet head) to give rise to secondary infections (comet tail).

While generating recombinant MVA (rMVA) this comet-shaped plaque phenotype was detected occasionally (Fig. 3), suggesting that this highly modified VV strain might produce significant amounts of EEV.

Figure 3: Comets of recombinant MVA on RK-13 cells. Cells were infected with a low MOI of MVA containing the K1L gene. At day 4 post infection this light microscopy picture was taken.

Several independent approaches were used to quantify the absolute and relative amounts of the different viral forms made in primary chicken embryo fibroblasts (CEF).

5.1.1 Determination of infectivity in the supernatant of infected cell monolayers

EEV is the infectious virus form that is released into the medium without necessity of cellular lysis of the host cell. Back titration of the medium collected at different time points after infection of subconfluent cell monolayers directly reflects therefore the amount of infectious viral progeny in the EEV form.

5.1.1.1 MVA produces extracellular enveloped virus

CEF cells grown in 6-well plates were infected with MVA at a m.o.i. of 0.05. Infecting cells with a low m.o.i. is a very sensitive method to determine the ability of the virus

to productively spread in target cells. Virions can undergo several rounds of DNA replication, assembly, and dissemination. The experiment was carried out in triplicate. It was found that MVA indeed produced detectable amounts of EEV (Fig. 4). The amount of infectious virus detected in the supernatant showed a 4.5 $\log_{10}$ step increase over a 48 hours period. Thus at the end of the experiment more than 30.000 times more virus was present in the supernatant than at the beginning. When comparing the infectivity detected in the cell lysate to the one in the supernatant, the former fraction had 1.5 to 2 $\log_{10}$ step more infectivity, meaning that 30 to 100 times more intracellular than extracellular virus is produced. This result suggests that compared to WR, that has been shown to produce 40 to 300 times more intracellular than extracellular virus progeny, depending on the cell lines tested, MVA in CEFs may produce only slightly more EEVs. In contrast, IHD-J may produce 6 to 14 times more intracellular than extracellular virus and may therefore be a much better EEV-producer than both WR and MVA (Payne, 1980) see also below.

5.1.1.2 MVA produces the highest amount of EEV in CEF cells

Ever since the study by Payne (Payne, 1979) it is clear that the efficiency of EEV-formation depends on the virus strain as well as the cell type used. By comparing for instance IHD-J infection in HeLa to RK-13 cells, Payne concluded that whereas in the former cells only 7% of the total infectivity was EEV, in the latter it was 25 to 40%. Since MVA is severely host range restricted and replicates only efficiently in CEF and BHK-21 cells (Drexler et al., 1998), a recombinant MVA was used for comparative studies. MVA-ssP-K1L-gfp has the VV host range gene K1L and the green fluorescent protein (gfp) incorporated into its genome. This recombinant virus was generated and described previously (Staib et al., 2000). The K1L gene product enables the virus to grow permissively in RK-13 cells, which allows to include this cell line for comparison of EEV production.

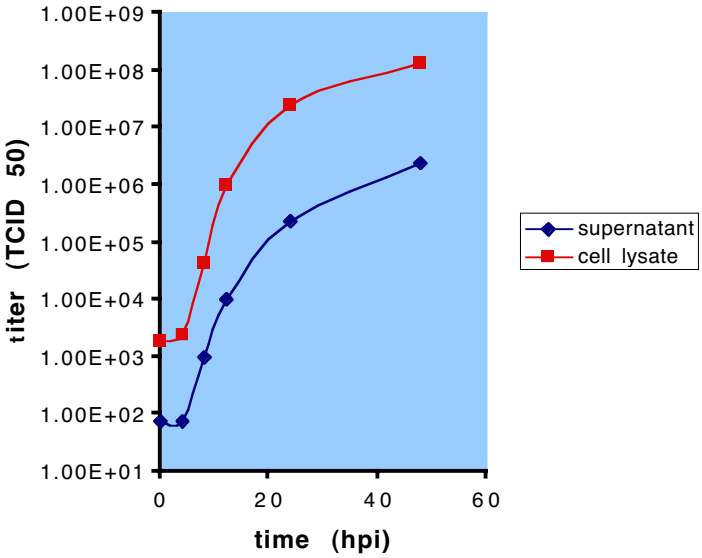


Figure 4: Growth of MVA in CEF cells. Subconfluent monolayers of primary CEF grown in 6-well plates were infected with 0.05 m.o.i. wtMVA. Supernatant and cell lysate were harvested separately at 0, 4, 8, 12, 24, and 48 hours post infection and back titrated (see 4.14).

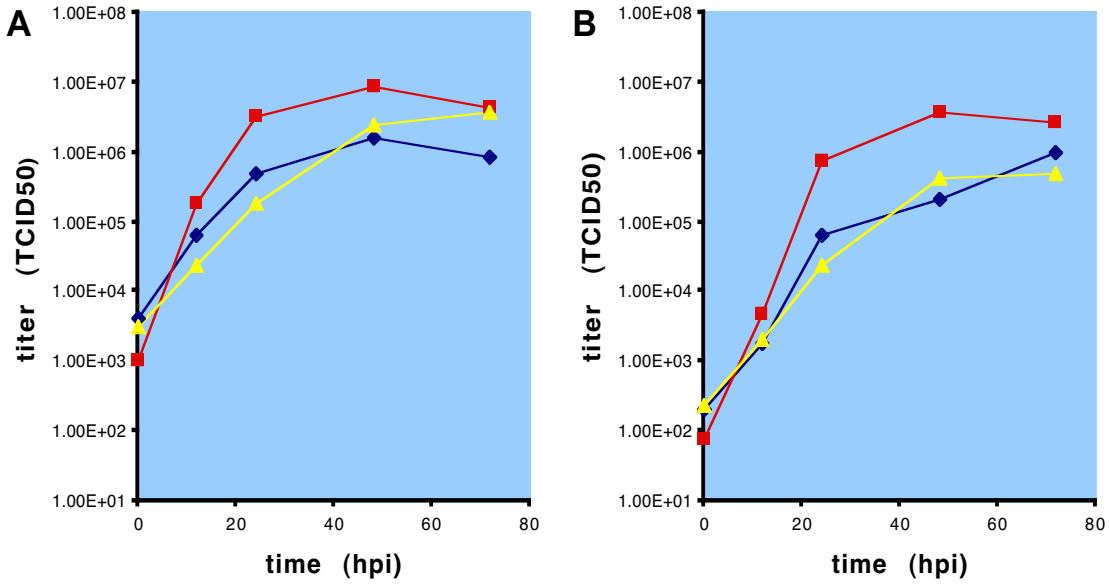


Figure 5: Growth of MVA-ssP-K1L-gfp in RK-13 (blue), CEF (red), and BHK-21 (yellow) cells. Cells were infected with m.o.i. 0.05, cell lysate (A) and supernatant (B) were harvested at 0, 12, 24, 48, and 72 h.p.i., and back titrated separately.

BHK-21, CEF, and RK-13 cells were infected with MVA-ssP-K1L-gfp at a low moi, cell lysates and supernatants were back titrated separately, as described in the previous experiment. This comparison revealed that MVA resulted in the highest intracellular and extracellular titers in CEFs (Fig. 5). While there is no significant difference in the ability of the virus to produce intracellular virus in the 3 cell lines tested (Fig. 5A), there is a clear difference in the production of extracellular virus. Surprisingly, MVA produces most EEV in CEF cells, being 10 times higher than the production in RK-13 and BHK-21 cells (Fig. 5B). These results indicate that CEF cells were most suitable for further studies of MVA envelopment. All further studies were carried out with wtMVA.

5.1.2 Quantification of the different viral forms of MVA

However, these experiments do not allow discrimination between IMV, IEV, and CEV that are all cell associated. Therefore, the different viral forms, both intracellular and extracellular were separated by CsCl gradients (see 4.15).

5.1.2.1 Purified MVA virions show a high percentage of envelopment

To distinguish between the cell associated CEV and IEV one further step of separation was included. After removal of the medium the cells were not simply scraped off, but trypsinized after a brief wash with PBS. Trypsin treatment has been shown to release CEV from the cell surface (Blasco and Moss, 1992). Using this method it was possible to work with 3 different virus fractions: the extracellular fraction (containing EEV that is by definition free in the medium), the cell associated fraction (containing CEV removed by trypsinization), the intracellular fraction (containing IMV and IEV). These 3 fractions were purified separately as described in methods (see 4.15).

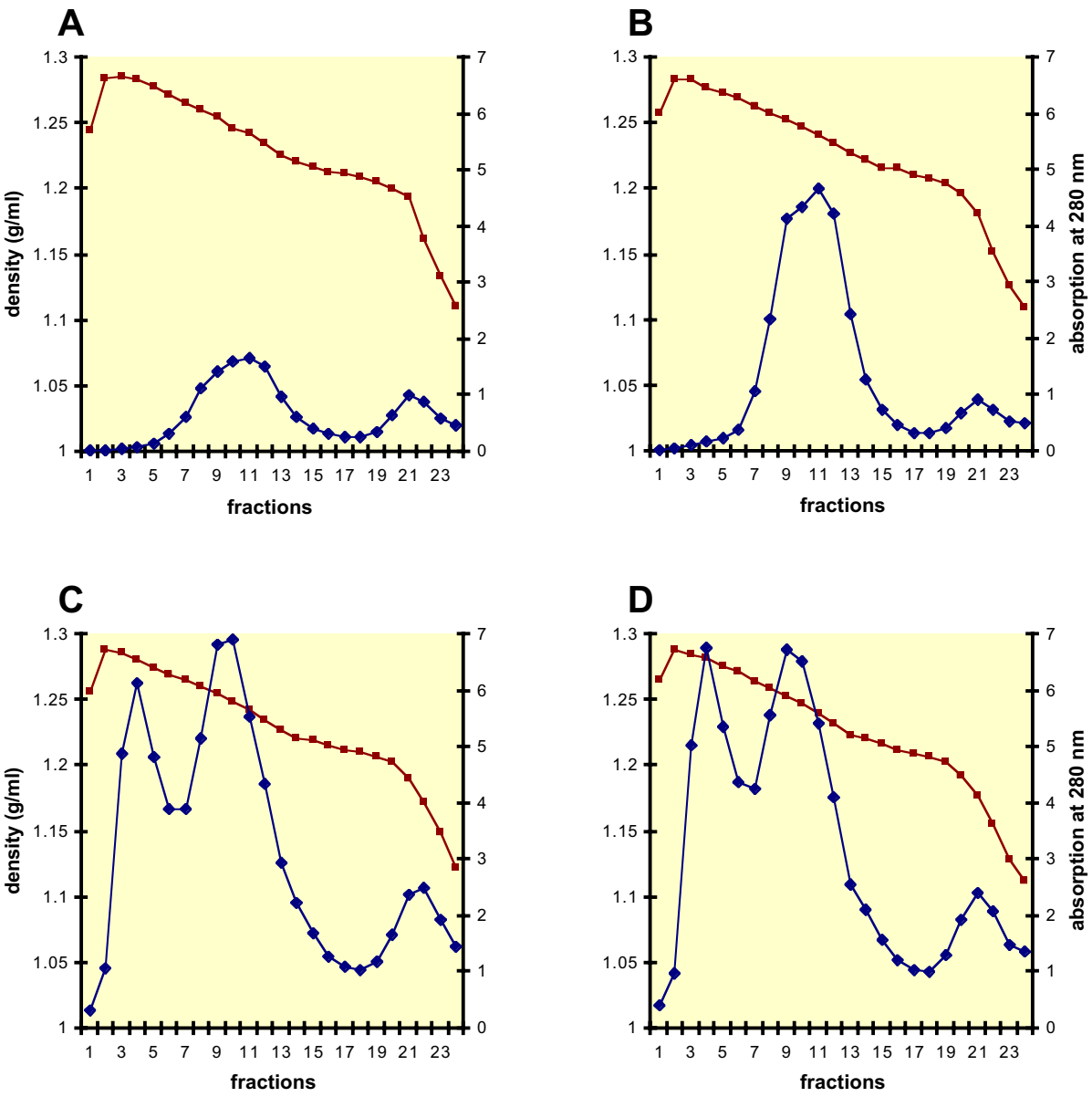
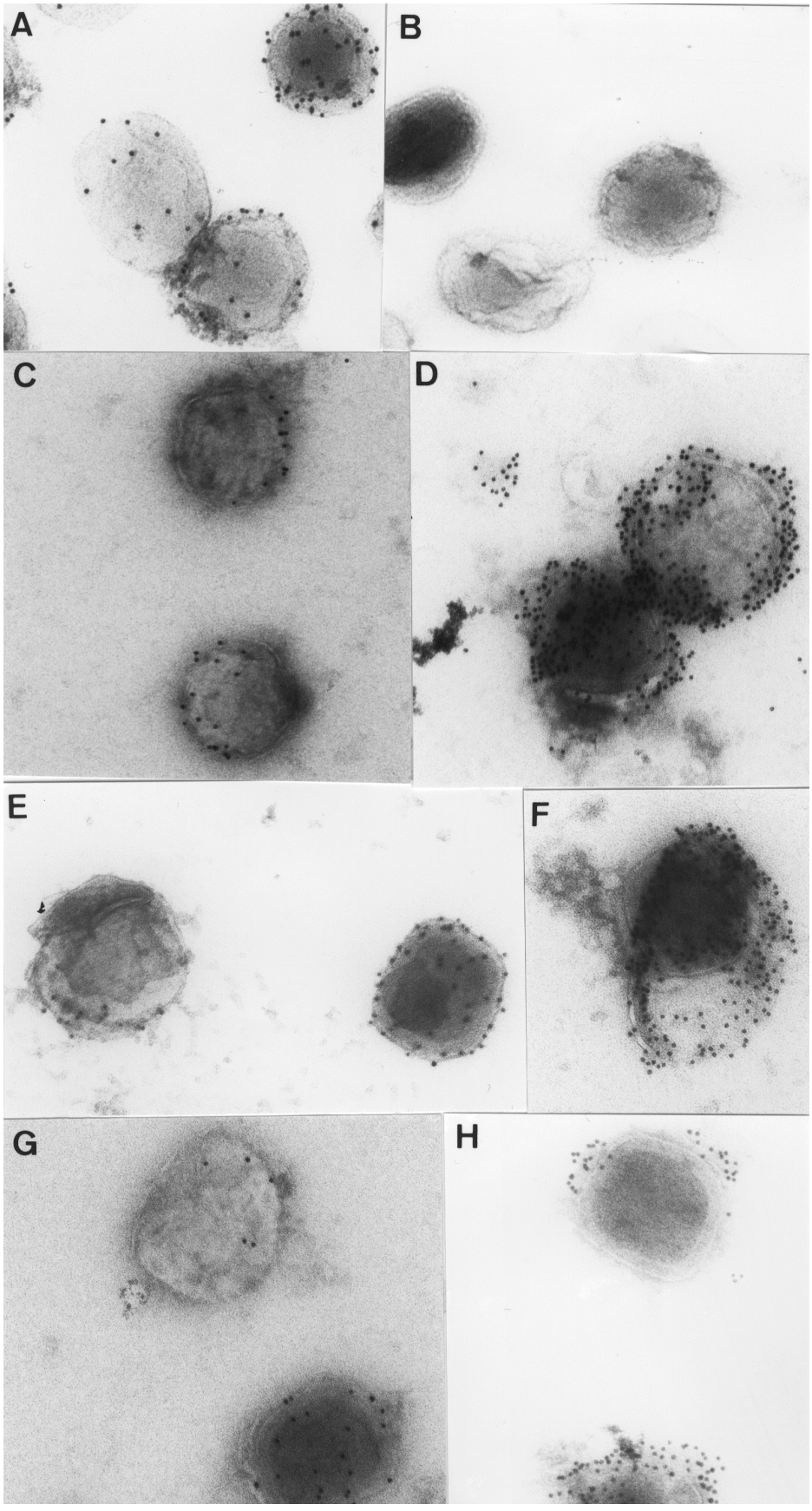


Figure 6: Separation of different viral forms produced in MVA infected CEFs by CsCl-gradients. Confluent monolayers of CEF cells grown in 7 225 cm^2 flasks were infected with m.o.i. 1 wtMVA. At 40 h.p.i. supernatant (containing EEV), cell-attached virions (CEV), and cells (containing IEV and IMV) were harvested and purified as described in methods The cellular fraction had to be divided to avoid overloading of the gradient (C and D). After centrifugation the gradients were fractionated from the bottom to the top of the tube and the presence of viral particles was determined by OD_{280} measurements (blue graph). The optical density of each fraction was subsequently determined (red graph). Peaks are corresponding to EEV (A), CEV (B) IMV (fraction 4 in C and D) and IEV (fractions 9 and 10 in C and D each).

Virus particles can be separated according to their buoyant density by sedimentation in CsCl gradients (Payne and Norrby, 1976). IMV bands in the CsCl gradient at 1.26-1.27mg/ml. Since EEV consists of an IMV with one additional membrane it is less dense and bands at 1.23-1.24mg/ml. Measurement of the absorbance at 280 nm was used to detect the virus peaks in the gradient fractions. The peaks detected at that density in the extracellular and cell associated fraction correspond to EEV and CEV respectively (Fig. 6A and B). Notably, the CEV peak was about 3 times higher than the EEV peak, indicating that the majority of enveloped virus is not released in the medium, but remains attached to the cell surface. The intracellular virus fraction was so concentrated, that the sample had to be divided in two to avoid overloading the gradient. In this intracellular fraction more than half of the virus progeny banded as the enveloped form (Fig. 6C and D), whereas less than half of the intracellular virus banded as IMV.

Virions contained in the peak fractions were subsequently analyzed by negative staining EM to confirm the identity of the different viral forms (see 4.16.1). For this, antibodies to B5R and A17L were used; the gene product of B5R, p42 is an EEV specific protein that is absent from the IMV (Engelstad and Smith, 1993; Isaacs et al., 1992). A17L is a major membrane protein of the IMV (Jensen et al., 1996). The A17L protein p21 is therefore covered by additional membranes in EEV, CEV and IEV particles, and is not accessible for antibody labeling.
Virions corresponding to the IMV fraction labeled heavily with anti-p21 (Fig. 7A), whereas the EEV specific protein p42 was absent from these particles (Fig. 7B), defining those clearly as IMV. The outer envelope of CEV, IEV and EEV appeared partially destroyed, most likely due to the virus purification procedure (Smith and Vanderplasschen, 1998). Consequently, IMV particles were visible underneath the disrupted outer membrane, showing a positive labeling with anti-p21 (Fig. 7C, E, G). Nevertheless, labeling with anti-p42 showed the existence of an additional EEV-specific membrane, confirming that the particles are enveloped (Fig. 7D, F, H).

Figure 7: (next page) EM-analysis of negative stained virus particles. Viral particles out of the peak fractions were labeled with antibodies against A17L (anti-p21) and B5R (anti p-42), respectively. The presence of those proteins is shown by gold particles (10 nm). A: IMV labeled with anti-p21, B: IMV labeled with anti-p42, C: EEV labeled with anti-p21, D: EEV labeled with anti-p42, E: CEV labeled with anti-p21, F: CEV labeled with anti-p42, G: IEV labeled with anti-p21, H: IEV labeled with anti-p42.


