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Vaccinia virus host range factor C7L plays an important regulatory role in the MVA life cycle

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Summary

The highly attenuated modified vaccinia virus Ankara (MVA) serves as a candidate recombinant vaccine against infectious diseases and cancer. MVA was derived from chorioallantois vaccinia virus Ankara by over 570 serial passages in chicken embryo fibroblast cells (CEF). During this attenuation process, the virus has lost 31 kbp of DNA of the parental genome including several immunomodulatory and regulatory genes. The resulting MVA is highly attenuated and unable to replicate in human and most other mammalian cells but has still full replicative capacity in permissive CEF and baby hamster kidney (BHK) cells. However, several regulatory gene sequences of vaccinia virus are still conserved within the MVA genome including the vaccinia virus host range gene C7L. Previously, C7L has been characterized as crucial viral factor for the replication of vaccinia virus in human cells, though its exact function remains unclear.

In this work, the relevance of C7L in the MVA molecular life cycle was investigated using a MVA-C7L deletion mutant virus (MVA-ΔC7L). Initial experiments demonstrated an essential role of C7L to allow for completion of the MVA molecular life cycle upon infection of human and murine cells. Infection of non-permissive mammalian cells with MVA activates the cascade-like viral early, intermediate and late gene expression. In contrast, the deletion mutant virus MVA-ΔC7L was found defective in late protein synthesis in human and murine cells but not in permissive CEF and BHK cells. These results were confirmed by a β-galactosidase reporter gene assay using the recombinant viruses MVA-P11LZ and MVA-ΔC7L-P11LZ. Both viruses contain the *E. coli* LacZ gene under control of the strictly late vaccinia viral promoter P11. Strong β-galactosidase expression was observed upon infection of human and murine cells with MVA-P11LZ whereas infection with MVA-ΔC7L-P11LZ resulted in drastically reduced levels of β-galactosidase expression.

Analysis of viral DNA synthesis and monitoring for signs of apoptosis revealed no differences in human cells infected with MVA or MVA-ΔC7L suggesting that the block of late protein synthesis is regulated at a step succeeding viral DNA-replication and independent of the induction of apoptosis. Furthermore, the phosphorylation levels of the eukaryotic translation initiation factor 2 (eIF2α) were compared in MVA and MVA-ΔC7L infected human cells. Phosphorylation of eIF2α is one of the major cellular stress responses during viral infections leading to the global down regulation of translation initiation. No difference was observed

between both viruses. Additionally, it could be shown that C7L function in the MVA life cycle can be complemented by the vaccinia virus host range gene K1L.

To identify functional domains of C7L that would be sufficient to overcome the block of viral late gene expression in non-permissive cells, a transient transfection reporter gene assay was established. Defined deletion mutants of C7L were constructed and analyzed for their ability to restore late protein synthesis of MVA-ΔC7L-P11-LZ. It was observed that the whole N-terminus of the protein is required and only the terminal 12 amino acids are dispensable to mediate the host range function in human and murine cells.

Furthermore, to investigate how C7L could mediate its host range function, a yeast-two-hybrid screen of a cDNA library derived from EBV immortalized human lymphocytes was performed and several putative C7L-interacting proteins were identified. These potential interaction partners were expressed through *in vitro* translation and tested in Co-immunoprecipitation assays.

To evaluate the role of C7L for the immunogenicity of vaccines based on MVA vaccination studies were conducted. Consistent with the *in vitro* data, cellular and humoral immune responses against late viral and recombinant antigens were abrogated in mice vaccinated with viruses lacking C7L, confirming that the C7L gene has an essential role for the expression of late viral antigens *in vivo*.

1. Introduction

1.1. Vaccinia virus

Vaccinia virus (VACV) is a member of the genus *Orthopoxviridae* belonging to the large family of poxviruses (Moss 2001). During the smallpox eradication campaign VACV was used as a life vaccine against variola virus, another member of the genus *Orthopoxviridae* and the causative agent of smallpox. The origin of VACV is not completely understood and there is no known natural host. Wild-type VACV strains show a broad host and cell type tropism and therefore are capable of replicating in most cell lines of both avian and mammalian origins. VACV has a 192 kb linear double-stranded DNA genome encoding over 200 genes that are transcribed from both DNA-strands (Antoine et al., 1998; Goebel et al., 1990; Moss 2001). The open reading frames are mostly non-overlapping and tend to occur in groups: highly conserved genes with essential replication functions are usually located in the central region, whereas variable open reading frames involved in virus-host interactions are mostly located at the left and right termini (Moss 2001). Poxviruses are among the largest known viruses with capsid diameters of 300-400 nm and are visible even under the light microscope.

1.2. Vaccinia virus replication cycle and gene expression

Poxviruses are unique among DNA viruses because their entire life cycle, including transcription, genome replication and virus assembly, occurs exclusively in the cytoplasm of the host cell (Goebel et al., 1990; Antoine et al., 1998; Moss 2001). This is possible as the viruses encode virtually all of the proteins required for these processes which endows the virus with considerable autonomy with respect to cellular functions (Moss 2001). This special feature may in part explain the broad host range displayed by some poxviruses.

1.2.1. Vaccinia virus replication cycle

As illustrated in Fig. 1, the VACV replication cycle is a complex sequence of cytoplasmic events that begins with binding to the cell surface and subsequent fusion of virus and mammalian cell membranes. Two distinct infectious virus particle types, the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV), can initiate the infectious cycle (Smith et al., 2002). The IMV and EEV virions differ in their surface glycoproteins and in the number of wrapping membranes, and they are thought to enter cells by different mechanisms (Vanderplasschen et al., 1998; Vanderplasschen and Smith, 1997; Locker et al., 2000). So far, several virion proteins have been shown to be crucial for binding of the virion to the cell surface. The cell determinants of binding are thought to be ubiquitously expressed glycosaminoglycans or components of the extracellular matrix (Blasco et al., 1993; Lin et al., 2000; Hsiao et al., 1998 + 1999; Chung et al., 1998). After binding, the fusion event between the virion and the host cell membranes is still poorly understood, but at least one conserved virion protein has been linked to this fusion/entry event that ultimately releases the virion core structure into the cytoplasm (Senkevich et al., 2004). Until now no specific host cell receptors are known to be required for virion fusion and entry. Subsequently after the release of the viral core into the cytoplasm the viral RNA polymerase and transcription factors that were packaged into the virion during morphogenesis initiate the first cascade of viral gene expression, which synthesizes viral mRNA under the control of viral early promoters (Moss 2001; Broyles 2003). Then, yet unidentified host and/or viral factors induce the dissolution of the core structure, a poorly understood process known as core uncoating. This uncoating step releases the viral DNA into the cytoplasm, where it functions as a template for DNA replication. The subsequent waves of intermediate and late transcription require newly synthesized DNA as template. Unlike early transcription, which is believed to be exclusively under the control of viral factors that are packaged within the core, the subsequent intermediate and late transcription stages require cooperation with host-derived factors (Rosales et al., 1998 a,b; Sanz and Moss, 1998; Gunasinghe et al., 1998; Wright et al., 1998; Broyles et al., 1999; Katsafanas and Moss 2004).

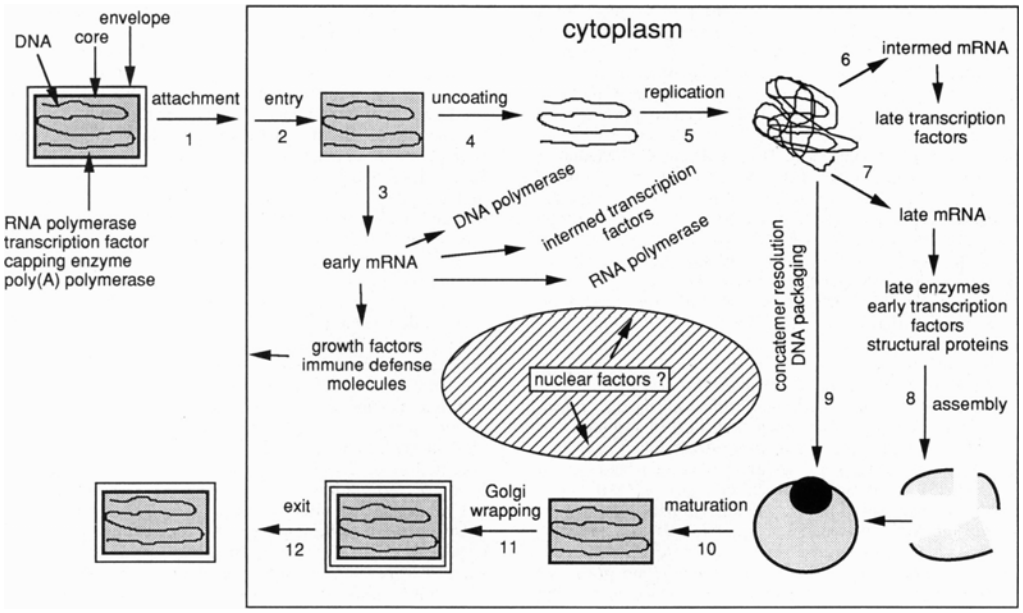


Fig. 1: Life cycle of VACV in permissive cells (Moss 2001).

Concomitant with the accumulation of late viral gene products morphogenesis and assembly of infectious virus particles begins. The first infectious mature virus form that is assembled is designated intracellular mature virus (IMV). It consists of the viral core and an envelope. The IMVs migrate via microtubule-mediated trafficking to the Golgi where they are wrapped with two additional Golgi-derived membranes to form intracellular enveloped virus (IEV). The IEV form loses its outermost membrane as it fuses with the cell membrane to form the cell-associated enveloped virus (CEV), which is either propelled towards neighbouring cells by actin-tail polymerization, or is released directly as free extracellular enveloped virus (EEV) particles (reviewed in Smith et al., 2002). It is thought that the CEV and EEV forms are particularly important for rapid cell-to-cell spread, whereas the IMV form probably contributes to virus dissemination only after late stage cell death and membrane rupture (Smith et al., 2003; Smith and Law, 2004).

Poxviruses express an array of modulatory proteins that modify both the intracellular and extracellular environments of infected cells to facilitate viral replication. These virus-encoded proteins collectively counteract a wide range of antiviral defence responses including those mediated by the interferon system, apoptosis pathways, stress-induced signalling cascades, antigen presentation and pro-inflammatory pathways (Seet et al., 2003).

1.2.2. Temporal regulation of gene expression

Like most other classes of viruses, poxviruses coordinate the process of genome replication and virion assembly through regulated timing of gene expression. Proteins participating in DNA replication (Jones and Moss, 1984; Lee-Chen and Niles, 1988; Smith et al., 1989) and intermediate gene transcription (Jones et al., 1987; Lee-Chen and Niles, 1988; Ahn et al., 1990; Broyles and Pennington, 1990; Sanz and Moss, 1999) are synthesized as early class genes and those participating in virion morphogenesis and assembly are expressed as post-replicative intermediate and late class gene products (Rosel and Moss, 1985). Proteins involved in the evasion of host defences tend also to be early class gene products (Kotwal et al., 1989; Moore and Smith, 1992; Ng et al., 2001).

The temporal control of gene expression is determined through a cascade-like initiation of transcription: the transcription factors required for intermediate genes are expressed as early proteins, factors required for late genes are intermediate gene products and those required for transcription of early genes are late gene products packaged inside progeny virions for the use in the next cycle of infection. Thereby the switch from early to late gene expression requires genome replication. Inhibition of DNA synthesis results in the persistence of early gene transcription and the inhibition of intermediate and subsequent late gene transcription (Vos and Stunnenberg, 1988). Without DNA synthesis no transcriptional switch occurs as only newly synthesized DNA can serve as template for intermediate and late transcription.

All three classes of VACV genes are transcribed by the virus encoded multisubunit DNA-dependent RNA polymerase which shares a high degree of amino acid similarity to the eukaryotic cellular RNA polymerase (Broyles and Moss, 1986; Patel and Pickup, 1989).

1.2.2.1. Early gene expression

VACV early class mRNA is synthesized within minutes after virus entry and is extruded in the host cytoplasm through pores in the core surface. The virion core particle apparently retains much of its structural integrity after cell entry (Kates and McAuslan, 1967; Munyon et al., 1967). This is possible because all the enzymes and other proteins required to synthesize mature mRNA are packaged within the virion core along with the DNA genome. These proteins include RNA modification enzymes such as the mRNA capping enzyme, poly(A) polymerase and a 2`-O-methyltransferase in addition to the RNA synthesis machinery.

Approximately half of the VACV genes belong to the early class (Oda and Joklik, 1967). To initiate early mRNA synthesis apparently only two factors are required, the viral RNA polymerase containing an associated polypeptide encoded by the viral H4L gene and a single transcription factor, called VACV early transcription factor (ETF) (Broyles et al., 1988). ETF is a heterodimer of the viral D6R and A7L gene products (Broyles and Fesler, 1990; Gershon and Moss, 1990). The H4L polypeptide subunit of the RNA polymerase is required specifically for early transcription. (Ahn and Moss, 1992; Ahn et al., 1994; Deng and Shuman, 1994).

Transcription of VACV early genes terminates just downstream of open reading frames in response to the sequence TTTTTNT on the non-template strand of the DNA (Yuen and Moss, 1987). At least two trans-acting factors are required to induce termination and transcript release by the RNA polymerase. The capping enzyme, which is presumably carried with the RNA polymerase as an elongation complex induces the RNA polymerase to cease transcription and release the template (Deng and Shuman, 1997). Another factor, a ssDNA-dependent ATPase with nucleic acid helicase motifs called NPH I was identified following the demonstration of an ATP requirement for the termination process (Deng and Shuman, 1997). NPH I drives release of the transcript through hydrolysis of ATP.

1.2.2.2. Intermediate gene expression

The viral intermediate transcription factors (VITFs) are products of early class genes, allowing the second stage of transcription to commence when viral DNA replication occurs. The required viral proteins include the viral RNA polymerase, the viral capping enzyme (Vos et al. 1991 a,b;), VITF-1 and VITF-3. VITF-1 is the 30 kDa subunit of the viral RNA polymerase (Rosales et al. 1994a) and VITF-3 is a heterodimer of viral A8L and A23R gene products (Sanz and Moss, 1999).

VITF-2 was identified in nuclear extracts from uninfected HeLa cells (Rosales et al., 1994b), documenting the first known VACV transcription factor that is not encoded by the virus. In addition some intermediate promoters have eukaryotic transcription factor Yin-Yang1 (YY1) binding sites and YY1 accumulates in the cytoplasm of VACV infected cells (Broyles 2003; Broyles et al., 1999). While there are no data linking VITF-2 and YY1, the Ras-GTPase-activating protein SH3 domain-binding protein and the cytoplasmic activation/proliferation-associated protein-1 were shown to co-purify with VITF-2 activity from HeLa cells (Katsafanas and Moss, 2004).

It has been suggested that de novo synthesis of the viral RNA polymerase is required for intermediate and late gene transcription (Hooda-Dhingra et al., 1989). In addition, proteins functioning in intermediate transcription may require phosphorylation by the B1R serine/threonine protein kinase (Traktman et al., 1989; Banham and Smith, 1992; Lin et al., 1992).

1.2.2.3. Late gene expression

Three intermediate stage proteins (G8R, A1L and A2L) and one early protein (H5L) have been identified as having late transcription stimulatory activity (Keck et al., 1990 + 1993; Zhang et al., 1993; Wright and Coroneos, 1993; Hubbs and Wright, 1996; Passarelli et al., 1996; Kovacs et al., 1994; Kovacs and Moss, 1996). In addition, the host proteins heterogeneous nuclear riboproteins A2/B1 and RBM3 were shown to stimulate late stage transcription *in vitro* (Wright et al., 2001). The failure to identify any VACV-encoded factors with promoter-binding activity prompts speculation that host factors may target the viral promoters, forming a nucleation site for virus-encoded factors that eventually recruit the RNA polymerase to the site of initiation.
Probably transcription termination of intermediate and late genes is induced by an active mechanism as the VACV RNA polymerase appears not to respond to specific termination signals in intermediate and late genes (reviewed by Condit and Niles, 2002). Northern blotting of these two classes of RNA revealed that their 3`termini are extremely heterogeneous (Xiang et al., 2000). The A18R was shown to play a role in supporting transcript release (Xiang et al., 1998; Lackner and Condit, 2000) whereas G2R and J3R support transcription elongation by the RNA polymerase (Black and Condit, 1996). An interaction between G2R with the late factor H5R was also detected in a yeast two-hybrid screen (McCraith et al., 2000). There are hints that a host protein of unknown identity may be crucial for the termination process.
Late gene products include proteins required for virion morphogenesis and factors that are packaged into new virions including the RNA polymerase, early gene transcription factors and a poly(A) polymerase.

1.3. Modified vaccinia virus Ankara

Modified vaccinia virus Ankara (MVA) is an attenuated strain of VACV that was initially developed for the use as a safer vaccine during the last decades of the smallpox eradication campaign.

1.3.1. Development and general features of MVA

MVA was attenuated by growing the parental strain chorioallantois vaccinia virus Ankara (CVA) by 516 serial passages on primary chicken embryo fibroblast (CEF) cells (Mayr et al., 1978). During the course of attenuation, 15% of the parental viral genome was lost reducing the genome size from 208 kilo bases (kb) to 177 kb (Antoine et al., 1998). The resulting virus was highly attenuated and displayed a phenotype that in part can be attributed to the disruption of at least two host range genes as well as other genes possibly involved in the evasion of the host immune response (Antoine et al., 1998).
MVA can still infect mammalian cells but is unable to productively replicate in human and most mammalian cells due to a block of virion morphogenesis at a very late stage of the virus life cycle (Stickl et al., 1974; Blasco and Moss, 1991). The virus can be amplified on CEF or baby hamster kidney (BHK) cells (Carroll and Moss, 1997; Drexler et al., 1998; Meyer et al., 1991). MVA infection of non-permissive human cells induces the complete cascade-like transcription of early, intermediate and late gene classes and is characterized by an unimpaired late protein synthesis (Sutter and Moss, 1992). Importantly, for the use as a vaccine, the block to form viral particles does not affect the expression of viral or recombinant genes under the control of viral promoters (Sutter and Moss, 1992). The avirulence of MVA has been demonstrated by inoculation of various animals including newborn, irradiated and SCID mice as well as immune-suppressed macaques (Meyer et al., 1991; Stittelaar et al., 2001; Wyatt et al., 2004). The safety of MVA for application in humans has been demonstrated when over 120.000 individuals including immunocompromised patients were vaccinated during the smallpox eradication campaign without any report of the adverse side effects associated with replication competent strains of VACV (Mayr et al., 1978; Stickl et al., 1974).

1.3.2. MVA today: next generation smallpox vaccine and viral vector

After the world health organization declared the eradication of smallpox in 1980, a risk of deliberate release of variola virus, for example as bioterrorist attack remains. Due to their safety record and immunogenicity replication-deficient VACV, like MVA are considered as the next generation smallpox vaccines (Earl et al., 2004; Stittelaar et al., 2005; Wyatt et al., 2004). Furthermore, poxvirus vectors are considered to be promising candidates for the use as recombinant vaccines due to their efficient expression of foreign antigens and unique immunological properties in eliciting long-term protective humoral and cell-mediated immune responses (Moss 1996). MVA has been proven to be equivalent to replication competent VACV in its ability to induce cellular as well as humoral immunity to a variety of antigens (Sutter and Staib, 2003). Furthermore the packing size of the MVA genome for recombinant genes is large, reaching a hypothetical value of ~50 kb (Sutter and Staib, 2003) and thus allowing for the expression of full-length antigens or the co-expression of two or more foreign genes. MVA can be handled under bio safety level 1, recombinant viruses are easy to manufacture and are stable over time when frozen.

Therefore, recombinant MVA are promising vector vaccines and are now widely used in clinical studies including therapeutic and prophylactic vaccination protocols against infectious diseases (HIV, Malaria and Tuberculosis) and cancer (melanoma, prostate cancer, colon cancer and cervical cancer) (Dorell et al., 2006; Goonetilleke et al., 2006; Harrop et al., 2006; Imoukhuede et al., 2006; McShane et al., 2004; Meyer et al., 2005; Peters et al., 2007).

1.4. Orthopoxvirus host range genes

Although orthopoxviruses are closely related the host species tropism of the individual viruses are quite distinct (Esposito and Knight, 1985; Fenner et al., 1989; Buller and Palumbo, 1991). Cowpoxvirus has been isolated under natural conditions from rodents, cows, humans, cats and elephants (Marennikova et al., 1977; Ladnyi et al., 1975; Bennett et al., 1986), whereas others such as ectromelia and variola are restricted to mouse and man, respectively (Fenner 1982; Buller et al., 1986). Although both ectromelia virus, the causative agent of mousepox, and variola virus have a very restricted host range they are highly contagious diseases that results in high mortality of infected individuals.

For many other viruses, tropism specificity in cultured cells is mainly determined by specific receptors for virus binding and entry (Dimitrov 2004; Smith and Helenius, 2004) but for poxviruses no specific host-cell receptors have been identified. Poxviruses bind and enter both permissive and restrictive cells, but in restrictive cells the virus fails to complete its life cycle as a result of cell-type-specific blockages (Johnston et al., 2003; McFadden 2005). The isolation of mutants which are unable to multiply in some cell types has suggested that viral functions are involved in host range determination. At least 90 of the open reading frames are conserved among the various poxviruses which are required for replication and morphogenesis. The remaining open reading frames are more divergent due to differences in adaptation to the various hosts (Upton et al., 2003; McLysagh et al., 2003; Gubser et al., 2004). These non-conserved genes give each poxvirus its unique characteristics of host range, immunomodulation and pathogenesis (Seet et al., 2003). At least four genes, C7L (Perkus et al., 1990; Oguiura et al., 1993), K1L (Drillien et al., 1981, Gillard et al., 1986; Perkus et al., 1990), E3L (Chang et al 1995; Beattie et al., 1996) and CP77 have been identified in VACV and cowpoxvirus which are important for virus host range in tissue culture (Gillard et al., 1986; Spehner et al., 1988; Perkus et al., 1990; Takahashi-Nishimaki et al., 1991). The mechanism by which each viral host range protein overcomes host restriction could be unique to each protein and its cellular context.

1.4.1. Vaccinia virus host range gene K1L

Initial studies with a VACV host range mutant (VVhr) that is unable to replicate in human cells identified the K1L gene as being essential for VACV growth in human cells (Drillien et al. 1981; Gillard et al., 1985 + 1986). VVhr was derived from the Copenhagen strain of VACV and has 18 kbp deleted near the left end of the genome (Drillien et al., 1981). Insertion of the early expressed K1L gene (Gillard et al., 1986), which is among the deleted open reading frames restored growth of VVhr on human (Drillien et al., 1981; Gillard et al., 1985 + 1986) and on RK13 cells (Perkus et al., 1990; Sutter 1994; Ramsey-Ewing and Moss 1996). However, some cell lines like BHK and CEF cells are completely permissive for the VVhr deletion mutant indicating that K1L expression is not required for VACV replication in these cells (Drillien et al., 1981).
The K1L coding sequence was partially deleted from the MVA genome during the attenuation process (Altenburger et al., 1989). Restoration of the K1L ORF into MVA extends the host range to otherwise non-permissive RK13 cells but not to human cells (Sutter et al., 1994; Wyatt et al., 1998; Meyer et al., 1991; Carroll and Moss, 1997). It has also been shown that MVA can grow in RK13 cells stably transfected with the K1L gene (Sutter et al., 1994). The host range restriction of VACV and MVA lacking the K1L gene in RK13 cells is the result of a translational block. In the absence of K1L there is transcription of early genes but neither intermediate RNA nor DNA-replication are detectable. Viral peptides are only synthesized in the first hour followed by a total block in cellular and viral protein synthesis.
A yeast-two-hybrid assay indicated that K1L interacts with C10L, another VACV protein (McCraith et al., 2000) whereas another study identified the rabbit homologue of human ACAP2, a GTPase-activating protein as a cellular protein interacting with K1L (Bradley and Terajima, 2005), but no biological significance has yet been attributed to one of these interactions.
K1L contains multiple ankyrin repeats (ANKs), a common 33-residue protein motif that has been found in many different proteins. ANK repeats were shown to mediate protein-protein interactions (Sedgwick and Smerdon, 1999). An examination of all K1L ANKs for their contributions towards VACV replication in both human HeLa cells and rabbit RK13 cells showed that K1L supports viral replication in human and rabbit cells through a cell-type-specific set of its ankyrin repeat residues. Furthermore the ANK residues needed for viral replication are distinct from the binding site for ACAP2 demonstrating that K1L may mediate the host range function independent from the ability to bind ACAP2 (Meng and Xiang, 2006).

Until now, the only molecular function described for K1L is its ability to inhibit the activation of the host transcription factor NF-κB by preventing the degradation of its inhibitor, IκBα (Shisler and Jin, 2004). NF-κB regulates the expression of genes involved in immune responses, inflammation, proliferation and apoptosis (for Reviews: Gosh and Karin, 2002; Karin and Ben-Neriah, 2000).

1.4.2. Vaccinia virus host range gene C7L

Unexpectedly, when the K1L gene was deleted from wild type VACV, the virus retained the ability to multiply in human cells but lost the capacity to multiply in RK13 cells (Perkus et al., 1990; Wild et al., 1992). Subsequent experiments identified the early expressed 18 kDa polypeptide C7L as functionally equivalent to K1L for multiplication in human cells (Perkus et al., 1990; Oguiura et al., 1993). C7L is also among the open reading frames deleted from VVhr and insertion of the C7L coding sequence into VVhr also restores the ability of VVhr to productively replicate in human cells (Oguiura et al., 1993).
In human cell lines like MRC-5- or HeLa-cells C7L and K1L behaved as functionally equivalent genes despite the fact that they display no similarity at the amino acid level. Loss of both C7L and K1L expression from VACV abrogated the virus ability to replicate in human cells, while deletion of one gene could be compensated by the presence of the other. In other cell lines, such as RK13 and hamster Dede, either the K1L (RK13) or the C7L (Dede) gene alone was critical for viral multiplication. In rat NRK cells, VACV required both C7L and K1L for efficient multiplication (Oguiura et al., 1993).
Experiments with the attenuated NYVAC strain of VACV that has targeted deletions of the C7L and K1L genes indicated that C7L might play a role in apoptosis inhibition in HeLa cells. Furthermore C7L was shown to prevent the phosphorylation of the eukaryotic translation initiation factor eIF2α induced by NYVAC infection. Reintroduction of C7L also rescued the ability of NYVAC to replicate in HeLa cells (Najera et al., 2006).

1.4.3. CP77, a host range gene from Cowpox

Cowpox virus, the member of the orthopoxvirus family with the largest genome, probably encodes more genes involved in cell adaptation than any of the other members. In contrast to VACV, Cowpox virus can multiply in Chinese hamster ovary (CHO) cells (Drillien et al., 1978; Hruby et al., 1980). VACV infection of CHO cells leads to a very extensive and early shutoff of both host cell and viral protein synthesis (Drillien et al., 1978; Njayou et al., 1982). A cowpox 77 kDa protein encoded by the CP77 gene was discovered to be required for cowpox and VACV replication in CHO cells (Spehner et al., 1988; Kotwal and Moss, 1988; Perkus et al., 1990; Ramsey-Ewing and Moss, 1995; Shchelkunov et al., 1998;). Although VACV is closely related to Cowpox virus, the CP77 gene orthologue in the VACV genome is either deleted (strain Copenhagen) or fragmented (strain WR). Insertion of the CP77 coding sequence into the VACV genome allows replication in CHO cells (Spehner et al., 1988).
Without CP77, the growth of VACV in CHO cells is blocked at the level of translation of viral intermediate stage mRNA (Hsiao et al., 2004; Ramsey-Ewing and Moss, 1995 + 1996). Further, it has been demonstrated that CP77 could substitute for the VACV host range genes C7L and K1L in permitting replication of the virus on human cells (Perkus et al., 1990). Additionally, the three unique host range genes C7L, K1L, and CP77 were functionally equivalent for VACV replication on pig kidney cells, but not on RK13 (Perkus et al., 1990). Here, either K1L or CP77 permits replication of VACV (Perkus et al., 1990). Until now, the only obvious similarity between the three proteins is the presence of ANK repeats in K1L and CP77. Despite their obvious importance in antagonizing host restriction, the molecular basis of the host range activity of the orthopox host range genes CP77, K1L and C7L remains unclear.

1.5. Restriction events in poxvirus-infected cells

The regulatory factors and intracellular steps that are involved in limiting poxvirus replication are examined very poorly. As all of the currently known cellular determinants that are required for VACV binding and entry into cells are ubiquitous surface elements, binding and entry into non-permissive cells is also efficient (Lin et al.; 2000; Hsiao et al., 1998 + 1999; Chung et al., 1998; Senkevich et al., 2004). Therefore, restriction events that limit viral replication in non-permissive cells seem to occur after viral entry and initiation of the replication cycle.

Poxviruses are less S-phase dependent than other viruses but nevertheless they are able to mitogenically stimulate quiescent cells to increase viral replication. The poxviral expression of growth factors that are homologous to cellular ones like epidermal- or vascular endothelial-growth factor stimulate the onset of mitosis in neighbouring cells. Deletion of growth factor genes was shown to result in severe attenuation in infected animals (Seet et al., 2003; Johnston and McFadden, 2004; Turner and Moyer, 2002; Smith and Kotwal, 2002). Poxviruses might also interfere with the activity of specific cell-cycle components in the infected cells, but it is not known if there is a direct link with host restriction (Wali and Strayer, 1996 + 1999; Santos et al., 2004).

Although VACV encodes most of the factors needed for its replication, some factors must be recruited from the host cell to complete the replication cycle. These factors include the cellular translational machinery and host derived transcription factors for intermediate and late transcription, such as VITF-2 (Rosales et al., 1994a+b; Sanz and Moss, 1998; Katsafanas and Moss, 2004). Furthermore poxviruses use the cellular microtubule- and actin-based motility machinery for their morphogenesis and egress of infectious virus. One factor that directly modulates poxvirus propagation is the molecular chaperone Hsp90 that associates with the viral factories and regulates the efficiency of VACV replication by interacting with the viral core protein 4a, which is crucial for virion assembly (Hung et al., 2002).

Cells have evolved diverse intracellular anti-viral signalling pathways responding to infection. Almost all viruses have invented defence mechanisms against the cellular interferon-mediated antiviral state (reviews: Sen 2001; Samuel 2001; Katze et al., 2002). The anti-interferon strategies of VACV include inhibitors of interferon induction, expression of viral interferon-receptors, phosphatases that block the STAT-mediated signal-transduction pathway and inhibitors of the

interferon-induced protein mediators of the antiviral state, such as protein kinase R (PKR) (reviews: Sen 2001; Samuel 2001; Katze et al., 2002; Seet et al., 2003).

In contrast to the well-studied interferon system, the mechanisms by which some of the other signalling pathways can fight poxvirus replication are less well understood. For example, the activation of host cell p21-activated kinase 1 (PAK1) is required for optimal replication of myxoma virus (Johnston et al., 2003) and extracellular signal-regulated kinases 1 and 2 (ERK1,2) activation is necessary for optimal VACV infection (Andrade et al., 2004).

Poxviruses are also able to prevent the activation of pro-inflammatory signalling cascades, like those mediated through NF-κB, by encoding multiple signalling inhibitors (Harte et al., 2003; Ole and Pickup,2001;Gil et al., 2001; Shisler and Jin, 2004; DiPerna et al., 2004; Camus-Bouclainville et al., 2004)

All poxviruses encode a wide range of apoptosis inhibitors, a process that is frequently induced during poxvirus infection (Everett and McFadden, 2002; Shisler and Moss, 2001; Barry et al., 2004).

1.6. Aim of the thesis

The Orthopoxvirus host range genes K1L, C7L and CP77 were identified more than 10 years ago as genes that are required for VACV to replicate productively *in vitro* in many cell lines (Gillard et al., 1986, Perkus et al., 1990 and Spehner et al., 1988). The deletion of K1L and C7L from VACV results in abortive replication in most mammalian cells (Drillien et al., 1981; Gillard et al., 1986; Perkus et al., 1990). This block in replication occurs at the translation of intermediate stage viral mRNA in human HeLa cells (Hsiao et al., 2004). Reinsertion of K1L, C7L or CP77 was demonstrated to be sufficient to fully rescue viral replication in human cells (Perkus et al., 1990; Oguiura et al., 1993; Hsiao et al., 2004). However, the exact nature of the host restriction in non-permissive cells and the mechanisms by which it is overcome through the function of these host-range genes remain elusive today.

Although MVA is highly attenuated, several important regulatory gene sequences of VACV are still conserved in the MVA genome, including VACV genes K3L, E3L and C7L (Antoine et al., 1998; Moss and Shisler, 2001). The aim of this study was to investigate the role of C7L for the MVA life cycle in permissive and non-permissive cells. Therefore, essential steps of the viral life cycle like viral gene expression and viral DNA expression should be investigated using MVA-C7L deletion mutant viruses (MVA-ΔC7L). Major cellular stress responses during viral infections such as the down-regulation of global translation initiation through phosphorylation of eIF2α or the induction of apoptosis through activation of caspase-3 should be characterized for MVA- and MVA-ΔC7L-infected cells.
In addition, to investigate how C7L mediates its host range function, a yeast-two-hybrid screen should be established and conducted during this work in order to identify putative C7L interacting proteins. Based on these results, potential interactions should be further tested in co-imunoprecipitation assays. With the aim to identify essential regions of the protein that could contribute to the host range function defined C7L deletion mutant sequences should be constructed and tested in a reporter-gene-assay.
Finally, as MVA is a promising candidate vector for the development of new recombinant vaccines, the consequences of C7L-deletion for the immunogenicity of MVA *in vivo* should be analyzed.

2. Materials

2.1. Chemicals

Product	Manufacturer
2-β-Mercaptoethanol	Sigma *(Munich)*
Acrylamid/Bisacrylamid (30%)	National Diagnostics *(Atlanta, GA, USA)*
Agarose	Gibco/BRL *(Eggenstein)*
Ammoniumperoxodisulfat (APS)	Merck *(Darmstadt)*
Bacto Agar	Difco Laboratories *(Detroit, MI, USA)*
Bacto Tryptone	Invitrogen *(UK)*
Bacto yeast-extract	Invitrogen *(UK)*
Brefeldin A	Sigma *(Munich)*
Bromphenolblue	Serva *(Heidelberg)*
CPRG (chlorophenol red-β-D-galactopyranoside)	Roche *(Mannheim)*
Difco Yeast Nitrogen Base without amino acids and ammonium sulfate	BD Pharmingen *(Hamburg)*
DMSO	Merck *(Darmstadt)*
DTT	Serva *(Heidelberg)*
EDTA	Sigma *(Munich)*
Ethanol	Merck *(Darmstadt)*
Ethidiumbromide	Serva *(Heidelberg)*
Glycerol	Roth *(Karlsruhe)*
Human lymphocyte MATCHMAKER cDNA library	Clontech *(USA)*
LMP-Agarose (Low Melting Point Ultra Pure)	Life Technologies^{TM}
Methanol	Merck *(Darmstadt)*
NP-40	Serva *(Heidelberg)*
o-dianisidine	Sigma *(Munich)*
Phenol	Roth *(Karlsruhe)*
Ponceau S	Sigma *(Munich)*
Paraformaldehyd (PFA)	Sigma *(Munich)*
Psoralen (4`-aminomethyl-trioxsalen)	Calbiochem *(La Jolla, CA, USA)*
Salmon testis carrier DNA 10,4 mg/ml	Sigma-Aldrich *(USA)*
Select Agar	Invitrogen *(UK)*
Select peptone 140	GIBCO *(UK)*
Skim milk powder	Töpfer *(Dietmannsried)*
SDS	Serva *(Heidelberg)*
Sucrose	Sigma *(Munich*
TEMED	Bio-Rad *(Munich)*
Tris	Roth *(Karlsruhe)*
Triton X-100	Sigma *(Munich)*
Tween 20	Sigma *(Munich)*
Uracil	Sigma-Aldrich *(USA)*
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	Promega *(Madison)*

2.2. Biochemicals

Product	**Manufacturer**
1 kb DNA ladder	Invitrogen *(Karlsruhe)*
1 kb DNA ladder (Gene ruler)	Fermentas *(St. Leon-Rot)*
Adenine-hemisulfate	Sigma-Aldrich *(USA)*
Amino acids	Sigma-Aldrich *(USA)*
Ampicillin	Serva *(Heidelberg)*
Beta galactosidase protein from E.coli	Sigma *(Steinheim)*
Bovine serum albumin (BSA)	Sigma *(Munich)*
Desoxyribonucleotides (dNTPs)	Roche *(Mannheim)*
Dulbecco`s Modified Eagle Medium (DMEM)	Cambrex, BioWhittaker *(Verviers, Belgium)*
Fetal Bovine Serum (FBS)	Biochrom KG *(Berlin)*
FuGENE	Roche *(Mannheim)*
Kanamycine	Serva *(Heidelberg)*
L-[^{35}S] Methionine cell lebelng mix	Amersham *(Little Chalont, UK)*
Na-Pyruvate	Cambrex, BioWhittaker *(Verviers, Belgium)*
P^{32} radioactive phosphor	Hartmann Analytic *(Braunschweig)*
Pen-Strep (10.000 U Penicillin/ml, 10 mg/ml Streptomycin)	Cambrex, BioWhittaker *(Verviers, Belgium)*
PMSF (100x)	*0,1742 g PMSF diluted in 10 ml isopropanol*
Prestained Protein Ladder "BroadRange"	NEB BioLabs *(Schwalbach)*
Protein G-Plus Agarose	SC Biotechnology *(Santa Cruz, CA, USA)*
Protein Kinase Inhibitor Cocktail (Mini-Complete)	Roche *(Mannheim)*
RNasin® Ribonuclease Inhibitor	Promega *(Madison)*
RPMI 1640 (1x)	Biochrom KG*(Berlin)*
Transfectin	Bio Rad *(Munich)*
Salmon testis carrier DNA 10,4 mg/ml	Invitrogen *(Karlsruhe)*

2.3. Buffers and Solutions

Buffer	**Composition**
Cracking buffer stock solution (for 100ml)	8 M Urea 5% SDS 40 mM Tris HCL pH 6,8 0,1 mM EDTA 0,4 mg/ml bromphenolblue
Cracking buffer working solution	1 ml cracking buffer stock solution 10 µl β mercaptoethanol 50 µl PMSF (from 100x stock) 1 complete mini tablet (roche)
DNA sample buffer (5x)	99,95% Glycerol (v/v) 0,01 NaP Buffer (v/v) 0,04% Bromphenol blue (w/v)
Dot-Blot-hybridization-buffer	7 % SDS 0,25 M Na_2HPO_4
Dot-Blot-wash buffer 1	5 % SDS 0,2 M Na_2HPO_4
Dot-Blot-wash buffer 2	1 % SDS 0,2 M Na_2HPO_4
ELISA blocking buffer	2% BSA 0,05% Tween 20 in PBS

ELISA coating buffer pH 9,6	70 mM $NaHCO_3$ 30 mM Na_2CO_3
ELISA stop solution	0,5 M NaOH
ELISA wash buffer	0,05 % Tween 20 in PBS
FACS buffer pH 7,4	1% BSA (w/v) 0,02% NaN_3 from 20% stock (w/v) in 1xPBS
LB agar	1,5% Agar in LB-medium
LB-medium pH 7,0	1% casein extract (w/v) 0,5% yeast extract (w/v) 0,5% NaCl (w/v) 0,1% glucose (w/v)
Paraformaldehyd (PFA)	2% Paraformaldehyd (w/v) in PBS buffer
PBS buffer pH 7,4	0,14 M NaCl 2,7 mM KCl 3,2 mM Na_2HPO_4 1,5 mM KH_2PO_4
PEG/LiAc-solution	8 ml 50% PEG 3350 1 ml 10 x TE-buffer 1 ml 1M LiAc
Plasmid purification buffer 1 pH 8,0	100 µg/ml RNase A 10 mM EDTA 50 mM Tris/HCl
Plasmid purification buffer 2	0,2 M NaOH 1% SDS (w/v)
Plasmid purification buffer 3	3 M potassium acetate 2 M acetic acid
Proteinase K	1 mg/ml Proteinase K 1,5 mM $CaCl_2$
RIPA immunoprecipitation buffer pH 7,4	50 mM Tris-HCl 1% NP-40 (v/v) 0,25 % Na-deoxycholate (w/v) 150 mM NaCl 1 mM EDTA
SDS-PAGE buffer pH 8,3 (10x)	25 mM Tris 192 mM Glycine 0,1% SDS (w/v)
SDS-PAGE fixing buffer	50 % Methanol (v/v) 40% H_2O (v/v) 10% acetic acid (v/v)
SDS-PAGE loading buffer pH 6,8 (2x)	50 mM Tris 2 % SDS (w/v) 0,04% Bromphenol blue (w/v) 84 mM 2-Mercaptoethanol 20% Glycerol (v/v)
SDS-PAGE Transfer buffer pH 8,3	25 mM Tris-Base 192 mM Glycine 20% Methanol
Sorbitol buffer pH 7,5	182 g/l sorbitol 37 g/l EDTA
Sucrose 36% pH 9,0	36% sucrose (w/v) in 10mM Tris
TAE buffer pH 8,0	40 mM Tris/HCl 1 mM EDTA
TAC medium	90% NH_4CL from 0,16 M stock 10% Tris ph 7,65 from 0,17 M stock

TBS buffer pH 7,6	50 mM Tris 150 mM NaCl
TE buffer pH 7,6	10 mM Tris/HCl 0,1 mM EDTA
TE/LiAc	8 ml H_2O 1 ml 10 x TE-buffer 1 ml 10 x LiAc
TEN buffer pH 7,4 (10x)	100 mM Tris 10 mM EDTA 1 M NaCl
1mM Tris buffer pH 9,0	1 mM Tris
10 mM Tris buffer pH 9,0	10 mM Tris
WB stripping buffer pH 6,8	100 mM 2-mercaptoethanol 2% SDS (w/v) 62.5mM Tris/HCl
Zymolyase solution	10 mg/ml Zymolyase (20.000 U/g) in sorbitol-buffer

Unless stated otherwise, buffers were prepared in ultrapure H_2O milliQ. The pH was adjusted with HCl or NaOH.

