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Generation of Transgenic Mice to Evaluate Promoter Activity and Specificity of Two Human Endogenous Retrovirus Long Terminal Repeats

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Untersuchungen zur Promotor-Aktivität und -Spezifität von zwei Long Terminal Repeats humaner endogener Retroviren in transgenen Mäusen

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Meiner Familie

CONTENTS

ABBREVIATIONS

AIDS	aquired immune deficiency syndrome
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BCA	bichioninic acid
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CCLR	cell culture lysis buffer
cDNA	complementary DNA
Chang liver	human liver cell line
CMV	cytomegalovirus
cm	centimeter
cpm	counts per minute
DAB	3,3´-diaminobenzidine
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide-tri-phosphates
DQB	class II gene of the major histocompatibility complex
DTNB	5,5´-dithio-bis-[2-nitrobenzoic acid]
DTT	dithiothreitol
EBFP	enhanced blue fluorescent protein
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescent system
ECFP	enhanced cyan fluorescent protein
E. coli	Escherichia coli
EGFP	enhanced green fluorescent protein
EMBL	european molecular biology lab
ES	embryonic stem cells
EtBr	ethidiumbromide
EYFP	enhanced yellow fluorescent protein
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
g	gram
g	gravity
GFP	green fluorescent protein
h	hour
HaCaT	human keratinocyte cell line
HC	high concentrated
HCG	human chorionic gonadotropin
Hela	human cervical adenocarcinoma cell line

Hep2	human adenocarcinoma cell line
HERV	human endogenous retrovirus
HIV	human immunodeficiency virus
Huh-7	human liver cell line
ICCD	intensified charged coupled device
ICOS	inducible T-cell co-stimulator
IDDM	insulin-dependent diabetes mellitus
IMR	induced mutant resource
kb	kilobases
kDa	kilo Dalton
LB medium	Luria Bertani medium
LC5	human lung fibroblast cell line
LC-5	human lung carcinoma cell line
LTR	long terminal repeat
MAR	matrix attachment region
Mb	megabases
MCF7	human mammary adenocarcinoma cell line
mg	milligram
MiaPaCa2	human pancreas carcinoma cell line
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
ms	milliseconds
M-MLV	moloney murine leukemia virus
NADH	nicotinamide-adenine dinucleotide (reduced form)
ng	nanogram
nm	nanometer
No.	number
NRL	nose rump length
NT	nuclear transfer
NTera2D1	human teratocarcinoma cell line
OD	optical density
PAC	P1-derived artificial chromosomes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative logarithm of the hydrogen ion concentration
PI	propidium iodide
PLB	passive lysis buffer
PMSG	pregnant mares serum gonadotropin
PP_i	inorganic pyrophosphate
Prnp	prion protein
PVDF	polyvinylidendiflouride
RFP	red fluorescent protein
RLU	relative light units
RNA	ribonucleic acid

rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s	seconds
SAR	scaffold attachment region
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SV 40	simian virus type 40
T47D	human breast cancer cell line
TBS	Tris-buffered saline
tg	transgenic
TNF-alpha	tumor necrosis factor alpha
tRNA	transfer RNA
TSE	transmissible spongiform encephalopathy
Tyr	Tyrosine
U	unit
U373	human astrocytoma cell line
UTP	uridine triphosphate
V	volt
wt	wildtype
YAC	yeast artificial chromosome
µg	microgram
µl	microliter
µm	micrometer

1 INTRODUCTION AND OBJECTIVES

Endogenous retroviruses and related elements are an integral part of the genome of many organisms, including primates and humans. Around 8% of the human genome is derived from retrovirus-like elements (Smith, 1999). They originate from ancient retroviral infections or are relics of transposomal activity in the germ-line cells (Paces et al., 2002).

Human endogenous retrovirus long terminal repeats (HERV-LTRs) are represented with 52.7 Mb (1.8%) in the human genome. These sequences contain all the signal structures required for the regulation of gene transcription, such as promoters, enhancers and transcription factor binding sites (Majors, 1990; Brosius, 1999; Schoen, 2001). They thus represent an enormous resevoir of regulatory sequences within the human genome. Could this potential of HERV-LTRs be utilized for regulation of therapeutic genes? To ensure efficiency of gene therapy, promoters that regulate therapeutic genes have to be highly active to create large numbers of transcripts in the target cell and also to compensate low efficiency of gene delivery systems. For safety reasons, tissue- or cell-type specificity of the promoter is desirable.

Two HERV LTRs were chosen on behalf of these criteria: HERV-L which showed high promoter activity and specificity *in vitro* and HERV-H-H6 which showed high promoter activity *in vitro*. The objective of the investigations reported here was to study expression level and pattern of these HERV-LTRs *in vivo* through the establishment of transgenic mouse models: HERV-L was cloned into a luciferase expression vector and HERV-H-H6 into an EGFP expression vector. Gene constructs were injected into the pronuclei of zygotes. Transgenic mice were examined for integration of the injected gene construct and for expression of the corresponding reporter gene in various tissues. While the pBL-HERV-L construct was not active in transgenic animals, pEGFP-HERV-H-H6 was expressed in selected organs. To consider species-specific effects, pEGFP-HERV-H-H6 and pBL-HERV-L were also used to generate transgenic rabbits. This work will be presented in the dissertation of Zoltan Hubbes at the Department for Molecular Animal Breeding/LMU Munich.

2 REVIEW OF THE LITERATURE

2.1 Genetic engineering of the mouse

2.1.1 Transgenic technology

Transgenesis, or the stable integration of foreign DNA into the host genome, has developed into one of the most powerful techniques for analysing gene function and regulation (Hammes and Schedl, 2000). Transgenic animals have recently been defined by Beardmore (1997) as: "organisms containing integrated sequences of cloned DNA (transgenes), transferred using techniques of genetic engineering (including those of gene transfer and gene substitution)". There are several techniques for the production of transgenic animals. The most commonly used methods are pronuclear DNA microinjection and embryonic stem (ES) cell manipulation. Other possibilities are nuclear transfer (NT), cytoplasmatic DNA injection, use of viral vectors, manipulation of primordial germ cells and sperm-mediated gene transfer (Mepham et al., 1998; Wolf et al., 2000). Consequences of gene transfer can include "gain of function" or "loss of function" phenotypes. Potential and actual applications of transgenic animals were summarized by Gordon (1996) as follows:

- Basic research
- Disease models
- Vaccine testing
- Toxicity testing
- Xenotransplantation
- Gene farming (production of therapeutic proteins)
- Manipulation of livestock production traits

The first transgenic animals that were generated were mice (Gordon et al., 1980). Since then, gene transfer has been established not only in mammalian species such as cattle, sheep, rabbits, goats, rats and pigs but also in fish and poultry (Brem et al., 1985; Campbell et al., 1996; Chen et al., 1996; Kubisch et al., 1997; Chan et al., 1998; Hong et al., 1998; Baguisi et al., 1999; Zakhartchenko et al., 1999).

2.1.2 Mouse models in genetics

Mice have become the mammalian model of choice for the application of genetics in biomedical research due to the evolutionary conservation of physiological systems and their attendant pathologies among all mammals as well as the exceptional power of genetic research technologies in this species (Paigen, 2002). Features of mouse reproduction physiology like short generation time (10 weeks from being born to giving birth), prolifical breeding in the lab (5-12 pups), immediate postpartum oestrus and deposition of a vaginal plug (for timing of pregnancies) reinforce this position (Silver, 1995). Until the 1980s, biomedical studies involving alteration of the mouse genome had to rely either on the appearance of spontaneous mutations or on the generation of chemically- and radiation-induced mutations and allophonic mice. These studies played a major role in biomedical research of the last century (Jonas, 1984). The discovery of the severe combined immunodeficiency (SCID) mouse became well known as model for a corresponding human disease (Custer et al., 1985). In 1981 Margaret C. Green listed hundreds of mouse strains and mutants attesting to the rich biological material available through genetic studies on mice.

