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MASTERARBEIT

Titel der Masterarbeit

Generation of avian sarcoma-leukosis viruses to achieve lymphatic-specific expression of a fluorescent marker in an *ex-ovo* CAM model.

verfasst von

Kathrin Barbara Bracher

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1. Table of contents

2. List of abbreviations

ALV	Avian leucosis virus
ASLV	Avian sarcoma-leucosis virus family
BS	Binding site
CAM	Chorioallantoic membrane
CAT	Chloramphenicol acetyltransferase
CEF	Chicken embryo fibroblast
CMV	Cytomegalic virus
FGFR3	Fibroblast growth factor receptor 3
GFP	Green fluorescent protein
LB	Luria broth
LDL	Low density lipoprotein
LTR	Long terminal repeat
MCS	Multiple cloning site
PCR	Polymerase chain reaction
RCANBP	**R**eplication **c**ompetent **s**arcoma-leucosis virus with **n**o splice acceptor, **B**ryan RSV **P**olymerase
RCASBP	**R**eplication **c**ompetent **s**arcoma-leucosis virus with a **s**plice acceptor, **B**ryan RSV **P**olymerase
RSV	Rous sarcoma virus
TK	Thymidine kinase
VEGFR3	Vascular endothelial growth factor receptor 3

3. Abstract

3.1. English

The lymphatic vasculature is involved in a number of pathological conditions such as neoplasm and metastasis. It is part of the circulatory system and built up from lymph vessels that carry a clear fluid called lymph, which is recycled blood plasma. To enable a study of metastasis, the chorioallantoic membrane (CAM) of chicken embryos, which constitutes a large membrane area nerved with blood and freely accessible lymphatic vessels, is used to allow *in-vivo* manipulation and imaging of the vessels without invasive interventions. To visualize the lymph vessels in the model organism a strategy involving a replication-competent avian sarcoma-leukosis virus with a splice acceptor, Bryan RSV Polymerase (RCASBP) and the same virus without the splice side (RCANBP) were used. The virus is able to spread horizontally to neighbouring cells after infection, as well as vertically to daughter cells, thus allowing ubiquitous infection and transgenesis of embryos in a single generation.

The main objective of this research was to construct a virus, which drives gene-expression exclusively in lymphatic cells. An appropriate marker for lymph vessels is Prox1, which is not expressed in in blood vessels except of the jugular vessel and strong conserved in most species. To achieve lymphatic specificity the binding motif of Prox1 was used six times consecutively, followed by different promoter elements and the eGFP gene, which served as fluorescent marker. As such promoter elements, CMV and thymidine kinase (TK) promoters were utilized to drive specific expression of eGFP in lymph endothelial cells. Methodically the project involved the construction of a specific plasmid using a subcloning vector, infection of chicken fibroblast cells to produce virus particles and concentration of the virus via ultracentrifugation. The concentrated virus was then injected into the germ discs of the chicken embryos. Following the embryos were screened from day eight to fourteen for lymph specific eGFP expression.

To conclude, the minimal CMV promoter is sufficient to strongly drive gene expression in chicken DF-1 cells, as well as ubiquitously in the chicken embryo and CAM, while using the minimal TK promoter led to markedly lower eGFP expression in DF-1 cells and embryo. Further, it was noticed that viruses with the fragment of the subcloning vector inserted inline with the viral genes showed a stronger expression of eGFP then the virus with the fragment integrated inversely *in vitro* (DF-1 cells) and *in vivo* (chicken embryos and CAM). However, it was not possible to achieve specific expression of eGFP in lymph endothelial cells, with any of the different vector constructs.

3.2. German

Das Lymphsystem ist an einer Reihe von pathologischen Prozessen, wie zum Beispiel an der Tumorentstehung und an der Metastasierung, beteiligt. Das lymphatische System ist Teil des Blutkreislaufes und besteht aus Lymphgefäßen, die eine klare Flüssigkeit, genannt Lymphe (recyceltes Blutplasma), transportieren. Um die Studie der Metastasierung zu ermöglichen, wurde auf die Chorioallantoic Membran von Hühnerembryonen zurückgegriffen, die eine große Membranfläche von frei zugänglichen Lymph- und Blutgefäßen besitzt, welche eine in-vivo Manipulation und die Abbildung von Gefäßen ohne invasive Eingriffe ermöglichen. Um die Lymphgefäße im Modelorganismus darstellen zu können wurde eine replikationskompetentes aviäres Leukosevirus mit einem splice acceptor (RCASBP) und das selbe Virus ohne einen splice acceptor (RCANBP) generiert. Den größten Vorteil bildet die Replikationskompetenz, die es dem Virus erlaubt, sich horizontal in Nachbarzellen sowie auch vertikal in Tochterzellen zu vermehren. Durch diese Eigenschaft wird die generalisierte Infektion und Transgenität innerhalb einer Generation ermöglicht.

Das Hauptziel dieser Diplomarbeit war es, einen Vektor zu konstruieren, der ausschließlich in Lymphgefäßen exprimiert wird. Als idealer Marker bietet sich Prox1 an, welcher in Blutgefäßen außer in den Jugulargefäßen nicht exprimiert wird und in den meisten Spezies hoch konserviert ist. Das Prox1 Bindungsmotiv wurde sechsmal hintereinander geschalten, gefolgt von verschiedenen Promoter Elementen und dem eGFP Gen, welches als fluoreszierendes Markerprotein fungiert. In diesem Fall wurden Promotorelemente vom Zytomegalievirus und der Thymidinkinase verwendet, um eine spezifische Expression von eGFP in Lymphangioblasten zu erzielen. Das Projekt umfasste methodisch die Konstruktion von spezifischen Plasmiden unter Verwendung eines Subcloning Vektors, die Infektion von Hühnerfibroblastenzellen die Viruspartikel produzieren und die Konzentration von Viren mittels Ultrazentrifugation. Das Viruskonzentrat wurde dann in die Keimscheiben der Hühnerembryonen injiziert und anschließend wurden die Embryonen vom achten bis zum vierzehnten Tag auf eine spezifische eGFP Expression hin untersucht.

Zusammengefasst war der minimal CMV Promoter ausreichend um eine starke Marker Genexpression in Hühner DF-1 Zellen auszulösen, genauso wie in Hühnerembryonen und deren CAM, während der minimal Thymidinkinase Promoter zu einer merklich geringeren eGFP Expression in den DF-1 Zellen und im Embryo führte. Des Weiteren wurde bemerkt, dass Viren mit dem Fragment, welches inline mit den viralen Genen eingebracht wurde, eine stärkere Expression von eGFP zeigte, als Viren, die das Fragment inverse integriert hatten, sowohl *in vitro* (DF-1 Zellen) als auch *in vivo* (Hühnerembryo und CAM). Allerdings war es nicht möglich eine spezifische Expression von eGFP in lymphendothelalen Zellen mit einem unserer verschiedenen Vektorkonstrukte zu erzielen.

4. Introduction

4.1. The lymphatic system

The word lymphatic originates from the Latin word “lympha”, which means clear water. The lymphatic system was first described independently in the seventeenth century by Olaus Rudbeck and Thomas Bartholin (Bartholin 1654). It is found in vertebrates, like teleost fish, amphibians, reptiles, avian and mammalians (Wilting, Papoutsi et al. 2002, Jeltsch, Tammela et al. 2003, Ny, Koch et al. 2005, Kuchler, Gjini et al. 2006, Yaniv, Isogai et al. 2006). The lymphatic system in avian embryos was first described by Budge in 1882 (Budge 1882, Budge 1887). As part of the circulatory system, the lymphatic system is built up of lymph vessels that carry the clear fluid called lymph, which is recycled blood plasma. Its main function is the transport of plasma from the tissue via lymph capillaries and the ductus lymphaticus back to the subclavian vein into the circulatory system. In average the lymph system in adult humans returns one to two litres of interstitial fluid in average per day (Levick 1999). The lymph fluid is filtered by lymph nodes, small oval-shaped organs. Several afferent lymph vessels carry the lymph fluid to the node, while only one efferent lymph vessel leads the collected lymph fluid back to the circulatory system.

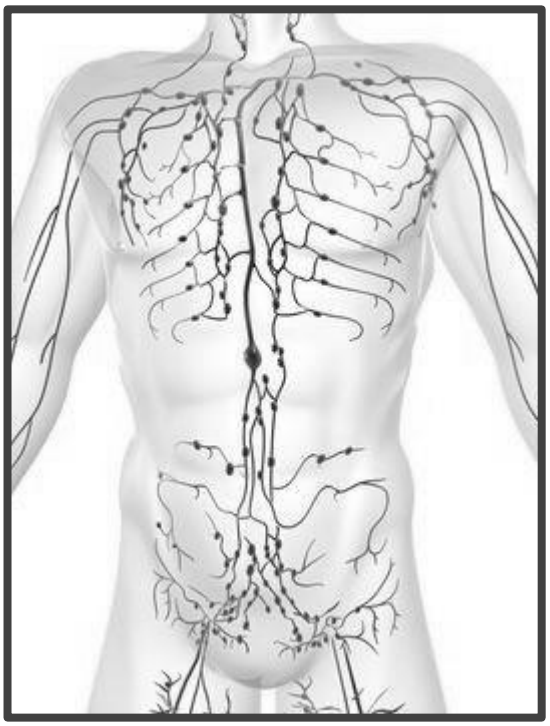


Fig. 1.: Lymphatic system in humans. http://de.depositphotos.com/2896646/stock-photo-lymphatic-system.html

Lymphatic capillaries and the thoracic duct are composed of lymphatic endothelial cells (LEC), a smooth muscles layer, which is important for contraction and an outermost fibrous membrane, which is called adventitia. The smaller lymphatic vessels are blind ended, thin walled and composed of lymphatic endothelial cells between 30-80 µm in diameter (Alitalo, Tammela et al. 2005). LECs are differentiated cells, phenotypically distinct from blood vascular endothelial cells. Lymph vessels differentiate from blood endothelial cells if Prox1 (Prospero-related homeodomain transcription factor 1) is expressed (Saharinen, Tammela et al. 2004). In *Prox1*$^{-/-}$ chicken embryos budding of lymphatic endothelial cells is arrested and cells fail to express lymphatic endothelial markers. Prox-1 is absent in blood vascular endothelial cells, except to cells of jugular vessels, hence it constitutes a major marker for lymphatic endothelial cells (Wilting, Papoutsi et al. 2002). The DNA binding

domain of Prox1 shows 100% homology between human, mouse and chicken. In addition to lymphatics, the protein is expressed in the neural tube, lens, retina and other neural placodes, liver, heart, spinal, trigeminal and sympatric ganglia (Wilting, Aref et al. 2006).

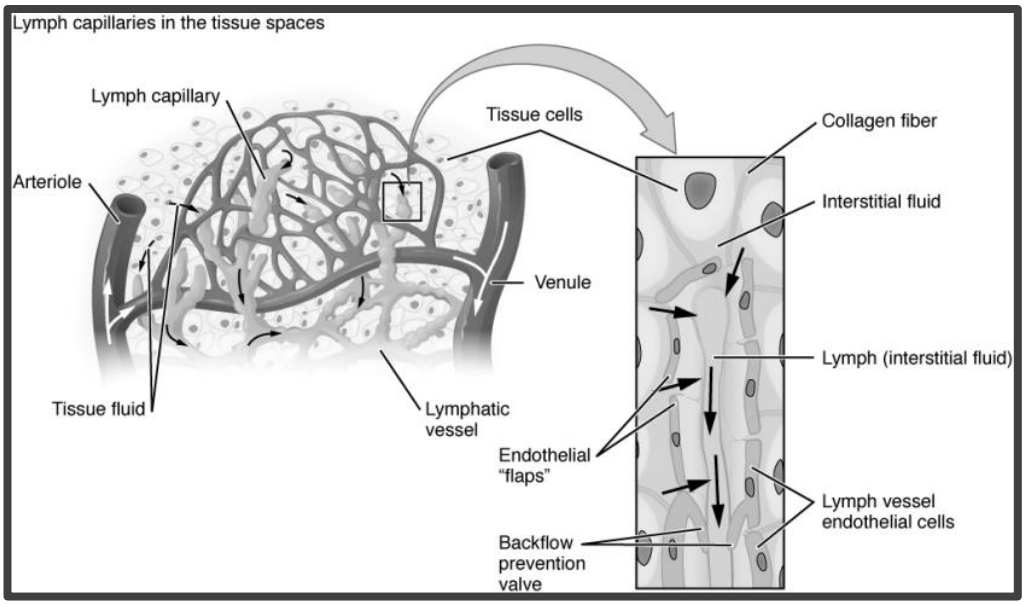


Fig. 2.: Composition of lymphatic capillaries, located in almost every tissue in the body, except the central nervous system, bone marrow, bones, teeth, and the cornea. http://cnx.org/content/m46563/latest/#fig-ch22_01_02

Another lymphatic endothelial marker is the VEGF-R3 (vascular endothelial growth factor), which is a member of the VEGFR family of growth factors (Alitalo, Tammela et al. 2005, Karpanen and Alitalo 2008). In early development, VEGF-R3 is present in all endothelia, although in later stages the VEG-FR-3 receptor is solely expressed in LECs, except due to fenestrated blood vessels in endocrine organs, e.g. pancreas (Tammela, Enholm et al. 2005).

The lymphatic vasculature is significantly involved in a number of pathological conditions, such as inflammation, lymphedema, tumour lymph angiogenesis and metastasis. Equally to blood vessels, lymphatic vessels can serve as conduits for metastasizing tumour cells to escape the primary tumour. Entering the blood circulation via the lymphatic system, the malignant cells can reach distant organs to form metastases. According to newer estimations, about 80% of all solid tumours spread via the lymphatic system reviewed by (Alitalo and Detmar 2012). The other two ways of metastasis are the haematogenous and the cavitous metastasis. The metastasis process proceeds in multiple steps, termed as metastasis cascade. The metastasis cascade is subdivided in intravasation, the procrastination of tumour cells and extravasation. The intravasation is characterised by the breakup of cell-cell connections, enzymatic degradation and remodelling of

extracellular matrix. There are different paths of entering the vessel called active and passive intravasation distinguished by the type of locomotion of tumour cells.

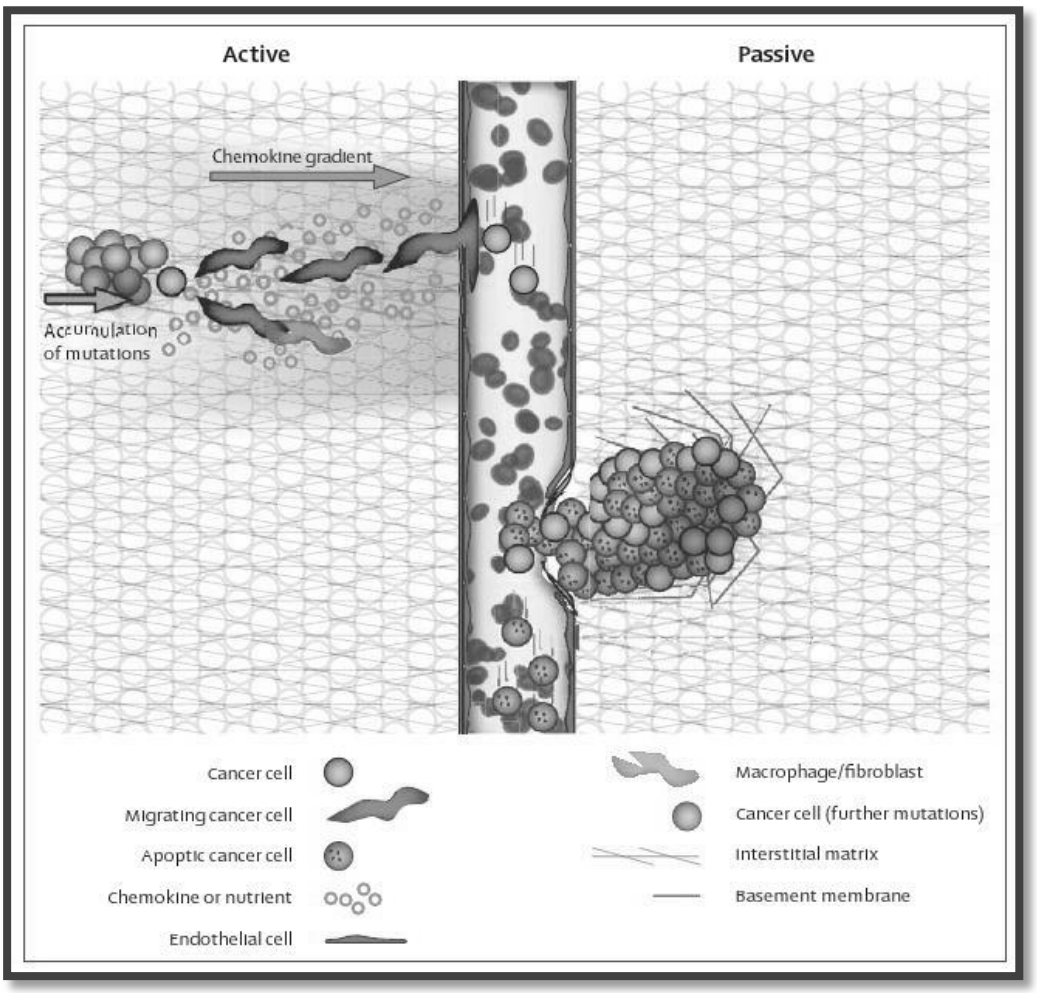


Fig. 3.: Active and passive mechanisms in initial steps of metastasis (Bockhorn, Jain et al. 2007).

Active intravasation is characterised by cancerous cells, which migrate actively towards lymphatic vessels, led by a nutrient and chemokine gradient, and in cooperation with stromal cells, e.g. fibroblasts, macrophage (Bockhorn, Jain et al. 2007). The precondition for active metastasis is the accumulation of mutations. (Bernards and Weinberg 2002, Ramaswamy, Ross et al. 2003).

Otherwise tumours metastasize through passive shedding, e. g. if the primary tumour experiences a trauma (Liotta, Saidel et al. 1976). Hence in tumour destroyed tissue the accommodative blood vessels have fragile walls (Chang, di Tomaso et al. 2000, Hashizume, Baluk et al. 2000) and therefore the vessels can collapse under solid stress (Helmlinger, Netti et al. 1997, Padera, Stoll et al. 2004).

However, the mechanism of how a tumour cell enters a lymphatic vessel is poorly understood, since it requires complex in vivo models to be studied. An attractive model organism

for observing metastasis is the *ex-ovo* chicken embryo, because it provides a large area of freely accessible CAM, with lymph and blood vessels.

4.2. The chicken embryo

4.2.1. Composition of a chicken egg

The chicken egg is composed of the egg shell (1), two different shell laminas (2, 3), the inner and the outer, and two chalazae (4, 13), which are spiral bands holding the egg yolk. The yolk is surrounded by the albumen, which is divided in the outer (5), the middle (6) and the inner egg white (12). The middle egg white is more viscous than the other two. The vitelline membrane is covering the yolk, which contains formative yolk (8), germinal disc and white (9) and yellow yolk (10). The egg contains an air cell (14) on the lager edge of the egg, which is increased by evaporation and therefore used as air supply in later embryonic stages. The outermost membrane is the cuticula (15), a thin membrane on the egg shell protecting the egg form dehydration and infections.

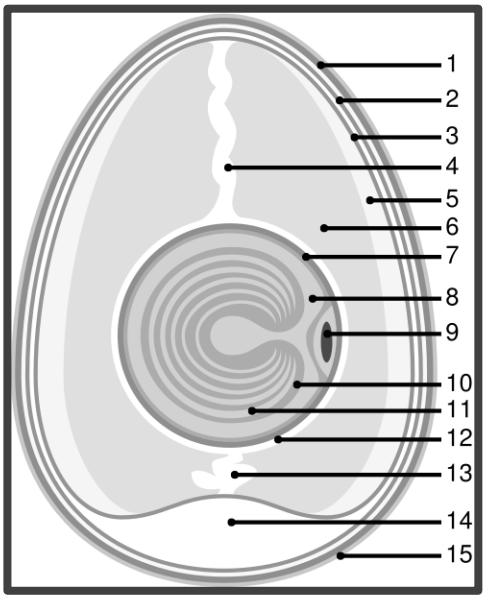


Fig. 4.: Composition of chicken egg (http://en.wikipedia.org/wiki/Egg_%28food%29).

4.2.2. Chorioallantoic membrane - CAM

To enable afore mentioned *in-vivo* metastasis studies, the *ex-ovo CAM* model offers several advantages. The CAM (chorioallantoic membrane) constitutes a large membrane area nerved with blood and lymphatic vessels freely accessible, allowing *in-vivo* manipulation and imaging of the vessels without invasive interventions. The first scientist, who published a paper about the use of the chicken chorioallotonic membrane was James B Murphy, from the Laboratories of the Rockefeller Institute for Medical Research of New York in 1913 (Murphy 1913). He recognised the advantages of the CAM and wrote: “For the general purposes of the experiment it has been found advantageous to use the outer membrane (fused allantois and chorion) for the reason that it lies just under the shell membrane and, therefore, can be inoculated with a minimum amount of trauma. Furthermore, this membrane is the respiratory organ of the chick at this period and is rich in lymphatics and blood vessels”. From this time point on the CAM has been established as an experimental system for research in tumour biology such as tumour invasion and metastasis (Quigley and Armstrong 1998).