A
B
C
D
E
F
G
H

5.1.2.2 Enveloped virus is the major viral form in MVA infected CEFs

The relative amounts of infectious virus progeny produced by MVA was determined by titration of the purified virions. CsCl fractions corresponding to viral peaks were pooled (Fig. 7: fractions 7-14 for EEV, 7-14 for CEV, 2-6 + 2-6 for IMV, and 7-15 + 7-15 for IEV) and back titrated. The relative amounts of infectious vaccinia virus virions is shown in Fig. 8. Whereas EEV represents only 4% of infectious progeny, 12% consist of CEV and 46% of IEV. This implies, that at the MOI used and at this time post-infection in CEFs infected with MVA, 62% of the infectivity is contained in the enveloped viral forms with only 38% in the IMV.

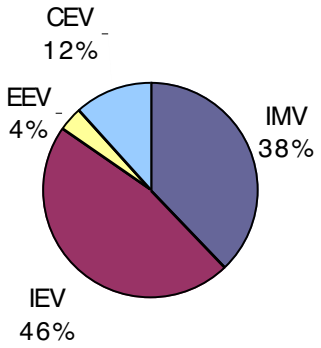


Figure 8: Relative amounts of virus forms in MVA infected CEFs estimated by their infectivity. The infectivity was determined by back titration of the peak fractions (fractions 7 to 14 (EEV) in A, fractions 7 to 14 (CEV) in B, fractions 2 to 6 (IMV) in C and D, fractions 7 to 15 (IEV) in C and D). The relative infectivity was calculated by dividing the infectivity measured in the peak fractions by the total infectivity contained in all fractions.

5.1.3 Effects of inhibitors of TGN-wrapping

Wrapping of Vaccinia virus was reported to be inhibited by Brefeldin A (Ulaeto et al., 1995) and IMCBH (Hiller et al., 1981; Kato et al., 1969; Payne and Kristenson, 1979). The target of Brefeldin A is the GTPase ARF-1; treatment of cells with this drug causes the ER to fuse with the Golgi complex and consequently membrane traffic is blocked. Because of this in VV infected cells EEV proteins fail to be targeted to the TGN and wrapping is blocked. The target of IMCBH is the EEV protein F13L. Since this protein is essential for TGN-wrapping, the drug inhibits the latter process. The aim of the following experiments was to see whether MVA envelopment was also affected by these drugs and if this treatment would facilitate the isolation of pure IMV (not contaminated by the IEV peak) needed for further experiments.

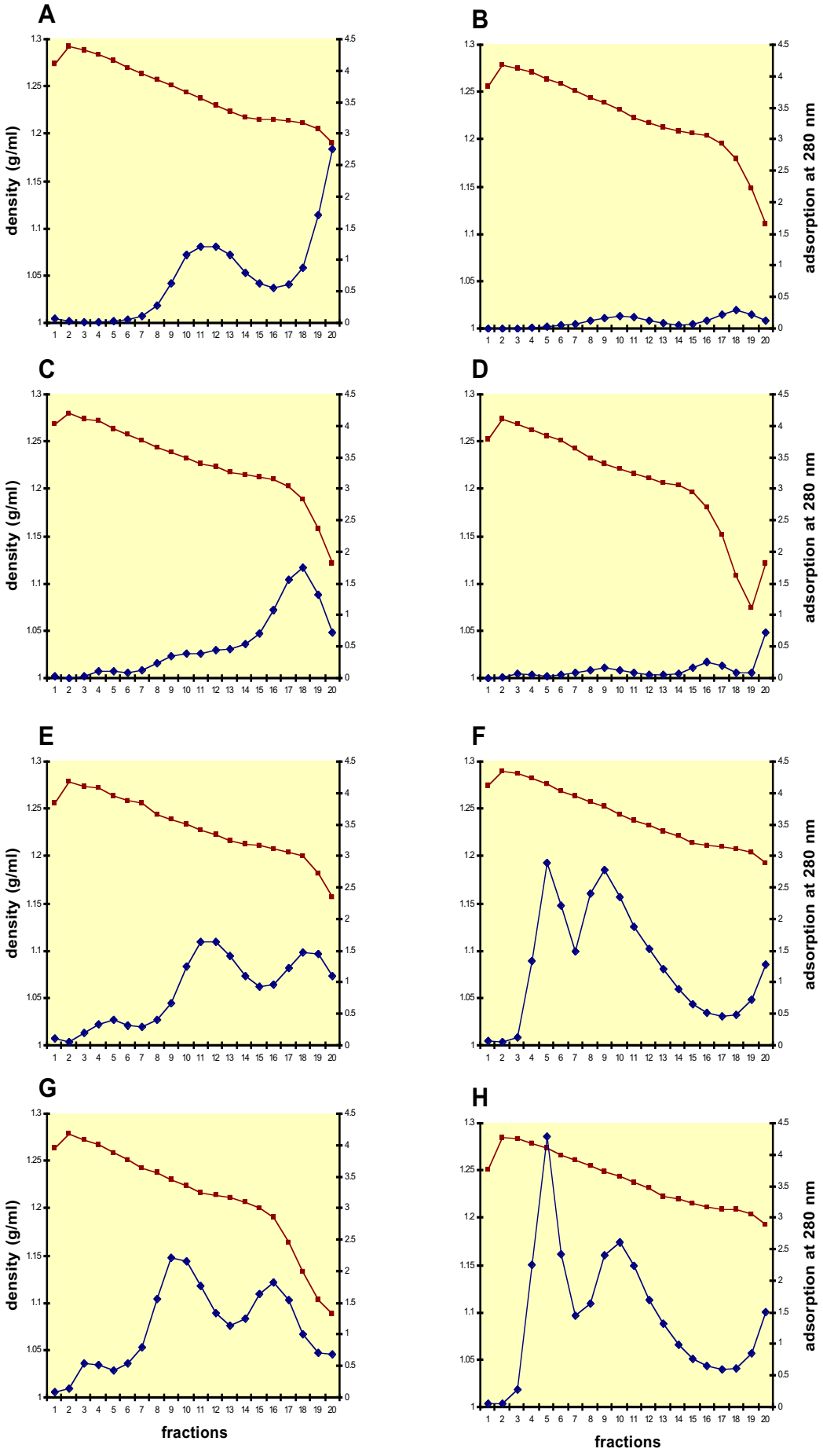
5.1.3.1 Inhibition of envelopment by Brefeldin A

Cells were infected with a m.o.i. of 0.1, harvested 50 h.p.i. and purified by CsCl gradient centrifugation as described above (see 4.5.6). In the case of Brefeldin A treatment the drug was added at 500 ng/ml at 14 h.p.i.. Concentrations of Brefeldin A higher than 0.1 μg/ml are toxic for CEFs when applied for more than 24 h (Boulanger et al., 2000). Therefore, the drug could not be given to the medium immediately after infection, but was added at 14 h.p.i. instead.

After Brefeldin A treatment (Fig. 9) three striking effects were visible. (1) The absorbance peaks at 280 nm for EEV and CEV decrease from 1.2 in untreated cells to 0.2 in treated cells and from 0.4 to 0.1 respectively. The latter values are very close to zero, indicating that virus release from the cell as EEV or CEV is inhibited in the presence of Brefeldin A. (2) The amount of IMV measured at 280 nm increases dramatically from 0.4 and 0.5 in untreated to 2.9 and 4.3 in treated cells. This indicates that in Brefeldin A treated cells viral particles accumulate inside the cell in the IMV form (3). The absorbance peak of the intracellular fraction of untreated cells that corresponds to the enveloped form of the virus, indicated by the buoyant density of its sedimentation of 1.23-1.24 mg/ml, shifts in the presence of Brefeldin A to a density of 1.24-1.25 mg/ml. This density is not characteristic for a specific viral form, but may indicate an incomplete wrapping of IMVs.

This suggestion was supported by negative staining Electron microscopy of particles from the shifted peak (Fig. 10). IMVs with which large membrane sheets were associated that labeled with anti-p42 were observed. This suggested that the IMVs associated with membranes containing EEV proteins, but failed to wrap themselves. This association of IMVs with membranes apparently resulted in a sedimentation in the gradients in peaks lighter than IMV but slightly heavier than IEVs. In contrast, completely wrapped enveloped particles showed a disrupted but always compact pattern when stained with anti-p42 (see Fig. 7H for comparison).

Figure 9: (next page) Comparison of CsCl gradient purified virions grown in presence (B, D, G, H) or absence (A, C, E, G) of 500 ng/ml Brefeldin A. CEFs were infected with a m.o.i. of 0.1 and EEVs (A and B), CEVs (C and D) and intracellular virus (E to F) harvested at 50 hours post infection. Density and absorption at OD_{280} were measured as in fig. 6.


A
B
C
D
E
F
G
H
density (g/ml)
adsorption at 280 nm
fractions

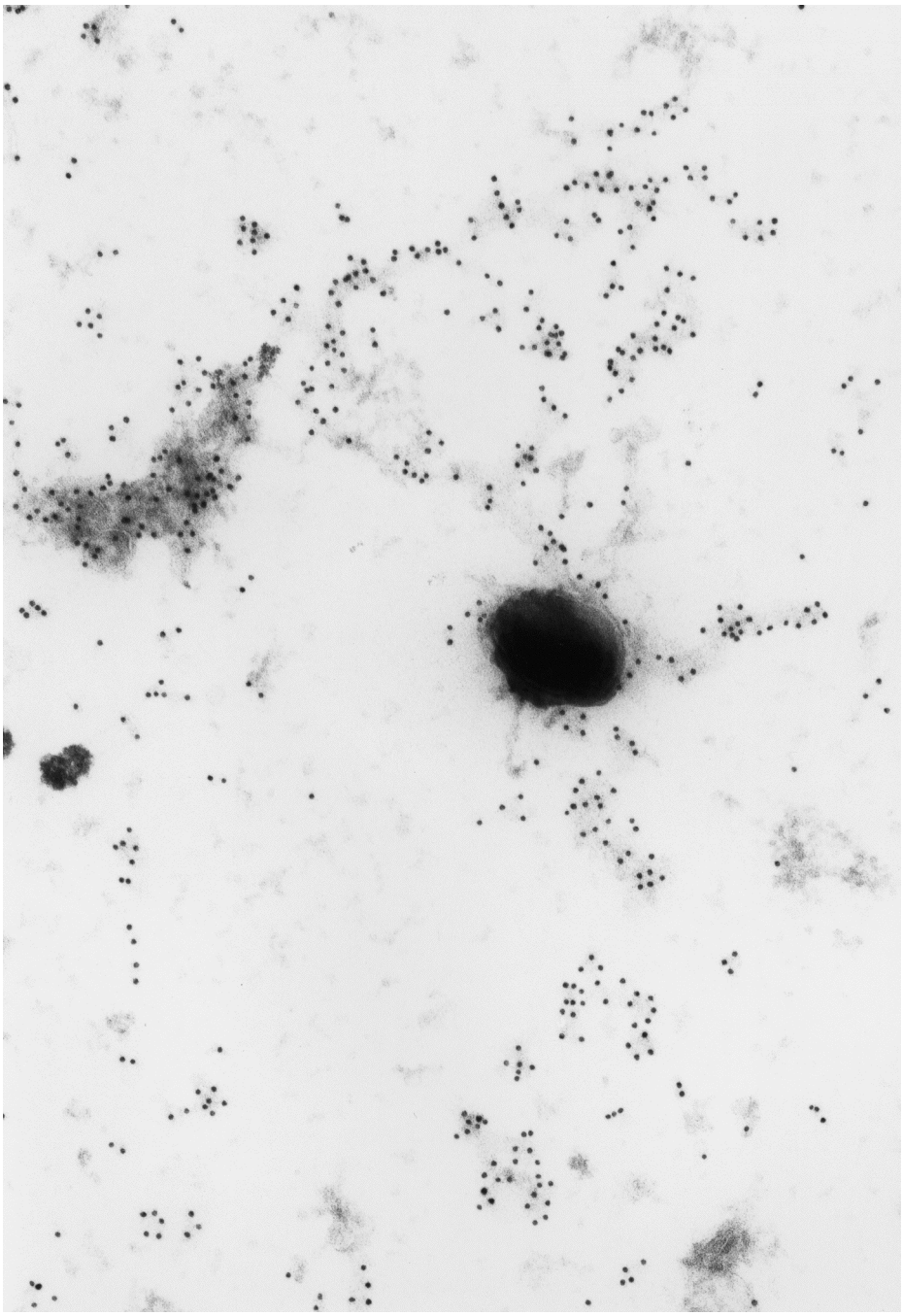


Figure 10: Incompletely wrapped IMV particle. Treatment with Brefeldin A caused an absorbance peak at a density of 1.24-1.25 mg/ml representing a density between the IMV and enveloped form. Virus particles out of this fraction were negative stained and labeled with an antibody against the EEV protein B5R. Membranes in close proximity to the IMV particle labeled heavily with anti-p42, indicating that IMV particles associated with membranes enriched in EEV proteins but did not wrap themselves completely.

The inhibition of wrapping by Brefeldin A was confirmed by EM studies of Epon embedded CEF cells infected with 0.1 m.o.i. of MVA in the presence and absence of the drug, and fixed 50 h.p.i. (see 4.6.2). An overview of characteristic cell profiles show the presence of viruses in all stages of maturation in untreated cells, although CEVs appeared to predominate (Fig. 11A and C). In Brefeldin A treated cells, however, IMVs accumulate and no obvious TGN-wrapping nor CEVs could be detected (Fig. 11B).

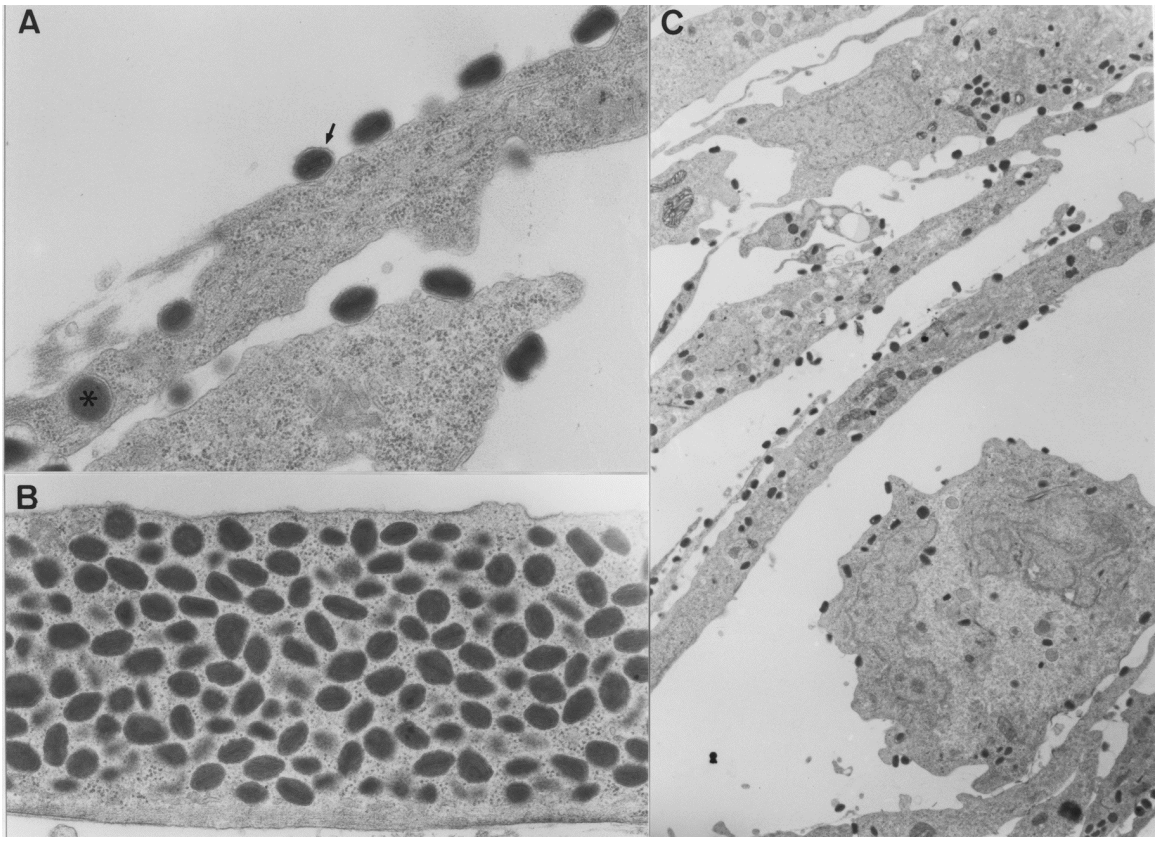


Figure 11: Inhibition of TGN wrapping by treatment with Brefeldin A. CEF cells were infected with moi 0.1 in presence (B) and absence (A and C) of Brefeldin A, and fixed 50 hours post infection. Without drug treatment the entire cells are covered with CEVs (C), in a higher magnification the outer envelope of these particles is visible (arrow), (*) indicates an IEV (A). In drug treated cells IMVs accumulate (B).

5.1.3.2 Inhibition of envelopment by IMCBH

CEF cells were infected with a m.o.i. of 10 with wtMVA, in presence and absence of 10µg/ml IMCBH, fixed 24 h.p.i. and prepared for EM analysis as described in methods (see 4.6.2). The different viral forms were counted in 15 profiles of infected cells and expressed as percentage of the total viral forms detected (Fig. 12).
It has to be mentioned, that EEVs can not be quantified by this method, since only cell associated virions are detectable.

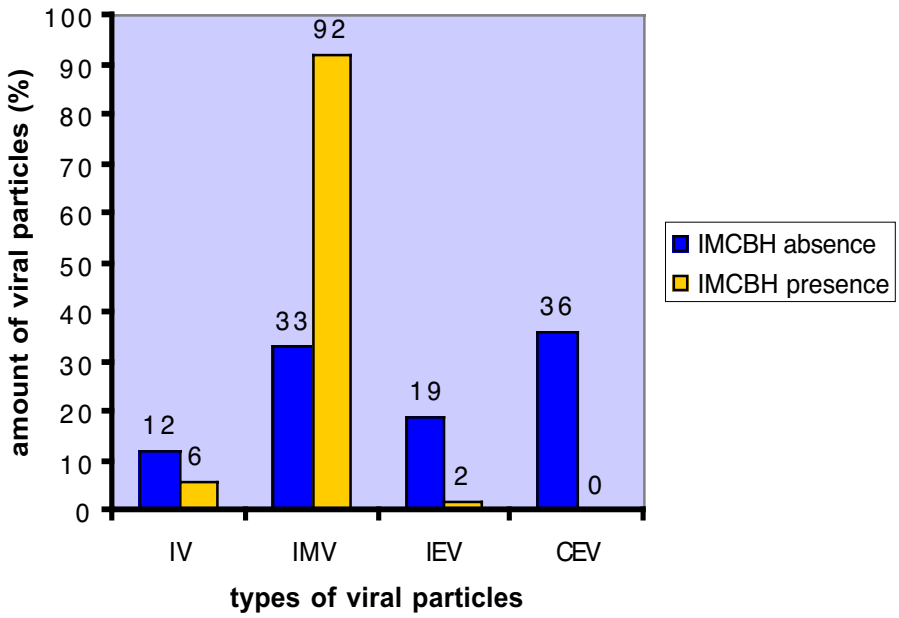


Figure 12: Inhibition of TGN wrapping of MVA in CEFs by IMCBH. CEF cells infected with MVA in presence (yellow) and absence (blue) of IMCBH. Treatment with IMCBH shows almost complete inhibition of envelopment.