Thus, when recombinant DNA methods merged with mammalian cell culture and embryo manipulation techniques in the 1980s, the mouse was the laboratory species most suitable for modern genetic engineering. Two mouse models played a major role in this development:

- Transgenic mice result from the introduction of either endogenous or exogenous gene sequences into the mouse genome by pronuclear injection. Subsequent overexpression of endogenous genes or expression of novel gene products, i.e. “gain of function “, is one possible phenotype. “Loss of function” is another possible phenotype resulting from insertional mutagenesis by the transgene. Targeted inhibition of gene activity can be achieved in transgenic mice by expression of antisense RNA (Katsuki et al., 1988; Pepin et al., 1992) or by dominant negative mutations (Hagenfeldt-Johansson et al., 2001).

- Knockout mice can be generated by directed mutagenesis in embryonic stem cells. When endogenous genes are disrupted, “loss of function “ of a defined endogenous gene is the resulting phenotype. The use of site specific Cre and Flp recombinases in gene targeting allowed the generation of conditional knock-outs, i.e. animals in which a gene knock-out is restricted to specific tissues (Lakso et al., 1992) or occurs in response to an exogenous induction signal (Metzger et al., 1995).

Over the last decade, many transgenic and knockout mutant mouse strains have been created. Models like *Prnp* knockout mice (Bueler et al., 1992) which play an essential role in TSE (Transmissible Spongiform Encephalopathy) research or the popular “green mice” generated by Okabe et al. (1997), illustrate the importance of genetically engineered mouse strains. They are documented in transgenic databases such as TBASE (Woychick et al., 1993) or Induced Mutant Resource (IMR) (Davisson, 1990): www.tbase.jax.org; www.jaxmice.jax.org/index.shtml.

Complementary to these “gene driven” approaches, in which mouse models are produced for those genes that we already know, the large-scale Munich ENU-mouse mutagenesis screen (Hrabe de Angelis and Balling, 1998; Rathkolb et al., 2000), which is part of the German Human Genome Project, offers a “phenotype driven” approach. What at first sight, appears to be solely a come-back of a well established technique for chemical mutagenesis (Russell et al., 1979), offers in fact in combination with current possibilities of genetic analysis a new dimension of functional genome analysis. In different mouse mutants that display one disease phenotype, the responsible mutated genes are identified. The study of divers mouse mutants with one phenotype but mutations in different genes is especially interesting for the understanding of pathogenesis of the corresponding disease. The mutant lines are freely accessible to non-commercial users. The strains are indexed under http://www.gsf.de/ieg/groups/enu/mutants/index.html.

2.1.3 Transgenic mice

The classical method, generation of transgenic mice by microinjection of DNA solution into the male pronucleus of fertilized oocytes, is described in detail in the corresponding manuals (Hogan et al., 1994; Pinkert, 1994). This method has remained basically unchanged since its development 20 years ago (Gordon and Ruddle, 1981). As donors for fertilized oocytes C57BL/6 x SJL hybrid mice have been shown to be more efficient than C57BL/6 mice. They delivered more fertilized oocytes, the percentage of eggs that survived injection and developed into pups was higher as well as the percentage of mice that retained the microinjected DNA (Brinster et al., 1985). After successful delivery of the DNA, the transgene integrates into the mouse genome and replicates with the endogenous chromosome. For a standard transgene, integration of 1-50 copies is normal, but integration of up to 1000 copies has been observed (Lo et al., 1987). The individual copies can be found in tandem arrays as head-to-tail fusion but also as head-to-head or tail-to-tail fusion. If transgene integration occurs at more than one site, the offspring of the transgenic founder are likely to carry only a subset of the copies integrated in the founder animal, due to segregation of chromosomes. In 10-30% of transgenic animals, integration does not occur during the one-cell stage, but later in development. As a consequence, the resulting mice will be mosaics and germ line transmission (Gordon and Ruddle, 1982) is not guaranteed. Transgenic mice of generation F0, so called founder mice, are hemizygous for the transgene (Wagner et al., 1983). Non mosaic founders with one integration site will pass on the transgene to 50 % of their offspring (Gannon et al., 1990). To eliminate doubts about mosaicism and genetic variability of the founders, F0 transgenic mice are mated with non-transgenic inbred mice. Expression studies are carried out from the F1 generation on.

The described characteristics of integration of the transgene already indicate the main problem of DNA microinjection: integration occurs randomly. Levels and spatial distribution of transgene expression are highly sensitive to transcriptional activators or silencers located in the vicinity of the integration site. This can lead to reduced, abolished or ectopic expression, a phenomenon called position effect. As position effects can

influence expression of the transgene, it is important to generate and examine several independent transgenic lines and to compare their phenotypes (Hammes and Schedl, 2000). A second negative aspect of random integration is that insertion of the transgene can occur within an endogenous gene, creating an insertional mutation and disrupting the locus. This can result in a distinct phenotype, especially in homozygous transgenic mice (Wagner et al., 1983). Another limitation of the DNA microinjection technique is that the number of integrated copies at one site can not be influenced. The presence of multiple copies integrated at a single site as concatemer has been shown to reduce the level of expression of the gene construct independently of the integration site (Garrick et al., 1998). Furthermore, the level of transgene expression can be negatively influenced by DNA methylation (Jaenisch, 1997). DNA methylation of endogenous genes is associated with the phenomenon of genetic imprinting. This modification of genetic information plays a decisive role in mammalian development when maternal and paternal genome have to contribute to embryonic development (Reik et al., 1987).

2.1.4 Design of the gene construct

Apart from the integration process, the design of the gene construct itself has major influence on its expression level and pattern. The DNA element to be transcribed can be complete or partial cDNA, or a genomic sequence. The transgene is cloned downstream of a suitable promoter element which may determine expression level and pattern. Including an intron downstream of a transcription start site has been shown to improve expression (Choi et al., 1991; Palmiter et al., 1991). A start codon preceded by a Kozak consensus sequence can also enhance expression (Kozak, 1987). Additionally, it has been demonstrated that efficient expression of a transgene requires polyadenylation of the transcribed product, for this reason it is advisable to clone a polyadenylation signal, e.g. from SV 40 or polyoma virus at its 3`end. Plasmid sequences flanking the transgene have shown to negatively influence expression (Chada et al., 1985).

In addition to the core techniques described above, further approaches have been made recently. To reduce position effects, transgenes consisting of large (>50kb) genomic fragments, can be obtained from yeast artificial chromosomes (YACs) (Chevillard et al., 2002), bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs). Another approach to enable position independent expression of transgenes is the use of matrix attachment regions (MARs) or scaffold attachment regions (SARs) (Mc Knight et al., 1996). Also conditional transgenic technologies (reviewed in Ryding et al., 2001) like the tetracycline transactivator system (Bujard and Gossen, 1992) and the ecdysone induction system (No et al., 1996, Saez et al., 2000) have been shown to be reliable tools for regulated transgene expression.