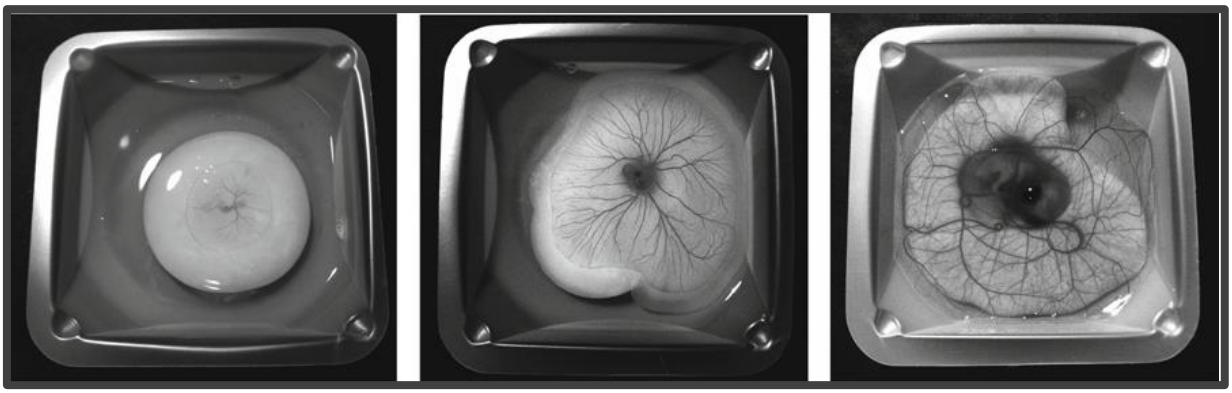


Fig. 5.: Development of cracked chicken embryos in a shell less *ex-ovo* culture on day 3, day 5 and day 10 (Deryugina and Quigley 2008).

The chorioallantoic membrane (CAM) — also called chorioallantois— is a vascular membrane found in eggs of e.g. birds and reptiles. The CAM consists histologically of three germ layers: the ectoderm, the mesoderm and the endoderm. The ectoderm faces the shell membrane and is subjacent by the respiratory capillary plexus. The angiogenesis and vasculogenesis of capillary plexus in the embryonic development is formed between days five and six after fertilization (Melkonian, Munoz et al. 2002). The mesoderm of the chorioallantois is a collagen-rich embryonic connective tissue, in which arteriole and venous blood vessels are embedded. The mesoderm is underlined with a thin endoderm layer, which separates the CAM from the allantoic cavity (Tufan and Satiroglu-Tufan 2005).

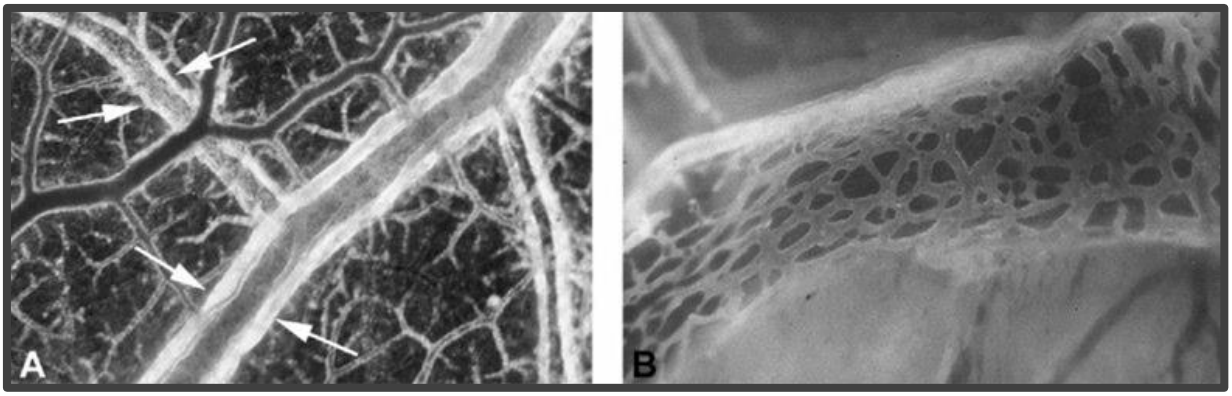


Fig. 6.: Differentiated chorioallantoic membrane (CAM) of a 13 day old chicken embryo, arrow: lymphatics (Papoutsi, Tomarev et al. 2001).

The CAM is evolved from the fusion of the mesodermal layers of two developmental structures: the allantois and the chorion. The allantois is mainly a reservoir for nitrogenous waste and also involved in the oxygenation of the embryo. The chorion, on the other hand, provides nutrients and protection needed for the embryo's survival. At 3.5 days after fertilization the chorioallantoic membrane is composed by three different layers: the chorionic and the allantoic epithelia of ectodermal and endodermal origin, in collaboration with the vascularized stroma of

mesodermal origin. Blood capillaries and sinuses are found between epithelial cells of the chorionic layer, the mesoderm (Leeson and Leeson 1963), which is in close contact with the shell membrane of the egg. As a result, the chorioallantoic membrane allows exchange of gases, such as oxygen and is involved in the transport of calcium carbonate from the eggshell, which is needed for bone formation.

Until day eight, the developed blood vessels, which descended from the mesoderm, grow very rapidly until reaching the base of the chorion. At day 14, the capillary plexus is located at the surface of the ectoderm, neighbouring the shell lamina. Rapid capillary proliferation continues until day eleven of 21. On day 18, the vascular system achieves its final arrangement (Ausprunk, Knighton et al. 1974). The first deep lymphatics are detectable during days four to five of incubation in the embryo (Clark and Clark 1920). In the CAM the large embryonic veins are accompanied by densed plexi of lymphatics, which are connected via anastomoses to larger lymphatic vessels. The lymphatics are drained into lymphatic trunks of the umbilicus and connected to the posterior lymph hearts in the chicken embryo (Wilting, Neeff et al. 1999). Hence the chorioalloantoic membranes of developing chicken eggs can be used for example to investigate development and tumour lymph angiogenesis.

The immune system in chicken at the embryonic stage is not fully developed like in other vertebrates (Leene, Duyzings et al. 1973). Therefore the manipulation of the CAM with tumour xenografts is enabled by the lack of adaptive immunity.

4.3. Rous Sarcoma Virus

The Rous Sarcoma Virus was discovered 1911 by Peyton Rous, a pathologist. He injected cell free extract of tumours gained from chicken into healthy conspecific, and noticed that new tumours originate (Rous 1910, Rous 1911). The Rous Sarcoma Virus (RSV) is an oncovirus – a virus causing cancer - belonging to the Retroviridae family. The RSV appertains to the genus of Alpharetroviridae like avian leukosis virus and avian myeloblastosis virus, both able to cause tumours in domestic birds and therefore the family is called avian-sarcoma leucosis virus.

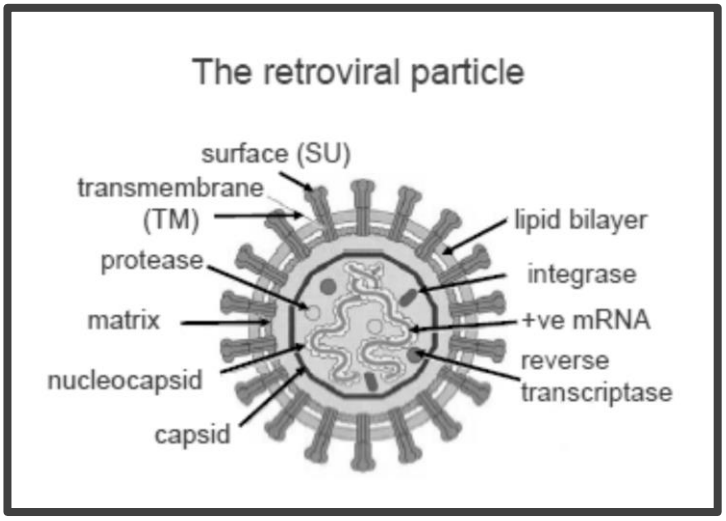


Fig. 7.: Retroviral particle (lecture VO 300347 Principles of Virology, Timothy Skern, University of Vienna).

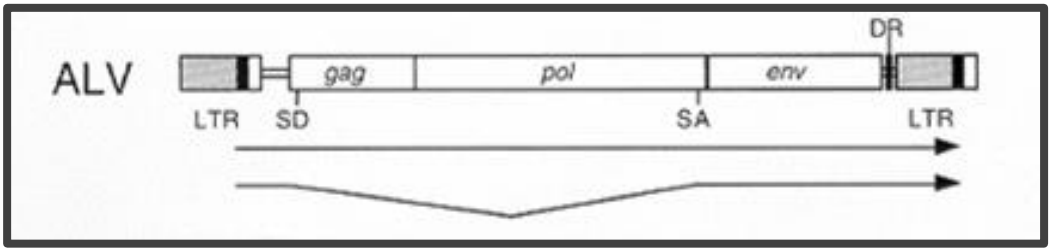


Fig. 8.: Organization of viral DNA genome of Avian leukosis virus, viral genes (gag, pol, and env), direct repeats (DR), the splice donor (SD) and splice acceptor (SA) sites (Coffin, Hughes et al. 1997).

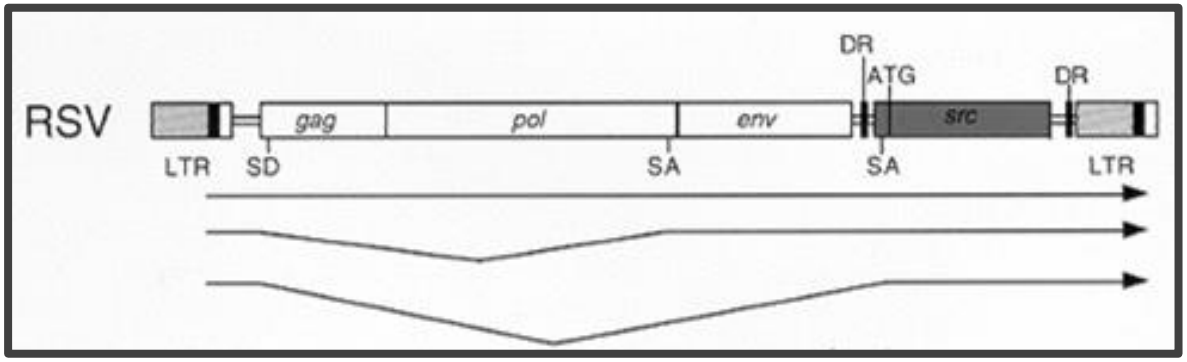


Fig. 9.: Organization of viral DNA genome of Rous Sarcoma Virus, viral genes (*gag, pol,* and *env, src* (contains initiator ATG)), direct repeats (DR), the splice donor (SD) and splice acceptor (SA) sites, (Coffin, Hughes et al. 1997)**.**

RSV is a class VI enveloped, icosahedral virus with a single stranded RNA genome of positive sense. As in other retroviruses, the RNA genome is replicated through a DNA intermediate (Flint

2009). The genome of the virus is approximately 13 kilo bases long. The transmembrane glycoproteins are directly involved in the fusion of the virus and host membranes for entry. The avian sarcoma-leukosis virus family includes a variety of enveloped virus subtypes, serially from envelope A to J. In laboratory mostly the subtypes A to E are used.

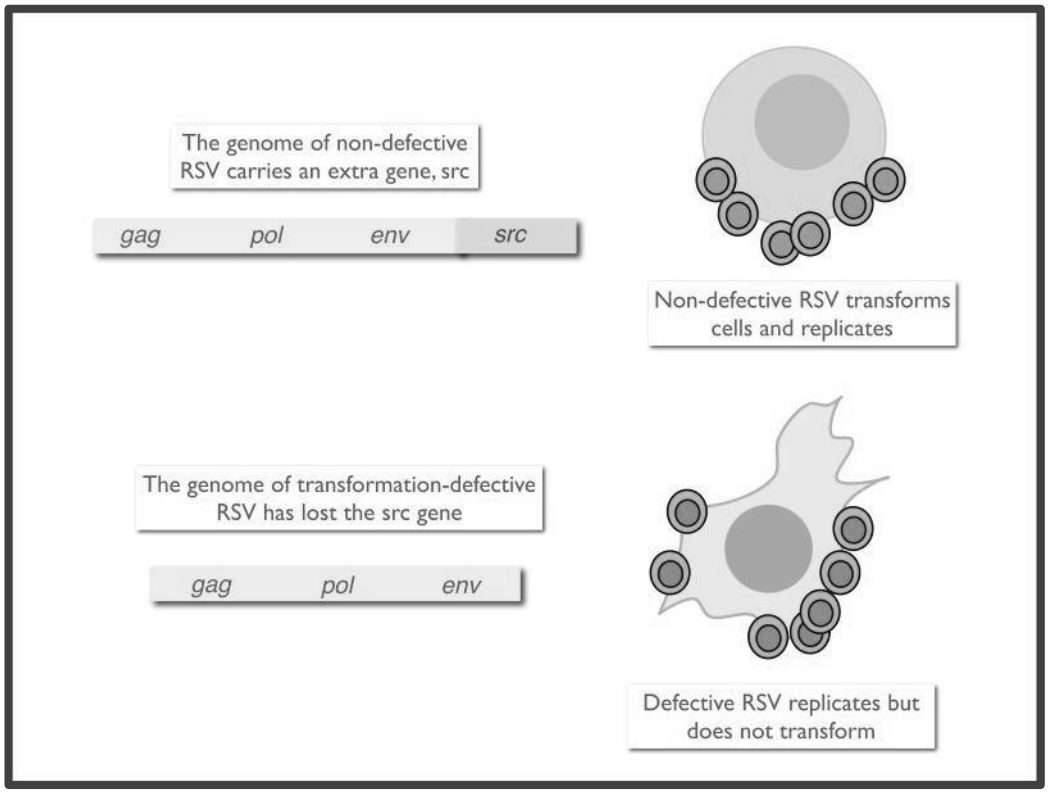


Fig. 10.: Non-defective RSV with information required for virus reproduction (*gag, pol, env*) and the information needed for the oncogenic transformation (*src*) (Vogt 2012).

The common retrovirus has three genes: *gag* – encoding capsid proteins and the protease, *pol* - encoding reverse transcriptase and integrase and *env* – encoding the surface glycoprotein. In RSV additionally an oncogene called *src* is encoded. V-src is a tyrosine kinase similar to the cellular counterpart c-src. The tyrosine kinase attaches phosphate groups to the amino acid tyrosine in host cell proteins and lacks a control region. Therefore, the tyrosine kinase is constantly active and the cell growth is accelerated (Vogt 2012). The first RSV has been exploited as a vector by replacement of the *src* gene with other cDNAs in 1984 (Hughes and Kosik 1984).

4.3.1. Cell cycle of retroviruses

The cell cycle of retroviruses is designated by two particular occasions, the reverse transcription, which is generating a DNA intermediate in the cytoplasm, and the integration of the generated DNA intermediate in the host genome.

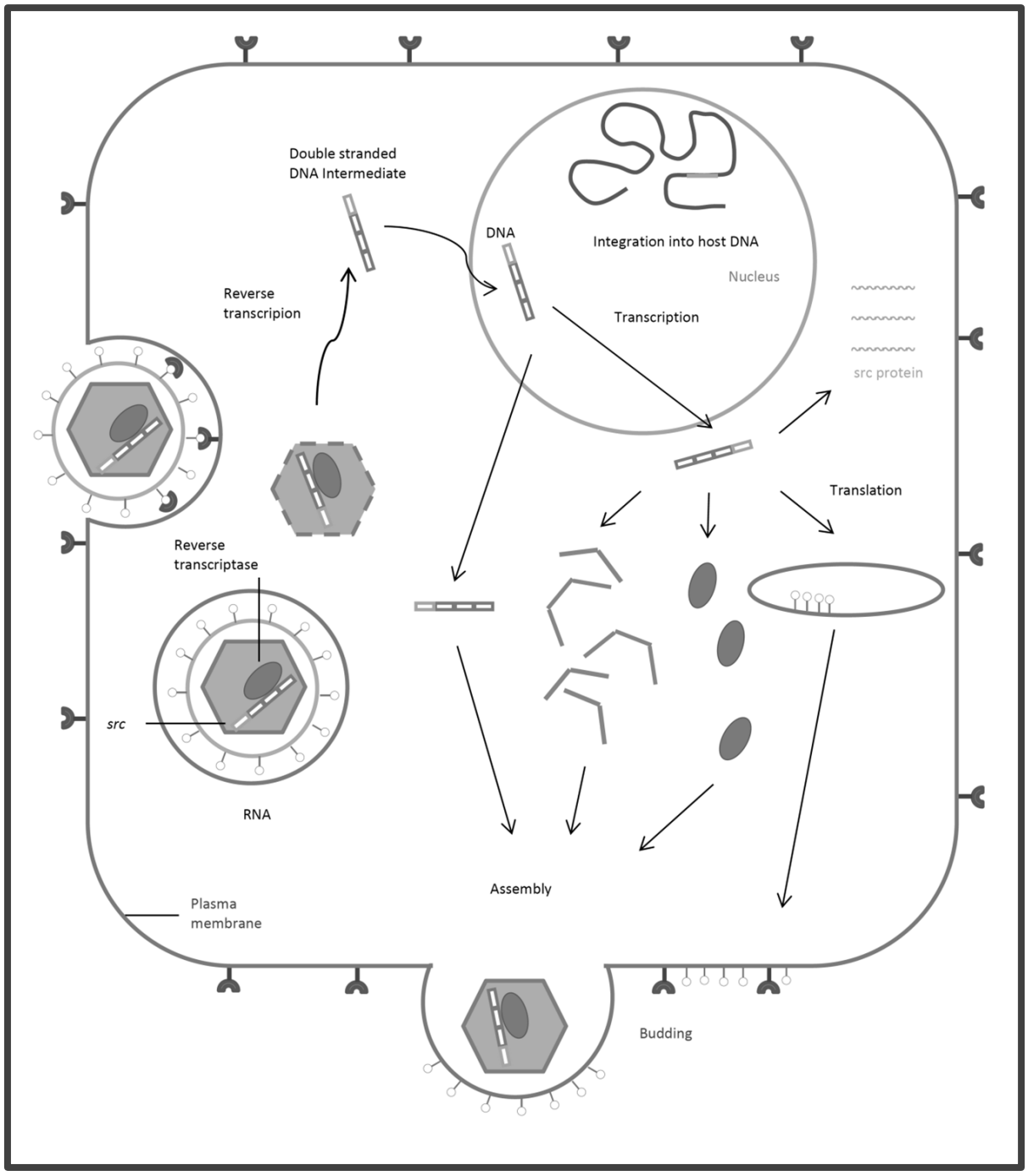


Fig. 11.: Retroviral life cycle (adapted from Hughes (Hughes 2004) and http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/RSV.html © Kathrin Bracher 2014).

First, the free RSV particle enters its target cell by its *envelope* glycoproteins *gp85* and *gp37*, interacting with its cognate receptor (tv-a). Thereafter the binding of the RSV particle leads to fusion of the viral and host membranes, which maintained importing the virus core, consisting of gag-derived proteins, full-length genomic RNA, and the reverse transcriptase protein. In case of the ASLV including the RCAS viruses, an intermediate step is necessary. The ASLV enters the cell using a

vesicle. The capsid is then set free via acidification (Hughes 2004). After introduction to the host cell, the nucleoprotein complex is converted to a double single stranded RNA molecule via reverse transcription into a double stranded DNA copy of the virus genome. The double stranded DNA intermediate is then locked in the cell nucleus. In the nucleus of the host cell, the retroviral genome is integrated into and covalently attached to the DNA of the host cell and therefore treated like a cellular gen, which is transcribed into RNA by host DNA-dependent RNA polymerase. Full-length genomic mRNA copies are initiated by the 5' Long Terminal Repeat. Spliced and unspliced RNAs are produced and used as mRNA and genomic RNA. A part of the RNA is then translated at the ribosomes for the necessary new virion elements, as the capsid proteins, the reverse trancriptase and the envelope proteins, and in case of the RSV, the oncogenic s*rc* protein. The virion is then assembled in cytoplasma and exported and released by budding of the host cell. The new virions are released as unprocessed particles needing protelytic processing to convert them into infectious viruses.

4.3.2. The RCAN/RCAS System

An appropriate strategy to generate a transgenic chicken embryos involves a **R**eplication-**C**ompetent **A**vian sarcoma-leukosis virus with a **S**plice acceptor, **B**ryan Rous Sarcoma virus **P**ol (RCASBP) and the same virus, where the splice side is deleted, the **R**eplication-**C**ompetent **A**vian sarcoma-leukosis virus with no **S**plice acceptor, **B**ryan RSV **P**olymerase (RCANBP) (Hughes, Greenhouse et al. 1987, Boerkoel, Federspiel et al. 1993, Hughes 2004). The major difference compared to lentiviral vectors is that these viruses are replication competent, able to spread horizontally to neighbouring cells after infection, as well as vertically to daughter cells, thus allowing ubiquitous infection and transgenesis of embryos in a single generation (G0). Only germ cells were found to be eGFP negative, compared to the somatic cells (Smith, Roeszler et al. 2009).