Clearly, these results show that IMCBH inhibits MVA TGN- wrapping. CEV represent 36% of infectious viral progeny in the untreated control cells, whereas no CEV was detected in IMCBH treated cells. Also, the amount of IEV decreases from 19% to 2%, whereas the amount of IMV increases dramatically from 33% in untreated cells to 92% in drug treated cells.

5.1.4 Influence of m.o.i. and time on the formation of enveloped virus

For detection of EEV and measurement of the efficiency of MVA to spread productively in target cell monolayers, the highly sensitive method of a multiple step growth curve was used, which means infection of the cells with a low m.o.i. and monitoring of the virus progeny 50 hours post infection. For EM analysis the cells were infected with a m.o.i. of 10 to make sure, that all cells are infected. Furthermore, the cells were fixed 24 h.p.i. since the cells are then still in a healthy state, and the different viral forms are well distinguishable from each other. Another classical time point for EM analysis of vaccinia virus infected cells is 16 h.p.i., since then all viral forms are present in the cell. By analyzing these data it could be shown that relative distribution of the different viral forms of vaccinia virus is time- (Fig. 13A) and moi dependent (Fig. 13B).

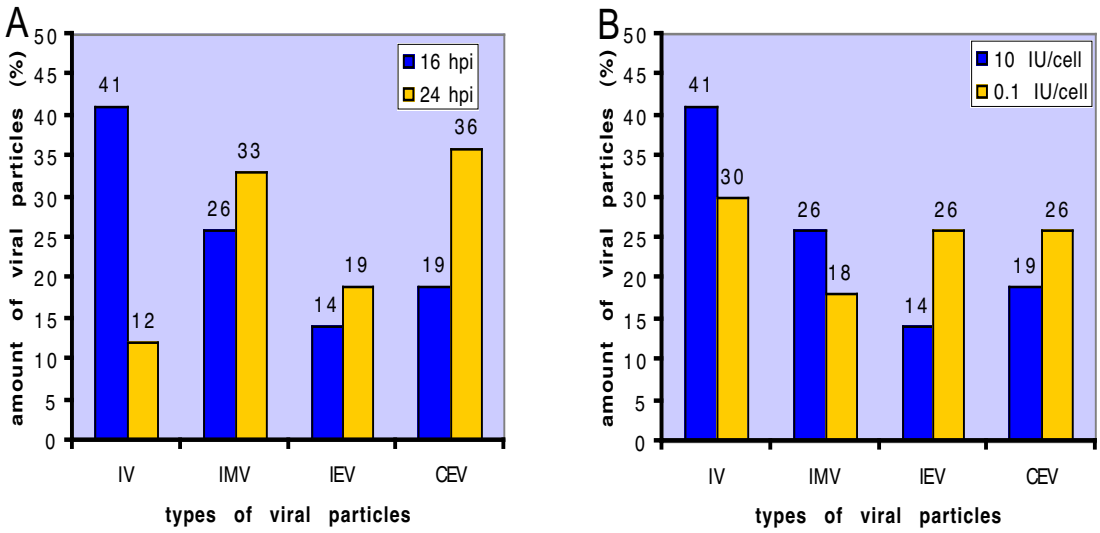


Figure 13: The different types of virus forms observed depend on time post infection and on input infectivity. CEF cells were infected with 10 MOI and fixed at 16 (blue) and 24 (yellow) h.p.i., respectively (A). CEF cells infected with 10 (blue) and 0.1 (yellow) IU/cell were fixed at 24 h.p.i. (B). Virus forms in 50 randomly chosen cell profiles each were counted.

At 16 hpi many immature virions or crescents were still detectable, whereas more of the infectious viral forms were detectable at 24 h.p.i. In particular the amount of CEV increases, reflecting that over time these virions accumulate on the surface of the infected cell. The multiplicity of infection seems to influence the efficiency of wrapping. The relative amounts of IEV and CEV are higher in cells infected with a low m.o.i. compared to higher m.o.i. at 16 h. post infection.

These results show that the wrapping events in MVA are not only cell type dependent, but are also dependent on the multiplicity of infection and on the time

post-infection. It could be shown that TGN-wrapping occurs in MVA and that, under the conditions tested, enveloped forms dominate over IMV. However, to be able to assess how efficient the wrapping in MVA infected CEFs really is, a comparison with well characterized and widely used vaccinia virus strains seemed necessary.

5.2. Efficiency of envelopment in MVA compared to the well characterized vaccinia virus strains WR and IHD-J

The most commonly used VV laboratory strains are IHD-J and WR. The two virus strains are closely related to each other and differ in their ability to produce and/or release extracellular enveloped viruses. Almost all studies concerning EEV production have been carried out with these two VV strains. IHD-J produces high yields of EEV, whereas WR releases little of this viral form. The above experiments suggested that MVA in CEFs produced high amounts of the TGN-wrapped forms, including CEVs. The next step was to compare EEV/CEV/IEV production by MVA in CEFs, to the well established strains WR and IHD-J.

5.2.1 Quantification of the viral forms made by MVA, WR, and IHD-J

All studies were carried out in CEF cells. The same growth curves as described above were used including trypsinization of the cell monolayer to release CEVs from the plasma membrane. This allowed assessment of the infectivity associated with EEV, CEV and the intracellular virions. To distinguish between IMVs and IEVs that are both intracellular, quantitative EM was used.

5.2.1.1 IHD-J produces most EEV

Subconfluent monolayers of CEF cells were infected with WR, MVA, and IHD-J at a m.o.i. of 10. At 6 different times post-infection EEV was collected from the medium, CEV was released from the cell surface by trypsinization, and IMV and IEV were set free from the cells by freeze/thawing and sonication. The experiment was carried out in triplicate and all samples were back titrated in duplicate. WR and IHD-J were titrated on BSC-40 cells by cristal violet staining (see 4.4.3.3), MVA was titrated on

CEF cells by immunostaining (see 4.4.3.2). It is shown in figure 14 at point 0 post infection that the input virus used for infection with MVA was higher than for WR and IHD-J. Nevertheless the results of this experiment show a clear tendency.

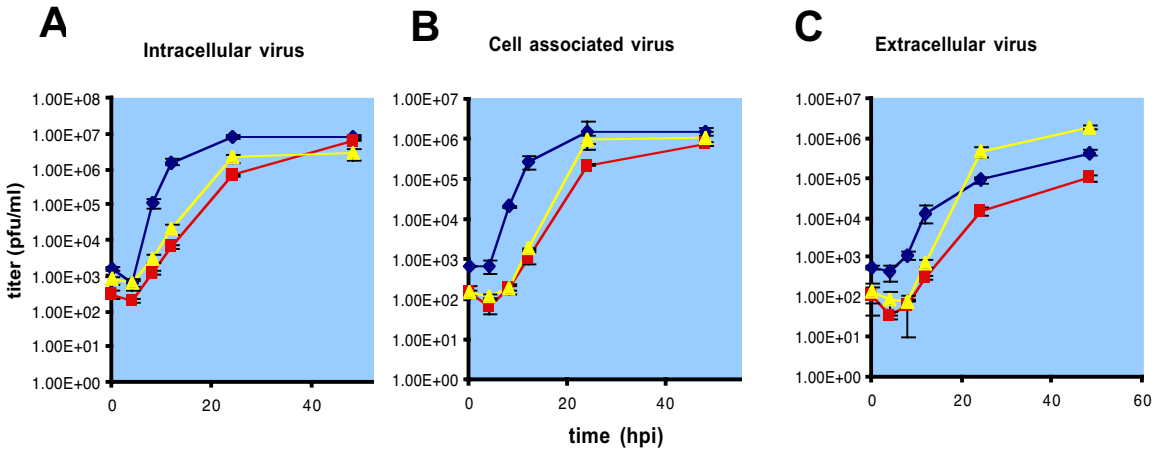


Figure 14: Time course of the production of infectious particles in CEFs infected with MVA, WR, And IHD-J. Monolayers of CEFs were infected for one hour with MVA (blue), WR (red), and IHD-J (yellow) at a moi of 10. Cells were washed and intracellular virus (A), CEVs (B), and EEVs (C) harvested at the indicated times post infection. The average infectivity contained in each sample is expressed as plaque forming unit (pfu) per ml. The standard deviation is shown as error bars. During the course of this experiment it appeared that the amount of infectivity measured at 0 h.p.i., which measures the virus that remains attached to the plasma membrane after absorption, was slightly higher (about 0.5 log) upon MVA infection. This is due to the fact that the MVA stock used throughout these experiments was titrated in a different way (by immunostaining, rather than by crystal-violet staining) compared to the two other viruses, making it difficult to directly compare the titers of the virus stocks. It thus appeared that the MVA time course was done by infecting with a slightly higher MOI than the two other viruses.

The results revealed a number of striking observations. First, IHD-J infection clearly resulted in the highest titers of EEV in the extracellular medium (Fig. 14C). Comparison of the intracellular titers revealed that MVA replicated with a faster kinetic than the two other viruses (Fig. 14A). At 8 h.p.i., for instance, the infectivity associated with lysates of MVA infected CEF was about 2 $\log_{10}$ higher than upon infection with the two other virus strains. This faster growth kinetics apparently also resulted in efficient CEV production (Fig. 14B) since at 8 and 12 h.p.i. MVA also produced about 2 $\log_{10}$ higher yields of the latter virus form compared to WR and IHD-J. At later time points this difference in CEV production was not so pronounced anymore and at 48 h.p.i. almost equal amounts of infectious CEV were detected in all three viruses.

When the production of CEV and EEV is compared per viral strain (Fig. 15), it is obvious that in IHD-J EEV production exceeds CEV infectivity. WR and MVA, however, produce more CEV than EEV. When the relative distribution of the

infectivity contained in the different fractions at 24hrs post-infection was compared (Fig. 16A) it was evident that IHD-J makes 13% EEV at that time of infection, while MVA and WR make only 1-2 % of this viral form. IHD-J and WR have a similar percentage (27% and 25% respectively) of the infectivity associated with CEVs, but in apparent contrast with the above, the relative infectivity contained in the CEV fraction is only 16% in MVA. Instead it appeared that the bulk of the infectivity of this virus was associated with intracellular virus (containing IMV and IEV). In fact, the relative infectivity for the different fractions correlated with the results obtained by back titration of CsCl gradient purified virions (Fig. 8): 85 and 84% of the infectivity was associated with intracellular virus, 15 and 12% associated with CEVs and 2 and 4% with EEVs in figure 16A and figure 8, respectively.

The combined results, however, show that EEV production by MVA is not particularly high when compared to the classically used laboratory strains IHD-J and WR.

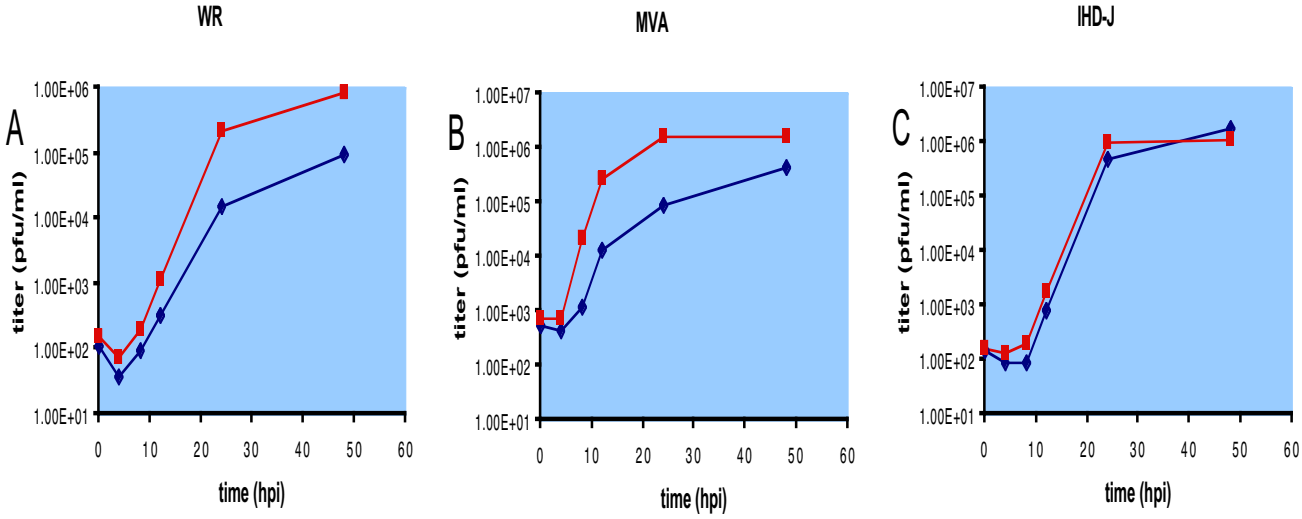


Figure 15: Comparison of EEV (blue) and CEV (red) production in CEF cells by WR (A), MVA (B) and IHD-J (C). Data was obtained as described in fig. 14.

5.2.1.2 MVA produces most CEV

The infectivity time course experiment does not allow to discriminate between the amounts of IMVs and IEVs made upon infection of CEF cells. Therefore, the extent of envelopment of intracellular viruses was investigated by EM. For this CEF cells grown in 6 mm petri dishes were infected with a m.o.i. of 10 with MVA, WR, and IHD-J, respectively. Cells were fixed at 24 h.p.i. and embedded in Epon for EM analysis (see 4.6.2). Obviously, this EM assay does not allow to quantify EEVs. Per VV strain used, the three different viral forms (IMV/IEV/CEV) were counted in 50 randomly

chosen profiles of infected cells and the absolute and relative amounts were calculated.

Counts of the total amounts of the different viral forms produced at 24 h.p.i. revealed that MVA infection resulted in the highest number of intracellular viruses. In 50 cell profiles a total of 553 IMV and IEV were counted in MVA infected cells, but only 309 and 383 in IHD-J and WR infected cells, respectively (Tab. 3). The most striking difference was observed in the amounts of CEV produced. MVA infection appeared to result in about 3.5 fold more of these viruses at the plasma membrane compared to IHD-J and WR, both of which produced similar amounts of CEV. Finally, upon IHD-J infection 1.5 and 2 fold more IEV could be counted compared to MVA and WR infections, respectively.

Table 3: Total amounts of IMV, IEV and CEV counted in 50 cell profiles at 24 hours post infection in CEFs infected with WR, IHD-J and MVA.

VV strain	IMV	IEV	CEV
WR	280	103	163
IHD-J	118	191	157
MVA	416	137	555

It was found that the relative amount of IMV is highest in WR (51%), intermediate in MVA (38%) and lowest in IHD-J (25%). IEV, the precursor of CEV and EEV was with 41% very high in IHD-J, more than double of the percentages obtained in WR and MVA infected CEFs, with 19% and 12% IEV, respectively. Most strikingly it was found that for MVA 50% of the viral forms counted was CEV, considerably higher than WR and IHD-J, that with percentages of 30% and 34% CEVs, respectively, produced similar amounts of this viral form.

The EM analysis had shown that MVA produced the highest amounts of CEV contradictory to the data obtained by measuring the relative amounts of infectivity, where most of the infectivity appeared to be associated with intracellular (IMV and IEV) virus upon MVA infection (Fig. 8 and Fig. 16A). These results could indicate that perhaps CEVs produced by MVA were less infectious (CEV had a relatively low pfu per particle ratio). Alternatively, the trypsin treatment was maybe less efficient in removing CEVs from the plasma membrane of MVA infected cells compared to IHD-J and WR infection. The latter possibility could be consistent with a number of observations suggesting that the EEV envelope around the MVA particle is more tightly attached to the underlying IMV and more resistant to rupture, compared to the

two other viral strains (Sancho, 2002; Spehner et al., 2000). Since MVA infection results in substantially more CEVs, failure to remove all of them from the cell surface could result in higher titers of virus associated with the cell.

By comparing the EM data to the infectivities measured above, a general pattern became discernable. IHD-J was the most efficient in the production of all of the TGN-wrapped forms. This was not only shown by the fact that at 24 h.p.i. by EM 75% of the virus was either IEV or CEV (with only 62% and 36% in MVA and WR, respectively), but also that this virus resulted in the highest amounts of EEV production as measured by plaque assay. Compared to WR MVA was also efficient in TGN-wrapping, but off the released particles the bulk remained attached to the plasma membrane. Finally, WR appeared to be least efficient in producing all of TGN-wrapped forms.

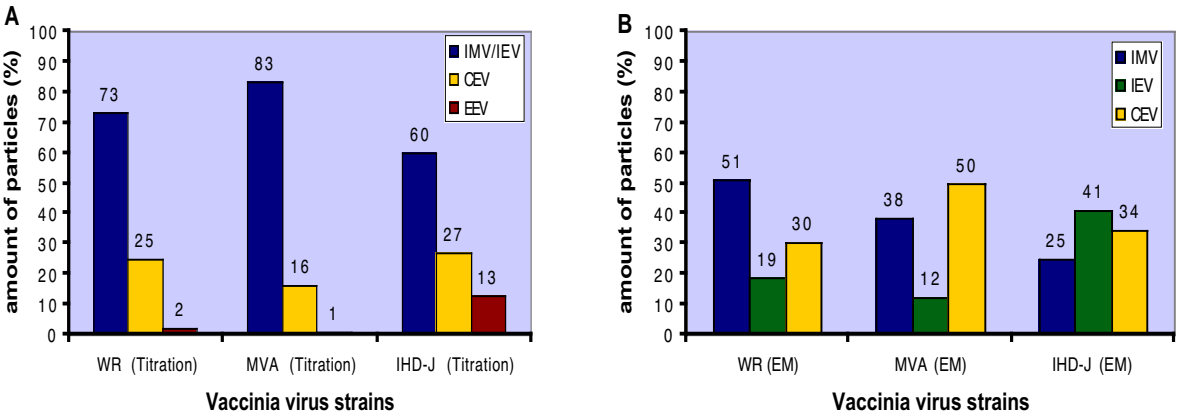


Figure 16: Comparison of the relative amounts of the different viral forms detected by EM and by titration, in CEFs infected with WR, IHD-J and MVA at a MOI of 10 and at 24 h.p.i. In panel A the relative infectivities contained in intracellular virus (blue bars) , CEV (yellow bars), and EEVs (red bars) was calculated at 24 h.p.i. using the values obtained in figure 14. In panel B CEFs were infected with WR, IHD-J and MVA and fixed at 24 h.p.i. Fixed cells were embedded in Epon and the different viral forms, IMVs (blue bars), IEVs (green bars), and CEVs (yellow bars) counted in 50 randomly chosen sections of infected cells. The values represent the percentage of different viral forms relative to the total viruses counted.

5.2.2 MVA makes actin tails

The high amount of CEV detected by EM at the plasma membrane of MVA infected CEF led to the question whether these viruses were able to induce actin tails as shown extensively for WR and IHD-J.

The A36R protein, which is characterized by its long cytoplasmic domain, is required for actin-based motility and virus spreading. For intracellular transport and egress of vaccinia virus a two-step model was proposed: IEVs are transported to the periphery along microtubules, and actin tails form at the plasma membrane after IEV fusion with the plasma membrane. Actin tail formation occurs from single viral particles, which always remain at the tip of these structures. CEVs attached to the outside of the cell continue to extend on those cellular projections, which can reach lengths of over 20 μm.

