Cell tropism of maedi-visna virus

Eydís Þórunn Guðmundsdóttir

Ritgerð til meistaragráðu
Háskóli Íslands
Læknadeild
Heilbrigðisvísindasvið

HÁSKÓLI ÍSLANDS

Frumusækni mæði-visnu veirunnar

Eydís Þórunn Guðmundsdóttir

Ritgerð til meistaragráðu

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Ágrip

Mæði-visnuveiran (MVV) er lentiveira sem sýkir sauðfé og veldur lungnabólgu og heilabólgu. Aðal markfrumur veirunnar *in vivo* eru átfrumur, en sumir stofnar MVV geta einnig vaxið í öðrum frumugerðum bæði *in vitro* og *in vivo*. Áður hefur verið sýnt fram á að endurtekning á basaröð í LTR (long terminal repeat) MVV veldur því að veiran getur vaxið í æðaflækjufrumum, liðþelsfrumum og bandvefsfrumum auk átfrumna. Einnig hefur verið sýnt fram á að heilasækni MVV tengist þeim stofnum sem eru með tvöfaldaða röð í LTR. Á tvöfaldaða svæðinu eru efliraðir sem umritunarþættir úr frumunni þekkja, því er líklegt að munur sé á stjórn umritunar. Þennan mun sem við fundum á vexti veiranna eftir því hvort þær höfðu einfalda eða tvöfalda röð í LTR var ekki hægt að finna í tjáningarvektorum þar sem LTR með einfalda eða tvöfalda röð var skeytt fyrir framan merkigen. Markmið þessarar rannsóknar var að komast að því á hvern hátt MVV veirur, sem ekki hafa tvöföldun í LTR, eru hindraðar í æðaflækjufrumum. Í því skyni var víxlritun, innlimun og mRNA myndun strax eftir sýkingu borin saman milli stofna með einfalda eða tvöfalda röð í LTR.

LTR með einfalda eða tvöfalda röð var klónað í sýkingarhæfa MVV klóninn KV1772 (stofnar VA3 og VA4). Æðaflækjufrumur voru sýktar með þessum veirum og sýni tekin á nokkrum tímapunktum fyrstu klukkustundirnar eftir sýkingu. Rauntíma PCR var notað til að fylgjast með víxlritun og mRNA myndun í sýktu frumunum. Innlimun var athuguð með því að nota Fluorescent *in situ* hybridization (FISH).

Enginn munur fannst á víxlritun á milli stofnanna, þar sem DNA myndun beggja stofna jókst jafn hratt fyrstu 12 tímana eftir sýkingu. Þegar athugað var hvort munur væri á innlimun VA4 og VA3 kom í ljós að báðar veirugerðir ná að innlima genamengi sitt í litninga hýsilfrumunnar. Munur fannst á mRNA myndun, þar sem mRNA myndun var meiri í VA4, sem hefur tvöföldun í LTR, en í VA3, sem ekki hefur tvöföldun í LTR, eftir 24 og 30 tíma sýkingu.

Þessar niðurstöður benda til þess að frumusækni mæði-visnuveiru sé stjórnað á umritunarstigi. Líklega er hér um að ræða stjórn á litni, sem ekki finnst með tjáningarvektorum.

Abstract

Maedi-visna virus (MVV) is a lentivirus of sheep, mainly affecting the lungs (maedi) and the CNS (visna). The primary target cells of MVV infection *in vivo* are cells of the monocyte/macrophage lineage. However, certain MVV strains can infect various other cell types *in vitro* and by experimental infection *in vivo*. It has been shown previously that a repeated sequence in the LTR of MVV extends the cell tropism of the virus from being strictly macrophage tropic to being able to grow in a variety of cell types, and that this extended cell tropism is associated with neurovirulence. LTR is the virus promoter region and contains enhancer sequences that cellular transcription factors can bind to. It is therefore likely that this difference between the two virus strains is because of transcription regulation. The loss of replication activity in SCP cells that we see in the viruses without a repeat in the LTR was not detected in the context of transcription of a reporter gene in transient transfection. The aim of this study was to find where in the replication cycle of MVV without duplication in the LTR the barrier is. This is done by comparing reverse transcription, integration and mRNA synthesis between virus strains with and without duplication in the LTR.

LTR with and without the duplication was cloned into the molecular clone KV1772. Sheep choroid plexus (SCP) cells were infected with the two virus strains and samples were taken at several time points the first hours after infection. Real-time PCR was used to monitor reverse transcription and mRNA synthesis in infected cells. Integration was detected by using fluorescent *in situ* hybridization (FISH).

No difference was detected in reverse transcription between the two viruses, where the two virus strains produce equal amounts of DNA the first 24 hours of infection. When integration was examined it was shown that both virus strains are capable of integrating the viral DNA into the host chromosome. However, MVV mRNA synthesis was reduced in the cells infected with MVV without the repeat in the LTR.

It therefore appears that the loss of virus replication that we detected in this study is determined by regulation of mRNA, possibly at the level of chromatin that is not detected by transient transfection.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
BBB	Blood brain barrier
bHLB	basic helix-loop-helix
BIV	Bovine immunodeficiency virus
CA	Capsid
CAEV	Caprine arthritis-encephalitis virus
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EIAV	Equine infectious anaemia virus
env	Envelope
ER	Endoplasmic reticulum
FIV	Feline immunodeficiency virus
H1	Histone 1
HAART	Highly active antiviral therapy
HAD	HIV-associated dementia
HAT	Histone acetyltransferase
HDAC	Histonedeacetylase
HIV-1	Human immunodeficiency virus-1
HIV-2	Human immunodeficiency virus-2
HTLV-1	Human T-lymphotropic virus 1
IN	Integrase
IκB	Inhibitor of κB
LTR	Long terminal repeat
MA	Matrix protein
miRNA	micro RNA
MLV	murine leukaemia virus
mRNA	messenger RNA
MVV	maedi-visna virus
NC	Nucleocapsid
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
ORF	Open reading frame
PBMC	Pheripheral blood mononuclear cells
PBS	Primer binding site
PBS	Phosphate buffered saline
PIC	Pre-integration complex
Ppt	Polypurine tract
Pol	Polymerase
p-TEFb	Positive transcription elongation factor b
Rev	Regulator of virion protein expression
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
RNase H	Ribonuclease H
RRE	Rev responsive elements
RT	Reverse transcriptase
RTC	Reverse transcription complex

SCP	Sheep choroid plexus cells
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SU	Surface glycoprotein
TAR	Transactivating responsive element
Tat	Trans-activation protein
TM	Transmembrane glycoprotein
tRNA	transfer RNA
Vif	Virion infectivity factor
VPA	Valproic acid
Vpr	Viral protein R

x

1   Introduction

1.1   Historical background of maedi visna in Iceland

Maedi visna virus (MVV) was introduced to Iceland in 1933, when 20 Karakul sheep where imported from Halle, Germany. According to standard procedures at the time these sheep were certified free of known infectious diseases. It seems that at least two of these twenty sheep were latent carriers of the virus which gave rise to the outbreak in Iceland, in two widely separated areas. In 1939 the disease was recognised by Gíslason and in 1954 Björn Sigurðsson described and classified the disease in a new category of diseases, "slow infections". Slow infections are separated from acute and chronic infections, where it may be years from the time of initial contact between the host and the infectious agent until the first clinical signs are seen. After the first clinical signs the symptoms increase and finally the disease leads to death of the host (Palsson, 1976; Sigurðsson, 1958).

Because the annual mortality rate from maedi was often 20-30% year after year and there was no evidence suggesting that the epidemic was going to subside, the government decided to make an attempt to eradicate the disease. All sheep in the affected areas were slaughtered and replaced with young stock from uninfected areas. This eradication scheme was carried out between 1944 and 1952 and was successful. Since 1965 Iceland has been free of this disease (Palsson, 1976; Pálsson, 1972).

1.2   Transmission and symptoms in sheep

The most important mode of transmission is considered to be from mother to offspring by colostrum and milk. The virus is also transmitted via the respiratory route, associated with close contact, particularly under intensive housing or grazing conditions (Peterhans et al., 2004; Torsteinsdottir et al., 2003).

MVV infection in sheep causes a multi-organ disease. The clinical signs are pneumonia (maedi), encephalitis (visna), mastitis and arthritis. In the epidemic in Iceland maedi was the main clinical symptom, but in a restricted area visna was found in sheep flocks where maedi had already been causing losses. Therefore a certain relationship was suspected from the beginning.

Visna only occurred in the south-western part of the country and was never observed in the north-eastern part where maedi was prevalent for many years (Palsson, 1976). In many foreign sheep breeds a maedi-like disease has been described but clinical visna is rarely seen in other sheep breeds. It seems that the Icelandic sheep are more susceptible to encephalitis than other sheep breeds (Georgsson, 1990; Palsson, 1976; Pétursson, 1994).

1.3 Viruses

Viruses are classified by two systems, the classical system and the Baltimore classification system. The classical system uses four characteristics to classify viruses, the nature of the nucleic acid (DNA or RNA), the structure of the protein shell (icosahedral or helical), presence or absence of an envelope, and the size of the virion and the capsid. All viruses have to produce mRNA that can be translated by cellular ribosomes. The Baltimore classification system is based on this fact and classifies viruses based on the genetic system of each virus and the pathway viruses use to produce mRNA.

Retroviruses are the only members of Baltimore class VI, see figure 1. Retroviruses have a single stranded +RNA genome and each virion contains two genomic copies, which is unique to the *retroviridae* family*,* where all other viruses have only one genome copy in each virion. Retroviruses have their own polymerase that can produce DNA from an RNA template (Coffin, 1997; Flint, 2004).
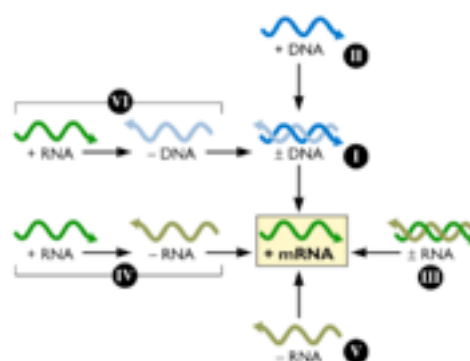
Figure 1. The Baltimore classification system. Retroviruses are the only members in the VI group in the Baltimore classification system (Flint, 2004).

The virus family *Retroviridae* is divided into three main groups, *Oncovirinae, Spumavirinae* and *Lentivirinae* see figure 2. *Oncovirinae* or Oncoviruses are capable of forming tumours and are divided into five subdivisions, *alpha* (α)*, beta* (β), *delta* (δ), *gamma* (γ), and *epsilon (ε)*. Retroviruses can also be classified into two groups, simple and complex. The *alpha*, *beta*, *gamma* and *epsilon* retroviruses are simple but the *delta* retroviruses, lentiviruses, and spumaviruses are complex (Weiss, 2006).
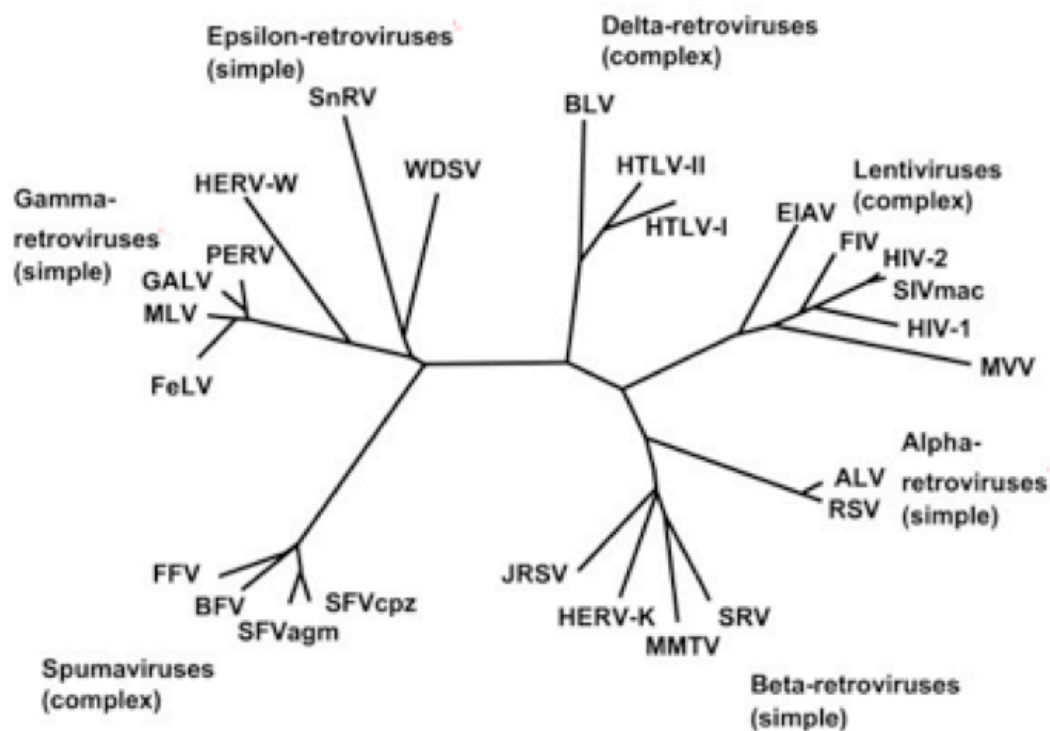
Figure 2. Retrovirus phylogeny. Phylogenetic tree of retroviruses (Weiss, 2006).

1.3.1 Lentiviruses

Lentivirinae or lentiviruses form a double stranded DNA intermediate that is produced from the genomic RNA by an RNA dependent DNA polymerase called reverse transcriptase (RT). Independently two different research groups discovered the reverse transcriptase enzyme in 1970 (Baltimore, 1970; Temin and Mizutani, 1970). After reverse transcription the viral DNA needs to be integrated into the host cell genome for efficient expression. The integrated viral DNA then serves as the template for viral mRNA and genome RNA synthesis by cellular enzymes (Carey and Dalziel, 1993; Flint, 2004). Unlike other retroviruses, lentiviruses are capable of

infecting non-dividing cells because the viral genome is actively transported into the nucleus of infected cells (Clements and Zink, 1996; Fassati and Goff, 2001).

The first lentivirus to be identified was the Equine infectious anemia virus (EIAV). EIAV causes chronic relapsing anemia in horses (Clements and Zink, 1996). Lentivirues in sheep have been detected in many countries, but never in Australia and New Zealand. A variety of names have been used for this disease, maedi-visna in Iceland but also ovine progressive pneumonia virus (OPPV) and ovine lentivirus (OvLV). These infections are very common in sheep although epidemics similar to that in Iceland are rare and symptoms of visna are unusual (Palsson, 1976). Caprine arthritis encephalitis virus (CAEV) is closely related to MVV and causes arthritis in adult goats and leukoencephalomyelitis in kid goats (Clements and Zink, 1996; Pepin et al., 1998).

The other known lentiviruses cause immunodeficiency, two types in humans, human immunodeficiency virus type 1 (HIV-1) and 2 (HIV-2), simian immunodeficiency virus (SIV) in nonhuman primates, feline immunodeficiency virus (FIV) in cats, and bovine immunodeficiency virus (BIV) in cattle (Clements and Zink, 1996).

Multi-organ disease is the characteristic of all lentiviral infections. Lentiviruses either replicate mainly in cells of the monocyte/macrophage lineage, or both in macrophages and CD4$^+$ lymphocytes (Clements and Zink, 1996; Gendelman et al., 1986). The diseases caused by lentiviruses are characterized by the relatively slow onset and progressive debilitating course with the exception of EIAV where most animals progress from a chronic stage characterized by recurring peaks of viremia and fever to an asymptomatic stage of infection (Carey and Dalziel, 1993; Clements and Zink, 1996).

1.4 Maedi Visna Virus (MVV)

1.4.1 Structure

The maedi-visna virion (MVV) is ~ 100 nm in diameter (Thormar, 1961). The structure of MVV is shown in figure 3. The virus particle is composed of two units, the envelope and the core. The envelope is made of a lipid bilayer

originating from the host cell membrane. The lipid bilayer is covered with spikes made of two glycoproteins, surface (SU, gp 135) and trans-membrane (TM, gp 44). The matrix (MA, p16) protein forms a layer beneath the evelope (Pepin et al., 1998). The virion core is cone shaped and is about 40 nm in diameter (Thormar, 1961). The core consists of capsid protein (CA, p25) and inside the capsid there are two single stranded 9.2 kb RNA strands bound to the nucleocapsid proteins (p14). In addition, cellular tRNA^{lys}, protease (PR, p11), reverse transcriptase (RT, p66/p51) and integrase (IN, p34) are inside the capsid (Gonda, 1994; Pepin et al., 1998).
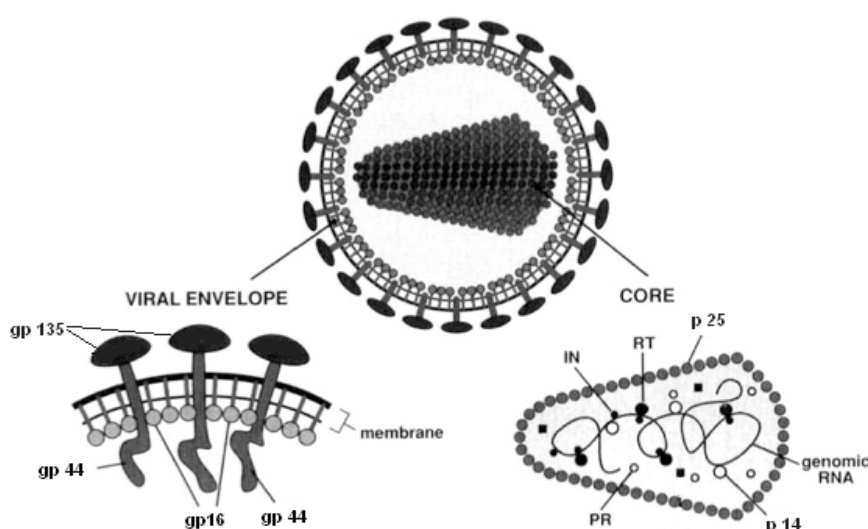
Figure 3. MVV structure. The location of MVV structural proteins, modified from (Gonda, 1994).