2.4. Kits

Product	**Manufacturer**
BD Cytofix/Cytoperm Kit	BD Pharmingen *(Hamburg)*
Lumi-Light®	Roche *(Mannheim)*
PCR master	Roche *(Mannheim)*
p-Nitrophenyl phosphate tablet sets ELISA substrate for AP	Sigma *(Steinheim)*
QIAGEN Plasmid Maxi Kit	QIAGEN *(Hilden)*
QIAquick Gel Extraction Kit	QIAGEN *(Hilden)*
QIAquick PCR Purification Kit	QIAGEN *(Hilden)*
RediprimeTM Random Prime Labelling System	Amersham *(Little Chalont, UK)*
TNT® Coupled Reticulocyte Lysate System	Promega *(Madison)*
TNT® T7 Quick for PCR DNA	Promega *(Madison)*

2.5. Enzymes

Product	Manufacturer
Alcaline Phosphatase – Calf Intestine (CIAP)	Roche *(Mannheim)*
PWO DNA Polymerase	Roche *(Mannheim)*
Proteinase K	Sigma *(Munich)*
Restriction enzymes	NEB BioLabs *(Schwalbach)*
	Roche *(Mannheim)*
T4-DNA-Ligase	Roche *(Mannheim)*
Trypsin-EDTA	Invitrogen *(Karlsruhe)*
Zymolyase	MP Biomedicals *(Germany)*

All enzymes were used in combination with the buffers recommended and provided by the manufacturer

2.6. Synthetic Oligonucleotides (Primers)

All oligonucleotides were synthesized at Operon.

Primers for deletion III

NIH-GS83: 5`- GAA TGC ACA TAC ATA AGT ACC GGC ATC TCT AGC AGT - 3`

IIIf-1B: 5`- CAC CAG CGT CTA CAT GAC GAG CTT CCG AGT TCC – 3`

Primers for GAL4 BD-C7L fusion protein ("bait") construction

C7L-NdeI: 5´- CAT ATG GGT ATA CAG CAC GAA TTC G – 3´

C7L-NotI: 5`- GCG GCC GCA TCC ATG GAC TCA TAA TCT C – 3`

Primers for library plasmids (addition of T7-promoter and HA-tag)

AD-HAforw: 5`- AAA ATT GTA ATA CGA CTC ACT ATA GGG CGA GCC GCC ACC ATG TAC CCA TAC GAC GTT CCA GAT TAC GCT CCA CCA AAC CCA AAA AAA GAG – 3`

AD-rev: 5`- ACT TGC GGG GTT TTT CAG TAT CTA CGA T – 3`

Primers for construction of C7L deletion mutants

C7L-BstXI: 5`- CCA ACG CGT TGG ATG GGT ATA CAG CAC GAA TT – 3`

C7L-NsiI: 5`- ATG CAT TTA ATC CAT GGA CTC ATA ATC – 3`

C7muforw32: 5`- CCA ACG GCT TGG ATG ATT ATT TCG AAT GAT TAC AAG A – 3`

C7muforw16: 5`- CCA ACG CGT TGG ATG AGA AAT TTA CAG TTA CAT AAA G – 3`

C7muforw8: 5`- CCA ACG CGT TGG ATG ATT ATT AAT GGA GAT ATC GCG – 3

C7muforw4: 5`- CCA ACG CGT TGG ATG GAA TTC GAC ATC ATT ATT AAT G – 3`

C7murev32: 5`- ATG CAT TTA TAG ACT GAT GTA TGG GTA ATA – 3`

C7murev16: 5`- ATG CAT TTA CGG GGA TGA GTA GTT TTC – 3`

C7murev7: 5`- ATG CAT TTA ATA CGG GAT TAA CGA ATG TT – 3`

C7murev8: 5`- ATG CAT TTA CGG GAT TAA CGG ATG TTC T – 3`

C7murev9: 5`- ATG CAT TTA GAT TAA CGG ATG TTC TAT ATA – 3`

C7murev10: 5`- ATG CAT TTA TAA CGG ATG TTC TAT ATA CG – 3`

C7murev12: 5`- ATG CAT TTA ATG TTC TAT ATA CGG GGA TG – 3`

C7muint1: 5`- GGT CGG AAA TCG ACG AGG TCT TAA CCG TAT TTG CAA ACA A – 3`

C7muint2: 5`- GAC CTC GTC GAT TTC CGA – 3`

C7muglyc1: 5`- AGT ATA AAG TTA AAG AAG AAT CCC CGT ATA TAG AAC ATC C – 3`

C7muglyc2: 5`- TTC TTC TTT AAC TTT ATA CTT TTT – 3`

C7mupho1: 5`- TCA TTA TAC GCC CAG ATT GGG AGG TCA AAG GAT TAA CCG T – 3`

C7mupho2: 5`- CCA ATC TGG GCG TAT AAT GA – 3`

Primers for construction of K1L-HA expression plasmid

K1L-XhoI: 5`- CTC GAG ATC TGT CAC GAA TTA ATA C – 3`

K1L-NotI: 5`- GCG GCC GCT TAG TTT TTC TTT ACA CAA TTG – 3`

K1L-BstXI: 5`- CCA ACG CGT TGG ATG TAC CCA TAC GAT GTT C – 3`

K1L-NsiI: 5`- ATG CAT TTA GTT TTT CTT TAC ACA ATT G – 3`

2.7. Plasmids

Plasmid	Antibiotic resistance	Source
pIIIΔHR-P7,5	100 µg/ml ampicilin	Staib et al.
pIIILZ-P11	100 µg/ml ampicilin	Sutter and Moss, 1992
pACT	50 µg/ml ampicilin	Clontech *(USA)*
pCMV-HA	100 µg/ml ampicilin	Clontech *(USA)*
pGADT7	100 µg/ml ampicilin	Clontech *(USA)*
pGADT7-CBF1	100 µg/ml ampicilin	Gift from S. Petermann
pGBKT7	50 µg/ml kanamycin	Clontech *(USA)*
pGBKT7-EBNA3a	50 µg/ml kanamycin	Gift from S. Petermann
pRBK1Ldel	100 µg/ml ampicilin	Staib et al.

2.8. Synthetic Peptides

All synthetic peptide were purchased from Biosynthan (Berlin). Peptides were diluted in DMSO (1 mg/ml) and stored at -80°C. For peptide coating of cells (ICS, see 3.7.5.) stocks were used 1:1000, resulting in 1µg/ml final concentration.

Peptide	MHC restriction	Amino acid sequence	Origin	Reference
$A6L_{6}$	HLA-A*0201	VLYDEFVTI	117L-A6L	Oseroff et al., 2005; Pasquetto et al., 2005
$B22R_{79}$	HLA-A*0201	CLTEYILWV	189R-B22R	Terajima et al., 2003
$C7L_{74}$	HLA-A*0201	KVDDTFYYV	018L-C7L	Terajima et al., 2003
$H3L_{184}$	HLA-A*0201	SLSAYIIRV	093L-H3L	Drexler et al., 2003
$I1L_{211}$	HLA-A*0201	RLYDYFTRV	026L-I1L	Oseroff et al., 2005; Pasquetto et al., 2005
$A3L_{270}$	H2-K^{b}	KSYNYMLL	122L-A3L	Moutaftsi et al., 2006
$B8R_{20}$	H2-K^{b}	TSYKFESV	176R-B8R	Tscharke et al., 2005
$K3L_{6}$	H2-K^{b}	YSLPNAGDVI	024L-K3L	Tscharke et al., 2005
β-Gal_{96}	H2-K^{b}	DAPIYTNV	β-Galactosidase	Overwijk et al., 1997

2.9. Antibodies

Specifity	Conjugate	Purpose	Source	Working dilution
Anti-eIF2α-phospho	-	Primary antibody for Western blot	Cell Signaling Technology *(Beverly, USA)*	1:1000
Anti-β actin	-	Primary antibody for Western blot		1:10.000
Anti-cleaved caspase 3	-	Primary antibody for Western blot	Cell Signaling Technology *(Beverly, USA)*	1:1000
Anti-C7L	-	Primary antibody for Western blot; Immunoprecipitation	E. Kremmer *(GSF-insitute for , Munich)*	1:10
Anti-CD8	APC	ICS staining	Caltag/Invitrogen *(Karlsruhe)*	1:250
Anti-CD62L	PE	ICS staining	Caltag/Invitrogen *(Karlsruhe)*	1:250
Anti-c-myc	-	Primary antibody for Western blot; Immunoprecipitation	Clontech *(USA)*	1:1000
Anti-HA	-	Primary antibody for Western blot; Immunoprecipitation	Clontech *(USA)*	1:1000
Anti-IFNγ	FITC	ICS staining	BD Pharmingen, *Heidelberg*	1:500
Anti-mouse IgG	Peroxidase	Secondary antibody for Western blot	Dianova *(Hamburg)*	1:3000
Anti-mouse IgG and IgM	Alkaline Phosphatase	Secondary antibody for ELISA		1:1000
Anit-rabbit IgG	Peroxidase	Secondary antibody for Western blot	Dianova *(Hamburg)*	1:3000
Anti-rat IgG	Peroxidase	Secondary antibody for Western blot	Dianova *(Hamburg)*	1:3000
Anit-vaccinia virus	-	Immuno staining	Quartett *(Berlin)*	1:1000

2.10. Fluorescent dyes

Dye	Stock concentration	Final concentration	Manufacturer
EMA (Ethidium Monazide Bromide)	2 mg/ml	1 µg/ml	Sigma

2.11. Viruses

The viruses used in is work were already present in the laboratory. Fresh virus preparations were made (see 3.5.3 and 3.5.4.) and the titers were determined by immunostaining as described in 3.5.5.

Virus	Description
MVA-II$_{new}$ (MVA)	Wildtype virus
MVA-ΔC7L	Recombinant MVA with deletion of C7L open reading frame
MVA-P11-LacZ (MVA-P11-LZ)	Recombinant MVA expressing β-galactosidase under control of vaccinia virus promoter P11
MVA-ΔC7L-K1L	Recombinant MVA with deletion of C7L open reading frame and insertion of K1L open reading frame
MVA-ΔE3L	Recombinant MVA with deletion of E3L open reading frame

2.12. Cell lines

Cell line	Description	ATCC number
B-LCL	Human HLA-A*0201 positive lymphoblastoid B cells	-
B16-F1	Murine melanoma cells (C57BL/6)	ATCC CRL-6323
BHK-21	Baby hamster kidney fibroblasts	ATCC CCL-10
CEF	Primary chicken embryo fibroblasts	-
HEK 293T	Human kidney	45504
HeLa	Human epithelioid carcinoma, cervix	ATCC CCL-2
MRC-5	Human lung embryo fibroblasts	ATCC CCL-171
NIH-3T3	Murine fibroblasts	CRL-1658

2.13. Bacteria- and yeast-strains

E. coli DH10b: F$^-$ *mcrA* Δ(mrr-hsdRMSmcrBC) Φ80d*lac*ZΔM15 Δ*lacX74 endA1 recA1 deoR Δ(ara, leu)7697 araD139 galU galK nupG rpsI*

Table 1: Bacteria Phenotypes

Marker	Description
F$^-$	Contains no F chromosome; no conjunction possible
mcrA	Mutation prevents mcrA restriction of methylated DNA
Δ(mrr-hsdRMSmcrBC)	Deletion of a gene cluster of six restriction enzymes
Φ80d*lac*ZΔM15	Carries the defect lambda pro-phage Φ80 who carries the d*lac*ZΔM15 allel, d*lac*ZΔM15 encodes the ω-fragment of β-galactosidase
Δ*lacX74*	Deletion of *lac* operon
endA1	Mutation of unspecific endonuclease 1
recA1	Mutation prevents homologous recombination
deoR	Mutation of a repressor of the deoCABD operons
Δ(ara, leu)7697	Deletion of a gene cluster from ara- to leu-operon
araD139	Mutation of L-ribulose 5-phosphatase 4-epimerase
galU	Mutation of UDP-glucose pyrophosphorylase
galK	Mutation of galaktokinase
nupG	Mutation of a nucleoside transporter
rpsI	Mutation in protein S12 of 30S ribosomal subunit → streptomycine resistance

AH109: *MATa; trp1-901; leu2-3; 112; ura3-52; his3-200; gal4Δ, gal80Δ; LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2; URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ;* (James et al., 1996)

Table 2: Yeast Phenotypes

Wildtype	Mutant	Phenotype
HIS3	his3-200	His$^-$ requires histidine (His) for growth
LEU2	leu2-3, 112	Leu$^-$ requires leucine (Leu) for growth
TRP1	trp1-901	Trp$^-$ requires tryptophane (Trp) for growth
URA3	ura3-52	Ura$^-$ requires uracil (Ura) for growth
GAL4	gal4Δ or gal80-542	Gal$^-$ deficient for regulation of genes for galactose-metabolism
GAL80	gal80Δ or gal80-538	Gal$^-$ deficient for regulation of genes for galactose-metabolism

Table 3: AH 109 reporter genes and their phenotypes

Reporter gene	Gene product	Positive phenotype
ADE2	Phosphoribosylaminoimidazol-Carboxylase	Ade$^+$: Adenine-prototrophie
HIS3	Imidazolglycerolphosphat-Dehydratase	His$^+$: histidine-prototrophie
lacZ	β-galactosidase	LacZ$^+$: positive for β-galactosidase activity
MEL1	α-galactosidase	MEL 1$^+$: positive for α-galactosidase activity

2.14. Mice

All mice were derived from in-house breeding under specific pathogen-free conditions at the GSF animal facility in Neuherberg following institutional guidelines.

Strain	MHC Restriction	Reference
HHD	HLA-A*0201	Pascolo et al., 1997
C57BL/6	H2-K^b and H2-D^b	http://jaxmice.jax.org

HHD II is an inbred strain of transgenic mice on a C57BL/6 background. The endogenous H-2 D^b and β2-microglobulin (β2m) gene loci are disrupted and a chimeric human (α1, α2 and mouse α3) HLA-A2.1 heavy chain covalently linked to the human β2m light chain (together called the HHD molecule) is introduced. As the export of MHC molecules to the cell surface requires association with β2m CD8+ T cells of these mice are educated on and restricted to the HHD molecule. This animal model allows the study of CTL dependent immunity to HLA-A2.1 restricted antigenic determinants in mice (Pascolo et al., 1997).

2.15. Cell culture media

Medium	Composition
Freezing Medium	90% FBS (inactivated at 56°C) 10%DMSO
DMEM 10%	10 % FBS (inactivated at 56°C) in DMEM medium
M2 Medium	10 % FBS (inactivated at 56°C) 50 µM 2-Mercaptoethanol in RPMI medium
RPMI 10%	10 % FBS (inactivated at 56°C) in RPMI medium
RPMI 2%	2 % FBS (inactivated at 56°C) in RPMI medium

2.16. Yeast culture media

Medium	**Composition**
YPDA-Medium, (Agar)	20g/l Select Peptone 10g/l Yeast Extract 0,2 % Adenine-Hemisulfate (20 g/l Agar) 2 % glucose was added after autoclavation
SD-Medium (Agar)	1,7 g/l yeast nitrogen base without AA and $(NH_4)SO_2$ 5 g/l $(NH_4)_2SO_4$ 10 % 10 x dropout solution (20 g/l Agar) pH 5,8; 2 % glucose and 3-AT (optional) were added after autoclavation
10x dropout-solution (DO)	200 mg/l L-adenine-hemisulfate 200 mg/l L-arginin HCL 200 mg/l L-histidine HCL monohydrate 300 mg/l L-Isoleucine 1000 mg/l L-leucine 300 mg/l L-lysine HCL 200 mg/l L-methionine 500 mg/l L-phenylalanine 2000 mg/l L-threonine 200 mg/l L-tryptophane 300 mg/l L-tyrosine 200 mg/l L-uracil 1500 mg/l L-valin one or more amino acids were excluded to obtain a certain selective minimal medium

2.17. Consumables

Product	Manufacturer
3MM-Filter paper	Whatman *(Maidstone, UK)*
Cell culture flasks (T185, T225)	Greiner *(Nürtingen)*, Corning *(New York, NY,USA)* Nunc *(Wiesbaden)*
Cell culture plates 6-, 12-, 96-well	Corning *(New York, NY, USA)*
Cell lifter	Corning *(New York, NY, USA)*
Cell strainer 100 µm	BD Pharmingen *(Hamburg)*
FACS tubes	Bio-Rad *(Munich)*
Falcon tubes (15 ml, 50 ml; PS, PP)	BD Pharmingen *(Hamburg)*
GenePulser cuvettes	Bio-Rad *(Munich)*
Glass beads (425-600 µm ; 1 mm)	Sigma-Aldrich *(USA)*
Gloves	Kimberly-Clark *(Mainz)*
Hyperfilm^{TM} ECL	Amersham *(Little Chalfont, UK)*
Nitrocellulose membrane	Bio-Rad *(Munich)*
NUNC-immuno-plate (Maxi-sorb)	Nunc *(Wiesbaden)*
Nylon membrane	Bio-Rad *(Munich)*
PCR reaction tubes	Eppendorf *(Hamburg)*
Petri dishes	Nunc *(Wiesbaden)*
Pipette tips	Molecular Bioproducts *(San Diego)*
Pipettes “Cellstar” (1-25ml)	Corning *(New York, NY, USA)*
Reaction tubes (0,5 ml, 1,5 ml, 2 ml)	Eppendorf *(Hamburg)*
Sterile filters (Minisart 0,2-0,45 µm)	Sartorius AG *(Göttingen)*
Syringes (5, 10, 20 ml)	BD Pharmingen *(Hamburg)*
Syringes (Omnifix-F 1 ml)	Braun *(Melsungen)*
Ultracentrifuge tubes (Ultra clear)	Beckmann *(Munich)*

2.18. Software

Product	Manufacturer
FacsDIVA	Becton Dickinson, Heidelberg
FlowJo 6.4.2	Treestar, Ashland
GraphPadPrism 4	Graph Pad Software, San Diego
Quantity One 4.1.1	Bio Rad, Munich
MS Office	Microsoft, Redmond

2.19. Laboratory equipment

	Model/type	Manufacturer
Block thermostat	BT 1302	HLC BioTech *(Bovenden)*
Centrifuge	Avanti J-25	Beckman *(Munich)*
	Megafuge 1.0R	Heraeus *(Hanau)*
	Biofuge fresco	Heraeus *(Hanau)*
	Biofuge pico	Heraeus *(Hanau)*
	Optima LE-80K	Beckman *(Munich)*
CO_2 Incubator	FunctionLine Hera Cell 150	Heraeus *(Hanau)*
Cup sonicator	Sonopuls HD200	Bandelin (*Berlin)*
Dot blot apparatus	Minifold ® I Dot Blot System	Schleicher-Schuell
Electro-blotting System	Panther™ SemiDry	Owl Scientific *(Portsmouth, NH, USA)*
Electrotransformator	E.coli pulser	Bio-Rad *(Munich)*
Flow cytometer	FACS Canto	Becton Dickinson *(Hamburg)*
Film processor	Curix 60	Agfa *(Köln)*
Freezer (-20°C)	Excellence	Bauknecht *(Stuttgart)*
Freezer (-80°C)	Hera freeze	Heraeus *(Hanau)*
Fridge (4°C)	UT6-K	Bauknecht *(Stuttgart)*
Gel Dryer	Model 583	Bio-Rad *(Munich)*
Haematocytometer	Neubauer counting chamber	Karl Hecht KG *(Sondheim)*
Horizontal Electrophoresis Sytem	A1Gator	Owl Scientific *(Portsmouth, NH, USA)*
	A2Gator	
Hybridization Oven	6/12	UniEquip (Martinsried,Germany)
Ice machine	AF 200	Scotsman *(Milan, Italy)*
Incubation shaker	Innova 4430	New Brunswick Scientific *(Nürtingen)*
Laminar flow	HERAsafe HS 12	Heraeus *(Hanau)*
Micropipette	Pipetman P10-1000	Gilson *(Middleton, USA)*
Microplate reader	Microplate Reader Model 550	Bio-Rad (*Munich)*
Microscope	Axiovert 25	Carl Zeiss *(Oberkochen)*
Microwave	900 W	Siemens *(Munich)*
Multi channel pipette	Transferpette-12 (20-200 µl)	Brand *(Wertheim)*
	Calibra 852	Socorex *(Ecublens, Switzerland)*
PCR Cycler	GeneAmpR PCR System 2700	Applied Biosystems *(Foster City, CA, USA)*
Phospho Imager	Molecular Imager PharosFX	Bio-Rad (*Munich)*
Phospho Imager Screen	Imaging Screen-K	Bio-Rad (*Munich)*
Phospho Screen Eraser	Screen Eraser-K	Bio-Rad (*Munich)*
UV/VIS Bio-photometer	GeneQuant II	Pharmacia Biotech *(Uppsala, Sweden)*
Pipettor	Easy jet	Eppendorf (Hamburg)
Power supply unit	Model 200/2.0	Bio-Rad *(Munich)*
Rotor	Typ 19, SW28, SW 41	Beckmann *(Munich)*
Stratalinker 1800 UV crosslinking unit	Crosslinking unit with five 365 nm long wave UV bulbs	Stratagene *(La Jolla, Ca, USA)*
Thermomixer/ -block	Thermomixer 5436	Eppendorf *(Hamburg)*
Ultracentrifuge	Optima LE-8K	Beckmann *(Munich)*
Universal Hood	Gel Doc 2000	Bio-Rad *(Munich)*
Vacuum aspirator	Unijet II	Uniequip *(Martinsried)*
Vortexer	Vortex Genie 2	Scientific Industries *(Bohemia, NY, USA)*
Waterbath	U3	Julabo *(Seelbach)*

3. Methods

3.1. Bacteriological techniques

3.1.1. Culture of *E.coli*

E. coli were cultured on agar plates or in liquid culture under shacking at 37 °C. Table lists the different culture strategies employed.

Table 4: Culture techniques used for growth of *E. coli*

Culture	Medium	Antibiotic	Volume
After transfection (1h pre-culture)	LB-Medium	-	1 ml
After transformation (over night)	Bacto-Agar Plates	100 µg/ml ampicillin 50 µg/ml ampicilin 50 µg/ml kanamycin	
Analytical plasmid preparation	LB-Medium	100 µg/ml ampicillin 50 µg/ml ampicilin 50 µg/ml kanamycin	3 ml
High yield plasmid preparation	LB-Medium	100 µg/ml ampicillin 50 µg/ml ampicilin 50 µg/ml kanamycin	400 ml
Generation of electrocompetent cells (preculture)	LB-Medium	-	100 ml
Generation of electrocompetent cells	LB-Medium	-	2 x 250 ml

3.1.2. Generation of electro-competent bacteria

The production of electro-competent bacteria was carried out under sterile conditions using only autoclaved equipment and solutions. About 10 µl of a glycerin culture of the *E. coli* strain DH10B were transferred to a 100 ml LB-medium pre-culture without antibiotic and grown over night under vigorous shacking. For the main culture, 2 x 250 ml of LB medium without antibiotic were inoculated with 1-2 ml of the pre-culture and incubated at 37 °C under vigorous shacking. Bacterial growth was monitored by determining the optical density at a wavelength of 600 nm (OD600) at intervals. The bacteria were harvested in their exponential growth phase at an OD600 of about 0,6. The cell suspension was cooled in ice water for 15 min. The equipment and solutions were pre-cooled to ensure that all the following steps of the protocol could be carried out at nearly 0°C. The bacteria were centrifuged for 15 min at 5000 rpm, washed three times in 500 ml ddH_2O and resuspended in 10 ml 10% glycerin. After centrifugation for 12 min at 5000

rpm the bacterial sediment was resuspended in 1,5 ml 10% glycerin and shock frozen in liquid nitrogen in 50 µl aliquots. The bacteria were stored at -80°C.

3.1.3. Transformation

To generate bacterial clones containing the plasmid of interest, electro-competent cells were transformed by electroporation. 25 µl electro-competent bacteria (stored at -80°C) were thawed on ice and subsequently mixed with 5 µl DNA solution purified from a ligation reaction or from yeast DNA preparation. This mix was applied to a pre-cooled electroporation cuvette and pulsed at 1.8 kV, 200 Ω and 25 µF in the *E. coli* pulser. Pulsed cells were immediately taken up in 1 ml LB-medium without antibiotic and incubated in a shaker at 37°C. After 1 h of incubation the bacteria were carefully pelleted using a centrifuge (4000 rpm, 4 min) and resuspended in a small amount of fresh LB-medium. 10% and 90% of the solution were applied onto LB-Agar plates containing antibiotic and incubated over night at 37 °C.

3.1.4. Isolation of plasmid DNA

After successful transformation colonies were picked and further cultured in LB-medium containing antibiotic. Plasmid DNA was isolated and analyzed for presence of the plasmid of interest by restriction digestion. One positive clone was selected for a larger culture to isolate plasmid DNA for future use.

3.1.4.1. Isolation of plasmid DNA for analytical purpose (Mini-Prep)

For plasmid isolation 3 ml of antibiotic-containing LB-medium were inoculated with bacteria derived from one colony grown on agar plates. Bacteria were cultured overnight in a shaker at 37°C and 170 rpm. The next day, bacteria were pelleted by centrifugation (13.000 rpm, 5 min). The bacterial pellet was resuspended in 100 µl buffer P1. Subsequently 200 µl of buffer P2 were added and the mix was incubated 5 min on ice. After addition of 150 µl buffer P3 the solution was mixed and incubated on ice for 5 min. Cellular debris and proteins were visible as a white fluffy precipitate which was removed by a centrifugation step (13.000 rpm, 10 min). The supernatant was mixed with 800 µl of phenol/chloroform/isoamylalcohol (25:24:1) to clear the DNA from residual proteins. After a centrifugation step (13.000 rpm, 5 min), the DNA was precipitated from the supernatant by addition of 2,5 Vol 100% Ethanol and incubation for 30 min at -80°C. The precipitated DNA was pelleted by centrifugation (13.000 rpm, 20 min) and washed with 70% ethanol. The DNA pellet was air-dried and subsequently resolved in 18 µl H_2O and 2 µl RNase-A-solution (10 mg/ml). A fraction (5-10 µl) was used for restriction enzyme digestion.

3.1.4.2. High yield isolation of plasmid DNA (Maxi-prep)

For high yield plasmid isolation 500 ml antibiotic-containing LB-medium were inoculated with 2 ml of a bacteria overnight culture. The culture was shaken (110 rpm) at 37°C overnight. The next day 700 µl of this culture were removed and mixed with glycerin (50%) and stored at -80°C as a back-up stock. Plasmid isolation was conducted using the Quiagen Plasmid Maxi Kit according to the manufactures instructions. Isolated DNA was taken up into 500 µl of H_2O and subsequently the DNA concentration was measured.

3.2. Techniques for Molecular Biology

3.2.1. PCR reactions

The Polymerase chain reaction (PCR) is a method used to specifically amplify target genes or gene fragments. All PCR reactions instead of the deletion III PCR were performed using the PWO-polymerase that contains a 3`-5`exonuclease activity (proofreading activity). The PCR for the deletion III was performed using the PCR Master Kit (Roche). Reaction conditions and temperature settings are listed in Table 5 to Table 9.

Table 5: Reaction conditions for PCR with PWO-Polymerase

Mix I	Mix II
2 µl dNTP (10 mM)	10 µl 10 x PCR Puffer (+$MgSO_4$)
1 µl primer 1 (5 pmol/µl)	0,5µl PWO-polymerase
1 µl primer 2 (5 pmol/µl)	39,5 µl H_2O
1 µl MVA DNA	
45 µl H_2O	

For one PCR reaction 50 µl Mix II were added into 50 µl of Mix I, containing the template of interest, immediately before placing the reaction vessels into the PCR cycler.

Table 6: Temperature setting for PCR used to isolate K1L, C7L and C7L-deletion mutant sequences

I	2`	94°C	
II	15``	94°C	
	30``	54°C	x 30
	1`	72°C	
III	7`	72°C	
IV	∞	4°C	

Table 7: Temperature setting for PCR used to add T7-promotor- and HA-tag-sequences to Y2H-clones

I	1`	94°C	
II	15``	94°C	
	5`	72°C	2x
III	15``	94°C	
	5`	70°C	2x
IV	15``	94°C	
	5`	68°C	21x
V	7`	68°C	
VI	∞	4°C	

Table 8: Reaction conditions for PCR with PCR master kit

2 µl DNA
5 µl Primer 1 [5 pmol/µl]
5 µl Primer 2 [5 pmol/µl]
1 µl DMSO
50 µl PCR-Master-Mix (inkl. Taq-DNA-Polymerase, dNTPs, $MgCl_2$)
ad 100 µl ddH_2O

Table 9: Temperature setting for deletion III PCR

I	2`	94°C	
II	30``	94°C	
	40``	55°C	
	3`	72°C	30x
III	7`	72°C	
VI	∞	4°C	

3.2.2. Analytical gel electrophoresis

To verify the sizes of PCR products or fragments resulting from restriction digestion electrophoresis in 1% agarose gels was performed. Gels were prepared using 1x TAE buffer and the fluorescent, DNA-intercalating dye ethidiumbromide (5 µg/100 ml gel) was added for visualization of DNA. The DNA-solution was mixed with loading buffer. 6 µl of a premixed 1 kb ladder were used as a mass standard and for estimation of DNA concentrations of vector and insert prior to ligation reactions. Electrophoresis was conducted at 75-85V for 30-50 min. After electrophoresis the gel was analyzed and photographed under UV-excitation (312 nm).

3.2.3. DNA purification from agarose gels

Gel electrophoresis was also used to separate one DNA fragment from another or to clean DNA from other reaction components such as enzymes prior to further use. The fragment of interest was excised from the agarose gel after electrophoresis with a scalpel and DNA was extracted using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. DNA was eluted in 30 µl H_2O.

3.2.4. Restriction enzyme digestion

Restriction digestions of DNA were performed at 37°C or at 55°C (BstXI) for 2 hours. All enzymes were used with the buffer and BSA concentration recommended by the manufacturer. Generally, 1 U enzyme was used to digest 1 µg of DNA. After digestion reaction vessels were incubated at 65°C for 10 minutes in order to stop the reaction. To prevent unspecific cleavage DNA was either purified using the QIAquick PCR purification kit or purified by gel electrophoresis and DNA extraction from the gel.

3.2.5. Dephosphorylation

Vector DNA used for ligation reactions was dephosphorylated after restriction digestion. Removal of the free phosphate at the 3` end of the vector prevents relegation of the linearized vector. 3 µl (9 U) alkaline phosphatase (AP) and 10 µl 10x AP buffer were added to the vector restriction reaction. Water was added to achieve a total volume of 100 µl. The reaction was incubated at 37 °C for 1 h. After 30 minutes incubation, another 3 µl of AP were added. To stop the dephosphorylation 5 µl EDTA were added and the reaction tubes were incubated at 70°C for 20 min. To achieve a complete inhibition of the reaction, vector DNA was immediately purified by gel electrophoresis and DNA extraction from the gel.

3.2.6. Ligation

Ligations of DNA fragments were combined at a vector to insert ratio of 1:3. Concentrations were estimated from agarose gels under consideration of the different sizes of vector and insert. Per 1 µg of DNA 1 unit ligase was used. Ligation reactions were always carried out in 20 µl reaction volume containing 2 µl 10x ligase buffer. The ligation reactions were incubated overnight at 14 °C. The reaction was stopped by addition of 5 µl EDTA and a 10 minute heating step at 65°C. Ligated DNA was subsequently precipitated by addition of 40 µl H_2O, 4 µl 3 M NaAc and 150 µl ethanol. After incubation for 30 min at -80°C the DNA was purified by centrifugation (13.000 rpm, 20 min). The DNA pellet was washed with 70% ethanol. The air-dried pellet was diluted in 15 µl H_2O and used for transformation of competent cells.

3.2.7. Determination of DNA concentration

Concentrations of solutions containing double stranded DNA were photometrically measured by their adsorption of light at a wavelength of 260 nm. Since an adsorption of 1 equals a concentration of 50 µg/ml dsDNA, the concentration of the solution was calculated as follows:

$$Adsorption\ at\ OD_{260}\ x\ 50\ \mu g/ml\ x\ dilution\ factor\ =\ concentration\ of\ dsDNA$$

3.2.8. Sequencing

For each of the constructed plasmids a sample was analyzed by commercial sequencing in order to verify the inserted sequence. Primers used for sequencing are listed in Table 10.

Table 10: Primers used for plasmid sequencing

Plasmid	Sequencing primer	Sequence 5`→ 3`
P7,5-C7L and all P7,5-C7L mutants	T7	TAA TAC GAC TCA CTA TAG G
pACT library plasmids forward	GAL-4ADfor	TAC CAC TAC AAT GGA TGA TG
pACT library plasmids reverse	pGAL4AD-3`	GAA CTT GCG GGG TTT TTC
pCMV-HA-K1L	CMV-profor	ATG GGC GGT AGG CGT G
pCMV-HA-C7L	CMV-profor	ATG GGC GGT AGG CGT G
pCMV-HA-133	CMV-profor	ATG GGC GGT AGG CGT G
pGBKT7-C7L	T7	TAA TAC GAC TCA CTA TAG G

3.2.9. Dot blot southern hybridization

Confluent cells grown in 6-well plates were infected with 10 pfu per cell and incubated for 30 min at 4°C. The medium was removed and substituted with 1ml pre-warmed RPMI 2% per well (timepoint t = 0) and subsequently incubated at 37°C and 5% CO_2 atmosphere. At 0h, 2h, 4h and 8h post infection 600 µl of the medium were removed and the cells were harvested in the remaining 400 µl. The viral DNA was isolated as described in 3.5.2. and diluted in 50 µl H_2O. 350 µl TE 10/1 (pH 7,0) and 40 µl 3M NaOH were added to the DNA solution and incubated at 65 °C for 45 min. Subsequently, after cooling on ice, 400 µl 2M NaOAc were added. A nylon membrane and Whatman papers corresponding to the size of the dot blot apparatus were equilibrated in H_2O and placed into the dot blot apparatus. 400 µl of each probe were transferred to the membrane by aspiration. Subsequently the membrane was dryed at 80°C for 30 min. To detect the bound DNA, the membrane was pre-hybridized for 45 min at 65 °C with 20 ml dot blot hybridization buffer. In the meantime, 25 ng of plasmid DNA containing the MVA-H3L open reading frame were mixed with 43,5 µl TE 10/1 (pH 8,0), incubated for 5 min at 95°C to denature the DNA and subsequently mixed with rediprime and 5 µl αP^{32}dCTP and heated to 37°C for 10 min. By addition of 5 µl 0,2 M EDTA and heating to 95°C for 5 min the reaction was stopped and the ^{32}P labeled DNA was diluted in 20 ml pre-warmed hybridization buffer and incubated with the membrane over night at 65°C to detect the bound DNA. Finally, the membrane was washed for ten minutes at 65°C with dot blot wash buffer 1 and dot blot wash buffer 2. Analysis of radioactivity was visualized on a phosphor imager plate.

3.3. Techniques for protein analysis

3.3.1. Western blot

Western blotting is an antibody-based method that can be used to detect and quantify proteins that have been separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight.

3.3.1.1. Preparation of cell lysates

To isolate proteins the medium was removed from the culture dish and cell monolayers were washed with cold PBS. Subsequently 300 µl (for 6-wells) or 150 µl (for 12-wells) NP40-lysis buffer were added to the monolayers and the dishes were incubated on ice for 20 min. After centrifugation (13.000 rpm, 10 min) the supernatants were used for analysis.

3.3.1.2. SDS-PAGE and Semi-Dry-Transfer of proteins

15 µl to 50 µl of supernatant prepared as described under 3.3.1.1. were mixed with an appropriate amount of protein loading buffer and incubated for 5 min at 95°C to denature proteins. Subsequently, samples were applied to the pockets of the stacking gel and proteins were separated at 80 Volt (stacking gel) to 130 Volt (resolving gel).

Table 11: Composition of 20 ml stacking gels for SDS-PAGE

H_2O	13.6 ml
1M Tris ph 6,8	2,5 ml
Bis/Acrylamid (30%)	3,4 ml
SDS (10%)	0,2 ml
Ponceau S solution	0,2 ml
APS (10%)	0,2 ml
TEMED	0,02 ml

Table 12: Composition of 30ml 12% resolving gels for SDS-PAGE

H_2O	9,9 ml
1,5 M Tris pH 8,8	7,5 ml
Bis/Acrylamid (30%)	12 ml
SDS (10%)	0,6 ml
APS (10%)	0,6 ml
TEMED	0,024 ml

After electrophoresis, the gel, a nitrocellulose membrane (0,45 µl pore size) and Whatman papers were equilibrated for 10 minutes in transfer buffer. Gel and membrane were placed between 12 layers of Whatman paper and into a semi-dry-blotting apparatus. Blotting voltage was set to be a maximum of 0,5 mA/cm^2 gel size. Blotting was carried out for 30 minutes.

3.3.1.3. Detection of proteins on the membrane

After blotting, the nitrocellulose membrane was incubated in blocking buffer for 2 h at room temperature or over night at 4°C. After blocking the membrane was washed 3 times for 10 minutes in PBS-T or TBS-T, and then incubated for 2 h with the primary antibody diluted in blocking buffer. Unbound antibody was removed by washing as described above. The membrane was then incubated for 1 hour with the secondary antibody diluted in blocking buffer and subsequently washed again. Depending on the size of the membrane 1-2 ml substrate solution (a 1:1 mix of Lumi-Light solution A and B) were used to cover the membrane. The membrane was incubated for 5 minutes with the substrate solution. Protein-specific signals were detected by placing a photographic film on the membrane and exposing it for 30 sec to 5 minutes. After exposure, the film was developed and proteins were analyzed for correct molecular weight by comparison with the marker proteins.

Table 13: Corresponding blocking buffers, primary and secondary antibodies and washing agents

Blocking solution	Primary antibody (dilution)	Secondary antibody (dilution)	Washing agent
5% Milk in PBS-T	α C7L (1:10)	α rat-PO (1:3000)	PBS-T
5% Milk in PBS-T	α HA (1:1000)	α rat-PO (1:3000)	PBS-T
5% Milk in PBS-T	α myc (1:1000)	α mouse-PO (1:3000)	PBS-T
5% Milk in TBS-T	α eIF2α (1:1000)	α rabbit-PO (1:2000)	TBS-T
5% Milk in TBS-T	α cleaved caspase (1:1000)	α rabbit-PO (1:2000)	TBS-T
5% Milk in TBS-T	α β-actin (1:10000)	α mouse-PO (1:2000)	TBS-T

PBS-T: PBS + 0,1% Tween 20 TBS-T: TBS + 0,1% Tween 20

3.3.2. *in vitro* transcription/translation reactions

3.3.2.1. *in vitro* transcription/translation with plasmid template

To perform *in vitro* coupled transcription/translation reactions from a plasmid-template the TNT® Coupled Reticulocyte Lysate System was used. In the TNT Lysate System used in this work ^{35}S-methionine radiolabeled proteins are produced of genes cloned downstream from the T7 RNA polymerase promoter in a single-tube reaction. To generate radiolabeled myc-tagged C7L protein the required components were assembled in a 1,5 ml reaction tube as shown in table 14.