Frischknecht et al. (Frischknecht et al., 1999b) have shown, that mutation of Tyr 112 and Tyr 132 in the A36R protein abrogates actin tail formation, and residues 71-100 (Rietdorf et al., 2001) are sufficient for IEVs to reach the cell periphery. In MVA all those crucial amino acids and protein domains are unaltered, but the A36R gene contains two in frame deletions (residues 139-147 and 216-219). Therefore, the ability of MVA to induce actin tails was investigated.

CEF cells grown on cover slips and infected with MVA at m.o.i. 5 were fixed 16 hours post infection. Before permeabilization, CEVs present on the cell surface were labeled with an antibody to B5R, an EEV-specific membrane protein and a FITC-conjugate. Afterwards, cells were permeabilized and intracellular actin stained with phalloidin-rhodamine. In agreement with the EM observations, showing that some cell profiles contained many CEVs at the plasma membrane, by immunofluorescence the entire cell surface appeared covered with B5R positive CEVs (Fig. 17A). Some of theses viruses were clearly present on the tip of an actin tail (Fig. 17B). Attempts to quantify by immunofluorescence how many of the CEV were attached to an actin tail and to compare this number to WR infection were unsuccessful. It was found that the MVA infection resulted in so many CEVs at the plasma membrane that the actin tails were no longer visible and could not be counted accurately.

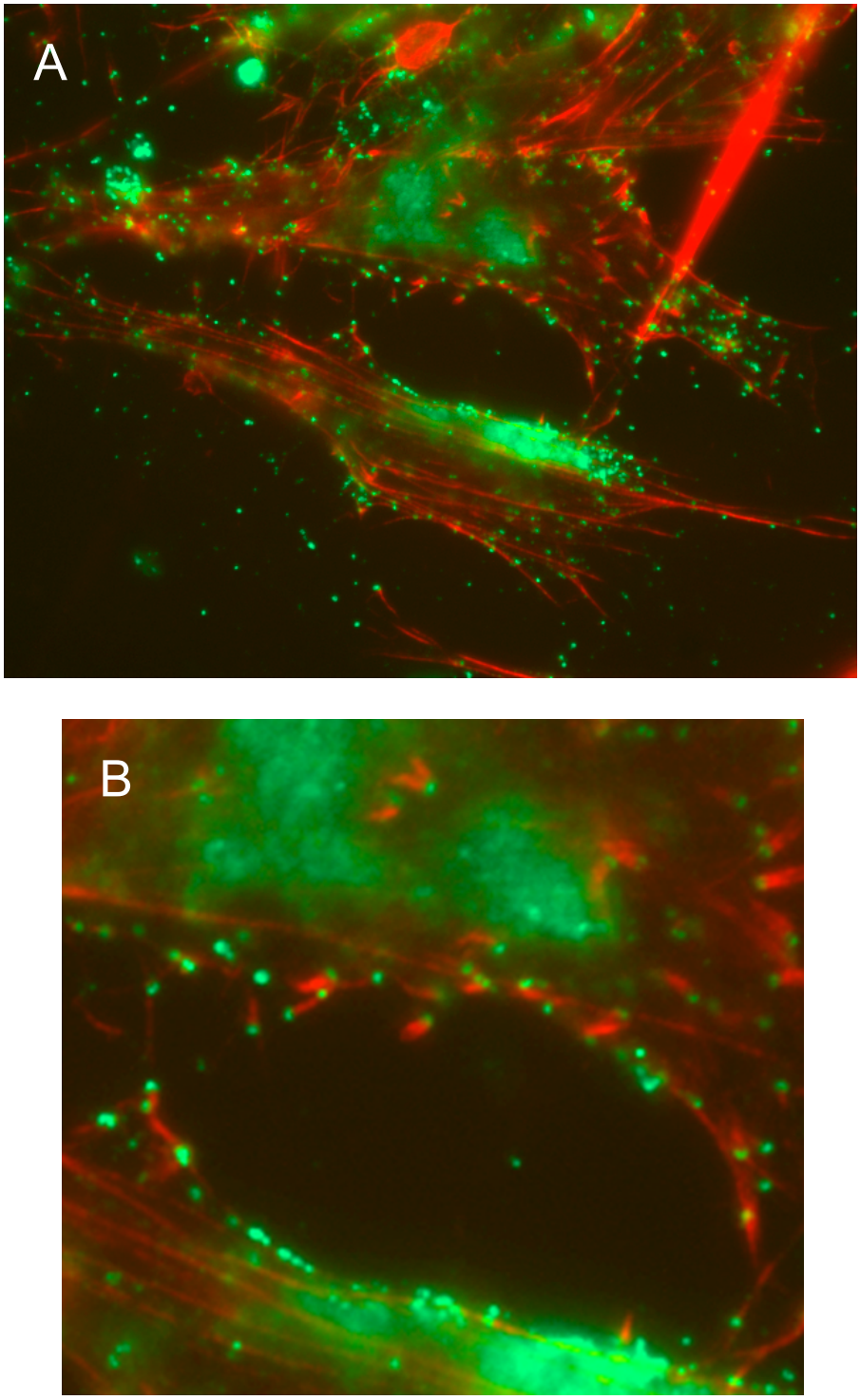


Figure 17: Immunofluorescence microscopy of CEVs on actin tails in MVA infected chicken embryo fibroblasts. Cells grown on cover slips infected and fixed as described above. CEVs on the surface only were labeled with rat anti B5R and incubated with goat anti rat FITC (shown as green dots in panel A). Cells were subsequently permeabilized with TX-100, and actin was stained with phalloidin rhodamine (shown in red). Panel B shows a selected area of panel A in a higher magnification. Arrow heads point to CEVs on tips of actin tails.

5.3 Targeting a foreign protein to the outer envelope of MVA extracellular enveloped virions

As outlined before EEV was chosen as a model of integration. The initial model antigen consisted of the hemagglutinin of Canine Distemper Virus (CDV.H) of the virulent A75/17 strain (Summers et al., 1984). CDV.H is an immunodominant antigen, capable of eliciting fully protective antibody response against CDV (Pardo et al., 1997; Welter et al., 1999).

Two different strategies were followed to target the recombinant protein to the surface of extracellular enveloped virions:

Since it was shown, that non-VV proteins can be incorporated to some extent into the EEV (Schmelz et al., 1994; Vanderplasschen et al., 1998b) native CDV.H (using the full coding sequence of the hemagglutinin from the CDV strain A75/17) was inserted into the MVA genome.

To increase the chance of incorporation of the CDV.H protein into the EEV, different fusion-proteins were engineered, in which the transmembrane domain of CDV.H was replaced by transmembrane anchors of known EEV membrane proteins (Roper et al., 1998). Those proteins were expected to be targeted to the trans-Golgi network (TGN), where the IMVs obtain their additional membranes to form the IEV and then EEV. This method was used successfully for targeting a chimeric human immunodeficiency virus type 1 glycoprotein to the outer envelope of nascent vaccinia virions, using the transmembrane and cytoplasmic domains of the vaccinia virus EEV protein B5R (Katz et al., 1997). In this work the transmembrane domains of the EEV specific genes A33R, A34R, and A36R were used.

5.3.1 Generation of recombinant MVA

During the attenuation process the MVA genome has suffered 6 major deletions. Those naturally occurring sites of deletion have been used as insertion sites for foreign genes. In this work the target genes were inserted into the deletion III site in the MVA genome (Fig. 19).

Insertion plasmids flanked by sequences of MVA DNA allow precise insertion of target genes into the selected site within the MVA genome by homologous recombination (Fig. 19). Additionally, the plasmids contain the coding sequence of the VV host range gene K1L under transcriptional control of its authentic promotor to be integrated as a transient selectable marker to facilitate isolation of recombinant

MVA (rMVA) by growth selection on RK-13 cells. Multiplication of MVA in rabbit kidney-derived RK-13 cells, but not in other nonpermissive cells, can be restored by insertion of the vaccinia virus K1L gene into the MVA genome. During nonproductive infection of MVA in RK-13 cells, viral early mRNA is transcribed and persists for at least several hours, but synthesis of virus-induced polypeptides occurs only during the first hour and is followed by abrupt inhibition of all protein synthesis (Sutter et al., 1994). Insertion of the K1L gene allows MVA infection to proceed to late stages of viral protein synthesis in RK-13 cells leading to effective production of infectious progeny (Sutter et al., 1994). Repetitive DNA sequences flanking the K1L gene were designed to remove the marker gene from the viral genome by homologous recombination under nonselective growth conditions in BHK-21 cells. The principle of this procedure is depicted in fig. 19, and the procedure is described in detail in 4.5.
CDV.H is a type II membrane glycoprotein. Group II integral membrane proteins have the N-terminal domain on the cytosolic side and expose the C-terminal domain on the extracellular side. These proteins possess a signal sequence at or near the N-terminus, which is combined with an anchor sequence for integration into the plasma membrane. Plasmids containing genes for chimeric proteins were obtained form Riccardo Witteck, Switzerland. The chimeric genes consisted of the extracellular domain of CDV.H and the transmembrane domain of the EEV specific VV proteins A33R, A34R and A36R, respectively (see Fig. 18), all of which were thought to be group II transmembrane proteins too. The idea was that the signal sequence in the transmembrane domain of the EEV specific proteins should target the extracellular domain of CDV.H to the surface of EEV particles.

Figure 18: schematic presentation of chimeric proteins. TM = tansmembrane domain of VV EEV proteins. A33R TM = AA1-AA60 of VV strain IHD-J, A34R TM = AA1-AA34 of VV strain IHD-J, A36R TM = AA1-AA25 of VV strain WR. Extracellular domain of CDV.H = AA56-AA608 of CDV.H strain A75/17. Constructed by Paul Howley, Institut de Biologie Animale, Universite Lausanne, Switzerland.

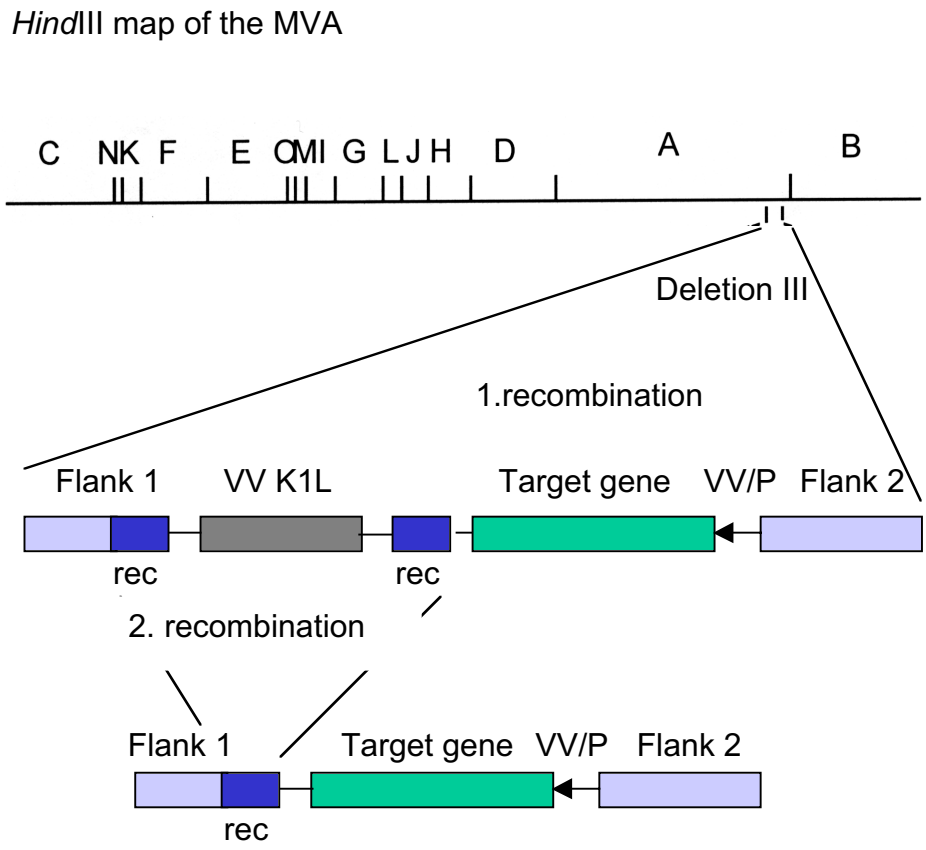


Figure 19: Generation of recombinant MVA by homologous recombination. The vector plasmids pIIIdHR are created to insert foreign proteins into the deletion III of the MVA genome (schematic shown as a HindIII restriction map). Flank 1 and flank 2 refer to MVA-DNA sequences upstream and downstream the deletion III allowing the insertion of the target genes into the MVA genome by homologous recombination. Rec2 indicates the position of a 283-bp repetitive MVA-DNA fragment homologous to the right end of flank 1, which allows the deletion of the K1L expression cassette in a second recombination event, resulting in a recombinant MVA containing only the target gene.

5.3.1.1 Construction of insertion plasmids

A double set of plasmids was constructed, placing the recombinant genes under transcriptional control of either the natural vaccinia virus specific early/late promotor P7.5 or the strong synthetic promotor sP. The plasmids were obtained by opening the MVA insertion vectors pIIIdHR-P7.5 and pIIIdHR-sP with PmeI in the multiple cloning site (Staib et al., 2000). Blunt inserts A33R-CDV.H, A34R-CDV.H, and CDV.H were cloned into the dephosphorylated vector DNA (see 4.2.1). The insertion of the target genes into the expression vector and the correct orientation of the insert were confirmed by restriction analysis. For each insertion plasmid two positive clones were amplified.

Before generating the recombinant viruses the synthesis of the foreign proteins was checked by transient expression experiments. Therefore, MVA infected BHK-21 cells were transfected with equal amounts of the different insertion plasmids. Cell lysates from these cells were assessed by western blot analysis using a rabbit serum against CDV.H (Fig. 20). The specificity of the antibody was confirmed by transfection with the empty plasmids and by a mock control.

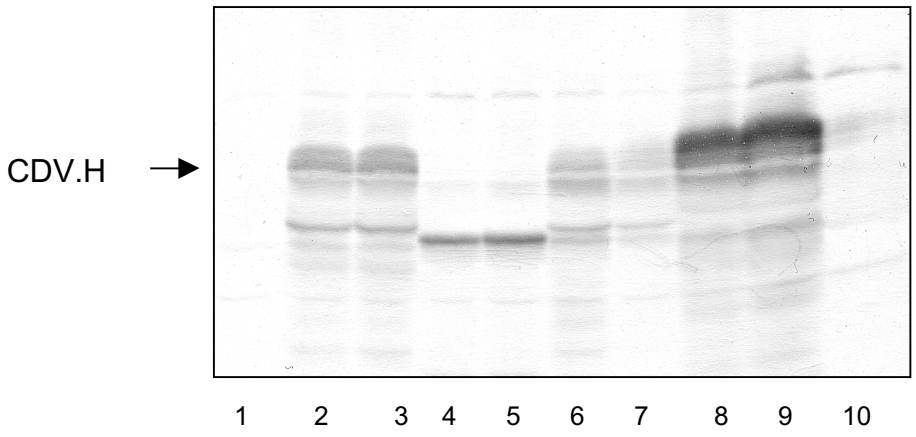


Figure 20: Transient protein expression. BHK-21 cells grown in 6-well plates were infected with m.o.i. 10 MVA and transfected with 15 µg plasmid DNA each: pIIIdHR-sP (lane 1), pIIIdHR-sP-CDV.H (lane 2+3, pIIIdHR-sP-A36R-CDV.H (lane 4+5), pIIIdHR-sP-A34R-CDV.H (lane 6+7), pIIIdHR-sP-A33R-CDV.H (lane 8+9), and mock (lane 10). Bands are detected with an antibody against CDV.H.

Specific staining of a band of the predicted molecular weight of ca. 75 kDa was detected for CDV.H wt (Fig. 20). The different fusion constructs generated different results. Clearly the highest expression was achieved using the transmembrane anchor of A33R. In contrast, either lesser amounts of recombinant protein (A34R-CDV.H) or recombinant protein of a different molecular weight (A36R-CDV.H) were obtained using the other VV EEV transmembrane anchors. Corresponding results were achieved by immunostaining of RK-13 cell monolayers after infection with the cell lysate of infected and transfected BHK-21 cells. The ratio of detectable cytopathic effects (CPE) in total versus CDV.H-positive stained CPEs was best for the A33R-CDV.H fusionprotein. Generally, the VV early/late promotor P7.5 provided a higher transfection efficiency than the strong synthetic promotor sP. The A36R-CDV.H construct was not used in the further experiments because of the expression of a truncated protein. Some time later it was published that A36R is a type Ib membrane protein, anchored with its N-terminus in the membrane but exposing the

C-terminus into the cytosol (Rottger et al., 1999). Furthermore, A36R was shown to be present only on the outer envelope of IEV particles but not on CEVs (van Eijl et al., 2000), and was therefore not suitable for this project.

5.3.1.2 Plaque purification of recombinant MVA

BHK-21 cells were infected with a low m.o.i. of wtMVA and transfected with a surplus of plasmid DNA. This combination allows the highest percentage of recombinant MVA. The cell lysates of infected and transfected BHK-21 cells were diluted on RK-13 cell monolayers to select for recombinant MVA. Wild type MVA is not able to grow efficiently in RK-13 cells, only rMVA expressing the K1L gene product can replicate. Cell aggregates, the typical cytopathic effect of MVA on RK-13 cells, were picked in the highest dilution and distributed to a new round of plaque purification. During RK-13 passages the expression of the proteins was routinely checked by immunostaining (Fig. 21), and the absence of remaining wtMVA by PCR analysis (Fig. 22).

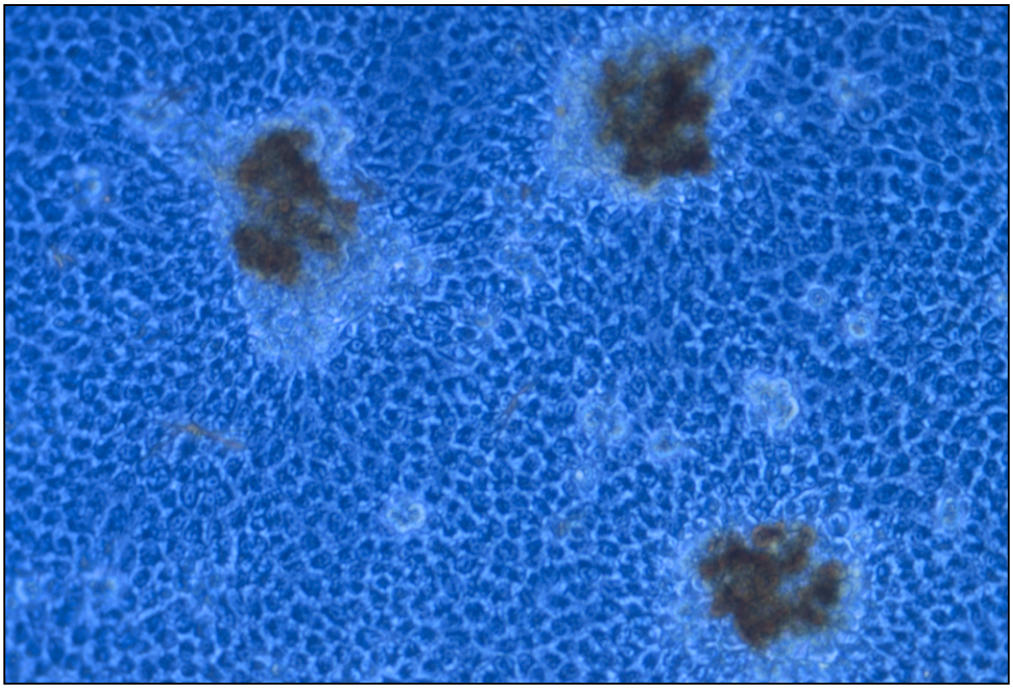


Figure 21: Microscopy of fixed RK-13 cells infected with recombinant MVA expressing CDV.H. Cell aggregates form on RK-13 cells upon infection with MVA expressing the K1L gene. Cells were fixed on day 3 post infection and stained with anti-CDV.H (see 3.13.2).