1.4.2 Genomic organization

MVV carries its genomic information in two identical single stranded positive RNAs. The RNA genome is reverse transcribed to DNA with reverse transcriptase (Carey and Dalziel, 1993; Lin and Thormar, 1970).

The MVV has typical lentivirus genetic organization, see figure 4. The genome is comprised of three major genes that encode structural proteins and enzymes that are necessary for virus replication. These major genes are: *gag* (group-specific antigens)*, pol* (polymerase), and *env* (envelope)*.* In addition to these three genes MVV has three regulatory or accessory genes, *vif* (viral infectivity factor)*, tat* (trans-activating protein) and *rev* (regulator of virion protein expression)*.* The long terminal repeats (LTR) flank the viral

coding regions. The LTR provides the cis signal required for transcription, integration and polyadenylation of viral RNA (Clements and Zink, 1996; Pepin et al., 1998).
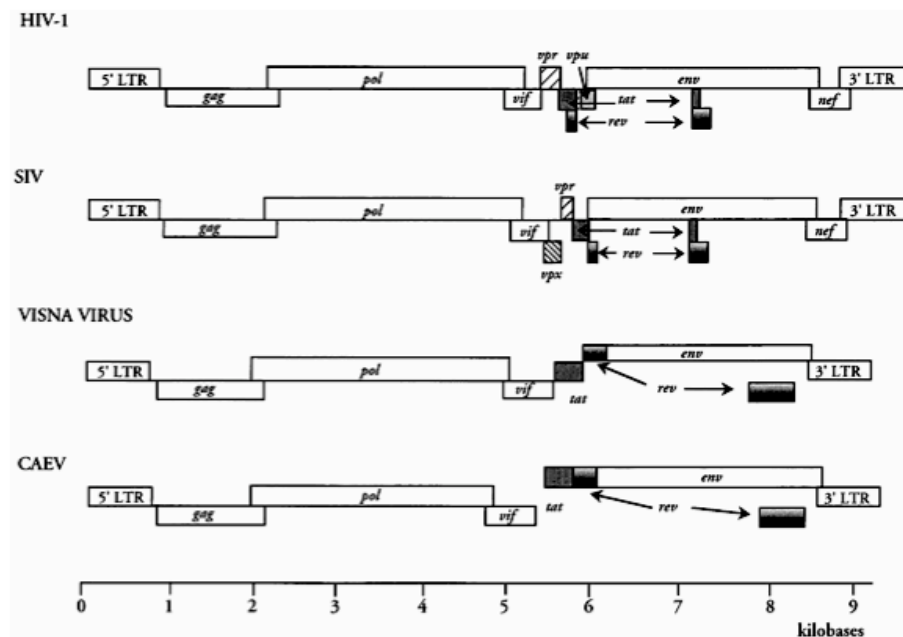
Figure 4. Genetic organization of lentiviruses. Comparison of HIV-1, SIV, MVV (visna virus) and CAEV genomes (Clements and Zink, 1996).

1.4.3 Genes and proteins

All retroviruses contain the three genes *gag, pol* and *env*. Lentiviruses such as MVV are more complex and encode three additional genes, *tat, vif* and *rev*. HIV-1 contains these six genes and the genes *vpu* (viral protein U)*, vpr* (viral protein R) and *nef* (negative regulatory factor) in addition. A genome comparison of the lentiviruses: HIV-1, SIV, MVV, and CAEV, is shown in figure 4 (Clements and Zink, 1996).

1.4.3.1   Gag (group-specific antigens)

The major structural proteins of the MVV are encoded by the *gag* gene. The Gag precursor protein (Pr55^{gag}) is cleaved into 3 proteins: the capsid (CA; p25), the nucleocapsid (NC; p14) and the matrix (MA; p16) (Sonigo et al., 1985). The CA protein forms the hydrophobic core of the virion. During infection the CA protein elicits a strong antibody response that is important for diagnosis. Inside the CA protein core the RNA genome is connected to NC proteins. The MA protein is localized between the viral membrane and the CA protein core (Pepin et al., 1998).

1.4.3.2   Env (envelope)

Retroviruses have two envelope proteins called surface (SU) and
transmembrane (TM). These two proteins are derived from a precursor
polypeptide (in HIV-1 gp160). The SU protein forms the spikes that stick out of
the lipid bilayer covering the virion (see figure 3). In MVV the TM protein is 44
kDa and is connected to the matrix layer. The TM glycoprotein is anchored in
the lipid bilayer and the SU glycoprotein (135 kDa) is non-covalently linked to
the TM (Pepin et al., 1998). Each spike on the virion surface is made of a SU
trimer connected to a TM trimer. The Env proteins are the main determinant of
receptor binding upon infection (Freed, 2001; Vogt, 1997).

1.4.3.3   Pol (polymerase)

The viral enzymes are encoded by the *pol* gene. The Gag-Pol precursor (pr
$150^{Gag-Pol}$) is cleaved into reverse transcriptase (RT), integrase (IN), protease
(PR), and dUTPase (Pepin et al., 1998).

   Reverse transcriptase (RT) is a RNA-dependent DNA polymerase. This
enzyme reverse transcribes viral RNA into viral DNA. RT also has RNase H
activity which removes RNA from the RNA-DNA hybrids and removes the 5'
capped nucleotide and the 3' poly A tail from the ends of viral RNA (Luciw,
1992; Pepin et al., 1998).

   Integrase (IN) is part of the preintegration complex that transfers the
viral DNA into the nucleus. IN recognizes long terminal repeats (LTRs) at the
ends of the newly synthesized viral DNA duplex. It has been shown *in vitro*
that the IN enzyme is necessary and sufficient for integration (van Gent et al.,
1991). IN cleaves two bases from the 3' end and ligates the 3' end into the
cellular DNA (Turner and Summers, 1999).

   Protease (PR): After the virus particle buds from the host cell the PR
cleaves the Gag-Pol polyprotein precursor into final enzyme forms (Pepin et
al., 1998).

   The dUTPase gene in lentiviruses is located in the *pol* gene between
the RNase H and Integrase. dUTPase activity has been demonstrated in
MVV, CAEV, FIV and EIAV but not in HIV and other primate lentiviruses
(Pepin et al., 1998; Petursson et al., 1998). dUTPase lowers the ratio of
dUTP/TTP. The cellular dUTPase activity is higher in dividing cells than

nondividing cells. MVV lacking a functional dUTPase gene can replicate in sheep macrophages *in vitro* and *in vivo* (Petursson et al., 1998).

1.4.3.4   Vif (Viral infectivity factor)

Vif is a 29 kDa protein and the *vif* gene in MVV is located in a similar position as in HIV-1 (Andresson et al., 1993; Sonigo et al., 1985). Vif is conserved among all lentiviruses except EIAV. The Vif amino acid sequence is conserved among closely related lentiviruses for example the amino acid sequence in MVV and CAEV is similar but in distantly related lentiviruses the amino acid sequences are very different. There is a conserved sequence, SLQXLA, in the carboxyl end of the protein (Oberste and Gonda, 1992). This sequence has been associated with replication of all the lentiviruses except EIAV and it has been shown to incrase HIV-1 infectivity up to 1000 fold (Desrosiers et al., 1998; Harmache et al., 1995; Kristbjornsdottir et al., 2004; Lockridge et al., 1999; Strebel et al., 1987). Normally, a functional *vif* gene is required for the production of infectious HIV-1 viruses. Compared to wild type viruses, viruses lacking a functional *vif* gene are severely restricted in their ability to replicate in most cells (non-permissive cells) (Borman et al., 1995; Gabuzda et al., 1992; Kristbjornsdottir et al., 2004). After fusion of non-permissive and permissive cell lines, the non-permissive phenotype was dominant over the permissive one (Harmache et al., 1995; Madani and Kabat, 1998; Simon et al., 1998). This suggested that there was an anti-viral factor activity expressed in the in the non-premissive phenotype and that Vif counteracted this activity. This anti-viral factor was then identified as the APOBEC3G protein that is a cytosine deaminase (Sheehy et al., 2002).

1.4.3.5   Tat (trans-activating protein)

Both MVV and CAEV have an open reading frame (ORF) called *tat*, between the *pol* and *env* genes, which encodes a 10 kDa protein. The Tat protein of HIV-1, SIV, BIV and EIAV is essential for efficient viral replication and trans-activates the LTR strongly by binding to a transactivating responsive element (TAR). TAR is a stem-loop structure located at the 5' end of all initiated viral transcripts. MVV, CAEV and FIV do not have a TAR sequence but the Tat proteins of MVV and CAEV have been shown to have a weak *trans*-activating

effect on the corresponding LTR. The Tat protein of FIV has no such trans-activating effect (Gonda, 1994; Villet et al., 2003). Since trans-activation was detected and the gene location was similar to the *tat* gene in HIV-1 the MVV gene was named *tat.* The basal LTR promoter activity in MVV and CAEV is very high compared to HIV-1, or at least 40 times higher (Davis and Clements, 1989; Saltarelli et al., 1993). This high promoter activity might be the reason why MVV and CAEV do not need the *trans*-activation from the Tat protein unlike HIV-1 which needs it for efficient replication. Recent evidence shows that Tat in MVV is structurally and functionally similar to Vpr in HIV-1 (Villet et al., 2003).

1.4.3.6 Rev (regulator of virion protein expression)

Rev is a 19 kDa polypeptide that is encoded by two exons which are located at the 5' and 3' ends of the *env* gene. *Rev* is expressed early and the Rev protein enables the export of unspliced and partially spliced viral RNA from the nucleus to the cytoplasm and stimulates the association of these viral RNAs with the polyribosomes. Rev expression is required for cytoplasmic expression of incompletely spliced mRNA that encodes the viral envelope protein (Clements and Zink, 1996; Tiley et al., 1990). In MVV Rev binds to the rev response element (RRE), which is a stem-loop structure in the RNA encoding the N terminus of the TM protein (Tiley and Cullen, 1992). The Rev protein function is conserved among lentiviruses and is essential for viral infectivity (Tiley, Malim, and Cullen, 1991).

1.4.3.7 The Long Terminal Repeat (LTR)

The long terminal repeats (LTRs) are located on the 5' and 3' ends of the viral DNA. The LTR is divided into three regions: R, U3 and U5. The R region is common to both the 3' and the 5' ends of the virion RNA and contains the cap cite, polyadenylation signal and the termination signal for viral RNA transcription. The U3 region is located on the 3' end of the viral RNA and contains the enhancer and promoter elements for RNA transcription initiation. The U5 region is located on the 5' end of the viral RNA and the nucleotide sequence immediately downstream of the U5 region is complementary to mammalian lysine tRNA and serves as a primer binding site for the reverse

transcription from RNA to DNA (Gonda, 1994; Hess, Small, and Clements, 1989; Narayan and Clements, 1989).

1.5 Replication cycle

1.5.1 Viral attachment and uncoating

The virion surface glycoprotein attaches to specific receptors on the target cell surface. The specific receptor for MVV is not known. Research has shown that MVV can enter many different sheep cell types and also cells from other species (Clements and Zink, 1996).

HIV-1 binds the surface (SU) envelope (env) glycoprotein to the CD4 receptor (Clements and Zink, 1996; Freed, 1998). Binding to the CD4 receptor is not sufficient for infection, HIV-1 and other primate lentiviruses require additional cell-surface proteins to promote fusion. In HIV-1 the co-receptor is different for different cell types. In T lymphocytes the co-receptor is usually CXCR4 and normally CCR5 in macrophages. After this connection the entry is mediated through fusion of the viral envelope with the cellular membrane. After entry the virus is uncoated (Clements and Zink, 1996; Turner and Summers, 1999). A schematic picture of the lentiviral replication cycle is shown in figure 5.
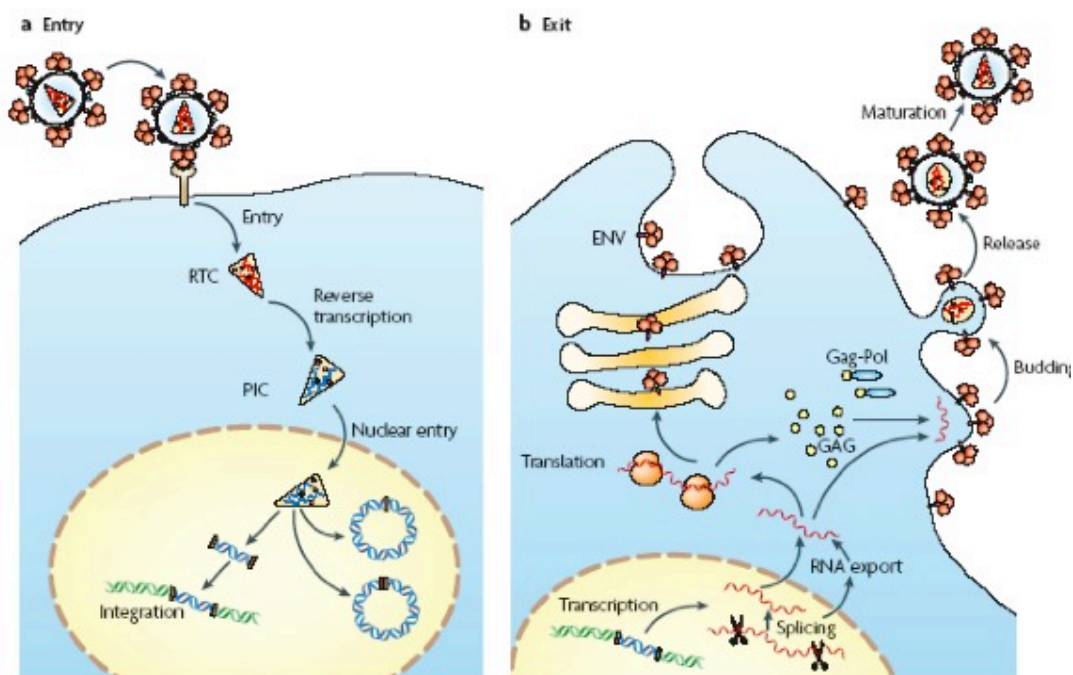
Figure 5. The lentiviral replication cycle. Events in HIV-1 life cycle a) entry and b) exit (Goff, 2007).

1.5.2 Reverse transcription

It is believed that the reverse transcription starts in the virus particle and is not completed until after virus entry into the cytoplasm of the target cell (Le Rouzic and Benichou, 2005). The viral RNA genome enters the cytoplasm as a part of a large nucleoprotein complex, called reverse transcription complex (RTC) (Fassati and Goff, 2001; Telesnisky, 1997). In HIV-1 the RTC is composed of two copies of viral RNA and the viral proteins: RT, IN, NC, Vpr and a few molecules of the matrix protein (Fassati and Goff, 2001; Nermut and Fassati, 2003).

The retroviral DNA synthesis depends on two distinct enzymatic activities of the RT protein. The DNA polymerase that can use either RNA or DNA as a template, and its nuclease activity, ribonuclease H (RNase H) which degrades the RNA from the RNA:DNA duplex. For effective reverse transcription two template "jumps" or strand transfer reactions have to take place (Telesnisky, 1997). Reverse transcription is initiated by binding of tRNA^{lys} to the primer binding site (PBS) adjacent to the U5 near on the 5' end of the viral RNA (Sonigo et al., 1985).