Table 14: Components of an *in vitro* transcription/translation reaction using TNT® Coupled Reticulocyte Lysate System and pGBKT7-C7L as template

Component	
TNT® Rabbit Reticulocyte Lysate	25 µl
TNT® Reaction Buffer	2 µl
TNT® T7 RNA Polymerase	1 µl
Amino Acid Mixture, minus methionine, 1 mM	1 µl
[^{35}S] methionine	2 µl
RNasin® Ribonuclease Inhibitor (40u/µl)	1 µl
DNA template pGBKT7-C7L	2 µl (= ng)
Nuclease-Free Water	16 µl

All handling of the lysate components should be performed at 4°C or on ice.

After addition of all components the lysate was mixed and incubated for 90 min at 30°C.
To analyze the results of the transcription/translation reaction a 5 µl aliquot was removed from the reaction, mixed with SDS-PAGE loading buffer, heated to 100 °C for 2 min and separated by 12% SDS-PAGE. Gels were fixed for 20 min in fixation buffer and then dried. Analysis of radioactivity was visualized on a phosphor imager plate.

3.3.2.2. *in vitro* transcription/translation with a PCR product template

To generate ^{35}S-methionine radiolabeled HA-tagged cDNA-library proteins, the PCR products generated as described in 3.2.1. were subjected to *in vitro* coupled transcription/translation reactions using the TNT® T7 Quick for PCR DNA Kit from Promega. To generate the radiolabeled proteins the required components were assembled in a 1,5 ml reaction tube as shown in table 15.

Table 15: Components of an *in vitro* transcription/translation reaction using TNT® T7 Quick for PCR DNA and library PCR products as template

Component	
TNT® T7 PCR Quick Master Mix	20 µl
[^{35}S] methionine	2 µl
DNA template PCR product	3 µl

All handling of the lysate components should be done at 4°C or on ice.

After addition of all components the lysate was mixed and incubated for 90 min at 30°C. To analyze the results of the transcription/translation reaction a 3 µl aliquot was removed from the reaction, mixed with SDS-PAGE loading buffer, heated to 100 °C for 2 min and separated by 12% SDS-PAGE. Gels were fixed for 20 min in fixation buffer and then dried. Analysis of radioactivity was visualized on a phosphor imager plate.

3.3.3. Co-immunoprecipitation with *in vitro* generated proteins

The *in vitro* generated radiolabeled HA/myc tagged proteins as described in 3.3.2.1. and 3.3.2.2. were subjected to co-immunoprecipitation reactions.
10 µl of *in vitro* translated, ^{35}S-methionine-labeled myc-tagged C7L protein was mixed with 10 µl of *in vitro* translated, ^{35}S-methionine-labeled HA-tagged cDNA-library protein and incubated at room temperature for 2 h. Then either 100 µl of anti-C7L monoclonal antibody, 10 µl of anti c-myc monoclonal antibody or 20 µl of anti-HA polyclonal antibody were added to the protein mix and incubated at room temperature for 2 h. It was important to add only one of the antibodies to the reaction sample. Meanwhile the Protein-G-agarose beads were prepared. For each reaction 30 µl of the beads were transferred to a new tube and were pelleted by centrifugation (2500 rpm, 2 min). The supernatant was discarded and the beads were washed 3 times with IGEPAL buffer. Finally, the beads were resuspended to their original volume and 30 µl of the bead suspension

together with 500 µl of RIPA buffer were added to each co-immunoprecipitation reaction. To ensure adequate mixing the reaction tubes were rotated at room temperature for 2 h. Subsequently the beads were pelleted by centrifugation (2500 rpm, 2 min) and washed 4 times with RIPA buffer. Finally the precipitates were boiled in loading buffer at 85°C for 5 min and separated by 12% SDS-PAGE. Gels were fixed for 20 min in fixation buffer and then dried. Analysis of radioactivity was visualized on a phosphor imager plate.

3.3.4. β-galactosidase reporter gene assay

Cells were seeded into 96-well dishes and grown to sub-confluence. Subsequently the cells were infected with MVA-p11-LacZ or MVA-ΔC7L-LacZ with 10 pfu/cell in a volume of 100 µl per well. For each virus 4 wells were infected. If desired, the cells were transfected 1 h post infection as described in 3.4.5. The cells were incubated for 16 h at 37°C and 5%-CO_2 atmosphere. Then the infection medium was replaced by 200 µl β-galactosidase lysis buffer per well and incubated for 5 min at room temperature. Per well, 100 µl cell lysate were transferred to a new 96-well dish and mixed with 100 µl β-galactosidase reaction buffer. 5–30 min after addition of the reaction buffer the β-galactosidase activity was quantified with a microplate reader for 96 well dishes *(Microplate Reader, Bio-Rad)* at 570 nm.

Table 16: Buffers used for β-galactosidase reporter gene assay

buffer	**composition**
β-galactosidase lysis buffer	2,4 ml 1M Na_2HPO_4 1,6 ml NaH_2PO_4 0,4 ml 1M KCL, 0,4 ml 1M $MgSO_4$ 0,4 ml 25mM EDTA 0,5 ml 10% NP40 34,16 ml H_2O 120 µl β-Mercaptoethanol
β-galactosidase reaction buffer	9,0 ml H_2O 800 µl 1M Phosphatpuffer pH 7.4 50 µl 2M $MgCl_2$ 7 µl β-Mercaptoethanol 100 µl 600 mM CPRG

3.4. Techniques for cell culture

3.4.1. Mammalian cell culture

Mammalian cells were cultured under sterile conditions and only handled under a clean bench. Culture was carried out at 37°C in incubator providing a 5% CO_2 atmosphere and 95% humidity. All cell lines used except for HEK 293T were grown in RPMI medium supplemented with 1% penicillin-streptomycin and 5% to 10% fetal calf serum (FCS). HEK 293T were grown in DMEM supplemented with 10% fetal calf serum (FCS). Cell lines were grown in monolayers in T185 flasks. When cells had reached approximately 90% confluence they were split at a ratio of 1:2 to 1:10 depending on their growth kinetics and intended use. Medium was removed and cells were washed 2 times with PBS. After washing, the monolayer was covered with 3 ml trypsin-EDTA solution and incubated at 37°C for approximately 3 minutes to detach the cells. 7 ml fresh medium were added to the trypsin-solution and cells were singularized by re-suspension and required fractions were transferred into a T185 flask with fresh medium or plated onto cell culture plates

3.4.2. Cryo conservation of eukaryotic cells

Only cells in their exponential growth phase were subjected to freeze storage. Cells cultivated in a T185 cell culture flask were harvested by trypsination and pelleted for 5 min at 4°C and 1400 rpm. The cell pellet was resuspended in cold freezing medium and transferred to sterile cryopreservation tubes in 1ml aliquots. The cells were frozen slowly by storing them over night in slow-cooling containers at – 80°C. After 24 h the tubes were transferred to liquid nitrogen (-196 °C) for long term storage.

3.4.3. Thawing of cryo conserved eukaryotic cells

To re-cultivate deep frozen cells the cell suspension was thawed in a 37°C water bath and transferred into 10 ml of pre-heated RPMI 10%. The cell suspension was transferred into a T185 cell culture flask and cultivated at 37°C.

3.4.4. Infection of cells with MVA or MVA recombinants

For infection with MVA mammalian cell lines were grown to 80% confluence in 6-well, 12-well or 96-well plates. Virus (stored at -80°C) was thawed and sonicated for 30 seconds to singularize viral particles. For each well an M.O.I. between 10 and 20 was taken up in 1 ml (6-well), 500 µl (12-well) or 100 µl (96-well) RPMI medium containing 2 % FCS. Medium was removed from the monolayer and substituted with the RPMI/virus mix. Cells were then incubated at 37°C.

3.4.5. Transient transfection of cells with plasmid DNA

Some plasmids used for transfection in this work contain the target gene constructs under the control of the VACV early/late promoter P7.5. Expression of target genes is therefore only possible if cells are infected with MVA/recombinant MVA prior to transfection to allow transcription of the virally regulated genes. Infection with MVA or recombinant MVA was carried out as described under 3.4.4. 12-well plates containing approximately 400.000 cells and 96-well plates containing approximately 8000 cells were incubated with 500µl (12-well) or 100µl (96-well) RPMI medium containing the desired virus at an MOI of 10 for 60 minutes under standard culture conditions. Transfection reagents were prepared using serum-free RPMI medium in separate 1,5 ml reaction tubes for each well. Mix A and B were prepared in separate tubes as listed in table 17, mixed and subsequently incubated for 20 minutes at room temperature.

Table 17: Solutions used for transfection of mammalian cells with plasmid DNA

Culture dish	Mix A	Mix B
One well of 12-well dish	1 µg DNA in 100 ml serum-free RPMI	3 µl Transfectin in 100 ml serum-free RPMI
One well of 96-well dish	0,1 µg DNA in 100 ml serum-free RPMI	0,2 µl Transfectin in 100 ml serum-free RPMI

After sufficient infection time, the cells were left in the infection medium and the transfection mix was added drop wise to the wells and culture was continued under standard conditions. Transfected cells were harvested 16-24 hours post transfection using a cell scraper and cell lysates were prepared as described under 3.3.1.1.

3.5. Virological Methods

3.5.1. Generation of recombinant MVA

3.5.1.1 Stable transfection for homologous recombination

To generate recombinant viruses by homologous recombination permissive CEF cells were infected with MVA-ΔC7L and transfected with the MVA transfer vector pIIILZ-P11. Infection with MVA-ΔC7L was carried out at MOI 0,01 in one well of a 6-well cell culture dish as described 3.4.4. 90 min post infection the cells were transfected with plasmid DNA using the FuGENE 6® transfection reagent following the manufacturer's instructions. 6 µl *FuGENE6* were diluted in 100 µl serum free RPMI and incubated for 5 min at room temperature. Subsequently 1,5 µg plasmid DNA was added, mixed and after incubation for 15 min at room temperature the transfection solution was added drop wise to the infected cells. Transfected cells were harvested 48 hours post transfection using a cell scraper. Viruses were released from the cells by freeze-thawing and sonication.

3.5.1.2. Isolation of recombinant MVA virus

The *E.coli* LacZ gene of the insertion cassette allows for isolation of recombinant MVA on CEF cells by plaque purification and staining with X-gal. Only those recombinant MVA viruses that have integrated the insertion cassette containing the LacZ gene into their genomes are able to convert the colorless sugar equivalent X-gal into a blue dye. The virus suspension obtained from transfection and infection of CEF cells was diluted in 10^{-1} steps in RPMI 10% to result in dilutions of 10^{-1} to 10^{-4}. CEF cells were grown in 6-well plates to 80% confluence. Growth medium was removed and the cells were infected with the virus suspension by adding 2 ml of each dilution to two wells, respectively. The infected cells were incubated for 4 hours at 37°C and 5% CO_2 atmosphere. Subsequently the medium was removed and substituted with 1 ml of a 1:1 mix of in ddH_2O melted 2% LMP-agarose and 2 x MEM. After 30 min at room temperature the agar became solid and the cell culture dish was incubated for 48-72 hours at 37°C and 5% CO_2 atmosphere. Then a new 1:1 mix of 2% LMP-agarose and 2 x MEM was prepared. To this mix

0,01 Vol of a X-gal solution was added. 1 ml was put to each well and after the agar became solid again incubated at 37°C. After 2-6 hours blue stained plaques became visible on the agar caused by cells that were infected with β-galactosidase expressing viruses. The plaques were isolated under sterile conditions from high dilutions using a 200 µl pipette tip, transferred into an Eppendorf tube and resuspended in 500 µl RPMI 10%. The isolated cells were freeze-thawed and sonicated three times for one minute. To obtain wildtype free recombinant virus preparations, the isolated plaques were further passaged on CEF cells. To screen for wildtype free virus, viral DNA was isolated as described in 3.5.2. and analyzed by a deletion III PCR as described in 3.2.1. The isolated recombinant virus was subsequently amplified and purified.

3.5.2. Extraction of DNA from infected cells

Tissue culture cells were infected at an MOI of 10 in a 6-well dish. Cells were harvested with a cell scraper and pelleted at 2000 rpm for 2 min. The pellet was resuspended in 400 µl of 1xTE buffer (pH 7,6) and freeze-thawed three times. 50 µl of 10x TEN buffer, 50 µl of proteinase K and 23 µl of 20% SDS were added to the virus suspension, the sample was mixed and incubated for 2 to 4 hours at 56°C. The DNA was extracted with 600 µl of phenol-chloroform. The samples were vortexed and centrifuged at 13000 rpm for 10 min at room temperature. The aqueous upper phase was transferred into a fresh tube. The extraction procedure was repeated up to three times. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The sample was frozen at -80°C for 30 min to aid precipitation and DNA pelleted by centrifugation at 13000 rpm for 15 min at 4°C. The pelleted DNA was washed with 250 µl of 70% ethanol and centrifuged at 13000 rpm for 15 min at 4°C. The pellet was air dried and resuspended in 50 µl of 1x TE buffer (pH 7,6).

3.5.3. Virus amplification and crude stock preparation

For large scale MVA virus preparations primary CEF cells were cultivated in 10-40 T225 cell culture flasks at 37°C. After 2-3 days, the medium was changed and the 80% confluent cell layer was infected at an MOI 0,3 by adding the calculated volume of virus suspension diluted in 1 ml medium to each flask. The cells were cultivated for another 2-3 days until a cytopathic effect was recognizable. The cells were harvested by freezing at -80°C and subsequent thawing. During the

thawing process, the flasks were shaken regularly to completely abrade the cell layer with the pieces of frozen medium. The cell suspensions were transferred to 250 ml ultra centrifugation cups and centrifuged for 90 min at 4°C and 13500 k. The cell pellet was resuspended in a suitable volume of 10 mM Tris buffer pH 9,0 (max. 30 ml for further purification and 6 ml for crude stock preparations), freeze thawed three times and sonicated three times for one minute in ice water. This crude stock virus preparation was either stored at -80°C or further purified.

3.5.4. Virus purification

MVA crude stock preparations were purified from cellular debris and proteins by sucrose-cushion-ultra-centrifugation. To break down the cells and to separate virus particles from the cell walls, crude stock preparations were homogenized by sonication. Cellular debris was pelleted by centrifuging the suspension for 5 min at 4000 rpm and 4°C. The virus containing supernatant was transferred to a new 50 ml Falcon tube and the pellet was resuspended in 25 ml of cold 10 mM Tris buffer (pH 9,0) and again sonicated. The procedure was repeated three times and resulted in a maximum of 80 ml virus suspension. Ultracentrifugation cups were prepared with 25 ml of 36% sucrose in 10 mM Tris buffer and the virus suspension was carefully laid onto the cushion. Virus particles were pelleted by ultracentrifugation for 90 min at 13500 k at 4°C. The supernatant was removed and the pellets resuspended in a maximum volume of 12 ml 1 mM Tris pH 9,0. For the second purification step, ultracentrifugation cups were prepared with 9,5 ml of 36% sucrose in 10 mM Tris buffer and the purified virus suspension was again carefully laid onto the cushion. Virus particles were pelleted by ultracentrifugation for 90 min at 13500 k and 4°C. The supernatant was removed and the virus pellet was re-suspended in 3-6 ml 1 mM Tris pH 9,0 and stored at -80°C.

3.5.5. Virus titration

The infectivity of a MVA suspension was determined by titration and plaque formation frequency. The virus stock was freeze thawed three times and sonicated three times for one min in ice water in a cup sonicator. The virus suspension was serially diluted in RPMI 2% medium to obtain dilutions from 10^{-1} to 10^{-10}. Primary CEF cells in 6-well culture plates were infected in duplicates with the different virus dilutions (1 ml per well). The infected cells were incubated for 48 h at 37°C and 5% atmosphere. Then the medium was removed and cells were fixed by adding

1 ml per well of a 1:1 mix of aceton/methanol for 2 min. Subsequently the culture dishes were air dried. Each well was incubated for 1 h at room temperature with 1ml of PBS containing 3% FKS and anti-VACV antibody (1:1000). Subsequently the cells were washed three times with PBS and incubated for 45 min with an anti-rabbit, peroxidase conjugated secondary antibody 1:1000 diluted in PBS (1ml per well). After washing with PBS, the virus plaques were visualized by the addition of a substrate solution (1ml per well). To prepare the substrate solution, o-dianisidine was diluted to saturation into 100% ethanol. 240 µl of the saturated solution were mixed with 12 ml PBS and 12 µ H_2O_2. During the incubation period (15-45 min) the virus-infected plaques stained red. To determine the titer, the stained plaques of a suitable dilution were counted and the mean value of both wells was calculated. To express the titer in IU/ml, the mean value was divided with the dilution level.

3.5.6. PUVA-induced VACV inactivation

The desired amount of MVA was suspended in PBS and incubated with 10 µg/ml psoralen (4`-aminomethyl-trioxsalen) at room temperature for 10 min in a 6-well plate. Subsequently, the mix was irradiated for 5 min in a Stratalinker 1800 UV crosslinking unit. Then the mixture was used for vaccination of mice.

3.6. Yeast techniques

3.6.1 Cultivation and long-time storage of yeast cells

For the long term storage of yeast cells glycerol-stock were prepared. A single colony was plated thinly on YPDA medium or on suitable SD selective medium and incubated overnight at 30°C. The cells were harvested in 1 ml of YPDA-or SD- medium and mixed with 50% glycerol to a final concentration of 25% and stored at -80°C. To re-cultivate the frozen cells, a small amount of the glycerol-stock was plated on YPDA- or suitable selective SD- medium and incubated for 2-3 days at 30°C until the colonies reached 1-2 mm in size. To re-cultivate the yeast cells in liquid culture, 1 ml of YPDA- or SD-medium was inoculated with one or more colonies and mixed.

3.6.2. Yeast transformation with the LiAc-method

To obtain a high transformation efficiency it is important to transform only yeast cells that are in the logarithmic growth phase because in this phase the cells are able to incorporate several plasmids. Also the quality of the carrier DNA is of importance, because the DNA must have a high molecular weight and must be completely denatured. The competence to incorporate DNA is determined by the lithium-ions and PEG.

3.6.2.1 Method with medium efficiency

This method was used if only one particular plasmid or a small amount of plasmids were transformed into yeast and a high transformation-efficiency was not of importance. Some yeast-colonies were plated thinly on YPDA- or corresponding SD- selective medium and incubated overnight at 30°C. The cells were harvested, resuspended in 500 µl H_2O and subsequently collected by centrifugation (13.000 rpm, 30 sec). The pellet was washed with 1 ml of sterile TE/LiAc and finally resuspended in 750 µl of TE/LiAc. 1µg DNA and 47,5 µg salmon-testes carrier-DNA (denature at 95°C for 5 min before use) were mixed and subsequently 50 µl of competent yeast-cells and 300 µl of PEG/LiAc were added and mixed again. The mix was incubated with continuous shacking at 30°C for 30 min followed by a heat shock at 42 °C for 15

min. Then the cells were harvested by a short centrifugation and resuspended in 50 µl of H_2O. The complete suspension was plated on a selective-dish were only this cells are able to grow that had incorporated the plasmid. The cells were incubated for 3-4 days at 30 °C.

3.6.2.2. Method with high efficiency

One or more yeast-colonies were diluted into 50 ml YPDA- or SD-medium and incubated overnight at 30 °C under shacking. The overnight-culture was then diluted into 300 ml YPDA-medium until the culture reached an OD_{600} of 0,1-0,2 (logarithmic growth phase). Subsequently the cells were harvested by centrifugation (5 min; 1000 x g). The pellet was resuspended in TE-buffer to a final volume of 25-50 ml. The centrifugation step was repeated and the cells were resuspended in 1,5 ml TE/LiAc. 1µg DNA and 95 µg salmon-testes carrier-DNA (denature at 95°C for 5 min before use) were mixed. Subsequently 100 µl of competent yeast-cells and 600 µl of PEG/LiAc were added and incubated with continuous shacking at 30°C for 30 min. Before the heat shock (42 °C; 15 min), 70 µl of DMSO were added to the mix. Subsequently the cells were put on ice for 1-2 min followed by a short centrifugation (30 sec.; 13.000 upm). The pellet was resuspended in 500 µl TE-buffer. The transformed cells were plated on permissive or selective medium and incubated for 3-4 days at 30 °C.

3.6.2.3 Transformation of a cDNA-library

150 ml of a suitable selective SD-medium were inoculated with a yeast-clone that was pre-transformed with the bait and incubated overnight at 30 °C until an OD_{600} of 1,0. The overnight-culture was mixed with YPDA-medium to an OD_{600} of 0,15. The culture was incubated for 3-5 h to a final OD_{600} of 0,4-0,5. Subsequently the cells were harvested by centrifugation (5 min, 1000 g), the pellets were washed in 40 ml sterile H_2O and then resuspended in 5 ml TE/LiAc.
In 50 separate 1,5 ml reaction-tubes 9,8 µg of cDNA and 95 µg salmon testes carrier DNA (denature at 95°C for 5 min before use) were mixed. The following procedure was performed as described in 3.7.2.2. The 50 separate transformation-mixes (500 µl) were plated on –Trp, -Leu, -His (1mM 3-AT) selective-dishes using glass beads. Only cells that had incorporated a pACT cDNA library plasmid and where the cDNA encoded protein interacts with the bait protein should be able to grow. The dishes were incubated for 3 days at 30 °C. After this incubation time a

background of small colonies became visible. The dishes were cleaned with sheets of velvet until the colonies had disappeared and the dishes were incubated for another 3-4 days. After this treatment only some single colonies that have had activated the reporter gene should grow. These colonies were plated on a permissive dish and stored at 4°C.

3.6.3. Amplification of the pACT cDNA library

The cDNA library derived from human lymphocytes contained $1x10^6$ independent cDNAs cloned into the vector pACT. The library was available as an already pre-transformed glycerol stock of *E. .coli* (strain BNN132) with a titer of 1×10^8 cfu/ml. This stock was plated on 60 dishes (13 cm in diameter) containing ampiciline (50 µg/ml) with a density of 20.000 cfu/plate. Under consideration of the titer 9ml of LB-Medium were mixed with 12 µl glycerol stock. On each dish, 150 µl of the mix were seeded using glass beads (1mm in diameter). The dishes were incubated at 30°C for 40 h. The cells were harvested with 5 ml LB-Medium per dish and the bacterial cells were obtained by centrifugation (5000 rpm, 30 min, 4 °C). Subsequently, with the bacterial pellet a high yield plasmid DNA isolation as described in 3.1.4.2. was performed.

3.6.4. Test for activation of reporter genes HIS3 and ADE2

The transformants that were examined for reporter gene activation were plated on a permissive dish (-Leu, -Trp) and incubated overnight at 30°C. Then the clones were replica-plated on –Trp, -Leu, -Ade-dishes or –Trp, -Leu, -His-dishes with varying amounts of the inhibitor 3-AT. The cells were incubated at 30 °C for 4-5 days and monitored for growth.

3.6.5. pACT cDNA library plasmid-isolation from yeast cells

The yeast colonies were resuspended in 500 µl of SD-medium without leucine. The transformed yeast cells contain two plasmids, the bait plasmid and one of the cDNA pACT library plasmids. To isolate only the pACT cDNA plasmid, the cells were grown in 500 µl SD-medium(–Leu) that is selective for the pACT plasmids. The cells were incubated overnight at 30 °C under shacking and harvested by centrifugation (14.000 upm; 5 min). The supernatant was decanted and the pellet was resuspended in the remaining medium (about 50 µl). 20 µl of Zymolyase-solution were added, mixed and incubated for 30-60 min at 37 °C with shacking. Subsequently, 10 µl of 20% SDS were added and the mix was frozen at -20°C. After thawing the solution was mixed again to obtain complete cell lysis. The samples were mixed with TE-buffer to a final volume of 200 µl and the proteins were precipitated by mixing with 200 µl of phenol-chloroform-isoamylalkohol (25:24:1). The upper organic phase contained the plasmid DNA which was precipitated by addition of 8 µl 10 M ammonium-acetate and 500 µl 100 % ethanol at -80 °C for 1 h. Following a centrifugation at 13.000 rpm for 10 min the pellet was washed with 70 % ethanol, dried and resuspended in 20 µl H_2O.

3.6.6. Transformation of yeast plasmids into electro-competent *E. coli* DH10b

To use plasmid DNA isolated from yeast cells for subsequent sequencing reactions, restriction enzyme digests or re-transformations into yeast the plasmids have to be amplified in bacteria first. 5 µl of yeast-plasmid solution were mixed with 25 µl competent bacteria and electro-pulsed. Then 1 ml of LB-Medium was added and the bacteria were incubated 1 h at 37 °C under shacking. Subsequently the bacteria were harvested by centrifugation (2500 upm; 5 min) and the pellet resuspended in a small amount of medium. The bacteria were plated on selective-dishes containing ampicilline (50 µg/ml) and incubated at 37 °C for 24 h.

3.6.7. Preparation of yeast protein extracts for Western blot analysis

Yeast cells were diluted in 5 ml selective SD-medium and grown overnight at 30°C. The overnight culture was diluted in 50 ml YPD-medium and grown under shacking to an OD_{600} of 0,4–0,6. Subsequently the cells were harvested by centrifugation (1000 rpm, 5 min, 4°C) and washed with 50 ml ice-cold H_2O. The resulting pellet was shock frozen in liquid nitrogen. The cells were thawed and resuspended in 340 µl pre– warmed (60°C) cracking buffer. 15 min after thawing, 3,4 µl PMSF were added. This was repeated every 7 min. Glass beads were added and the suspension was heated to 70°C for 10 min. Subsequently, the suspension was vortexed for 1 min and the cellular debris was pelleted by centrifugation (13.000, 5 min, 4°C). The supernatant was stored on ice and the cellular pellet was heated to 100°C for 5 min before a second centrifugation step. The second supernatant was united with the first and 15 µl were mixed with SDS sample buffer, heated to 95°C for 5 min and run on a denaturing SDS gel (see 3.3.1.2.) followed by Western blot analysis (see 3.3.1.3.).

3.7. Immunological Methods

3.7.1. Collection of blood sera

Blood sera of C57BL/6 mice were obtained by scarification with a scalpel at the tail vein of the mice. After 4 h incubation at room temperature and over night incubation at 4°C blood samples were centrifuged for 10 min at 4000 rpm and 4°C. The supernatants were centrifuged again under the same conditions. Subsequently blood sera were stored at -20°C until β-galactosidase specific ELISA was performed as described in 3.7.2.

3.7.2. β-galactosidase specific ELISA

100 µg/µl of β-galactosidase protein were diluted in coating buffer and 100 µl of the solution were applied to each well of a 96 well NUNC-immuno-plate (Maxi Sorb). The plate was coated over night at room temperature. After coating, the plate was washed two times with ELISA-washing buffer. To block non-specific binding, 200 µl of blocking buffer were applied to each well and incubated for 1 h at 37°C followed by four washing steps.
Blood sera were diluted in washing buffer in steps from 1:50 to 1:6400. 100 µl of each dilution were transferred to the wells of the 96 well plate and incubated fro 1 h at 37°C. Subsequently the wells were washed four times with washing buffer and 100 µl of secondary AP-labeled anti-mouse antibody solution (1:1000) were added to each well and incubated for 30 min at 37°C. Then the antibody solution was removed and the wells were washed as described above. During this time a p-nitrophenyl phosphate substrate solution was prepared according to the manufacturers kit instructions and 75 µl of the solution were applied to each well the 96-well plate and incubated for 30 min at 37°C. The enzyme-substrate reaction was stopped by the addition of 0,25 µl stop solution per well and the extinction was measured at a wavelength of 405 nm using a microplate reader.

3.7.3. Preparation of splenocytes

Spleens were removed and homogenized with a syringe plunger over a cell strainer into RPMI 10% medium. After centrifugation of the homogenate (1500 rpm, 5 min) the erythrocytes were lysed with 3 ml TAC buffer (2 min, 37 °C) and washed with 40 ml RPMI 1%. The cells were again filtered over a Nylon filter and counted.

3.7.4. Cell counting

Cells were counted at a 1:40 dilution. 50 µl of cell suspension was mixed with 450 µl PBS. From this dilution 50 µl were mixed with 50 µl of Trypan blue solution (0,4 %) and 100 µl PBS to result in a 1:40 dilution (total). Cells were counted in a Neubauer counting device. Two quadrates were counted and the cell number was calculated using the following formula:

$$N\ (cells/ml) = mean\ of\ two\ quadrates \times dilution\ factor \times 10^4$$

3.7.5. Intracellular Cytokine Staining (ICS)

Peptide stimulation of lymphocytes

For peptide stimulation for each sample 200 µl RPMI 10% containing 4 x 10^6 splenocytes were transferred to flat-bottomed 96-well plates. For each peptide a master mix containing 5 µl vortexed and sonicated peptide (from 1 mg/ml stock) and 5 µl of brefeldin A (1 mg/ml stock) per 1 ml RPMI 10% medium was prepared and thoroughly mixed. 50 µl of this master mix were added to each well and mixed. Cells were incubated with the peptides for 5 hours at 37 °C in a 5% CO_2 atmosphere.

EMA-Staining and Fc-Block

Cells were transferred to a 96 well V-bottom plate, washed and then incubated for 20 min under light with 50 µl Fc block (1:100) and EMA (1:1000) to block Fcγ receptors and stain dead cells. Cells were washed twice with FACS buffer in a total volume of 200 µl (2 min, 1500 rpm).
The anti-CD16/CD32 antibody serves to block cellular Fc receptors. This blockage prevents unspecific signals generated by antibodies that fail to specifically bind their target and are instead bound by the Fc receptors on leucocytes.
EMA staining is used for live/dead discrimination, since this photo-activated molecule can enter only dead or damaged cells that no longer have intact membranes. Upon entering these cells EMA can form stable links to nucleic acids present in the cell. This reaction requires the presence of visible light and is irreversible, allowing specific detection of EMA stained cells by excitation with a 488 nm laser.

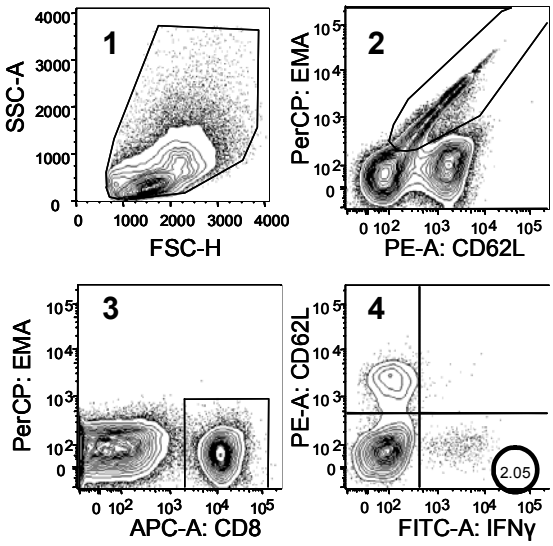
Surface markers and intracellular cytokine staining

After EMA stain and Fc-block, washed cells were stained with 50 µl of the surface markers CD8a and CD62L (30 min in the dark on ice). Following the cells were again washed three times with FACS-buffer. Cells were then treated with 100 µl Cytofix/Cytoperm to permeabilize the cell walls (15 min in the dark on ice): Cells were washed three times with PermWash buffer before they were stained with 50 µl of intracellular antibodies (1:500 dilution of αIFNγ FITC-labeled antibody in 50 µl Perm-Wash buffer (30 min in the dark on ice). Finally, cells were washed again three times, fixed with 1% PFA and stored until used for analysis.

3.7.6. Flow cytometry

Flow cytometry allows for analysis of cells on a single cell level. By aspiration through a fine needle and hydrodynamic focusing cells successively enter a detection channel, where they pass through a set of laser beams. Analysis of cell size, granularity and protein expression is based on the forward light scatter (FSC), the sideward light scatter (SSC) and the emission of light by laser-activated fluorochromes, respectively. The usage of several lasers and different fluorochromes with distinct emission spectra allows for the simultaneous analysis of a variety of different markers. Analysis is possible for proteins expressed at the cell surface, as well as for intracellular proteins after permeabilization and fixation of the cell. The optical readout from

analyzed cells is converted to digital information in a detector system and can be visualized and analyzed using specific software such as FACS-Diva or FloJo. Since emission spectra of some fluorochromes show partial overlaps each experiment contained single colour samples for each used fluorochrome to be able to define cells that are truly positive and to adjust instrument settings in order to substract signal overlaps for each detection channel.



Schematic: FACS-Analysis. A representative gating strategy for ICS samples is depicted.
Analyzed cells were identified by typical FSC/SSC parameters (1), discriminated from dead cells (2: EMA-) and then analyzed for surface (3: CD8+) and expression markers (4: IFNγ+).

3.8. Statistical analysis

All statistical analysis was performed using GraphPad Prism4 Software. If not stated otherwise, results are generally expressed as Mean+_ SEM. Differences between groups were analyzed for statistical significance using two-tailed student t-test.

4. Results

4.1. The C7L gene is required for MVA late protein synthesis

MVA has been generated through attenuation in CEF cells. During this process MVA has lost many genes implicated in the regulation of virus-host interactions, including the host range gene K1L. C7L is among the few conserved open reading frames that were described to play a role for the host range of VACV. Deletion of C7L and K1L from VACV results in non-permissive infection of human cells while loss of only one gene could be compensated by the presence of the other (Oguiura et al., 1993; Perkus et al., 1990). The infectious cycle of VACV lacking both K1L and C7L is blocked at the stage of intermediate gene translation in human HeLa cells (Hsiao et al., 2004). Until now, C7L has only been studied in the context of replication competent VACV. Therefore it was of interest which role C7L would play in the MVA life cycle.
The C7L of replication competent VACV has been characterized to belong to the early expressed class of genes (Oguiura et al., 1993). Western blot analysis of MVA infected BHK cell lysates with a monoclonal antibody recognizing the C7 protein at different time points post infection revealed that during MVA infection the C7L gene is also expressed as an early gene. The C7L gene product was detectable as early as two hours post infection and was also synthesized in the presence of AraC, an inhibitor of VACV DNA replication demonstrating that it is an early gene product (Fig. 2).

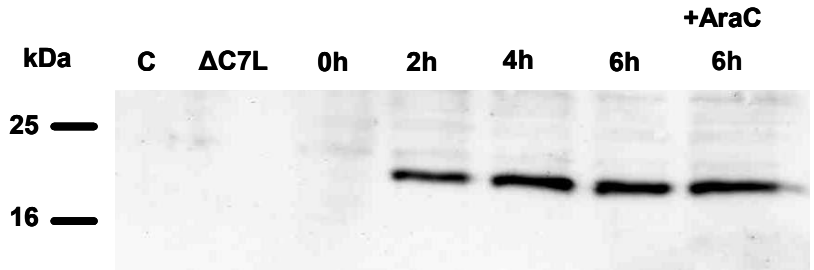


Fig. 2: The C7L gene is expressed early during MVA infection
BHK cells were infected with MVA or MVA-ΔC7L in the presence or absence of AraC. The cells were harvested at the indicated time points and the lysates were separated on a 12% reducing SDS PAGE, transferred on a PVDF-membrane and detected by Western blot analysis with an antibody specific for C7L.

Initial experiments indicated that human and murine cells infected with a MVA-C7L deletion mutant virus (MVA-ΔC7L) display diminished or nearly abolished viral late gene expression in human or murine cells, respectively. For theses experiments, MVA-permissive CEF- and BHK-cells as well as non-permissive human and murine cells were infected with MVA or MVA-ΔC7L and the synthesized viral proteins were labeled at different time points post infection with radioactive ^{35}S-methionine. While there was no difference in the protein expression pattern in CEF- and BHK-cells displayed by both viruses, human HeLa-cells showed only residual late protein synthesis. This observation was even stronger in murine cells (NIH-3T3) infected with MVA-ΔC7L where nearly no late protein synthesis was detectable (Fig. 3).

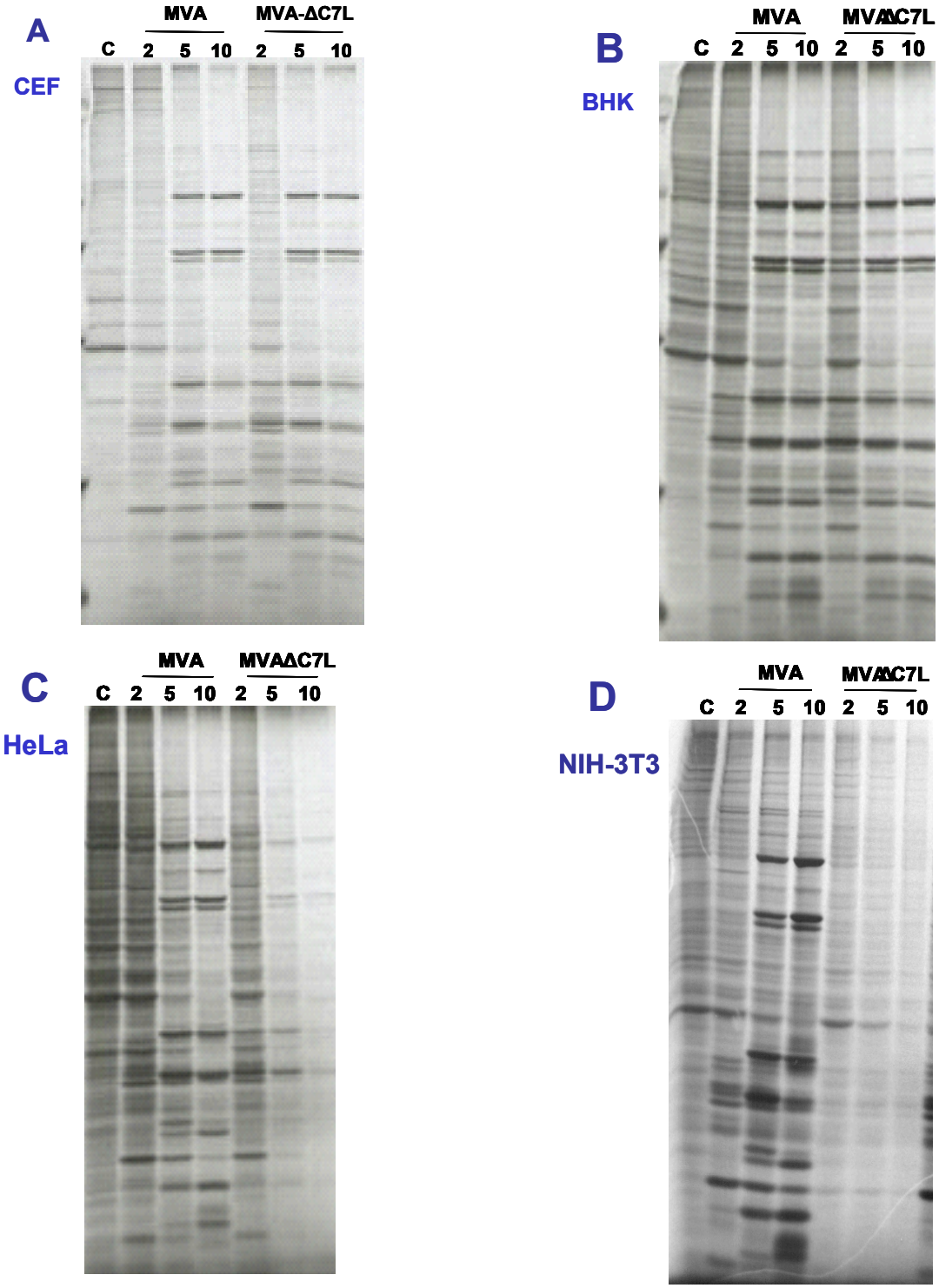


Fig. 3: Analysis of viral protein synthesis of cells infected with MVA or MVA-ΔC7L
CEF- **(A)**, BHK- **(B)**, Hela- **(C)** and NIH-3T3- **(D)** cells were infected with MVA or MVA-ΔC7L at a MOI of 20. At various times after infection, cells were labeled with (^{35}S)-methionine for 30 min. Cells were then harvested and the lysates were resolved by electrophoresis in a SDS-12 % polyacrylamide gel. Labeled proteins were visualized by autoradiography of the dried gel. Numbers above the lanes indicate the time (hours) after infection when proteins were radiolabeled. C indicates uninfected cells.