Recombinant MVA was plaque purified by serial passages on RK-13 until elimination of wtMVA. Wild type free recombinant MVA was plaque purified by passages on the

MVA permissive cell line BHK-21 to delete the K1L gene in the absence of selection pressure by an intramolecular recombination event (Fig. 19).
Recombinant MVA with the native CDV.H (MVA-P7.5-CDV.H) and the A33R-CDV.H fusion protein (MVA-P7.5-A33R-CDV.H), both under transcriptional control of the vaccinia virus early/late promotor P7.5, were successfully generated. The construction of a recombinant virus containing the A34R-CDV.H fusion protein failed as well as the construction of rMVAs with the target genes under the control of the strong synthetic promotor sP, which was designed to overexpress the target proteins to obtain a high abundance of foreign protein on the surface of EEV. During the plaque purification all of them lost their ability to grow on RK-13 cells after a few passages and/or did not express the foreign gene any more.
MVA-P7.5-A33R-CDV.H was obtained after 7 passages on RK-13 cells to eliminate wtMVA and 6 passages on BHK-21 for deletion of the K1L gene. MVA-P7.5-CDV.H was obtained after 3 passages on RK-13 and 4 passages on BHK-21, respectively.

5.3.1.3 Characterization of recombinant MVA

The obtained recombinants were characterized for genetic stability and clonal purity by PCR analysis of the viral DNA using primers complementary to the flanking regions of the insertion site (see 4.5.3). Hereby, the presence of a stably integrated recombinant DNA was confirmed as well as the absence of any wildtype DNA and the selective marker gene K1L (Fig. 22A). The original K1L gene of Vaccinia virus Ankara was only partially deleted during the attenuation process that had lead to the generation of MVA. Remains of the gene are still present at the deletion II site. In some cases recombination events occur between the K1L sequence in the plasmid DNA and the remaining K1L sequence in the MVA genome leading to a genomic aberration. Therefore, the region of the deletion II site was tested for changes in size by PCR analysis, using primers complementary to MVA sequences upstream and downstream the deletion II (Fig. 22B).

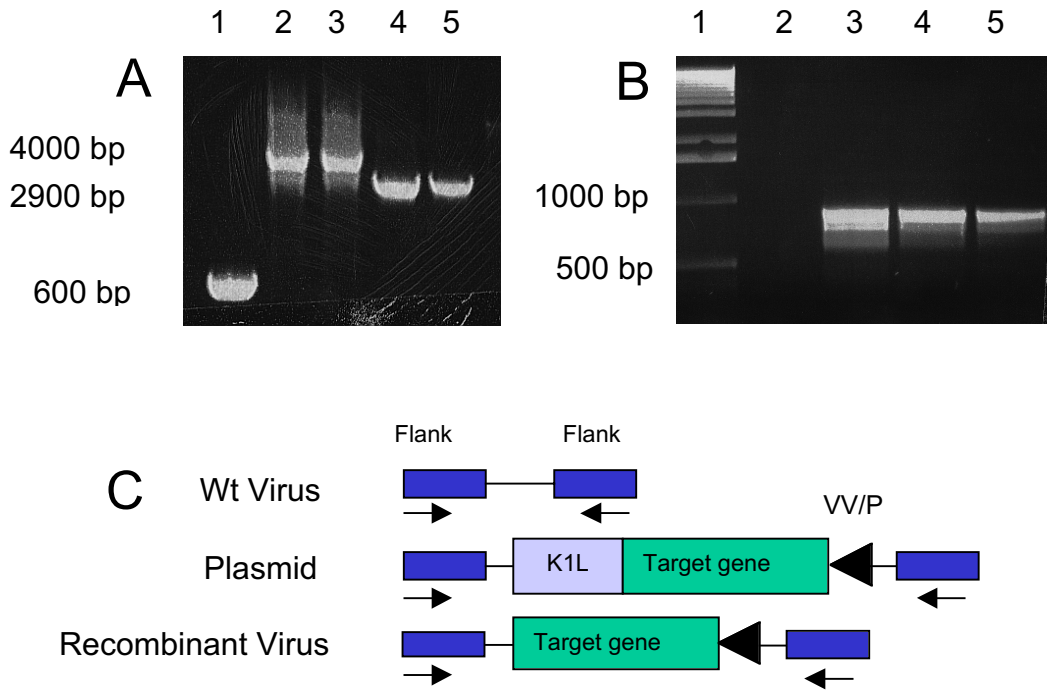


Figure 22: PCR analysis for insertion of the foreign protein into the deletion III of the MVA genome and deletion of the marker gene K1L (A), and unaltered site of deletion II (B). A: Amplification of the deletion III using the primers NIH-GS83 and IIIfl1b results in a PCR product of approximately 600 bp for the wtMVA. (lane 1). Amplification of the complete insertion cassette including the K1L and the target gene with the same primers results in a PCR product of about 4000 bp (pIIIdHR-P7.5-A33R-CDV.H in lane 2, pIIIdHR-P7.5- CDV.H in lane 3). Recombinant MVA with the inserted target gene and the deleted marker gene show a band of about 2900 bp after amplification of the genomic deletion III (lane 4: MVA-P7.5-A33R-CDV.H, lane 5: MVA-P7.5-CDV.H). A schematic overview is depicted in panel C. Arrows indicate primers. B: Amplification of the deletion II using the primers II 0011b-5' and II 0011b-3' shows no genomic aberration of MVA- P7.5-A33R-CDV.H (lane 4) and MVA-P7.5-CDV.H (lane 5) compared to wtMVA (lane 3). Lane 2 contains the H_2O control and lane 1 the 1kb ladder.

The unaffected ability of the recombinant viruses to productively grow in permissive cells in comparison to wtMVA was confirmed in a multiple step growth curve (Fig. 23). Of special interest was the unaltered production of enveloped virions present in the supernatant of the infected cells.

DNA of the recombinant MVA was isolated and the inserted genes including the promotor region were sequenced to confirm the absence of any mutation. The expression of the recombinant proteins was again verified by western blot analysis. A kinetic study was performed to obtain more detailed information of the protein production (Fig. 24). Although the cells were infected with the same moi of rMVA, the fusion protein was produced more efficiently than the native CDV.H. The same effect was detected by comparative immunostaining of infected CEF monolayers (Fig. 25).

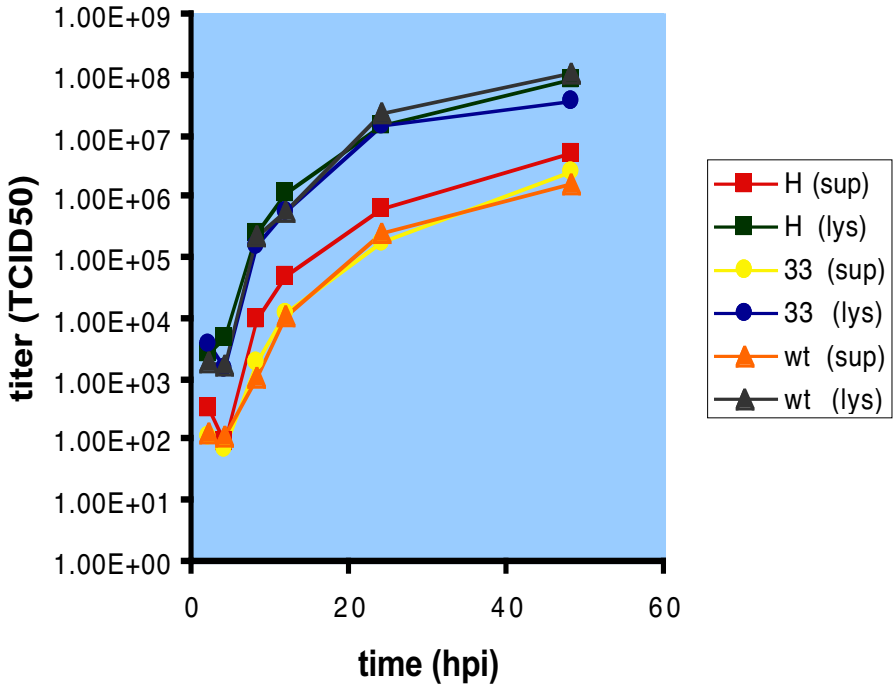


Figure 23: Productive replication of rMVA shown in a multiple step growth curve. CEF cells grown in 6-well plates were infected with wtMVA (▲ wt), MVA-P7.5-A33R-CDV.H (● 33), and MVA-P7.5-CDV.H (■ H) at m.o.i. of 0.05. Supernatant (sup) and cell lysate (lys) were harvested separately at 0, 4, 8, 12, 24, and 48 hours post infection.

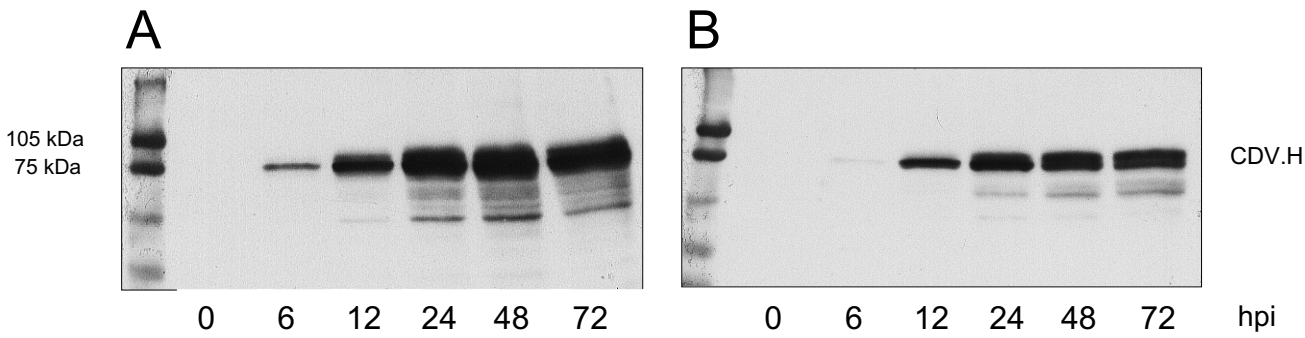


Figure 24: Expression of recombinant proteins in a time course experiment. BHK-21 cells grown in 6-well plates were infected with 10IU/cell of MVA-P7.5-A33R-CDV.H (A) and MVA-P7.5-CDV.H (B) for 1 h. Cell lysates were harvested at 0, 6, 12, 24, 48, and 72 h.p.i. 1/15th of the total volume was applied to each slot. All the samples were treated equally at any time of the experiment. The proteins were detected in western blot analysis by a rabbit anti-CDV.H serum.

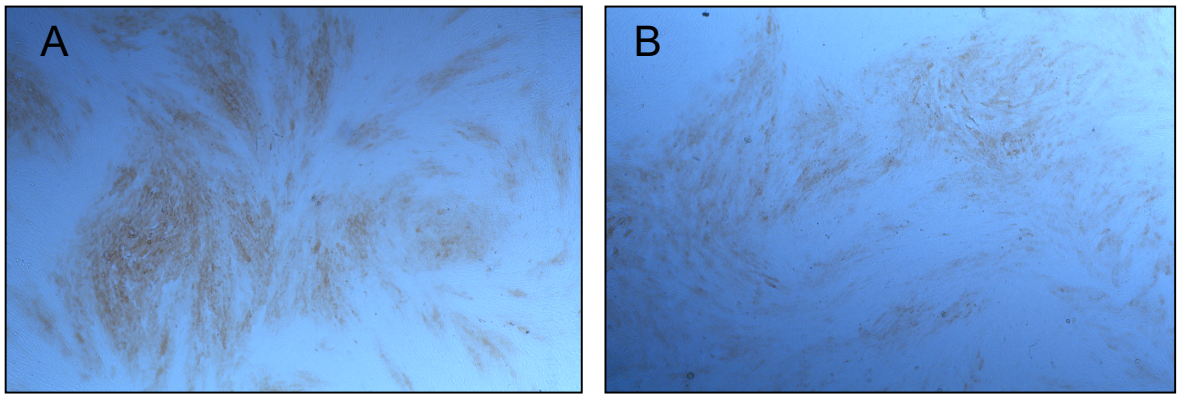


Figure 25: Immunostaining of fixed BHK-21 cells infected with MVA-P7.5-A33R-CDV.H (A) or MVA-P7.5-CDV.H (B). The recombinant proteins were detected by a rabbit anti-CDV.H serum.

The functional integrity of the early and late expression of the promotor was tested by western blot analysis in a time course experiment, where AraC, an inhibitor of DNA synthesis, was added to the infected cells. Since vaccinia virus late gene expression is dependent on DNA synthesis, no late genes are made in the presence of AraC. In Fig. 26 the amount of protein detected in presence or absence of AraC reflects the expression of the proteins early and late or early only, convincing the functional ability of the promotor.

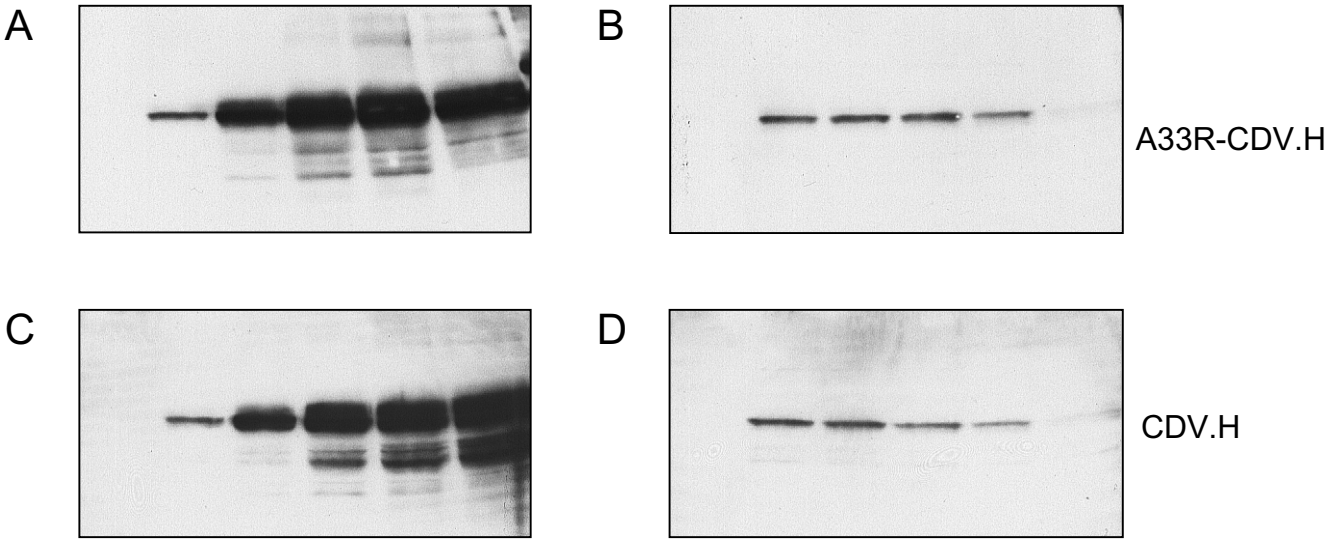


Figure 26: Expression of recombinant proteins in presence (B, D) or absence (A, C) of AraC. Cells were infected with MOI 10 MVA-P7.5-A33R-CDV.H (A, B) or MVA-P7.5-CDV.H (C, D) and harvested as in the time course experiment described above. In the case of drug treatment 40 µg/ml AraC were added directly after the infection.

This data shows that the recombinant proteins are expressed, the promotor is functional in both early and late phase and the DNA sequence of the genes is unmodified. But for unknown reasons the fusion protein is expressed to a higher extend than native CDV.H.

5.3.2 Localization of the recombinant protein

5.3.2.1 Recombinant proteins are present in the supernatant of infected cells

A first attempt to determine the localization of the proteins was made by testing the supernatant of infected cell monolayers for the presence of the foreign protein in westernblot analysis (Fig. 27). Presence of the foreign proteins in the supernatant can be an indication for the incorporation in EEV particles, also present in the supernatant. As a control a rabbit serum against B5R, the most abundant protein in EEV, was used. The B5R protein has the size of 42 kDa and forms also dimers in the envelope (Engelstad et al., 1992). High amounts of B5R were found in the supernatant, confirming the presence of EEV particles. Taking the same samples and using the antibody directed against CDV.H, specific bands for CDV.H could be detected for both rMVA, but not for wtMVA. Although the same quantity of material was used, the staining for the native CDV.H turned out to be stronger than for the fusion protein, which was designed to increase the efficiency of integration. This result can be due to the fact that not only EEV particles are present in the supernatant, but also cellular debris.

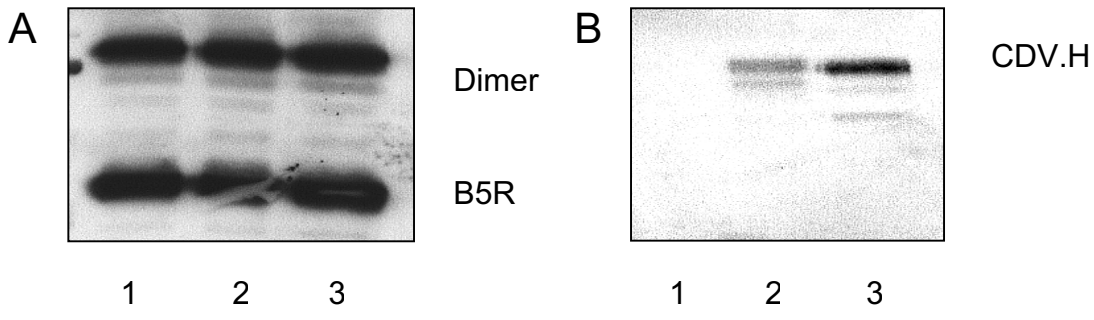


Figure 27: Western blot analysis of supernatant of BHK-21 cells infected with wtMVA lane 1), MVA-P7.5-A33R-CDV.H (lane 2), or MVA-P7.5-CDV.H (lane 3) using serum against B5R (A) or CDV.H (B).