Figure 6 shows a schematic overview of reverse transcription. **Step 1** shows how the (-) strand DNA synthesis proceeds to the 5' end of the RNA

genome ending at the R region (100-150 bp). This short minus strand is called (-) strong-stop DNA (-ssDNA) (Flint, 2004; Telesnisky, 1997). In **step 2** RNase H degrades the RNA from the RNA:DNA hybrid, resulting in a single stranded DNA product. **Step 3**, single stranded DNA hybridizes with the R region at the RNA 3' end. This region can be on the same or second RNA genome. This is called the first strand transfer jump. This transfer is mediated by identical sequences called repeated (R) sequences at the 5' and 3' ends of the RNA genome. The annealing reaction appears to be facilitated by NC proteins. This jump allows the RT to continue DNA synthesis on the RNA template (Flint, 2004; Telesnisky, 1997). **Step 4** when the RT transcribes the polypurine tract (ppt) region near the 3' end of the RNA genome, the RNA of the RNA:DNA hybrid escapes digestion by RNase H. This RNA serves as a primer for the synthesis of (+) strand DNA using the (-) DNA strand as a template (Flint, 2004). **Step 5** shows how RT continues -ssDNA synthesis and RNase H continues removing the RNA from the RNA:DNA hybrid. **Step 6:** In some retroviruses RNase H leaves additional primers for (+) strand DNA synthesis. When –ssDNA synthesis is finished and after + strand synthesis is ensued the RNase H activity of RT removes the tRNA and ppt primers, this is shown in **step 7**. **Step 8** the removal of the tRNA primer exposes the (+) DNA pbs sequence for pairing to its complement on the (-) strand DNA. **Step 9** shows the second strand transfer. **Step 10** Circular DNA intermediate is formed. Repair with host enzymes can produce a dead-end product, a circular DNA molecule with a single LTR. Linear molecule with two LTRs is produced. Repair of the discontinuities in the (+) strand is mediated by host enzymes, **step11** (Flint, 2004).
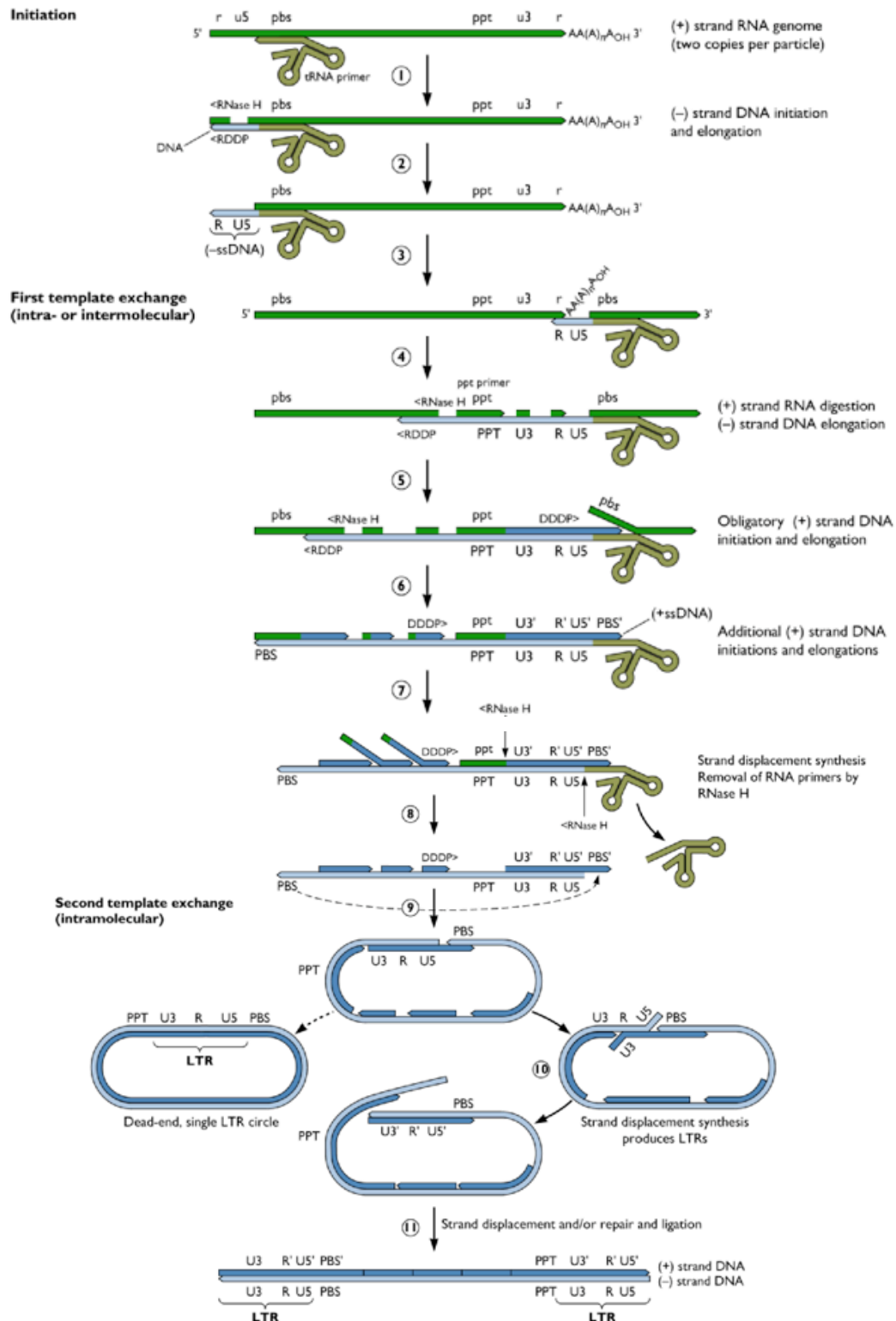
Figure 6. Reverse transcription. The reverse transcription is described in the text above (Flint, 2004).

1.5.3 Transport into the nucleus

The linear double stranded DNA is transported to the nucleus as a component of the pre-integration complex (PIC) through the nuclear pore. Due to this nuclear transport lentiviruses are therefore not dependent on the dissolution of the nuclear membrane during mitosis like other retroviruses. The structure of the PIC is not completely known, but HIV-1 PIC is known to contain viral DNA, IN, Vpr, MA, NC, RT proteins and the HMG I(Y) protein from the host cell (Clements and Zink, 1996; Farnet and Bushman, 1997; Sherman and Greene, 2002).

1.5.4 Integration

The LTR is involved in the integration of the viral DNA into the host chromosome. Viral integrase recognizes the LTR ends of the viral DNA. During the integration 2 bases from each end of the viral DNA are removed exposing recessed 3' hydroxyl groups. These 3' hydroxyl groups attack the phosphodiester bonds on each target DNA strand, staggered by 4-7 bp, leading to a short duplication of the host DNA upon repair, the duplication length varing with different viral IN. Cellular enzymes are thought to mediate repair of the gaps caused by the staggerd integration (Farnet and Bushman, 1997; Hindmarsh and Leis, 1999; Katzman and Sudol, 1994). Integration is shown in figure 7.

It seems that HIV-1 integration *in vitro* occurs randomly but *in vivo* the viral DNA is preferentially integrated upstream of active genes. It has also been shown that HIV-1 integrates into intronic regions of actively transcribed genes and genes that are activated after HIV-1 infection (Farnet and Bushman, 1997; Schröder et al., 2002).
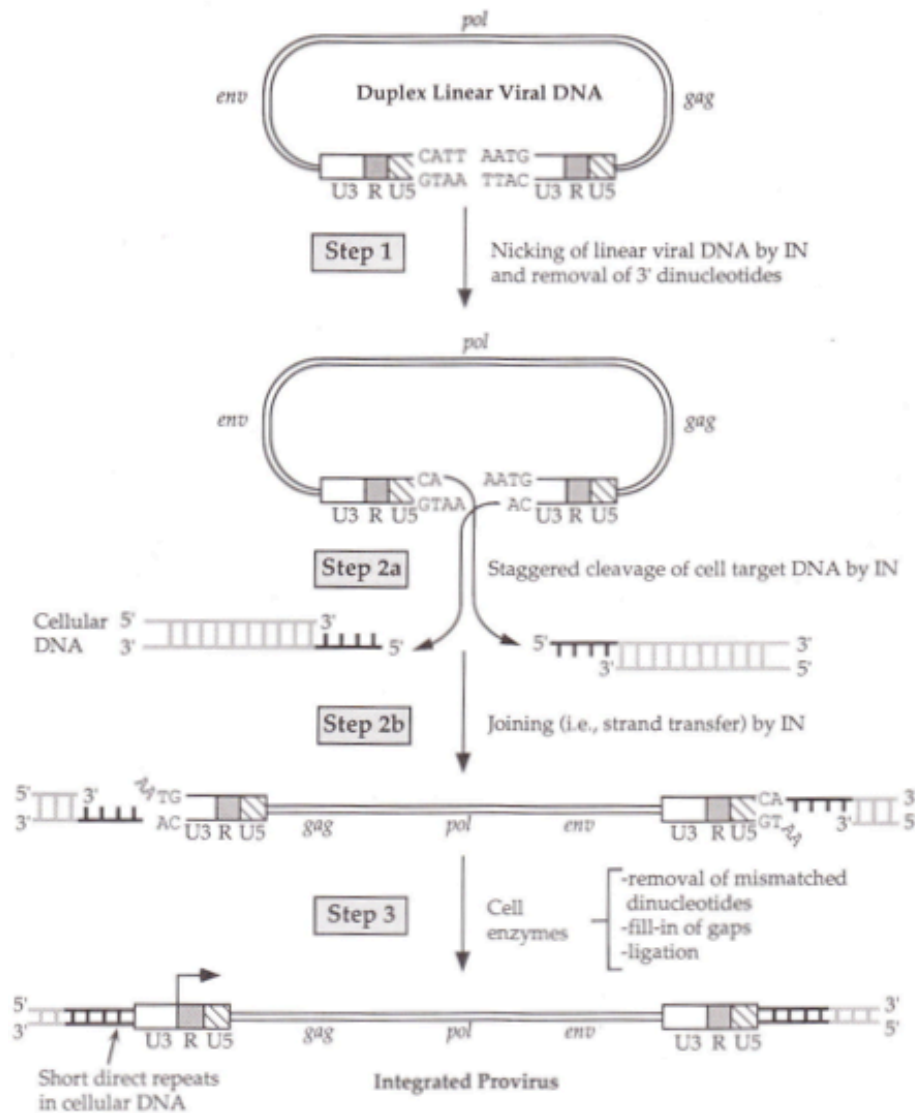
Figure 7. Integration. Step 1: The TT bases on the 3' end of viral DNA are removed. Step 2a: The target DNA is cleaved by IN the cut is staggered to generate a short overhang with phosphorylated 5' ends. Step 2b: The phosphorylated 5' end of the target DNA and the 3' hydroxyl ends of the viral DNA are joined. Step 3: Host enzymes remove mismatched bases, fill in the single-stranded gap and ligate the remaining ends (Luciw, 1992).

1.5.5 Transcription

When the retrovirus has integrated the viral DNA into the host genome and formed a provirus, transcription of viral genes starts. The virus depends on the host transcription machinery system where transcription factors bind to the LTR (Luciw, 1992). Lentiviral transcription is divided into two phases. In the first phase low levels of multi spliced *tat* (Gourdou et al., 1989) and *rev* (Tiley et al., 1990) mRNA are produced in MVV. A critical level of Rev is requied to shift from the early to late phase of viral replication. In the later phase the

other viral proteins and the genome are produced (Gourdou et al., 1989; Tiley et al., 1990).

1.5.6 Translation

Translation of unspliced mRNA yields the proteins Pr55^{gag} and Pr160$^{gag-pol}$. 5-10% of the unspliced mRNA is translated into Gag-Pol polyprotein as a result of a frameshifting event where ribosomes jump back one nucleotide at the 3' end of *gag* and carry on in the *pol* reading frame. This frameshifting ensures the expression of the Pol proteins: RT, PR, dUTPase, and IN (Freed, 2001; Swanstrom, 1997).

The Env precursor glycoprotein is trafficked to the cell membrane through the rough endoplasmic reticulum (ER). The protein is cotranslationally glycosylated and inserted into the lumen of the ER. After translation the glycoprotein monomers form oligomers, which is thought to be important for transport from the ER to the golgi system. In the golgi the glycoprotein is cleaved by a host cell protease, into two protein subunits: surface glycoprotein (SU) and transmembrane glycoprotein (TM) (Freed, 2001; Freed and Martin, 1995; Pepin et al., 1998; Swanstrom, 1997).

1.5.7 Assembly

The assembly of lentiviruses takes place at the plasma membrane. The Pr55^{Gag} polyprotein of MVV assembles at the plasma membrane of the host cell. The N-terminus of the MA domain of the polyprotein is mainly responsible for this attachment. The N-terminus of MA is co-translationally modified at its N-terminus by myristic acid. Research has shown that in HIV-1 the folding of MA exposes a path of highly basic residues next to the plasma membrane. When the Gag protein has reached the plasma membrane it engages in Gag-Gag (as well as Gag-lipid and Gag-RNA) interactions enabling assembly of the virus. The C-terminus of the CA protein is important for dimerization of Gag proteins. It is not completely understood how Env glycoproteins are incorporated into virions but it seems that the cytoplasmic tail of TM protein interacts with the MA domain of the Gag (Freed, 1998; Freed, 2001; Luciw, 1992).

In retroviruses the ψ sequence is responsible for directing packaging of two single stranded copies of RNA into virions. In MVV the encapsidation determinants are located between the major splice donor site (MSD) and the gag initiation codon (Bjarnadottir et al., 2006). The interaction between the ψ sequence and the NC domain of Gag mediates encapsidation of the retroviral RNA (reviewed in (Freed, 2001)).

1.5.8 Budding

After assembly the virion buds or pinches off from the host cell plasma membrane. In many retroviruses a late (L) domain is located in Gag and this domain seems to stimulate virus release from the host cell. In HIV-1 the amino acids near the C-terminus of the p6 domain of Gag seem to be very important for budding (Gottlinger et al., 1991). This amino acid motif is also located in the C-terminus of the Gag in MVV (Andresson et al., 1993). The host factor Tsg101 which is a subunit of mammalian ESCRT-1 (endosomal sorting complexes required for transport) and is involved in vesicle budding is exploited by the lentiviruses (Bieniasz, 2006).

1.5.9 Maturation

Protease PR-mediated Gag processing leads to virus maturation, which is essential for virus infectivity. Maturation usually takes place at the plasma membrane. The viral PR cleaves the Gag and GagPol polyprotein precursors to generate mature Gag and Pol proteins. The viral capsid does not gain its right shape until after budding from the cellular membrane, see Figure 8 (Coffin, 1992).
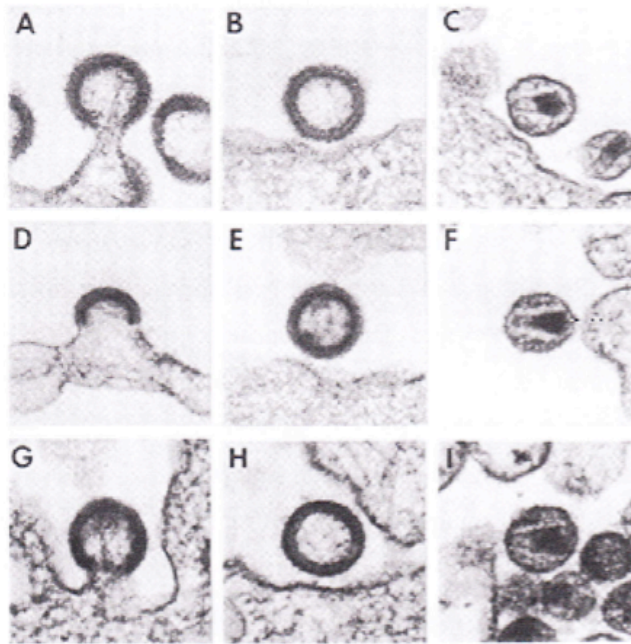
Figure 8. Lentivirus budding and maturation. The viral capsid gains its right shape after budding. A-C. HIV-1; D-F. HIV-2; G-I. BIV (Coffin, 1992).

1.6 Transcription control in retroviruses

Retroviruses use the 5' LTR to control transcription of viral genes. They use the cellular RNA pol II and transcription factors from the host cell. These cellular transcription factors bind to the LTR and modulate the basal level of gene expression. The initiation of transcription is directed from the 5' LTR (Klaver and Berkhout, 1994; Ou, 1995). Retrovirus LTRs vary from about 300bp in Rous sarcoma virus (RSV) to 1200 bp in mouse mammary tumor virus (MMTV) (Luciw, 1992). The LTR of MVV is over 400 bp (Andresson et al., 1993; Sonigo et al., 1985). The LTR is divided into three regions: U3, R and U5. The U3 region contains both enhancer sequences and the promoter. The MVV LTR contains several binding sites for transcription factors.

Transcription is initiated at the junction of U3 and R on the 5´LTR. In HIV-1, transcription factor II D (TFIID) binds to the TATA box and the transcription factors nuclear factor κB (NF-κB) and SP1 bind to their promoter sequences (Clements and Zink, 1996). The MVV LTR contains other binding sites than the HIV-1 LTR. The MVV U3 region contains several potential AP-1 binding sites (Andresdottir et al., 1998; Andresson et al., 1993). Only one of these potential AP-1 sites is identical to the consensus sequence, see figure 9 (Agnarsdottir et al., 2000). The MVV LTR also contains incomplete AP-2, AP-

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3, (Andresson et al., 1993) and AP-4 binding sites (Hess, Small, and Clements, 1989). It has been shown that AP-1 and AP-4 are necessary for MVV transcription in macrophages (Clements and Zink, 1996). The transcription factors c-Fos and c-Jun bind to AP-1 (Carruth, Morse, and Clements, 1996; Clements et al., 1994; Shih et al., 1992).


```
1071VA3  ...............G..C........................................A..T...A...................................T..........................
1071VA4  ...............G..C........................................A..T...A...................................T..........................
Kv1772   CACTGTTACCAGAAATCATAGTCAGGATGACACAGCAAATGTAACCGCAAGTTCTGCTTTTTTGCGCTGAGTCATGTAGCAGCTGATGGTTAAGTCATAACCGCAGATGTAAACAAGTTGCCTATATAAGCC
                        -------  ------                                         -------   --------
                         AP-1     AP-1   E box  PEA-2                             Ap-1      AP-4       AP-1        E box              TATA
                                                                                           E box
```
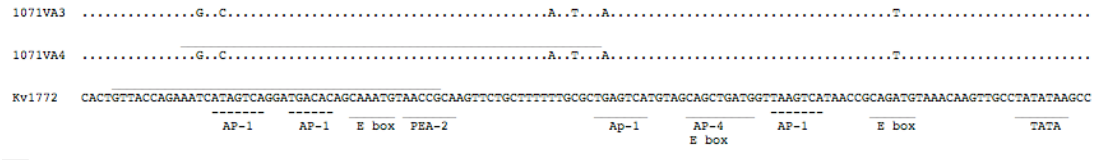
Figure 9. Comparison of the U3 region of viruses: 1071VA3, 1071VA4, and KV1772. Duplicated sequences are shown once with a bar above the sequence. Transcription factor binding sites are shown by a solid line, where the sequence is identical to the consensus sequence, broken lines indicate sites that deviate from the consensus sequence by one nucleotide.