To confirm this phenotype of the C7L deletion mutant virus and to study it in more detail, a β-galactosidase reporter gene assay was developed. Therefore a recombinant MVA expressing the *E.coli* LacZ gene under the control of the vaccinia viral late promoter P11 (Bertholet et al. 1985) was constructed (MVA-ΔC7L-P11LZ). A corresponding C7L containing virus (MVA-P11LZ) already existed in our laboratory.

To construct MVA-ΔC7L-P11LZ, the MVA transfer plasmid pIIILZ-P11 (Sutter und Moss 1992) was used. pIIILZ-P11 contains two MVA sequences, Del III flank-1 and Del III flank-2, that are identical to the neighboring sequences of the deletion III in the MVA genome (Fig. 4).

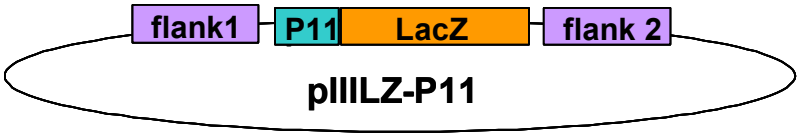


Fig. 4: Simplified illustration of the MVA transfer plasmid pIIILZ-P11
pIIILZ-P11 contains two MVA sequences homologous to the neighboring sequences of deletion III (flank1 + 2) and the *E.coli* LacZ gene under the control of the late vaccinia viral promoter P11.

By intergenomic homologous recombination, the LacZ-gene sequences between the flanks can be stably integrated into the deletion III locus of the MVA genome. Since the transcription of poxviral genes in the cytoplasm of infected cells is controlled by the viral transcription machinery, recombinant genes have to be placed under the control of a VACV promoter. The VACV promoter P11 integrated in the vector plasmid pIIILZ-P11 controls the transcription of the recombinant gene and allows only for late gene expression.

CEF cells were transfected with the MVA transfer plasmid pIIILZ-P11 and simultaneously infected with MVA-ΔC7L to allow for homologous recombination to generate MVA-ΔC7L-P11LZ. Recombinant MVA were isolated through plaque purification by passaging on CEF cells. Plaques formed by recombinant viruses were visualized by addition of the reagent X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). X-gal is an inert chromogenic substrate for β-galactosidase. The β-galactosidase hydrolyzes X-Gal into a colorless galactose and 4-chloro-3-brom-indigo which results in blue staining of cells that are infected with the recombinant virus (Fig. 5).

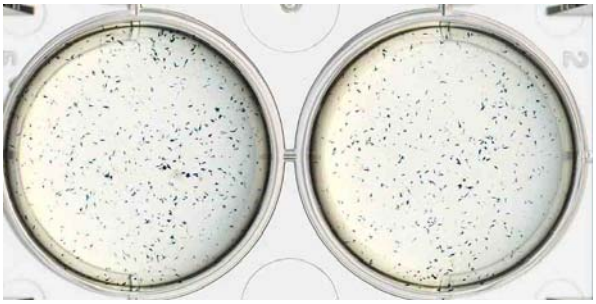


Fig. 5: MVA-ΔC7L-P11LZ infected CEF cells after X-gal staining.
Expression of the LacZ gene allowed the conversion of the colorless sugar-equivalent X-gal into a blue dye. The visualization of blue stained virus plaques allowed isolation of recombinant MVA.

DNA was extracted from selected stained virus plaques and genomes of recombinant viruses were analyzed by PCR with specific primers (NIH-GS83 / IIIf-1B) for the insertion site of the LacZ gene sequence (deletion III). The expected fragment sizes for the amplified DNA fragments were 4764 bp in the case of a correct integration of the LacZ gene sequence and 762 bp without integration of the LacZ gene sequence (empty deletion III). Four virus isolates displayed insertion of LacZ into deletion III (Fig. 6). Furthermore these isolates represented pure recombinant clones because no residual DNA fragments for the empty deletion III were amplified.

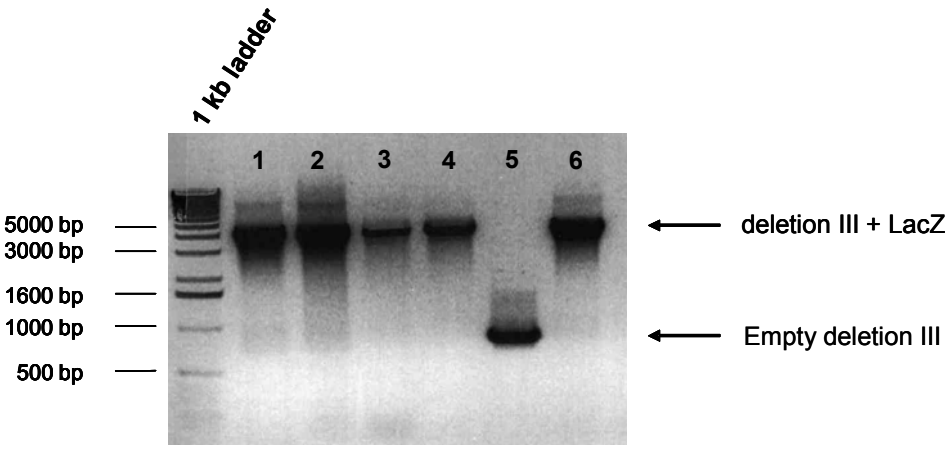


Fig. 6: PCR-analysis of selected plaque isolates
Plaques were isolated from CEF cells and viral DNA was extracted. A PCR specific for deletion III was performed with primers NIH-GS83 / IIIf-1B to analyze the correct insertion of the LacZ gene into MVA-ΔC7L (lanes 1-4). DNA isolated from MVA wt infected cells (lane 5) and the plasmid pIIILZ-P11 (lane 6) served as control templates. 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

Virus clone 4 was chosen for preparation and purification of a viral stock. The resulting stock was titrated on CEF cells in parallel to MVA-P11LZ.

The MVA-P11LZ and MVA-ΔC7L-P11LZ viruses were used to establish a β-galactosidase reporter gene assay. Both viruses contain the LacZ gene under control of the viral late promoter P11 and therefore allow detection and quantification of viral late protein synthesis. The LacZ gene encodes for the β-galactosidase, an enzyme that catalyzes the hydrolysis of β-galactosides including lactose. The β-galactosidase gene functions well as a reporter since the protein product is extremely stable and resistant to proteolytic degradation in cellular lysates. Furthermore the enzyme activity can be easily measured. Cell lysates are incubated with a reaction buffer containing the substrate CPRG (chlorophenol red-β-D-galactopyranoside). The yellow galactoside analog CPRG is converted by the β-galactosidase into galactose and the chromophore chlorophenol red, yielding a dark red solution. β-galactosidase activity of the solution is quantitated using a microplate reader to determine the amount of substrate converted at 570 nm. BHK-21-, HeLa-, MRC-5- , NIH-3T3- and B16 cells were infected with MVA-P11LZ or MVA-ΔC7L-P11LZ and 16 hours post infection the lysates were analyzed for β-galactosidase expression by addition of CPRG. Fig. 7 shows the detected β-galactosidase activity in all examined cell lines. MVA permissive BHK cells displayed no differences in β-galactosidase synthesis of both viruses suggesting that the C7L gene product is not essential for late protein synthesis in this cell line. On the contrary, human HeLa and MRC-5 infected with MVA-ΔC7L-P11LZ showed drastically reduced β-galactosidase activity compared to MVA-P11LZ. This effect was even stronger in murine NIH-3T3 and B16 cells where late protein synthesis of MVA-ΔC7L-P11LZ was reduced nearly to background levels.

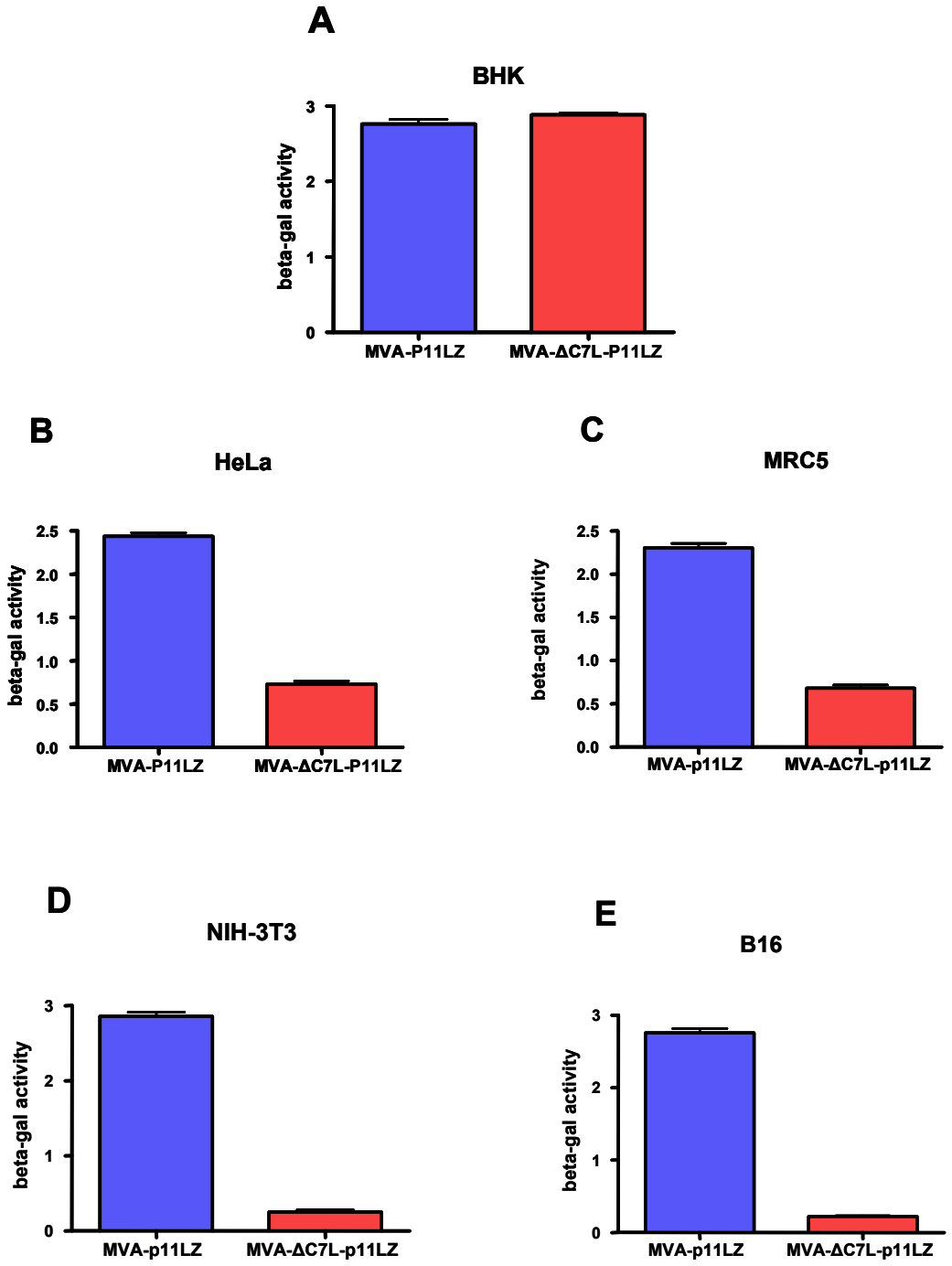


Fig. 7: Viral late gene expression in BHK-21-, HeLa-, MRC-5- NIH-3T3- and B16 cells.
MVA-P11LZ or MVA-ΔC7LP11LZ infected (MOI 10) BHK-21- **(A)**, HeLa- **(B)**, MRC-5- **(C)** NIH-3T3- **(D)** and B16- **(E)** cells were assessed 16 h post infection for β-galactosidase expression. After addition of CPRG the lysates of infected cells were analyzed with a photometer.

The results obtained with this experiment confirm the observations of the previously performed ^{35}S-methionine metabolic labelling experiments suggesting that late viral protein synthesis of MVA in human and murine cell lines requires expression of the VACV host range gene C7L.
To confirm that this conclusion would also apply to primary cells the β-galactosidase reporter gene assay was repeated with primary murine embryo fibroblasts (MEF). Again, in MVA-ΔC7L-P11LZ-infected cells LacZ was not synthesized whereas strong late gene expression was detectable in MVA-P11LZ-infected cells (Fig. 8).

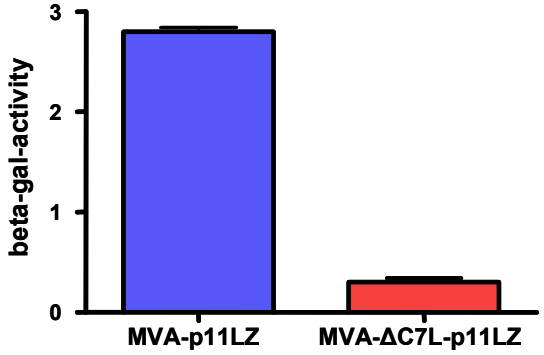


Fig. 8: Viral late gene expression in primary murine embryo fibroblasts (MEF)
MEF cells were infected with MVA-P11LZ or MVA-ΔC7L-P11LZ (MOI 10) and were assayed 16 h post infection for β-galactosidase expression. After addition of CPRG the lysates of infected cells were analyzed with a photometer.

These experiments suggested that also *in vivo* no substantial amounts of late viral proteins should be synthesized upon infection with MVA lacking the C7L gene.

4.2. Viral DNA replication is not impaired in MVA-ΔC7L-infected HeLa cells

VACV gene expression is regulated in a cascade-like manner where late protein synthesis is dependent on previous viral DNA replication. Therefore it was investigated whether the lack of MVA-ΔC7L viral late protein synthesis in HeLa cells was due to an indirect consequence of an earlier blockage of viral DNA replication. To determine accumulation of viral DNA in MVA- and MVA-ΔC7L-infetced BHK and HeLa cells dot-blot assays were performed. For this, viral DNA was isolated at 0h, 2h, 4h and 8h post infection and applied to a nylon membrane, which then was hybridized with a viral DNA probe (H3L open reading frame) labelled with radioactive phosphor (^{32}P). Exposition of the membrane to a phosphorimager plate and quantification of detected DNA revealed accumulation of viral DNA in MVA-ΔC7L-infected BHK and HeLa cells that was even higher than after infection with MVA (Fig. 9), suggesting that the diminished viral late protein synthesis of MVA-ΔC7L in HeLa is not the consequence of a defect in viral DNA replication.

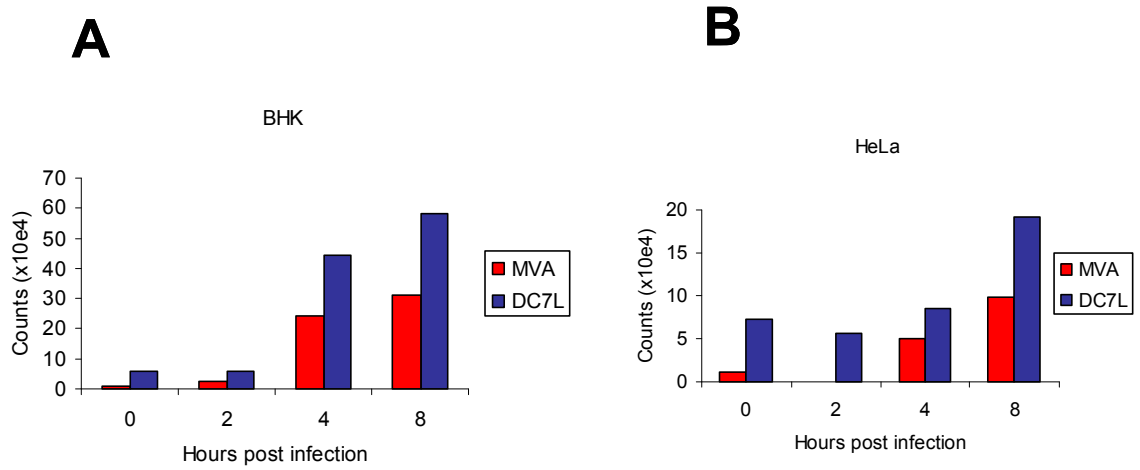


Fig. 9: Viral DNA replication is not impaired in MVA-ΔC7L-infected HeLa cells.
BHK **(A)** or HeLa **(B)** cells were infected with 10 PFU of MVA or MVA-ΔC7L per cell. At the indicated times, cells were harvested, lysed and DNA samples were applied to a nylon membrane using a dot blot manifold. The membrane was hybridized with ^{32}P-labelled MVA DNA and radioactivity was quantitated using a PhosphorImager.

4.3. MVA-ΔC7L does not induce caspase-3 dependent apoptosis in HeLa cells

Host restriction of some orthopoxvirus mutants in certain cell lines has been associated with induction of apotosis in these cells (Turner and Moyer 1998). In the context of theses results, it was of interest if deletion of C7L from MVA would induce early caspase dependent cell death and thus explain the lack of late gene expression. HeLa cells were infected with MVA, MVA-ΔC7L and MVA-ΔE3L and lysates were analyzed at 0 h, 3 h and 6 h post infection by Western blot analysis for the activation of caspase-3. Activation of caspase-3 requires proteolitic processing of its inactive precursor into activated p17 and p12 fragments. The antibody specific for cleaved caspase-3 used for this experiment specifically detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 that is produced upon cleavage. This antibody does not recognize full length caspase-3.

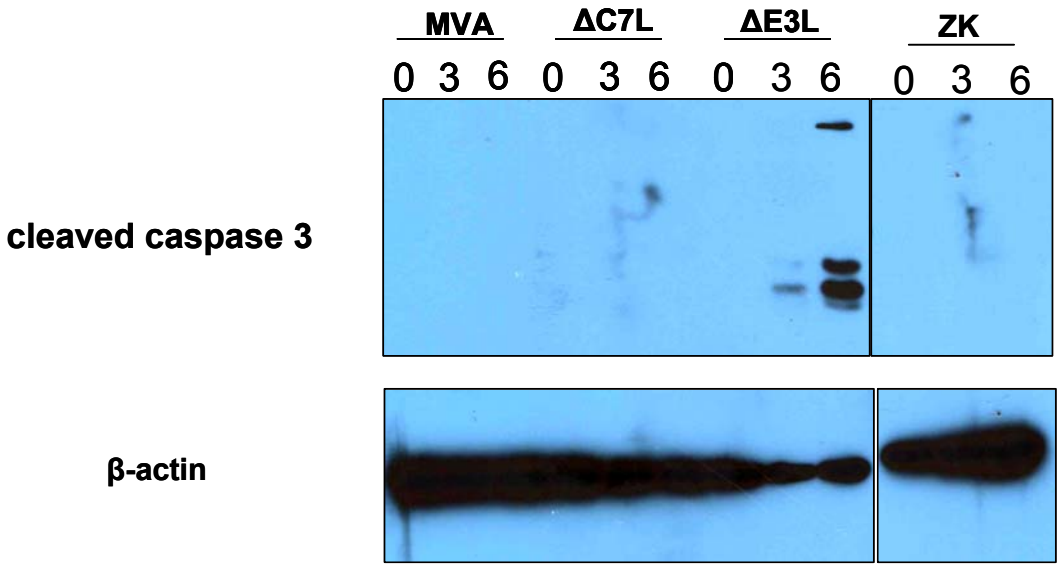


Fig. 10: MVA-ΔC7L infection of HeLa cells does not induce caspase-3 cleavage
Lysates of mock-, MVA-, MVA-ΔC7L- and MVA-ΔE3L-infected HeLa cells (MOI 10) were prepared at 0h, 3h and 6h post infection, separated on a 12% reducing SDS PAGE, transferred on a PVDF-membrane and detected by Western blot analysis with an antibody specific for the large fragment (17/19 kDa) of cleaved caspase-3.

As shown in Fig. 10, Cleaved caspase-3 could not be detected in MVA-ΔC7L, MVA and mock infected cells indicating that MVA-ΔC7L infection of HeLa cells does not induce caspase 3 dependent apoptosis. MVA-ΔE3L-infection, however, serving as positive control, clearly induced cleavage of caspase-3 as early as 3 h post infection which is consistent with previous observations (Ludwig et al., 2006). This result suggests that the aborted late protein synthesis in MVA-ΔC7L infected HeLa cells is not due to apoptosis.

4.4. MVA-ΔC7L does not induce phosphorylation of eIF2α in HeLa cells

The phosphorylation of the α subunit of the eukaryotic translation initiation factor 2 (eIF2 α) by the protein kinase R (PKR) is known to be a cellular major stress response during viral infections that leads to the downregulation of global translation initiation (Rowlands et al., 1988). Thus, the levels of phospho-eIF2α-S51 in HeLa cells infected with MVA and MVA-ΔC7L were determined by Western blot analysis with a specific antibody against eIF2α–S51-phospho recognizing only the phosphorylated form of eIF2α. As shown in Fig. 11, no phosphorylated eIF2α was detectable in mock, MVA and MVA-ΔC7L-infected cells whereas in MVA-ΔE3L-infected cells, that served as positive control, eIF2α was phosphorylated as early as 3 h post infection and remained phosphorylated over the course of infection. The results obtained for MVA and MVA-ΔE3L controls are consistent with previous observations (Ludwig et al., 200; Najera et al., 2006). This experiment suggests that the lack of MVA-ΔC7L late protein synthesis is not induced by a global inhibition of translation initiation.

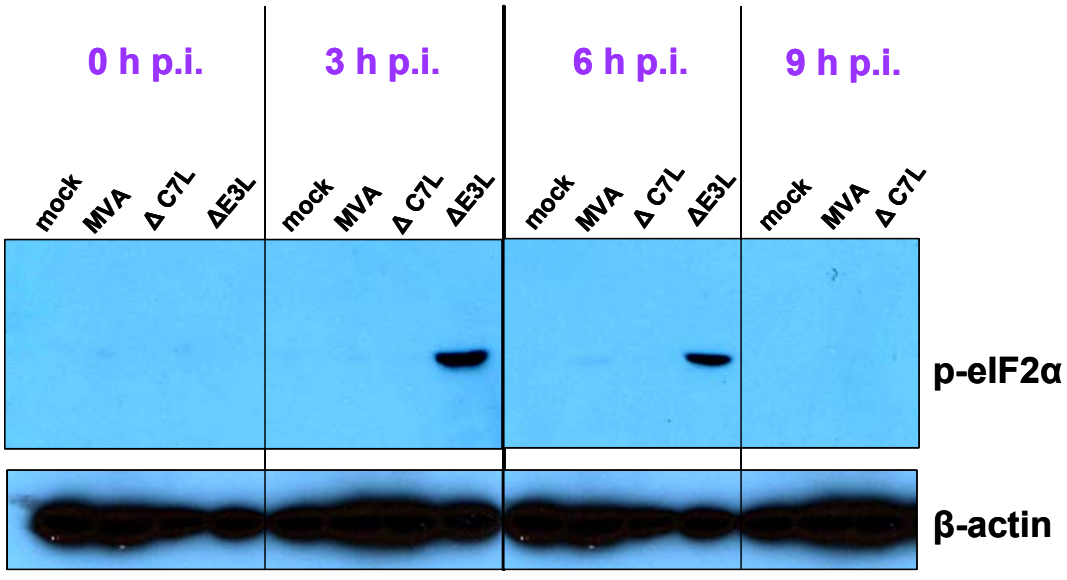


Fig. 11: The α subunit of eIF2 in HeLa cells is not phosphorylated upon MVA-ΔC7L infection
Lysates of mock-, MVA-, MVA-ΔC7L- and MVA-ΔE3L-infected HeLa cells (MOI 10) were prepared at 0h, 3h and 6h post infection, separated on a 12% reducing SDS PAGE, transferred on a PVDF-membrane and detected by Western blot analysis with an antibody specific for the phosphorylated form of eIF2α.

4.5. Determination of essential C7L regions for viral late gene expression

The orthopoxvirus host range genes K1L and CP77 contain ankyrin repeats and for both proteins the ankyrin repeats that are essential to promote viral replication in different cell lines have recently been determined (Meng and Xiang, 2006; Hsiao et al., 2006). C7L is highly conserved among different strains of VACV. On the amino acid level, the C7 proteins of MVA and of replication competent VACV strains display 100% identity. Furthermore, the C7 proteins of various orthopoxviruses exhibit 99% to 95% identity (Fig. 12).

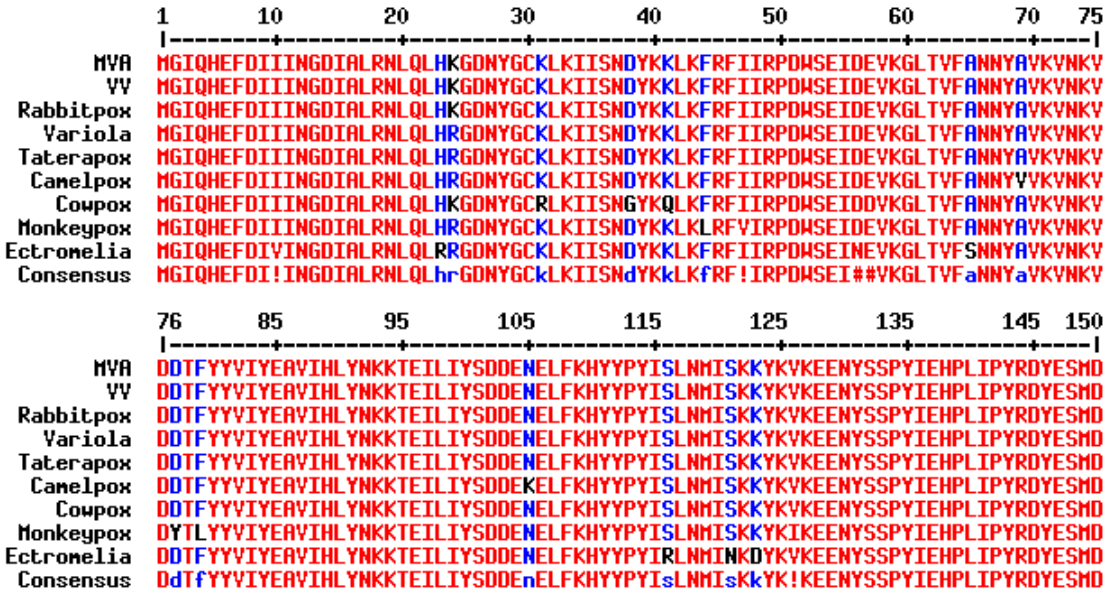


Fig. 12: Alignment of C7L amino acid sequences from various orthopoxviruses
The alignment was created using http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html

However, the C7 protein does not contain any yet described domain or sequence motif as determined by performing a PROSITE domain search. Nevertheless, this domain search revealed six potential phosphorylation- and one potential glycosylation-site (Fig. 13) contained within the C7 protein but these patterns are found frequently in many proteins.

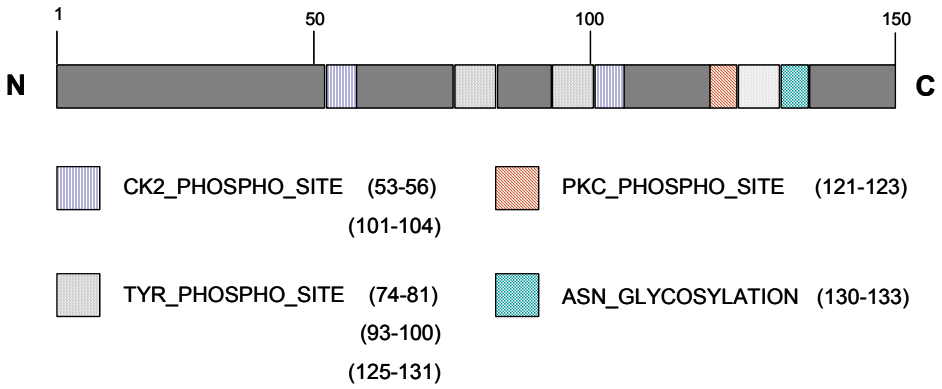


Fig. 13: Potential phosphorylation- and glycosylation-sites of C7L
The potential phosphorylation- and glycosylation-sites of C7L were identified using the PROSITE domain search on www.expasy.ch/prosite/.

To identify essential regions of the C7 protein which are necessary for promoting MVA late protein synthesis in human and murine cells, a transient-transfection assay was developed.

4.5.1. Development of a transient-transfection reporter gene assay for rescue of MVA-ΔC7L late protein synthesis

To circumvent the need for construction of recombinant viruses containing mutated C7L sequences and analysis of viral late gene expression a transient-transfection reporter gene assay was developed. Using this assay, deletion mutant sequences of C7L that are tested for their ability to rescue MVA late gene expression can be expressed from a plasmid template. This transient-transfection reporter gene assay was developed on the basis of the β-galactosidase reporter gene assay (see 4.1.).

The idea behind the transient-transfection reporter gene assay was that MVA-ΔC7L-P11LZ infected cells are transfected with plasmids expressing full-length or mutated C7L sequences. These co-expressed sequences are thought to provide the C7L deletion mutant virus with C7 protein to rescue the viral late gene expression. As the MVA-ΔC7L-P11LZ virus contains the LacZ reporter gene under the control of the viral late promoter P11 the restoration of late protein synthesis can be easily detected.

As a prerequisite it was tested if exogenously expressed C7L in principle could promote the late viral protein synthesis of a C7L deletion mutant virus. Murine NIH-3T3 cells were infected with

MVA-ΔC7LP11LZ at a MOI of 10 and 1h post infection the cells were infected with MVA (MOI 10) to provide C7 protein that could rescue the late gene expression of the deletion mutant. 18 h post infection the cells were monitored for β-galactosidase synthesis.

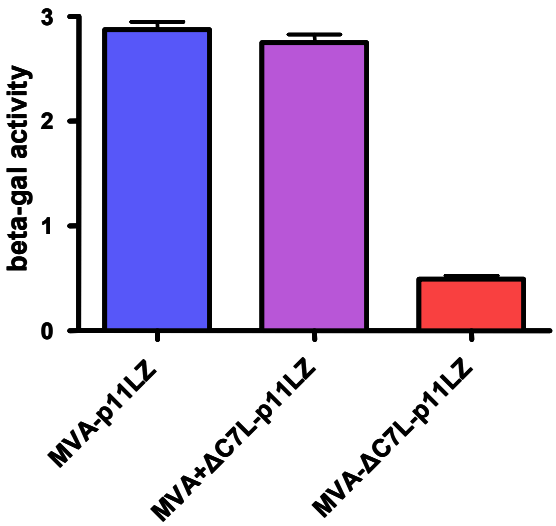


Fig. 14: MVA expressed C7L can rescue late protein synthesis of MVA-ΔC7L-P11LZ
NIH-3T3 cells were infected with MVA-P11LZ (blue), MVA-ΔC7L-P11LZ (red) or superinfected with MVA-ΔC7L-P11LZ and MVA (violet). At 18 h postinfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.

Fig. 14 demonstrates that MVA-infection of MVA-ΔC7L-P11LZ-infected NIH-3T3 cells restores viral late gene expression as indicated by β-galactosidase synthesis that is under the control of a viral late promoter. The β-galactosidase activity displayed by the superinfected cells is comparable to the activity of MVA-P11LZ infected cells. NIH-3T3 that were infected with MVA-ΔC7L-P11LZ alone synthesized only residual levels of β-galactosidase. This result suggests that exogenously expressed C7L in principle is able to rescue the late viral protein synthesis of MVA-ΔC7L-P11LZ to wild type levels.

To test whether late viral gene expression could also be restored by C7L expressed from a plasmid template transfected into MVA-ΔC7L-P11LZ-infected cells. A plasmid was constructed that expresses full length C7L under the control of a viral promoter. To express the C7L coding sequence under the control of the VACV early/late promotor P7.5, the P7.5 promoter was isolated by restriction enzyme digest with NotI and BamHI from the plasmid pIIIΔHRP7.5 (Staib et al.) (Fig. 15 B) and subsequently ligated into the plasmid pRBK1Ldel (Fig. 15 B) that was also digested with NotI and BamHI.

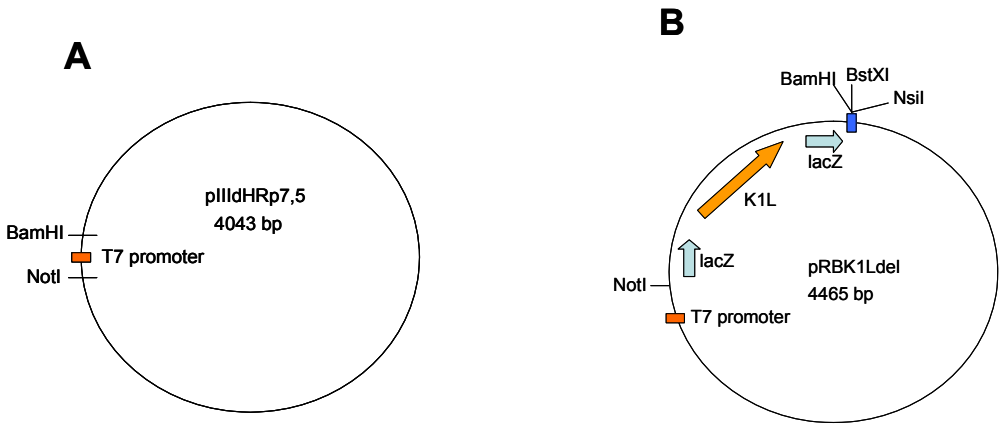


Fig. 15: Map of vector pIIIΔHR (A) and map of vector pRBK1Ldel (B)
The vector pIIIΔHR **(A)** contains the P7.5 promoter. The vector pRBK1Ldel **(B)** contains the K1L cds with LacZ flanks and a T7 promoter.

The digestion of pRBK1Ldel with NotI and BamHI generated two fragments: A 1514 bp fragment containing the K1L coding sequence with flanking sequences derived from the *E.coli* LacZ gene and a 2951 bp fragment containing a T7 promoter, the ampicilin resistance gene and a multiple cloning site for the insertion of the P7.5 promoter and the C7L coding sequence (Fig. 16).

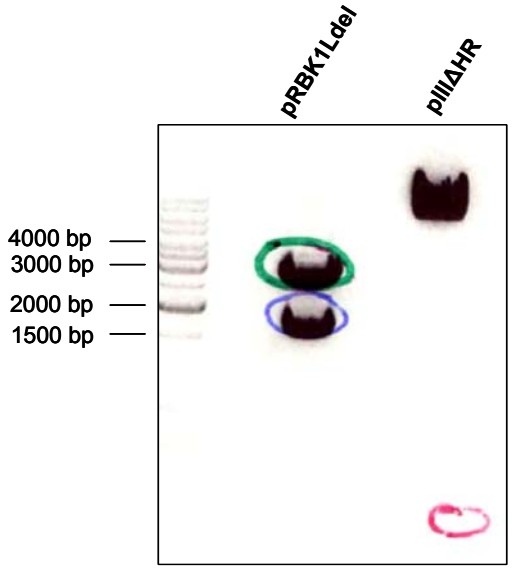


Fig. 16: Isolation of P7.5 promoter from pIIIΔHR by restriction enzyme digest and digest of pRBK1Ldel
The plasmids pRBK1Ldel and pIIIΔHR were digested with NotI and BamHI, separated on a 1% agarosegel and the resulting fragments were visualized by ethidiumbromide staining. The fragment representing the P7.5 promoter is indicated in red and the fragment indicated in blue represents the K1L coding sequence with the lacZ flanks.

The 165 bp restricition fragment corresponding to the fragment containing the P7.5 promoter and the fragment corresponding to pRBK1LdelΔK1Lflanks were isolated by excision and gel-purification and then ligated and transformed into *E.coli* DH10b. The resulting plasmid was called P7.5-empty. Subsequently the full length C7L coding sequence was isolated from a MVA DNA template by PCR-reaction using the primer pair C7L-BstXI / C7L-NsiI that added a BstXI (5`end) and a NsiI (3`end) restriction site to the C7L coding sequence (Fig. 17). The 453 bp-PCR-product was digested with BstXI and NsiI and subsequently ligated into the BstXI/NsiI digested vector P7.5 empty. The resulting plasmid P7.5-C7L expresses the C7L gene under the control of the vaccinia virus early/late P7.5 promoter.

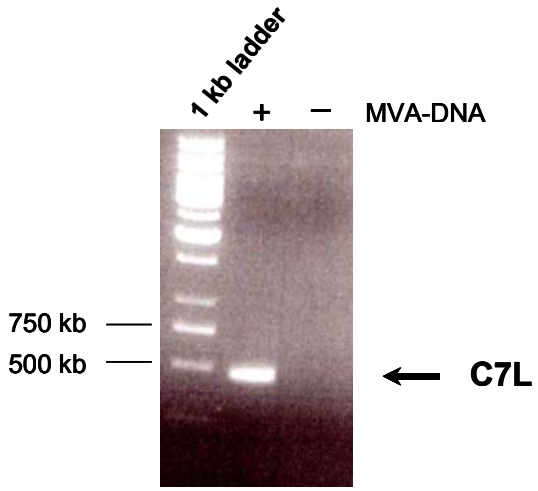


Fig. 17: Isolation of C7L cds from MVA-DNA
The C7L cds was amplified by PCR using specific primers with restriction sites for the enzymes BstXI (forward) and NsiI (reverse). As template served MVA total DNA. 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

The expression of C7L from P7.5-C7L was examined by Western blot analysis. Vaccinia viral promoters are not recognized by the cellular transcriptional machinery as the virus replicates in the cytoplasm and encodes for its own RNA polymerase and transcription factors. Therefore, NIH-3T3 cells were infected with MVA-ΔC7L and 1h post infection transfected with the P7.5-C7L plasmid. 18 h post transfection cell lysates were analysed for C7L expression using an antibody specific for C7L.

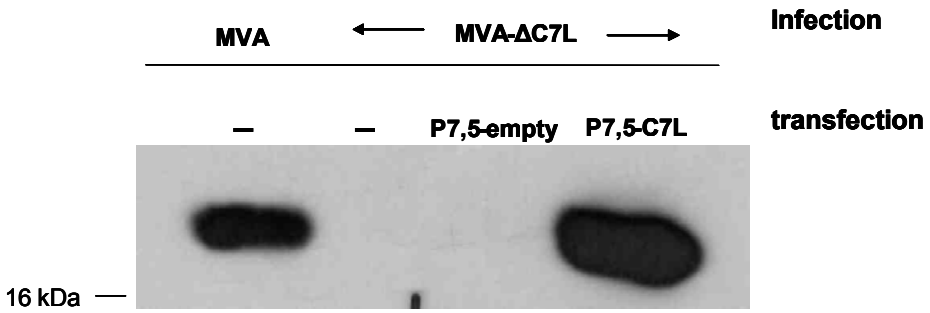


Fig. 18: Expression of C7L from the plasmid P7.5-C7L
NIH-3T3 cells were infected with MVA or MVA-ΔC7L at a MOI of 10 and 1h post infection the MVA-ΔC7L-infected cells were transfected with the plasmid P7.5-C7L or P7.5-empty. The cell-protein-extracts were separated on a reducing SDS PAGE, transferred on a PVDF-membrane and detected by Western blot analysis with an antibody specific for C7L.

As indicated in Fig. 18, the C7L-expression levels that are obtained with the P7.5-C7L plasmid construct are similar to those obtained during MVA-infection. Subsequently, the construct was tested for its ability to rescue late protein expression of MVA-ΔC7L-P11LZ.
Subconfluent monolayers of human HeLa cells were infected with either MVA-P11LZ or MVA-ΔC7L-P11LZ (MOI=10) and 1 h post infection the cells were transfected with the plasmid P7.5-C7L. The analysis of β-galactosidase synthesis 18 h post infection demonstrates that C7L expression from a plasmid template transfected into MVA-ΔC7L infected cells is able to rescue late gene expression of the deletion mutant nearly to wild type level (Fig. 19).