5.3.2.2 Recombinant proteins are present on CsCl gradient purified enveloped particles

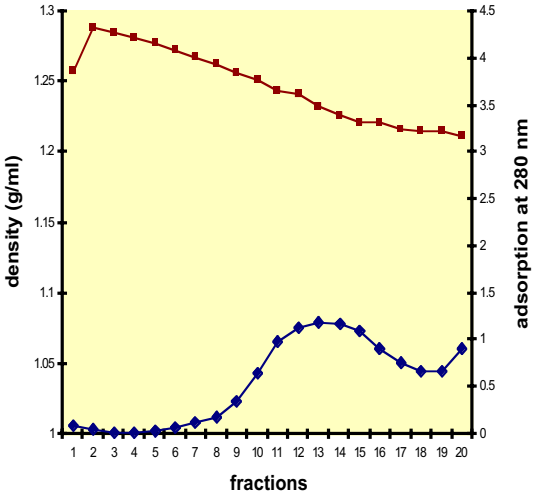
Since the detection of the foreign proteins in the supernatant of infected cell monolayers was just a hint for their presence also on EEV particles, virus particles were purified by CsCl gradients (Fig. 28). To obtain as much EEV as possible, cells were infected at a low MOI, and the material was harvested 50 h.p.i. In parallel, virus was grown in presence of Brefeldin A as described in 5.1.3.1 (Fig. 29). In these conditions, enough material of all viral forms for further analysis was obtained. Particles out of the peak fractions were then used in western blot analysis (Fig. 30).

Like in the western blot analysis carried out before, wtMVA showed no labeling for CDV.H in any of the analyzed fractions, confirming the specificity of the antibody. Heavy labeling was found in the IEV fraction of both recombinants. The fusion protein was detected in a single prominent band of about 75-80 kDa, native CDV.H on the other hand was also detected in a form of lower molecular weight, probably a degradation product. EEV particles of both recombinants also showed labeling with anti CDV.H, to a lower extent as the IEV particles, but with the same expression pattern. Notably, CEVs were almost not stained, only a very faint band of the predicted molecular weight could be detected for the fusion protein. This was also true for the IMV particles of MVA-P7.5-A33R-CDV.H. Morphologically, CEVs and EEVs are identical particles but the trypsinization used to isolate the CEVs might have had an influence on the integrity of the protein leading to a changed accessibility of the antibody.

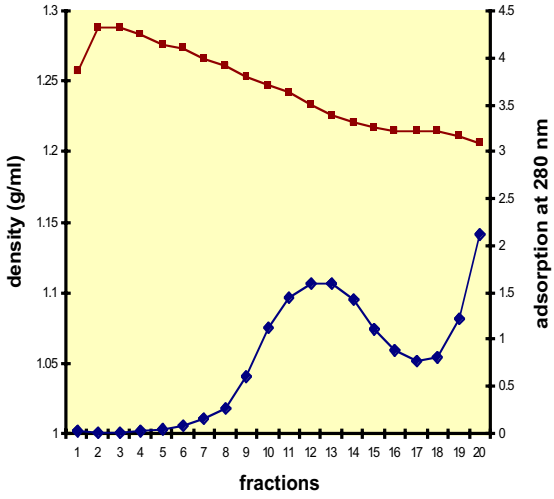
The localization of the proteins was then studied by negative staining EM of purified particles (Fig. 31). This technique is highly sensitive and allows the detection of single proteins on individual virus particles. The wt control confirmed the absence of any background staining in the CEV and EEV fraction (Fig. 32). In the IMV and IEV fraction a minor cross reaction was detectable.

Figure 28: (next page) CsCl gradients of recombinant MVA. CEF cells were infected at a MOI of 0.05 with MVA-P7.5-A33R-CDV.H (A, C and E) and MVA-P7.5-CDV.H (B, D and F), respectively. At 50 h.p.i. EEV (A and B), CEV (C and D), and intracellular virus (E and F) were harvested as described in fig. 6. Again the intracellular virus had to be divided and loaded on two gradients each, but only one of those is shown for each recombinant.

A


density (g/ml)
adsorption at 280 nm
fractions

B


density (g/ml)
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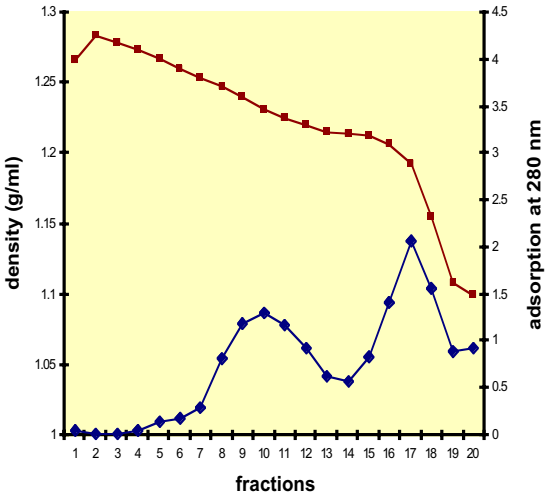
C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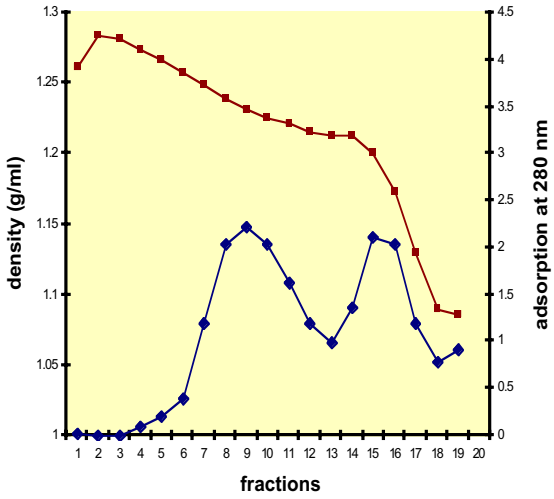
fractions

D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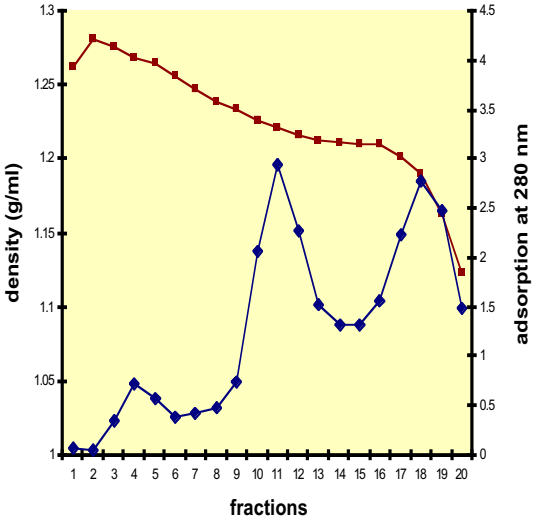
E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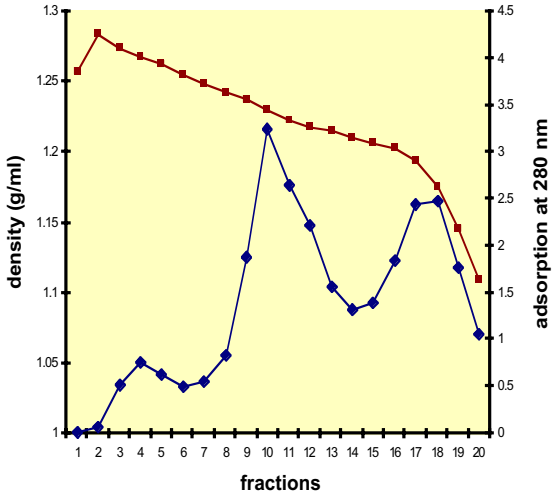
F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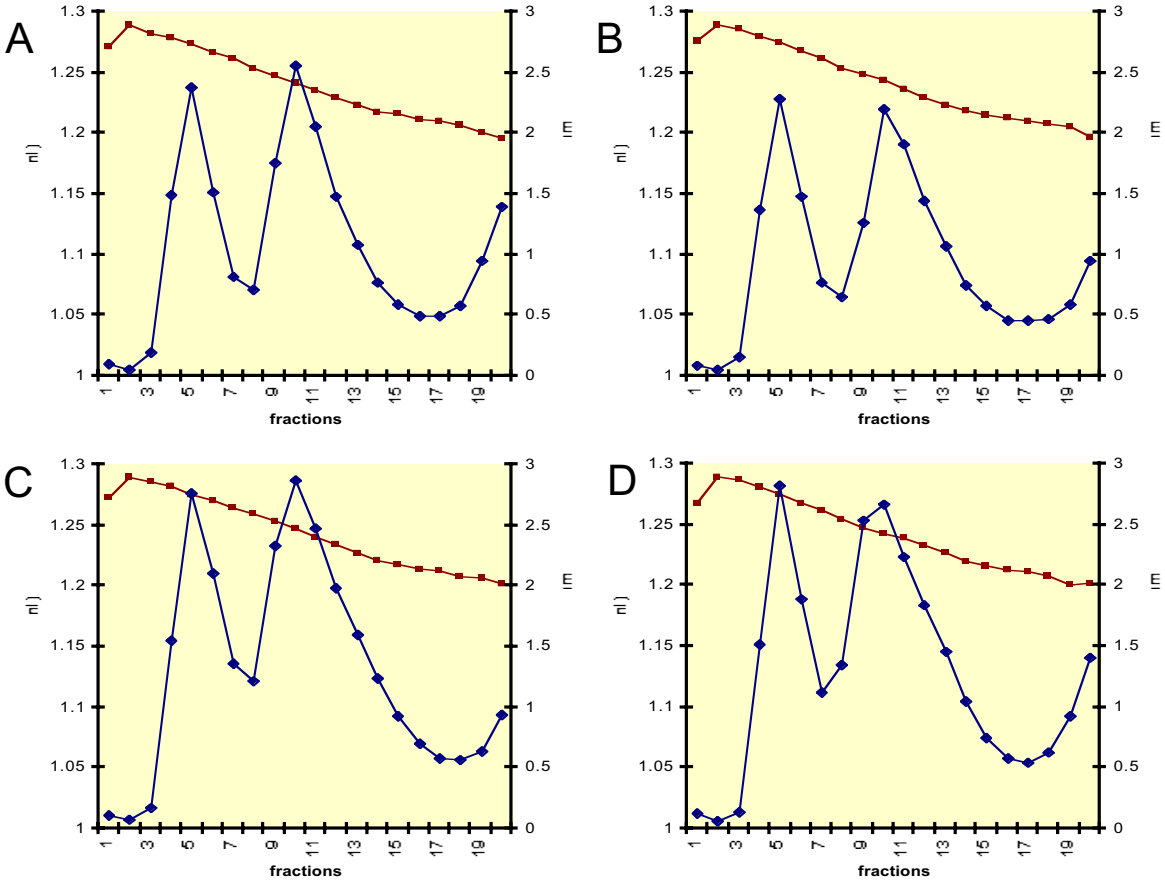


Figure 29: CsCl gradients of the intracellular fraction of MVA-P7.5-A33R-CDV.H (A and B) and MVA-P7.5-CDV.H (C and D) after treatment with Brefeldin A.

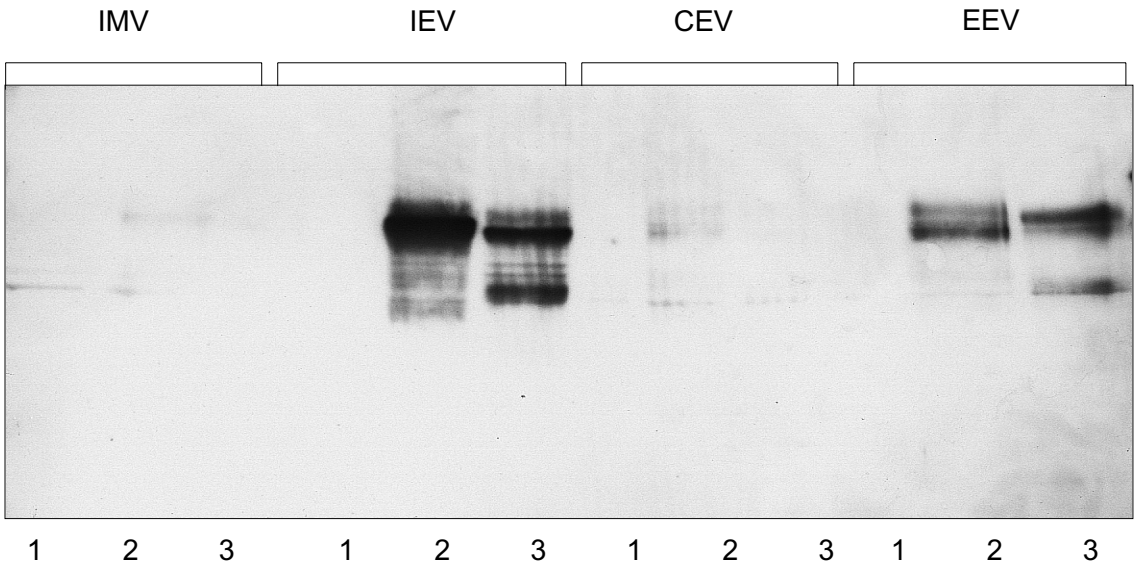


Figure 30: Detection of the recombinant proteins in CsCl-gradient purified particles. Virus particles out of the peak fractions of CsCl-gradients corresponding to IMVs, IEVs, CEVs and EEVs were measured due to their protein content. 7µg protein was applied per slot. The recombinant protein was detected by a rabbit serum anti CDV.H (1 = wtMVA, 2 = MVA-P7.5-A33R-CDV.H, 3 = MVA-P7.5-CDV.H).

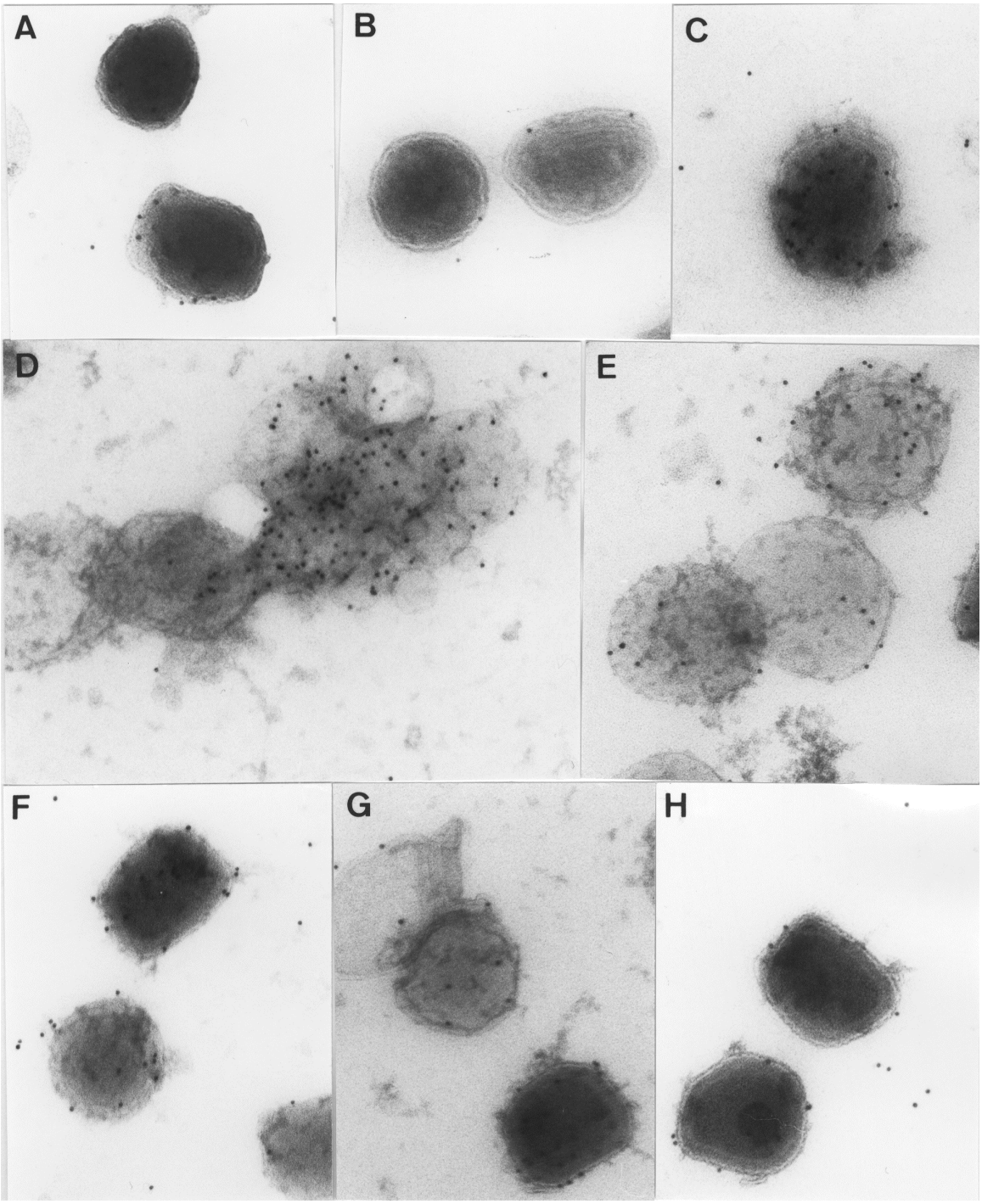


Figure 31: Micrographs of CsCl gradient purified virus particles of rMVA labeled with anti CDV.H. The recombinant protein on IMVs (A and B), IEVs, (C and D), CEVs (E and F) and EEVs (G and H) of MVA-P7.5-A33R-CDV.H (A, C, E and G) and MVA-P7.5-CDV.H (B, D, F and H) was detected by a rabbit serum against CDV.H and 10 nm protein A gold.

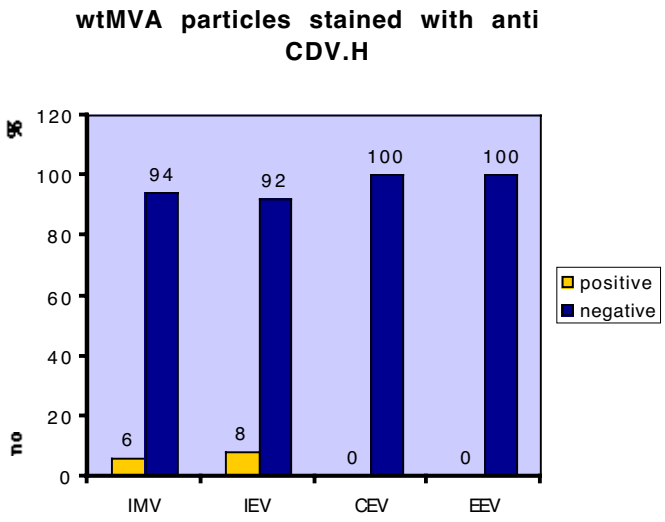


Figure 32: wtMVA particles stained with anti CDV.H. Virus out of the peak fractions of CsCl gradient purified viruses was negative stained and labeled with rabbit serum against CDV.H and incubated with 10 nm protein A gold. 100 randomly chosen particles were counted for the presence or absence of gold particles.