A PEA2 binding site is located in MVV LTR (Agnarsdottir et al., 2000). This binding site has been shown to be important for EIAV transcription in various cell types (Maury et al., 2000; Payne et al., 1999). In MVV it has been shown that the PEA2 protein binds to the PEA2 binding site but it is not known what effect it has on MVV transcription (Sutton et al., 1997). MVV also contains incomplete SP-1 and NF-κB binding sites (Andresson et al., 1993). These binding sites are important in HIV-1 where there are three SP-1, and two NF-κB binding sites (Kurth et al., 1996). It is believed that interaction between SP-1 and NF-κB is necessary to induce HIV-1 gene expression (Majello et al., 1994; Perkins et al., 1993).

This multiplicity of different transcription factors enables retroviruses to control transcription by using specific combinations of activators and repressors. Some transcription factors are only expressed in certain cell types or at a certain time in cell maturation. This controls which cell types viruses are able to grow in. The role of LTR in the cell specificity of the murine leukaemia virus (MLV) has been well investigated. Friend-MLV primarily induces erythroleukemias in NFS mice whereas Moloney-MLV induces T-cell lymphomas. Recombinant viruses were made where the LTR of the Friend-MLV replaced the corresponding region of the Moloney-MLV and vice versa. The recombinant virus, where the genome was primarily from Moloney-MLV induced erytholeukemias in NFS mice and the recombinant virus containing

the genome mainly from Friend-MLV induced T-cell lymphomas (Chatis et al., 1983; Chatis et al., 1984). Research has shown that the Friend-MLV has an erythroid cell specific transcriptional enhancer in the U3 region of the LTR. The Moloney-MLV transcriptional enhancers are active in more cell types (erythroid and lymphoid cells) than the Friend-MLV enhancer (Bosze, Thiesen, and Charnay, 1986; Li et al., 1987).

Whether viruses are able to grow in a certain cell type can depend on where in the cell maturation stage the host cell is. MVV mainly infects cells of the monocyte/macrophage lineage. MVV infects monocytes but the provirus remains silent until the monocytes mature into macrophages. When the monocyte matures the expression of the transcription factors Fos and Jun are induced. They bind to AP-1 binding sites in cellular promoter regions resulting in maturation of the monocyte into a macrophage. As mentioned earlier the MVV has several AP-1 binding sites that can bind these cellular transcription factors (Clements et al., 1994). HIV-1 also infects cells of the monocyte/macrophage lineage, and similarly to MVV the provirus is silent until the expression of NF-κB is initiated in monocyte maturation (Griffin et al., 1989). Variations in the LTR sequence of HIV-1 have also been associated with expanded cell tropism (Ait-Khaled et al., 1995; Corboy and Garl, 1997; Jeeninga et al., 2000; van Opijnen et al., 2004).

1.6.1 Duplicated sequences in retrovirus LTRs

In the cloned MVV KV1772kv72/67 and KV1514 there is a 43 bp duplication in the U3 region of the LTR (Andresson et al., 1993; Staskus et al., 1991). The MVV strain 1071VA4 has a 54 bp duplication in the LTR that overlaps the 43 bp repeat in KV1772 (Agnarsdottir et al., 2000). This duplicated region contains several transcription factor binding sites: AP-1, AML/PEA-2, and a CAAATG sequence which constitutes a consensus E box (CANNTG) (Oskarsson et al., 2007) It has been shown that E box motifs interact with basic helix-loop-helix (bHLH) proteins (reviewed in (Massari and Murre, 2000).

HIV-1 contains certain duplications in the LTR. HIV-1 has three binding sites for SP-1, and two binding sites for NF-κB. HIV-2 has only 1 binding site for NF-κB (Markovitz et al., 1990). T cell activation is thought to be one of the

mechanisms that lead to high levels of HIV-1 replication. HIV-1 responds better to T cell activation than HIV-2 (Tong-Starksen, Welsh, and Peterlin, 1990). NF-κB is kept in the cytoplasm by the inhibitor of κB (IκB), when T cells are not activated. During T cell activation IκB is targeted for degradation in the proteasome. When NF-κB is released from IκB, NF-κB is able to enter the nucleus and stimulate transcription of target genes (Janeway, 2008). This could be one of the factors that makes HIV-2 less virulent and may account for the longer period of viral latency in HIV-2 than HIV-1.

In HIV-1, the number of SP1 binding sites in the LTR seems to be important. Normally HIV-1 contains three SP1 binding sites (Kurth et al., 1996). An HIV-1 strain with four SP1 binding sites has been isolated from an HIV-1 infected patient. Cell culture experiments showed that this promoter, containing four SP1 binding sites, had slightly higher promoter activity and viral replication rate than the isogenic control strain containing three SP1 binding sites and outgrew the control strain in 35 days (Koken et al., 1992).

In the U3 region of the LTR of human T-lymphotropic virus (HTLV-1) there are three 21 bp repeats. These repeats contain binding sites for transcription factors from the ATF/CREB family (Franklin et al., 1993; Seiki et al., 1983; Shimotohno et al., 1986; Yin, Paulssen, and Gaynor, 1996). These binding sites are important for the HTLV-1 transcriptional activator protein Tax (Paskalis, Felber, and Pavlakis, 1986; Shimotohno et al., 1986). Tax does not bind directly to these binding sites, but it is thought that the Tax protein binds to the transcription factors ATF and CREB (Brauweiler et al., 1995; Franklin et al., 1993). When Tax is present it stabilises the binding of CREB to the 21 bp repeats which might lead to more gene expression and therefore control the switch from latency to replication (Brauweiler et al., 1995). The transcriptional activity of these repeats is proportional to the number of copies of the 21 bp sequence (Shimotohno et al., 1986).

MLV has several repeated sequences in the LTR. The Moloney-MLV has a 75 bp repeat in the U3 region of the LTR. This repeat seems to control the length of the latent period of the disease induction. Viruses containing only one copy of the sequence in the LTR have a longer latent period than the viruses that have the duplication (Li et al., 1987). This also applies for the mouse virus Graffi-MLV. Two clones of the Graffi-MLV: GV1.2 and GV1.4 are

identical except for the presence of a 60 bp duplication in the U3 region of the GV1.2. The GV1.2 clone has shorter latent period than the GV1.4 clone (Barat and Rassart, 1998). Mink cell focus-forming retrovirus (MCF-MLV) such as MCF 247 can replicate in the thymus and sometimes cause a leukemogenic phenotype. MCF 30-2 can replicate in the thymus but does not cause leukemia. The virus MCF 247 has a duplicated enhancer sequence in the LTR that seems to make the MCF 247 virus more oncogenic than the MCF 30-2 virus (Holland et al., 1989).

The virus SRS 19-6 belongs to the MLV family. SRS 19-6 can grow in many cell types and unlike most other MLV, SRS 19-6 can induce leukaemia in multiple cell lineages. It seems that the SRS 19-6 needs to have a certain region repeated in the U3 region of the LTR for the ability to grow in T-cells, these repeats were not found in proviruses isolated from other tissues (Granger, Bundy, and Fan, 1999).

1.7 Gene expression

1.7.1 Chromatin control of gene expression

The eukaryotic genome is packed into a structure called chromatin. The basic repeating unit of chromatin is the nucleosome. The nucleosome core particle consists of approximately 147 bp of DNA wrapped 1,67 turns around a histone octamer complex. The octamer complex is composed of two H2A-H2B heterodimers and two H3-H4 heterodimers. The core histones cores consist of a predominantly α-helical, globular domain that is necessary for octamer assembly and DNA binding. Histone 1 (H1), called the linker histone, binds to the DNA bridging the nucleosomes. The DNA is wrapped around the nucleosome and H1 folds the chromatin into a more compact structure. The chromatin structure is shown in figure 10. Nucleosomes are thought to negatively regulate gene expression by restricting access of factors that bind to DNA (Marzio and Giacca, 1999; Watson, 2004).
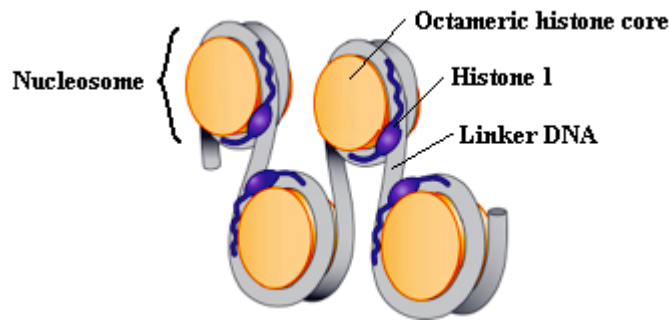
Figure 10. Chromatin structure. The chromatin consists of nucleosomes. The DNA is wrapped around an octameric histone core. Histone 1 (H1) binds to the DNA and folds the chromatin into a more compacted structure. Adjusted from (Watson, 2004).

The incorporation of DNA into nucleosomes has a profound impact on gene expression. It is very important that the nucleosomes are mobile and that the grip they have on the DNA can be loosened so that specific regions on the DNA can be made accessible for factors determining gene expression. In eukaryotes most transcriptional regulation is mediated by changes in the chromatin structure. These changes in chromatin structure can either be mediated by ATP-dependent chromatin remodelling factors or posttranslational modifications of the histones (Williams and Tyler, 2007).

The histones' N-terminal tails are typically modified with a variety of small molecules. Lysines are frequently either modified with acetyl groups or methyl groups. Serines are often modified with phosphate. When the histones H3 and H4 are acetylated the nucleosomal structure cannot be folded into highly compact structures making the chromatin (DNA) more accessible to proteins. As a consequence the transcription machinery may have more access to the promoters and therefore transcription is more frequently initiated. The acetylation of specific lysine residues of the the N-terminal domains of the core histones can therefore reverse the negative effect that packaging of the DNA has on gene expression (reviewed in (Eberharter and Becker, 2002)).

Generally the histoneacetyltransferases are not able to bind to a specific DNA sequence, rather several transcription factors recruit them to the promoters (Marzio and Giacca, 1999).

1.7.2 HIV-1 gene expression

In cells infected with HIV-1 the proviral genome is integrated into the host genome and the HIV-1 LTR is packed into two nucleosomes called nuc-0 and nuc-1. The viral LTR is a very strong promoter when analyzed as naked DNA *in vitro* but is almost silent when integrated into the host genome. Nuc-0 and nuc-1 encompass nt 40-200 and 452-596 (Williams and Greene, 2007). The region between nuc-0 and nuc-1 remains nucleosome free even though an additional nucleosome would fit there (Steger and Workman, 1997). This region is the transcription start site and because it is nucleosome free, multiple cellular transcription factors are able to bind to the area. Cellular proteins are constitutively bound to this area and are also thought to induce marked promoter bending. This bending could be the reason why this site is disfavoured for nucleosome assembly (Marzio and Giacca, 1999; Wu, 2004). Nuc-1 is located at the transcription start site. When transcription of the HIV-1 promoter is activated nuc-1 is rapidly disrupted suggesting that when nuc-1 is present on the LTR it may maintain the promoter silent (Marzio and Giacca, 1999).

For efficient activation of the LTR, HIV-1 needs the Tat protein to be translated. Tat binds to the TAR element and interaction between Tat and TAR is necessary for increasing RNA polymerase II (Pol II) activity and the production of full-length viral transcripts. Tat binds to cyclin-dependent kinase 7 (CDK7), leading to interaction between Tat and the CDK-activating kinase (CAK) complex. *In vitro* Tat activates the phosphorylation of the carboxyl-terminal domain of RNA Pol II by CAK. This phosphorylation is essential to recruit transcriptional complexes to the LTR promoter (Cujec et al., 1997). Tat binds to histone acetyltransferase (HAT) proteins p300 and CBP. P300 and CBP are also recruited to the LTR promoter when virus activation is initiated. It is not clear whether the HAT proteins are recruited to the LTR promoter by Tat or by other transcription factors that bind to the LTR promoter. Tat also interacts with NF-κB. In summary Tat affects the LTR promoter activity through several indirect pathways (Easley et al., 2009; Marzio and Giacca, 1999).

1.7.3 HIV-1 latency

HIV-1 replicates preferentially in activated CD4$^+$ T cells. These cells generally only survive for a few days after they get infected but in resting memory CD4$^+$ T cells HIV-1 can establish a latent infection. This viral latency makes the virus able to evade the host immune response. Latent HIV-1 has the viral genome integrated in the host genome but is transcriptionally silent. Latent infected cells are very rare, only about 1 in 10^6 resting CD4$^+$ T cells. These cells are considered to be the reason why antiviral drugs are not capable of eradicating the virus (Han et al., 2007).

Both preintegration and postintegration HIV latency has been detected. Preintegration latency is common when HIV infects resting, unactivated cells. This state is a consequence of the APOBEC3G protein that leads to ineffective reverse transcription, also the import of reverse transcribed HIV genome into the nucleus is restricted in resting T-cells. The long-term latency of HIV cannot be explained by preintegration latency because the half-life of incompletely reverse transcribed RNA genome is only about one day. The reason for postintegration HIV latency is not as clear as preintegration. It is believed that postintegration latency is established during the shut-off of T-cell activation (reviewed in (Williams and Greene, 2007)).

For maintaining postintegration HIV latency it seems to be important that the histones in nuc-0 and nuc-1 are deacetylated. It has been shown that several transcription factors such as NF-κB p50 homodimer, LSF1, YY1 and thyroid hormone receptor, bind to the HIV promoter. They bind to the promoter between nuc-0 and nuc-1, see figure 11, and recruit histone acetylase 1 (HDAC1) to the HIV-1 promoter (Coull et al., 2000; Jiang et al., 2007; Williams and Greene, 2007).
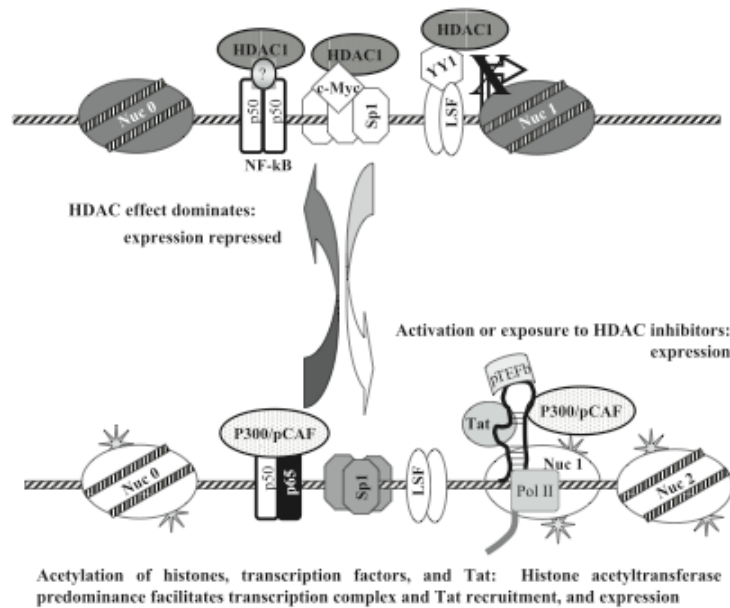
Figure 11. Acetylation of histones. Proviral latency is maintained by several transcription factors that bind to the HIV-1 promoter and recruit HDAC1 to the HIV-1 LTR (Jiang et al., 2007).

Other factors that contribute to HIV-1 latency are for example cellular barriers that the virus overcomes by using the viral protein Tat. In HIV-1, Tat works as an activator for virus expression. Tat transcriptional activity is dependent on the interaction with positive transcription elongation factor b (P-TEFb) kinase. P-TRFb phosphorylates serine-2 residues in the C-terminal domain of the largest subunit of RNA polymerase II. P-TRFb kinase activity is restricted by 7SK, a cellular RNA that serves as a scaffold for HEXM1. HEXM1 is a cellular protein that inhibits P-TEFb. Another cellular factor that contributes to viral latency is NF-κB. NF-κB/Rel factors can both promote positive and negative effects on transcription depending on which family member of the Rel family is bound to the HIV-1 LTR. In resting T cells the NF-κB p50 homodimers bind to the HIV-LTR and promote recruitment of the HDAC1 to the HIV-1 LTR, see figure 11. In activated T- cells NF-κB p50-RelA heterodimer binds to the HIV-LTR and recruits histone acetyltransferase p300, leading to localized histone acetylation that promotes initiation of transcription, see figure 11.