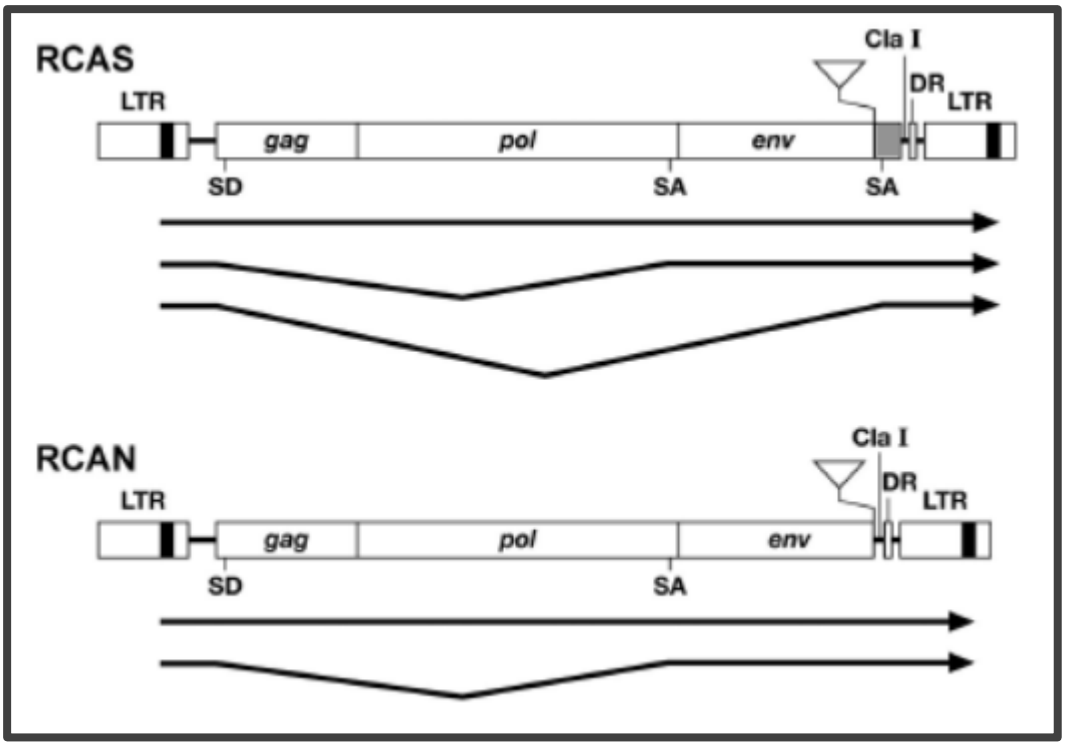


Fig. 12.: Blueprint of RCAS and RCAN, the upstream direct repeats (DR) has been deleted from RCAS and RCAN, the *src* gene including the splice acceptor has been deleted from RCAN and replaced by a *ClaI* site (Hughes 2004).

The RCASBP/RCANBP virus is only competent to infect avian cells like chicken and quail. Therefore it bears little risk to human (Biosafety level 1) and can be handled in common cell culture laboratories. As afore mentioned the RCASBP vector requires the avian-specific tv-a or tv-b receptor to enter the cell, depending on the envelope subtype (Payne and Venugopal 2000). The cellular receptor tv-a is both in sequence and structure related to an LDL receptor repeat (Bates, Young et al. 1993). The tv-b membrane protein of the host cell serves as receptor for the B, D and E envelopes. Tv-b is a member of the fas ligand receptor family (Brojatsch, Naughton et al. 1996, Adkins, Brojatsch et al. 2000). The receptor for the C envelope is closely related to mammalian butyrophilins, which are members of the immunoglobulin superfamily (Munguia and Federspiel 2008).

The vector is able to carry a fragment up to 2.5 kilobases. Larger fragments are not tolerated, even if they are folded very compactly, since the virus capsid limiting the space for extra RNA. All viruses have two copies of their genomes, together with host tRNAs and some other small RNAs of host origin.

4.3.3. Blueprint of the RCAS virus

The DNA genome of the SR-A strain of the Rous Sarcoma Virus was modified by deleting the *src* oncogene in lieu thereof a restriction site (Hughes and Kosik 1984). The splice acceptor used to generate the spliced mRNA of the *src* gene is derived from a cellular *src* gene (figure 9)

The adapter plasmid to introduce the required genes was constructed by Hughes et al. (Hughes, Greenhouse et al. 1987) and it is called Cla12-apaterplasmid. It can be replicated in *E. coli* because it carries an ampicillin-resistance marker. The entire plasmid is approximately 13 kilobases long.

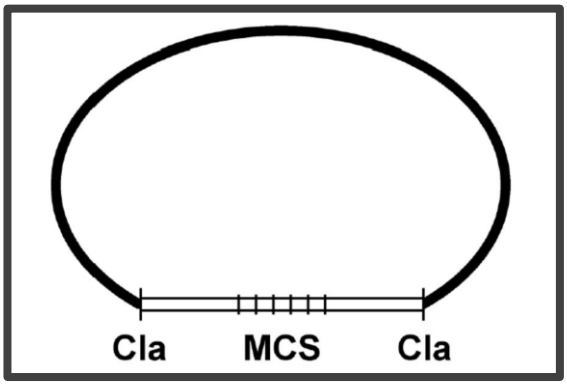


Fig. 13.: Cla12-adapterplasmid with two *ClaI* restriction sites (Hughes 2004)**.**

Two hypothesis were postulated, how the src oncogene was acquired. The first postulated hypothesis assume that an ALV provirus integrated into the *src* gene and that thereupon the RSV was generated from an RNA arising from this integrated ALV provirus (Swanstrom et al., 1983). The second hypothesis supposes that c-RSV was adventitiously packed into the ALV virion, which does not explain the presence of the splice acceptor.

Moreover, the *src* region of Rous sarcoma virus is flanked by direct repeats, which are approximately 100 base pairs long. These two direct repeats enable the reverse transcriptase to jump between them, but they as well pose the problem that any insert is quickly lost. To solve this problem the upstream copy of the direct repeat was deleted. The second repeat is necessary and sufficient for the viral replication in ALVs. (Sorge, Ricci et al. 1983). The direct repeats may account for a role in the viral transport and/or in the packing of viral RNA into the core. RCAN vectors differ from the corresponding RCAS vectors only in case of lacking the *src* splice acceptor, as mentioned before. The *src*-derived splice acceptor site is retained in the RCAS vectors and therefore the expression of spliced messages from inserted genes via the viral long terminal repeat (LTR) is possible. In contrast the RCAN vectors can express an inserted gene from an appropriate internal promoter by using e.g.

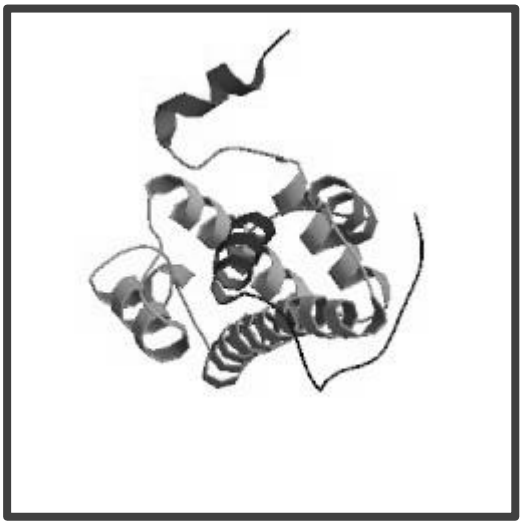


Fig. 14.: Prospero homeobox protein 1 from the protein data base bank.

CMV promoter or a tissue specific promoter. The idea of using such a vector construct to drive expression in cultured cells and in specific tissues of avian model organisms was tested by different research groups. Functional expression from an extra promoter was shown before. Petropoulos revealed that a bacterial gene - chloramphenicol acetyltransferase (CAT) using a muscle specific chicken promoter - alpha sk-actin - is working if a retrovirus is used (Petropoulos, Payne et al. 1992). High levels of CAT in striated muscle were detected after infection with this retroviral construct.

Two elements in the avian vector besides the required promoter contribute to the replication rate. The LTR is a strong enhancer, which is expressed in high doses in avian cells. Additionally, the *pol* region affects the replication and expression in avian cells. In case of RCASBP and RCANBP, the two vectors used in this study, the *pol* region was substituted with a Bryan high-titre strain resulting in a replication rate increased about one log. Therefore, these enhanced vectors were used to achieve a higher expression in the specifically infected cells.

4.3.4. DF-1 – Chicken Fibroblast cell line

In cell culture, chicken embryo fibroblasts (CEFs) exhibit a limited lifespan of 20 to 30 passages, like most primary cells. Newly cultured cell lines have additionally the problem of endogenous proviruses, which are closely related to the avian sarcoma – leucosis virus family. Therefore, the recombination between these viruses is able and likely. To overcome these difficulties a new cell line of spontaneously immortalized chicken embryo fibroblasts, DF-1 was used to produce the RCASBP and RCANBP viruses (Himly, Foster et al. 1998, Schaefer-Klein, Givol et al. 1998). This cell line is derived from an EV-0 embryo. Compared to primary embryo fibroblasts, they have enhanced growth potential. The cells are free of endogenous sequences related to avian sarcoma and leucosis viruses and have normal fibroblastic morphology. They differ from primary CEF cells in growth rate, mitochondrial function and response to oxidative stress. Therefore, these cells are a valuable tool for the generation of the virus.

Schaefer-Klein et al showed that the titre of the virus produced by DF-1 chicken fibroblasts is 2-fold higher than the titre of the same virus produced by CEF (Chicken embryo fibroblast). Hence DF-1 cells are better virus producer than CEF, regardless which virus vector was used. The titres of the subgroup (B) and (C) viruses were one log lower than were the titres of the subgroup (A) virus. Subgroup (B) viruses showed signs of this cytotoxic effect in both cell cultures, if high levels of subgroup (B) virus was produced (Schaefer-Klein, Givol et al. 1998). As expected, the titres of RCAS on DF-1 cells were 10–15-fold lower than the titres of RCASBP (Federspiel and Hughes 1997).

4.3.5. eGFP (enhanced fluorescent protein)

The three scientist Martin Chalfie, Osamu Shimomura, and Roger Y. Tsien were awarded the Nobel Prize in Chemistry for their discovery and development of the green fluorescent protein (GFP) (Shimomura, Johnson et al. 1962). GFP is a protein extracted from the jellyfish Aequoria victoria and exhibits bright green fluorescent light when exposed to ultraviolet light. It has the major excitation peak at a wavelength of 395nm. The first crystal structures of a GFP were reported of an mutant and one month later oft the wild type GFP in 1996 from two independent groups (Ormo, Cubitt et al. 1996, Yang, Moss et al. 1996). In 1995 the today used enhanced GFP was discovered, which allowed the first time the usage of eGFP in mammalian cells (Cormack, Valdivia et al. 1996, Thastrup, Tullin et al. 2001-01-09). The eGFP monomer has its major excitation peak at a wavelength at 488nm.

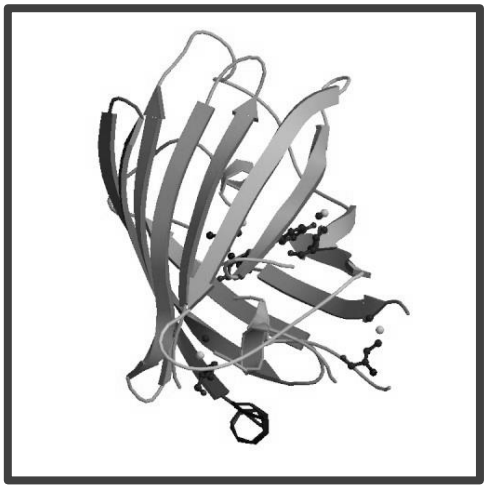


Fig. 15: The fluorescent protein of Aequoria victoria from the protein data bank.

5. Aims and Approaches

The aim of the master thesis was to construct a set of viruses, which are able to replicate in chicken embryos. Therefore the avian sarcoma-leukosis virus vectors RCASBP and RCANBP were chosen to generate transgenic *ex-ovo* chicken embryos. The replication competent viruses should have the quality to be expressed in lymph endothelial cells only, expressed under a promoter with a six times Prox1 binding site similar to the mechanism described by Shin et. al. (Shin, Min et al. 2006). According to the method used by Shin et. al. the six times prox1 binding site alone, was not able to mediate any transcriptional activation. It was decided to use additionally a promoter. Due to the problem that the used promoter-reporter assay using FGFR-3 promoter and firefly luciferase is not working in chicken, the decision was made to replace the mouse FGFR-3 by the CMV Promoter and the firefly luciferase was substituted by the marker protein eGFP.

At the beginning a vector composed of the six times prox1 binding site and the eGFP promoter was generated and tested in D1-cells and chicken embryos. In both eGFP expression was not detectable; hence it was necessary to use a promoter element. The decision was made to use the strong CMV promoter and a shortened CMV, as weaker promoter element. After the first transfections it was recognised that the CMV promoter showed no expression, due to a problem in the gene sequence. The miniCMV promoter in contrast showed a very strong expression of eGFP in every cell. Therefore the miniCMV construct was used as positive control. Additionally the six times prox1 BS was deleted in order to observe if the eGFP expression is changing.

In the following step a weaker promoter element, called minimal Thymidine kinase was chosen to achieve a specific expression of eGFP in the lymph endothelial cells. The miniTK promoter in combination with the six times prox1 BS was able to express eGFP in the DF1 cells, but in a lower amount than the miniCMV element. Therefore this vector construct was produced via cell culture and the concentrated virus was then injected into germ discs of fertilized chicken embryos. This transgenic chicken showed better results than the before tested vector constructs. It was not possible to achieve a content result.

As final try a vector with a Prox1 promoter, without the six times prox1 BS was constructed and tested in the chicken embryos, because this promoter element should be specifically expressed in lymph endothelial cells.

Furthermore the vector construction and virus production should be optimized and transformation techniques of embryos should be raised to achieve ubiquitously transgenic embryos at a high rate. The infected chicken embryos should serve as *ex-ovo* model organism, which is easy to transfect. The *ex-ovo* model organism can be used with its freely accessible membrane as ideal research objective were the lymph vessels can be depicted.

6. Methods and Materials

6.1. Retroviral vectors

All of the vectors used in these experiments are derivatives of the RCAS family (Hughes *et al.,* 1987; Petropoulos *et al.*, 1991; Federspiel and Hughes, 1997). RCAS**BP** (**B**ryan **P**olymerase) was prepared from RCAS by replacing the *pol* region with the corresponding segment from the Bryan high titre strain of Rous sarcoma virus (Federspiel and Hughes, 1997). The subgroup B *env* was used.

6.1.1. Schema of vector construction

First a fragment was amplified from a plasmid including the desired fragment, using primers including restriction sites for two enzymes settled in the multiple cloning site. The gained fragment was then cut by the desired restriction enzymes.

Fig. 16.: The PCR-generated fragment (green) and the subcloning plasmid Cla12 (blue).

The obtained cut and purified fragment was integrated into the Cla12 subcloning vector, which was modified by adding the reporter gene eGFP and a multiple cloning site, using a ligase. To elicit, if a fragment was integrated, a colony PCR was made. The isolated plasmid DNA from the fluid culture was then digested with restriction enzymes as described subsequent in 5.2.. One of the positive clones was then selected for the next working step, cloning the new fragment into the virus.

The newly created plasmid was then cut with *ClaI*, purified and cloned into the virus RCANBP.

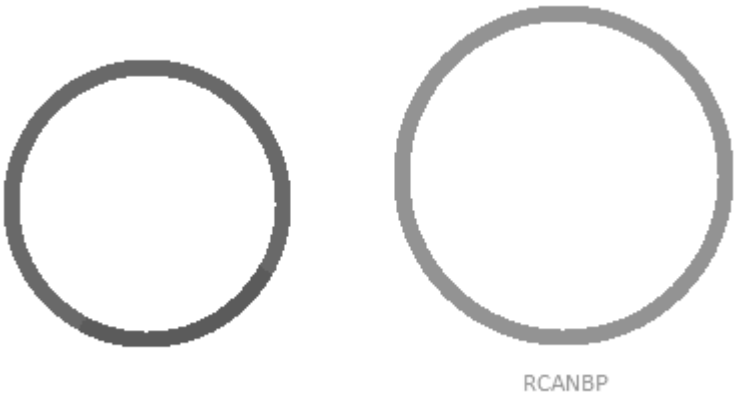


Fig. 17.: Cla12 plasmid with generated fragment (blue and green) and virus RCANBP (orange).

The fragments were randomly integrated “inline” or “inverse”, in the correct or wrong direction, because of using a single restriction enzyme. To elicit, if a fragment had been integrated and, if so, in which direction the integration occurred, a colony PCR was made as in the first cloning step. The newly created plasmids were additionally verified afterwards via restriction digest and a second control PCR.

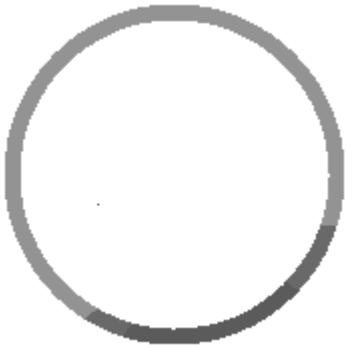


Fig. 18.: Fully synthesised virus plasmid.

6.2. Cloning

To construct the viral plasmids, standard cloning techniques were used. First using PCR, DNA fragments (e.g. CMV, miniCMV, TK,…) were synthesised (Q5 High Fidelity Polymerase, NEB, Ipswich, MA, USA) using specific primers containing restriction sites (HVD Life Science, Vienna, Austria) from plasmids containing the desired gene region.

DNA fragment with Q5 High Fidelity Polymerase	
Q5 Reaction Buffer	1 µl
10mM dNTPs	0,1 µl
10µm *BamHI* fwd Primer	0,2 µl
10µm *SalI* rev Primer	0,2 µl
0,5 µg Plasmid DNA	0,5 µl
Q5 Polymerase	0,1 µl
ddH_2O	up to 10 µl
Total volume	**10 µl**

Thermo cycler conditions		
98°C	4 min	
98°C	1 min	
Annealing temperature -5°C	30 sec	40x
72°C	1 min	
72°C	10 min	

Subsequently the PCR products were cut with two different restriction enzymes (Fast Digest Enzymes, Fermentas, Vienna).

Fast Digest of	**PCR Product**	**Plasmid**
10x Fast Digest Green Buffer	5 µl	2 µl
Fast digest enzyme	5 µl	2 µl
DNA	5 µg	2 µg
ddH_20	up 50 µl	up 20 µl
Total volume	**50 µl**	**20µl**

The reagents were mixed gently and spun down. Following the reaction mix was incubated for twelve minutes at 37°C using the heat block. In case of the vector 1µl FastAP thermo sensitive Alkaline Phosphatase (Fast Digest Enzymes, Fermentas, Vienna) to a volume of 20µl was added, to prevent the recircularisation during ligation. For inactivation of the enzymes a certain temperature and time, as predetermined in the manual is needed.

Gründemann and Schömig showed that the usage of Ethidiumbromid and UV light leads to solid DNA damage (Grundemann and Schomig 1996). They exhibit that after 20 to 45 seconds less than 1% of the tested DNA was intact. Different other groups showed before that the UV light may harm the DNA fragments and therefore the efficiency of the following ligation was affected. (Cariello, Keohavong et al. 1988, Hartman 1991, Maueler, Kyas et al. 1994). By using methylene blue it was possible to avoid the usually used UV-light-based detection of the fragment. So the breakup of the generated fragments, due to the UV light could be eliminated. The newly generated fragments, which have to be eluted from an agarose gel, were stained with 0,01% methylene blue for 15 to 20 minutes as described in the method and materials part. Methylene blue carries a positive charge, thus it binds to the negatively charged DNA fragments. The ionic binding of methylene blue to phosphoric acid of nucleic acids results in weak binding, which can be removed by elution or filtration kits. The only disadvantage is that we were not able to detect bands, which contain less than 0,2 µg DNA.

Then the cut fragments were separated and purified by using an 1% agarose gel and following an elution kit (QIAEX II Gel Extraction Kit, Hilden, Germany) to elute the desired fragment. To visualize the fragments 0,01% methylene blue was used. The gel was incubated for 15 till 20 minutes and following the gel was washed with distillate water till the bands became visible. First the desired DNA band were exercised with a clean scalpel, put into 1,5ml tubes and weighted. The following steps were done as described in QIAEX II Gel Extraction Kit. The DNA from the pellet was eluted by vortexing the pellet with 40 µl ddH_2O and subsequently incubated for five minutes on the shaker. The NanoDrop 8000 (NanoDrop Technologies, Wilmington, Delaware, USA) was used to determine the concentration of the eluted DNA.