2.2 Human endogenous retroviruses

2.2.1 Characteristics and biological significance of human endogenous retroviruses

Approximately 2.7% (78.9 Mb) of the human genome consist of proviral structures called human endogenous retroviruses: HERVs (Smit, 1999). These proviruses most probably originate from primary infections of germ line cells by ancient retroviruses. In some cases they were amplified by retrotransposition or reinfection in the course of evolution (Wilkinson et al., 1994; Löwer et al., 1996; Parseval et al., 2001) as presented in Figure 1. The current HERV database contains 39 HERV families colinear with the typical retroviral genome: LTR-*gag*-*pol*-(*env*)-LTR. 1.8% (52.7 Mb) of the human genome is derived from HERV-LTRs and 0.9% of the genome (26.2 Mb) are internal HERV sequences (Paces et al., 2002). A proviral long terminal repeat (LTR) contains the functional regions U3, R and U5. U3 and U5 are unique sequences derived from the 3´ and 5´ ends of the viral RNA, while R is a short sequence present at both termini of the RNA genome. The strongest viral transcriptional enhancer sequences are typically found in U3 as are promoter signals such as the TATAA box (Feuchter and Mager, 1990; Majors 1990). The structural genes are *gag*, which codes for the proteins of the viral capsid, *pol* which codes for the viral enzyme reverse transcriptase, integrase and protease and *env* which codes for a transmembrane glycoprotein and the surface protein

(Wilkinson et al., 1994). HERVs are classified according to sequence homologies, but the nomenclature is not yet stabilized (Parseval et al., 2001).

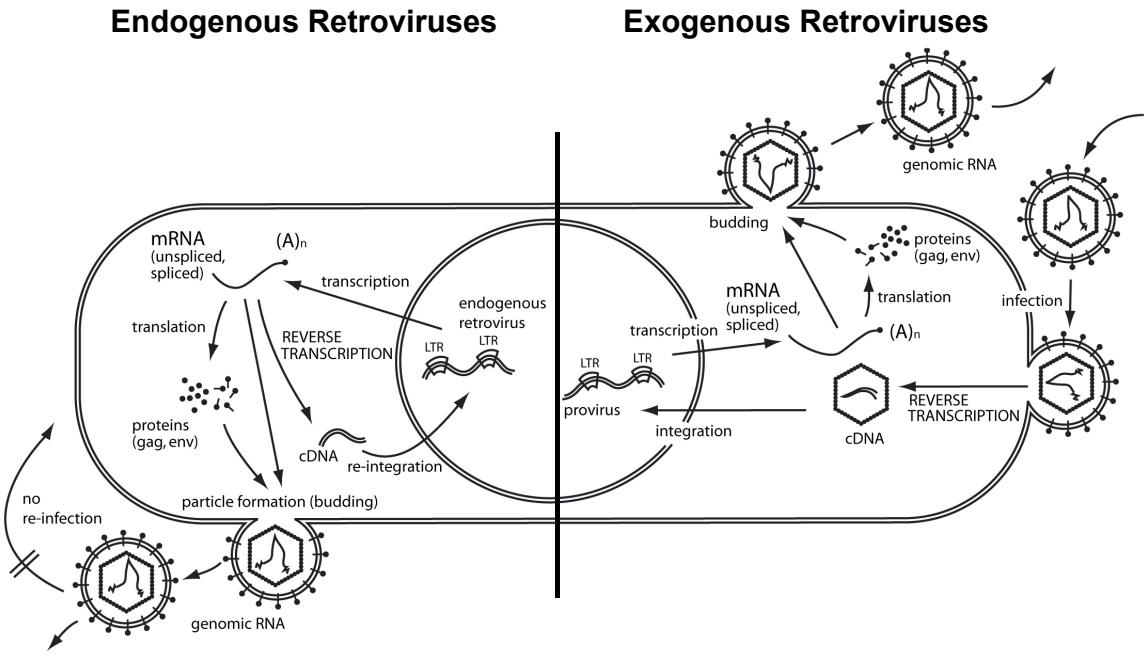

Figure 1: Life cycles of endogenous retroviruses in comparison to exogenous retroviruses modified from Löwer et al. (1995)

Different elements of these proviruses play a role in physiological as well as in pathological processes. HERV LTRs can serve as transcription regulators, alternative promoters and polyadenylation signals for cellular genes. A LTR sequence of the HERV-E family is involved in the tissue-specific expression of human salivary amylase. The insertion of this HERV element upstream of the human amylase gene complex is correlated with a change from pancreatic to parotid expression (Samuelson et al., 1988). In the human cytochrome c1 gene, part of a HERV-I LTR was found to be involved in transcriptional regulation (Suzuki et al., 1990). A HERV-H LTR is reported to promote the expression of a protein related to phospholipase A2 by intergenic splicing of two

adjacent human genes (Feuchter et al., 1993; Kowalski and Mager, 1998; Kowalski et al., 1999). A HERV-H LTR seems to be involved in the regulation of the ICOS gene (Inducible T-cell CO-Stimulator) on the human chromosome 2q33, which plays an important role in autoimmune diseases (Ling et al., 2001). A possible involvement of LTR 13, in association with certain DQB 1 alleles (genes of the major histocompatibility complex) in type I diabetes (IDDM) was suggested by Pascual et al. (2001). 14 complete HERV-K LTRs are scattered all over chromosome 19, all in vicinity (<100 bp) of zinc-finger related genes (Vinogradova et al., 1997), while HERV-R is reported to regulate expression of the human Krüppel-related zinc finger gene (H-plk) in different organs such as placenta, adrenal cortex and testis (Kato et al., 1990; Abrink et al., 1998).

Examples for activity of other HERV elements within the human genome are described as well. The HERV-W *env* gene on chromosome 7 encodes syncytin: a protein essential for the formation of the syncytiotrophoblast layer of the placenta (Blond et al., 2000; Mi et al., 2000). In patients with multiple sclerosis, AIDS and Alzheimer´s dementia with concomitant elevated TNF-alpha levels, HERV expression was found in monocytes and brain tissue (Johnston et al., 2001). HERV-H envelope genes are associated with immunosuppressive properties (Mangeney et al., 2001; Parseval et al., 2001). The presence of autoantibodies crossreacting with retroviral *gag* proteins can be associated with the autoimmune disease Lupus erythematodes (Hishikawa et al., 1997). HERV-RNA can be found in cerebrospinal fluid of patients suffering from schizophrenia (Karlsson, 2001). Retrovirus-like particles are expressed in steriod-dependent manner in the human mammary carcinoma cell line T47D (Ono et al., 1987; Seifarth et al., 1995; Seifarth et al., 1998). HERV-K RNA expression is detectable in teratocarcinoma, i.e germ cell tumor cell lines (Löwer et al., 1993). Most patients with germ cell tumors, seminomas as well as ovarian germ cell tumors, display antibodies against HERV-K *gag* and *env* proteins (Herbst et al., 1998; Boese et al., 2000). High level expression of HERV-R in the adrenal cortex as well as in the placenta was described by Katsumata et al. (1998).

2.2.2 *In vitro* and *in vivo* models for testing promoter activity of HERV-LTRs

Promoter activity and specificity of isolated HERV LTRs in heterologous reporter systems have been investigated in various cell culture models. Data is indicating that HERV-LTRs drive expression of reporter genes in celltype-specific manner. Table 1 gives an overview on studies testing HERV LTR promoter activities *in vitro*. A single *in vivo* model has been published so far: the endogenous retroviral like element AMY1C, associated with the human salivary amylase gene complex, drove expression of a reporter gene in transgenic mice exclusively in the parotidea (Ting et al., 1992).