T cell receptor ligation additionally induces nuclear factor of activated T cells (NFAT). NFAT enhances NF-κB to positively regulate HIV-1 gene

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expression. In resting T cells NF-κB and NFAT are excluded from the nuclei and therefore unavailable to promote HIV-1 replication. RelA can also bind to the p-TEFb and initiate RNA pol II dependent synthesis of HIV-1 Tat mRNA when Tat is not available (Han et al., 2007; Richman et al., 2009; Williams and Greene, 2007).

It seems that the RNA pol II complex is located at the HIV-1 promoter and initiates the synthesis of RNA transcripts. However when the viral protein Tat or the cellular activating signals are not available RNA pol II can only produce short viral transcripts. When Tat is available it binds to the TAR and recruits the p-TEFb complex to the C-terminal domain of RNA pol II. These events encourage the RNA pol II to synthesize full-length HIV transcripts (Williams et al., 2006).

At last the initial blocks of Tat production could be the consequence of impaired HIV-1 mRNA export out of the nucleus because of low levels of poly-pyrimidine tract-binding protein (PTB) in resting T cells or cellular miRNAs binding to viral mRNA that is expressed early and prevent Tat production (reviewed in (Williams and Greene, 2007).

1.7.4 Reactivation of latent HIV proviruses

The best treatment for people infected with HIV-1 is highly active antiviral therapy (HAART). This treatment suppresses the viral replication and has to be maintained for life because treatment interruption leads to rapid replication of the virus. People on HAART have increased rates of heart disease, diabetes, liver disease and cancer. As mentioned above the proviral latency is the result of multiple restrictions on HIV-1 expression. If the resting HIV-1 latently infected CD4$^+$ T cells are activated it might be enough to eliminate the virus (Richman et al., 2009; Williams and Greene, 2007).

Histone deacetylases (HDACs) are important for maintaining HIV-1 latency. They are recruited to the HIV-1 promoter by several transcription factors. Valproic acid (VPA; 2-propyl-pentanoic acid) is a histone deacetylase inhibitor. VPA has been used as a well establised therapy for seizures and to treat people with bipolar disorder and other psychiatric disorders (Gurvich et al., 2004; Haddad PM, 2009; Jiang et al., 2007). VPA might be able to inhibit HDAC from deacetylating histones on the HIV-1 promoter and initiate HIV-1

expression. First experiments pointed to the direction that using VPA and HAART resulted in reduction of latent HIV-1 infected CD4$^+$ T cells in vivo (Lehrman et al., 2005; Ylisastigui et al., 2004). Further study showed that VPA did not significantly reduce the number of latent HIV-1 infected CD4$^+$ T cells (Sagot-Lerolle et al., 2008; Siliciano et al., 2007).

Other factors that could be used to reactivate a latent HIV-1 provirus are HMBA (hexamethylene bisacetamine) and prostratin. HMBA can stimulate the release of P-TEFb from ribonucleoprotein complexes containing HEXIM1 and 7SK snRNA (small nuclear RNA) in the absence of Tat and activate intracellular signalling cascades that can increase HIV-1 expression in latently infected cells. Prostratin has been used in cell-line model to stimulate HIV-1 expression though the protein kinase C mediated activation of NF-κB, see figure 12 (reviewed in (Williams and Greene, 2007)).
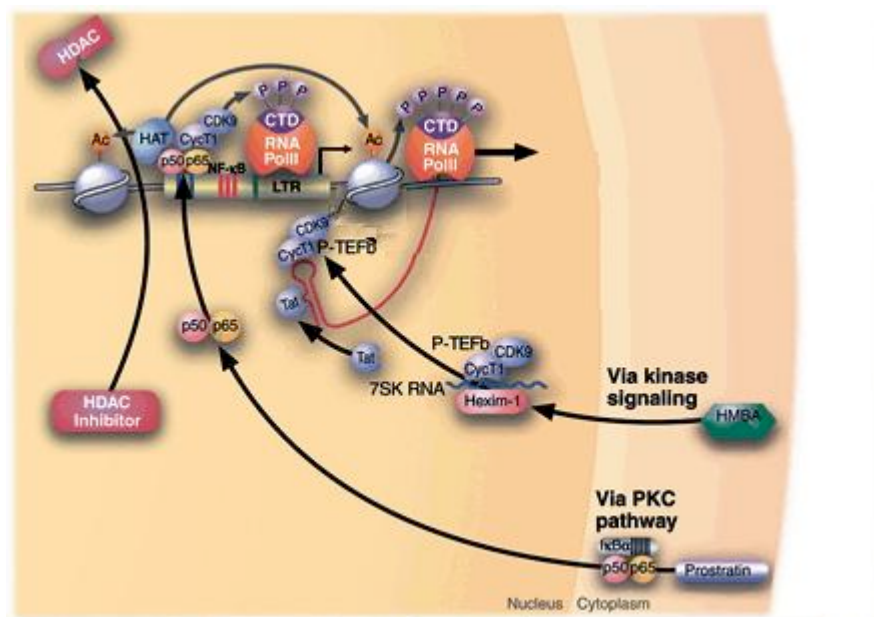
Figure 12. Reactivation of latent HIV-1 provirus. The use of HDAC inhibitor allows the acetylation of histones on the LTR promoter and can release the repressive effect of HDACs have on the HIV-1 expression. The use of HMBA stimulates the relese of P-TEFb from ribonucleoprotein complex containing HEXIM and 7SK snRNA. Prostratin stimulates HIV-1 by releasing active NF-κB. Adjusted from (Williams and Greene, 2007).

1.8   Retrovirus neurovirulence

Many retroviruses invade the nervous system and cause neurological symptoms. In addition to MVV neurological symptoms have been described

for HIV-1, SIV-1, HTLV, CAEV, FIV and MLV (González-Scarano, 1995).
Reports of an unusual encephalopathy in HIV-1 positive patients appeared
shortly after the recognition of acquired immune deficiency syndrome (AIDS),
the late stage of HIV-1 infection (Snider et al., 1983; Whelan et al., 1983). In
AIDS HIV-1 has infected T4 helper cells and severely affected the immune
system. At this stage the patients are more susceptible to opportunistic
infections like cytomegalovirus (CMV), Epstein Barr virus (EBV), other viral
and bacterial infections and also more likely to develop uncommon types of
tumours. Those opportunistic infections may affect the central nervous system
(CNS). HIV-1 can cause neurological syndromes in the brain that are not
related to these opportunistic infections (Kaul and Lipton, 2006; Owe-Larsson
et al., 2009; Price et al., 1988).

Damage in the CNS can be seen in up to 35-70% of AIDS patients
(Budka, 1991; Glass et al., 1995; Johnson et al., 1996; Navia, Jordan, and
Price, 1986). Among HIV-1 patients HIV-associated neurocognitive disorders
(HAND) are common. HIV-associated dementia (HAD) is a serious condition
during the AIDS stage of HIV-1 infection. HAD has three main clinical
features: cognitive impairment, behavioural abnormalities and disturbed motor
function (Owe-Larsson et al., 2009). Since 1997 HAART has been used to
treat HIV-1 infected patients and has increased their life expectancy.
Following the use of HAART the incidence of HAD has decreased but HAART
does not provide complete protection from HAD and in most cases does not
reverse the disease (Clifford, 2008; Kaul and Lipton, 2006).

HIV-1 invades the CNS early following infection (Gabuzda and Hirsch,
1987). Even though HIV-1 causes significant neurological impairments, cells
of neural origin (neurons, oligodendrocytes and astrocytes) are rarely infected
in ADIS patients (Ghorpade et al., 1998). The cells that are most often
infected are mononuclear phagocytes, microglia and blood derived brain
macrophages. Microglia are the resident macrophages of the brain (Bagasra
et al., 1996; Gabuzda et al., 1986; Koenig et al., 1986). Microvascular
endothelial cells and dendritic cells in the choroid plexus can also be infected
in the brain of HIV-1 infected patients (Bagasra et al., 1996; Hanly and Petito,
1998; Moses et al., 1993; Wiley et al., 1986). HIV-1 infects choroid plexus
cells both *in vivo* (Bagasra et al., 1996) and *in vitro* (Harouse et al., 1989).

It is not known how HIV-1 invades the CNS but there is a possibility that the virus passes the blood-brain barrier by infection of the microvascular endothelial cells and the choroid plexus (Bagasra et al., 1996; Falangola et al., 1995; Harouse et al., 1989; Moses et al., 1993; Moses and Nelson, 1994; Petito et al., 1999; Poland, Rice, and Dekaban, 1995). It is also possible that HIV-1 enters the CNS by migration of previously infected macrophages through the blood brain barrier (BBB). This hypotheses is called the "Trojan horse" hypothesis (Price et al., 1988).

When macrophages, microglia and astrocytes are either activated or infected by HIV-1 they induce activation of inflammatory mediators, cytokines, chemokine receptors, extracellular matrix-degrading enzymes and glutamate receptor-mediated excitotoxicity which can initiate downstream signaling pathways and disturb neuronal and glial function. The viral proteins, envelope glycoprotein (gp160) and Tat have potential for neurotoxicity. All these factors can cause neurodegeneration and neuronal death, see figure 13 (Kaul and Lipton, 2006).
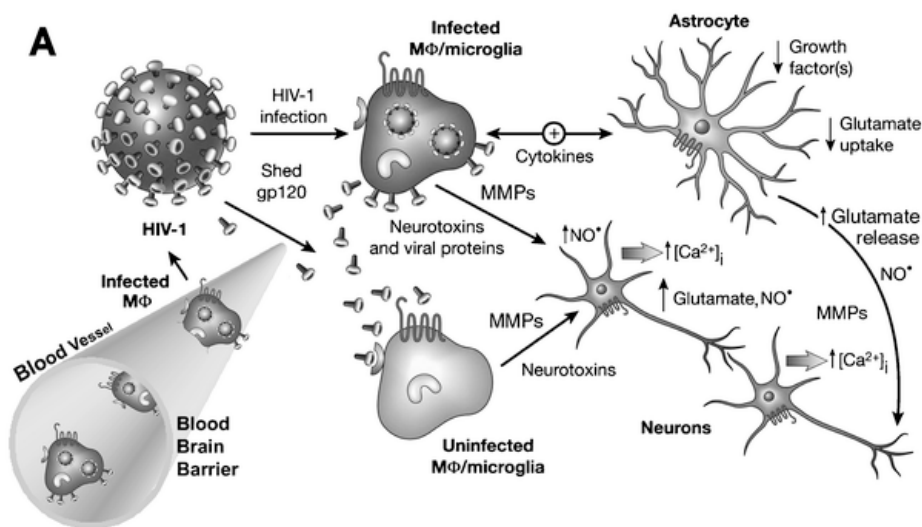
Figure 13. Model of HIV-1 neuropathology. Overview of the factors that can cause neurodegeneration and neuronal death (Kaul and Lipton, 2006).

Certain strains of HIV-1 are neurotropic and they are capable of infecting and replicating in brain cells of the monocyte/macrophage lineage. They can cause a productive infection in primary human brain explant culture and cause a lytic infection of normal microglia cells which T-cell adapted virus

strains generally can not replicate in (Koyanagi et al., 1987; Li et al., 1991; Watkins et al., 1990). MVV is also macrophage tropic and causes severe brain damage, which makes it a good model system to investigate retrovirus neurovirulence.

1.8.1 The role of LTR in MVV neurotropism

In the MVV epidemic in Iceland maedi was the main clinical symptom, but visna was only found in flocks were maedi had been prevalent for several years. Visna was sometimes the main clinical symptom (Palsson, 1976). The reason for these different clinical signs from the same virus infection is not likely to be the effect of host genetic variation because there is only one sheep breed in Iceland. It is more likely that viral factors are responsible for these different clinical signs. MVV infected cells in the brain are macrophages, plasma cells, endothelial cells, fibroblasts, and cells in the choroid plexus which is also seen in brains of HIV-1 positive patients (Georgsson, 1989).

Previously it has been shown that the cell tropism of MVV strains isolated from a sheep suffering from visna was different than MVV strains isolated from sheep with clinical signs of maedi (Andresdottir et al., 1998). The isolate from the brain grows faster in sheep choroid plexus cells than the lung isolate.

It has been shown that a specific duplication in the U3 region of the MVV LTR determines the cell tropism of the virus. The visna virus strain KV1772 is a descendant of a virus that was originally isolated from the brain of a visna-affected sheep. LTR with and without a repeat was cloned into the molecular clone KV1772 and they were named VA4 with a 54 bp repeat and VA3 without the repeat. These molecular clones are identical except for the repeat in the LTR of VA4. This repeat overlaps the 43 bp repeat in the KV1772 molecular clone, see figure 14. The importance of the extended cell tropism in MVV was observed when DNA was extracted from formalin-fixed, paraffin embedded samples of field cases. Four visna cases and eight maedi cases were sequenced. Three of the field cases of visna had the same 43 bp repeat but the fourth sample came from a different farm and had a different 34 bp repeat that overlapped the 43 bp repeat by 14 bp. The samples of maedi field cases did not have a repeat except for one sample, sample 13, where

two virus types were detected, one containing a 43 bp repeat whereas the other did not, see figure 14 (Oskarsson et al., 2007).
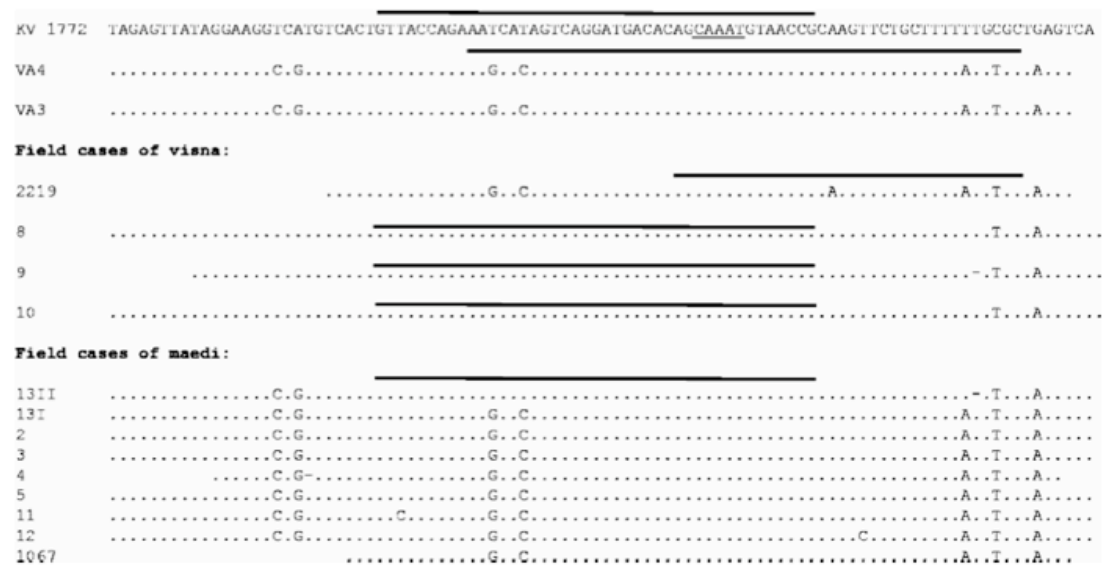
Figure 14. LTR duplications in MVV clones and from field cases of visna and maedi. Duplications are shown with a thick black line above the sequence. Identical nucleotides are indicated with dots and deletions are indicated with dashes. The CAAAT sequence is underlined.

The region that is essential for effective virus replication in sheep choroid plexus (SCP) cells contains a number of known transcription factor binding sites. The CAAATG sequence is a consensus E box sequence (CANNTG). When the CAAAT sequence was deleted from either copy of the duplication in the LTR it caused a defect in virus replication in SCP cells but not in macrophages. When other transcription factor binding sites were deleted it did not have any effect on virus replication in SCP cells, see figure 15 (Agnarsdottir et al., 2000; Oskarsson et al., 2007).
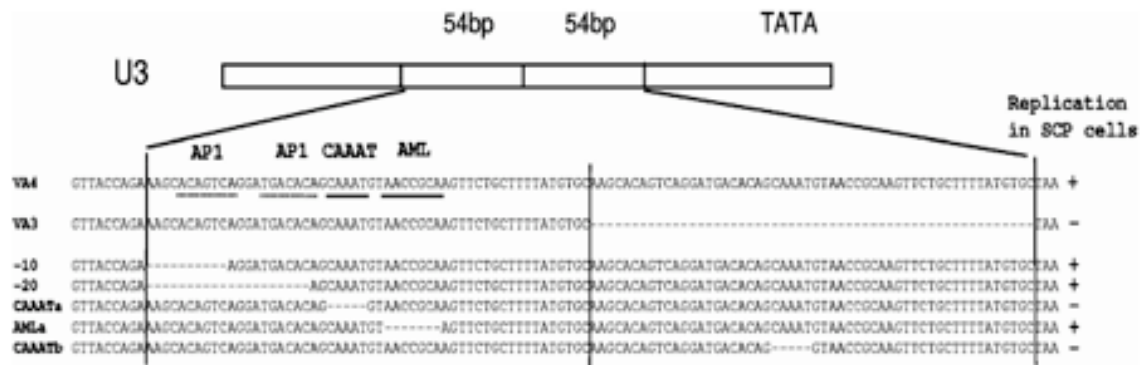
Figure 15. Deletions in the duplicated region in the LTR of the molecular clone VA4 and replication in SCP cells. The virus can not replicate in SCP cells when the CAAAT sequence is deleted from the either duplication.