Subsequently the cut fragments were ligated (T4 Ligase, NEB) with the already cut and dephosphorylated subcloning vector Cla12 in a rate of 1:3 of insert and vector at least overnight. Furthermore the ligation preparation were transformed into *E.coli* using a 5µl ligation mix and 50µl competent *E.coli* (10 beta competent *E.coli*, NEB). The colonies were grown over night at 37°C on luria broth agar with ampicillin. On the next day a colony PCR was performed and positively tested clones were selected.

Colony PCR with Q5 High Fidelity Polymerase	
Q5 Reaction Buffer	1 µl
10mM dNTPs	0,1 µl
10µm fwd Primer	0,5 µl
10µm rev Primer	0,5 µl

bacterial solution	1µl
Q5 Polymerase	0,1 µl
ddH_2O	up to 10µl
Total volume	**10 µl**

The colonies were picked from the agar plates using sterile pipette tips and putting them in 20 µl sterile ddH_2O. 1µl of the bacterial solution was used for the colony PCR

Thermo cycler conditions		
98°C	4 min	
98°C	1 min	
Annealing temperature -5°C	30 sec	30x
72°C	1 min	
72°C	5 min	

The positive tested clones were grown in three millilitres overnight, shaking at 1000rpm in liquid LB with Ampicillin to conduct MiniPrep (EndoFree Plasmid Maxi Kit, Qiagen) on the following day.

Modified Mini Prep protocol (EndoFree Plasmid Maxi Kit)

1,5 ml bacterial suspension was filled in a 1,5 ml tube and centrifuged at 3000 x g for 15 minutes in a 4°C precooled centrifuge. The pellet was resuspended in buffer1 using the vortexer. Following buffer2 was added, the tubes were inverted five times and incubated on room temperature. After five minutes precooled buffer3 was added, the mixture was blended immediately by inverting the tube five times and the mix was put five minutes on ice. The tube was centrifuged at full speed for ten minutes. Afterwards the supernatant was twice pipetted to a new tube to remove the bacterial remnants. The gained solution was precipitated with one millilitre 96% ethanol for one hour at room temperature and centrifuged for 15 minutes at 4°C at full speed. Following the supernatant was discarded and the pellet was washed with 70% ethanol, till the pellet detached. The ethanol was removed by drying with open lid. The pellet was resuspended with 40µl distillate water and the purified plasmid was heated five minutes at 55°C. Following the DNA concentration was determined via NanoDrop measurement.

- **Buffer1 – resuspension buffer**
  50 mM Tris·Cl, pH 8.0
  10 mM EDTA
  100 µg/ml RNase A

- **Buffer2 – lysis buffer**
  200 mM NaOH, 1% SDS (w/v)

- **Buffer3 – neutralization buffer**
  3.0 M potassium acetate, pH 5.5

Afterwards the clones were cut again with restriction digest enzymes to verify the clones a second time. After selecting successfully a positive clone, the procedure was repeated to transfer the fragment to the virus backbone. The large viral genome only provides one unique standard restriction enzyme site (*ClaI*), therefore the cassette is able to integrate in two directions and give two different constructs. The insertion direction was determined with two different sets of primers (HVD Life Science, Vienna, Austria), which synthesised fragments in variable length.

6.3. Cell culture

The DF-1 cell were cultivated in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS and 50µg/ml Gentamycin.

6.3.1. Splitting

The cells were split when the flasks got confluent normally in the ratio 1:3. The old medium was aspirated using a vacuum pump. Following the cells were washed twice with buffer without phosphate, which enables a faster detachment of the cells. Then three millilitres trypsin were added and aspirated after the cells contracted. DMEM medium with supplement FCS was added and the cells were detached mechanically with a hit on one side of the flask. The solution was split at three flasks or the whole amount of cells was added to a 175cm^2 flask.

6.3.2. Viral production

10 ng of plasmid were used to transfect DF-1 cells of a middle flask (75cm^2) maintained in DMEM supplemented with 10% FCS without antibiotics, at 80% to 90% confluence using Lipofectamine (Lipofectamine®2000 Reagent, Invitrogen, Carlsbad, CA, USA) in DMEM. After 18h, fresh medium was added and cells were split 1:3 at confluence. After three days of incubation the cells were controlled under fluorescent microscope, if they show an eGFP expression and subsequently passaged to a 175cm^2 flask. After further three days the confluent cells were split 1:3 on three further bottles. When the cells grow confluent, medium with 1% FCS was added, and the

supernatant was harvested once per day for three days, filtered through 0,45 µm and centrifuged as described previously (Morgan and Fekete 1996, Logan and Tabin 1998). Briefly, 36ml medium from each day of harvest was ultra-centrifuged at 50,000g for 2.5 h at 4°C, using a swing out rotor. The supernatant was then carefully poured off, leaving a small pellet in approximately 150 ml residual medium. Following the tubes were vortexed gently and then shaken upright in the cold storage room for one hour to resuspend the viral pellet, which was then aliquoted into sterile tubes (15 µl/tube) and stored at 80°C until use. The virus was kept chilled throughout this entire procedure. After the procedure the virus is concentrated to a titre between $1x10^5$ IU/µl to $1x10^7$ IU/µl. The virus titre is determined with titration in case of RCASBP and RCANBPs, which are expressing eGFP in transfected DF-1 fibroblasts. Additionally the titre was ascertained for all RCASBP/RCANBP constructs via RNA isolation (RNeasy Mini Kit, Qiagen) out of the virus concentrate, transcribed in cDNA (RevertAid H Minus First Strand cDNA Synthesis Kit, Invirtogen) and finally a qPCR, (QuantiFast SYBR Green PCR Kit and Rotor-Gene Q, Qiagen) out of cDNA was made.

6.4. Virus titre determination

6.4.1. Virus production including RNA isolation and Reverse Transcription

First 3 x 10^5 DF-1 cells per millilitre were seeded in each well of the 96well plate. Four hours after cell seeding different virus – DMEM mixtures were added. The starting mixture consisted of 5µl virus suspension and 45µl DMEM and was diluted in each step one to ten. After about three days first fluorescence patches were apparent, by RCANBP vectors which are expressing eGFP.

Following a modified protocol (RNeasy Mini Kit (Qiagen cat# 74106) including the Qiashredder (Qiagen cat# 79656)) for RNA isolation was used. As described in the manual (RNeasy Mini Kit) 350µl buffer were mixed with 3,5µl (1/100) ß – mercaptoethanol. 10µl concentrated virus were added to the prepared virus buffer. The buffer-virus mixture was transferred to the shredder and centrifuged at full speed for two minutes. The shredded lysate is then placed in the Qiacube automat and the standard protocol as in the standard manual is used (QIAcube, Qiagen).

Reverse Transcription

RevertAid H Minus First Strand cDNA Synthesis Kit	
RNA preparation	11 µl
primer	1 µl

The mixture was gently mixed and spun down. Following the mixture was incubated at 65°C for five minutes. Then the samples were put on ice to break down the secondary structures in GC-rich samples.

5x reaction buffer	4 µl
RNAse inhibitor	1 µl
dNTPs	2 µl
reverse transcriptase	1 µl
Total volume	**20 µl**

The mixture was gently mixed and spun down. Following the mixture was incubated at 25°C for five minutes. Subsequently the sample were incubated for one hour at @ 42°C and then for five minutes at 70°C. For longer storage use the -80°C freezer.

6.4.2. qPCR: Master Mix (Applied Biosystems cat# 4324018)

TaqMan® Universal PCR Master Mix, Applied Biosystems® (Thermo Scientific Fisher, Waltham, MA, USA)	
8 µl	Universal PCR Master Mix
4 µl	ddH_2O
2 µl	primer (1/20)
2 µl	cDNA[1]

The qPCR was performed with the PCR cycler StepOnePlus™ Real-Time machine (Carlsbad, CA, USA).

6.5. Determination of protein concentration

To determine the protein concentration the Bradford reagent (Protein Assay Reagent Dye Concentrate, BioRad, Hercules, CA) was used. To dilute the standard curve a BSA Standard with the concentration of 2mg/ml was used, id est 1,8µl BSA (50mg/ml) were diluted with 43,2µl Aqua dest.. Hence the dye solution is diluted 1:5 in Aqua dest..

[1] For the qPCR do not use more than 1/10 of cDNA synthesis template for the PCR reaction volume.

Standard sample	µg BSA	µl BSA [2mg/ml]	RIPA Buffer	Fill up with Aqua dest. to 50µl
1	1 µg	0,5 µl	5 µl	44,5 µl
2	2 µg	1,0 µl	5 µl	44,0 µl
3	5 µg	2,5 µl	5 µl	42,5 µl
4	10 µg	5,0 µl	5 µl	40,0 µl
5	15 µg	7,5 µl	5 µl	37,5 µl
6	20 µg	10,0 µl	5 µl	35,0 µl
7	30 µg	15,0 µl	5 µl	30,0 µl
Blank	-	0,0 µl	5 µl	45,0 µl
Sample	-	5,0 µl	-	45,0 µl

2,5ml standard dilution was added to every sample and afterwards the mixture was filled to a cuvette. The mixture was incubated for five minutes at room temperature. Then the samples were measured at 595nm and the standard curve was calculated with the scheduled Excel table.

6.6. Western blot

For western blotting analysis scraped and lysed cells were centrifuged for ten minutes, 18000rcf, 4°C and incubated in 6x non reducing sample buffer at 95°C for five minutes. The amount of protein was determined using a Bradford assay (BioRad, Hercules, CA). Lysates were loaded onto 15% 1,5mm thick polyacrylamide gels, electrophoresed, and blotted on polyvinylidene difluoride membranes (BioRad, Hercules, CA).

Afterwards the membranes were incubated with mouse anti 19 antibody, which is specific for all strains of the avian sarcoma/leukaemia virus, infected avian cells and avian cells harbouring Rous sarcoma or avian leukaemia derived vectors. The detection was carried out using a secondary antibody HRP-labelled goat anti-mouse antibody (BioRad, Hercules, CA). The bound antibodies were visualized using ECLplus system (Amersham Pharmacia, Little Chalfont, U.K.) and exposed on hyper film (Amersham Pharmacia, Uppsala, Sweden). A standard incubation time of five minutes was used for developing the hyper film. To determine the loading level of protein, the housekeeping gene ß-actin (antibody: A2228, Sigma Aldrich, St. Louis, MO) was used as loading control.

6.6.1. Buffers

The separating and stacking buffer for gel pouring are diluted from 10x stocks (BioRad, Hercules, CA). The electrophoresis and blotting buffer were diluted form 10x stocks (BioRad, Hercules, CA). Following buffers were produced as described.

- **10% Ammonium Persulfate**

Ammonium persulfate	1g
Aqua dest.	up to 10ml

- **10x TBS**

1M Tris/HCl	100ml (pH 7,2 – 7,6)
NaCl	43,83g (1,5 M)
Aqua dest.	Up to 500ml
Set pH with NaOH	

- **1xTBSTween**

10x TBS	50ml
0,1% Tween	0,5ml
Aqua dest.	Up to 500ml

- **6x sample buffer – non reducing**

Stacking gel buffer	7 ml
Glycerol	3 ml (3,8g)
SDS	1g
Bromphenyl blue	1,2 mg
Aqua dest.	up to 10ml

6.7. Chicken embryos

Pathogen-free, fertilized chick eggs were washed after delivery with 5% H_2O_2 in water. Then the eggs were dried and rested upside down for one to two hours, helping the blastula to float up. To detect the air pouch on the upper edge of the chicken egg a torch was used and following the egg was candled. The *ex-ovo* embryos were prepared as previously described in detail (Deryugina and Quigley 2008, Deryugina and Quigley 2008, Bekes, Schweighofer et al. 2011) and given to small, ethanol washed plastic dishes.

6.8. Imaging

An inverse cell fluorescence microscope (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany) was used to monitor eGFP expression in DF-1 cells. To show the eGFP expression in the CAM model a fluorescence stereomicroscope was used (SteREO Lumar.V12, Carl Zeiss).

7. Results

7.1. Construction of virus plasmids

To achieve lymphatic specific transgene expression, the Prox1 responsive six times Prox1 binding site in combination with a different promoters, similar as published by Shin et. al. (Shin, Min et al. 2006) was exploited. This method is based on a promoter assay by McEwen, showing that a three kilobase promoter fragment is able to mediate transcriptional activation of the firefly luciferase reportergen using Prospero homebox protein (McEwen and Ornitz 1998). Shin et. al. shortened the promoter to different length fragments (Shin, Min et al. 2006). These were tested if they drive any expression. They showed that a Prox1 binding site containing nine nucleotides (CACGCCTCT, (Hassan, Li et al. 1997)) arranged in six tandem repeats in combination with the FGFR3 promoter is sufficient to mediate transcriptional activation by Prox1, compared to one repeat, which was not able to mediate any expression. The luciferase of the original plasmid, which was a kind gift of Y.K. Hong, was replaced by eGFP and the mouse FGFR3 promoter, which has not worked in the chicken embryo, was substituted by CMV, miniCMV, or miniTK. The necessary flanking ClaI sites were provided by the subcloning vector pU-Cla12. To facilitate cloning of the respective promoters, pU-Cla12 was previously modified to contain the 6x PBS (prox1 binding site) before an MCS, followed by the eGFP open reading frame. Three different chicken specific viruses were constructed utilizing this modified Cla12-adapter plasmid.

7.2. Detailed workflow of vector construction using RCANBP_miniTK as example

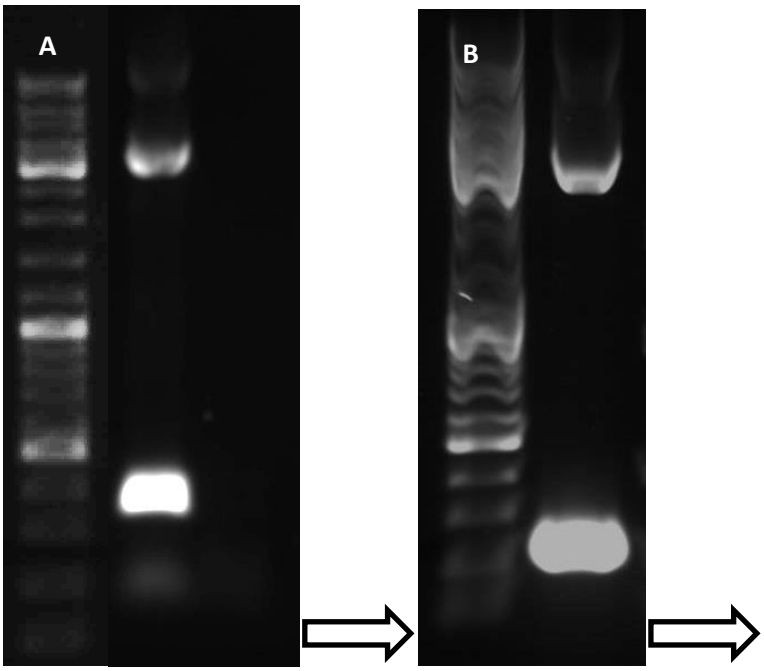


Fig. 19.: Amplified miniTK PCR product, lane1: GeneRuler, lane2: 220 base pair long generated PCR product of miniTK from a plasmid including the designated PCR sequence, lane3: ddH_2O, (A), cut MiniTK fragment, lane1: GeneRuler, lane2: with *BamHI* and *SalI* cut miniTK fragment (B).

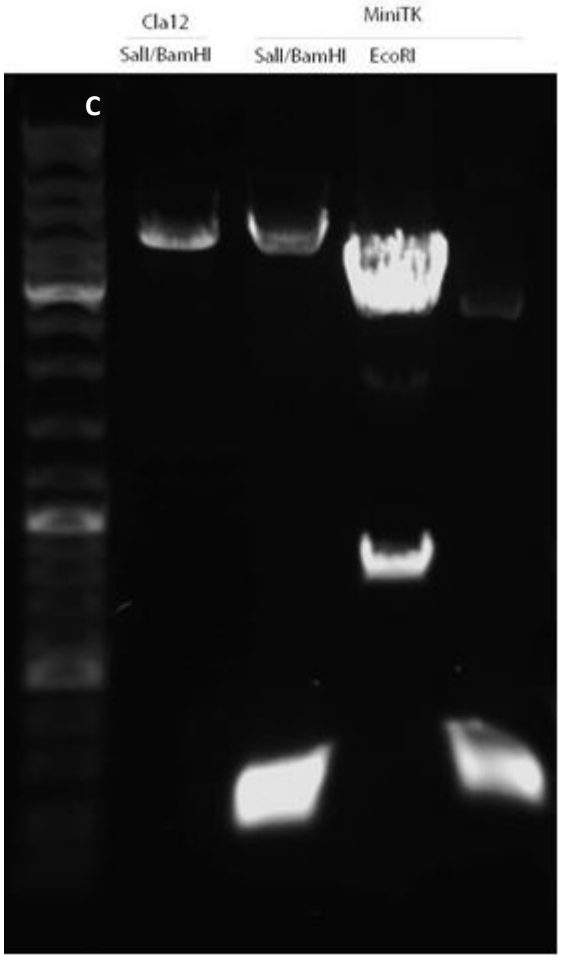


Fig. 20.: Cut vector plasmid Cla12 and cut miniTK fragments, lane1: GeneRuler[2], lane2: *Cla12*plasmid cut with *SalI* and B*amHI*, lane3: miniTK cut with *SalI* and B*amHI*, miniTK cut with *EcoRI,* lane4: miniTK undigested.

The miniTK PCR product was synthetized from a templateTK plasmid using two cloning primers with attached restriction sites, the cloningprimer_miniTK_forward with an added *SalI* restriction site (BS45)[3] and cloningprimer_miniTK_reverse with an added *BamHI* (BS46). The proper size of the synthesised fragment was then checked on an agarose gel. Subsequently the 220 base pair long PCR product was eluted and cut with the *SalI* and *BamHI* as described in the method and materials part. The gel-purified miniTK fragment was then ligated into the gel-purified modified Cla12-vector.

[2] GeneRuler™ DNA ladder mix (Thermo Scientific) was used for all agarose gels
[3] List of primers 8.1.

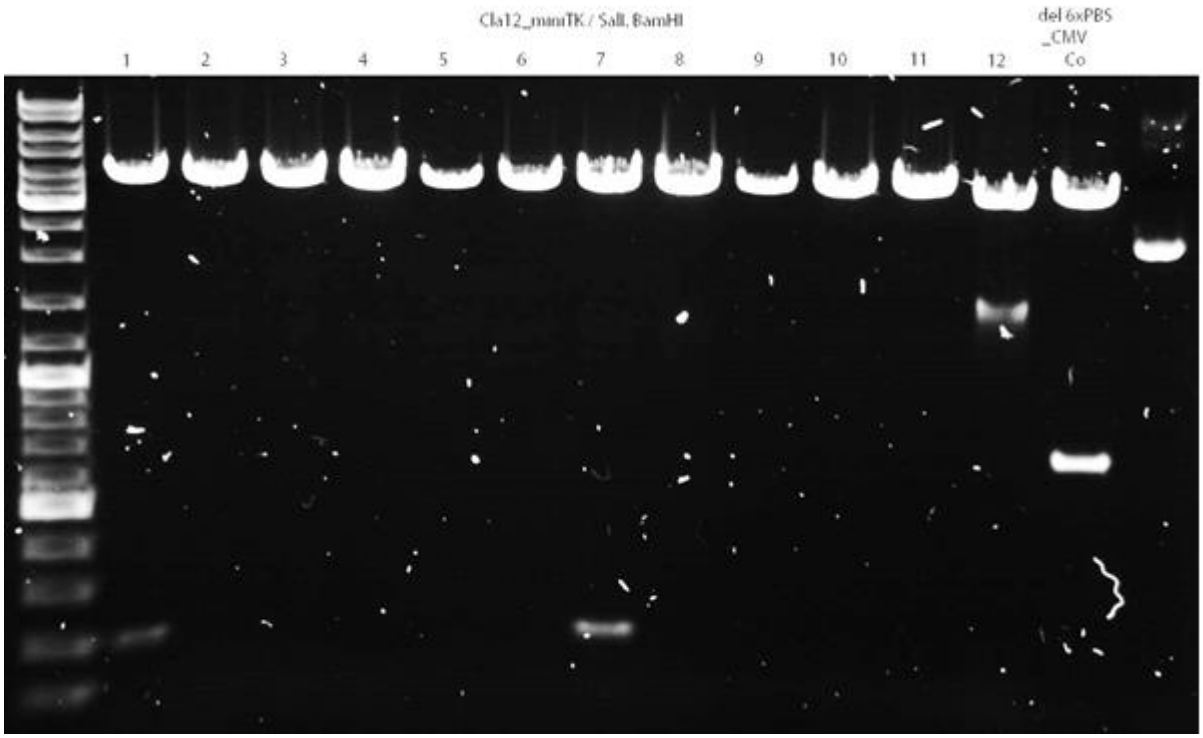


Fig. 21.: Picked clones from the agar plate cut with *SalI* and *BamHI*, lane1: GeneRuler, lane2-13: Cla12_miniTK clone 1-12, lane14: Cla12_del6xPBS_CMV as control, lane15: plasmid undigested.