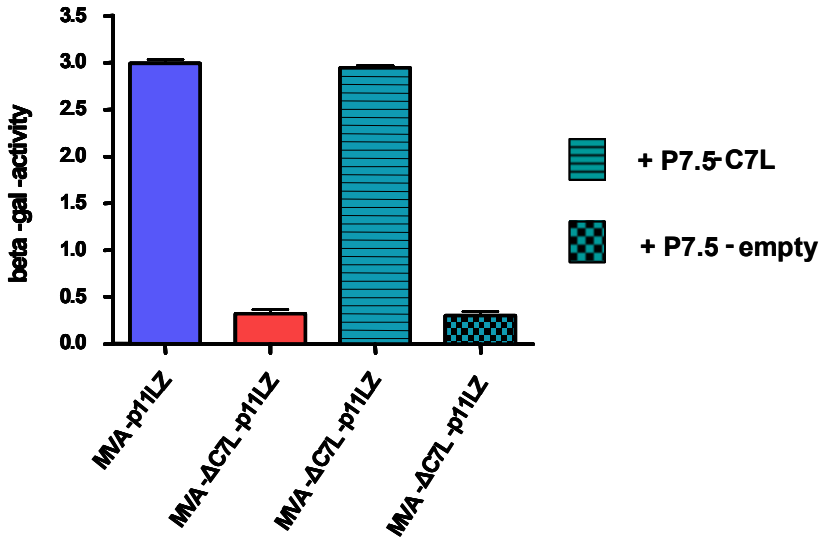


Fig. 19: Rescue of MVA-ΔC7L-P11LZ late protein synthesis in HeLa cells by transiently expressed C7L
Subconfluent monolayers of HeLa cells were infected with MVA-P11LZ (blue) or MVA-ΔC7L-P11LZ (red). MVA-ΔC7L-P11LZ infected cells were transfected 1h p.I. with P7.5-empty or P7.5-C7L as indicated or were left untransfected. At 18 h post transfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.

4.5.2. Role of C-terminus and N-terminus of C7L for viral late gene expression

As mentioned above, the C7 protein is highly conserved among different members of orthopoxviruses (Fig. 12) but does not contain any yet identified sequence motif. Therefore it was not possible to predict the region of the protein that could be necessary for promoting late viral gene expression. For this, defined parts of the C- and N- terminus of the protein were deleted and their ability to restore late gene expression of MVA-ΔC7L-P11LZ was determined using the transient transfection assay.

4.5.2.1. Construction of plasmids containing C7L- C-terminal or N- terminal deletion mutant sequences

To construct plasmids that express C7L C-terminal or N-terminal deletion mutant sequences specific primers were constructed to isolate mutant sequences from a MVA DNA template by PCR reaction. The primer pairs, the size of the corresponding PCR products and the names of the resulting plasmids are listed in tables 17 and 18. The obtained ethidiumbromide stained PCR products are shown in Fig. 20.

Table 17: Primers used for isolation of C7L C-terminal deletion mutant sequences and the resulting plasmids

Forward primer	Reverse primer	Size PCR product	Resulting plasmid
C7L-BstXI	C7murev32	353 bp	ΔC32
C7L-BstXI	C7murev16	403 bp	ΔC16
C7L-BstXI	C7murev12	417 bp	ΔC12
C7L-BstXI	C7murev10	423 bp	ΔC10
C7L-BstXI	C7murev9	426 bp	ΔC9
C7L-BstXI	C7murev8	429 bp	ΔC8
C7L-BstXI	C7murev7	432 bp	ΔC7

Table 18: Primers used for isolation of C7L N-terminal deletion mutant sequences and the resulting plasmids

Forward primer	Reverse primer	Size PCR product	Resulting plasmid
C7muforw32	C7L-NsiI	353 bp	ΔN32
C7muforw16	C7L-NsiI	403 bp	ΔN16
C7muforw8	C7L-NsiI	429 bp	ΔN8
C7muforw4	C7L-NsiI	441 bp	ΔN4

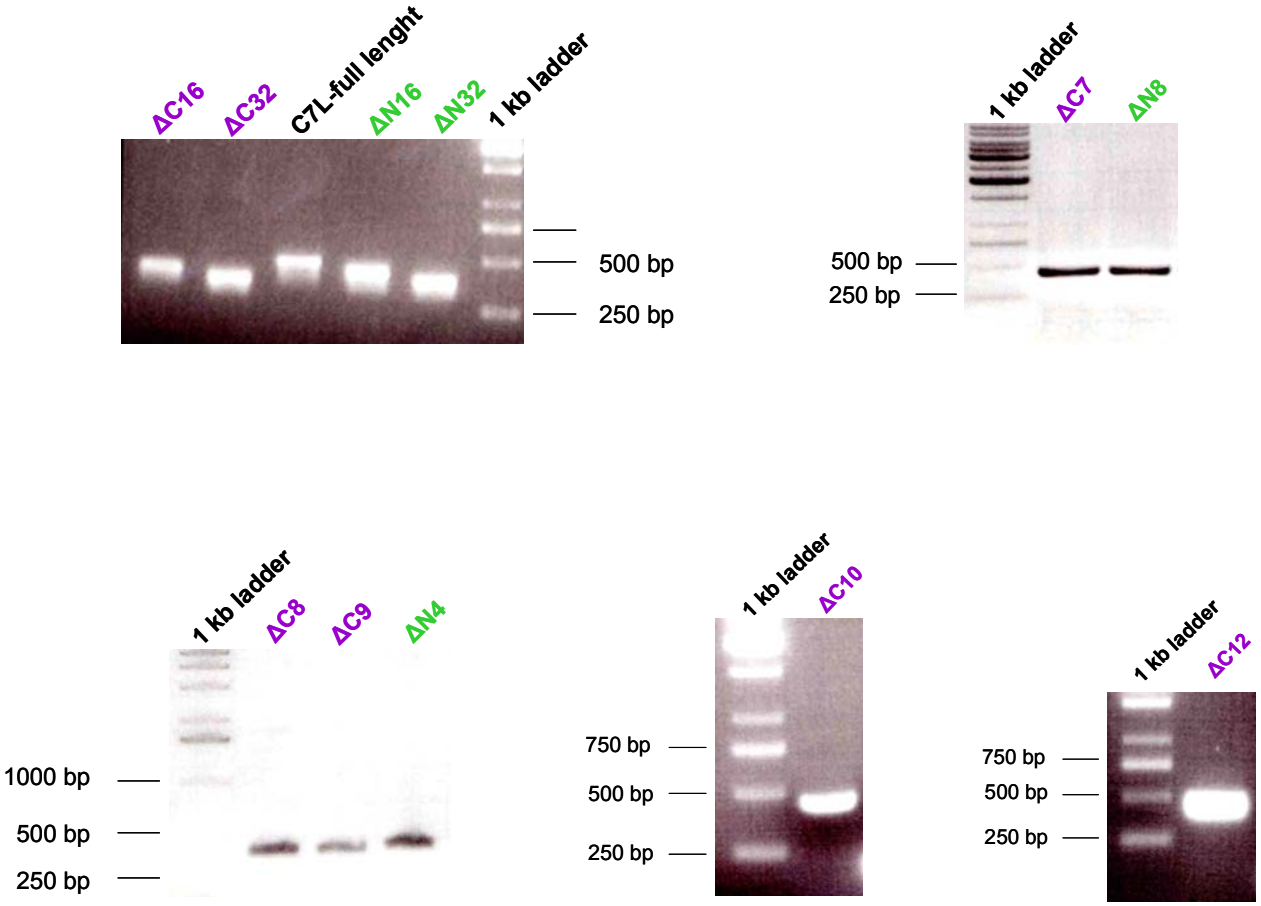


Fig. 20: Isolation of C7L C- and N-terminal deletion mutants from MVA-DNA
The C7L C- (violet) and N- (green) terminal deletion mutant sequences were amplified by PCR using specific primers with restriction sites for the restriction enzymes BstXI (forward) and NsiI (reverse). As template served MVA total DNA. 10 µl of the 100 µl total PCR reactions were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

All forward primers added a 5` BstXI- and a 3`NsiI- restriction site to the mutant sequences. The PCR products were digested with BstXI and NsiI and ligated into BstXI / NsiI digested P7.5-empty. The resulting plasmids expressed the C7L mutant sequences under the control of the viral early/late promoter P7.5. Alignments of the resulting C- and N-terminal C7L amino acid sequences are shown in Fig 21.

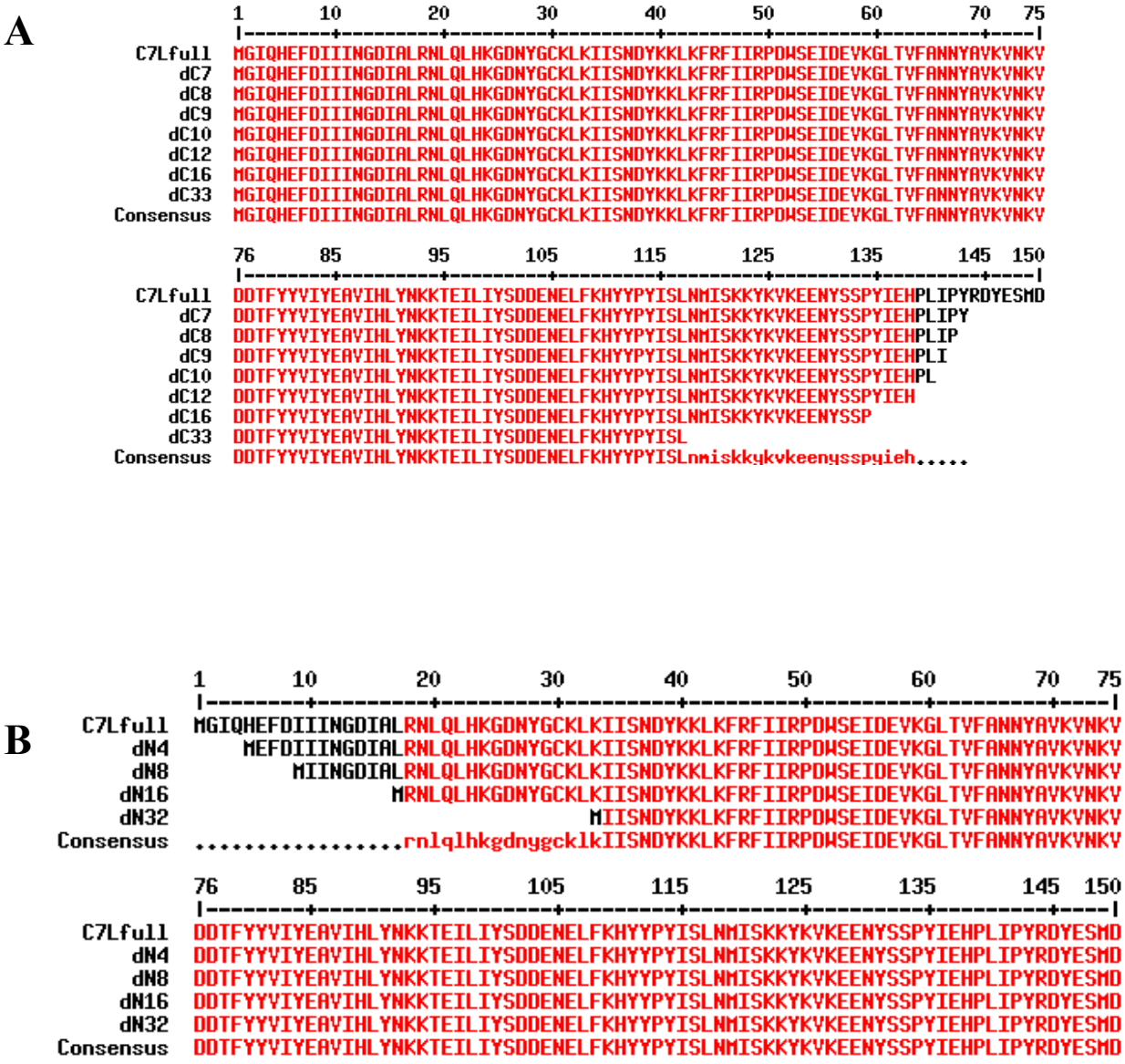


Fig. 21: Amino acid sequences encoded by P7.5-C7L and deletion mutant constructs
Alignments of C7L C- **(A)** and N- **(B)** terminal deletion mutant sequences. The alignments were created using http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html

4.5.2.2. The N-terminus of C7L is necessary for promoting late gene expression

The different plasmids encoding C7L N-terminal deletion sequences were used in the transient transfection assay to analyze if the N-terminal part of the C7 protein is necessary for the late gene expression of MVA-ΔC7L-P11LZ.
Human HeLa and murine NIH-3T3 cells were infected with either MVA-P11LZ or MVA-ΔC7L-P11LZ (MOI 10) and 1 h post infection transfected with deletion mutant constructs ΔC4, ΔC8, ΔC16 and ΔC32. Synthesis of β-galactosidase was analyzed 18 h post infection.

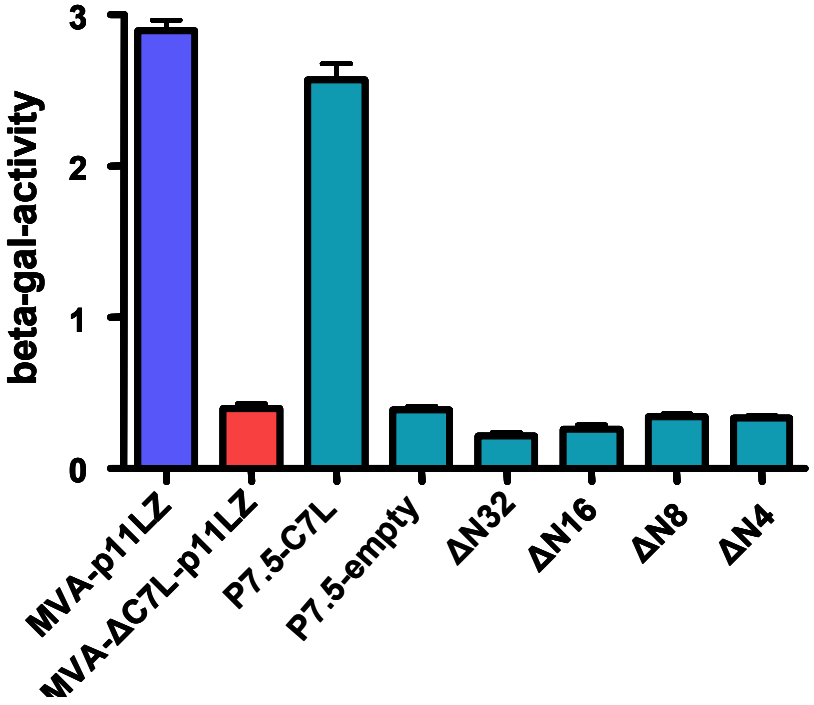


Fig. 22: Rescue of viral late protein synthesis by transiently expressed N-terminal mutants of C7L in MVA-ΔC7L-P11LZ infected NIH-3T3 cells
Subconfluent monolayers of NIH-3T3 cells were infected with MVA-P11-LacZ (blue) or MVA-ΔC7L-P11L (red) and 1 h p.I. transfected with P7.5-empty, P7.5-C7L**,** ΔN4, ΔN8, ΔN16 or ΔN32 as indicated (green). At 18 h posttransfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.

Full length C7L rescued late protein synthesis of MVA-ΔC7L-P11LZ nearly to wildtype level whereas neither of the N-terminal deletion mutant constructs was able to produce any effect indicating that the whole N-terminus of the C7 protein is required to promote viral late protein expression in human and murine cells (Fig. 22). Experiments performed with HeLa cells yielded the same result.

4.5.2.3. Amino acids 1-138 of C7L are essential to promote C7 late viral gene expression in human and murine cells

The results obtained with the N-terminal deletion mutant constructs showed that the N-terminus of C7L is important for allowing late protein synthesis of MVA-ΔC7L-P11LZ. To analyze if the C-terminus is dispensable for this function, the same assay was also performed with the C-terminal deletion mutant constructs (ΔC7, ΔC8, ΔC9, ΔC10, ΔC12, ΔC16 and ΔC32) in HeLa and NIH-3T3 cells.

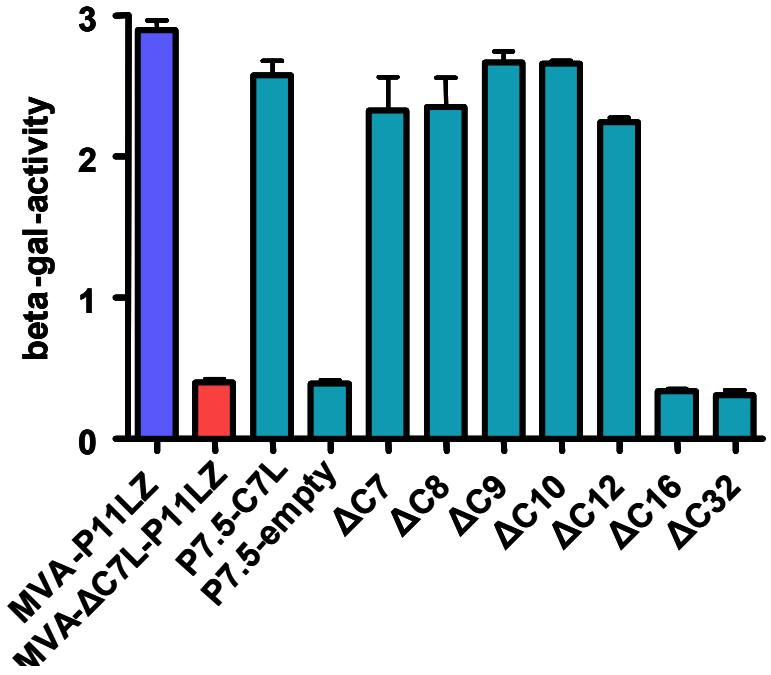


Fig. 23: Rescue of viral late protein synthesis by transiently expressed C-terminal mutants of C7L in MVA-ΔC7L-P11LZ infected NIH-3T3 cells
Subconfluent monolayers of NIH-3T3 cells were infected with MVA-P11-LacZ (blue) or MVA-ΔC7L-P11L (red) and 1 h p.I. transfected with P7.5-empty, P7.5-C7L**,** ΔC7, ΔC8, ΔC9, ΔC10, ΔC12, ΔC16 or ΔC32 as indicated (green). At 18 h posttransfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.

Transfection of MVA-ΔC7L-P11LZ-infected NIH-3T3 with the deletion mutant constructs ΔC7, ΔC8, ΔC9, ΔC10 and Δ12 rescued the late protein synthesis of the deletion mutant virus. Deletion constructs ΔC16 and ΔC32 were not sufficient to promote late expression (Fig. 23). Infection and transfections of HeLa cells yielded the same results suggesting that the C-terminal 12 amino acids are dispensable for promoting late protein synthesis. Deletion of 16 amino acids from the C-terminus abrogates β-galactosidase activity. It seems that only a small part of the C-terminus of C7L is dispensable to still allow late protein synthesis.

4.5.2.4. Expression of C7L deletion constructs

All deletion mutant constructs with exception of ΔC7, ΔC8, ΔC9, ΔC10 and ΔC12 were unable to rescue late protein synthesis of MVA-ΔC7L-P11LZ. Therefore the expression levels of the different mutants were determined to analyze if they were indeed unable to promote late gene expression or if they were not or not to a sufficient level expressed in the transfected cells.
To verify the expression of the deletion mutant constructs, BHK cells were infected with MVA-ΔC7L-P11LZ, transfected 1 hour post infection and harvested after 18 hours. The cell extracts were separated on a 12 % denaturating, reducing SDS-polyacrylamidegel and the C7L-deletion mutant proteins were detected by Western blot analysis using an antibody specific for C7L.

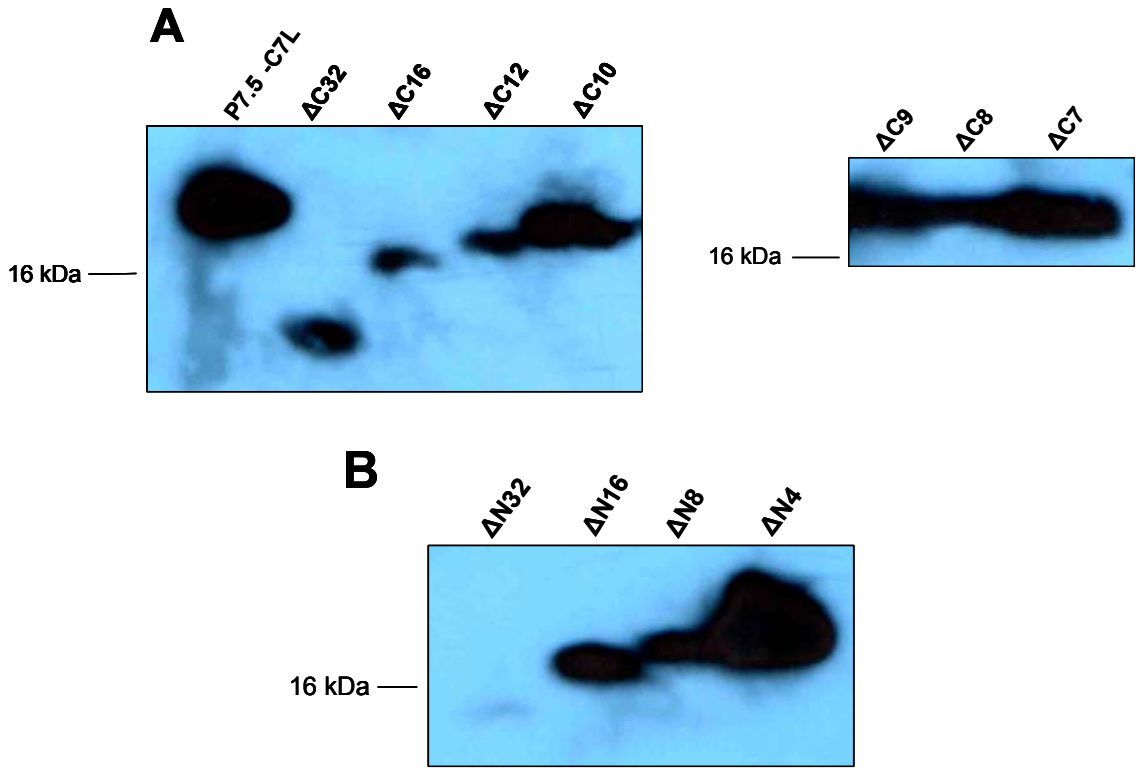


Fig. 24: Expression of C7L-N- and C-terminal deletion mutant constructs in BHK cells
BHK cells were infected with MVA-ΔC7L at a MOI of 10 and transfected 60 min post infection with the indicated C- **(A)** and N- **(B)** terminal P7.5-C7L-deletion mutant plasmids. The protein extracts were separated on a reducing 12% SDS PAGE, transferred on a PVDF-membrane and detected by Western blot analysis with an antibody specific for C7L. As control served a protein extract from MVA-ΔC7L infected and P7.5-C7L transfected cells.

The expression of all C7L C- and N- terminal deletion mutant constructs could be demonstrated (Fig. 24) although ΔN32 is expressed only to a very low level. The expression levels of all the mutants with exception of ΔN4 are lower than the expression strength of full length C7L. Nevertheless, the amount of synthesized protein of the mutants ΔC7, ΔC8, ΔC9, ΔC10 and ΔC12 seems to be sufficient to carry out the function of rescuing late protein synthesis. It remains unclear if ΔN32 is unable to rescue the late gene expression of MVA-ΔC7L-P11LZ due to the absent expression of the construct. However, expression of ΔN4, ΔN8 and ΔC16 could be detected although these mutants did not promote late β-galactosidase synthesis. The results suggest that ΔC32, ΔC16, ΔN32, ΔN16 and ΔN4 do not rescue the function of C7L which is not due to absent expression of the constructs. In table 19 the results obtained concerning rescue and expression of the different constructs are summarized.

Table 19: Expression and rescue of deletion mutant constructs

plasmid	rescue	expression
ΔC32	No	+
ΔC16	No	+
ΔC12	+	+
ΔC10	+	+
ΔC9	+	+
ΔC8	+	+
ΔC7	+	+
ΔN32	No	Very low
ΔN16	No	+
ΔN8	No	+
ΔN4	No	+

4.5.3. The role of the central region of C7L for late gene expression

The results obtained with the C- and N- terminal deletion mutant constructs indicated that the whole N-terminus as well as nearly the complete C-terminus of C7L are important to promote late viral protein synthesis. Therefore it was of interest, if only the terminal regions are necessary and the central region of the protein would be dispensable. Therefore plasmids expressing mutants of C7L containing deletions in the central region of the protein were constructed and analyzed with the transient transfection assay.

4.5.3.1. Construction of C7L mutants with deletions in the central region

For the construction of C7L mutants with deletions in the central region new primers were designed that allowed the isolation of the mutant sequences by PCR reactions. Three constructs with deletions of the first CK2-phosphorylation site (amino acids 53-56), the ASN-glycosilation site (amino acids 130-133) and a small part without any predicted function (amino acids 59+60) were generated. Table 20 shows the primers used for each construct and the resulting products.

Table 20: Primers used for isolation of C7L mutant sequences with deletions in the central region and the resulting plasmids

Primer PCR A	Primer PCR B	Primer PCR C	Resulting plasmid
C7L-BstXI + C7muint2	C7L-NsiI + C7muint1	PCR-product A + PCR-product B	Δint
C7L-BstXI + C7muglyc2	C7L-NsiI + C7muglyc1	PCR-product A + PCR-product B	ΔASN
C7L-BstXI + C7mupho2	C7L-NsiI + C7mupho1	PCR-product A + PCR-product B	ΔCK2

The primers C7muint1, C7muglyc1, C7mupho1 used in PCR B contained the desired deletion. Each of these primers is 40 nucleotides in length, 20 nucleotides before and 20 nucleotides behind the desired mutation. The primers C7muint2, C7muglyc2, C7mupho2 used in PCR A are 20 nucleotides in length and complementary to the 5` part of the 40-mers
(Fig. 25, representative example for construction of ΔASN).

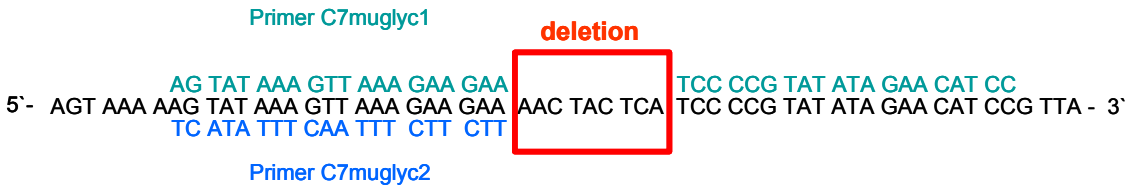


Fig. 25: Schematic example of primers constructed to introduce central deletions into C7L coding sequence
Primer C7muglyc1 (green) is a 40 mer and contains the desired deletion (red). Primer C7muglyc2 (blue) is a 20 mer and complementary to the 5`part of C7muglyc1.

In Fig. 26 the PCR strategy is shown that was used for the construction of the central deletion mutants . In PCR A the 5` part of the desired mutation is amplified whereas in PCR B the part of C7L is amplified that is intended to contain the deletion. For each construct, PCR A and PCR B were performed with MVA DNA as template. Subsequently the PCR products were separated on a 1% agarose gel (Fig. 27) and the fragments corresponding to the desired PCR products were excised and purified. Both PCR products contain an overlapping region. Therefore both PCR products are used in a further PCR as "primers" without any additional template (PCR C) because they hybridise and are subsequently elongated.

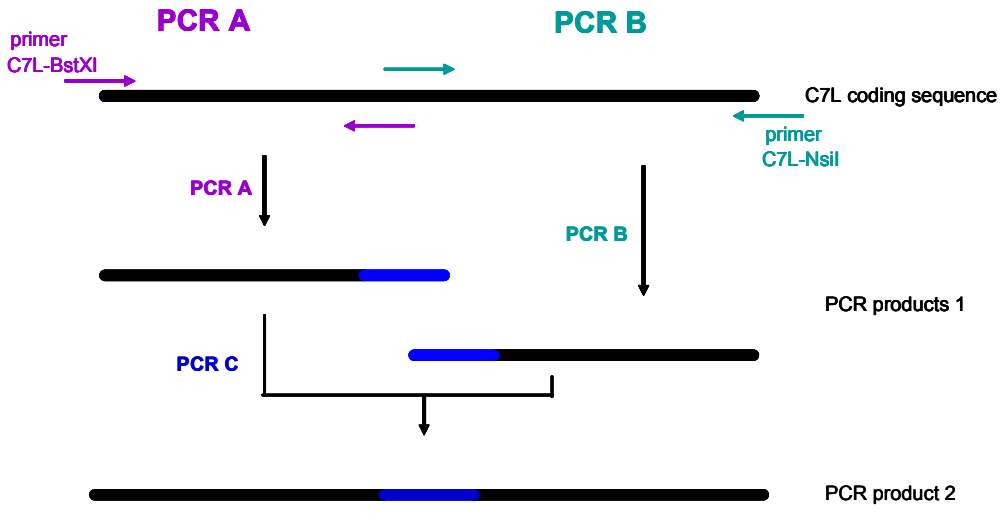


Fig. 26: PCR strategy for the construction of C7L central deletion mutants
PCR A and B are performed with specific primers listed in table and MVA DNA as template. Performing PCR A the 5` part of the deletion is amplified and with PCR B the 3`part that contains the desired deletion. Both PCR products contain overlapping sequences that hybridise and are elongated in PCR C. PCR C is performed without any additional template and primers. PCR product 2 contains the desired deletion.

The resulting PCR products containing the desired mutations were isolated, digested with BstXI / NsiI and ligated into BstXI/NsiI digested empty MVA transfer plasmid P7.5. Alignments of the resulting C7L amino acid sequences are shown in Fig. 28.

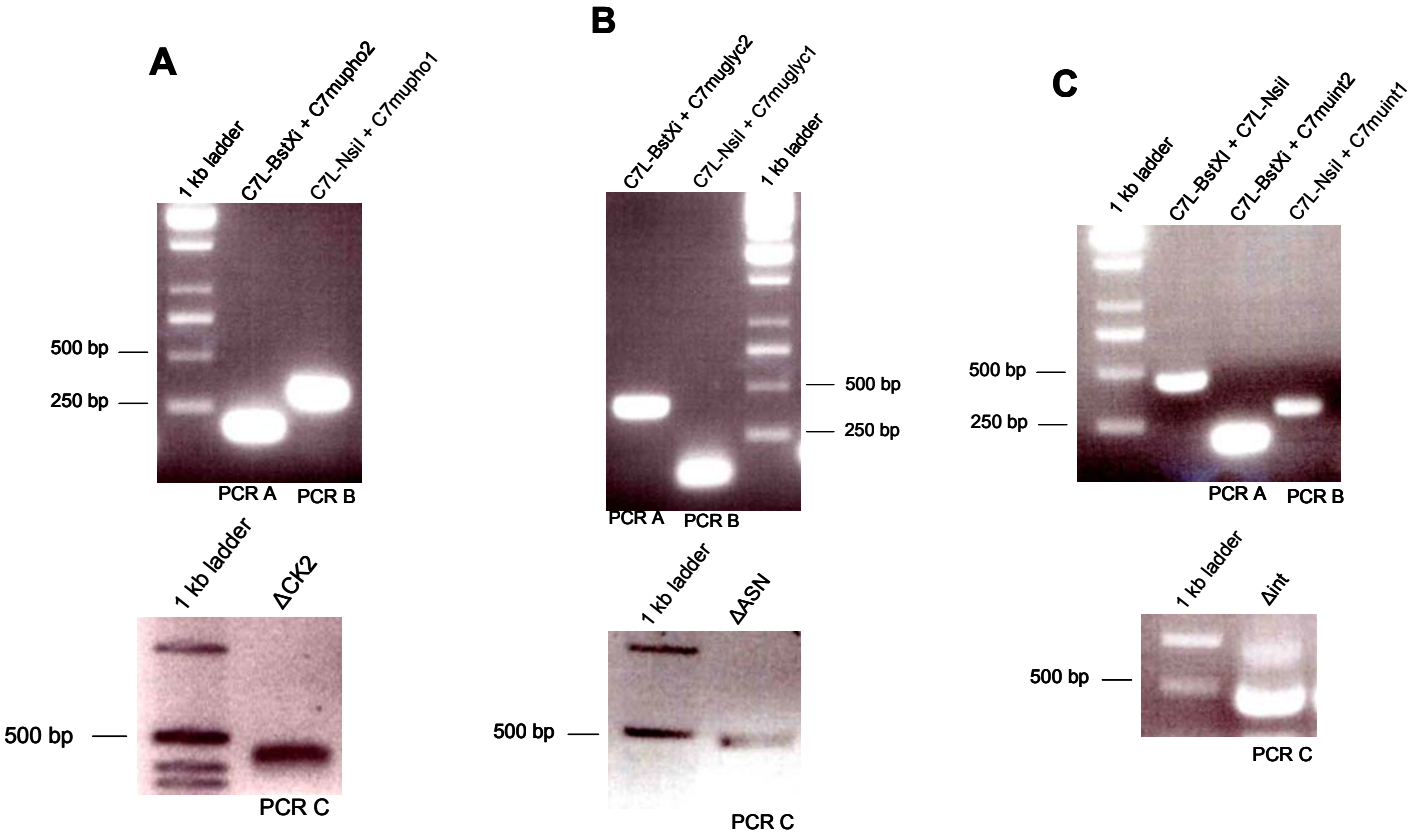


Fig. 27: Isolation of C7L central deletion mutants from MVA-DNA
The C7L mutants with deletions in the CK2-phospho site **(A)**, the ASN-glycosylation site **(B)** and an undefined site **(C)** were amplified by PCR using specific primers as indicated. As template served MVA total DNA. The total PCR reactions (100 µl) were separated by a 1% agarose gel and visualized by ethidiumbromide staining (PCR A +B). The PCR products obtained with PCRs A and B were used as primer / template for PCR C.

```
                1       10        20        30        40        50        60        70   75
                |-------+---------+---------+---------+---------+---------+---------+----|
C7Lfull         MGIQHEFDIIINGDIALRNLQLHKGDNYGCKLKIISNDYKKLKFRFIIRPDWSEIDEVKGLTVFANNYAVKVNKV
d59+60          MGIQHEFDIIINGDIALRNLQLHKGDNYGCKLKIISNDYKKLKFRFIIRPDWSEIDEV--LTVFANNYAVKVNKV
d130-132        MGIQHEFDIIINGDIALRNLQLHKGDNYGCKLKIISNDYKKLKFRFIIRPDWSEIDEVKGLTVFANNYAVKVNKV
d53-56          MGIQHEFDIIINGDIALRNLQLHKGDNYGCKLKIISNDYKKLKFRFIIRPDW----EVKGLTVFANNYAVKVNKV
Consensus       MGIQHEFDIIINGDIALRNLQLHKGDNYGCKLKIISNDYKKLKFRFIIRPDWseidEVkgLTVFANNYAVKVNKV

                76      85        95        105       115       125       135       145  150
                |-------+---------+---------+---------+---------+---------+---------+----|
C7Lfull         DDTFYYVIYEAVIHLYNKKTEILIYSDDENELFKHYYPYISLNMISKKYKVKEENYSSPYIEHPLIPYRDYESMD
d59+60          DDTFYYVIYEAVIHLYNKKTEILIYSDDENELFKHYYPYISLNMISKKYKVKEENYSSPYIEHPLIPYRDYESMD
d130-132        DDTFYYVIYEAVIHLYNKKTEILIYSDDENELFKHYYPYISLNMISKKYKVKEE---SPYIEHPLIPYRDYESMD
d53-56          DDTFYYVIYEAVIHLYNKKTEILIYSDDENELFKHYYPYISLNMISKKYKVKEENYSSPYIEHPLIPYRDYESMD
Consensus       DDTFYYVIYEAVIHLYNKKTEILIYSDDENELFKHYYPYISLNMISKKYKVKEEngsSPYIEHPLIPYRDYESMD
```

Fig. 28: Amino acid sequences encoded by P7.5-C7L and central deletion mutant constructs
Alignment of C7L mutant sequences with deletions in the central region. The alignment was created using http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html

4.5.3.2. The ASN-glycosylation site, the first CK2-phosphorylation site and amino acids 59+60 are necessary for promoting late gene expression

The above constructed plasmids encoding the C7L deletion sequences were analyzed as described for the C- and N-terminal mutants in the transient transfection assay using HeLa and HIH-3T3 cells. Data obtained from transfection/infection experiments in NIH-3T3 cells (Fig. 29) suggest that the constructed mutants with deletions in the first CK2-phospho-site, the ASN-glycosylation site or two undefined amino acids disrupt the ability to promote late gene expression of MVA-ΔC7L-P11LZ. Infection and transfections of HeLa cells yielded the same results.

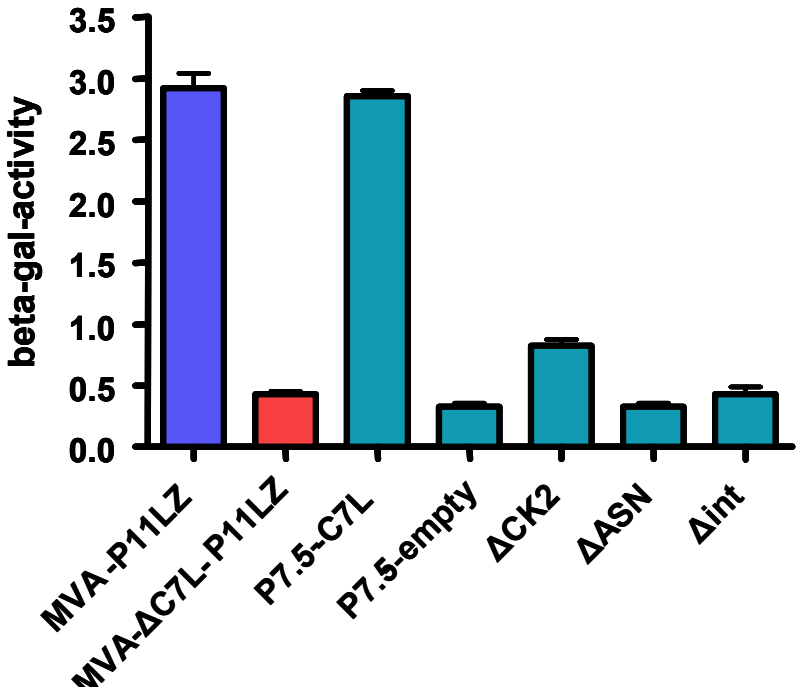


Fig. 29: Rescue of viral late protein synthesis by transiently expressed central deletion mutants of C7L in MVA-ΔC7L-P11LZ infected NIH-3T3 cells
Subconfluent monolayers of NIH-3T3 cells were infected with MVA-p11-LacZ (blue) or MVA-ΔC7L-p11L (red) and 1 h p.I. transfected with P7.5-empty, P7.5-C7L, ΔCK2, ΔASN and Δint as indicated (green). At 18 h posttransfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.

The expression of the central deletion mutant constructs was confirmed by Western Blot analysis of MVA-ΔC7L infected BHK cell lysates that were transfected with the deletion constructs (Fig. 30).

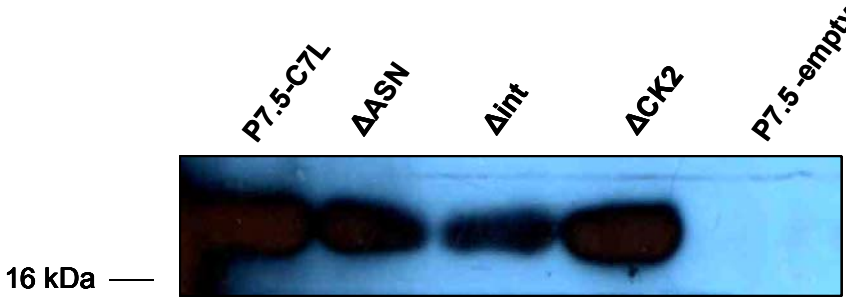


Fig. 30: Expression of C7L-central deletion mutant constructs in BHK cells
BHK cells were infected with MVA-ΔC7L at a MOI of 10 and transfected 60 min post infection with the indicated P7.5-C7L-deletion mutant plasmids. The protein extracts were separated on a reducing 12% SDS PAGE, transferred on a PVDF-membrane and detected by western Blot analysis with an antibody specific for C7L. As control served a protein extract from MVA-ΔC7L infected and P7.5-C7L transfected cells.

In summary, the results obtained with the transient-transfection reporter gene assay using different C7L deletion mutant constructs suggest that nearly the whole protein is essential to promote late gene expression of MVA in human and murine cells. Only a small part from the C-terminus (12 amino acids) is dispensable for this function. The central region seems also to be essential, as the three analyzed deletions here also lead to abrogation of late protein synthesis.