Previously the promoter activity of the LTRs with and without a repeat was tested in transient transfections with reporter plasmids. First, LTRs with the repeat and without the repeat were cloned into the pCATbasic plasmid and were transiently transfected into SCP cells. No difference could be detected between the LTR with and without a repeat, and prior infection resulted in 3-4 fold transactivation (Oskarsson et al., 2007). It has also been shown that the CAAAT and the AML sequences are important. When the CAAAT and AML sequence was deleted from the LTR without a repeat it resulted in less transcription, see figure 16 (Oskarsson et al., 2007). The loss of replication activity in SCP cells that we see in the viruses without a repeat in the LTR can therefore not be detected in the context of transcription of a reporter gene in transient transfection.
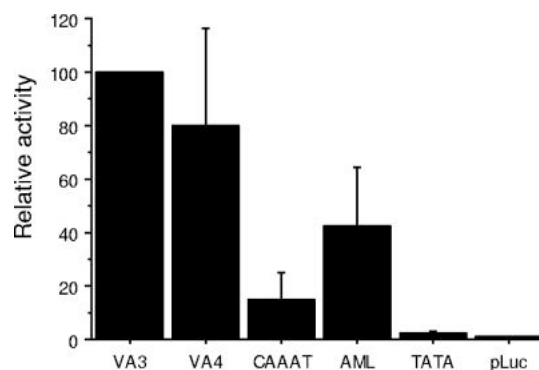
Figure 16. Relative luciferase activity. Lusciferase activity of the LTR with (VA4) and without (VA3) a repeat and with deletions of the CAAAT sequenca and the AML site from VA3 in SCP cells (Oskarsson et al., 2007).

2   Aims of the study

MVV without duplication of a CAAATG sequence in the LTR does not replicate in other cell types than macrophages. The aim of this study was to compare the two MVV strains VA3 (without the duplication) and VA4 (with the duplication) and find where in the replication cycle of VA3 the barrier is. The project can be divided into 3 parts:

2.1   *Reverse transcription*

To find if there is a difference in reverse transcription between the virus strains VA3 and VA4 in sheep choroid plexus (SPC) cells.

2.2   *Integration*

To find if there is a difference in integration of the two virus strains VA3 and VA4 in SCP cells.

2.3   *mRNA*

To find if there is a difference in mRNA production in the two virus strains VA3 and VA4 in SCP cells.

3   Material and methods

3.1   *Visna virus molecular clones*

The MVV molecular clone KV1772kv72/67 (1772) is derived from a plaque-purified biological clone of visna virus strain KV1772. KV1772 was selected for neurovirulence by serial passage of strain KV1514 in sheep (Andresson et al., 1993; Georgsson, 1989). The KV1772kv72/67 is contained in two plasmids, p8XSp5-RK1 and p67f. The p8XSp5-RK1 is the 5' visna molecular clone with a *Stu*I site in a 296 bp sheep flanking sequence from the 5'LTR of the virus through the *Xba*I site at position 7768. P67f is the 3' visna molecular clone with kv1772 sequence from the *Xba*I site at position 7768 to the end of the LTR (Skraban et al., 1999). The field strain 1071 has two size variants (Andresdottir et al., 1998). The larger one has a 54 bp duplication in the U3 region of the LTR. Previously the U3 region of the two size variants were cloned into a KV1772kv72/67 backbone. The clone without the duplication was called 1071VA3 (VA3) and the clone with the duplication was called 1071VA4 (VA4) (Agnarsdottir et al., 2000). VA4 and VA3 are used in this work.

3.2   *Cell work*

Two cell types were used: Sheep choriod plexus (SCP) cells and sheep macrophages. Macrophages were used to grow viruses and SCP cells were used in infection studies.

3.2.1 Cell culture

SCP cells were stored in liquid nitrogen until used. When used they were thawed and seeded on T75 flasks (Nunc™) in growth medium. Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Gibco™) supplemented with 2 mM glutamine, 100 IU per ml penicillin and 100 IU per ml streptomycin. SCP cell medium was either 10% lamb serum (growth medium) or 1% lamb serum (maintenance medium). Macrophage medium was 15% lamb serum (LS), with $5 \cdot 10^5$ M 2-mercapto ethanol added. Cells were always cultured at 37°C in a humidified atmosphere of 5% CO_2. Before infection cells were seeded on 12 well multidishes (Nunc™) in growth

medium. When cells were 80-90% confluent the growth medium was replaced with maintenance medium.

3.2.2 Macrophage isolation

Between 200 and 250 ml of blood from healthy sheep was collected in two glass flasks which contained 2,5 ml of heparin (LEO®) to prevent blood clotting. The blood was then diluted 1:1 with PBS that contained 100 IU/ml of penicillin and 100 IU/ml of streptomycin. Twenty five ml of diluted blood was put carefully on top of 15 ml of Ficoll-Plaqe™ plus sugar gradient (Amersham Biosciences), that had been placed in a 50 ml centrifuge tube. The tube was centrifuged for 30 min at 2100 rpm and 20°C. Four layers were formed: serum at the top, then a white layer with lymphocytes and monocytes, then PBS, and at the bottom were red blood cells and phagocytes with cleaved nucleus. The white blood cell layer was sucked up with a pipette and put in a new centrifuge tube, filled up to 40 ml with PBS mixed and then centrifuged for 15 min at 2000 rpm and 20°C. The supernatant was poured off and the cells were resolved in lysis solution (0,14 M HN$_4$Cl + 0,02 M Tris-HCL. pH 7,2), which causes lysis of the red blood cells. The tube was incubated for 2 minutes before adding PBS and centrifuged in 15 min at 12000 rpm and 20°C. The supernatant was poured off and the pelleted cells were washed in PBS several times until the supernatant was clear. Then the cell pellet was dissolved in small amount of PBS and mixed with 20 ml of macrophage medium. Cells were counted on a haemacytometer and about 100 million cells were put in each T25 flask. After one day the supernatant was replaced with new macrophage medium. Macrophages were cultured for one week before they were infected.

3.2.3 Chromosome collection

SCP cells were seeded in T-25 flasks containing 5ml of growth medium. After 24 hours the cells were infected with the virus strains VA3 or VA4. After 27 h infection 100 µL of Colcemid Karyo Max®colcemid® (10µg/ml) (Gibco ™) was added to the cell culture. The colcemid stops the cell cycle in metaphase. After 3 hours the cells were treated with trypsin. When the cells were loose from the surface of the flask 5 ml of medium was added and then the cells

were transferred to 15 ml centrifuge tube. The tube was centrifuged at 1500 rpm for 10 min. The medium was removed except for about 0.5 ml of supernatant remaining above the cell pellet. The cell pellet was resuspended in the remaining medium. Five ml of prewarmed (37°C) 0,075 M KCl solution was added, drop-by-drop, while the tube was gently shaken. The cells were incubated at 37°C for 20 min and centrifuged at 1500 rpm for 10 min. The supernatant was removed and 8 ml of fresh and cold fixer (Methanol/Acetic acid, 3:1) was added slowly, the first 2 ml were added dropwise while the tube was placed on a shaker and shaken gently. The tube was incubated at room temperature for 15 min and centrifuged at 1500 rpm. The fixation was repeated two more times. After the last centrifugation the cells were resuspended in a small amount of fixative. One drop was then spread on a clean microscope slide. The microscope slides were stored at -20°C until they were used in fluorescent in situ hybridization (see 3.6).

3.2.4 Cell infection and VPA treatment

Cells were cultured in 12 well multidishes (Nunc ™) and infected with a multiplicity of infection (m.o.i) of 1-10 with a virus preparation that had been measured (see 3.4.1 and 3.4.2). At 2 h post-infection, the supernatant was replaced with fresh DMEM medium. VPA was added to the medium to a total concentration of 3 mM.

3.3   DNA and RNA methods

3.3.1 DNA isolation

DNA from cell culture was isolated with Gentra Purgene Cell Kit (Qiagen), according to the protocol for cultured cells.

3.3.2 Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were performed in a PTC-200 DNA Engine Thermal Cycler (MJ Research), and Thermal Cycler 2720 (Applied Biosystem™). Taq polymerase and Taq buffer (New England BioLabs) were used. The reactions were performed in 20 µl reaction volume.

PCR reaction solution

DNA	1-3 µl
10 x Taq Buffer	2 µl
dNTP mix (2mM)	2 µl
forward primer (20 µM)	1 µl
reverse primer (20 µM)	1 µl
Taq polymerase (500 U)	0.1 µl

Total reaction volume was brought to 20 µl with ddH$_2$O

PCR program

Step 1 (denaturing)	94°C	3 min
Step 2 (denaturing)	94°C	30 sec
Step 3 (annealing)	45-60°C	30 sec
Step 4 (elongation)	72°C	30 sec – 1 min

Cycle step 2-4 was repeated 29 -39 times

Step 5 (elongation)	72°C	5 min

3.3.3 Electrophoresis on agarose gel

PCR products were run on a 1% agarose gel, smaller fragments were run on a 2% agarose gel. Gels were made with "Agarose, For Routine Use" (Sigma). Dried agarose was dissolved in 0.5x TBE (Tris borate-EDTA, 0,045 M Tris borate and 0.001 M EDTA, pH 8.0). The agarose was melted in the TBE buffer until it boiled and then cooled down to 50-60°C and 2 drops of ethidium bromide (10 mg/ml) (Sigma) added. Before loading onto the gel one tenth volume of restriction buffer, 10x (RSB) (50% glycerol, 15 mM EDTA, 0.25 bromophenol blue), was added to the PCR product. Electrophoresis was carried out at 60-75 V for 30-60 min and 1 kb DNA ladder (Invitrogen) was used as a standard. The fragment was visualized under UV light in a White/UV Transilluminator (UVP, UK), photographed using the Grap-IT program (UVP, UK) and printed in a Sony Digital Graphic Printer (UP-D860E).

3.3.4 Cloning of ovine β-actin

RNA was isolated from non-infected SCP cells, see chapter 3.3.7 cDNA was generated from the RNA (chapter 3.3.9). PCR primers for ovine β-actin mRNA (GeneBank accession no. U39357) are listed in table 1. The β-actin mRNA was amplified with Taq polymerase (chapter 3.3.2) and cloned into a pBAD TOPO$^®$vector see chapter 3.3.5.

Table 1. Primers for amplification of β-actin

Names	Sequence	Tm	GC%	Annealing temperature
kinda b-actin for	5'- atg tac cct ggc atc gca ga -3'	59.4	55	60
kinda b-actin rev	5'- aca tct gct gga agg tgg ac -3'	59.4	55	60

3.3.5 TOPO cloning

TOPO cloning was performed with the pBAD TOPO® expression kit (Invitrogen™). The Taq polymerase creates A overhangs that are necessary for the pBAD TOPO® vector. In the Topo cloning reaction 4.5 µl of PCR product was used, 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 0,5 µl TOPO vector. The reaction mixture was mixed gently and incubated at room temperature for 5 minutes, after the reaction the mixture was put on ice and then used in heat shock transformation into DH5α cells. DH5α chemically competent *E.coli* cells were kept in -80°C and thawed on ice. Three µl of the TOPO cloning reaction was added to 50 µl of DH5α cells and incubated on ice for 10 min. The transformation was heat-shocked at 42°C for 30 sec and then incubated on ice for 2 min. LB-media, 250 µl (with 20 mM of sterile glucose), was added and pre-cultured, shaken at 37°C for 1 hour. The transformation mixture was spread on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C. The following day colonies were picked and cultured in 2 ml of LB amp medium (contaning 100 µg/ml ampicillin) overnight, shaking at 37°C.

3.3.6 Plasmid isolation

Plasmids were isolated with miniprep and midiprep kits from Qiagen (QIAprep®Spin Miniprep Kit, and HiSpeed®Plasmid Midi Kit). Colonies were cultured in 2 ml (miniprep) or 100ml (midiprep) of LB amp medium shaking at 37°C overnight. Isolation of plasmid was preformed the following day according to the manufacturer's instructions. Plasmid DNA was collected in 30-50µl (miniprep) and 500 µl (midiprep) of elution buffer (EB) and stored at -20°C.

3.3.7 RNA isolation

RNA from cells was isolated with Absolutley RNA RT-PCR miniprep kit from Stratagene, according to the protocol for cultured cells.

3.3.8 DNA and RNA quantification

Quantitation and quality assessment of DNA and RNA was carried out in NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.) according to the manufacturer's user manual.

3.3.9 cDNA preparation

cDNA was generated by using 100 ng of RNA in each cDNA reaction. The reverse primer -1818kpnI (listed in table 2) binds to the end of the *gag* RNA template and generates single stranded cDNA (see chapter 3.3.9.1). Ovine β-actin cDNA was produced by using the same RNA samples and oligo dT primers that bind to the poly A tail of genomic mRNA (see chapter 3.3.9.2).

3.3.9.1 cDNA preparation for MVV quantification

RNA, 100 ng, isolated (3.3.7 and 3.3.8) from infected cells was used in each cDNA reaction and filled up to 9μl with DEPC water. Two μl of the reverse primer -1818kpnI (20 μM) was added to the RNA in PCR tubes. The tubes were heated to 65°C for 5 min and 22°C for 10 min in Thermal Cycler 2720 (Applied Biosystem™). The reverse transcription reaction was carried out with addition of 4 μl 5×buffer (250mM Tri- HCl (pH 8.5), 250 mM KCl, 20 mM MgCl$_2$ 50 mM DTT) (Fermentas), 2 μl 10×dNTP (0.2 mM), 0.25 μl Rnase Out^{TM} Ribonuclease inhibitor (40U) (Invitrogen™) or 0.25μl Ribolock (40U) (Fermentas), 0.5 μl Revert Aid MuLV Reverse Transcriptase (50U)(Fermentas). The volume was brought to 20 μl with DEPC water. The reaction was heated up in thermocycler to 42°C for 40 min and then to 90°C for 5 min, to disable the enzyme, and cooled down to 4°C in Thermal Cycler 2720 from Applied Biosystem. Samples were then measured with quantitative real-time PCR (qrt PCR), Taq Man assay (see chapter 3.5.1).

3.3.9.2   cDNA preparation for quantification of β-actin

RNA, 100 ng, isolated (3.3.7 and 3.3.8) from infected cells was used in each cDNA reaction and filled up to 9 µl of DEPC water. Two µl of Oligo dT primer (0.5 µg/µl), was added to the RNA in PCR tubes. The tubes were heated to 65°C for 5 min and 22°C for 10 min in Thermal Cycler 2720 (Applied Biosystem™). The reverse transcription reaction was carried out with addition of 4 µl 5×buffer (250mM Tri- HCl (pH 8.5), 250 mM KCl, 20 mM MgCl$_2$ 50 mM DTT) (Fermentas), 2 µl 10×dNTP (0.2 mM), 0.25 µl Rnase Out^{TM} Ribonuclease inhibitor (40U) (invitrogen™) or 0.25µl Ribolock (40U) (Fermentas), 0.5 µl Revert Aid MuLV Reverse Transcriptase (50U) (Fermentas). The volume was brought to 20 µl with DEPC water. The reaction was heated up in thermocycler to 42°C for 40 min and then to 90°C for 5 min, to disable the enzyme, and cooled down to 4°C in Thermal Cycler 2720 from Applied Biosystems. Samples were then measured with qrt PCR, SYBR Green assay (see chapter 3.5.2).

3.4   Virus Quantification

After 7-8 days infection in macrophages the supernatant was collected and virus was quantified either by Real-time PCR (3.4.1) or Reverse transcriptase assay (3.4.2).

3.4.1 Virus quantification with qrt PCR

Cell free supernatant, 200 µl, from infected macrophages was centrifuged at 14000 rpm for 60 min at 4°C. The supernatant was removed and the sediment was dissolved in 10 µl of TNE (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA), with 0,1% Triton X-100. Nine µl of resolved sediment was mixed with 2 µl of 20 µM primer -1818KnpI (see table 2) that had been resolved in DEPC (diethylpyrocarbonat 0,1%) treated water, to increase the stability of RNA, placed in PCR tubes and heated at 65°C for 5 min and 22°C for 10 min in Thermal Cycler 2720 (Applied Biosystem™). In the reverse transcription reaction 4 µl 5×buffer (250mM Tri- HCl (pH 8.5), 250 mM KCl, 20 mM MgCl$_2$ 50 mM DTT) (Fermentas), 10×dNTP (0.2 mM), 0.25 µl Rnase Out^{TM} Ribonuclease inhibitor (40U) (invitrogen™) or 0.25µl Ribolock (40U) (Fermentas), 0.5 µl Revert Aid MuLV Reverse Transcriptase

(50U)(Fermentas) was used and total volume brought to 20 μl with DEPC water. The reaction was heated up in thermocycler to 42°C for 40 min and then to 90°C for 5 min, to disable the enzyme, and cooled down to 4°C in Thermal Cycler 2720 from Applied Biosystems. Samples were then measured with qrt PCR, Taq Man assay (see chapter 3.5.1).