Table 1: *In vitro* models for gene regulation by HERV LTRs in different cell types

LTR promoter	Activity	Cell type	Reporter	Reference
HERV-H-H6 **HERV-H-5´R2** **HERV-H-3`R1** **HERV-H-N10-14** **HERV-H-PB-3**	high activity in NTera2D1. 293, Hep2,COS-1 and NIH3T3; activity in COS-1; no activity; low activity in NTera2D1 and 293; activity in COS-1.	NTera2D1:human teratocarcinoma cell line; 293: human embryonal kidney cell line; Hep2: human adenocarcinoma cell line; NIH 3T3: mouse cell line; COS-1: monkey kidney cell line.	Chloramphenicol acetyltransferase assay	Feuchter and Mager, 1990
HERV-K Class I **HERV-K Class II** **HERV-K Class III** **HERV-K Class IV**	high relative activity low relative activity high relative activity lowest relative activity	-	*In vitro* transcription assay (^{32}P-UTP)	Mold et al., 1997
HERV-K-T47D **HERV-K-PI167** **HERV-L** **HERV-E** **HERV-H-H6** **HERV-T**	around 25% rel activity in HaCaT and Hela; relative activity under 4% in all lines tested; 250% rel. activity only in HaCaT; relative activity under 2% in all lines tested; relative activity from 20% to 100% in all lines tested; relative activity under 5% in all lines tested.	LC5: human lung fibroblast cell line; MiaPaCa2: human pancreas carcinoma cell line; U373: human astrocytoma cell line; 85HG66: human glioma cell line; ChangLiver: human liver cell line; HaCaT: human Keratinocyte cell line; Hela: human cervix adenocarcinoma cell line; MCF7: human mamma adenocarcinoma cell line.	Luciferase assay	Schoen et al., 2001
HERV-K-T47D-LTR-U3/R **L20-U3/R** **L48-U3/R** **L5-U3/R**	Highest rel. activity <10% in Huh-7 and 293; rel. activity <5% in all cell lines tested; highest rel. activity 8% in LC-5; High rel. activity in Chang liver (25%) and LC-5 (27%), medium rel. activity (>10%) in all other cell lines tested.	T47D: human breast cancer cell line; Hela: human cervix adenocarcinoma cell line; ChangLiver: human liver cell line; Huh-7: human liver cell line; 293: human kidney cell line; LC-5: human lung carcinoma cell line.	Luciferase assay	Baust et al., 2001

2.2.3 Characteristics and biological significance of the HERV-H-H6 LTR

The HERV-H family is one of the most widely represented among human endogenous retroviruses, with approximately 1,000 elements per haploid genome (Mager and Henthorn, 1984). Since they are present in the genome of New World monkeys, they are supposed to have entered the primate genome >40 million years ago. However, the majority of the HERV-H elements result from later expansion 30-35 million years ago (Mager and Freeman, 1995; Andersson et al., 1997). Expression of HERV-H elements was detected at low level in lung (Lindeskog et al., 1993) and peripheral blood mononuclear cells (Medstrand et al., 1992; Lindeskog et al., 1993; Kelleher et al., 1996) and at high level in normal placenta (Wilkinson et al., 1990). Significant amounts of HERV-H transcripts were also expressed in tumor cell lines, mainly bladder carcinomas, teratocarcinomas, testicular tumors and lung tumors (Wilkinson et al., 1990; Hirose et al., 1993). Only 10 % of the HERV-H proviruses are structurally intact with full length *gag*, *pol* and *env* domains interrupted by several stop codons and a total size of 8.7 kb. The majority is partially deleted and only about 5.8 kb long (Hirose et al., 1993; Wilkinson et al., 1993).

HERV-H-H6 is a member of the HERV-H family. Its LTR was first isolated by Feuchter and Mager (1990) from a Hep2 cDNA clone. Hep2 cells represent a subline of HeLa (human cervical adenocarcinoma) cells. Schoen et al. (2001) selected two active LTRs, HERV-H-CL1 type Ia and HERV-H-CL4 type Ia, from Chang liver cells (human liver cells) by a *pol*-expression array combined with RT-PCR. They proved to be 100% identical to the HERV-H-H6 LTR described by Feuchter and Mager (1990). Sequence data was deposited by Schoen et al. with the EMBL/GenBank Libraries under Accession number AF 315090. Fasta search in the HERVdatabase revealed 99.7% identity of AF 315090 with rv_062672 located on the human chromosome 13. This suggests that HERV-H-H6 is localized on the human chromosome 13.

The following structures were identified on the HERV-H-H6 LTR by Schoen et al. (2001): type I repeat, type II repeat and a unique region. In addition, the HERV-H-H6

LTR contains a GC-rich region downstream of the TATA box. This region has been identified in all HERV-H LTRs of type I and Ia characterized so far (Sjottem et al., 1996; Anderssen et al., 1997). It was found to contain binding sites for the transcription factors Sp1 and Sp3, which may be involved in the tissue-specific expression pattern of HERV-H elements (Nelson et al., 1996; Sjottem et al., 1996). Blast search revealed the presence of the steroid-regulatory sequence TGTTCT, which is also part of the HERV-R LTR. High level expression of HERV-R in the adrenal cortex is possibly related to steriod production in adrenocortical cells (Katsumata et al., 1998).

The HERV-H-H6 LTR has been tested for promoter activity in heterologous systems by two *in vitro* studies so far: Feuchter and Mager (1990) investigated the HERV-H-H6 LTR by chloramphenicol acetyltransferase assay and Schoen et al. (2001) examined the LTR by luciferase assay. Feuchter and Mager (1990) described strong promoter activity of the HERV-H-H6 LTR in human (NTeraD1, 293, Hep2), monkey (COS-1) and mouse (3T3) cells. Schoen et al. (2001) described strong relative promoter activity in human LC5 and U373 cells (100% and 70% respectively) and weaker relative promoter activity in human MiaPaCa2 (20%), 85HG66 (10%), ChangLiver (50%), HaCaT (30%), Hela (15%) and MCF7 (25%) cells. Relative promoter activity was standardized with the cotransfected plasmid pRL-TK (Promega), which contained the thymidine kinase promoter upstream of the renilla luciferase gene.

2.2.4 Characteristics and biological significance of the HERV-L LTR

Within the human genome, a group of 200 moderately reiterated elements, named HERV-L elements, was identified (Cordonnier et al., 1995). Disclosing similarities in their *pol* gene with the foamy retroviruses suggest a role as evolutionary intermediate between classical retrotransposons and infectious retroviruses. HERV-L are present among all placental mammals, suggesting that they were already present at least 70 million years ago (Bénit et al., 1999). The complete nucleotide sequence (6,591 bp) of one of these elements was determined from a PCR product of reverse-transcribed RNA from human placenta. It was termed HERV-L because primers were complementary to

leucine tRNA (Cordonnier et al., 1995). The complete HERV-L sequence was deposited by Cordonnier et al. (1995) with the EMBL/GenBank Library under the accession number X89211. HERV-L is characterized by the following features: bordered by LTRs, presence of *gag* and *pol* genes and of a dUTPase region, absence of the *env* gene (Bénit et al., 1999). The coding regions *gag* and *pol* contained several stop codons, indicating that the cloned HERV-L element could not code for a functional gene product (Cordonnier et al., 1995). HERV-L contains a distinct region disclosing 53% homology to a mouse mammary tumor virus sequence (Moore et al., 1987), which has been identified as dUTPase. A dUTPase sequence is found in type B and D oncoviruses, in nonprimate lentiviruses, in poxviruses and in herpesviruses (Mc Geoch, 1990).