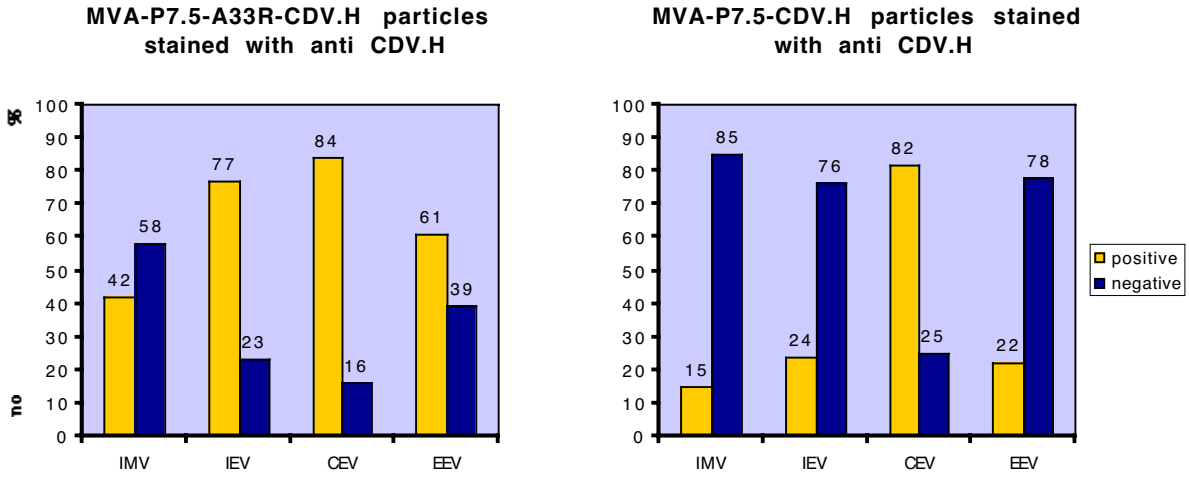


Figure 33: Virus particles of recombinant MVA labeled with anti CDV.H. 100 randomly chosen particles each were counted for the presence or absence of labeling.

Over 80% of the CEV particles of both rMVAs showed positive staining with anti CDV.H (Fig. 33). This result is contradictory to the data obtained by western blot where no notable band could be detected in the CEV fraction of any of the two recombinant MVAs. The main difference between A33R-CDV.H and CDV.H particles is, that the fusion protein was also highly present in the IEV and EEV fraction, whereas positive staining for CDV.H in those fractions was extremely low for the native CDV.H protein, also contradictory to the data obtained by western blot analysis. Fig. 34 shows a possible explanation. As outlined before only IMV and CEV

particles can be purified in a very clean way. The IEV and EEV fractions contain cellular debris. Cellular membranes and vesicles labeled heavily with anti CDV.H are frequently seen in those fractions. For MVA-P7.5-A33R-CDV.H also IMV particles showed a labeling with anti CDV.H. This data suggests that the prominent bands for the recombinant proteins, that were detected by western blot analysis, did not reflect the presence of that protein on those particles, but was mainly due to contamination with cellular debris despite the purification.

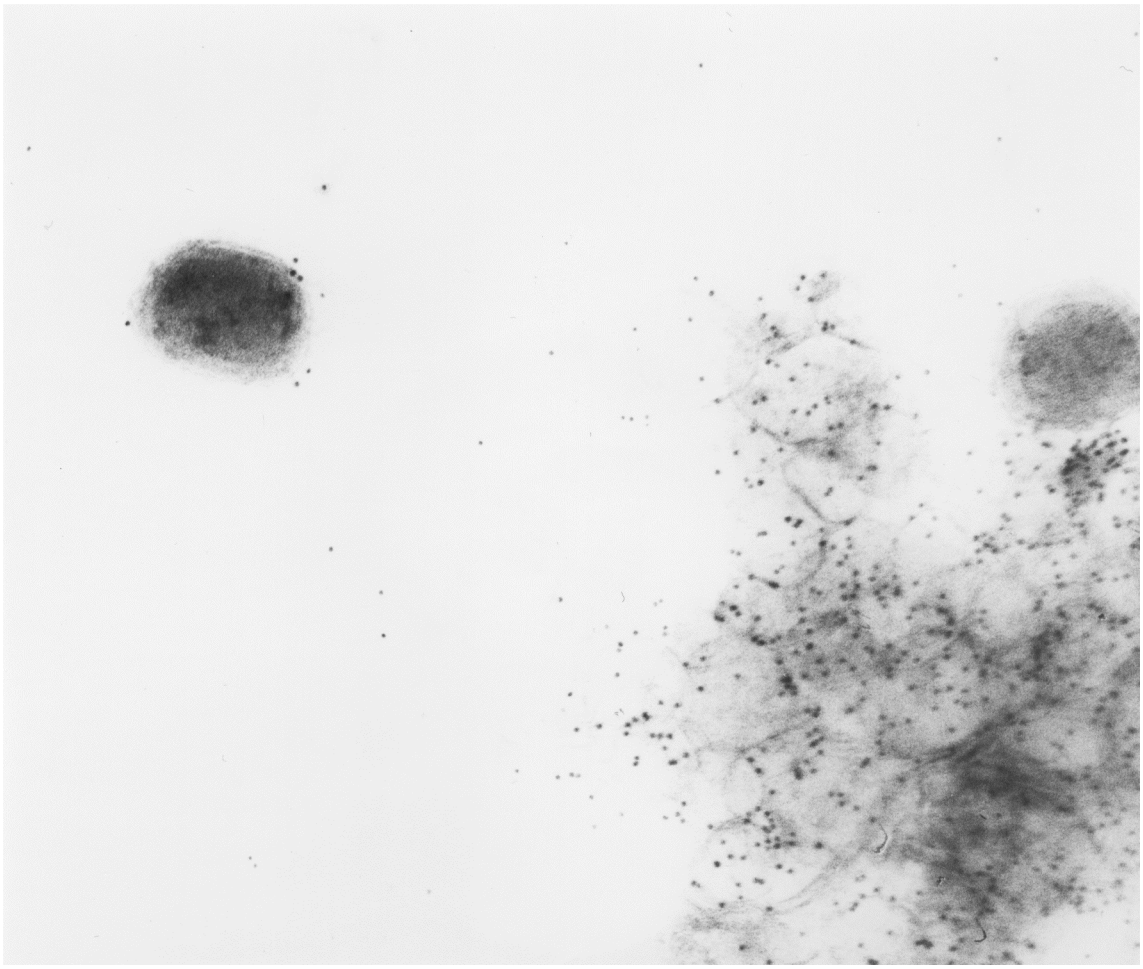


Figure 34: Micrograph of an EEV particle of MVA-P7.5-CDV.H labeled with serum against CDV.H and protein A gold. The EEV particle itself shows a poor labeling with CDV.H, whereas vesicles on the right side of the picture label heavily.

Since the outer envelopes of the viral particles suffer during the purification process, most of the particles have lost parts of their envelope or even lost it completely. Therefore, the relative distribution of CDV.H versus B5R as a marker for CEVs and EEVs was examined (Tab. 4). Furthermore, it was possible to distinguish between presence and absence of labeling, and the state of the envelopes and abundance of the proteins was also characterized by counting the gold particles on virus surfaces.

If CDV.H proteins are present on the envelope of EEV, the distribution pattern of CDV.H labeling should correspond to B5R labeling. This is not clearly the fact. The fusion protein seems to be abundant in IMVs, CEVs, and EEVs to almost the same extent. The native CDV.H in contrast is preferentially located on CEVs, particularly with respect to the weak labeling of this fraction with anti B5R. On the other hand, there is no explanation for the weak labeling of MVA-P7.5-CDV.H EEV particles with anti CDV.H.

	MVA-P7.5-A33R-CDV.H		**MVA-P7.5-CDV.H**	
	anti B5R	anti CDV.H	anti B5R	anti CDV.H
IMV	69	208	0	47
CEV	1371	345	269	307
EEV	935	229	269	40

Table 4: CsCl gradient purified viruses were negative stained and incubated with antibodies directed against B5R and CDV.H, respectively, followed by an incubation with protein A gold. Gold dots were counted on 50 randomly chosen particles per fraction of each virus.

5.3.2.3 In infected cells native CDV.H is localized predominantly in the cytoplasm and the A33R-CDV.H fusion protein in the ER

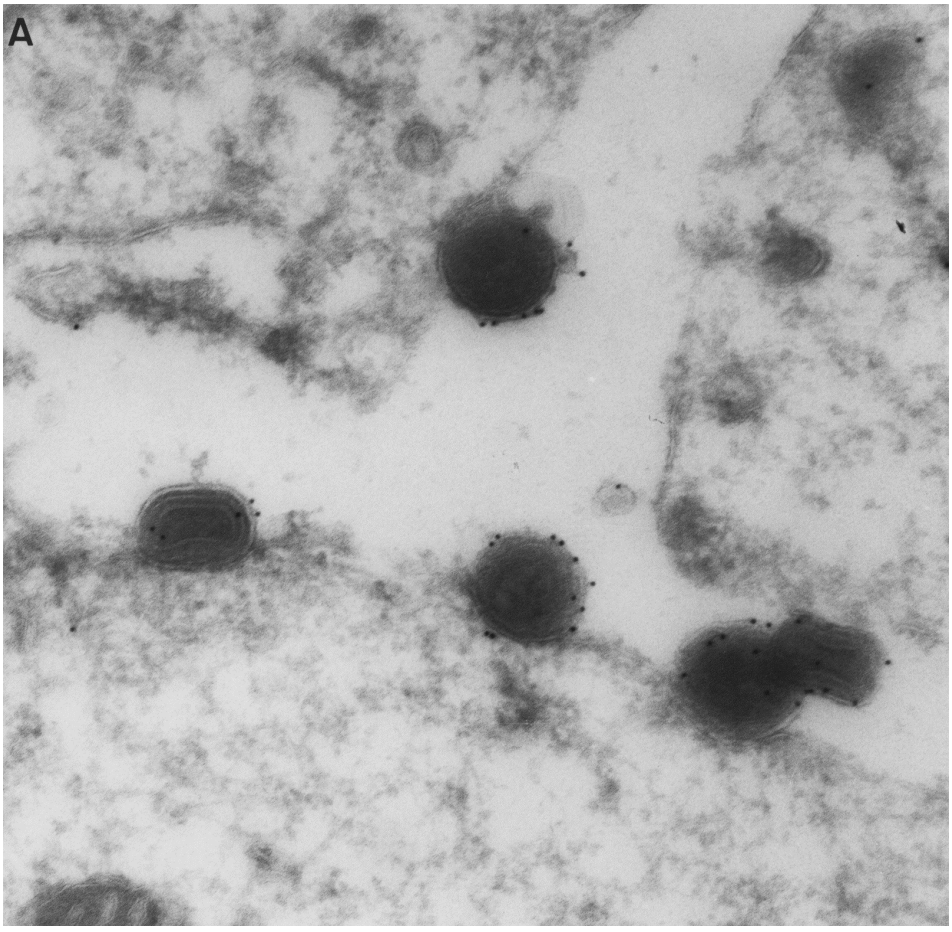
The localization of the recombinant proteins in infected cells was determined by cryo EM (Griffiths, 1993). These experiments were part of a collaboration between the group “viral vectors” from Gerd Sutter, GSF, Munich and the “vaccinia virus” group from Jacomine Krijnse Locker, Cell Biology programme, EMBL, Heidelberg, and were carried out by M.C. Sancho.

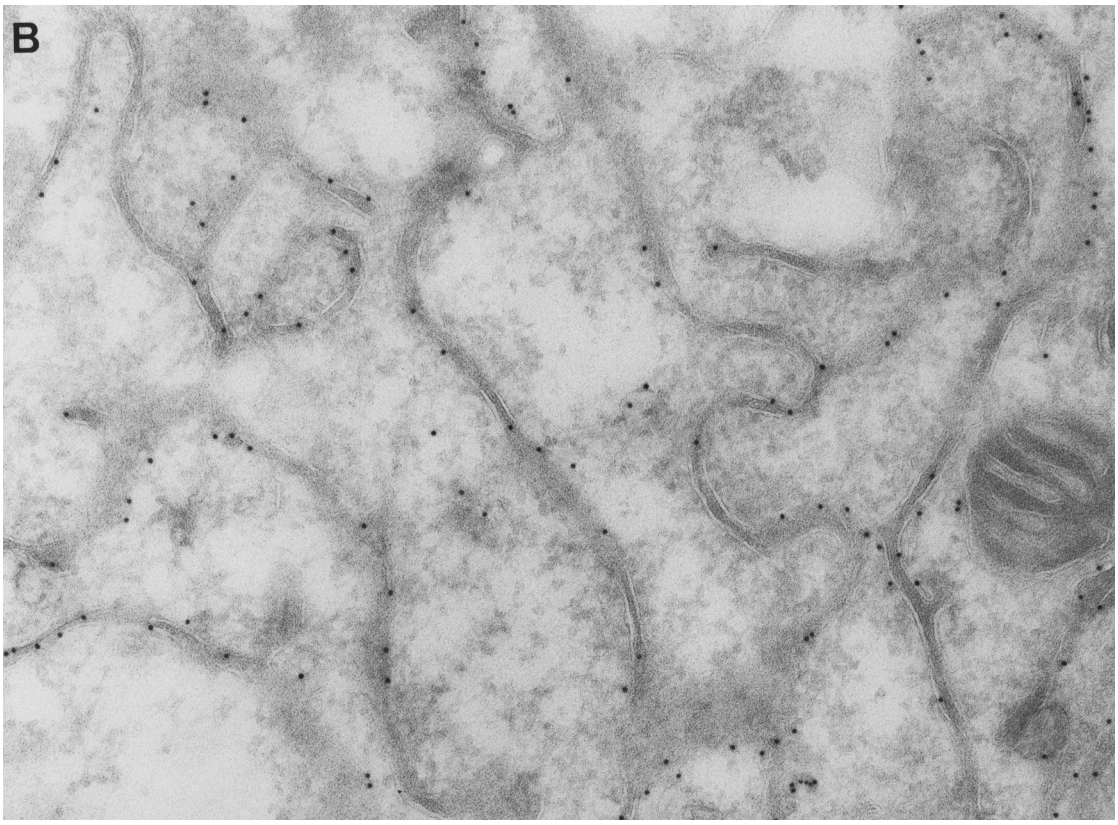
BHK-21 cells were infected with recombinant and wtMVA for 16 h, fixed and prepared for cryo EM (Griffiths, 1993). Ultra thin sections were labeled with the rabbit serum against CDV.H and B5R, respectively, and incubated with protein A gold. Fig. 35A shows a CDV particle labeled with anti B5R. A comparable abundance of recombinant protein on the surface of CEVs was not detected, but at least some CEV particles were decorated with a few gold dots confirming the presence of recombinant protein on these particles. Also in this experiment the native CDV.H appeared to be incorporated to a higher extent than the fusion protein. However, the vast majority of labeling was not found to be associated with enveloped viral particles. A33R-CDV.H was located predominantly in the ER (Fig. 35B) and in the plasma membrane. Native CDV.H, on the other hand, did not show any preferential

location, but seemed to be spread over the cytoplasm and cellular membranes and vesicles (Fig. 35C). Additionally, the labeling for native CDV.H was very weak.

Taken together these results show, that the foreign proteins are incorporated into the viral envelope to some extent, but not as specifically and not as dense as it was expected. The transmembrane domain of A33R did not greatly promote targeting of the foreign protein to the outer envelope of EEVs. The strong labeling for anti CDV.H obtained in western blot analysis seems to be in most part due to contamination with cellular membranes.

Figure 35: Micrographs of cryo sections. (A) CEV particles of wtMVA labeled with anti B5R. (B) Heavy labeling of the ER with anti CDV.H detecting the A33R-CDV.H fusion protein. (C) Native CDV.H located with cellular membranes and in the cytoplasm.

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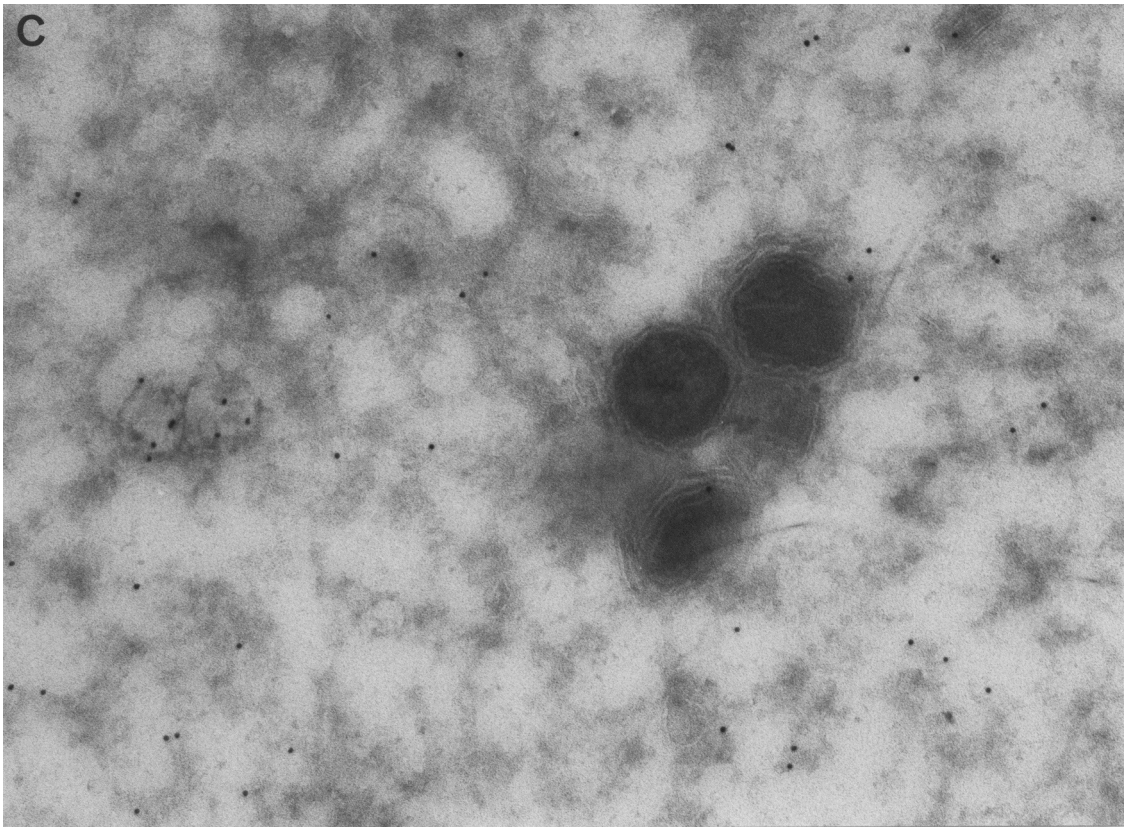
C

6. DISCUSSION

The aim of the current work was to test whether foreign proteins could be targeted to the surface of extracellular enveloped virus upon MVA infection in CEFs. For this, it was first tested whether CEFs infected with MVA produced significant amounts of EEV. Subsequently it was tested whether two proteins expressed by MVA-recombinants were incorporated into such EEVs and to what extent. Using a number of independent approaches the current study shows that MVA infected CEFs results in a moderate production of EEVs, but appears to make substantially more CEVs. The results also show that the replication competent VV strain IHD-J is more efficient in TGN-wrapping and in releasing EEVs in the extracellular medium than MVA, while WR is less efficient. Finally, the data show that the two proteins tested, CDV.H and A33R-CDV.H, were incorporated into the EEV in MVA infected CEFs, albeit with low efficiency. This may be in part due to the fact that both proteins were not efficiently targeted and/or retained in the TGN.