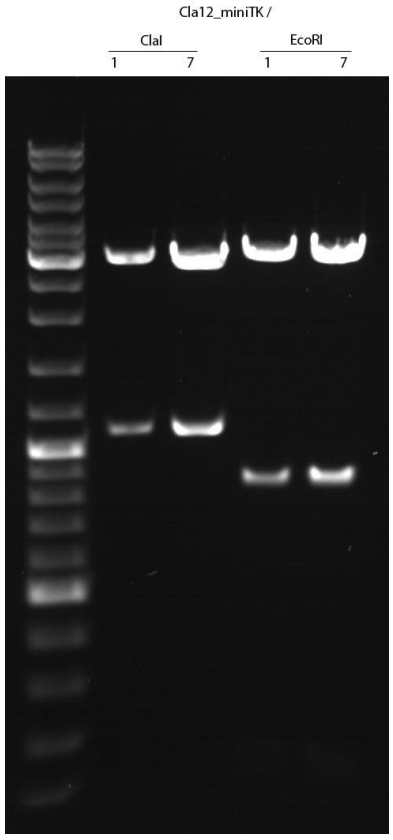
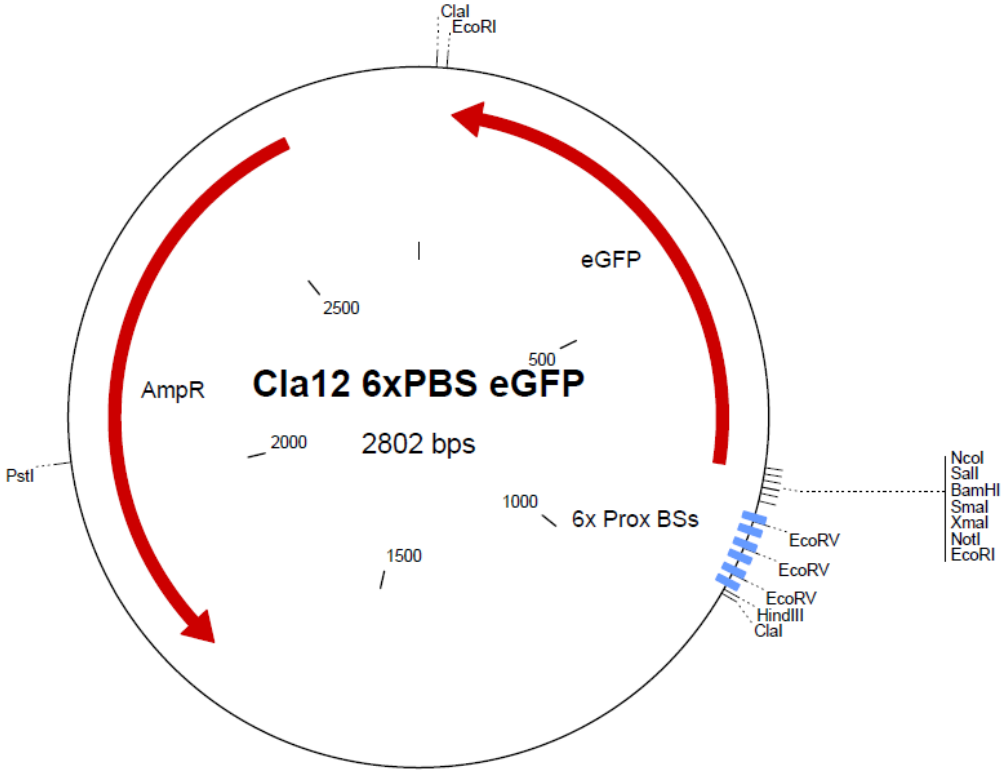


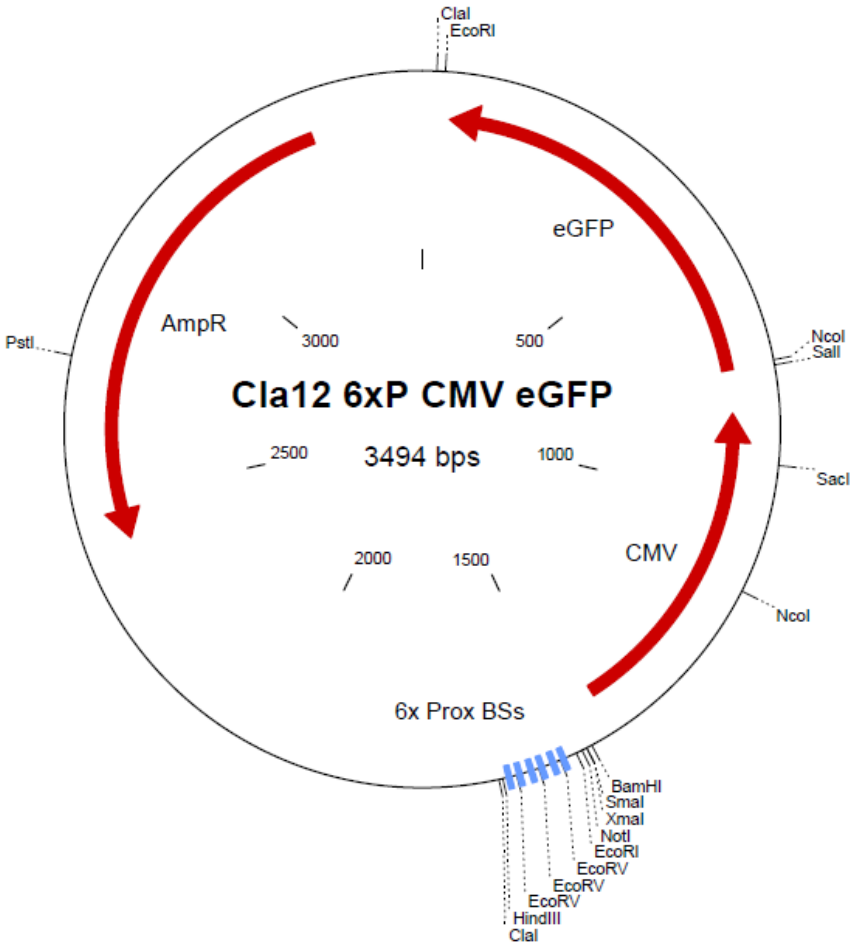
Fig. 22.: Control cut of Cla12_miniTK with *ClaI* and *EcoRI*, lane1: gene ruler, lane2+3: Cla12_miniTK 1+7 cut with *ClaI*, lane4+5: Cla12_miniTK 1+7 cut with EcoRI.

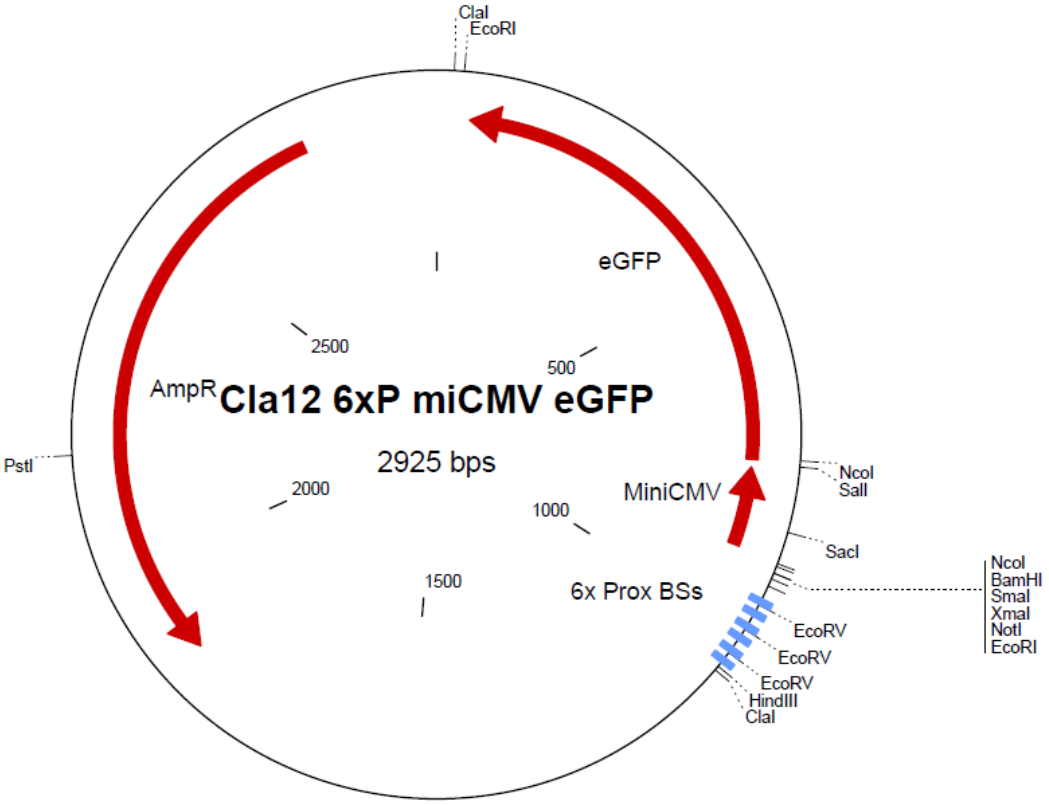
The Cla12_miniTK clones 1 and 7 have successfully integrated the desired 220 base pair long fragment. Therefore these two clones were checked with a second restrictions enzyme *EcoRI* (figure 23) and selected to continue the work.

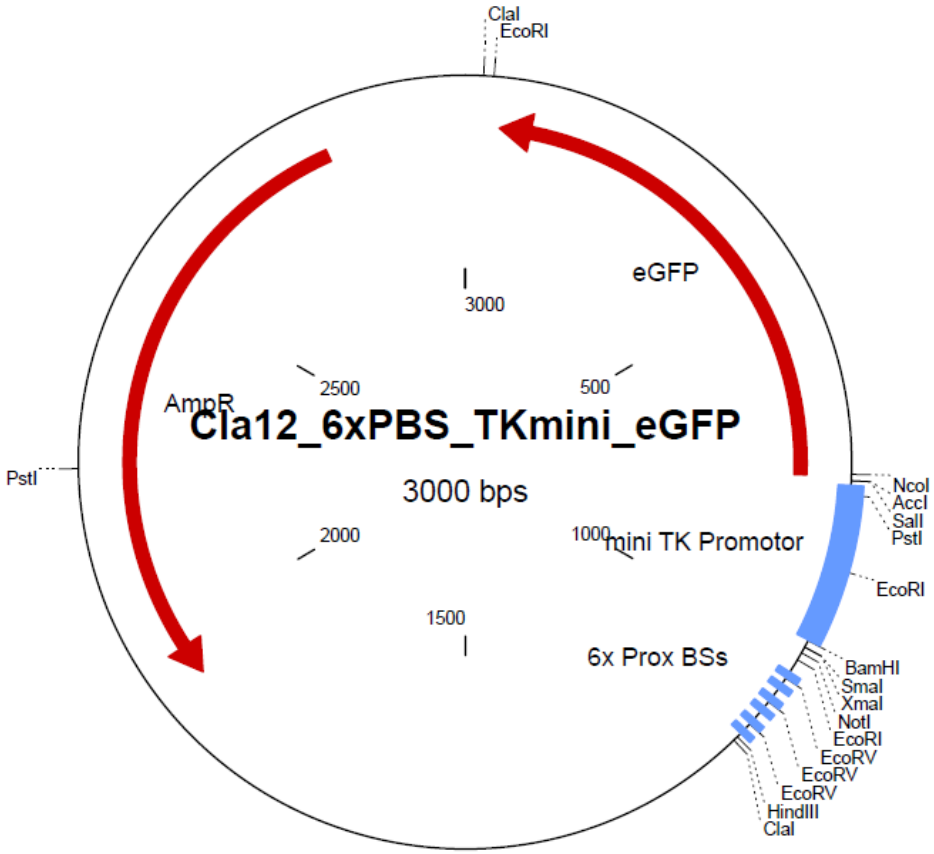
A similar procedure was chosen to clone miniCMV, CMV, and miniTK with and without a six-times Prox1 binding site into the Cla12 subcloning vector. All resulting constructs are depicted in paragraph 6.3..

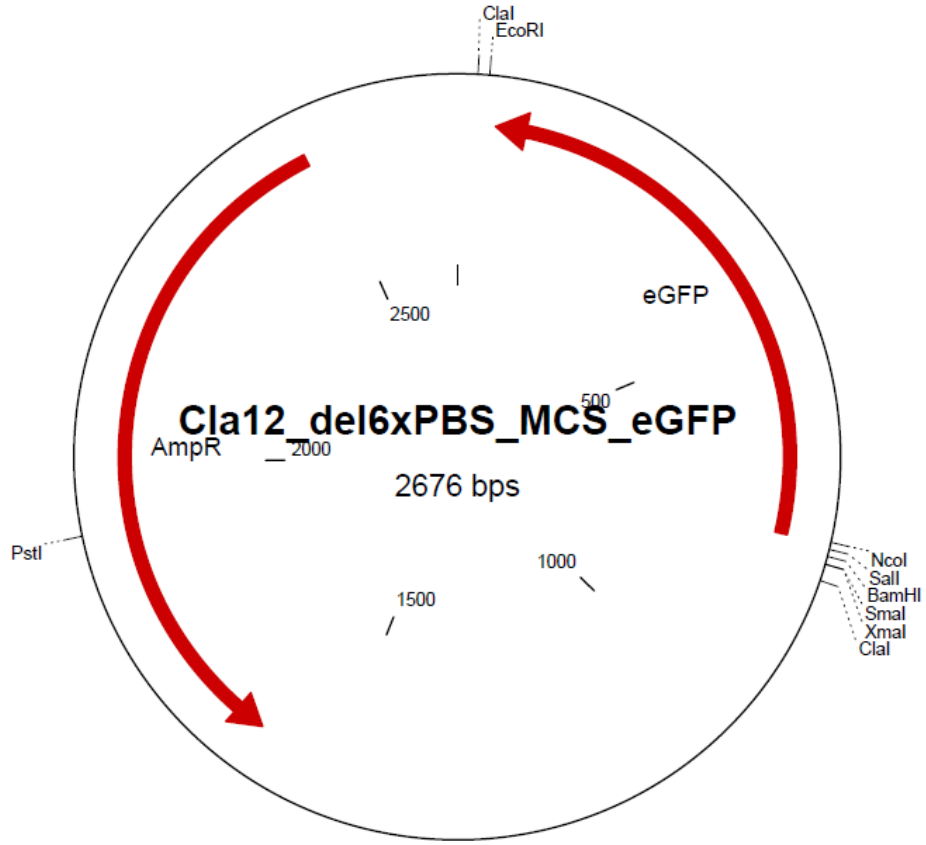
7.3. Generated plasmids

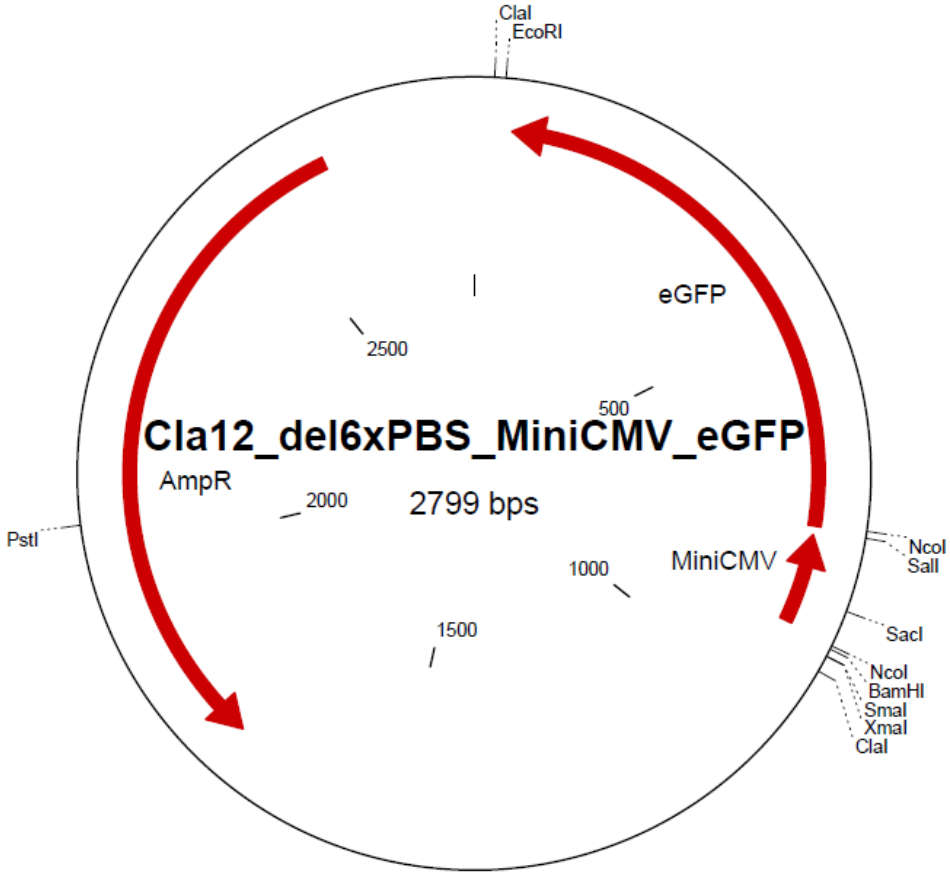





ClaI
EcoRI
eGFP
2500
500
AmpR
Cla12 6xP miCMV eGFP
2925 bps
PstI
2000
MiniCMV
1000
NcoI
SalI
SacI
1500
6x Prox BSs
NcoI
BamHI
SmaI
XmaI
NotI
EcoRI
EcoRV
EcoRV
EcoRV
HindIII
ClaI


ClaI
EcoRI
eGFP
3000
2500
500
AmpR
Cla12_6xPBS_TKmini_eGFP
3000 bps
PstI
2000
1000
mini TK Promotor
NcoI
AccI
SalI
PstI
EcoRI
1500
6x Prox BSs
BamHI
SmaI
XmaI
NotI
EcoRI
EcoRV
EcoRV
EcoRV
HindIII
ClaI


ClaI
EcoRI
eGFP
2500
500
Cla12_del6xPBS_MCS_eGFP
AmpR
2000
2676 bps
PstI
1000
1500
NcoI
SalI
BamHI
SmaI
XmaI
ClaI


ClaI
EcoRI
eGFP
2500
500
Cla12_del6xPBS_MiniCMV_eGFP
AmpR
2000
2799 bps
PstI
1000
MiniCMV
NcoI
SalI
1500
SacI
NcoI
BamHI
SmaI
XmaI
ClaI

7.4. Principal of “inline“ versus “inverse“ promoter constructs

The second cloning step following the generation of the different subcloning vectors the integration into the virus backbone is possible in two opposing directions, because of the necessity of using a single restriction site, such as *ClaI,* does not allow directional cloning. As we will see in the following results the direction of integration is important for the eGFP expression in DF-1 cells and in the model organism.

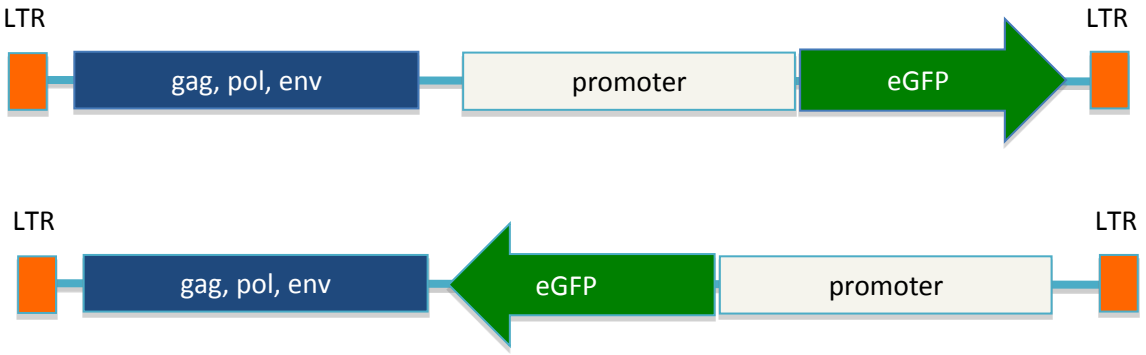


Fig. 23.: Virus construct “inline“ respectively “correct” and virus construct “inverse”, orange: LTR, blue: viral proteins, green: eGFP, white: different promoters.

The constructs inline with the viral genome generally showed a stronger expression of eGFP. On the contrary the inverse construct showed a clearly slighter expression of eGFP. As expected, this phenomena was observed in every vector which was constructed e.g. in chicken embryos transfected with 6xPBS_miniCMV and 6xPBS_miniTK (figures 30 and 32).

7.5. Verification of plasmids

A three-step procedure to select the correct-formed clones was used. After growing them over night on luria broth ager with ampicillin a colony PCR was performed to preselect potential correct clones. As a second step the prepped clones were digested to confirm if the whole fragment had been integrated. The third step, repeating the PCR identification on the prepped plasmid, was to ensure that the integration direction is content with the first result from the colony PCR.