The 5′ and 3′ LTRs are 82% identical (i.e. 462 identical bp). They are flanked by short inverted repeats (TGA...ACA). They contain a CAT box, a TATA box and a polyadenylation signal. Screening the LTR reveals the presence of a two AP-1 binding sites (Cordonnier et al., 1995; Leib-Mösch, 2000). AP-1 contributes to keratinocyte-specific expression of the human profiaggrin gene (Maurer et al., 1991). Two CACACCC motives were identified as indirect repeats within the HERV-L LTR. They are characteristic motives of the keratinocyte-specific EBV ED-L2 promoter and other keratinocyte-specific promoters (Leib-Mösch, 2000). Fasta search in the HERV database revealed 99.5% identity of the HERV-L LTR (X89211; nucleotide 2-397) with rv_071357 on human chromosome 16. The HERV-L LTR was tested for promoter activity in heterologous systems by Schoen et al. (2001). The LTR was examined by luciferase assay in different cell lines. Strong relative promoter activity (270%) was measured only in human HaCaT cells. In all other cell lines tested the relative promoter activity was weak (< 29%). Relative promoter activity was standardized with the cotransfected plasmid pRL-TK (Promega), which contained the thymidine kinase promoter upstream of the renilla luciferase gene.

2.2.5 Potential use of HERV-LTRs in gene therapy

Gene therapy involves the transduction of an active gene that may either be a functional homolog of a defective gene, or a nonrelated therapeutic gene (Salmons and Günzburg, 1993). Presently available gene delivery vehicles can be divided into two categories: viral and nonviral vectors. The nonviral vectors, also referred to as synthetic gene delivery systems, rely on direct delivery of either naked DNA or a mixture of DNA with liposomes. Viral vectors can be subdivided into two categories: a) integrating vectors, like retroviral and adeno-associated viruses and b) nonintegrating vectors, like modified adeno-associated viruses (Pfeifer and Verma, 2001). Major drawbacks of gene delivery systems are the efficiency and the safety of gene transfer. High expression level of therapeutic genes in the target cell and a distinct expression pattern in the organism are prerequisites for succesful clinical applications.

As vehicles in gene therapy, standard retroviral vectors can integrate into the host genome of the infected cell and deliver therapeutic genes. As consequence of the reverse transcription of the retroviral single-stranded RNA into double-stranded DNA in the infected cell, the U3 region of the LTR is duplicated and one copy is located at the 5´end of the provirus (Saller et al., 1998). The U3 region carries retroviral promoter and enhancer elements which control gene expression of the provirus (Yu et al., 1986). There is evidence that heterologous promoters, inserted into retroviral vectors to target expression, may interact with retroviral U3 sequences and influence the expression of therapeutic genes negatively (Wu et al., 1996). This problem can be avoided using Promoter Conversion (ProCon) retroviral vectors (Mrochen et al., 1997). ProCon vectors carry a heterologous promoter instead of the U3 region. After reverse transcription in the target cell, this heterologous promoter can control expression of the provirus. As HERV LTRs may exhibit tissue specific promoter activity, it might be interesting to insert them into ProCon vectors to control expression level and pattern of therapeutic genes. A comparison of the integration of standard retroviral vectors into infected cells to the integration of ProCon vectors is presented in Figure 2.

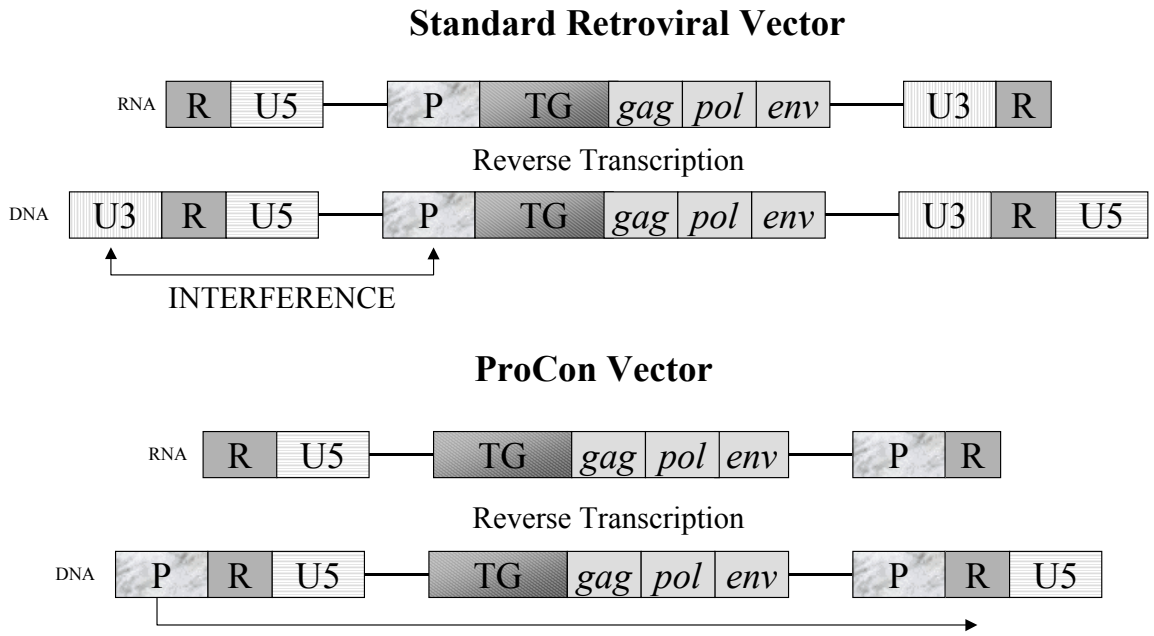

Figure 2: Reverse Transcription of retroviral single-stranded RNA to proviral double-stranded DNA in the infected cell. Comparison between a standard retroviral vector and a ProCon vector. R: repeat; U5: unique region 5´end; U3: unique region 3´end; P: heterologous promoter; TG: therapeutic gene; *gag, pol, env*: genes coding for retroviral enzymes.

Apart from genetic diseases, various forms of cancer and infective diseases, e.g. HIV are major targets for gene therapy (Salmons and Günzburg, 1993). Current clinical studies of gene transfer in humans, sorted by disease, are available under: http://www4.od.nih.gov/oba/rac/clinicaltrial.htm. Current clinical studies sorted by therapeutic genes are available under: http://137.187.206.75/oba/rac/gtbfgrep.htm.

2.3 The use of Green Fluorescent Protein (GFP) as reporter gene

In 1997 the popular german newspaper Bild reported: „Forscher züchten grüne Leuchtmäuse“ (scientists bred green, luminescent mice). This piece of news based on a publication by Okabe et al. (1997) in the FEBS Letters. They had generated transgenic mice with the reporter gene enhanced green fluorescent protein (EGFP) under the control of the chicken beta-actin promoter, a cytomegalovirus enhancer, a beta actin intron and the bovine globin poly-adenylation signal. All tissues from these transgenic mice, with exception of erythrocytes and hair, looked green under excitation light (Figure 3). In a short period of time, the GFP from the jellyfish *Aequorea victoria* had vaulted from obscurity to become one of the most widely studied and exploited proteins in cell biology and biotechnology (Tsien, 1998).