4.6. The VACV host range gene K1L can substitute C7L function in the MVA life cycle

Replication competent strains of VACV contain both the C7L and the K1L host range genes, whereas the K1L coding sequence is deleted from the MVA genome (Antoine et al., 1998). It has been demonstrated that C7L and K1L promote VACV replication in human cells. Deletion of both genes results in non-permissive infection whereas the insertion of one of the two genes is sufficient to allow replication of the virus. Insertion of K1L into MVA extends the host range only to RK13 cells but not to human cells (Meyer et al., 1991; Sutter et al., 1994). Therefore it was interesting if K1L could act as a functional homolog of C7L and allow the MVA-C7L deletion mutant virus the synthesis of late viral proteins in human and murine cells.
The transient transfection ß-Galactosidase assay (described in section 4.5.1.) was used for this purpose. The complete K1L coding sequence (855 bp) was isolated from VACV DNA (strain Western Reserve) with specific primers (K1L-XhoI and K1L-NotI) by PCR reaction (Fig. 31).

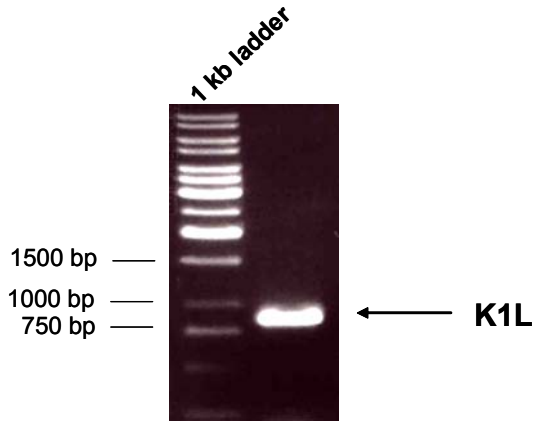


Fig. 31: Isolation of K1L coding sequence from VACV-DNA
The K1L coding sequence (855 bp) was amplified by PCR using specific primers with restriction sites for the enzymes XhoI (forward) and NotI (reverse). As template served VACV (strain Western Reserve) total DNA. 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

The isolated PCR product was digested with XhoI and NotI and subcloned into the XhoI / NotI treated plasmid pCMV-HA. The vector pCMV-HA contains an HA epitope tag sequence and allows the expression of the cloned gene as an HA-fusion protein under the control of the cellular CMV promoter (Fig. 32).

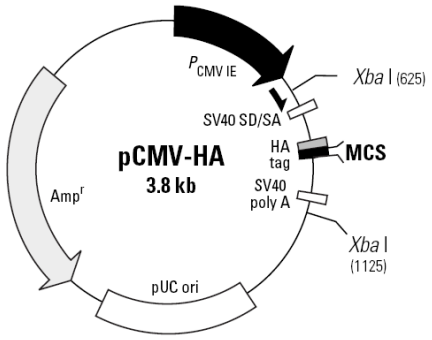


Fig. 32: Simplified map of pCMV-HA
The vector pCMV-HA expresses proteins containing an N-terminal HA-epitope-tag, an intron (SV40 splice donor/splice acceptor), a polyadenylation signal from SV40 and an ampiciline resistance gene for the selection in bacteria. Fusion proteins are under the control of the human cytomegalovirus immediate early promoter/enhancer ($P_{CMV\ IE}$)

The resulting plasmid was named pCMV-HA-K1L. In a second PCR using the primers K1L-BstXI and K1L-NsiI the K1L sequence fused to the HA-epitope tag was isolated and cloned into P7.5-empty (Fig. 33).

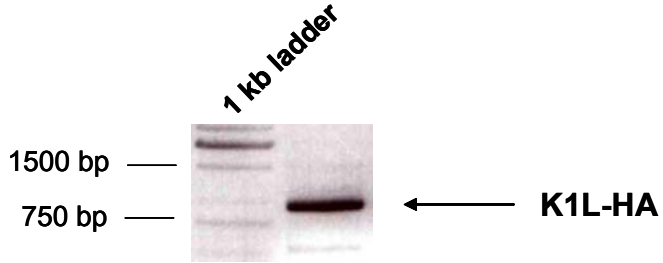


Fig. 33: Isolation of K1L-HA from pCMV-HA-K1L
The K1L-HA sequence was amplified by PCR using specific primers with restriction sites for the enzymes BstXI (forward) and NsiI (reverse). As template served the plasmid pCMV-HA-K1L. 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

The resulting plasmid P7.5-K1L-HA allowed the expression of K1L as a HA-fusion protein under the control of the VACV early/late promoter P7.5. The HA-epitope-tag was added to K1L for detection of the protein in Western blot analysis.

NIH-3T3 cells were infected with MVA-ΔC7L-P11LZ (MOI 10), 1 h post infection transfected with P7.5-K1L-HA and after 18 h analyzed for β-galactosidase synthesis. The transient expressed K1L was able to restore the late viral protein synthesis of the MVA-ΔC7L-P11LZ virus to the level of MVA-P11LZ in murine cells (Fig. 34 A), indicating that K1L and C7L can substitute for each other to allow viral late protein synthesis in the MVA context. Expression of K1L was verified by Western blot analysis using an antibody against the HA-epitope tag (Fig. 34 B).

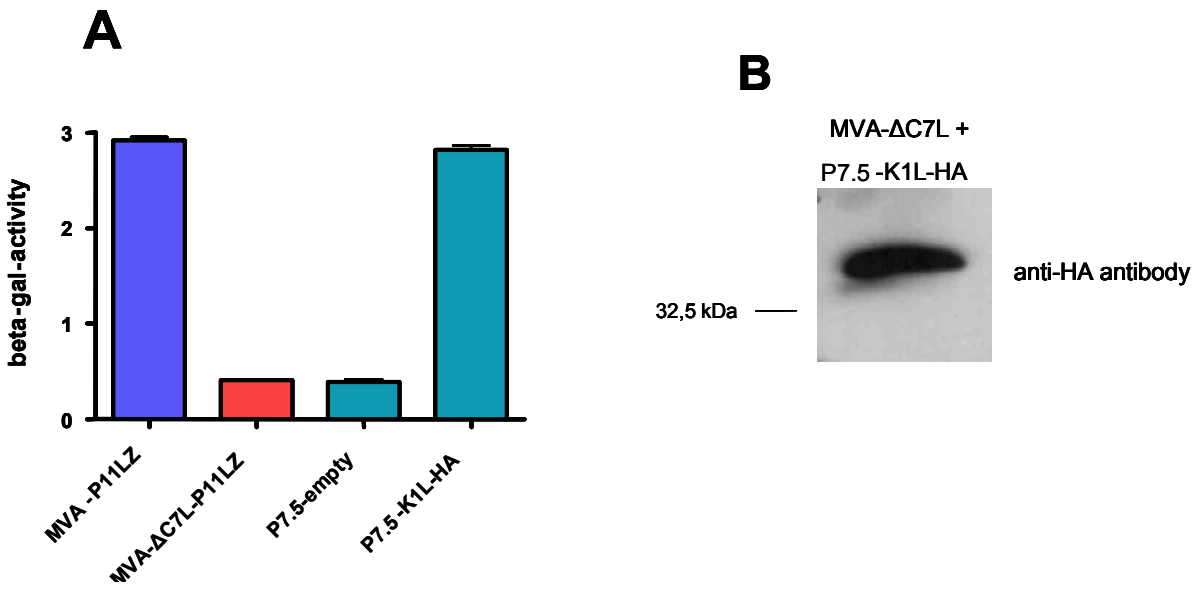


Fig. 34:Rescue of MVA-ΔC7L-P11LZ late protein synthesis in HeLa cells by transiently expressed K1L-HA (A) and expression of K1L-HA (B)
(A) Subconfluent monolayers of NIH-3T3 cells were infected with MVA-P11LZ (blue) or MVA-ΔC7L-P11LZ (red) and 1 h p.I. transfected with P7.5-empty or P7.5-K1L-HA as indicated (green). At 18 h post transfection cell lysates were assayed for β-galactosidase enzymatic activity by photometric analysis.
(B) Subconfluent monolayers of NIH-3T3 cells were infected with MVA-ΔC7L-P11LZ and 1 h p.I. transfected with P7.5-K1L-HA. At 18 h post transfection cell were harvested and the protein extracts were separated on a reducing 12% SDS PAGE, transferred on a PVDF-membrane and detected by western Blot analysis with an antibody specific for the HA-epitope-tag.

To confirm this result, BHK-, HeLa- and NIH-3T3- cells were infected with MVA, MVA-ΔC7L or MVA-ΔC7L-K1L and monitored for viral protein synthesis with ^{35}S-methionine labelling at different time points post infection (Fig. 35). The MVA-ΔC7L-K1L virus is deleted of the C7L open reading frame but contains the K1L open reading frame under the control of its endogenous promoter. Late protein synthesis could be restored in all murine and human cell lines tested, confirming that the VACV host range gene K1L is able to substitute C7L in the MVA life cycle.

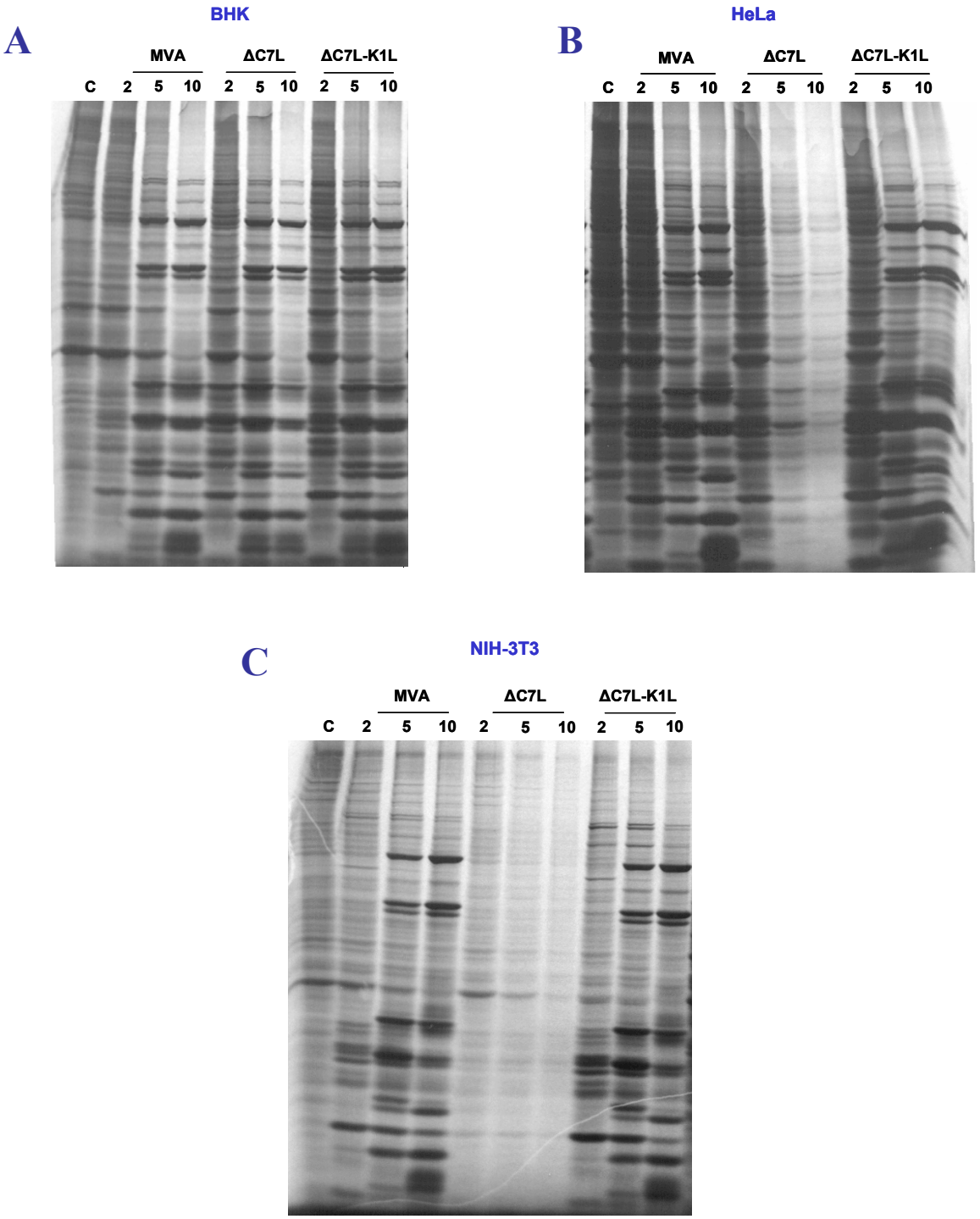


Fig. 35: Protein synthesis of cells infected with MVA, MVA-ΔC7L or MVA-ΔC7L-K1L
BHK- **(A)**, Hela- **(B)** and NIH-3T3- **(C)** cells were infected with MVA or MVA-ΔC7L at an MOI of 20. At various times after infection, cells were labelled with (^{35}S) methionine for 30 min. Cells were then harvested and the lysates were resolved by electrophoresis in a 12 % polyacrylamide gel. Labelled proteins were visualized by autoradiography of the dried gel. Numbers above the lanes indicate the time (hours) after infection when proteins were radiolabeled. C indicates uninfected cells.

4.7. Identification of potential cellular interaction partners of the MVA C7 protein with the yeast-two-hybrid system

One aim of this work was the identification of potential intracellular interaction partners of the MVA C7 host range protein. The yeast-two-hybrid method was chosen because the procedure is simple and has the important advantage that no previous knowledge about the interacting proteins is necessary to perform a screen (Fields and Song, 1989; Chien et al., 1991).
In the yeast-two-hybrid system interaction between two proteins is detected through protein-protein interaction-dependent reporter gene activation *in vivo*. This procedure is typically carried out by screening a protein of interest against a random library of potential protein partners. The method is based on the fact that many eukaryotic transcription activator proteins consist of two distinct functional domains. The yeast GAL4 transcription activator protein contains a DNA binding (aa 1-147) domain that binds to the promoter (Keegan et al., 1986) and an activation (aa 771-881) domain that directs the RNA polymerase to transcribe the downstream gene (Brent and Ptashne 1985). These two separate and independent Gal4 domains, which lack function alone, could be expressed as chimeric proteins with each GAL4 domain fused to one protein of interacting partner proteins (X and Y). The productive interaction between the partner proteins (X and Y) brings the separate activation domain (AD) into close proximity to the DNA binding domain (BD), thereby reconstituting the function of the GAL4 transcription activator protein and driving expression of a downstream reporter gene (Fig. 36).

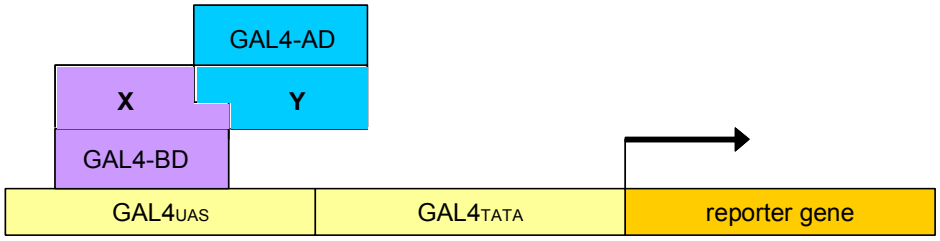


Fig. 36: Principle of the yeast-two-hybrid system
The fig. shows a construct of a GAL4-dependent promoter that is located upstream of a reporter gene. By interaction of the fusion proteins consisting of X-GAL4-BD and Y-GAL4-AD the transcription of the reporter gene is activated.

In this work the genetically engineered yeast strain AH109 was used. AH109 contains the prototrophic markers for the synthesis of the amino acids histidine and adenine (HIS3 and LEU2) as reporter genes. These reporter genes provide a positive selection of interaction on media deficient for the specific amino acids encoded by the reporter genes. Productive protein-protein interactions result in cell growth.

4.7.1. Construction of the C7L bait plasmid

For the construction of the GAL4 BD-C7L fusion protein ("bait") the yeast-expression-vector pGBKT7 was used. The plasmid pGBKT7 contains the DNA-binding domain of the transcriptional activator GAL4 (GAL4 BD) and appropriate restriction sites for constructing a translational fusion with C7L. Furthermore, the plasmid also contains a kanamycine (kan^r)-resistance gene for selection in bacteria and a gene allowing synthesis of tryptophane (TRP1) for the selection in yeast (Fig. 37).

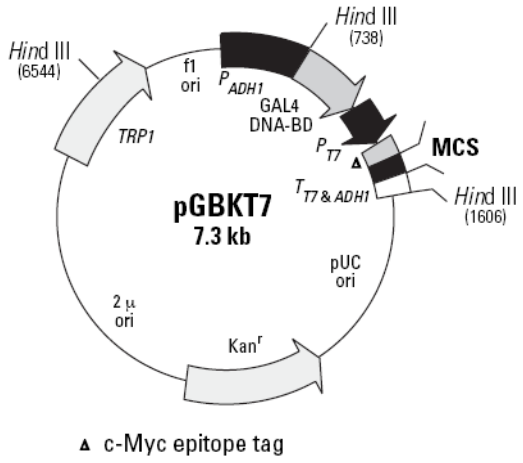


Fig. 37: Simplified map of the vector pGBKT7
The pGBKT7 vector expresses proteins fused to amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD). In yeast, fusion proteins are expressed from the ADH1 promoter (P_{ADH1}). pGBKT7 also contains the T7 promoter, a c-myc epitope tag, a kanamycine (kan^r) resistance gene for selection in *E. coli* and the TRP1 nutritional marker (TRP1) for selection in yeast.

Initially, a 453 bp DNA fragment encoding the full length C7L sequence was obtained by PCR amplification with specific primers (C7L-NdeI and C7L-NotI) using MVA DNA as template (Fig. 38). The PCR primer pair added NdeI and NotI restriction sites to the C7L coding sequence that allowed cloning into pGBKT7.

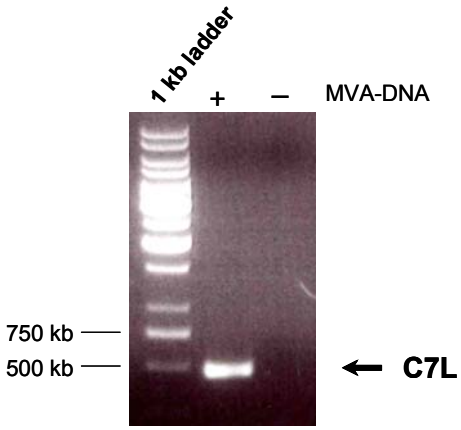


Fig. 38: PCR-amplification of C7L coding sequence
The C7L coding sequence was amplified by PCR using specific primers (C7L-NdeI and C7L-NotI). As template served MVA-DNA. 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

This PCR amplified product was digested with NdeI and NotI and was ligated with the NdeI/NotI-treated pGBKT7 plasmid to generate pGBKT7-C7L. This ligation resulted in the creation of an in-frame translational fusion of the GAL4 BD and C7L in plasmid pGBKT7. Correct insertion of C7L was confirmed by sequencing.

4.7.2. C7L has no self activating property of the reporter genes

The yeast strain AH109 used for the screen posses the reporter genes HIS3, ADE2, MEL1 and lacZ (James et al., 1996) (Fig. 39). By using different reporter systems for a two hybrid screen, promoter-specific artefacts should be excluded.

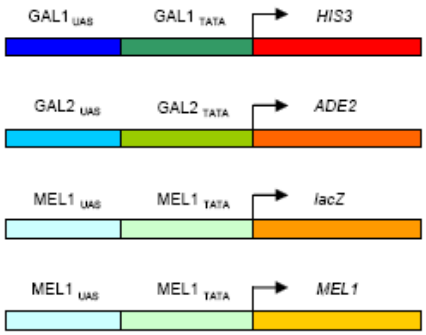


Fig. 39: Reporter gene constructs of the yeast strain AH109
The yeast strain AH109 posses the reporter genes HIS3, ADE2, lacZ and MEL1 which are under the control of different promoters (James et al., 1996).

In this work C7L was tested in the context of the promoters $GAL1_{UAS}$ and $GAL2_{UAS}$ controlling the HIS3 and ADE2 reporter genes.

The activation of the prototrophic reporter genes HIS3 and ADE2 can be confirmed by a change in yeast cell phenotype. Because AH109 is auxotroph for histidine and adenine synthesis, the activation of the HIS3- and ADE2- reporters can be proven by growth of the yeast cells on minimal medium lacking histidine or adenine. Furthermore the growth on histidine-free dishes can be regulated by addition of varying concentrations of the inhibitory agent 3-amino-1, 2, 4-triazol (3-AT). 3-AT is a competitor of the HIS3-geneproduct inidazoleglycerol-phosphate-dehydratase and limits the biosynthesis of histidine and growth of the cell. By growing yeast cells on histidine-free dishes with different concentrations of 3-AT the strength of the self-activating property or of the interaction between two proteins can be monitored.

The bait protein chosen for a yeast-two-hybrid screen should not be able to activate the reporter genes without the recruitment of an AD-fusion protein. If the bait displays self activating properties it is necessary to chose specific 3-AT concentrations that nevertheless allow for a screen on histidine-free dishes. Another possibility is to construct and test for self-activation of deletion mutants of the bait protein in order to get rid of the domain responsible for the self-activating property.

To check if the GAL4 BD-C7L-fusion protein displays self-activating properties, the bait construct was co-transformed with the empty plasmid pGADT7 into yeast cells. The plasmid pGADT7 contains the DNA-activation domain of the transcriptional activator GAL4 (GAL4 AD) and allows the construction of GAL4-AD fusion proteins. Furthermore it contains the nutritional marker LEU2 allowing the synthesis of leucine for the selection in yeast (Fig. 40).

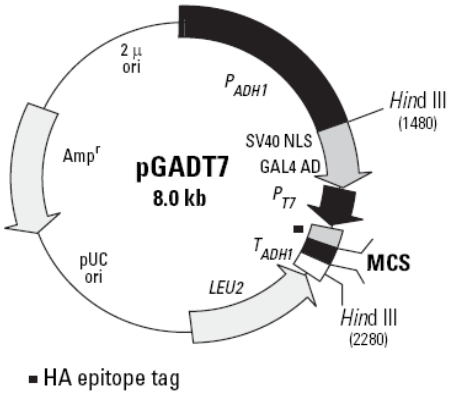


Fig. 40: Simplified map of the vector pGADT7
The vector pGADT7 expresses proteins fused to amino acids 768-881 of the GAL 4 activation domain (AD). The fusion protein is targeted to the yeast nucleus by the SV40 nuclear localization sequences that have been added to the activation domain sequence. pGADT7 also contains the T7 promoter, a HA epitope tag, a amp^r for the selection in bacteria and the LEU2 nutritional marker for selection in yeast.

The transformed cells were grown for 3 days on selective medium lacking leucine (Leu), tryptophane (Trp) and histidine (His) with various 3-AT concentrations (0 mM, 1 mM, 2,5 mM and 7,5 mM) to asses the activation of the HIS3 reporter or on selective medium lacking Leu, Trp and adenine (Ade) to test for the activation of the ADE2 reporter. AH109 is also auxotroph for the synthesis of leucine and tryptophane. Therefore only cells that are transformed with both plasmids are able to grow on the selective medium. Both empty vectors pGBKT7 and pGADT7 co-transformed into yeast cells served as negative control whereas co-transformation of pGBKT7-EBNA3a and pGADT7-CBF1 should activate the reporter genes. pGBKT7-EBNA3a contains an in-frame translational fusion of the GAL4 BD and the EBNA3a protein from Epstein Barr virus whereas pGADT7-CBF1 contains an in-frame translational fusion of the GAL4 AD and the cellular protein CBF1 which has previously been shown to interact with EBNA3a (Bourillot et al., 1998; Dalbies-Tran et al., 2001).

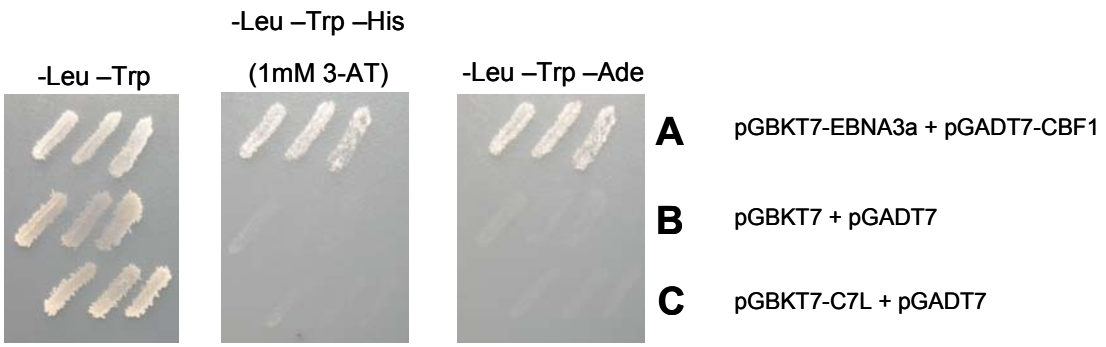


Fig. 41: The GAL4 BD-C7L-fusionprotein does not activate the HIS- and ADE- reporter genes
Yeast cells were co-transformed with pGBKT7-EBNA3a and pGADT7-CBF1 **(A)**, empty pGBKT7 and empty pGADT7 **(B)** or pGBKT7-C7L and empty pGADT7 **(C)** as indicated and grown for 3 days on medium lacking Leu and Trp, Leu, Trp and His (1 mM 3-AT) or Leu, Trp and Ade.

As shown in Fig. 41, the GAL4 BD-C7L-fusion protein is not able to activate expression of the HIS- and ADE- reporter genes without the recruitment of an AD-fusion protein because cells transformed with the bait construct are not able to grow on histidine-(1 mM 3-AT) or adenine-free medium.. The positive control cells transformed with pGBKT7-EBNA3a and pGADT7-CBF1 were able to grown on medium lacking Ade or His even up to a 7,5 mM 3-AT concentration (data not shown) because the interaction of EBNA3a and CBF1 reconstitutes the transcription factor and enables the cells to synthesize histidine and adenine. The results shown in fig. for growth on –His + 1 mM 3-AT medium are representative for the result obtained with higher 3-AT concentrations. In order to eliminate any background growth it was decided to perform the screen on selective medium containing 1 mM 3-AT.

4.7.3. Stable expression of the GAL4 BD-C7L-fusion protein in yeast

It was important to ensure that the GAL4 BD-C7L-fusion protein could be stably expressed upon transformation into yeast cells. Yeast cells were co-transformed with the pGBKT7-C7L bait construct and the plasmid pGADT7 that contained no insert. The transformed cells were grown on selective medium lacking Leu and Trp which only allowed growth of cells that had incorporated both plasmids. The cells were grown over night and yeast-cell-extracts were prepared. untransformed yeast cells served as negative control. The yeast-cell-extracts were separated on a 12% denaturating, reducing SDS-polyacrylamidegel. The GAL4 BD-C7L-fusion protein was detected by Western blot analysis using an antibody specific for C7L. Fig. 42 demonstrates the expression of the GAL4 BD-C7L fusion protein in yeast cells at the expected size of 40 kDa calculated as fusion of the C7 protein (18 kDa) and the GAL4 BD (21,8 kDa).

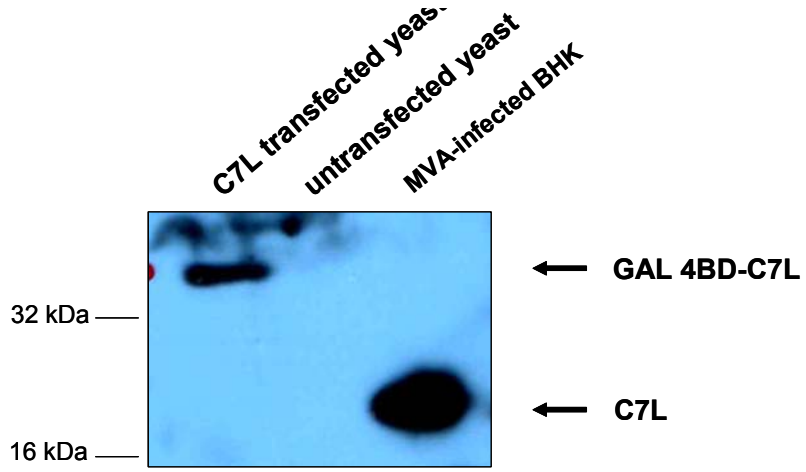


Fig. 42: The GAL4 BD-C7L-fusionprotein is stably expressed in the yeast strain AH109
The plasmid pGBKT7-C7L expressing the GAL4 BD-C7L-fusionprotein was co-transformed with pGADT7 into yeast strain AH109. Untransformed yeast cells as well as MVA-infected BHK cells served as controls. Yeast- and cell- protein extracts were prepared and separated on a 12% reducing SDS PAGE-gel, transferred on a PVDF-membrane and detected by Western Blot analysis with an antibody specific for C7L.

4.7.4. C7L is not toxic upon expression in yeast

Experience with yeast-two-hybrid screens showed, that some ectopically expressed proteins can be toxic for the yeast cells. This toxicity decreases the transformation efficiency and limits the results of the screen. Highly toxic baits are unsuitable for a yeast-two-hybrid screen.
The toxicity of the GAL 4 BD-C7L fusion protein for the yeast strain AH109 was tested by co-transforming the cells with pGBKT7-C7L and the empty-vector pGADT7. Co-transformation of the empty vectors pGBKT7 and pGADT7 as well as co-transformation of pGBKT7-EBNA3a and pGADT7-CBF1 served as controls. The transformed cells were expanded on –Trp, -Leu dishes that are permissive for cells that had incorporated both plasmids. The yeast cells were grown at 30°C for 3 days and then monitored for transformation efficiency and growth. Transformation of the yeast cells with the C7L expression construct displayed the same transformation efficiency and growth rate compared to the controls with the empty vectors and with the interacting proteins suggesting that C7L is not toxic upon expression in yeast.

4.7.5. Yeast-two-hybrid screen of a cDNA library derived from human lymphocytes for the identification of C7L interaction partners

The C7L gene has previously been shown to be required for efficient replication of a VACV-K1L deletion mutant on human cells (Perkus et al., 1990; Oguiura et al., 1993). Furthermore, as shown in Fig. 3 and 7, the C7 protein is also required for MVA expression of the late stage genes in human and murine cells. Therefore, as C7L is important for the viral life cycle in human cells, a cDNA library derived from human cells was used for the yeast-two-hybrid screen with the C7L bait construct. A commercially available cDNA library derived from EBV-immortalized human lymphocytes (Clontech: human Lymphocyte MATCHMAKER cDNA Library = HLM cDNA-library) was chosen. The cDNAs of the HLM cDNA library were cloned into the vector pACT by using two XhoI restriction sites. The GAL4-AD is fused to the SV40 NLS nuclear-localisation-signal for the transport of the fusion protein into the nucleus. The vector pACT contains an ampicillin-resistance gene (amp) for the selection in bacteria and a gene that enables the production of leucine (Leu) for the selection in yeast (Fig.43).

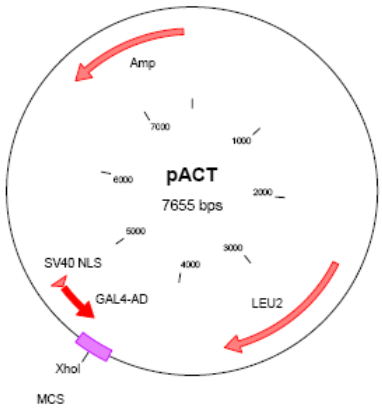


Fig. 43: Simplified map of the vector pACT
The vector pACT contains the gene for the GAL4 AD coupled to the nuclear localization-signal SV40 NLS. pACT contains an ampicillin-resistancegene (amp) for the selection in bacteria and a gene that enables for the production of leucine (Leu) for the selection in yeast. The map shows the restriction-site for the enzyme *XhoI* located in the MCS of the vector.

MVA is able to infect a broad range of cells including human B-lymphocytes, but it had not been demonstrated if expression of C7L is also necessary for MVA to complete late stage gene

expression in human B-lymphocytes. To test this, the β-galactosidase reporter gene assay was used. LCL cells were infected with the recombinant viruses MVA-P11LZ and MVA-ΔC7L-P11LZ and 16 h post infection the cells were assayed for β-galactosidase expression. Cells that were infected with MVA-ΔC7L-P11LZ showed a drastically reduced β-galactosidase activity as compared to MVA-P11LZ infected cells (Fig. 44) suggesting that C7L is also necessary for the expression of late viral genes in human B-lymphocytes and therefore it was assumed that the potential cellular interaction partners should be expressed in B cells.

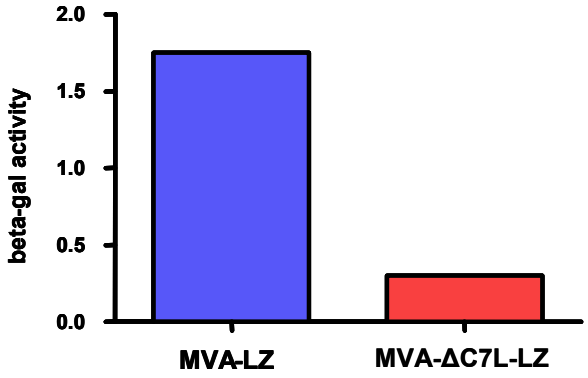


Fig. 44: β-galactosidase expression in B-cells
LCL cells were infected with MVA-P11LZ or MVA-ΔC7L-P11LZ. 16 h post infection the cells were lysed and incubated with the substrate CPRG. Production of β-galactosidase was quantified by a photometer using a 570 nm filter.

The HLM cDNA library was available as a pre-transformed glycerol stock of *E. coli* strain BNN132 and stored at -80°C. Before use, the library plasmids had to be amplified in BN132. Calculating from the titer of the library (1 x 10^8 cfu/ml), 12 µl of the glycerol stock were mixed with 9 ml LB-medium and dispersed on LB-amp (50 µg/ml) dishes. After two days the bacteria were harvested and the pACT cDNA plasmids were isolated.
The yeast two hybrid screen of the HLM cDNA library with the C7L-bait construct was performed as described in 3.6.2.3. Yeast cells that were already pre-transformed with the C7L-bait-construct were transformed with the pACT cDNA plasmids and dispersed on 50 selective dishes lacking Leu, Trp and His (1mM 3-AT) to allow only growth of cells that had incorporated both plasmids and that had activated the expression of the His reporter gene. During the whole screen pGBKT7-EBNA3a / pGADT7-CBF1 transformed yeast cells served as positive control and empty pGBKT7 / pGADT7 as well as pGBKT7-C7L / empty pGADT7 transformed cells as negative controls. 288 positive clones were obtained that also showed activation of the Ade

reporter on –Leu, -Trp, -Ade dishes. To confirm the activation of the reporter genes, the pACT cDNA library plasmids of the positive clones were isolated from the yeast cells, amplified in *E. coli* and again transformed into pGBKT7-C7L pre-transformed yeast cells which then were plated on selective reporter dishes. To increase the stringency of the His reporter activation, the transformed cells were plated in parallel on –Leu, -Trp, -His dishes with different 3-AT concentrations (2,5 mM, 5 mM and 10 mM). 238 of the 288 re-transformants showed again activation of the Ade reporter whereas 253 (1mM 3-AT), 221 (2,5 mM 3-AT), 191 (5mM 3-AT) and 119 (10 mM 3-AT) activated the His reporter again. A summary of the results is shown in table 21.

Table 21: Summary of activation of Ade and His reporter genes by different clones

Reporter gene	**Ade**	**His (1mM 3-AT)**	**His (2,5 mM 3-AT)**	**His (5 mM 3-AT)**	**His (10 mM 3-AT)**
activating clones	238	253	221	191	119

Regarding the results obtained with the different reporter gene constructs, it seems that the Ade reporter allows a more stringent detection of interactions than the His reporter. Some clones did not activate the Ade reporter but showed activation of the His reporter with 1 mM 3-AT. But this activation of the His reporter gene was abolished when higher 3-AT concentrations were used (2,5 to 10 mM).

The 119 clones that showed activation of the His reporter in presence of 10 mM 3-AT had also activated the Ade reporter gene. Therefore these clones were assumed to display positive interactions. To identify the potential C7L interaction partners the 119 positive clones were sequenced and their identity was determined with the NCBI data base. The results are shown in table 22.

Table 22: Potential interaction partners identified from the cDNA library

Clone number	Coding protein (NCBI BLAST)	GeneBank Accession number
1, 84, 149, 195, 216	Homo sapiens SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4)	NM_003072
3	Homo sapiens ELL associated factor 2 (EAF2)	NM_018456
4, 44, 90, 98, 275	Homo sapiens splicing factor 3b, subunit 2, 145kDa (SF3B2)	NM_006842
5, 75, 224 *	Homo sapiens ribosomal protein S27a (RPS27A)	NM_002954
6 *	Homo sapiens heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1)	NM_001017963
9	Homo sapiens eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa (EIF3S10)	NM_003750
10, 26, 153, 212, 213	chromosome 11 open reading frame 17 breast cancer associated gene 3; protein kinase A-interacting protein 1; koyt binding protein	NM_182901
15, 19, 21, 25, 63	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 12A (PPP1R12A)	
18 *	Homo sapiens ribosomal protein S25 (RPS25)	NM_001028
22, 112, 282	Homo sapiens Y box binding protein 1 (YBX1)	NM_004559
23	Homo sapiens peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PPRC1)	NM_015062
27	Homo sapiens MYC binding protein 2 (MYCBP2)	NM_015057
29	Homo sapiens solute carrier family 16 (monocarboxylic acid transporters), member 3 (SLC16A3)	NM_004207
30	Homo sapiens nuclear distribution gene C homolog (A. nidulans) (NUDC)	NM_006600
37, 232 *	Homo sapiens ribosomal protein S9 (RPS9)	NM_001013
39, 205	Homo sapiens chromobox homolog 4 (Pc class homolog, Drosophila) (CBX4)	NM_003655
45, 97	Homo sapiens Sin3A-associated protein, 30kDa (SAP30)	NM_003864
48	Homo sapiens general transcription factor IIIC, polypeptide 5, 63kDa (GTF3C5)	NM_012087
51, 79, 81, 140, 158, 191, 197, 222, 257, 261, 262	unknown	
53, 60,	Homo sapiens phosphorylase kinase, alpha 2 (liver)	NM_000292
58, 202 *	Homo sapiens ZFAT zinc finger 1 (ZFAT1)	NM_020863
62	Homo sapiens peptidylprolyl isomerase G (cyclophilin G) (PPIG)	NM_004792
65, 130, 271	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467

74	Homo sapiens NF-kappaB activating protein (NKAP)	NM_024528
76, 119	Homo sapiens copper metabolism (Murr1) domain containing 1 (COMMD1)	NM_152516
85,101, 103, 104. 105, 107, 114, 123, 128, 129, 166, 269, 270	Homo sapiens RNA binding protein S1, serine-rich domain (RNPS1)	NM_080594
87	Homo sapiens upstream binding transcription factor, RNA polymerase I (UBTF)	NM_014233
91, 180, 186, 204 *	Homo sapiens heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	NM_005347
99	Homo sapiens Josephin domain containing 3 (JOSD3)	NM_024116
111, 208, 230	Homo sapiens bromodomain containing 7 (BRD7)	NM_013263
113	Homo sapiens N-myc (and STAT) interactor (NMI)	NM_004688
131	Homo sapiens glioma tumor suppressor candidate region gene 2 (GLTSCR2)	NM_015710
144	Homo sapiens kinesin family member 3B (KIF3B)	NM_004798
148	Homo sapiens transcription termination factor, RNA polymerase I (TTF1)	NM_007344
172 *	Homo sapiens zinc finger, HIT type 4 (ZNHIT4)	NM_031288
184, 285	Homo sapiens RAN binding protein 9 (RANBP9)	NM_005493
185	Homo sapiens RAN binding protein 5 (RANBP5)	NM_002271
190, 214, 226, 279, 280 *	Homo sapiens ribosomal protein S7 (RPS7)	NM_001011
199, 264	Homo sapiens heterogeneous nuclear ribonucleoprotein A/B (HNRPAB)	NM_031266
200	Homo sapiens filamin A, alpha (actin binding protein 280) (FLNA)	NM_001456
203	Homo sapiens U2 small nuclear RNA auxiliary factor 1 (U2AF1)	NM_006758
215	Homo sapiens high-mobility group box 1 (HMGB1)	NM_002128
231	Homo sapiens ubiquitin A-52 residue ribosomal protein fusion product 1 (UBA52)	NM_001033930
258	Homo sapiens bromodomain containing 2 (BRD2)	NM_005104
265	Homo sapiens tousled-like kinase 1 (TLK1)	NM_012290
267	Homo sapiens serine palmitoyltransferase, long chain base subunit 2 (SPTLC2)	NM_004863

The cDNA clones indicated with * encode for ribosomal proteins (5, 18, 37, 75,190, 214, 224, 226, 232, 279, 286) heat shock proteins (6, 91, 180, 186, 204) and zink figer proteins (85, 172, 202). These proteins and some others are often identified in yeast-two-hybrid screens with various bait constructs but the interactions can not be proven using another method (Table 23) (Hengen, 1997; Golemis 1997). They are called "false positives". Possibly these proteins are frequently identified because they occur abundantly in cells or they interact directly with the GAL4 BD.