7.5.1. First step - Colony PCR with two sets of primers

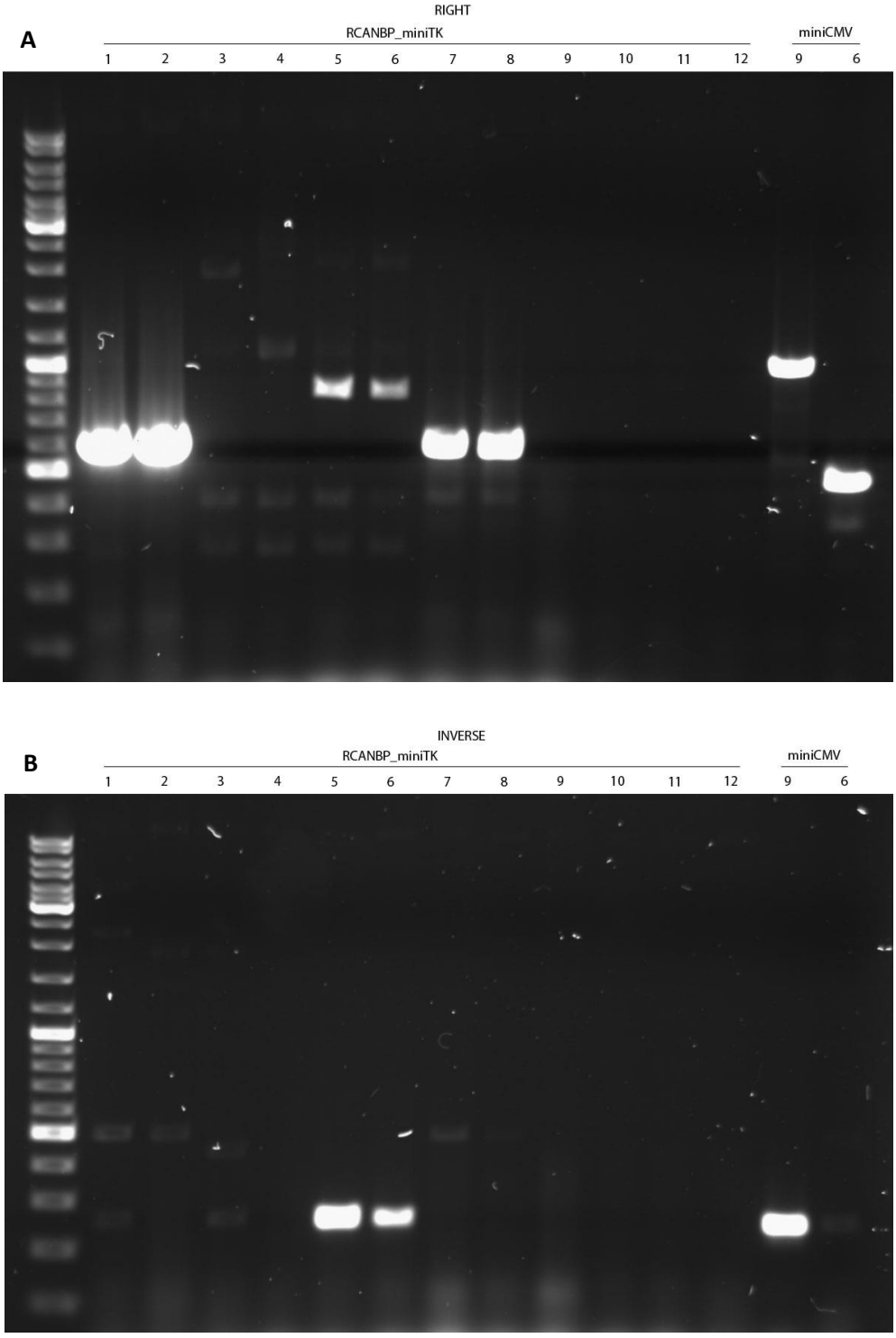


Fig. 24.: Colony PCR of RCANBP_miniTK in the inline direction, as controls the previous generated mini CMV 9+6 clones were used, lane1: GeneRuler, lane2-13: RCANBP-miniTK clone 1-12, lane14+15: controls miniCMV clone 9+6, Clones 1,2,7 and 8 show a band at about 600 base pairs and therefore the clones are positive, Clones 5+6 show unspecific products **(A), Colony PCR of RCANBP_miniTK in the inverse direction,** as controls the previous generated clones miniCMV 9+6 were used, lane1: GeneRuler, lane2-13: RCANBP-miniTK clone 1-12, lane14+15: controls miniCMV clone 9+6, Clones 5+6 show a band at about 250 base pairs and therefore these two clones are positive **(B).**

In case of the RCANBP_miniTK PCR product two sets of primers were used: The first set consisted of the primer RCANBP_forward (BS10) and in_eGFP_end (BS33) and synthesised a 594 base pair long PCR product using the annealing temperature of 55°C. This 594 base pair long product is only synthesised if the fragment has been integrated in the right direction and therefore indicates the positive clones RCANBP_miniTK 1,2,7 and 8. The clones RCANBP_miniTK 5+6 give an unspecific band, which turned out positive in the inline direction. The previously generated clone RCANBP_miniCMV inverse and inline were used as controls, which gave the prospect to check the PCR setup, by generated bands in the same or approximated length. Another set of primers was utilized to synthesise a product to the inverse direction using the primers RCANBP_forward (BS10) and eGFP_3'out (BS05) with an annealing temperature of 52°C to generate a 257 base pair long fragment. These two different PCR products helped to distinguish the inline inserted from the inverse inserted fragments. Attempts to combine the testing of inline and inverse clones, by using the primers RCANBP_forward (BS10), in_eGFP_end (BS33) and eGFP_3'out (BS05) in one reaction mix, did not exhibit clear results.

7.5.2. Second step - Control digest

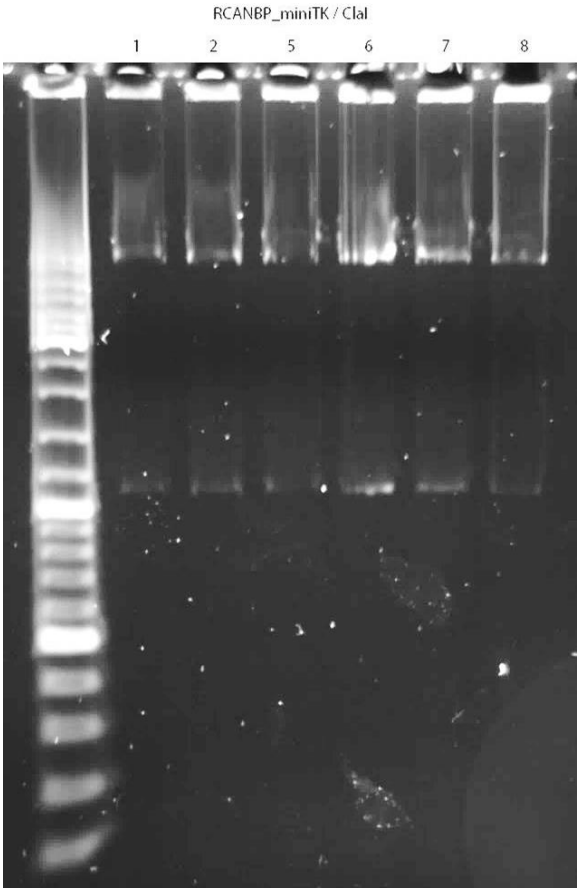


Fig. 25.: Digest of RCANBP_miniTK with the enzyme *ClaI*, lane1: GeneRuler, lane2-7: clones 1,2,5,6,7 and 8. All clones show a band at 1110bp and therefore they are positive.

After preparation, the positive tested clones were selected and digested with restriction enzyme *ClaI* to verify the correct size of the clones to make sure that the whole fragments had been integrated.

7.5.3. Third step - Control PCR with two primer sets

As performed in the colony PCR before two sets of primers were used again, which generate two different lengths of PCR products. The primers RCANBP_forward (BS10) and in_eGFP_end (BS33) were used to generate a PCR product of 594 base pairs, which is only generated if the fragment has been inserted in the right direction. An annealing temperature of 55°C was used. For the inverse inserted fragment the primers RCANBP_forward (BS10) and eGFP_3'out (BS05) were used to generate a PCR product of 257 base pairs length with an annealing temperature of 52°C. As obtained before the other tested clones show unspecific gene products.

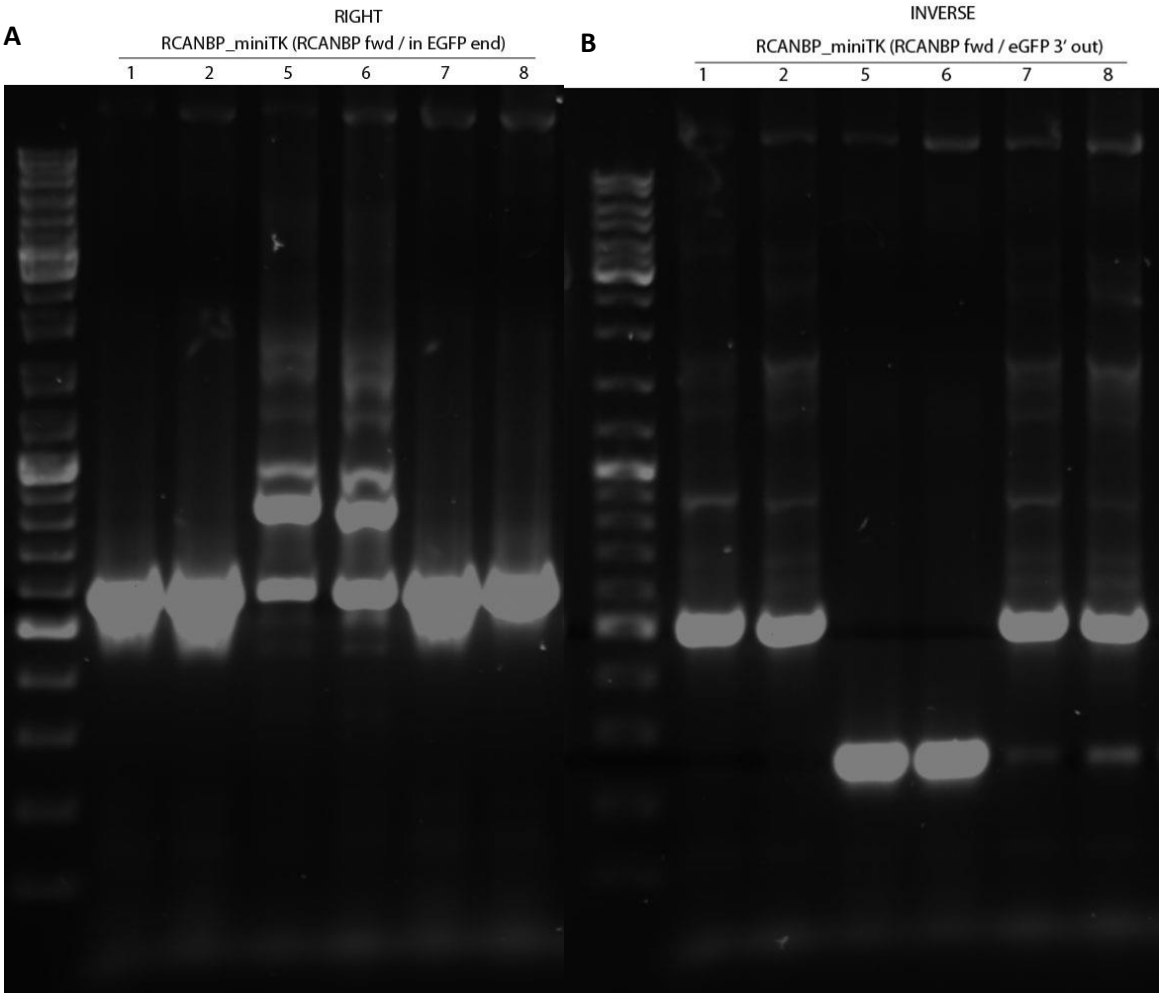


Fig. 26.: Control PCR of the preselected clones in both directions, results as in figure 24, lane1: GeneRuler, lane2-9: clones 1, 2, 5, 6, 7 and 8. The clones 1, 2, 7 and 8 in figure A show a specific band at 594 base pairs. The clones 5, and 6 show a specific band in figure B at 257 base pairs. Unspecific products were generated in the other lanes.

7.5.4. Ligation optimization by using methylene blue

The ligation is a complex process where the optimal temperature and especially the ratio of vector to insert is very important. An optimization of the ligation was necessary, due to the fact that two circumstances did not abet the ligation of the vector and the desired fragment. On the one hand a 13 kilo base large vector was used, which should ligate with a fragment length fewer than 1000 base pairs. On the other hand the restriction enzyme *ClaI* was used, producing a two base pair overhang, which is not conducive for a ligation. Therefore we decided to use a ligation technic based on the paper by Gründemann and Schömig (Grundemann and Schomig 1996).

7.6. Virus production including pretesting and titre determination

The DF-1 cells were transfected as described in methods and materials.

7.6.1. Pretesting

After transfecting the DF-1 cells the supernatant medium and the prepared centrifuged virus concentrate were tested and compared. For the first a set of primers consisting of RCANBP_forward (BS10) and in_eGFP_end (BS33) to produce a 359 base pair long PCR fragment were used. In the supernatant of transfected cells, the virus was detectable with the same primer combination using a common PCR. On the contrary, a common PCR of the virus supernatant of untransfected DF-1 cells gave no band. So a qPCR was used for the virus quantification. The procedure was done with the RCANBP_CMV plasmid.

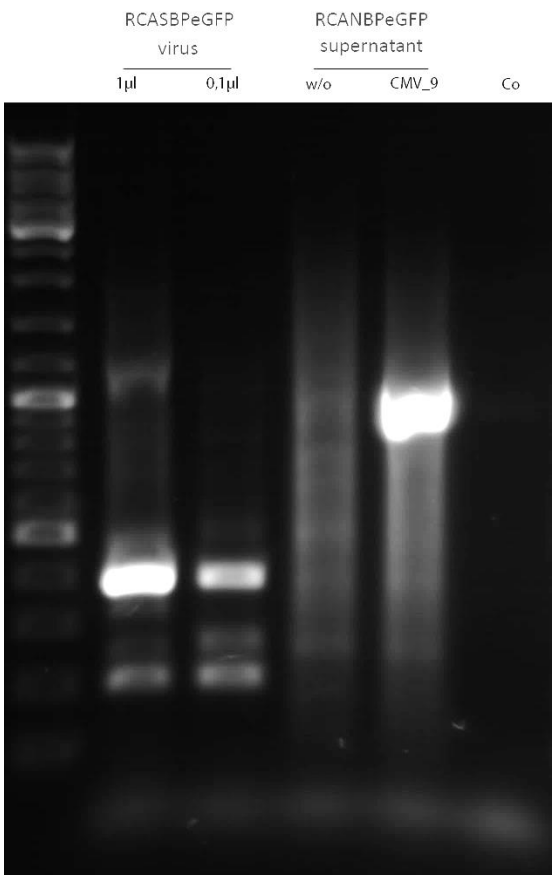


Fig. 27.: PCR products of RCASBP_eGFP cells and RCANBP_eGFP supernatant, lane1: GeneRuler, lane2+3: RCASBPeGFP 1µl and 0,1µl, lane4: supernatant without RCANBPeGFP, lane5: supernatant transfected with RCANBPeGFP_CMV, lane6: H_2O.

The infected DF-1 cells produced virus particles, which were concentrated via ultracentrifugation to a titre of average $1x10^{5-6}$ IU/µl. In case of the test virus RCASBP_eGFP, and RCANBP_miniCMV the titre can be determined with classic titration, because of the eGFP expression in the cells. Therefore, counting of infected cell patches is possible. For RCANBP viruses without a strong general promoter, RNA isolation and qPCR from cDNA is needed to roughly determine the titre level. First the RNA level of the RCASBP_eGFP and RCANBP was evaluated and qPCR results of all generated virus concentrates were matched to the results from previously titrated RCANBP_eGFP.

7.6.2. Virus titre determination via qPCR

The RCASBP vector had a higher ct- value than most of the vectors based on the RCANBP virus, which suggest that the virus construct RCASBP showed a lower virus titre, compared to RCANBP. In cell culture a contrary result was exhibit. A higher virus titre was achieved by the RCASBP virus, in all probability due to the more efficient transfection of cell patches in cell culture. So the RCANBP viruses, which showed no or a slight expression of eGFP were matched to the ct- value gained with cDNA from RCASBP. Two different amounts of

concentrated virus were used to correlate the titre to the results gained in cell culture to minimize the error rate (1µl and 0,1µl).

Virus titre 1µl					
Virus	**Promoter**	**6xPBS**	**direction**	**ct- value**	**standard deviation**
RCASBP				15,5301	0,1818
RCANBP	miniCMV	6xPBS	invers	10,9213	0,0760
		del 6xPBS	inline[4]	11,56906	0,0812
			Inline[4]	11,55496	0,0851
			inverse	10,50949	0,0031
	miniTK	6xPBS	inline	11,31809	0,048
		6xPBS	inverse	10,90513	0,2483
	Prox1		inline	16,86162	0,0805
			inverse	10,61111	0,5231

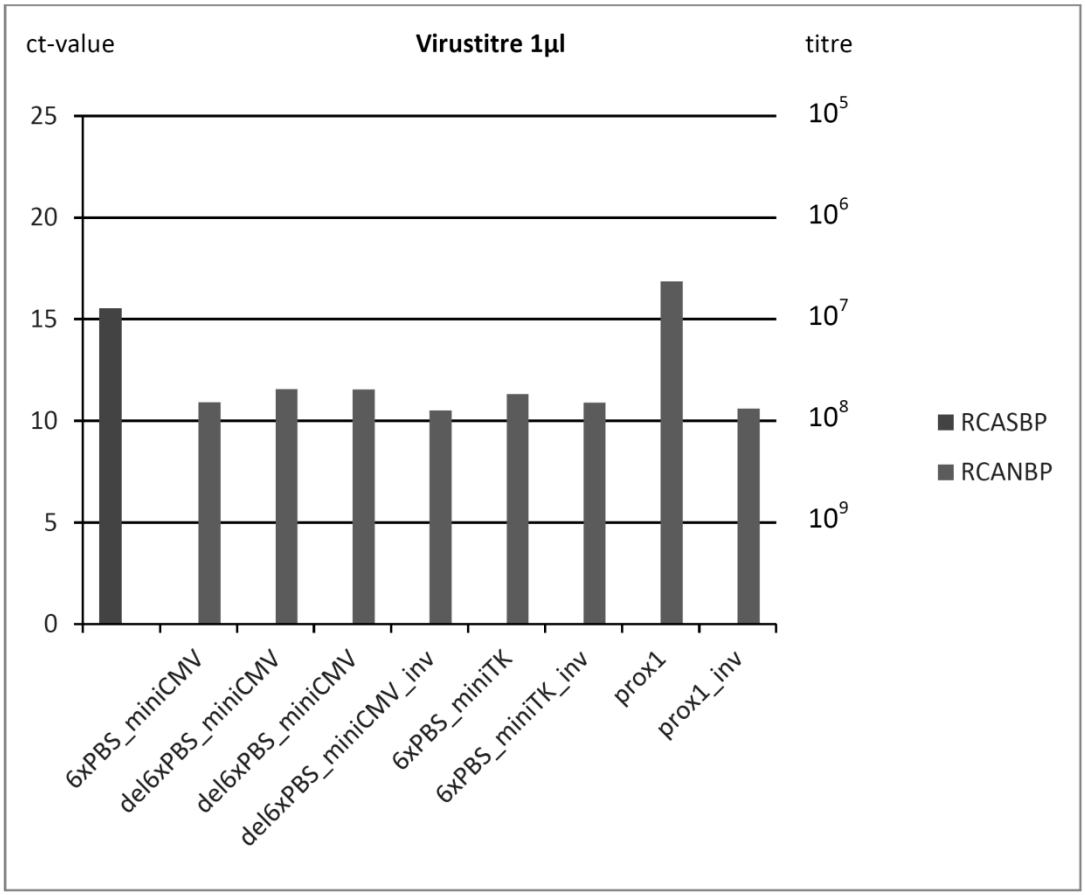


Fig. 28.: Bar chart of the ct- values of RCASBP and RCANBP correlated to a certain virus titre ascertained in cell culture via classical titration of fluorescent cells.

[4] two extra synthesised cDNAs from two different virus isolates.

To facilitate the comparison both viruses were blotted on the same diagram and matched to the correlating virus titre determined via classic titration using fluorescent RCANBP viruses.