6.1 The production of the different viral particles made by MVA in comparison to WR and IHD-J

The WR and IHD-J strains of VV have been extensively studied previously to compare the amounts of infectious virus associated with infected cells to the infectivity released into the extracellular medium. Payne (Payne, 1979) described for the first time that the production of EEV is virus strain and cell type dependent: whereas IHD-J infection in RK-13 cells resulted in the release of 25 to 40% of the total infectivity into the extracellular medium, in the case of WR this percentage was less than 1%. In HeLa cells, however, IHD-J released only 7% of the total infectious virus into the medium. Payne also showed that the amount of infectious virus associated with infected cells was similar in WR and IHD-J infected cells. Until now it is unclear why HeLa cells produce less EEVs compared to RK-13 cells. Two possible explanations come immediately to mind. First, TGN-wrapping may be much less efficient in these cells. Alternatively, the cytoskeleton-dependent transport of IEVs and/or subsequent fusion with the plasma membrane to release EEVs/CEVs may be inefficient compared to RK-13 cells. A thorough analysis comparing WR and IHD-J

infection in HeLa and RK-13 cells as described in this study is required to discriminate between these two possibilities.

In two subsequent studies Blasco et al. (Blasco et al., 1993) showed that the difference in EEV release between the WR and IHD-J strains may be partly due to the fact that upon WR infection EEVs are not being released but remained mostly attached to the plasma membrane in the form of CEVs. The authors showed that upon trypsin treatment of WR and IHD-J infected BSC-40 cells the amount of infectious virus that could be released from the cell surface upon WR infection almost equaled the amount of infectious virus released into the extracellular medium in IHD-J infected cells. These data strongly suggested that WR and IHD-J underwent TGN-wrapping to a similar extent but whereas IHD-J/EEV was then released from the cell, the same viral form in WR infection remained bound to the cell surface. The authors subsequently showed that whether EEV remains cell associated or is released into the medium is determined by a single amino acid (residue 151) difference in the EEV-specific A34R gene. If this amino acid was changed in the WR gene to the corresponding residue of the IHD-J gene, WR infection now resulted in substantial amounts of EEV in the extracellular medium (Blasco et al., 1993).

As mentioned above, in all of the studies in which WR and IHD-J were compared only two fractions were analyzed; the total virus associated with cells (including IMV, IEV and CEV) and released virus (EEV) and no distinction was made between IMV, IEV and CEV. The current study for the first time uses a number of independent approaches to determine the amounts of all four different viral forms made and compared these in three different virus strains. The only study comparing MVA to a replication competent VV strain is from Spehner et al. (Spehner et al., 2000) who used the Vaccinia Virus Copenhagen strain, which is less well characterized than WR or IHD-J for the production of the different types of particles. The current results therefore clearly extend previous data. They show that in CEFs the IHD-J strain is best at producing all of the TGN wrapped forms and releases the highest amounts of EEV. WR is least efficient in wrapping and more viruses accumulated intracellularly in the form of IMVs. Interestingly, MVA behaved intermediate between these two virus strains. Importantly, the results also show for the first time that different virus strains may also differ in their efficiency of TGN-wrapping. IHD-J was most efficient in this process, MVA intermediate and WR was clearly less good. This study thus does not confirm the data by Blasco et al. (Blasco et al., 1993) that showed that WR produced significantly more CEV than IHD-J, as we detected similar amounts of

these viruses in both viral strains. The current data should, however, be interpreted with caution as the efficiency of these processes may be cell type dependent.

In the study by Payne (Payne, 1980) the production of EEV and the relation to in vivo dessimination was compared in 13 different vv strains (see also below). Among those were also WR and IHD-J which were characterized by a low and high release of EEV, respectively. Unfortunately the Ankara strain, the origin of MVA, was not among the viruses tested. The reason why MVA in CEFs behaved intermediate between WR and IHD-J remains unclear. A possibility is that MVA initially had all the hallmarks of WR infection with relatively little TGN-wrapping and EEV release. During the repeated passaging of the virus it has adapted to undergo more efficient TGN-wrapping resulting in the production of more IEV and CEV compared to WR. An alternative, that appears less likely, is that Ankara behaved more closely to IHD-J but that during its passaging on chicken embryo fibroblasts has become less efficient in TGN wrapping and EEV release. Sequencing of the MVA genome [Antoine, 1998 #169] has shown that all of the known genes encoding VV envelope proteins are intact, with exception of the A36R ORF, which has two small internal deletions. A number of other genes encoding proteins of unknown function are deleted or altered in MVA and any of these or combinations of mutations may be responsible for the particular phenotype of MVA with respect to formation of enveloped particles.

The most striking observation made was that MVA resulted in a dramatic accumulation of CEVs at the plasma membrane of infected CEFs. This was not only shown by the infectivity time course, but also by EM in which in some cell profiles the entire cell surface appeared to be covered with these viruses. As mentioned above, whether extracellular virus is released as EEV or remains attached to the plasma membrane as CEV is determined by a single amino acid (Lys-151→Glu) in the A34R gene (Blasco et al., 1993). Comparison of the published A34R sequence of MVA to WR and IHD-J revealed that this amino acid at position 151 is the same as in the WR gene and differs from the IHD-J gene. These data confirm that MVA, having a A34R gene similar to WR, makes substantially less EEV and consequently extracellular virus accumulates as CEVs. As mentioned above, the accumulation of CEVs at the plasma membrane of infected CEFs could be the consequence of a relatively efficient TGN-wrapping (this study) and transport of IEVs, that subsequently accumulate at the cell surface upon fusion because they cannot be efficiently released from this cellular membrane. Whether microtubule mediated transport of

MVA/IEVs was more or less efficient compared to WR or IHD-J was not investigated in this study. The current study does indicate that MVA is capable of making actin tails. This is despite the fact that the A36R gene, the protein responsible for both kinesin dependent movement and actin tail formation lacks 13 residues (amino acid 139-147 and 216-219) compared to the WR and IHD-J genes. It should be mentioned, however, that the efficiency of tail formation was not investigated, nor was it compared to the two other virus strains.

Our data differ from the data by Spehner et al. (Spehner et al., 2000). In that study it was concluded that MVA produced significant amounts of all of the TGN-wrapped forms (74%) in CEF cells while under the same infection conditions the Copenhagen strain of VV produced only 22% of all of these forms. The latter results were obtained by separating the different viral forms by cesium chloride gradients and peak fractions were detected by following ^{3}H thymidine labeled particles. The amounts of the different viral forms made was thus calculated by measuring the amount of radioactivity contained in the peak fractions. In this study both infectivity as well as thin section EM were used to determine the relative amounts of the viral forms made. In this study MVA was compared to WR and IHD-J instead of the Copenhagen strain. In the Spehner et al. study only one time post-infection was studied, while the current data are based on detailed time course of infectivity contained in different viral forms. Throughout our study a moi of 10 was used most commonly, while in the Spehner et al. publication a moi of 0.1 was used. Data obtained in our study shows, that infection with a low moi leads to a higher extend of TGN wrapping and results in more enveloped virus progeny than infection with a high moi. The question is whether all of these differences can account for the fundamentally different conclusions the current and the Spehner et al. study arrives at. Copenhagen strain is not expected to behave fundamentally different from WR and IHD-J. It is difficult to conceive how the different detection methods used in the Spehner et al. study could influence the amount of IEV/CEV and EEV produced in CEFs. The most likely explanation for the discrepancy thus appears to be the use of only one moi and only one time post-infection.

The combination of the moi and the time post-infection used in the Spehner et al. study could have severely biased the amounts of TGN-wrapped forms produced by the Copenhagen strain in CEFs. A prediction is that Copenhagen behaves similar to either WR or IHD-J. Thus, in a proper time course experiment Copenhagen can be expected to produce similar amounts of the TGN-wrapped forms compared to WR or IHD-J. Finally, a much less likely explanation is that the TGN-wrapped forms of

IEVs, CEVs and EEVs made in MVA infected cells have a very low pfu per particle ratio compared to IHD-J and WR. Assuming that this is indeed the case, this would imply that the infectivity time course experiment severely underestimated the total amount of particles made upon MVA infection, providing perhaps an explanation for the discrepancy between our current and the Spehner et al. study. This possibility seems unlikely since a study by Sancho et al. (Sancho, 2002) strongly suggested that the pfu/particle ratios of purified WR and MVA is, in fact, very similar.

6.2 Is MVA infection a suitable system to target foreign proteins to the surface of extracellular particles?

While these data clearly showed that MVA in CEFs undergoes significant wrapping and CEV formation, they also show that this infection condition results in the release of only little EEV. The question therefore arises whether proteins targeted to the surface of CEVs are capable of inducing a humoral immune response and whether this putative response is just as efficient as when the same protein is exposed on EEVs.

In the study by Payne (Payne, 1980) the 13 different virus strains tested were classified into three groups. Group one viruses were characterized by a high EEV release and a high virulence in vivo, the representative being the IHD-J strain. Group two, comprising WR, released little EEV in vitro but was also highly virulent. Finally, a third group released little EEV and showed pour virulence as well. At the time when these data were published it remained unexplained how WR could be virulent while producing little EEV. Payne implied that in WR infected cells the virus must have evolved a way of spreading independently of EEV formation. It was subsequently shown that WR infection resulted in substantial amounts of virus attached to the cell surface as CEVs (Blasco and Moss, 1992) (Blasco et al., 1993). The combined data suggest that CEVs may be as efficient as EEVs in cell-to-cell spread. Although this point has not been tested directly, it can be envisioned that CEVs bound to the tip of a long actin tail containing plasma membrane protrusion, reviewed in Moss and Ward, (Moss and Ward, 2001) may be just as efficient in infecting neighboring cells as EEVs that are free to diffuse in the extracellular environment.

A study by Katz and Moss (Katz and Moss, 1997) suggests that this may indeed be so and that proteins exposed on CEVs are equally immunogenic as when exposed

on EEVs. In their study a chimeric protein containing the transmembrane and cytoplasmic portion of the B5R gene fused to the HIV-env protein was cloned into both IHD-J and WR. Recombinant viruses containing the wild-type HIV-env was used as a control. It was shown before that only the chimeric protein was efficiently incorporated into EEVs (Katz et al., 1997). It was subsequently tested whether these viruses induced antibodies to HIV-env. Whereas the recombinant carrying the wt HIV-env did not induce a humoral response, the WR and IHD-J recombinants carrying the chimeric protein were equally efficient in doing so. The authors therefore concluded that the induction of antibodies could be efficiently induced by exposure of the antigen on either EEVs or CEVs (Katz and Moss, 1997). Taking all of these data together and considering the fact that MVA results in the production of significant amounts of CEVs, the conclusion must be that indeed MVA could be a good candidate for targeting of foreign proteins to the surface of EEV/CEV. What should be kept in mind, however, is that the assembly of MVA may be blocked in most mammalian cells and therefore IEVs/CEVs in cell lines of interest may not be produced. In this respect it is worth mentioning that preliminary results in the Krijnse Locker laboratory indicated that HeLa cells, the most commonly used cell line of human origin, infected with MVA efficiently secrete immature viruses lacking MVA genomes. These viruses are secreted by virtue of the fact that they undergo TGN-wrapping and subsequent release in a way similar to EEV release. This observation opens up a unique opportunity to target TGN-derived foreign proteins to the surface of these non-infectious particles. It therefore also remains to be investigated in detail at what stage MVA is blocked in cells that are the target of infection during vaccination and whether those cells also secrete TGN-wrapped particles.

6.3 Targeting of CDV.H and A33R-CDV.H to the surface of EEV particles

The rational of using the EEV specific membrane for targeting of foreign proteins relies on a number of observations. First, EEV is the virus to be released into the extracellular medium and thus can be expected to expose antigens to the immune system. Second, in contrast to the IMV membranes that may exclude host proteins (Sodeik et al., 1993), the EEV wrapping membrane may be less selective. This was demonstrated by the fact that this membrane readily incorporated complement control proteins (Vanderplasschen et al., 1998b) and that by EM non-EEV membrane proteins that are retained in the TGN can be found in the viral wrapping membranes (Schmelz et al., 1994). Efficient targeting to the EEV may, however, require specific

targeting signals. In the above mentioned study by Katz et al. (Katz et al., 1997) , for instance, it was shown that the HIV-env protein fused to the transmembrane domain and cytoplasmic tail of B5R was incorporated significantly more efficiently into EEVs than wild-type env. These data were subsequently confirmed by Mathew et al., (Mathew et al., 2001). In that study they used a virus in which the wild-type B5R gene was deleted, which was shown before to result in a total block of EEV formation (Engelstad and Smith, 1993). It was then tested which part of the B5R gene was required to rescue EEV formation and it was found that these were the transmembrane domain and cytoplasmic tail of the protein. They also showed, in agreement with the data of Katz et al. (Katz et al., 1997) and Herrera et al. (Herrera et al., 1998), that the entire luminal domain of the B5R gene could be replaced by the corresponding luminal domain of the HA gene (A56R) attached to the transmembrane region and cytoplasmic tail of B5R. Rescue experiments comparing this chimeric protein to the full-length B5R gene showed that both proteins resulted in similar amounts of EEV. It should be noted that already in 1998 Herrera et al. (Herrera et al., 1998) had shown that the entire luminal domain of the B5R gene is dispensable for EEV formation and that thus this region represents an ideal domain to be swapped for foreign proteins (Herrera et al., 1998). The combined data thus indicate that the B5R gene may be an ideal candidate for targeting of foreign proteins to the surface of the EEV/CEV. Although less thoroughly investigated, one study suggested that the transmembrane domain and cytoplasmic tail of the HA protein (A56R) may also be a good candidate (Galmiche et al., 1997).

In the current study it was tested whether the transmembrane domains of the A33R, A34R and A36R genes could also be used for targeting to the EEV. The data show that the chimeric protein containing the membrane spanning domain of A36R resulted in a product with a molecular weight expected of a clipped form of the fusion protein. The reasons for this is unclear and was not further investigated. No recombinant virus could be obained with the A34R chimera. Again the reason for this is not clear at present; perhaps expression of the chimeric protein severely inhibited IMV formation, or recombination repeatedly occurred at the wrong site of the genome resulting in progeny that was no longer infectious. The chimeric A33R-CDV.H resulted in a viable recombinant with a proper expression of the fusion protein that was produced to high level and was stable. Unfortunately the data suggested that the chimeric construct was not properly targeted to the TGN and instead was predominantly retained in the ER. The most likely explanation for its failure to leave the ER was that the protein was not properly folded resulting in its retention (see

Doms et al., 1993 for a review). Consequently it was found that the wild-type CDV.H protein incorporated more efficiently into EEVs then the chimeric construct.

The question that arises from these and other data is whether incorporation into the IEV-wrapping membrane requires specific signals or whether any protein, that transiently travels through the TGN, will incorporate into IEVs. The current data suggested that a protein that is destined for transport to the plasma membrane can be incorporated to some extent into EEV/CEV. Furthermore the study by Vanderplasschen et al. (Vanderplasschen et al., 1998b) suggested that cellular proteins that are not specifically retained in the TGN readily incorporate into EEVs as well. Finally, EM data by Schmelz et al. (Schmelz et al., 1994) suggested that a TGN-retained non-EEV membrane protein can be found in the wrapping membranes, although it was not investigated if this protein was also present in the EEV.
A word of caution is required at this point. Most of the studies that tested whether specific proteins are targeted to the EEV are based on western blots only. This technique is prone to a number of problems. The least of those problems is that this technique does not allow proper quantitation. A more serious problem deals with the preparation of 'pure' EEV. It is clear that IMVs can be purified from infected cells to about 95% purity (Jensen et al., 1996) and contain very little cellular contamination. Consequently, by western blots the proteins of interest are never found in the IMV preparation, used as a control in the experiments; a result that authors readily use as a proof of specific incorporation into the EEV. It is not so clear, however, whether EEVs can be obtained with the same purity as IMVs. At first glance it seems easy to separate the extracellular particles from the rest of this same extracellular medium that supposedly contains no other membrane-containing components. However, upon negative staining EM, EEV preparations always contained substantial contamination, some of which was heavily labeled with antibodies to EEV membrane proteins. The origin of these EEV-positive membranes is not clear. However, preliminary thin section EM observation suggest that VV infected cells may secrete endosomal-like membranes in an exosome-like fashion, that contain EEV specific proteins and that co-purify with EEVs (Sancho, Meiser and Krijnse Locker, unpublished observations). Such contaminating membranes thus may severely bias the extent of incorporation of non-EEV proteins as they may contain cellular proteins unrelated to the EEV-wrapping process. Consequently, any study on the incorporation of foreign proteins into the EEV should be accompanied by a thorough EM negative staining inspection of EEV-preparations in which it should not only be

tested whether the protein of interest is indeed exposed on the EEV, but also if and how much contamination is present in the EEV preparation.

Another serious caveat of western blot analysis is its limitation in assessing how efficient incorporation into the EEV really is. In fact negative staining EM, that is often shown in parallel, does not help much. Such analyses often show a few gold particles, frequently enhanced by using a so-called 'bridging-antibody' (this means that the primary antibody was a mouse monoclonal, followed by rabbit anti-mouse and protein-A gold). Such a labelling procedure often leads to binding of several rabbit anti-mouse IgG to one IgG of the mouse monoclonal, leading to signal enhancement; examples of this may be found in Katz et al. (Katz et al., 1997) and in Vanderplasschen et al. (Vanderplasschen et al., 1998b), both groups used mouse monoclonal antibodies, while the significance of attachment of these gold particles on the surface of EEVs remains unclear. What is required is a thorough quantitation of the amount of the protein of interest present in IMVs, IEVs, EEV and other intracellular membranes. However, to perform such studies by western blotting is very difficult because with the exception of the IMV, none of these particles, including cellular membranes, can be purified without contaminating membranes. A possible solution to this problem would be to conduct a thorough quantitative EM study. In such a study a protein of interest is labeled with antibodies and the amount of labeling is then quantified over all membranes of interest such as the ER, the TGN, the wrapping membranes, the IEVs and CEVs. The comparative study will allow to directly estimate the density of labeling over all of these different membranes and eventually calculate the efficiency of incorporation into EEVs/CEVs by comparing the density of labeling on these particles to the labeling in the TGN. Such a study could be extended by assessing how well EEV membrane proteins are in fact incorporated into the EEV. Little is known about the efficiency of EEV-incorporation of EEV membrane proteins themselves. If substantial differences are found when comparing different EEV membrane proteins, such analyses could perhaps more directly predict which EEV proteins would be more or less suitable for the generation of chimeric proteins, carrying EEV specific targeting signals. Finally, each new chimeric protein that is constructed could be conveniently, directly and rather quickly tested with respect to its efficiency of incorporation into EEVs/CEVs using this method of quantitative EM. Using MVA infection in either BHK or CEF in such analyses would have the advantage that this virus results in a significant accumulation of CEVs at the plasma membrane allowing one to conveniently quantify labeling over the TGN/IEVs and CEVs in one section.

A start was made by quantifying by EM the labeling of the two different CDV.H proteins and a number of observations were made that would not have been made by any other technique. In particular it was found that the chimeric protein carrying the A33R transmembrane domain was mostly retained in the ER and therefore failed to be efficiently incorporated into the EEV. Other quantitative studies of this kind, in which it will in particular be assessed if and how much of cellular proteins are incorporated into IEVs and CEVs, are currently underway.

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