Table 23: "False positive" interaction partners often identified with the yeast two hybrid system

Heat shock proteins	tRNA synthetase
Ribosomal proteins	Collagen related proteins
Cytochrom C oxidase	Zn-finger proteins
Mitochondrial proteins	Vimentin
Proteasomal subunits	PCNA
Ferritin	Inorganic pyrophosphatase

Table is adapted from Golemis, 1997

Some proteins like for example the "Homo sapiens RNA binding protein S1" were isolated more than one time in the screen. Alignments of the cDNA inserts showed that all encoded for the identical cDNA. Probably these plasmids were overrepresented in the library due to the amplification of the cDNA library before the transformation.

4.7.6. C7L-depending reporter gene activation by the prey-proteins

In principle it is possible that the prey proteins alone are able to activate transcription of the reporter genes by direct binding to the promoter-region or to the GAL4-BD. Therefore the above described activation of the reporter genes with the cDNA clones listed in table 22 was tested for their dependence on C7L expression.

The plasmids encoding the potential C7L interacting proteins were co-transformed with the empty plasmid pGBKT7 containing only the GAL4 BD into AH109 and plated on –Leu, -Trp, -Ade and –Leu, -Trp, -His (1mM to 10 mM 3-AT) dishes. After four days the reporter gene activation was monitored. Proteins that did not activate the adenine or histidine reporter genes in presence of 1mM 3-AT without binding to C7L are listed in table 24.

Table 24: Proteins that do not activate the reporter genes without binding to C7L

Coding protein	Clone number
Homo sapiens heat shock protein 90kDa alpha (cytosolic), class A member 1 (HSP90AA1)	6
Homo sapiens peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PPRC1)	23
Homo sapiens ribosomal protein S9 (RPS9)	37, 232
Homo sapiens Sin3A-associated protein, 30kDa (SAP30)	45, 97
Homo sapiens NF-kappaB activating protein (NKAP)	74
Homo sapiens ribosomal protein S27a (RPS27A)	5, 75, 224
Homo sapiens Josephin domain containing 3 (JOSD3)	99
Homo sapiens glioma tumor suppressor candidate region gene 2(GLTSCR2)	131
Homo sapiens kinesin family member 3B (KIF3B)	144
Homo sapiens transcription termination factor, RNA polymerase I (TTF1)	148
Homo sapiens zinc finger, HIT type 4 (ZNHIT4)	172
Homo sapiens RAN binding protein 9 (RANBP9)	184, 285
Homo sapiens bromodomain containing 7 (BRD7)	208
Homo sapiens high-mobility group box 1 (HMGB1)	215
Homo sapiens bromodomain containing 2 (BRD2)	258

All the other tested proteins showed activation of the reporter genes independent of C7L expression. These clones were therefore excluded from further analysis.

4.7.7. Description of the proteins identified by the yeast-two-hybrid screen

A) Homo sapiens heat shock protein 90kDa alpha, class A member 1 (Hsp90)

Hsp90 is an abundant cytosolic ATP dependent molecular chaperone involved in folding, stability and activation of an unknown number of substrate proteins. Most known Hsp90 substrate proteins are transcription factors, key signaling proteins and protein kinases that regulate cell survival, proliferation metastasis, and angiogenesis (Richter and Buchner, 2001; Georgakis and Younes 2005). In presence of Hsp90 inhibitors many substrate proteins are destabilized and subsequently degraded via the ubiquitin proteasome pathway (Zhang and Burrows, 2004). The underlying chaperone mechanism is still poorly understood. Hsp90 was also shown to play a role in the life cycle of VACV as Hsp90 inhibition strongly inhibits VACV growth although the infection does not induce Hsp90 expression. In addition, Hsp90 interacts with a viral VACV core protein, A10L (Hung et al., 2002).

B) Homo sapiens peroxisome proliferator-activated receptor gamma, coactivator-related 1 (PRC)

PRC is a ubiquitously expressed transcriptional coactivator belonging to the PGC-1 gene family. Members of this family serve as master regulators of mitochondrial biogenesis and oxidative metabolism because they activate nuclear genes encoding mitochondrial proteins. (Handschin and Spiegelman, 2006; Andersson and Scarpulla, 2001).

C) Homo sapiens Sin3A-associated protein, 30kDa (SAP30)

SAP30 is a specific component of the Sin3A-histone deacetylase complex (Zhang et al., 1999). Gene expression is influenced by chromatin structure. Histone deacetylation converts chromatin to a 'closed state', leading to the silencing of gene transcription. Many studies suggest that SAP30 functions as a bridging and stabilizing molecule between the Sin3A complex and other corepressors such as CIR (Hsieh et al., 1991) or DNA-binding transcription factors like YY1 (Huang et al., 2003).

D) Homo sapiens ribosomal protein S9 (RPS9)

RPS9 encodes for a ribosomal protein essential for the assembly of the 30S ribosomal protein complex (Chan et al., 1993).

E) Homo sapiens NF-kappaB activating protein (NKAP)

NKAP is a novel nuclear regulator of TNF- and IL-1-induced NF-κB activation (Chen et al., 2003). The NF-κB/Rel transcription factor family regulates the expression of genes involved in immune responses, inflammation, apoptosis, and proliferation (Ghosh and Karin, 2002; Karin and Ben-Neriah, 2000).

F) Homo sapiens ribosomal protein S27a (RPS27a)

This gene encodes a fusion protein consisting of ubiquitin at the N terminus and ribosomal protein S27a at the C terminus. The protein is post-translationally processed, generating free ubiquitin monomer and ribosomal protein S27a. RPS27a is a component of the 40S subunit of the ribosome and belongs to the S27AE family of ribosomal proteins. It contains C4-type zinc finger domains and is located in the cytoplasm (Redman and Rechsteiner, 1989).
Ubiquitin, a highly conserved protein that has a major role in targeting cellular proteins for degradation by the 26S proteosome, is synthesized as a precursor protein consisting of either polyubiquitin chains or a single ubiquitin fused to an unrelated protein (Finley et al, 1989; Spence et al, 2000).

G) Homo sapiens Josephin domain containing 3 (JOSD3)

JOSD3 is a uncharacterized member of the Josephin domain family. Recently, it has been demonstrated that Josephin domain containing proteins display de-ubiquitination activity (Tzvetkov and Breuer, 2007).

H) Homo sapiens glioma tumor suppressor candidate region gene 2 (GLTSCR2)

GLTSCR2 was originally identified as a candidate tumor suppressor gene (Smith et al., 2000). GLTSCR2 is required for maintaining PTEN stability in cells (Okahara et al., 2004). PTEN negatively regulates PI3K/PIP_3 signals (Maehama and Dixon, 1998; Myers et al., 1998; Cantley and Neel, 1999) that play pivotal roles in regulating cell proliferation and apoptosis in response to various stimuli (Cantley 2002).

I) Homo sapiens kinesin family member 3B (KIF3B)

Microtubules are pipe-like structures that serve as tracks for intracellular transport (Scholey and Vale, 1993; Lodish et al., 1995; Alberts et al., 2002). Transport at the cellular microtubule network is mediated by kinesins, a superfamily of microtubule-based ATPase motors. (Dagenbach and Endow, 2004). KIF3B is a motor subunit of Kinesin-2 (Brown et al., 2005).

J) Homo sapiens transcription termination factor, RNA polymerase I (TTF-I)

RNA polymerase I synthesizes 35S rRNA which is processed into mature 25S, 18S and 5,8S rRNAs. (Reeder and Lang, 1994). Transcription of rRNA genes assembled into chromatin requires ATP-dependent remodeling of nucleosomes at the RNA polymerase I transcription start site. This remodeling reaction is triggered by the transcription termination factor TTF-I. By binding to its target site upstream of the gene promoter, TTF-I can activate transcription, presumably by recruiting remodeling complexes to the rDNA promoter (Längst et al., 1997, 1998).

K) Homo sapiens RAN binding protein 9 (RanBPM)

RanBPM was isolated as a binding partner of the small GTPase Ran in a yeast two-hybrid system (Nakamura et al., 1998). Recent studies have suggested that RanBPM is localized to the nucleus, the cytoplasm, and the plasma membrane (Nishitani et al., 2001; Ideguchi et al., 2002; Umeda et al., 2003; Denti et al., 2001). Other groups showed that Ran is important in the process of microtubule assembly (Carazo-Salas et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999).

L) Homo sapiens bromodomain containing 7 (BRD7)

BRD7 encodes a bromodomain protein that was first identified in nasopharyngeal carcinoma (Ying et al., 2000). Bromodomain is a 110 amino acid motif (Tamkun et al., 1992; Jeanmougin et al., 1997). Bromodomain proteins regulate signal-dependent, but not basal, transcription during active proliferation (Horn and Peterson, 2001; Patrizia at al., 2001). Studies have indicated that genetic aations of bromodomain genes contribute to the development of many human cancers by transcriptional dysregulation (French et al., 2001; Ohshima et al., 2001). BRD7 was shown to interact with nuclear transcription factor IRF2 (Ada et al., 2000) and form a triple complex adenovirus nuclear protein E1B-AP5 thereby affecting the transcription activity of E1B-AP5 (Kzhyshkowska et al., 2003).

M) Homo sapiens high-mobility group box-1 (HMGB1)

The HMGB1 protein is present in the nucleus and cytoplasm of almost all mammalian cells and is highly conserved between species (Vaccari et al., 1998; Bustin and Neihart 1979; Isackson et al., 1989). Within the nucleus, HMGB1 stabilizes nucleosomes and regulates transcription of many genes (Park et al., 2003; Stros et al., 2002). As a cytokine-like factor, HMGB1 is secreted by macrophages (Bonaldi et al., 2003; Wang et al., 2005), mature dendritic cells (Dumitriu et al., 2005), and natural killer cells (Semino et al.2005) in response to injury, infection, or other inflammatory stimuli.

N) Homo sapiens bromodomain containing 2 (BRD2)

BRD2, like BRD7, belongs to the bromodomain proteins. It is a cell cycle dependent transcription mediator that shows distinct protein kinase activation (Denis and Green 1996). BRD2 was found to interact with BRD7 (Zhou et al., 2006).

4.7.8. Co-immunoprecipitation of C7L with putative interaction partners

Co-immunoprecipitation experiments were performed to answer the question whether *in vitro* protein-protein interactions would corroborate the interactions found by the yeast-two-hybrid assay. For these experiments recombinant ^{35}S-methionine labeled proteins were produced by *in vitro* transcription-translation reactions. These reactions require a template where the desired sequence is under the control of a T7 promoter. The pGBKT7 plasmid (Fig. 37) contains an additional T7 promoter sequence located downstream of the yeast promoter sequence. Therefore, the pGBKT7-C7L bait construct could be used directly as template for the *in vitro* transcription-translation reactions for the generation of ^{35}S-methionine labeled and myc-tagged C7 protein (Fig 45 A). Immunoprecipitation reactions performed with the generated protein allowed precipitation with antibodies directed against C7L and against the myc tag indicating that the generated protein is indeed C7L that contains the correct inframe fusion to the myc-tag (Fig. 45 B).

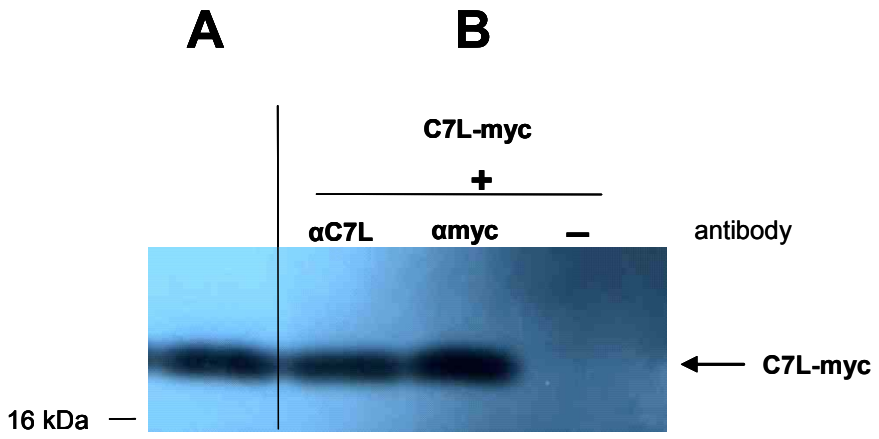


Fig. 45: *In vitro* coupled transcription/translation and immunoprecipitation of ^{3S}5-methionine radiolabeled myc-tagged C7L
(A) ^{35}S radiolabeld myc-tagged C7 protein was generated by *in* vitro transcription/translation reaction using the plasmid pGBKT7-C7L as template. **(B)** This protein was incubated with an antibody against C7L or against the myc-epiotpe tag and protein G-agarose beads. The proteins were analyzed by SDS-PAGE and autoradiography.

The cDNA sequences of the identified putative C7L binding partners are contained in the pACT cDNA library plasmid (Fig. 43). The pACT plasmid does not contain a T7-promoter sequence. Therefore, PCR reactions were performed with primers (AD-HAforw/AD-rev) that allowed the amplification of the cDNA sequences from pACT. The forward primer (AD-HAforw) contained additional to the T7-promoter sequence an HA epitope tag sequence (Fig. 46) to allow the precipitation of the generated putative binding partner proteins. Both primers are complementary to the pACT plasmid sequences flanking the contained cDNA. Thus, the primer pair can be used for all isolated plasmids containing potential C7L interaction partners.

5`- AAA ATT GTA ATA CGA CTC ACT ATA GGG CGA GCC GCC ACC ATG TAC CCA TAC GAC GTT CCA GAT TAC GCT CCA CCA AAC CCA AAA AAA GAG – 3`

Fig. 46: Sequence of primer AD-HAforw
The primer AD-HAforw contains a T7-promoter sequence (blue), an HA epitope tag sequence (yellow) and a sequence complementary to pACT (green). The ATG is located 5`of the HA-tag sequence.

RPS27A was isolated three times (clones 5, 75 and 224). An alignment of the cDNA inserts showed that all encode exactly for the same insert. Therefore, clone 75 was used representatively in the following experiments. For RPS9 (clones 37 and 232), SAP30 (clones 45 and 97) and RANBP9 (clones 184 and 285) clones 37, 45 and 184 were used representatively.
Using primers AD-HAforw and AD-rev, PCR reactions were performed using plasmids from the yeast clones 6, 23, 37, 45, 74, 75, 99, 131, 144, 148, 172, 184, 208, 215 and 258 (Fig. 47).

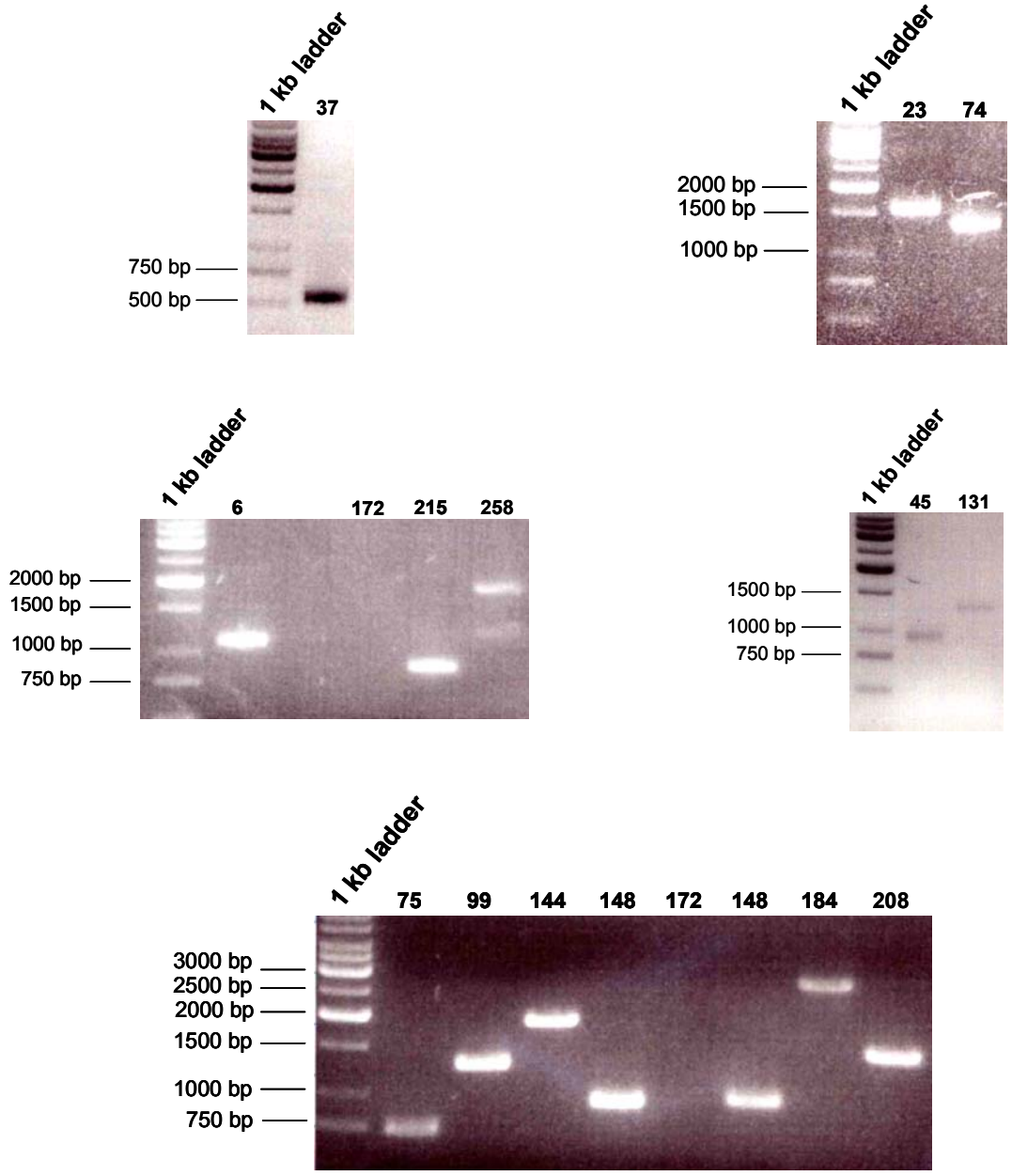


Fig. 47: Isolation of cDNA clone sequences from pACT library plasmids
The cDNA clone sequences were amplified by PCR using specific primers complementary to the pACT library plasmid. The forward primer AD-HAforw adds a T7-promoter sequence and an HA epitope sequence 5`of the cDNA. As templates served pACT plasmid DNAs from the yeast clones . 10 µl of the 100 µl total PCR reaction were separated by a 1% agarose gel and visualized by ethidiumbromide staining.

With plasmid DNA isolated from clone 172 no PCR product could be obtained although various PCR conditions were used (temperature, primer concentrations etc.). In table 25 the obtained PCR products and the expected molecular weights of the encoded proteins are summarized.

Table 25: Summary PCR products

Clone number	Size PCR product	Predicted mass corresponding protein
6	~ 1200 bp	~ 47 kDa
23	~ 1600 bp	~ 63 kDa
37	~ 550 bp	~ 22 kDa
45	~ 900 bp	~ 36 kDa
74	~ 1400 bp	~ 58 kDa
75	~ 750 bp	~ 27 kDa
99	~ 1300 bp	~ 54 kDa
131	~ 1300 bp	~ 54 kDa
144	~ 2000 bp	~ 81 kDa
148	~ 900 bp	~ 36 kDa
172	-	
184	~ 2600 bp	~ 103 kDa
208	~ 1300 bp	~ 54 kDa
215	~ 850 bp	~ 31 kDa
258	~ 1900 bp	~ 77 kDa

The generated PCR products were used directly as template for *in vitro* transcription-translation reactions with the TNT® Coupled Reticulocyte Lysate System for generation of ^{35}S-methionine labeled HA tagged proteins. These proteins and their putative binding partner, the c-myc tagged C7 protein were used in subsequent co-immunoprecipitation experiments (Fig. 48-61).

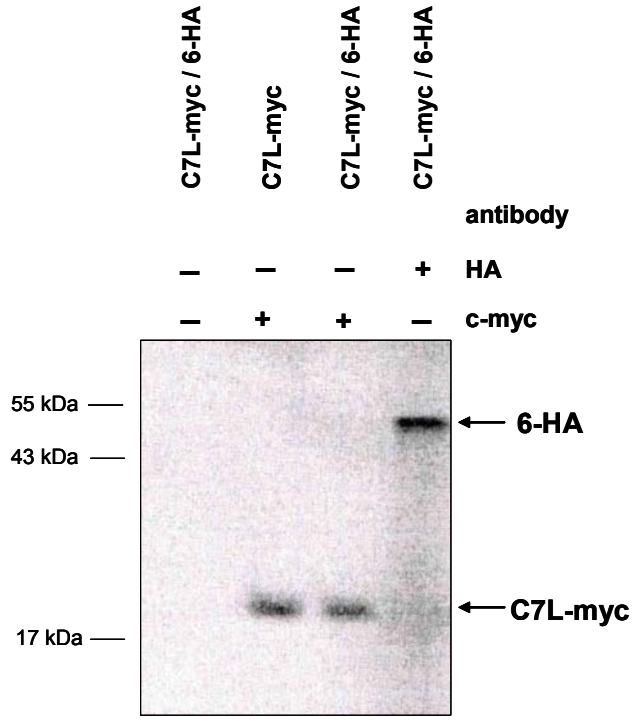


Fig. 48: Co-immuoprecipitation of *in* vitro generated C7L-myc and 6-HA
In vitro generated C7L-myc and 6-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 6-HA proteins are indicated on the right.

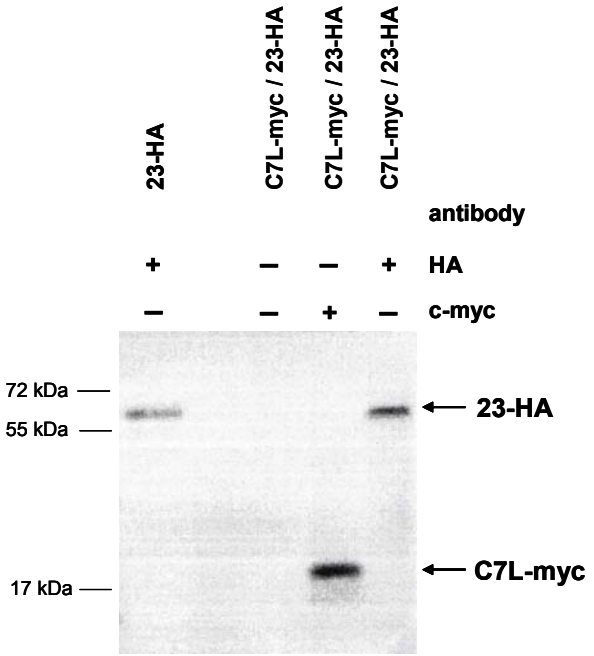


Fig. 49: Co-immuoprecipitation of *in* vitro generated C7L-myc and 23-HA
In vitro generated C7L-myc and 23-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 23-HA proteins are indicated on the right.

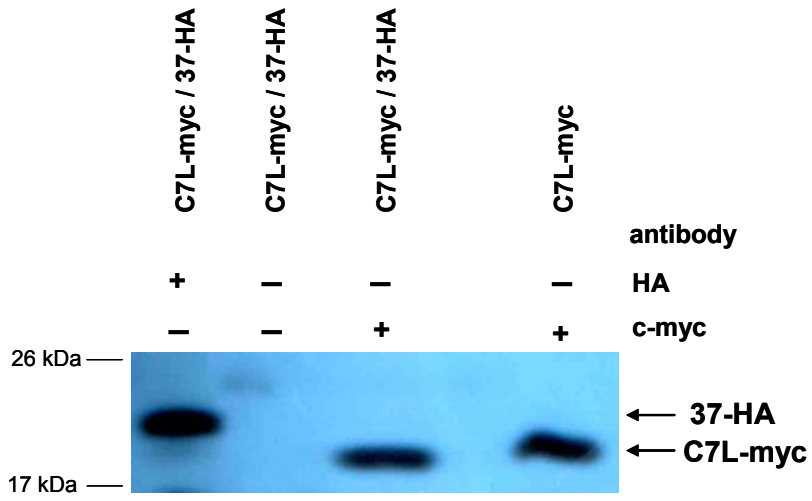


Fig. 50: Co-immuoprecipitation of *in* vitro generated C7L-myc and 37-HA
In vitro generated C7L-myc and 37-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 37-HA proteins are indicated on the right.

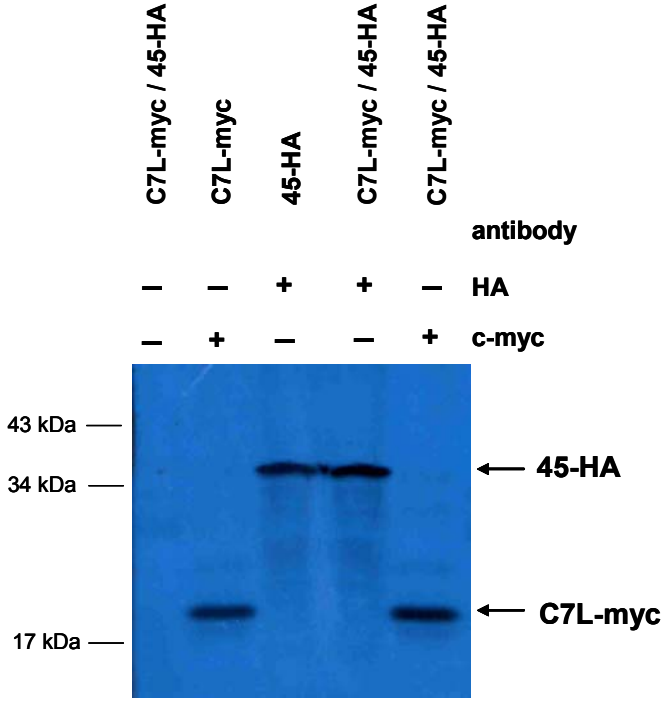


Fig. 51: Co-immuoprecipitation of *in* vitro generated C7L-myc and 45-HA
In vitro generated C7L-myc and 45-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 45-HA proteins are indicated on the right.

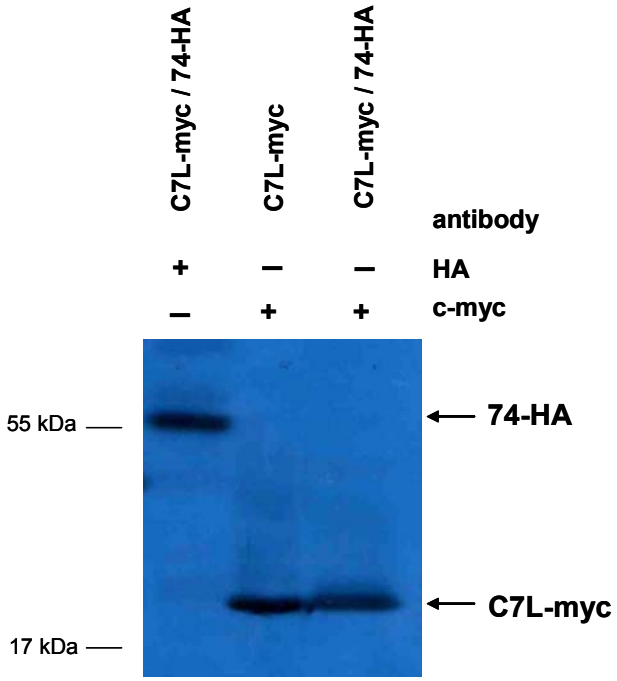


Fig. 52: Co-immuoprecipitation of *in* vitro generated C7L-myc and 74-HA
In vitro generated C7L-myc and 74-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 74-HA proteins are indicated on the right.

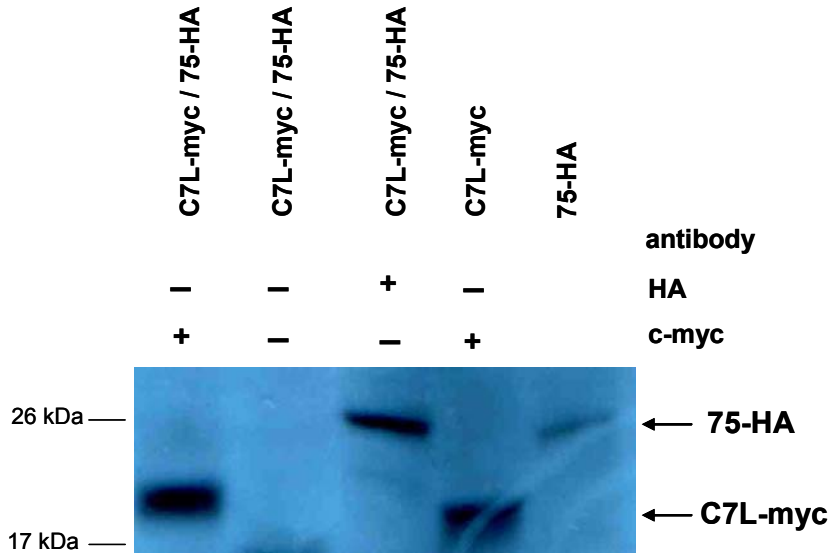


Fig. 53: Co-immuoprecipitation of *in* vitro generated C7L-myc and 75-HA
In vitro generated C7L-myc and 75-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 75-HA proteins are indicated on the right.

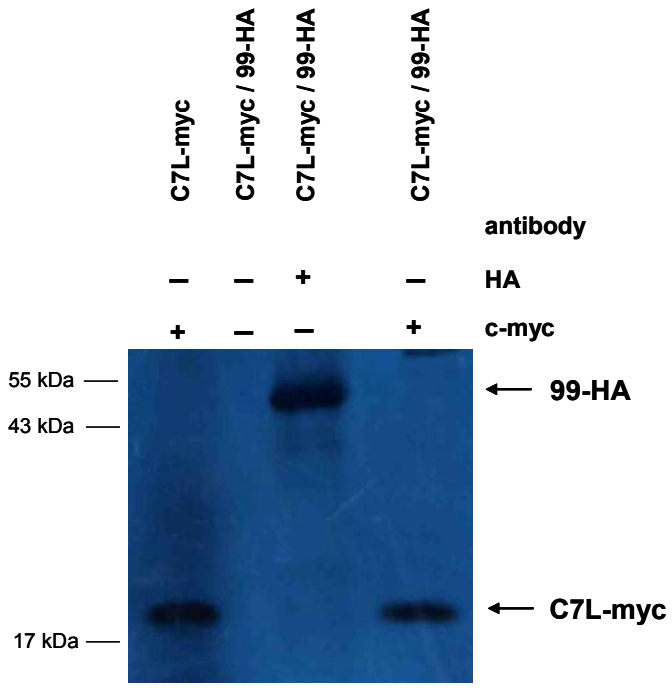


Fig. 54: Co-immuoprecipitation of *in* vitro generated C7L-myc and 99-HA
In vitro generated C7L-myc and 99-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 99-HA proteins are indicated on the right.

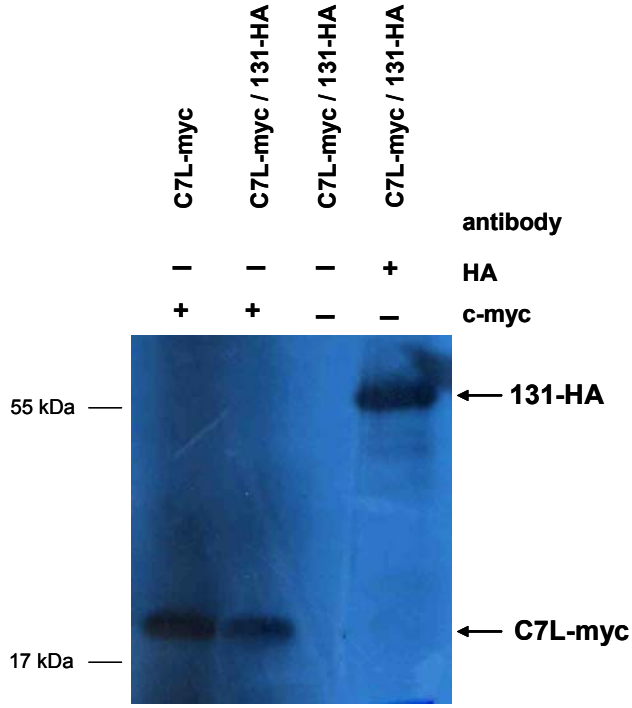


Fig. 55: Co-immuoprecipitation of *in* vitro generated C7L-myc and 131-HA
In vitro generated C7L-myc and 131-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 131-HA proteins are indicated on the right.

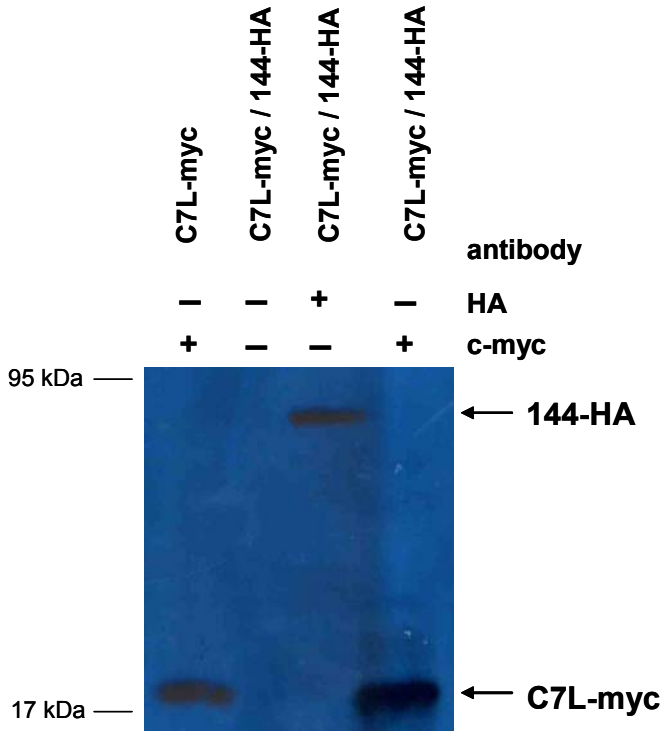


Fig. 56: Co-immuoprecipitation of *in* vitro generated C7L-myc and 144-HA
In vitro generated C7L-myc and 144-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 144-HA proteins are indicated on the right.

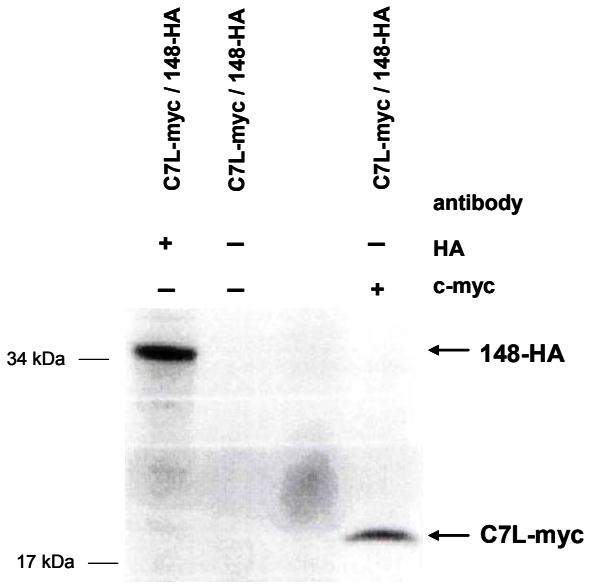


Fig. 57: Co-immuoprecipitation of *in* vitro generated C7L-myc and 148-HA
In vitro generated C7L-myc and 148-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 148-HA proteins are indicated on the right.

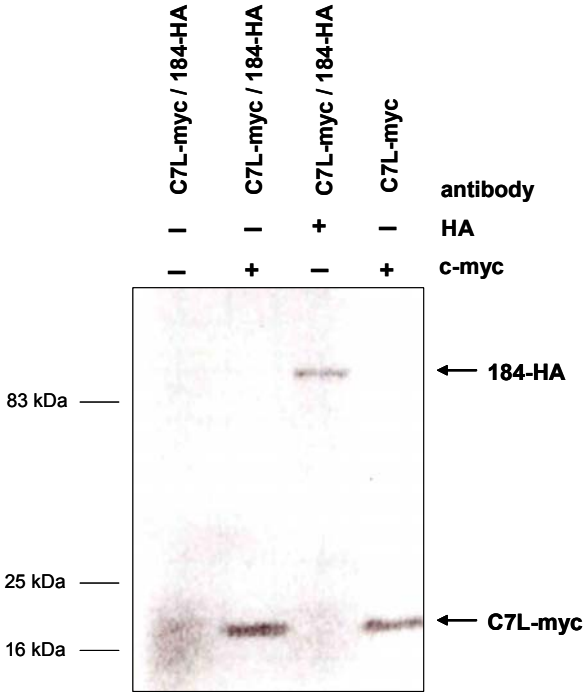


Fig. 58: Co-immuoprecipitation of *in* vitro generated C7L-myc and 184-HA
In vitro generated C7L-myc and 184-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 184-HA proteins are indicated on the right.

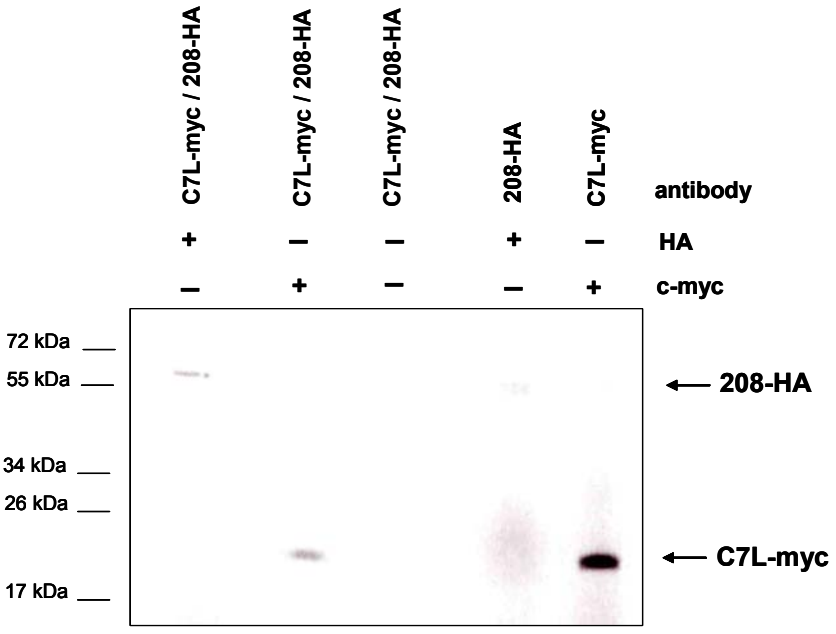


Fig. 59: Co-immuoprecipitation of *in* vitro generated C7L-myc and 208-HA
In vitro generated C7L-myc and 208-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 208-HA proteins are indicated on the right.

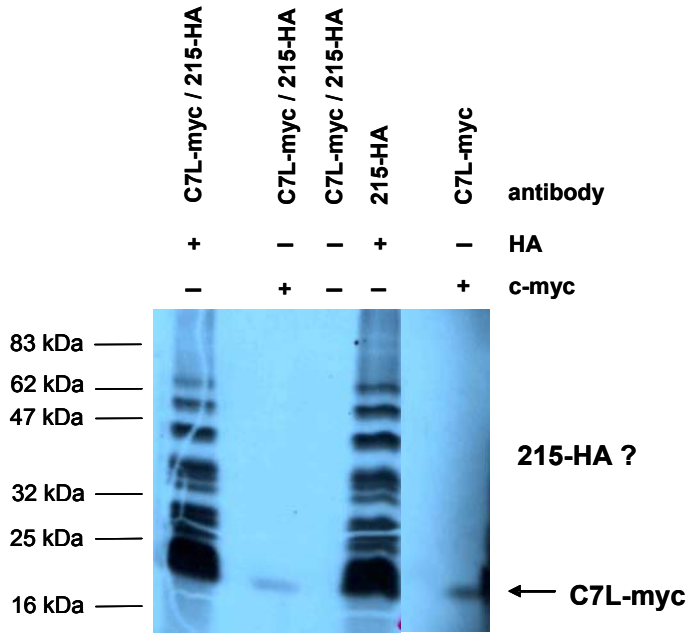


Fig. 60: Co-immuoprecipitation of *in* vitro generated C7L-myc and 215-HA
In vitro generated C7L-myc and 215-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 215-HA proteins are indicated on the right.