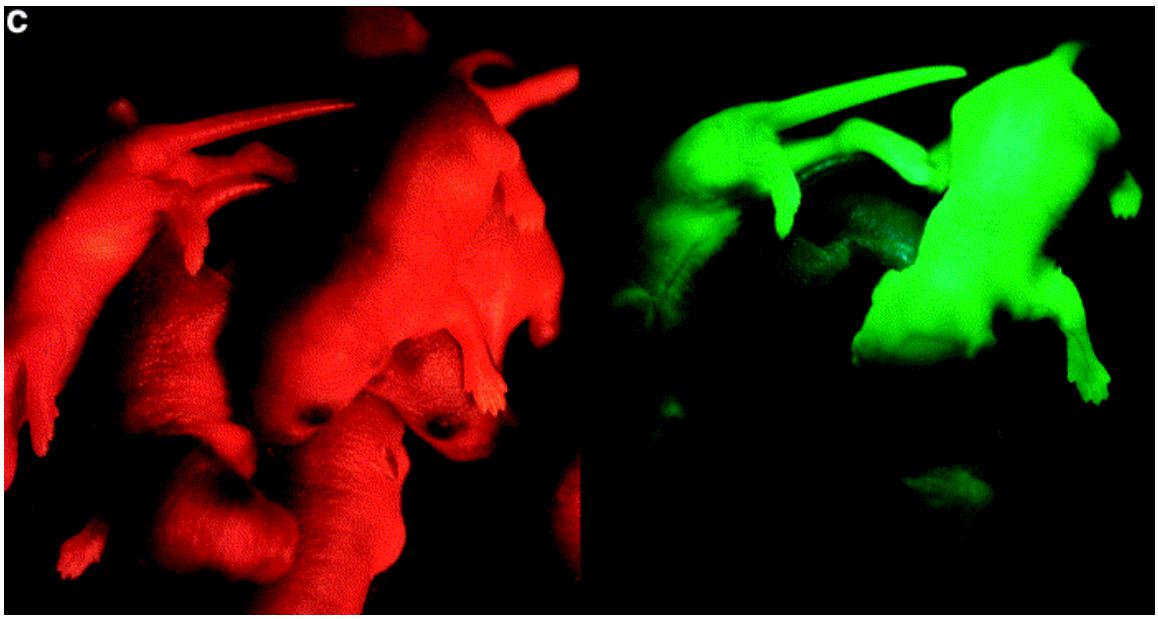

Figure 3: EGFP expression, visible to the naked eye under 360 nm UV light (Okabe et al., 1997).

2.3.1 The discovery of GFP

The green fluorescent protein was discovered by Shimomura et al. (1962). When they described isolation and purification of the aequorin protein from the *Aequorea* jellyfish he mentioned another substance: “a protein giving solutions that look slightly greenish in sunlight, though only yellowish under tungsten lights, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a mineralite, has also been isolated from the squeezates.” The emission spectra of the aequorin protein and the “green protein” were published shortly after (Johnson et al., 1962). The luminescence of the “green protein”, later termed GFP, peaked at 508 nm. Chemiluminescence of aequorin emitted blue light of a broad spectrum that peaked near 470 nm, which was close to one of the excitation peaks of GFP. The blue emission of aequorin was thus converted by GFP to the green glow in the intact animals. The same principle of color shift was discovered in related coelenterates containing GFP: in *Obelia* (hydroid), *Renilla* (sea pansy), *Phialidium* (jellyfish), *Mitrocoma* (jellyfish), *Cavernularia* (sea cactus), *Dicosoma* (sea anemone) and *Ptilosarcus* (sea pen) (Morin and Hastings, 1971; Ward, 1979; Tavaré et al., 2001). Apart from *Aequorea* GFP, only *Renilla* GFP has been well characterized (Ward, 1979). In the following, GFP refers to the *Aequorea* species except where another genus name is specifically indicated.

2.3.2 Structure of GFP and Enhanced Green Fluorescent Protein (EGFP)

The wildtype (wt) gfp 10 gene was originally cloned and sequenced from cDNA by Prasher et al. (1992) (GenBank accession No. M62653). Nucleotide sequences derived from *Aequorea* cDNA or exons indicated another 4 variants (Genbank accession No. L29345, X83959, X83960 and M62654). The variants generally differed by conservative amino acid replacements, suggesting that they might have nearly identical physical properties. In 1994, two groups showed that expression of cloned wt GFP in prokaryotes *(Escherichia coli*) and in eukaryotes (*Caenorhabditis elegans*) created fluorescence (Chalfie et al., 1994; Inouye and Tsuji, 1994). The gene contained all information necessary for the synthesis of the chromophore. No jellyfish-specific enzymes were

necessary for posttranslational processing. This led to extensive activity describing the use of wt-GFP (Cubitt et al., 1995). Limitations soon became obvious: photoisomerisation and autofluorescence when excited at the major peak i.e. 395 nm (Cubitt et al., 1995), slow formation of the chromophore (Heim et al., 1995) and precipitation in the cytoplasm as insoluble inclusion bodies. To overcome these deficiencies, various strategies have been used to generate and screen mutants of GFP (for review see Tsien and Prasher, 1998).

Cormack et al. (1996) identified the EGFP*mut*1 (F64L, S65T) in GFP transformed bacteria by fluorescence-activated cell sorting (FACS) analysis: GFP had been mutated by randomization of a predetermined, limited stretch of amino acid residues. Transformed bacteria had been screened by FACS analysis for increased brightness at 488 nm excitation light. The commercially available EGFP (Clontech, Palo Alto, CA) is based on the EGFP*mut*1 (F64L, S65T). In the EGFP*mut*1, insertion of the amino acid leucine at position 64 improved protein folding at 37°C, whereas the mutation of serine 65 to threonine promoted chromophore ionization. EGFP from Clontech is furthermore characterized by an optimal nucleotide sequence for translational initiation (Kozak, 1989) and a presumably neutral mutation of histidine 231 to leucine. The EGFP nucleotide sequence (4,151 bp) was deposited at GenBank under accession number CVU55761.

The GFP chromophore is a p-hydroxybenzylideneimidazolinone (Figure 4) formed from the residues 65-67 which are serine, tyrosine and glycine in the native protein (Prasher et al., 1992; Cody et al., 1993). The chromophore is formed by a cyclization reaction and an oxidation step that requires molecular oxygen (Cubitt et al., 1995).

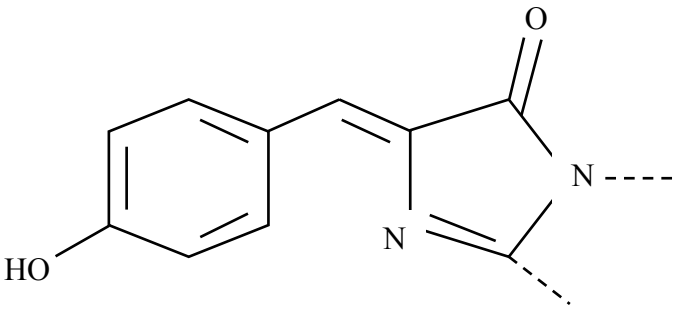

Figure 4: Structure of the GFP chromophore as proposed by Shimomura (1979)

Although GFP was cristallized in 1974 (Morise et al., 1974), the secondary, tertiary and quaternary structure were first solved in 1996 (Yang et al., 1996; Ormö et al., 1996). Under Protein Data Bank accession number 1EMA and 1GFL the amino acid sequence (236 amino acids) is provided. GFP is an 11-stranded ß-barrel threaded by an α-helix running up the axis of the cylinder. The chromophore is attached to the α-helix and is buried in the center of the cylinder: the so-called *ß-can* (Phillips, 1997).