Virus titre 0,1µl					
Virus	**Promoter**	**6xPBS**	**direction**	**ct- value**	**standard deviation**
RCASBP				18,36328	0,0443
RCANBP	miniCMV	6xPBS	invers	14,6307	0,4292
		del 6xPBS	Inline[5]	16,00406	0,0402
			Inline[5]	15,08411	0,1153
			inverse	13,83297	0,2054
	miniTK	6xPBS	inline	14,33911	0,0403
		6xPBS	inverse	13,44911	0,0798
	Prox1		inline	19,27465	0,1006
			inverse	14,46025	0,1336

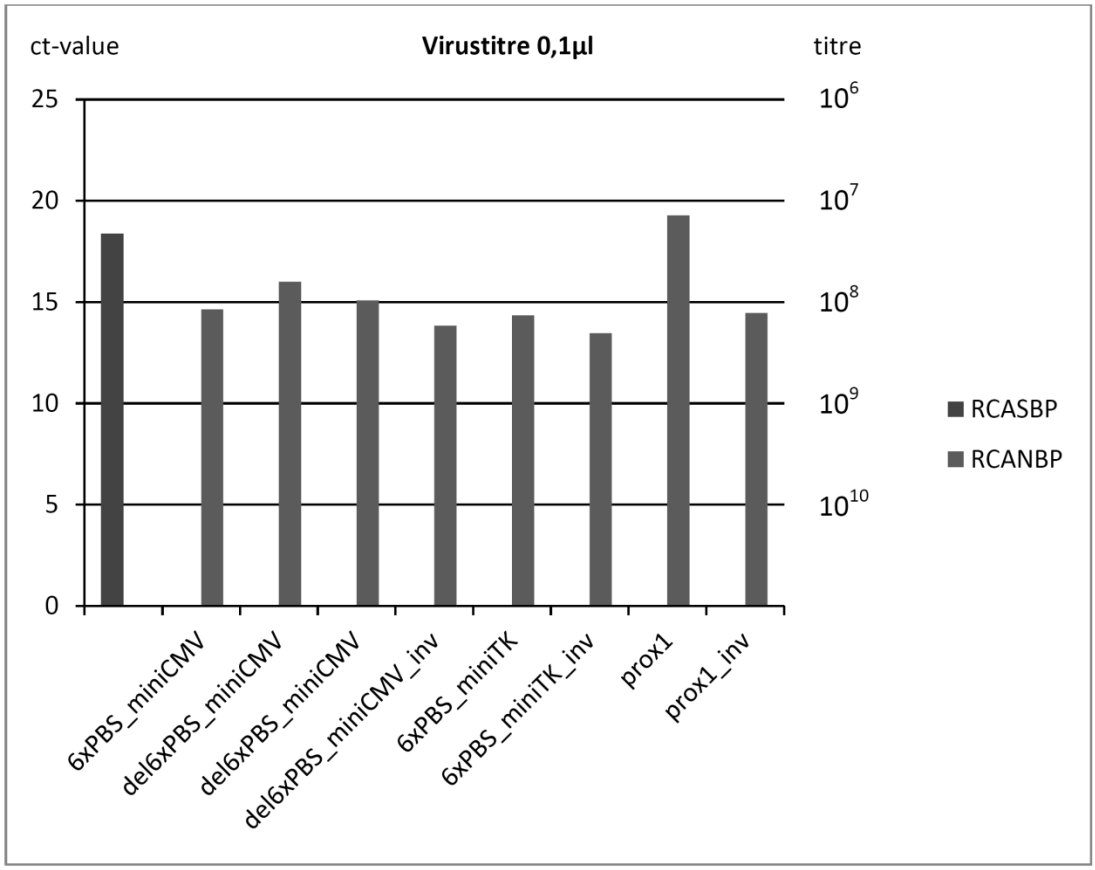
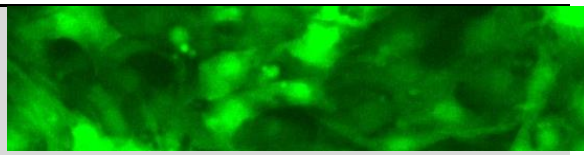
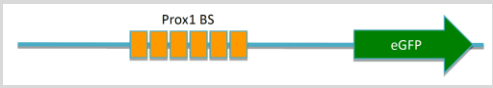
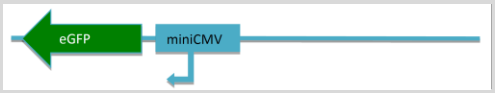
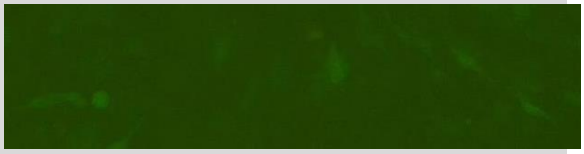
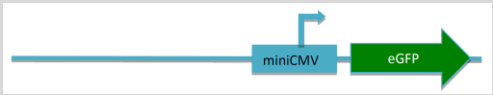
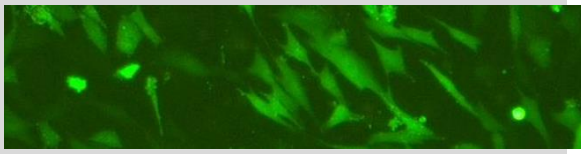
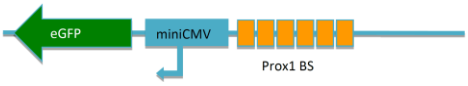
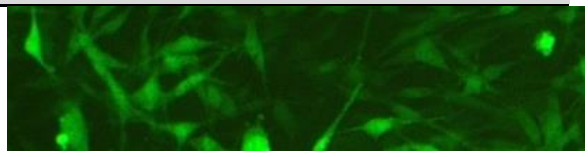
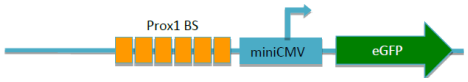
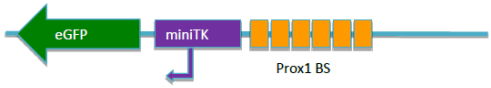
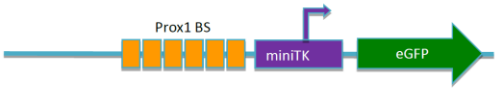
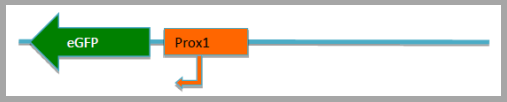
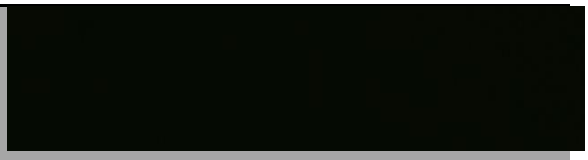
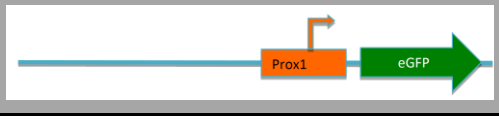
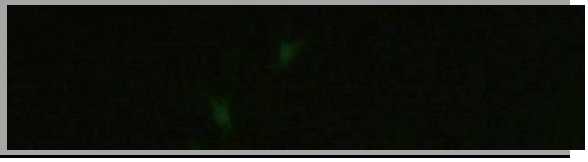


Fig. 29.: Bar chart of the ct-values of RCASBP and RCANBP correlated to a certain virus titre ascertained in cell culture via classical titration of fluorescent cells.

[5] two extra synthesised cDNAs from two different virus isolates.

7.7. Functional analysis of recombinant RCASBP/RCANBP viruses in vitro

	Virus Plasmid	Fluorescence in DF-1 cells
control viruses in DF1 cells	RCASBP_eGFP	
	RCANBP_6xPBS_MCS 	
	RCANBP_6xPBS_MCS 	
	RCANBP_MiniCMV 	
	RCANBP_MiniCMV 	
potential LEC specific 6xPBS based promoters	RCANBP_6xPBS_miniCMV 	
	RCANBP_6xPBS_miniCMV 	Toxic in DF-1 cells
	RCANBP_6xPBS_miniTK 	
	RCANBP_6xPBS_miniTK 	
chicken promoter fragment	RCANBP_2.3kb.chProx1 	
	RCANBP_2.3kb.chProx1 	

It was possible to generate a RCASBP viral vector, which is able to be expressed in all tissues of the CAM in an *ex-ovo* model, but it was not able to achieve a specific expression of eGFP in lymph endothelial cells in the CAM. Different strategies were tried to obtain a satisfactory result.

As promoter it was decided to utilize the cytomegalic promoter, which is commonly used to drive protein expression primally in mammalian cells, because eukaryotic promoters are poorly understood and span up to 100 kilo bases, which is far too big for the virus construct. Chicken promoter regions are barely investigated and therefore it was avoided to use such a hazardous promoter. As already mentioned, the RCANBP vector is a retroviral vector and therefore the nucleic acid is packed into an icosahedral capsid, which is limiting the size. In case of the RCANBP particle the maximum length of the uptaken nucleic acid can be 2.5 kilo bases, which is far too less capacity for a eukaryotic promoter.

The vectors were built up based on the vector construct published by Shin et al. (Shin, Min et al. 2006). In the publication, Shin et. al. showed that a 6xPBS-mFGFR3 core promoter construct linked with the marker protein Luciferase is highly Prox1 responsive. The Prox1 DNA binding domain is 100% conserved in chicken and in human and therefore an useful element in the vector construct. In contrast, the promoter had to be changed, because the mFGFR3 did not work in chicken. Hence the promoter element was substituted by the miniCMV promoter.

The generated plasmids were used to transfect DF-1 cells, which are chicken fibroblast cells, because it was not possible to isolate lymph endothelial cells from chicken. The DF-1 cells were appropriate to produce virus particles, but it was not possible to determine if the virus is functionally expressed in lymph endothelial cells of chicken embryos. Depending on eGFP expression in DF-1 chicken fibroblast cells an information about the virus specificity could be gained. If eGFP is expressed in DF-1 cells the promoter construct is too strong to be specifically expressed in lymph endothelial cells. If no eGFP is expressed there are two possibilities: either eGFP is not expressed in any chicken cell or the virus is specifically expressed in lymph endothelial cells.

The first vector which was constructed was a positive control vector RCASBP with a splice side, which was expressed in the whole chicken embryo. After checking that the procedure is working the first RCANBP vector without a splice site and a promoter was constructed, which gave no eGFP expression in chicken embryos.

At the outset a control vector using RCANBP, without a promoter was constructed, which gave as desired no fluorescence in the DF-1 cells and in the chicken embryo. Following two RCANBP vectors were constructed which should achieve an expression in lymph endothelial cells, using a CMV or a shortened CMV promoter (miniCMV). The virus with the CMV promoter and the 6xPBS was not able to achieve any expression of eGFP, due to a problem in gene sequence. However the miniCMV promoter and 6xtimes PBS showed a strong expression of eGFP in chicken fibroblast cells and in the embryo. Therefore, the decision to use the strong minimalCMV containing vector as positive control instead was made. In the following step, the 6xProxBS was deleted to check if the intensity of the eGFP expression is reduced by deleting the 6xProxBS. A minor difference in strength of eGFP expression was detectable. So it was suggested that the promoter element is too strong.

The second potential LEC specific vector construct used a minimal thymidine kinase instead of the minimal CMV promoter, which shows an unexpectedly strong expression of eGFP. Therefore, the thymidine kinase promoter was chosen, as less efficient than the Cytomegalic promoter. The core sequence of this promoter, the minimal TK promoter, is not expected to switch on gene expression without further enhancer elements. The last variant of the RCANBP_eGFP vector which was generated was a plasmid containing a Prox1 promoter without a 6xPBS. Due to a problem in virus production, it was not possible to control the produced virus in the chicken embryo (cp. 7.10).

7.8. Functional analysis of recombinant RCASBP/RCANBP viruses in vivo

The different concentrated viruses were injected into the blastula of fertilized chicken eggs using pulled glass capillaries. Then the injected eggs were sealed and incubated as described in the method and materials part (Bekes, Schweighofer et al. 2011). Using a fluorescence stereo microscope, eGFP expression in the embryos was controlled. Using different RCANBP_eGFP viruses inline with the viral genome, patches of fluorescent cells in the embryo were observed.

7.8.1. miniCMV promoters

As shown in the table, the DF-1 cells transfected with RCANBP_del6xPBS_miniCMV showed an expression of eGFP in the chicken fibroblast cells, leading to the conclusion that RCANBP_miniCMV deleted six times prox1 is not a proper promoter for the construct. So additionally a plasmid driven by a thymidine kinase promoter was constructed. Nevertheless the virus was tested in DF-1 cells and embryos.

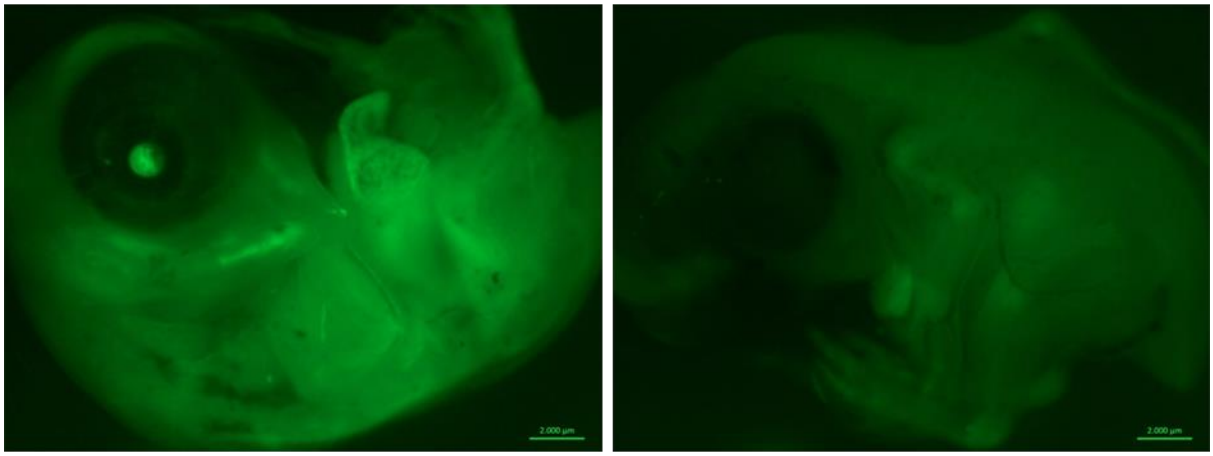


Fig. 30.: RCANBP_miniCMV binding site in E10 embryos, left: virus inline, right: virus inverse.

As observed before the fluorescence in the embryos injected with the virus inline showed a stronger fluorescence, than in embryos transfected with the virus where the cassette was inserted in the opposite direction (figure 30).

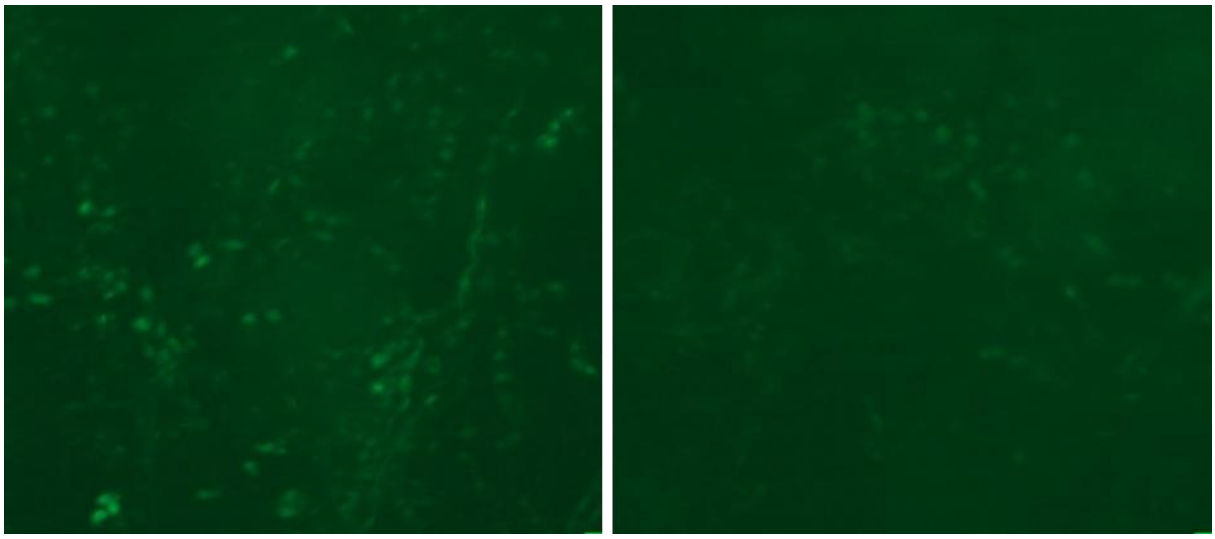


Fig. 31.: RCANBP_miniCMV site in CAM, left: virus inline, right: virus inverse.

The same phenomenon which was obtained in the cells and in the embryo before, was also recognized in the CAM. The virus RCANBP_miniCMV without the 6xPBS was far more efficiently expressed in the CAM, when the fragment had been integrated inline (figure 31).

7.8.2. Thymidine kinase promoters

Since the minimal CMV promoter turned out to unexpectedly strong expression a different promoter was designed. Therefore, the thymidine kinase promoter was chosen, as less efficient than the Cytomegalic promoter. The core sequence of this promoter, the minimal TK promoter, is not expected to switch on gene expression without further enhancer elements.

In the figure 32 different body parts of the chicken embryo are shown. As observed before in the DF-1 cells the embryos transfected with the virus inline showed a stronger expression of eGFP than embryos transfected with the virus inverse in the beak, the legs and the feathers.

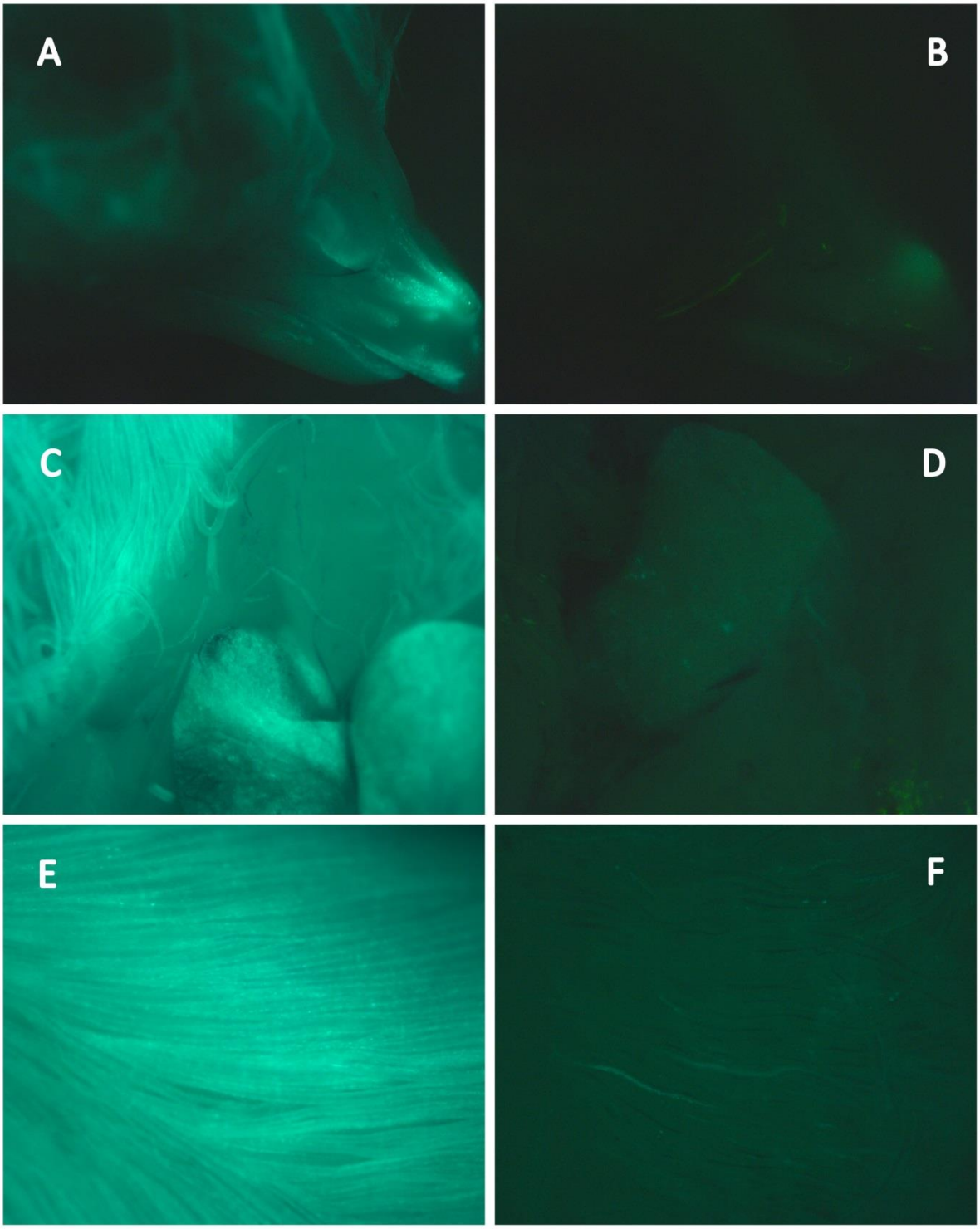


Fig. 32.: RCANBP_6xPBS_miniTK in E14 embryo, (A, B): beak, (C, D): body and leg, (E, F): feathers, left: body parts with RCANBP_miniTK_inline, right: with RCANBP_miniTK_inline.

Figure 33 shows that the CAM infected with the virus inline is much more fluorescent, than the CAM infected with the inverse integrated fragment. In both images a spreading of fluorescent cells around blood vessels is visible.