3.4.2 Reverse transcriptase (RT) assay

The EnzChek® Reverse transcriptase assay (invitrogen™) was used to measure reverse transcriptase activity. PicoGreen® is used to detect dsDNA and RNA-DNA heteroduplexes but not single stranded nucleic acid and free nucleotides. 200 μl of cell free supernatant from infected macrophages was centrifuged at 14000 rpm for 60 min at 4°C. The supernatant was removed and the sediment was dissolved in 10 μl of TNE (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA), with 0,1% Triton X-100. A mixture of long poly A template (1 mg/ml in 100 mM Tris-HCl, 0.5 mM EDTA, pH 8.1) diluted 1:5 in DEPC water and oligo-dT primer (50 µg/ml in 100mM Tris-HCl, 0.5 mM EDTA, pH 8.1) diluted 1:5 in DEPC water were incubated for 1 hour at RT to allow the primer to anneal to the template. Polymerase buffer, 390 µl (60mM Tris-HCl, 60mM KCl, 8mM MgCl$_2$, 13mM DTT, 100 µM dTTP, pH 8.1) was added to 10 µl of the primer and template solution. RNA-DNA heteroduplexes were formed by adding 5 µl of virus dissolved in TNE and 20 µl of the polymerase buffer solution. The reaction was kept at 27°C for 10-60 min. The reaction was stopped by adding 2 µl of 200 mM EDTA, and at last 4.5 µl of 400X PicoGreen® diluted in 1x TE buffer was added to the reaction. The RNA-DNA heteroduplexes were detected in a micro plate reader using a filter with fluorescent excitation and detection wavelengths at 485 nm and 585 nm respectively.

3.5   Real-time PCR

A real-time PCR assay measures the amount of a nucleic acid target during each amplification cycle of PCR. Both real-time Taq Man assay and SYBR green assy quantification was performed using a StepOne™ Real Time System (Applied Biosystems).

3.5.1 TaqMan assay

The TaqMan assay is based on the 5'-3' exonuclease activity of the Taq DNA polymerase. The probe is labeled with two dyes, a reporter dye (FAM) at the 5' end and a 3' quencher dye (BHQ-1). The 3' dye quenches the reporter dye. The probe anneals between the forward and reverse primer and during PCR amplification the probe is degraded by the 5'-3' exonuclease activity of the Taq DNA polymerase. Because the probe is broken down the two dyes are separated and the reporter dye is no longer quenched. Increasing amounts of the reporter dye can therefore be detected after each amplification cycle (Holland et al., 1991). A standard curve was made with ten-fold dilutions (10^5-10^2) of the plasmid p8XSp5-VR2 (Skraban et al., 1999). The real-time primers and the TaqMan probe are listed in table 2.

Table 2. cDNA primer, Real-time primers and TaqMan probe

Names	Sequence	Tm	GC%
-1818kpnl	5'- cgg ggt acc tta caa cat agg ggg cgc gg -3'	73.7	65.5
V1636	5'- taa atc aaa agt gtt ata att gtg gga -3'	55.8	25.9
V-1719	5'- tcc cac aat gat ggc ata tta ttc -3'	57.6	37.5
V1665 TaqMan	5'- Fam- cca gga cat ctc gca aga cag tgt aga ca – BHQ-1-3'	68.1	51.7

Taq Man real-time PCR reaction solution

DNA	5 µl
TaqMan gene expression Master Mix from ABI	10.0 µl
Forward primer V1636 (20 µM)	0.9 µl
Reverse primer V-1719 (20 µM)	0.9 µl
V1665 TaqMan probe (10 µM)	0.5 µl

Total reaction volume was brought to 20 µl with ddH$_2$O

Temperature cycling

Step 1 (holding stage)	50°C	2 min
Step 1 (polymerase activation)	95°C	10 min
Step 2 (denaturing)	95°C	15 sec
Step 3 (annealing and elongation)	60°C	1 min

Cycle steps 2-3was repeated 40 times

3.5.2 SYBR Green

SYBR Green binds to all double stranded DNA PCR products. An increase in double stranded DNA product during PCR leads to an increase in fluorescence intensity measured in each PCR cycle with the StepOne™ Real Time PCR System (Applied Biosystems). SYBR Green binds to DNA unspecifically so, if the primer anneals to non-target DNA sequences and primer-dimer artifacts it can hamper accurate quantification of the target DNA. A melting curve was done to assess the homogeneity and Tm of the products. SYBR Green was used to evaluate the β-actin expression in MVV infected cells.

SYBR Green real-time PCR reaction solution

DNA	5 µl
Power SYBR® Green PCR Master Mix	10.0 µl
Primer 1: Kinda b-actin for (20 µM)	0.9 µl
Primer 2: Kinda b-actin rev (20µM)	0.9 µl

Total reaction volume was brought to 20 µl with ddH$_2$O

Temperature cycling

Step 1 (polymerase activation)	95°C	10 min
Step 2 (denaturing)	95°C	15 sec
Step 3 (annealing and elongation)	60°C	1 min

Cycle steps 2-3was repeated 40 times

After 40 cycles a melt curve analysis from 60°C - 90°C was performed.

3.6 *Fluorescent in situ hybridization (FISH)*

The plasmid p8XS5-RK1, see chapter 3.1 (Skraban et al., 1999) was used as a probe to detect whether the MVV proviruses VA3 and VA4 were integrated into the chromosomes of the infected SCP cells. The plasmid was grown in DH5α cells and plasmid DNA was isolated with HiSpeed Midiprep kit (Qiagen) see 3.3.5. Then the probe was labelled with digoxigenin-11-dUTP (Enzo) by nick translation. The probe was diluted in t-DenHyb-2 hybridization buffer (Insitus Biotechnologies) and simultaneously denatured with the chromosomes and hybridized overnight at 37°C in a Hybridizer (Dako). After post-hybridization washes in 0.1x SSC at 60°C the digoxigenin was detected with anti-digoxigenin (Boehringer Mannheim) and Alexa 488 labeled

secondary mouse anti IgG1 (Molecular Probes). Chromosomes were counterstained with DAPI and visualized in a Leica epifluorescence microscope.

4   Results

4.1   Reverse transcription

To determine whether the block to VA3 replication in SCP cells was in reverse transcription, SCP cells were infected with VA3 and VA4 in 12 well plates at an m.o.i. of 1. DNA was isolated at several time points and viral DNA was detected by a TaqMan qrt PCR assay using a portion of the *gag* gene as a probe. This part of the MVV genome is replicated late in reverse transcription. It can be seen in figure 17 that reverse transcription starts immediately after infection and is completed in around 12 hours.
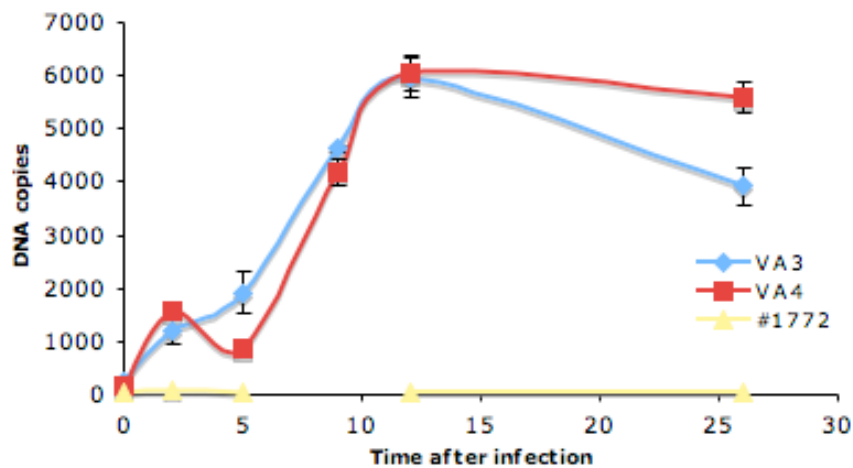
Figure 17. DNA copies of MVV during 26 hours of infection. #1772 is the virus strain 1772 that has been inactivated at 80°C for 15 minutes.

When DNA from infected cells was examined at several time points beyond 26 hours, VA4 produced more DNA than VA3 (see figure 18). This shows that VA4 was able to finish the replication cycle and produce infectious viruses that can infect new cells. Reinfection started after around 30 hours (figure 18).
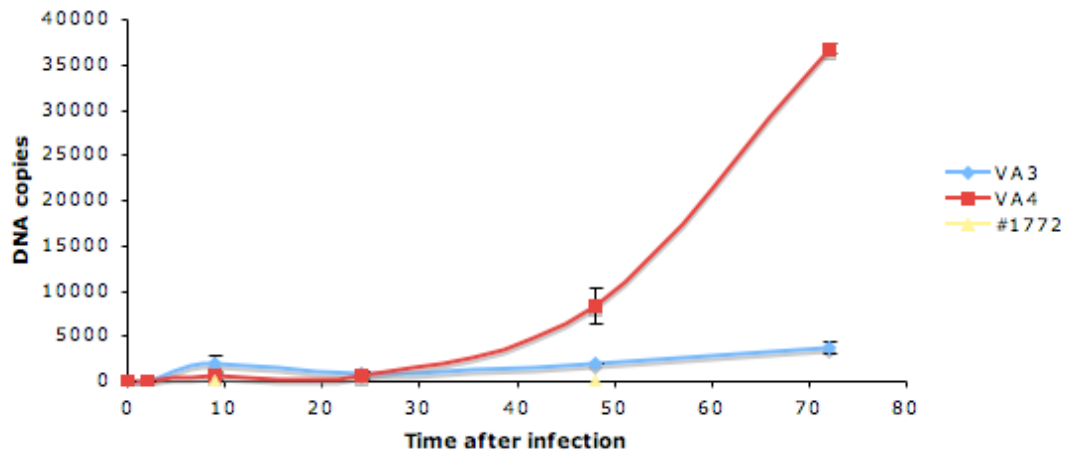
Figure 18. DNA copies of VA4 and VA3 on the first 72 hours of infection. #1772 is the virus strain 1772 that has been inactivated at 80°C for 15 minutes.

4.2 Integration

Cells in mitosis were checked for MVV provirus integrated into the host genome. Metaphase chromosomes were isolated from VA3- and VA4-infected SCP cells and analysed by fluorescent in situ hybridization (FISH) using a probe containing most of the MVV genome labeled with digoxigenin-11-dUTP. Thirty cells infected with VA4, 31 cells infected with VA3 and 10 non-infected cells were examined. Integrated provirus was found in all cells infected with VA3 and all except one in VA4, whereas the probe did not bind to the chromosomes in the negative control (table 3, figure 19). There were multiple insertions of provirus in each metaphase, and no specific integration site was detected (figure 19). No difference was thus found between integration of VA3 and VA4 into chromosomes.
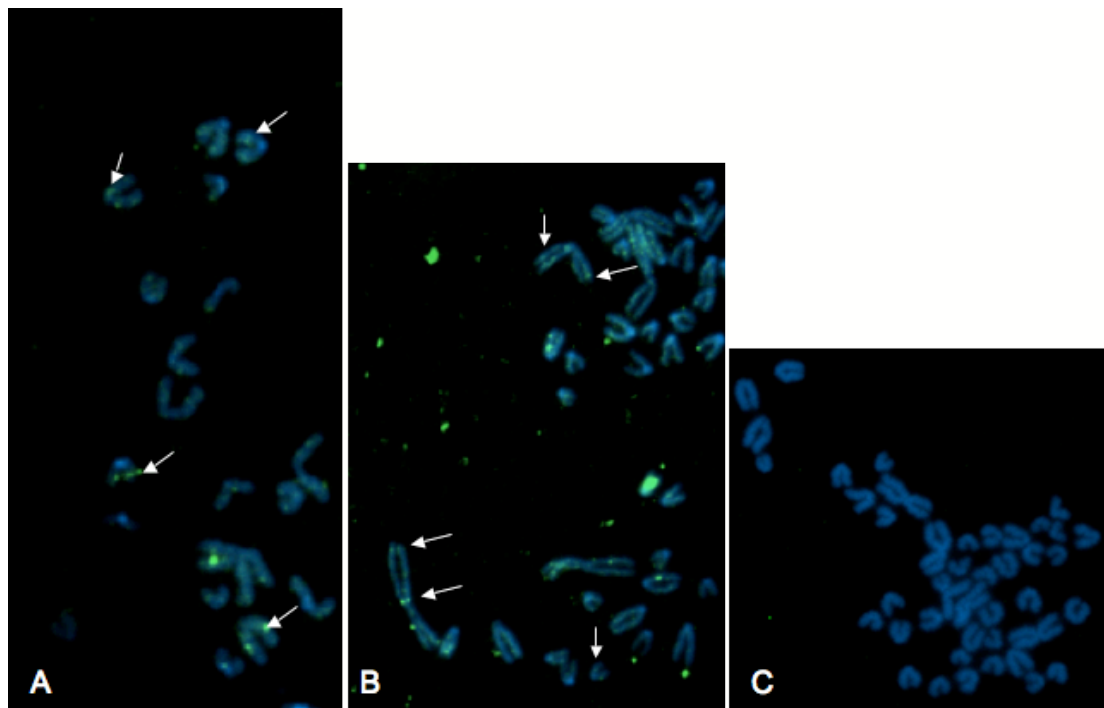
Figure 19. MVV integration into SCP cells chromosomes. A) SCP cells infected with VA4 MVV strain for 27 hours. B) SCP cells infected with VA3 MVV strain for 27 hours. C) Non infected SCP cells.

Table 3. Number of MVV insertions in SCP cells infected with VA4 and VA3.

Virus	Number of cells	Cells with MVV insertion
VA4	30	29
VA3	31	31
Non infected	10	0

4.3   mRNA synthesis

4.3.1 Generation of β-actin standard curve

β-actin mRNA was used as an internal standard for viral mRNA in quantitative real-time PCR. The β-actin forward primer 5'- ATGTACCCTGGCATCGCA-3' and reverse primer 5'-TAGGTGTAGACGACCTTCCACC-3' from (Bjarnadottir and Jonsson, 2005) were analyzed with the online Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu). The reverse primer showed potential hairpin formation. New β-actin PCR primers were designed based on the primers from (Bjarnadottir and Jonsson, 2005) see table 1.

β-actin PCR primers in table 1 were able to amplify β-actin cDNA. Figure 19 shows the β-actin product and that the primers are specific for β-actin and give only one PCR band. The primers do not form primer dimers. It is important that primers used in the qRT PCR SYBR Green assay do not form non-specific PCR products. The β-actin cDNA PCR product was cloned into a TOPO vector.
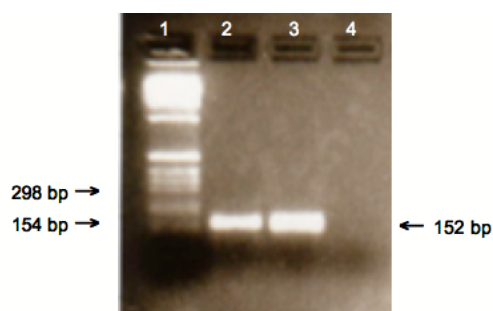
Figure 20. PCR on β-actin cDNA generated from RNA isolated from SCP cells. Lane 1. DNA ladder. Lane 2-3: **β**-actin product from two RNA samples and lane 4: negative control.

The β-actin primers bind to bases 999-1018 in exon 5 and 1152-1132 in exon 6. The PCR product amplified with these primers is shown in figure 19. This PCR produces one 152 bp cDNA product. The intron between exon 5 and exon 6 is 112 bp. Figure 20 shows that there is only one band of 152 bp suggesting that there is no DNA contamination since the PCR product from a genomic template should be 264 bp. The β-actin product, shown in lane 3 in figure 20 was cloned into a pBAD TOPO® plasmid.

When the β-actin had been cloned into pBAD TOPO®, ten-fold dilutions were used to make a standard curve, using a qRT PCR SYBR Green assay. The standard curve slope and the coefficient of determination (R^2) are shown in figure 21.
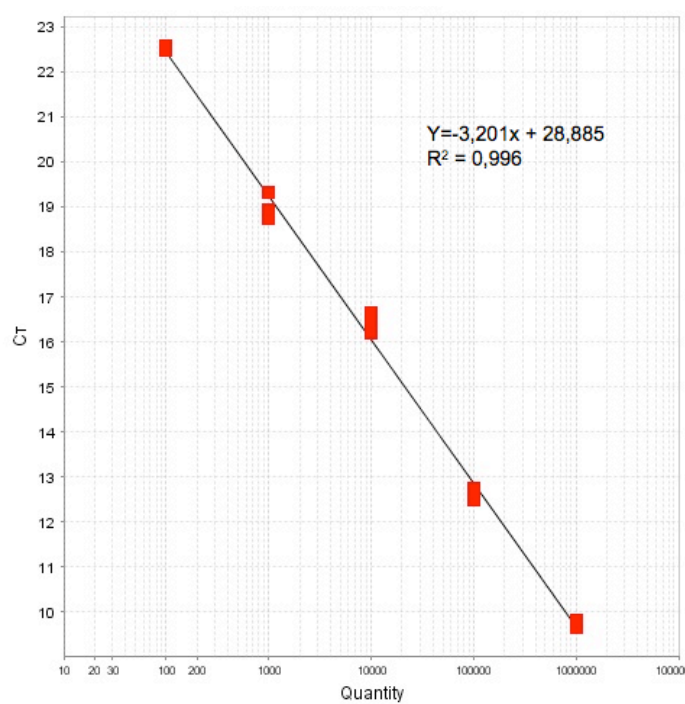
Figure 21. β-actin standard curve. Ten-fold dilutions (100 – 10^6) of β-actin pBAD TOPO$^®$ plasmid.