2.3.3 Biochemical and physical properties of EGFP

As a result of the chromophore mutation (serine 65 to threonine) in EGFP the GFP´s 395 nm excitation peak, due to the neutral phenol, is suppressed and the excitation peak, due to the phenolate anion (Figure 5) is enhanced five to six fold in amplitude and shifted to 488 nm (Cormack et al., 1996). The EGFP emission spectrum peakes at 507 nm.

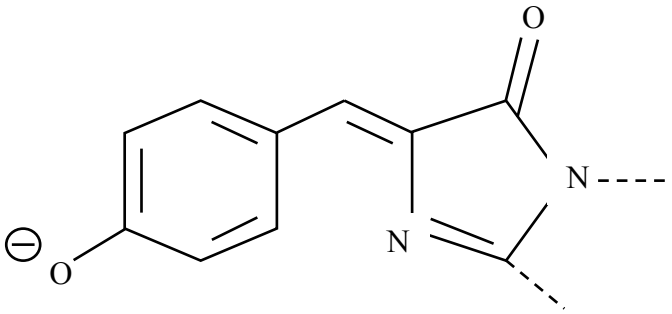

Figure 5: EGFP chromophore containing a phenolate anion

Maturation of the chromophore is about fourfold faster in EGFP than in GFP (Heim et al., 1995). Fluorescence is only emitted when the molecules have folded properly and remain in an oxidized state. Misfolded, reduced or fully denatured proteins are not fluorescent. To improve folding at 37°C, i.e. above jellyfish temperature, EGFP contains leucine at position 64 (Cormack et al., 1996). EGFP needs to be in an oxidized state to fluoresce because chromophore formation is dependent upon oxidation of Tyr-66. Strong reducing agents, such as 2 mM $FeSO_4$ or 5 mM $Na_2S_2O_4$, convert EGFP into a nonfluorescent form (Inouye and Tsuji, 1994). Fluorescence can be fully recovered after exposition to atmospheric oxigen. Weaker reducing agents, such as 2% ß-mercaptoethanol or 10 mM dithiothreitol (DTT), do not affect the fluorescence of EGFP (Inouye and Tsuji, 1994). EGFP retains its fluorescence in mild denaturants, such as 1% SDS and after fixation with formaldehyde. GFP fluorescence is irreversibly destroyed by 1% H_2O_2 and sulfhydryl reagents such as 1 mM DTNB (5,5´-dithio-bis-[2-nitrobenzoic acid]) (Inouye and Tsuji, 1994). Higher temperatures than 68°C cause denaturation of the protein with 50% of fluorescence lost at 78°C (Ward, 1982).

EGFP is acidic with a monomer molecular weight of 27 kDa. EGFP is expressed as monomer soluble in aqueous solution. It exhibits pH stability in the range between pH 7.0 and pH 11.5, the protein is 50% quenched at pH 5.5 (Patterson et al., 1997). EGFP is very resistant to photobleaching, perhaps because the fluorophore is so well shielded from chemical reactants, such as O_2, in the *ß-can* (Tsien, 1998). To protect GFPs from bleaching, cell-permeant antioxidants, such as vitamin E analogues, can be used. *In vivo* the protein has an estimated half life of >24 hours (Li et al., 1998), whereas in fixed cells, fluorescence can still be detected after three months when slides are kept in the dark at 4°C. EGFP can undergo photoconversion to a red fluorescent species under rigorously anaerobic conditions. The nature of this species emitting at 600 nm remaines to be clarified (Elowitz et al., 1997).

2.3.4 Application of GFPs as reporter gene

GFPs can be utilized either as fusion partners or as active indicators. The most successful and numerous class of GFP application has been as partner in gene fusion constructs to monitor host protein localization and fate. The gene encoding GFP is fused with the gene encoding the endogenous protein of interest and the resulting chimera is to be expressed in the same function and localization as the endogenous protein. GFP has been targeted successfully to numerous proteins in practically every major organelle of the cell (Tsien, 1998). A promoterless GFP gene can also be tagged to a heterologous promoter. EGFP expression level and pattern in the host organism thus describes promoter activity and specificity. As active indicator, GFP is less popular. The rigid protein shell protects the chromophore and hinders environmental sensitivity. The engineered fusion of GFP with the Shaker potassium channel is the first genetically encoded optical sensor of membrane potential (Siegel and Isacoff, 1997). Depolarization causes at most a 5% decrease in fluorescence in a time of approximately 85 ms. A more general way to make biochemically sensitive GFPs is to exploit fluorescence resonance energy transfer (FRET) between fluorescent proteins of different colors. This quantum mechanical phenomenon occurs when two fluorophores are in molecular proximity and the emission spectrum of one fluorophore (donor) excites the other fluorophore (acceptor). Any biochemical process changing the distance or orientation of the fluorophores modulates the efficiency of FRET (Tsien, 1993). GFP mutants with altered excitation and emission maxima are commercially available: enhanced blue fluorescent protein (EBFP), enhanced yellow fluorescent protein (EYFP), enhanced cyan fluorescent protein (ECFP) and red fluorescent protein (RFP) (CLONTECH, Palo Alto, CA).

2.3.5 Applications of EGFP in different species

GFP fluorescence is species-independent. One of the first proposed applications of GFP was to detect gene expression *in vivo* in the nematode *Caenorhabditis elegans*, whose cuticle hinders access of substrates required for detection of other reporter genes (Chalfie et al., 1994). Since then, GFP has been expressed successfully in microbes, vertebrates, invertebrates, plants and various cell lines. Since the discovery of the EGFP*mut*1 by Cormack et al. (1996), and the availability of the EGFP vector at Clontech (Palo Alto, CA), EGFP is employed in a universal manner in genetic engineering. In Table 2, some current examples illustrate the broad field of EGFP applications.

Table 2: Selection of current applications of EGFP

Organism		Reference
Escherichia coli	(gram $^-$ bacterium)	Cormack et al., 1996
Aspergillus oryzae	(funghi)	Maruyama et al., 2001
Drosophila melanogaster	(insect)	Sano and Nakamura, 2002
Anopheles albimanus	(insect)	Perera et al., 2002
Zebrafish	(fish)	Krovel et al., 2002
Transgenic mice	(mammals)	Okabe et al., 1997
Transgenic pigs	(mammals)	Cabot et al., 2001
Transgenic cattle	(mammals)	Funahashi et al., 2001

2.3.5 Sensitivity of GFP and EGFP as reporter gene

The sensitivity of GFP as reporter gene was described in a recent review as “somewhat disappointing” (Tsien, 1998). GFP´s independence from enzymatic substrates is on the one hand particularly useful for examination of intact organisms (Chalfie, 1994), but on the other hand allows no amplification of signals. A GFP molecule is not an enzyme that catalytically processes an indefinite number of substrates to form detectable chromophores: one GFP molecule containes at most one chromophore. As the ultimate sensitivity limit is set by cellular autofluorescence: at least 1 μM well folded GFP molecules are required in a mammalian cell to double fluorescence above background (Niswender et al., 1995). Mutant GFPs with higher extinction coefficients, like Clontech´s EGFP, improve this detection limit: 30 nM EGFP, which equals 4,000 molecules per cell, are detectable in the cell cytoplasm. When expressed on the cell surface, 700 molecules are sufficient for detection (CLONTECHniques, 1997). When targeted to a defined subcompartment of the cell and analyzed by fluorescence microscopy, the number of EGFP molecules required can be reduced further: 300-3,000 GFPs packed into a centrosome are readily visible as green dot inside a cell (Shelby et al., 1996). This estimate already assumes perfect GFP maturation.