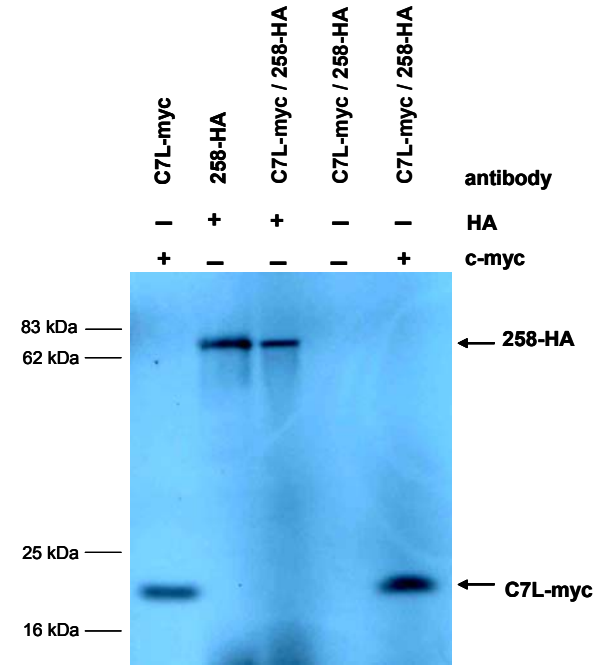


Fig. 61: Co-immuoprecipitation of *in* vitro generated C7L-myc and 258-HA
In vitro generated C7L-myc and 258-HA proteins were mixed, incubated with protein G agarose and immunoprecipitated with the indicated antibody directed against the c-myc- or the HA-tag antibody and analyzed by SDS-PAGE and autoradiography. The positions of the C7L-myc and 258-HA proteins are indicated.

By using the *in vitro* transcription/translation system it was possible to generate ^{35}S-methionine labeled putative C7L interacting proteins with the correct predicted sizes. Furthermore, the proteins were precipitated successfully with an antibody directed against the HA-tag indicating that the generated proteins contain the correct in frame fusion to the HA-tag.
However, by performing co-immunoprecipitation experiments with these *in vitro* generated proteins and *in vitro* generated C7L, it was not possible to confirm any interaction of C7L with a putative interacting protein that has been isolated with the yeast-two-hybrid method (Fig. 48-61).

4.8. The C7L gene is required for MVA late gene expression *in vivo*

It has been suggested that the induction of primary cytotoxic T cell responses to VACV protein antigens correlates with antigen expression and accumulation (Bronte et al, 1997, Gasteiger et al. 2007, Wyatt et al. 2008). Therefore, the specific immune response against late viral proteins should be significantly decreased after infection with MVA-ΔC7L as compared to MVA. To test this hypothesis, C57BL/6 mice were vaccinated with 10^8 IU of MVA-ΔC7L-P11LZ or MVA-P11LZ. Eight days post infection, splenocytes were harvested and the number of cytotoxic T cells specific for different early and late viral proteins was detected by intracellular cytokine staining. In both groups of mice similar frequencies and absolute numbers of CD8+ T cells specific for early viral antigens (B8R and K3L) were detected, indicating that both viruses had similar overall immunogenicity. In contrast, the T cell responses against late viral proteins were completely abrogated (A19L and the recombinant ß-galactosidase) or strongly reduced (A3L) in MVA-ΔC7L-P11LZ as compared to MVA-P11LZ infected mice (Fig. 62).

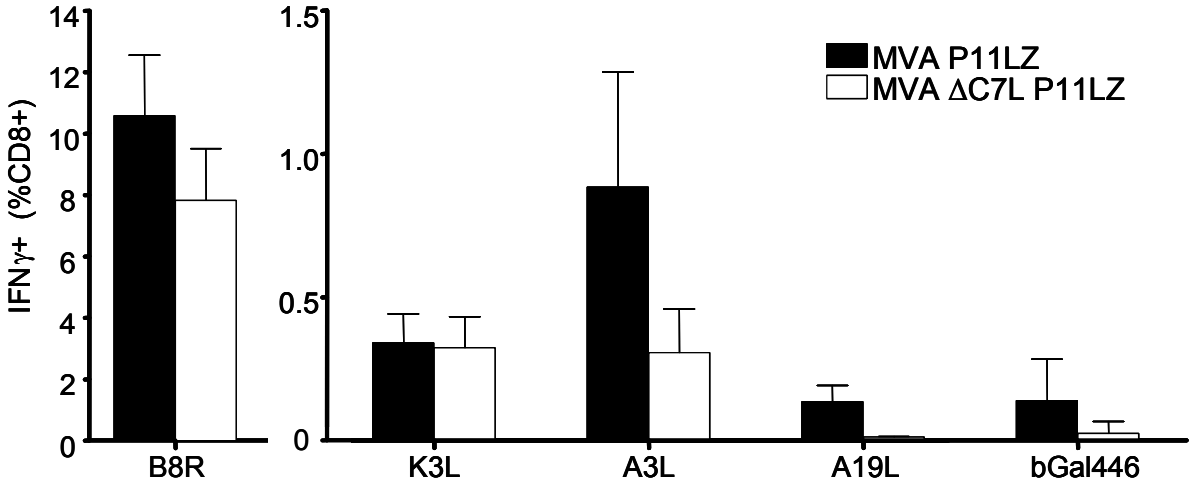


Fig. 62: Loss of T cell priming against late viral antigens upon vaccination with MVA-ΔC7L-P11LZ
C57 BL/6 mice were vaccinated i.p. with 10^8 IU of indicated viruses. Antigen-specific CD8+ T cell responses on day 8 post vaccination are indicated as the percentage of CD8+ splenocytes producing IFNγ in response to the indicated peptides.

This residual response against A3L raised the question whether there could be limited late gene expression in infected animals or whether the protein amount contained in the viral particles of MVA-ΔC7L-P11LZ was sufficient to induce the residual T cell response against the major viral core protein A3L (which accounts for 11% of the virion mass (Sarov and Joklik 1972)).To test this, the experiment was repeated with a third group of animals that received the same amount of MVA-ΔC7L-P11LZ particles but the viral particles were inactivated by psoralen-UVA-treatment (PUVA) to inhibit late viral gene expression which allows then to monitor for effects induced by viral protein input without gene expression (Tsung et al., 1996). The applied protocol achieved complete inactivation as no plaque formation was detectable when titrating PUVA-treated viruses on permissive CEF cells (data not shown). Further, kinetic FACS-analysis of cells infected with PUVA-treated or un-treated MVA-GFP revealed that early as well as late gene expression was efficiently inhibted by PUVA-treatment (data not shown)

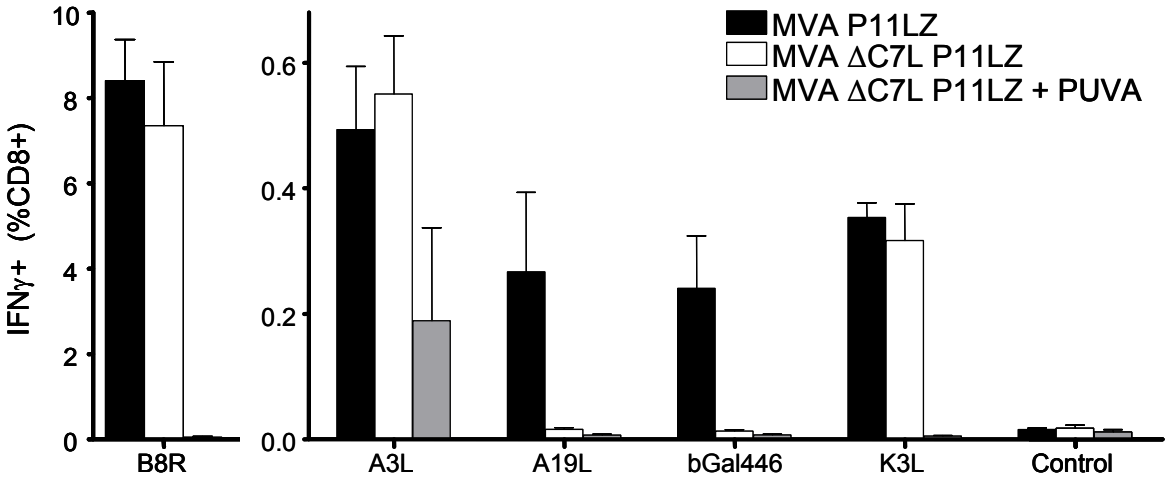


Fig. 63: T cell priming requires viral gene expression
C57 BL/6 mice were vaccinated i.p. with 10^8 IU of indicated viruses. Antigen-specific CD8+ T cell responses on day 8 post vaccination are indicated as the percentage of CD8+ splenocytes producing IFNγ in response to the indicated peptides.

Again, vaccination with MVA-ΔC7L-P11LZ induced T cell responses against early viral antigens (B8R, K3L) that were comparable to vaccination with MVA-P11LZ. In contrast, the responses against the late antigens A19L and β-galactosidse were completely abrogated when using the C7L-knock-out virus. In this experiment, the A3L-specific response was comparable for both viruses. Interestingly, PUVA-inactivated virus did only induce a A3L-specific T cell response (Fig. 63). This observation indicates that viral gene expression is necessary to induce efficient T cell induction with the potential exception for proteins that are highly abundant in the viral particles.

To circumvent the need for high viral input in order to induce robust T cell responses the experiment was repeated with HLA-A*0201-transgenic HHD mice. These mice have the advantage that a relatively low viral dose (10^7 IU) induces strong responses specific for late viral proteins (A6L, I1L and H3L). Indeed, vaccination with MVA-P11LZ induced strong T cell responses against late viral proteins. When mice were infected with MVA-ΔC7L-P11LZ, no responses against late viral proteins were detectable. Consistent with the previous experiments, the response against an early protein (B22R) was comparable in both groups, albeit low. Similar to C57BL/6 mice, HHD mice showed a comparable increase in the number of splenocytes per organ, the frequency of CD8+ T cells and the expression of activation markers in both groups when compared to naïve mice (Fig. 64).

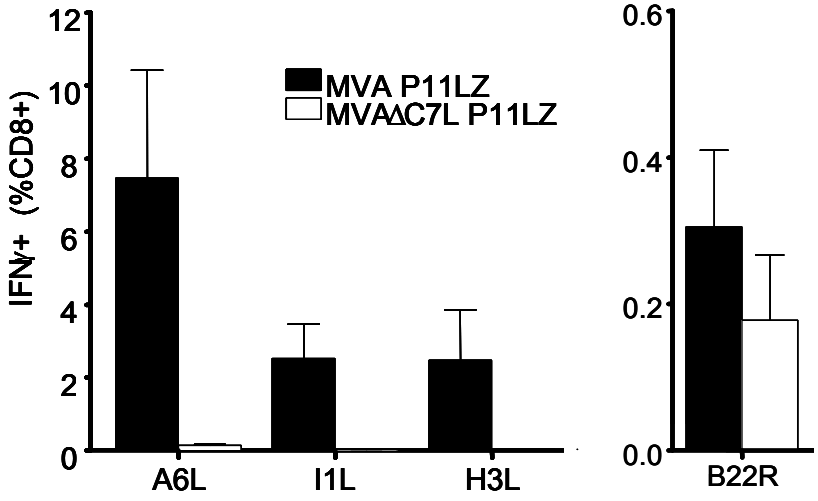


Fig. 64: Lack of C7L specifically abrogates T cell priming for late viral antigens
HHD mice were vaccinated i.p. with 10^7 IU of indicated viruses. Antigen-specific CD8+ T cell responses on day 8 post vaccination are indicated as the percentage of CD8+ splenocytes producing IFNγ in response to the indicated peptides.

To test for humoral immunity, an ELISA was established to detect ß-galactosidase-specific antibodies in the sera of mice that were vaccinated with gradient-purified MVA-P11LZ, MVA-ΔC7L-P11LZ or PUVA-inactivated MVA-ΔC7L-P11LZ. C57BL/6 mice were vaccinated i. p. with 10^8 IU of each virus and 21 days post infection blood was collected. Production of β-galactosidase-specific immunoglobulins (IgM/IgG) was detectable only in sera from mice that were vaccinated with MVA-P11LZ but not in sera from mice vaccinated with MVA lacking C7L or PUVA inactivated virus (Fig. 65).

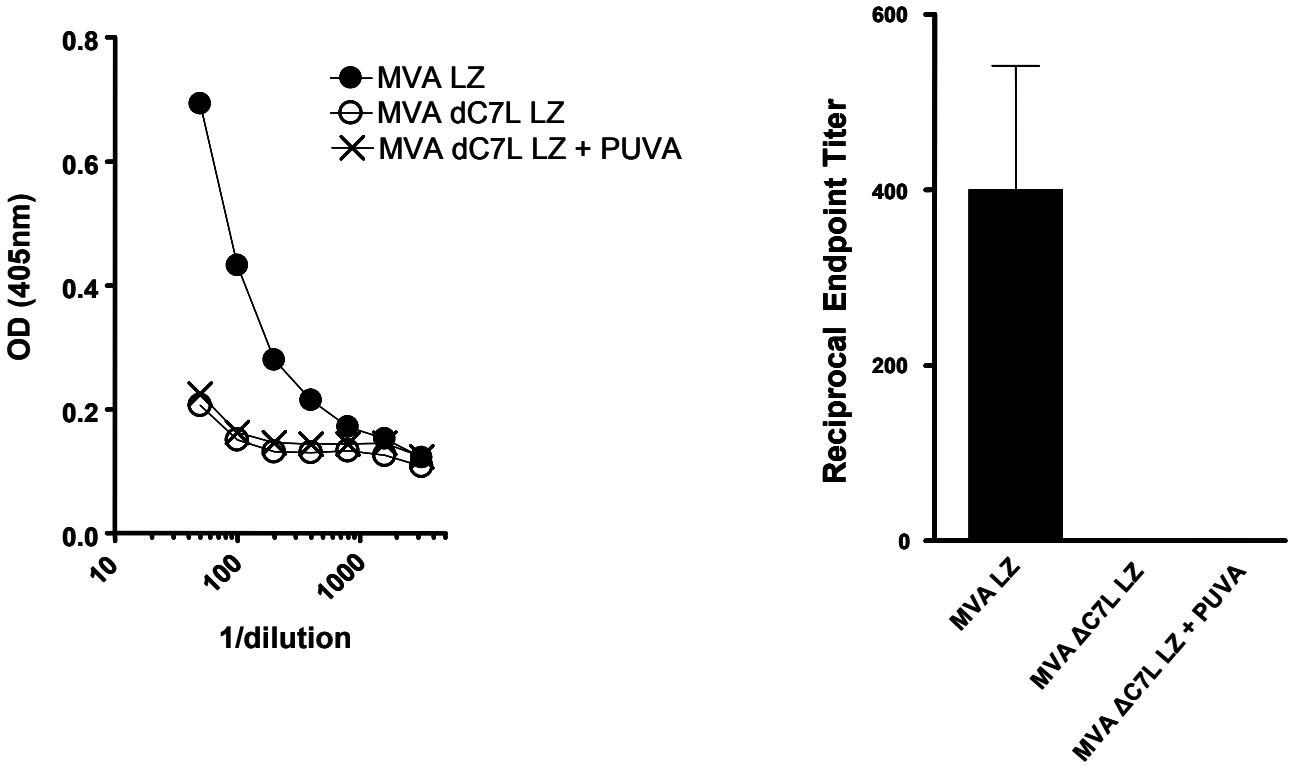


Fig. 65: Induction of β-galactosidase-specific antibodies
C57 BL/6 mice were vaccinated i.p. with the indicated viruses and sera were collected at day 21 p.i.. ELISA was conducted at serial dilutions (left) and reciprocal endpoint titers (right) were determined as the highest dilution resulting in an OD over the mean ODs of wt and naïve sera (not shown). No β-galactosidase-specific IgM/IgG was detectable in sera from mice vaccinated with MVA lacking C7L or PUVA inactivated virus.

5. Discussion

The attenuated vaccinia virus strain MVA is a promising candidate for a safe smallpox vaccine as well as for the development of new recombinant vaccines against infectious diseases and cancer. Due to its ability to induce an efficient immune response against recombinant and viral antigens, MVA is among those viral vectors that have been studied most extensively in preclinical and clinical research (Harari et al., 2008; Dorrell et al. 2006; McShane et al. 2004, Amara et al. 2001; Ramirez et al., 2000; Sutter et al., 1994). Vaccination with MVA protected monkeys and mice against a lethal poxvirus challenge (Belyakov et al., 2003; Earl et al., 2004). Importantly, the avirulence of MVA has been documented by infection of newborn, irradiated and SCID mice as well as immune-suppressed macaques (Meyer et al., 1991; Stittelaar et al., 2001; Wyatt et al., 2004). Most convincingly, MVA has been safely administered to more than 100,000 humans during the smallpox vaccination program, including individuals considered at high risk for conventional smallpox vaccination (e.g. immunocompromised, elderly, patients with atopic skin diseases) without any report of the adverse effects associated with other vaccinia virus vaccines (Mayr et al., 1978; Stickl et al., 1974). Similarly, the therapeutic administration of high-dose recombinant MVA to HIV-infected individuals without any documented complications further outlines the excellent safety profile of MVA vaccines (Cosma et al., 2003; Dorrell et al., 2006; Harrer et al., 2005).

Rationales for a further development of vaccines based on MVA might be deducted from a thorough understanding of the viral strategies for infecting cells and exploiting the cellular machinery for production of virally encoded proteins. In this process, the viral host range genes play critical roles. Until now, the only well-characterized VACV host range gene is the early expressed E3L (Chang et al., 1992). E3L encodes a double-stranded RNA (dsRNA)-binding domain that is required for inhibition of the cellular dsRNA-dependent antiviral enzymes PKR and 2`5`oligo-adenylate synthetase (Chang and Jacobs, 1993; Rivas et al., 1998; Watson et al., 1991). C7L is among the few conserved open reading frames which were described to play a role for the host range of VACV in human and murine cells (Oguiura et al., 1993; Perkus et al., 1990; Meng et al., 2007). However, C7L has only been studied in the context of replication competent VACV and no detailed phenotype was described so far. Therefore the present work aimed to characterize the role of C7L within the replication cycle of vaccinia virus MVA in permissive and

non-permissive cells. Several strategies were used to investigate the function of C7L. First, the temporal expression of C7L was analyzed. Second, it was tested, at which stage the block of viral replication occurs (viral gene expression, viral DNA replication, translation initiation, apoptosis). Next, it was investigated, whether defined functional domains of C7L would be sufficient to overcome this replicative block. Then potential interaction partners were identified by a yeast-two-hybrid screen and subsequently tested for interaction in co-immunoprecipitation experiments. Ultimately, consequences of C7L expression for *in vivo* expression of viral genes and immunogenicity of MVA were studied.

Temporal Expression of the C7L protein within the MVA lifecycle

Initial experiments performed in this work with MVA C7L-deletion mutant viruses indicated that C7L is required to promote expression of late viral proteins of MVA in non-permissive human and murine cells. Presence of C7L was not required for productive replication of MVA in permissive CEF and BHK cells. This result is consistent with the role of C7L in replication competent strains of VACV where C7L is also required to allow late protein synthesis and productive infection of a K1L deletion mutant VACV in human and murine cells (Meng et al., 2007; Perkus et al., 1990; Oguiura et al., 1993). Analysis of lysates of MVA infected cells revealed that C7L is detectable as early as two hours post infection and in the presence of AraC, an inhibitor of VACV DNA replication and therefore of late protein synthesis, confirming that C7L belongs to the early expressed class of genes (Oguiura et al. 1993). Moreover, C7L is still detectable six hours post infection. If C7L is continuously synthesized during the infectious cycle or if the C7 protein is relatively stable in infected cells requires further experiments.

DNA-Replication of MVA and MVA-ΔC7L

Replication of VACV requires successful progression through a series of temporally regulated steps where viral late gene expression is dependent on the previously completed DNA replication of the viral genome. Until now, several defects in protein synthesis during abortive replication of Orthopoxvirus host range mutants in a variety of cell lines have been reported. Hsiao et al. (2004) found that a VACV mutant with deletions of both C7L and K1L (VACV-ΔK1LΔC7L) was blocked at the stage of viral intermediate gene translation in HeLa cells. Viral DNA replication occurred unimpaired but levels of intermediate and late genes were greatly reduced. In RK13 cells deletion of the K1L open reading frame from VACV is sufficient to block viral DNA replication and synthesis of viral late mRNAs or proteins (Ramsey-Ewing and Moss 1996), suggesting a difference from the host restriction phenotype obtained with VACV-ΔK1LΔC7L in HeLa cells. To more precisely determine at which stage the life cycle of MVA-ΔC7L is blocked the viral DNA synthesis of MVA and MVA-ΔC7L in HeLa cells was determined. The conducted experiments showed that viral DNA-replication occurred in MVA-ΔC7L-infected human HeLa cells at comparable levels to MVA-infected cells, demonstrating that the replicative block induced by deletion of C7L occurs at a step post DNA replication.

MVA-ΔC7L is not an Inducter of Apoptosis

Infection of HeLa cells with a VACV host range mutant containing deletions of K1L and C7L genes results in caspase-2 and caspase-3 activation and induction of apoptosis (Hsiao et al., 2004). Furthermore, it has been demonstrated that C7L is able to inhibit caspase dependent apoptosis in HeLa cells induced by the NYVAC strain of VACV (Najera et al., 2006). The attenuated NYVAC virus was derived from the Copenhagen strain of VACV by the targeted deletion of 18 genes including K1L and C7L and is not able to grow on cell lines of human origin, as well as RK13 and pig kidney cells (Tartaglia et al., 1992a+b, 1994). Re-insertion of the C7L coding sequence into the NYVAC genome resulted in inhibition of caspase-3 dependent induction of apoptosis in HeLa cells (Najera et al., 2006). Caspase-3 is one of the key executioners of apoptosis, as it is responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri et al., 1994). Analysis of caspase-3 activation in MVA-ΔC7L infected HeLa cells revealed no difference to MVA infected cells suggesting that the replicative block displayed by MVA-ΔC7L is independent of the induction of apoptosis in human cells.

Influence of C7L on Initiation of Cellular Translation

The phosphorylation of the α subunit of the eukaryotic translation initiation factor 2 (eIF2α) by the protein kinase R (PKR) is known to be a cellular major stress response during viral infections that leads to the down regulation of global translation initiation (Rowlands et al., 1988). During eukaryotic translation initiation, the pre-initiation complex consisting of methionyl initiator tRNA, the 40S ribosomal subunit, eIF2 and GTP binds to the mRNA and scans for the AUG start codon. After base pairing between Met-$tRNA_i$ and AUG the GTP bound to eIF2 is hydrolyzed. Subsequently eIF2-GDP is released and the 60S ribosomal subunit binds to form the 80S initiation complex. The released eIF2-GDP must be recycled back to eIF2-GTP by the guanine nucleotide exchange factor eIF2B before it can be used in the formation of another pre-initiation complex (Hinnebusch, 2000; Krishnamoorthy et al., 2001). This process of translation initiation is controlled by a conserved mechanism involving the phosphorylation of the α subunit of eIF2. Phosphorylated eIF2α has an increased affinity for eIF2B, and this high affinity binding prevents the recycling process (Kimball, 1999 and Krishnamoorthy et al., 2001). Four different eIF2α kinases have been identified in mammalian cells including the dsRNA-dependent PKR (Kaufman, 1999). In response to the dsRNA synthesized during many viral infections, PKR becomes activated, phosphorylates eIF2α, and prevents viral replication by blocking translation initiation. In the context of VACV, it has been shown that infection of HeLa cells with NYVAC results in a translational block late in infection that correlates with an increase in eIF2α phosphorylation. Upon re-introduction of the C7L gene into the NYVAC genome the levels of phosphorylated eIF2α decreased in HeLa cells followed by restoration of late protein synthesis. Furthermore, NYVAC retained the ability to replicate on HeLa cells (Najera et al., 2006). Similar results were obtained with the VACV K1L and C7L deletion mutant virus (Hsiao et al., 2004). Nevertheless, MVA-ΔC7L infection of HeLa cells did not induce eIF2α phosphorylation. Western blot analysis of MVA and MVA-ΔC7L infected cells performed in this work revealed no differences in the level of eIF2α phosphorylation between both viruses. This result may be explained in part by the different genetic backgrounds of MVA, NYVAC and VACV.

Compensation of MVA-ΔC7L Phenotype by VACV K1L

C7L and K1L were reported as functionally equivalent host range genes that regulate VACV replication on human cells despite the absence of any evident sequence similarities (Perkus et al., 1990; Oguiura et al., 1993; Ramsey-Ewing and Moss, 1996). Infection of HeLa cells with a VACV mutant deleted of both genes results in a replicative block at the stage of viral intermediate gene translation (Hsiao et al., 2004). Insertion of K1L or C7L into the mutant is sufficient to overcome the replicative block and allow permissive replication. MVA lacks a functional K1L gene (Antoine et al., 1998) but insertion of K1L into MVA extends the host range of the virus only to RK13 cells and not to human cells although MVA-K1L also contains a functional C7L gene. It was interesting to investigate, if K1L expression would be sufficient to restore viral late gene expression of MVA-ΔC7L in human and murine cells. Experiments revealed that cellular and viral expression of K1L restored late gene expression of C7L deletion mutant viruses demonstrating that K1L can also compensate for C7L function in the MVA life cycle in human cells. Furthermore, this compensatory function of K1L could be extended to murine cells, which has not been investigated before.

Identification of Essential Domains for C7L function

The orthopoxvirus host range genes K1L and CP77 contain ankyrin repeats and for both proteins the ankyrin repeats that are essential to promote viral replication in different cell lines have recently been determined (Meng and Xiang, 2006; Hsiao et al., 2006). A data bank search for identification of domains using "PROSITE" did not identify any yet described domain or sequence motif contained within the C7 protein although C7L is highly conserved among orthopoxviruses (95-100% homology on amino acid level). The domain search revealed six potential phosphorylation- and one potential glycosylation-site contained within the C7 protein but these patterns are found frequently in many proteins. In order to identify essential region(s) of the C7 protein that mediate late gene expression in human and murine cells, distinct mutations were introduced into C7L and mutant constructs were investigated for their ability to rescue late gene expression in the transient-transfection reporter gene assay established in this work. Results indicate that nearly the whole protein is required to allow late gene expression of MVA, with the exception of the C-terminal 12 amino acids. Although all constructs displayed lower expression levels than the full length C7L, the mutants ΔC7, ΔC8, ΔC9, ΔC10 and ΔC12 were able to rescue late gene expression of MVA-ΔC7L-P11-LZ. However, the possibility that higher expression levels of ΔC16, ΔC32, ΔN4, ΔN8, ΔN16, ΔN32 could have resulted in rescue could not totally be ruled out. In contrast to C7L, a screen performed to identify essential parts of the host range gene K1L revealed that the protein possesses a distinct set of ankyrin repeats that alone are essential for replication of VACV in human or rabbit cells (Meng and Xiang, 2006). All the C7L deletion mutants constructed in this work showed a comparable phenotype in human and murine cells. Meng et al. (2007) analyzed distantly related C7L homologues from diverse mammalian poxviruses for their abilities to regulate VACV cellular tropism. Interestingly, all functionally equivalent C7L homologues that were identified in this study were more conserved in the N-terminal 10 amino acids than functionally non-equivalent homologues. Here, it was found that all the N-terminal deletion mutant constructs tested were unable to mediate late protein synthesis, which further suggests that these N-terminal residues could have a functional role.

Identification of Potential Cellular Interaction Partners for the MVA C7 Protein

Part of this work was the performance of a yeast-two-hybrid screen for identification of putative C7L interacting proteins. The yeast-two-hybrid assay proved to be one of the most efficient techniques for detection of new interactions (Fields and Song 1989; Drees 1999; Frederickson 1998). The procedure is simple, inexpensive and detects protein interactions *in vivo*. Furthermore, it has the important advantage that no previous knowledge about the interacting proteins is necessary for a screen to be performed. Therefore, the yeast-two-hybrid system seemed to be the method of choice to identify putative C7L interacting proteins.

Initial experiments that were performed with the constructed GAL4 BD-C7L fusion protein indicated that C7L is a suitable bait for a yeast-two-hybrid screen. It was demonstrated that the C7L fusion protein is stably expressed within yeast cells. Furthermore, C7L displayed no toxicity or auto-activation of the reporter genes upon expression in yeast cells. Under consideration of the study of McCraith et al. (2000) it was particularly important to exclude any self activating feature of C7L. McCraith et al. expressed all open reading frames that are encoded by the VACV genome as two-hybrid bait and prey proteins and tested most of the ~70000 pairs by yeast-two-hybrid assay. In their system C7L was found to be a strong auto-activator when fused to the GAL4 DNA-binding domain. However, they used different plasmids for the construction of the GAL4 BD fusion proteins and a different yeast strain.

As C7L is essential for the viral life cycle of MVA in human cells, a cDNA library derived from human cells was utilized for the yeast-two-hybrid screen. Using a library generated from cells in which the bait protein is known to be relevant will reduce the number of false-positive signals and produce more biologically relevant interactions. Therefore it was confirmed that C7L is required to promote late viral gene expression in human B-LCLs and then a cDNA library derived from EBV-immortalized human lymphocytes was chosen to identify putative C7L interacting proteins.

The yeast-two-hybrid screen of this cDNA library with the C7L-bait construct resulted in 119 clones that displayed activation of both reporter genes. The isolated clones were sequenced and subsequently analyzed for their auto activating property. 14 independent clones displayed no reporter gene activation without co-expression of the C7L bait construct.

The yeast-two-hybrid system is frequently producing a significant number of false-positives. The exact rate of false positive results is not known, but it has been estimated that as much as 50% of the identified interactions may be unreliable (Deane et al., 2002). False-positives may include activation domain fusion proteins that directly bind to promoters and activate the reporter gene expression. In this work the use of two different reporter gene constructs with different promoter structures should have eliminated many of these false-positive results. For this, in this work, only those clones were considered as positive that showed activation of both reporter genes.

Furthermore, auto-activators were excluded by co-transforming the plasmids encoding the potential C7L interacting proteins together with a plasmid containing only the GAL4 DNA-binding domain. In addition, it would have been good to test the interactions with reciprocal hybrids. This means to analyze if the interaction between C7L and the putative interaction partners is still detectable if C7L is fused to the AD and the library clones to the BD. Unfortunately, this experiment could not be performed because a library derived from EBV-immortalized human lymphocytes with cDNAs fused to the GAL4 DNA-binding domain was not available.

Moreover, one could test whether the proteins isolated from the library screen do interact with proteins unrelated to the test protein to eliminate “sticky” proteins. However, a yeast-two-hybrid screen of the same library performed under the same experimental conditions with the EBNA3a protein of Epstein Barr virus as bait did not identify any overlapping cDNA clones, excluding unrelated interactions.

For the HIS3 reporter gene, the assay was also optimized to reduce false-positives by the inclusion of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the HIS3 gene product in the media to reduce background due to basal HIS3 expression. Other false-positives that could not be excluded by the measures discussed above may include proteins that contain domains commonly involved in forming protein-protein interactions, but for which there is no physiological context for an interaction with C7L.

Most false-positive interactions cannot be identified without additional experiments. Therefore, alternative methods are required to confirm true protein-protein interactions that were detected using the two-hybrid system. In this work, co-immunoprecipitation experiments with *in vitro* translated proteins were performed for verification of the identified interacting proteins. Unfortunately, it was not possible to confirm any putative interaction partner with this method. As the *in vitro* generated proteins used in this work do not contain post-translational modifications, additional co-immunoprecipitation experiments with *in vivo* expressed proteins could potentially reveal interactions.
As C7L does contain some putative phosphorylation and glycosylation sites and some, but not all post-translational modifications do occur in yeast, interactions that depend on such modifications would be missed in the applied approaches.

Although the yeast-two-hybrid system offers many advantages over biochemical methods, it still has several intrinsic drawbacks. Another main problem besides the generation of false-positive results is the use of fusion proteins. By fusing a protein to the GAL4-BD or –AD the conformation of the protein may be altered and binding sites may become inaccessible. In most two-hybrid vector systems the DNA-binding domain and the activation domain are fused to the amino-terminal end of the test protein. In some cases, where interactions occur at the N-terminus of the test proteins, the presence of the DNA-binding or activation domain may cause problems. Therefore, the bait construct should be analyzed for its biological active conformation upon expression in yeast cells. This is usually done by testing the bait protein with a known interaction partner for interaction in the yeast-two-hybrid assay. This will only work if both proteins are folded correctly. Unfortunately, this control could not be performed as no interaction partner of C7L has been reported until today. Additionally, a known interaction partner of C7L could have served as a positive control throughout the whole screen. Isolation of that known interaction partner out of the cDNA library clones would have indicated that the screen did work satisfactory.

Another disadvantage of the two-hybrid method is the identification of non-relevant interaction partners. By screening all combinations of protein interactions it is possible that two proteins are able to interact although under physiologic conditions they are never expressed at the same time or in the same cellular compartment.

Furthermore, two-hybrid screens are critical due to a high frequency of false-negative results or missed interactions. This is emphasized by the minimal overlap of published datasets generated for the same organism (Li et al., 2004; Rual et al., 2005; Giot et al., 2003; von Mering et al., 2002). One reason for this incomplete coverage is that the clone sets used in the screens are incomplete. A second factor is that compared to *E. coli* transformations, yeast transformations are generally inefficient. Nevertheless, the yeast-two-hybrid system offers a very sensitive method because transient and weak interactions, which are often important in signaling cascades, are more easily detected in two-hybrid systems since the genetic reporter gene strategy results in significant signal amplification (Estojak et al., 1995). Possibly the interactions identified with the two-hybrid system in this work are too weak to be confirmed with the co-immunoprecipitation experiments.

Considering the identified clones and their published functions it is difficult to speculate if they could indeed interact with C7L. Despite its apparent important function in the MVA or VACV life cycle only little information exists about C7L. For example, the sub-cellular localization is not yet known but could help to exclude some of the identified partners.

Among the identified putative interaction partners are two ribosomal proteins (RPS9 and RPS27a) and one Heat shock protein (Hsp90). These proteins and some others are classified as bait-unspecific false-positives identified multiple times in various two-hybrid screens (Hengen, 1997; Golemis, 1997). One reason that argues towards the inclusion of Hsp90 to the putative interaction partners is provided by the results of Hung et al. (2002). They showed that Hsp90 is important to allow VACV the completion of its life cycle in infected cells. But they also identified a viral interaction partner of Hsp90 in their study by co-immunoprecitpitation, the VACV core protein A10.

Another interesting protein, that has been detected is KIF3B. VACV was shown to utilize the cellular microtubular network to co-ordinate its complex life cycle (Schramm and Krijnse Locker, 2005). Movement along microtubules is mediated by different motor proteins like kinesin-1 and kinesin-2 (Hirokava 1998; Vale 2003). For kinesin-1 it has already been demonstrated that it contributes to VACV morphogenesis. KIF3B is a motor subunit of kinesin-2 (Brown et al., 2005). Therefore, one can speculate that KIF3B could also play a role for VACV replication.

Nevertheless, in this work it was not possible to confirm an interaction of KIF3B with C7L by co-immunoprecipitation.

Some identified clones are described to be present in the nucleus of cells (TTF1, BRD2 and BRD7). Although VACV replication occurs entirely in the cytoplasm of the host cell relatively independent of host proteins it becomes more and more evident that the virus recruits some host nuclear proteins to the cytoplasm (Oh et al., 2005; Hsiao et al., 2006; Katsafanas and Moss 2007). Therefore, also proteins with a primary nuclear localization could represent potential C7L interaction partners.
Unfortunately, some of the identified putative C7L interacting proteins remain relatively uncharacterized until today. Therefore, it is quite difficult to speculate about the biological relevance of some of the recovered interactions.

Relevance of C7L expression for *In vivo Life Cycle of MVA* and Immunogenicity

The *in vitro* data obtained with the ß-Galactosidase-assay and the ^{35}S-methionine labeling experiments showed that a MVA mutant lacking the C7L gene is unable to promote late gene expression in the tested human and murine cell lines. To assess whether late gene expression was also abolished *in vivo*, the immunogenicity of the C7L knockout viruses was tested. Mice were immunized and analyzed for the production of antibodies and cytotoxic T cells against early and late viral antigens. These approaches were chosen because it is generally accepted that the induction of antibodies correlates with the availability of viral antigen. Moreover, two recent studies showed, that the primary induction of cytotoxic T cells correlates with the level of viral antigen synthesis (Gasteiger et al. 2007, Wyatt et al. 2008). Consistent with the prediction that mice vaccinated with MVA-ΔC7L-P11-LZ would synthesize only early viral proteins these mice had unaltered T cell responses against early viral antigens but failed to prime T cells against late viral antigens. This phenotype was consistently observed in wt and HLA-transgenic mice for multiple viral antigens with the notable exception of the A3 protein, which is expressed late during the infection cycle. Mice were able to mount A3-specificT cell responses irrespective of the presence of the C7L gene in the applied viruses. A3 is the major core protein and is abundantly contained in the viral particles (~11% of the virion mass) (Sarov and Joklik 1972). Therefore it was hypothesized that in the case of such abundant proteins present in the viral input in combination with the concurring viral infection could be sufficient for T cell induction without

the need for antigen neo-synthesis. This hypothesis is supported by the observation that mice vaccinated with psoralen/UV-inactivated virus (i.e. with viral input leading to no antigen expression) did induce T cell responses only against the A3 protein and not against any of the other tested early or late antigens. This data suggested that it should be more accurate to correlate immunogenicity with *in vivo* expression levels for model antigens that are not included into the viral particles, or at least not in significant amounts. Therefore, an ELISA was established to detect ß-galactosidase-specific antibodies and mice were vaccinated with gradient-purified viruses containing the LacZ-reporter-gene under strict control of a late viral promoter. As observed with the T cell assays, immunogenicity of ß-Gal was lost when infecting with the C7L-deficient virus.

Although the methods that were available to estimate viral antigen synthesis *in vivo* provide rather indirect evidence, the results convincingly show that the C7L gene is required to produce at least significant amounts of late viral proteins. Together with the stringent *in vitro* data the results obtained from live viral infections strongly suggest that MVA lacking the C7L gene is unable to promote late viral gene expression in infected mice.

List of Abbreviations

3-AT	3-Amino-1, 2, 4-triazol
Ade	Adenine
Amp	Ampiciline
AP	alkaline phosphatase
APS	Ammoniumperoxodisulfate
bp	Base pairs
BHK-21	Baby hamster kidney cells
BSA	Bovine serum albumin
cDNA	Copy DNA
CEF	Chicken Embryo Fibroblasts
Ci	Curie
CTL	Cytotoxic T cell
CVA	Chorioallantois vaccinia Ankara
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide tripohosphate
EBV	Epstein-Barr Virus
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked-Immunosorbent-Assay
EEV	Extracellular enveloped virus
EMA	Ethidium monazide
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAL4-AD	Activation domain of transcription factor GAL4
GAL4-BD	DNA-binding domain of transcription factor GAL4
GFP	Green fluorescent protein

His	Histidine
h.p.i.	Hours post infection
i.p.	Intraperitoneal
Kan	Kanamycin
kb	kilo bases
kDa	kilo dalton
ICS	Intracellular cytokine stain
IEV	Intracellular enveloped virus
IFNγ	Interferon γ
Ig	Immunoglobulin
IMV	Intracellular mature virus
IU	Infectious units
kDa	Kilo Dalton
LCL	lymphoblastoid cell line (primary EBV infected B-cell)
Leu	Leucine
M	Mol
Min	minute
mM	millimolar
mRNA	messenger ribonucleic acid
MOI	Multiplicity of infection
MVA	Modified Vaccinia virus Ankara
MW	Molecular weight
NLS	Nuclear localization signal
OD	optical density
ORF	open reading frame
PEG	Polyethyleneglycol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PMSF	Phenylemethylsulfonylfluoride
PO	Peroxidase

RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
s.c.	Subcutaneous
SD	yeast selective medium
SDS	Sodium dodecyl sulphate
SSC	Sideward scatter
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N, N, N‘, N‘-Tetramethylethylendiamine
TRP	Tryptophane
UV	Ultraviolet
VACV	Vaccinia virus
wt	Wild type
YPDA	Adenine-supplemented yeast medium

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