4.3.2 mRNA synthesis quantification

To evaluate the mRNA synthesis of the two virus strains VA3 and VA4, the gag cDNA was measured with a qrt Taq Man assay.
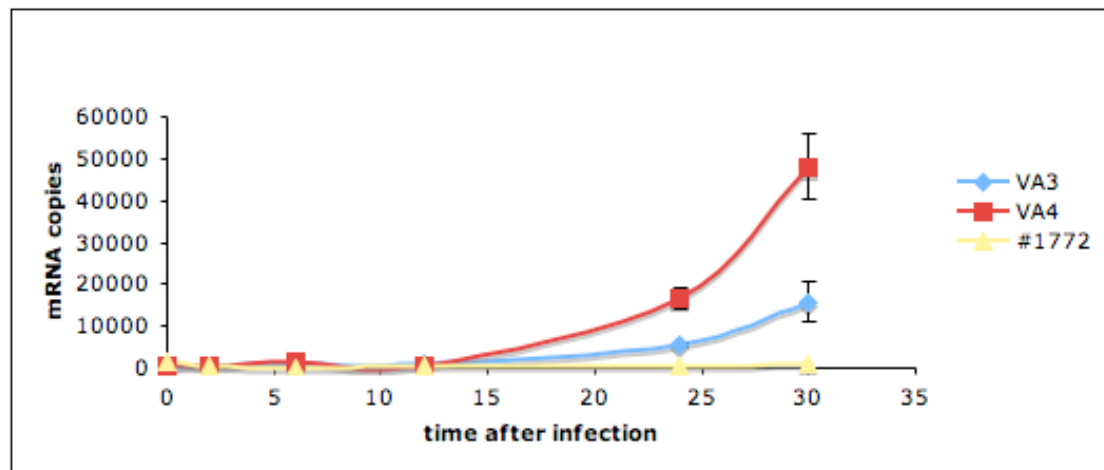
Figure 22. RNA copies of MVV during the first 30 hours of infection. #1772 is the virus strain 1772 that has been inactivated at 80°C for 15 minutes.


The virus strain VA3 did not produce mRNA at the same level as VA4 as shown in figure 22. mRNA was not produced until after 12-24 hours which

is consistent with the reverse transcription results (figure 18) where reverse transcription is not finished until after 12-24 hours.

β-actin mRNA was also evaluated in the RNA samples to see if there was a comparable amount of RNA in each sample, see figure 23. There is a comparable amount of β-actin RNA in the RNA samples at the same time, but the β-actin mRNA varies over the course of infection.
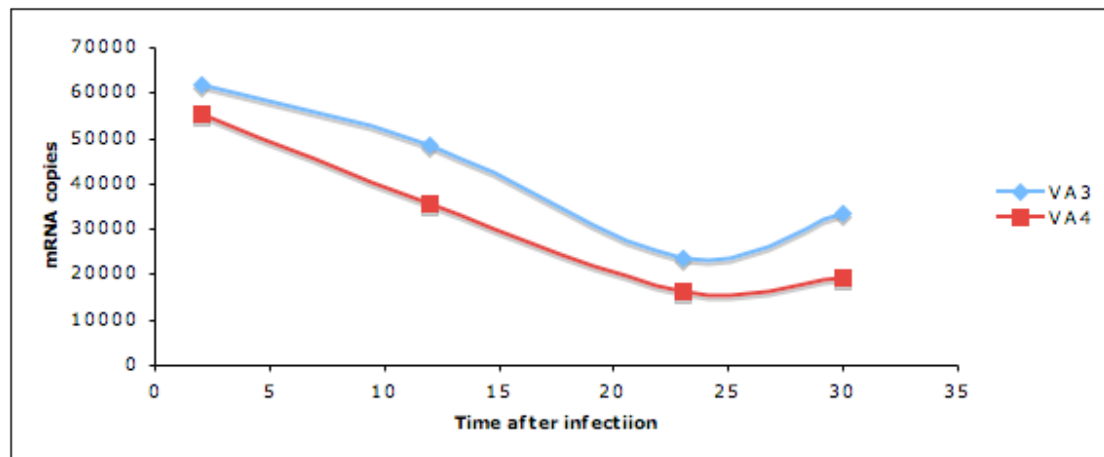
Figure 23. RNA copies of β-actin in cells infected with VA3 and VA4.

Figure 24 shows a single peak melting curve indicating that there is only one PCR product. This indicates that there is no DNA contamination in the RNA sample because genomic template in the RNA sample would give an additional peak in the melting curve.
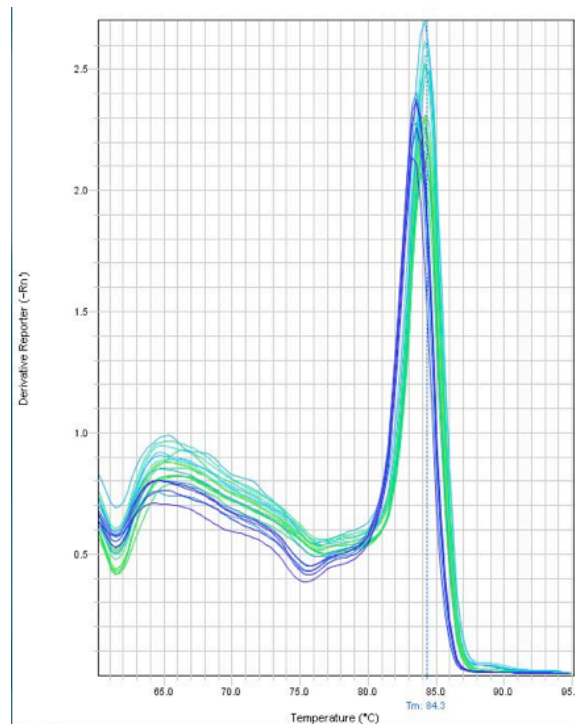
Figure 24. Melting curve of the β-actin rt PCR product. A single peak in the melting curve indicates that only one product was amplified

To see if the HDAC inhibitor VPA had any effect on the viral replication of the virus strain VA3, VPA in the concentration 3 mM was added to the medium of SCP cells infected with VA3. RNA was collected and MVV mRNA copies compared between the virus strains VA3, VA4 and VA3 treated with VPA, see figure 25. There was no difference in MVV mRNA copies when cells infected with VA3 where treated with VPA or not.
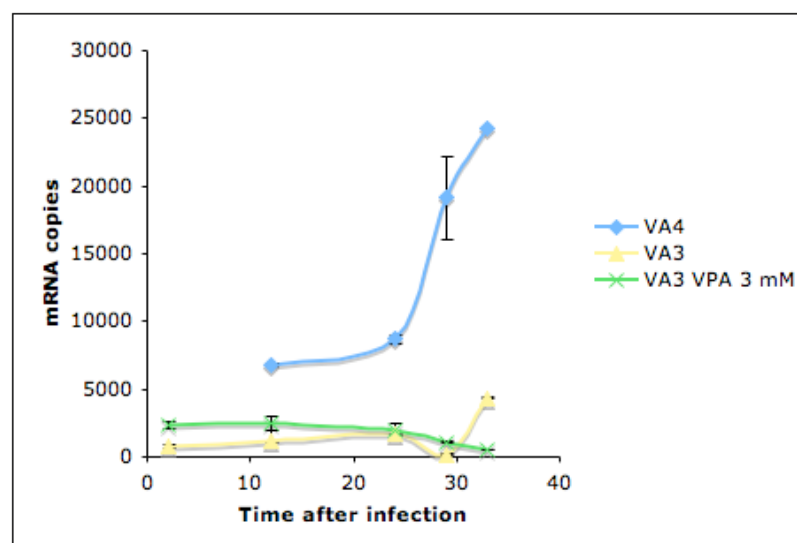
Figure 25. mRNA copies of MVV after infection with VA4 and VA3. VA3 infected cells were also treated with 3 mM VPA.

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5   Discussion

It was previously shown that a repeat sequence in the LTR of MVV broadened the cell tropism of the virus from being strictly macrophage tropic to being able to grow in a number of cell types, including SCP cells.

In this study different stages in the replication cycle of MVV strains with and without the repeat in the LTR were compared. The two virus strains, VA3, a strain without a repeat in the LTR, and VA4, a strain with a 54 bp repeat in the LTR were grown on SCP cells and reverse transcription, integration and RNA production were analyzed.

5.1   *Reverse transcription*

The reverse transcription in MVV started directly after infection and was completed 12 -24 hours after infection. Research on HIV-1 has shown similar results, where reverse transcription is completed after 24 hours (Butler, Hansen, and Bushman, 2001). Reverse transcription starts in the virion before infection (Le Rouzic and Benichou, 2005) or directly after the genetic material enters the host cytoplasm. The tight packaging of the NC protein molecules, the genomic RNA and the RT enzyme is loosened after entry into the cytoplasm and the RT enzyme is able to complete reverse transcription (Houzet et al., 2008; Thomas et al., 2008).

Reverse transcription starts using the tRNA primer near the U5 on the 5' end of the LTR, and to complete reverse transcription the virus has to make a transfer jump.The template transfer and completion of the first strand DNA synthesis is impeded by tight packaging in the virion but strong stop reverse transcription may start at this stage. To be certain that the cDNA that was measured in the real-time PCR was synthesized after infection of the host cell, primers and probe from the *gag* gene were used. The *gag* gene is replicated late in reverse transcription, well after the first strand transfer jump.

In the first 24 hours, before VA4 could re-infect the cell culture, there was no difference in reverse transcription between the two virus strains VA3 and VA4 indicating that virus entry and reverse transcription in VA3 was not impeded. The difference in virus replication in SCP cells can therefore not be

the result of a difference in reverse transcription between the two virus strains. After around 30 hours, VA4 reinfected new cells, and replication took off, whereas VA3 did not replicate further, confirming previous results that the replication of VA3 is attenuated in SCP cells.

5.2 Integration

As shown earlier, MVV has a fully functional retroviral integrase (IN) (Katzman and Sudol, 1994). In figure 19 it is shown that both virus strains, VA4 and VA3, were capable of integrating the viral DNA into the host chromosome, and no specific chromosome integration could be detected. In HIV-1 it has been shown that the HIV-1 virus integrates proviral DNA into the host chromosome in active genes and regional hotspots (Schröder et al., 2002). The MVV IN has been shown to have basic functional similarities to HIV-1 IN (Stormann, Schlecht, and Pfaff, 1995). Therefore it is likely that MVV is integrated into similar regions on the host chromosome. Since the sheep genome has not been sequenced and annotated it is not feasible to determine whether the integration sites are in active genes.

5.3 mRNA synthesis

In figure 22 it is shown that the virus strain VA4 produces more viral mRNA than the virus strain VA3. The viral mRNA production started around 12 hours after infection which is consistent with the results from the first part of this project where reverse transcription was finished 12-24 hours after infection.

β-actin mRNA was used as an internal reference in the qrt PCR. The amount of β-actin mRNA was similar in cells infected with VA3 and VA4 but there was a difference in the amount of β-actin mRNA at different time points after infection. The amount of β-actin mRNA was highest shortly after infection, when the cells had recently formed a confluent cell layer. This could indicate that β-actin mRNA, although very useful in our comparison of VA3 and VA4, is not as suitable as an internal reference in qrt-PCR as generally thought (Selvey et al., 2001).

Monocytes/macrophages are the main target cells of MVV infection and virus replication is restricted until differentiation of the monocytes to

macrophages (Gendelman et al., 1985; Gorrell et al., 1992). AP4 and AP1 sequences close to the TATA box have been shown to be important for MVV transcription in macrophages. The transcription factors c-Fos and c-Jun bind to AP-1 sites which leads to the activation of MVV gene expression as monocytes mature into macrophages (Shih et al., 1992).

The sequence that has been shown to be important for efficient MVV replication in SCP cells is the E box sequence CAAATG (Oskarsson et al., 2007). E- boxes are considered to be binding sites for basic helix-loop-helix DNA binding proteins (Massari and Murre, 2000). In HIV-1 four E-box motifs have been described in the LTR and one in HTLV-1 LTR. These E-box sequences are located close to the TATA region (Terme et al., 2009). In the visna virus molecular clone Kv1772 there are four E box sequences located upstream of the TATA box. (Andresson et al., 1993). This also applies for the MVV clone VA4 but because one of the E-box sequence is located in the duplication of the VA4 virus strain, the MVV clone VA3 has only three E box sequences upstream the of the TATA box (Agnarsdottir et al., 2000).

It was shown previously that there was no difference in transcription from the VA3 and VA4 LTRs in a luciferase expression plasmid in SCP cells. It is therefore likely that the difference in virus replication between the two virus strains is controlled at the level of the chromatin structure of integrated proviral DNA and can not be detected with transient transfection of plasmid constructs.

In HIV-1 it has been shown that the binding of AP-4 (and possibly other bHLH proteins) to the E- box can exclude binding of TATA binding proteins to the TATA box and inhibit LTR mediated HIV-1 transcription and recruit HDAC to the promoter. AP-4 mediated repression was inhibited when an HDAC inhibitor was used (Imai and Okamoto, 2006). The E-box sequence in the LTR of HTLV-1 binds to the bHLH protein E47 and represses both basal and Tax-induced LTR activity. E47-mediated repression in HTLV-1 was inhibited by the bHLH protein TAL1 (Terme et al., 2008). In HIV-1 and HTLV-1 the binding of bHLH proteins to E box sequences in the LTR may inhibit binding of TATA binding proteins to the TATA box and therefore repress proviral transcription. E-boxes in the LTR may account for the establishment and maintenance of latency in HIV-1 infected cells (Terme et al., 2009). In HIV-1 there are also

several other factors known to induce HIV-1 latency by altering chromatin structure.

The HDAC inhibitor VPA was used in an attempt to answer the question whether chromatin remodeling was involved in the transcriptional control of MVV expression. In figure 25 it is shown that there was no difference in MVV mRNA copies with the MVV strain VA3 upon treatment with VPA or not. Therefore the use of the HDAC inhibitor VPA did not seem to increase the viral replication of the MVV strain VA3, i.e. relieve possible transcriptional inhibition by allowing acetylation of an inhibiting nucleosome. VPA has been shown to inhibit multiple HDACs but not HDAC6 and HDAC10 (Gurvich et al., 2004). It can not be ruled out that the concentration of VPA was too low to inhibit the relevant HDACs or that the remodeling factors are not inhibited by VPA. The duplicated E-box most likely recruits a protein factor(s) stimulating transcription, and it has not been ruled out that this activation is via modification of an inhibitory nucleosome (see figure 26).
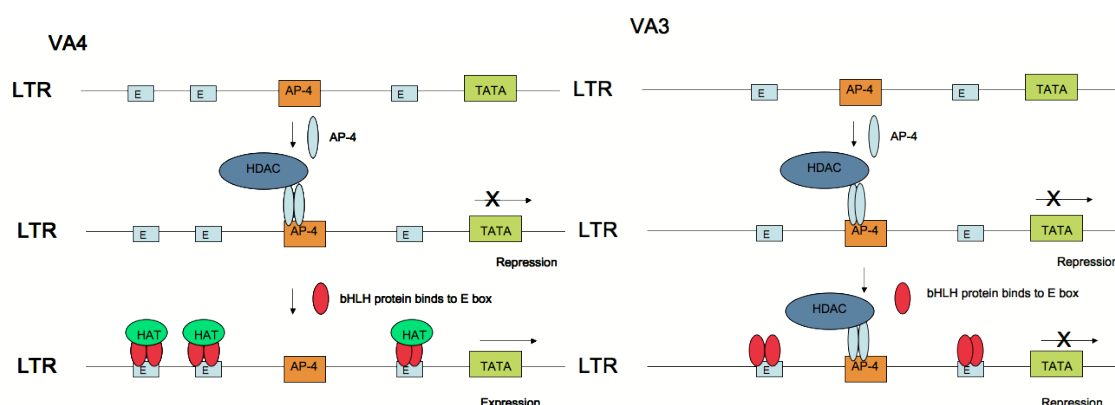
Figure 26. Possible model for MVV gene expression in other cell types than macrophages. AP-4 recruits HDAC to the MVV promoter, preserving the deactylation of histones. VA4 which has 3 E box sequences near the TATA box binds to a bHLH protein that recruits HAT to the promoter, which acetylates histones and facilitates gene expression. VA3 has only 2 E box sequences near the TATA box that are not able to release the AP-4 repression of gene expression.

6   Conclusion

The aim of this study was to find where the barrier is in the replication cycle of MVV without duplication in the LTR. The results show that there is no difference in reverse transcription and both viruses can integrate the viral DNA into the host chromosome. There is a difference in mRNA production, where VA4 produces more mRNA than VA3. The barrier in the replication cycle of MVV without duplication is therfore in the mRNA production. It is most likely that the control is at the level of chromatin remodeling. This is in accordance with the growing evidence for chromatin effect on retroviral gene expression.

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