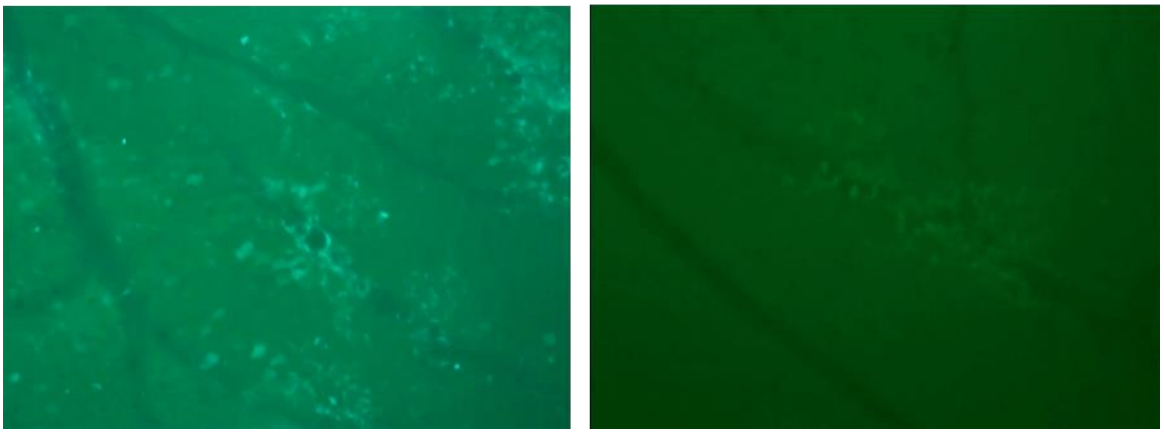


Fig. 33.: RCANBP_6xPBS_miniTK in CAM, left: RCANBP_6xPBS_miniTK inline, right: RCANBP_6xPBS_miniTK inverse.

7.9. Virus production in cell culture of RCANBP_prox1_eGFP

To indicate if the generation of virus is working proper, a PCR from viral cDNA, as described in the method and materials part, was necessary. For generation of cDNA RCASBP.B_reverse (BS11) in the correct and inverse integrated viruses was used. The generated cDNAs were checked with different primer pair variations over four days. The first primer was selected to control the integrated PCR fragment, the second is settled in the marker eGFP. The transfection mix was discarded one day after transfection and therefore day one is the day after the first medium change. For this experiment RCANBP_prox1_eGFP_inverse and RCANBP_prox1_eGFP_inline were used, because the western blot showed a lack in virus production.

Finally a gel with cDNA samples gained over four consecutive days was made, to be able to compare the results.

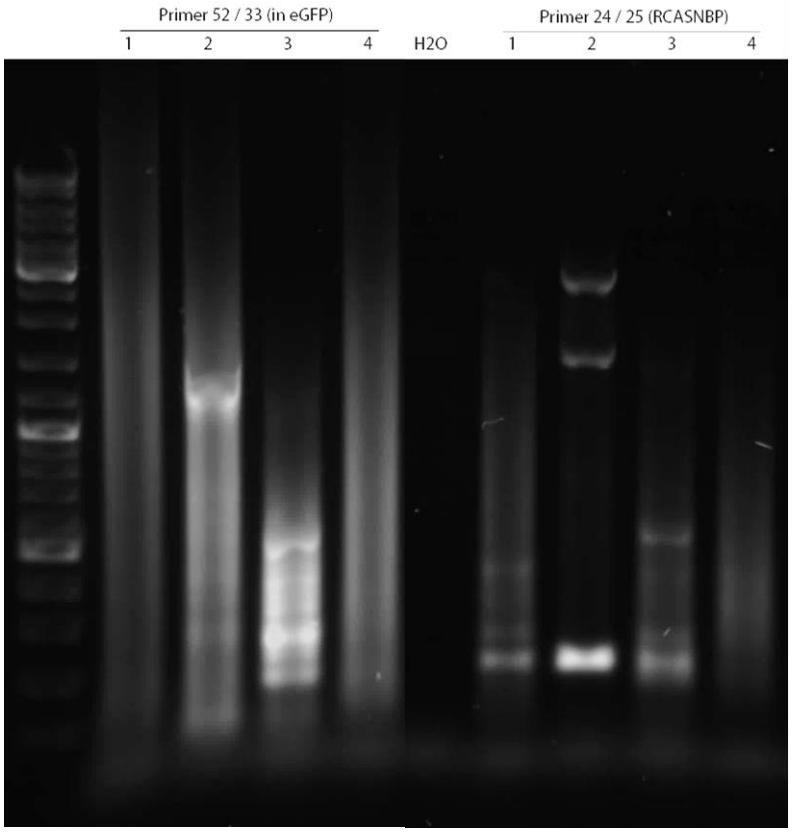


Fig. 34.: RCANBP_prox1_eGFP inline integration, PCR out of cDNAs using the cDNA generated with the primer RCASBP:B reverse, lane1: GeneRuler, lane2-5: PCRs of day 1-4 using primers BS52 and BS33, lane 2: no product visible, lane 3: PCR product of 1624 base pairs, lane4: product in different length shorter than 1624, lane 5: no product visible, lane6: H_2O, lane7-10: PCRs of day 1-4 using the primers BS24 and BS25, lane7: slight band at 259 base pair plus two additional bands, lane8: three bands one specific at 259bp, lane9: slight band at 259 base pairs and smear of unspecific bands, lane10: no product visible.

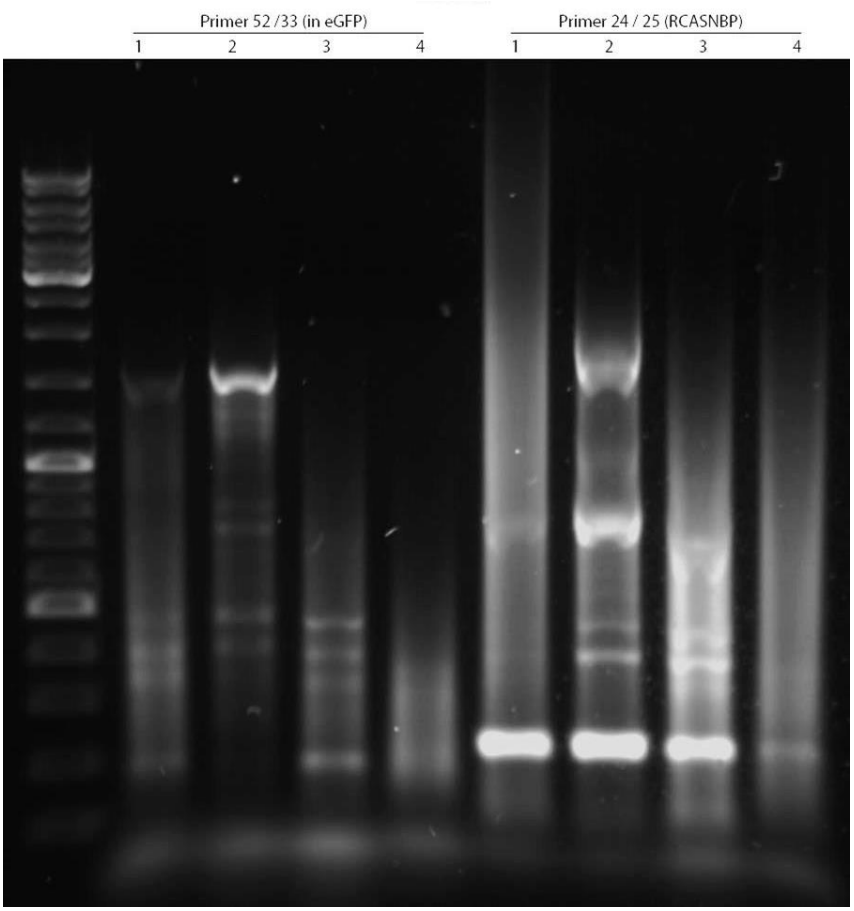


Fig. 35.: RCANBP_prox1_eGFP inverse integration, PCR out of cDNAs using the cDNA generated with the primer RCASBP.B reverse, lane1: GeneRuler, lane2-4: PCRs of day 1-4 using primers BS52 and BS33, lane 2: smear, no product visible, lane 3: PCR product of 1624 base pairs, lane4: product in different length shorter than 1624, lane 5: no product visible, lane6-9: PCRs of day 1-4 using the primers BS24 and BS25, lane6: strong band at 259 base pair plus, lane7: four bands and one specific at 259bp, lane8: band at 259 base pairs and smear of unspecific bands, lane9: only a slightly product visible.

The gels show clearly that the lager DNA product is only visible on the second day without degeneration. Subsequent the amplification of a whole DNA product was not possible. The second PCR product was easier to amplify because of the shorter length but also showed strong degradation on the fourth day. Due to this result we decided to use the cells for virus production as short as possible.

7.10. Western blot analysis of isolated virus

To determine the amount of virus, medium supernatant with virus was used to perform a western blot as described in method and materials. The monoclonal antibody is directed against the retroviral structural protein gag. (Potts, Olsen et al. 1987, Liu, Laufer et al. 2001, Zhang and Yang 2001, Kothlow, Schenk-Weibhauser et al. 2010, Kowalik and Hudspeth 2011, Mwizerwa, Das et al. 2011). Before loading the probes on the polyacrylamide gel the protein samples were measured using Bradford reagent to provide equal amounts of supernatant protein in every slot as described in method and materials.

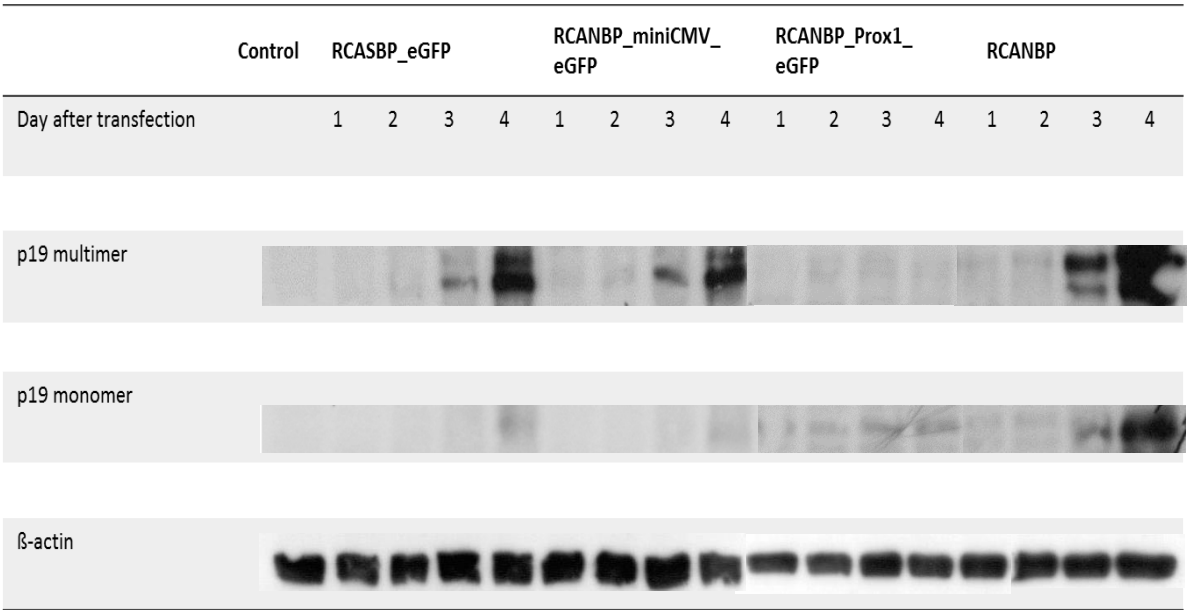


Fig. 36.: Time course of viral production of different RCASBP/RCANBP virus derived vectors.

The intensity of the bands increased every day in most of the virus samples. Only in case of the virus RCANBP_prox1_eGFP this gain of gag protein was not observed, which is inline with the PCR results (cp. 6.6.2). All over, more gag-protein was observed in the p19 multimeric bands than the monomeric bands.

7.11. Summary of functional analysis of the RCANBP promoter

In the following table the fluorescent expression of DF-1 cells and embryos in different plasmids in cells and embryos are summarized.

Virus Plasmid	Direction	Fluorescence in DF-1 cells	Fluorescence in avian embryo, lymph cells	Fluorescence in avian embryo, all tissues
RCASBP_eGFP		+++	-	+++
RCANBP_6xPBS_MCS	inline	-	-	-
RCANBP_6xPBS_MCS	inverse	-	-	-
RCANBP_MiniCMV	inline	++	-	++
RCANBP_MiniCMV	inverse	+	-	+
RCANBP_6xPBS_miniCMV	inline	toxic in cells	not tested[6]	not tested[6]
RCANBP_6xPBS_miniCMV	inverse	+++	+	+++
RCANBP_6xPBS_miniTK	inline	++	+	++
RCANBP_6xPBS_miniTK	inverse	+	+	+
RCANBP_prox1	inline	+	-	+
RCANBP_prox1	inverse	-	-	-

[6] Not tested, because the virus production was impossible.

8. Discussion

The constructed vectors were first tested *in vitro* in cell culture, if eGFP is expressed. Due to the fact that there was no cell line of chicken lymph endothelial cells available, and that we technically could not overexpress prox1 in this cell line, we had to test vector efficacy *in vivo* using *ex-ovo* chicken embryos. In the *ex-ovo* model –using the RCASBP.eGFP vector- the eGFP expression was detected in the chicken embryo, in different body parts and in the CAM, indicating that infecting the embryo results in efficient vector expression in all chicken cells.

The difficulties started, when we tried to build a construct that has cell type specific expression. We first used a promoter containing prox1-binding sites fused to the core FGFR-3 promoter which did not show LEC-specific expression. Subsequently, we constructed ten different RCANBP vectors with different promoters to generate transgenic *ex-ovo* chicken embryos, none of which showing the desired LEC-specific expression. Among these the six times Prox1 binding site was combined with the minimal thymidine kinase promoter. Especially the inverse inserted cassette showed no expression of eGFP in the chicken fibroblast cell line DF-1, compared to the correctly inserted cassette, which showed a slightly expression of eGFP. The virus of these transfected cell lines were then concentrated via virus ultracentrifugation and injected into germ discs of the chicken embryos. During virus production, including ultracentrifugation, we limited the virus preparation to day seven to prohibit virus particle degradation shown in 6.9. First fluorescence was detected in the eyes of the chicken embryo, where Prox1 is strongly expressed. After about twelve days some structures of the CAM showed a green fluorescence. Upon further analysis of the potentially positive lymphatic structures by imaging, whole amount stainings of RCANBP_6xPBS_miniTK transfected CAM in combination with anti_Prox1 antibodies with a confocal microscope (data not show) were made, it was noticed that, only a few lymphatic ECs actually expressed eGFP.

As last strategy it was decided to use a chicken Prox1 promoter fragment to achieve specific expression of eGFP in LECs. The viral transfection with the Prox1 promoter resulted in very few positive single cells, which was reflected in the chicken embryos. As shown in the western blot (6.10.) the virus production of RCANBP with the Prox1 did not work and therefore an expression in the chicken embryo is not expected. On the contrary the other virus supernatants showed a clear increase of produced virus over the tested period. In an additional experiment the processing of the RCANBP_Prox1 virus in cell culture was tested and it was asserted that the virus is fully produced only on day two. The reason for this could

be that the Prox1 promoter is extremely AT rich and therefore a random polyadenylation, which interferes with viral replication, is possible. Additionally the virus titre of RCANBP_Prox1 with 10^{6-7} is lower and underlined the result that functional virus production is not efficiently produced by cells, compared to the other RCANBP viruses, which achieve a titre of 10^{8}.

The only positive result was the successful *ev-ovo* infection and eGFP transfer of a chicken embryo including the CAM by injecting RCASBP virus into the chicken embryo germ discs. So far it was only possible to generate a transgenic chicken embryo in an *in-ovo* model. So this was the first time that it was able to generate an *ex-ovo* model in one generation. The aim to express specifically eGFP in lymph endothelial cells failed.

The challenge was that there were no cultured chicken lymph endothelial cells available to test the generated virus right at the beginning in the cell culture. So the complete working process was done with every generated virus, because in the vitro test system only the leakiness and not the specificity could be tested. The used virus is described to be able to spread horizontally and vertically, but was not able to express eGFP in every lymph endothelial cell over the whole embryo and in the CAM; therefore, a different better spreading virus should be invented. Another possibility is to generate a transgenic chicken embryo. The disadvantage of this strategy is that at least two generations of transgenic chickens are needed, and this is connected with a high effort.

9. Supplement

9.1. Primer

name	description	sequence
BS01	Pr in 6xProxBS to Fluor	CGCCTCTTCGGAGATCATCG
BS02	Pr in eGFP towards 3'	AAGATGGTGCGCTCCTGGAC
BS03	Pr in mCherry towards 3'	AGCCGTACATGAACTGAGGG
BS04	Pr in 6xProxBS to Fluor mcherry	TCGCCACCTCTGACTTGAGC
BS05	Primer eGFP 3' out	GGATCACTCTCGGCATGGAC
BS06	Primer mcherry 3' out	TCCCACAACGAGGACTACAC
BS07	Pr in 6xProxBS to out	GCGTGGGATCAAGCTTATCG
BS10	RCANBP(B) fw	GACTGCTTTTGGGGCTTGTA
BS11	RCASBP.B rev (7109-7092)	TTTCGCCTAAACACACCC
BS12	EGFP_N_Primer	CTGGTCGAGCTGGACGGCGACG
BS13	EGFP_2 Primer (389-408)	ACGACGGCAACTACAAGACC
BS20	MiniCMV Cloning fwd	CTTAGGATCCACGCAAATGGGCGGTAGGC
BS21	MiniCMV Cloning rev	CTTAGTCGACCTGGATCGGTCCCGGTGTCT
BS22	CMV Cloning Primer fwd	GATAGGATCCCGTAGTACGAGGCCCTTTCA
BS23	CMV Cloning Primer rev	GATAGTCGACAATTCCGGACCGGTACCA
BS24	RCASNBPfw (6509)	GAGCGGCTATTGACTTCTTG
BS25	RCASNBPrev (6723)	CAAATGAACGGCCCATTCTC
BS32	Cla12 to eGFP	TTATGCTTCCGGCTCGTATG
BS33	in eGFP end	TGAACAGCTCCTCGCCCTTG
BS34	Cla12 to 6xPBS	TAATCGCCTTGCAGCACATC
BS34A	Cla12 to 6xPBS corr	GATGTGCTGCAAGGCGATTA
BS40	chprox1 prom fwd FLANKING	AGGAGCCGGTAACACAAACA
BS41	chprox1 prom rev FLANKING	TGGCTTTTCCCCATCTGCAT
BS42	ClP prox1 fwd BamHI	CTTAGGATCCAGCCACAGATGCAGTCCTAGGG
BS43	ClP prox1 rev SalI	CTATGTCGACTGTCAGCTGGGCTCAGCTCAAG
BS45	Clp_miniTK_SalI	CTATGTCGACTTAAGCGGGTCGCTGCAGGGT
BS46	Clp_miniTK_BamHI_2	CTTAGGATCCCGTTTGCTGGCGGTGTCCC
BS47	Clp_FullTK	TGCTGGCCTTTTGCTCACATGG
BS48	chProx1 prom2 large fw FLANK	TAGGCAGCGTCTTGGGAATG
BS49	ClP prox1_sup fwd BamHI	GAACGGATCCTCATCCTGGTTGCAAACT
BS50	chProx1 prom2 rev FLANK	AGCAGCTTGCGGAGTACATT
BS51	ClP prox1_sup rev SalI	GAACGTCGACTGTGCCATCTTCAAAAGC
BS52	chProx1 prom2 fw FLANK	AAGCGTGGACTCCTGTCTTG
BS55	chProx1 prom middle to 3'	ATGCCTCGTTTCTGCTATGC

10.Curriculum vitae

Name:	Bracher Kathrin Barbara
E-Mail:	kababs@gmx.at

Education:

2011-2014:	University Vienna, Master studies in molecular biology
2/2012:	Wahlbeispiel Kuchler Group, MFPL, University of Vienna
3-4/2012:	Wahlbeispiel Kovarik Group, MFPL, University of Vienna
5-6/2012:	Wahlbeispiel Schneider Group, MFPL, University of Vienna
2-12/2013:	„Großpraktikum“ and master thesis, Skin and Epithelium Research Division, Medizinische Universität Wien
2011:	Admission for Master studies in molecular biology
2009-2011:	University Vienna, Bachelor studies in biology
2009:	Diploma in biomedical science
2006-2009:	Akademie für den medizinisch-technischen Laboratoriumsdienst on the general hospital in Vienna
1998-2006:	Bundesrealgymnasium Krems an der Donau, Ringstraße with focus on mathematical skills, Matura
1994-1998:	elementary school Spitz an der Donau

Interests and Skills:

Languages:	German and English
Interests:	Music: clarinet (member of a traditional Austrian brass band)
	Paramedic on the ambulance for the Austrian Red Cross
	Alpine skiing, swimming and inline skating

11.Figure table

12.Reference

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