Obviously the stronger the promoter/enhancers driving this transcription, the more protein will be detectable per cell. Most published reports of GFP or EGFP expression have used strong constitutive promoters from viruses such as the cytomegalovirus (CMV), SV40 or HIV long terminal repeats, or strong exogenous regulators such as the tetracycline transactivator system (Anderson et al., 1996; Gervaix et al., 1997; Okabe et al., 1997).

2.3.6 Qualitative and quantitative analysis of GFP and EGFP expression in transgenic mice

The first GFP transgenic mouse was generated by Ikawa et al. (1995). They announced fast, simple and non-invasive analysis of CMV-GFP integration and expression by observation of tails or fingers of newborn pups under a fluorescent lamp. Since then, numerous approaches have been made to examine integration and expression of GFPs in mouse tissue. While DNA integration can be routinely investigated by PCR (Table 3) and Southern blot, examination of protein expression has to cope with two delicate issues at a time:

i) precise localization of expression

ii) exact quantification of expression.

In the following, different techniques of detection and quantification of GFP and EGFP in transgenic mouse tissue are displayed.

Table 3: After extraction and purification of genomic DNA from mouse tissue according to laboratory manuals (Sambrok and Russel, 2001), EGFP (CVU55761) transgenic mice can be identified performing PCR.

Primer sequence: sense (s) and antisense (as)	Annealing conditions	Reference
5´-TCGAGCTGGACGGCGACGTAAA-´3 (s) 5´-TAGTGGTTGTCGGGCAGCAGCA-´3 (as)	-	Nolte et al., 2001
5´-ACCCCGACCACATGAAGCAGC-´3 (s) 5´-CGTTGGGGTCTTTGCTCAGGG-´3 (as)	22 sec 55 °C / 30 cycles	Kaneko et al., 2001
5´-TGACCTACGGCGTGCAGTGC-´3 (s) 5´-TCACCTTGATGCCGTTCTTCT-´3 (as)	-	Pfeifer et al., 2002
5´-ACGGCAAGCTGACCCTGAA-´3 (s) 5´-GGGTGCTCAGGTAGTGGTT-´3 (as)	60 sec 60°C / 45 cycles	Kato et al., 1999

Fluorescence microscopical analysis is an appropriate mean to exactly refer GFP expression to a specific cell type or cell organelle. Autofluorescence represents the main problem in fluorescence microscopy: many cell types or organs (kidney, hair follicles) display autofluorescence likely due to flavin coenzymes or mitochondria-bound NADH (Aubin, 1979; Lohmann, 1989). The use of the appropriate filter sets (common FITC filter sets for detection of EGFP) reduces autofluorescence, but improvement is limited. Tissue fixation techniques also play an important role.

Ikawa et al. (1995) simply squeezed a piece of tissue under a coverglass or prepared 10 µm thick cryosections. The freezing procedure turned out to increase background in fluorescence microscopy. Lathi et al. (2001) fixed testis tissue for 2 hours in 4% paraformaldehyde at room temperature (RT) and cut 70 µm thick sections on a vibratome. The sections were mounted with PBS and evaluated under the microscope. A broad organ spectrum was prepared for fluorescence microscopy by Kondoh et al. (1999). Mice were deeply anesthetized with phenobarbital and fixed by perfusion with 4% (w/v) paraformaldehyde-PBS via the left ventricle of the heart. Excised tissues were fixed again in 4% paraformaldehyde-PBS overnight at 4°C and then incubated in 20% sucrose-PBS for 48 h at 4°C. Pieces were then embedded in Tissue-Tek O.C.T. compound (Sacura Finetek, Torrance, CA), quickly frozen on dry ice and sectioned on a cryostat to 5-10 µm thickness. Although paraffin-embedded sections are reported to increase background fluorescence (Ikawa et al., 1999) and GFP is unlikely to withstand the complete dehydration required for paraffin or plastic embedding (Living Colors® User Manual, 2001), Walter et al. (2000) published the detection of EGFP in 5 µm thick paraffin sections. The method was established in CMV-EGFP transgenic mice. It includes a comparison between native preparations, propidium iodide and immunohistochemical stainings (ANIMALS, MATERIALS AND METHODS, chapter 3.6.2). Transgenic mouse embryos expressing EGFP can also be selected by fluorescence microscopy (Kato et al., 1999). After DNA microinjection, embryos were cultured *in vitro* to morula- or blastocyst-stage. In a microdrop of M2 medium, fluorescence could easily be observed under the microscope.

Whole-body optical imaging of green fluorescent protein can be a non-invasive approach to localize protein expression (Yang et al., 1999). Intact transgenic mice are exposed to a 470 nm UV lamp and the emitted fluorescence is collected through an external long pass filter. GFP fluorescence shines through structures like the flank or the skalp (Yang et al., 1999). The technique also offers the possibility to visualize GFP expressing organs in opened body cavities of anesthetized mice (Pfeifer et al., 2001).

The first quantification of GFP expression was achieved by *fluorometry*. Ikawa et al. (1995) homogenized tissue of different organs in PBS. After centrifugation of the homogenates, fluorescence was measured in the supernatants using a fluorometer. Protein concentrations in the supernatants were measured as well using a standard protein assay. Fluorescence at 509 nm was specified as fluorescence/(mg protein/ml). Recombinant GFP as standard for measurements is commercially available (http://www.turnerdesigns.com/).

Because the excitation optimum for EGFP is close to 488 nm, EGFP expressing cells are suitable for *fluorescence-activated cell sorting (FACS).* A method to isolate individual cells from transgenic mouse tissue and to sort out EGFP expressing cells was described by Hadjantonakis and Nagy (2000). Dissected organs were stored in DMEM supplemented with 10% fetal calf serum (FCS). After rinsing the organ with PBS, it was exposed to a drop of trypsin solution (0.25% trypsin in Tris-saline) in a petri dish. After 1-2 min incubation at 37°C, the sample was dissociated by trituration using a fine drawn glass pasteur pipette. When a single-cell suspension was achieved, 0.2-0.5 ml ice-cold DMEM was added and the cell-containing solution was placed on ice. Samples could be used directly for cell sorting.

The *multiplex real time EGFP-PCR* simultaneously calculates the cell number and the present EGFP gene copy numbers (Klein et al., 2000). Although the paper refers to cell culture, the method is also applied to transgenic mice (Hohenadl, VU Wien, personal communication).

A semiquantitative method for protein detection is *Western blotting* (Sambrok and Russell, 2001). In the Living Colors® User Manual (2001) SDS-PAGE is recommended with a 10% separation gel and 25-75 µg of lysate protein per lane on a minigel apparatus. Antibodies are commercially available from CLONTECH and Molecular Probes. Immunoprecipitation and subsequent SDS-PAGE of EGFP has been described for cells (Miserey-Lenkei et al., 2001).

Table 4: Selected methods to analyze EGFP transgenic mice

Analysis	Method	Literature
EGFP gene copy number/ cell	Multiplex real time PCR	Klein et al., 2000
EGFP expression pattern	Whole body imaging, Western Blot	Yang et al., 1999 ; Living Colors®User Manual, 2001.
EGFP expression level	Fluorometry, Western Blot	Ikawa et al., 1995; Living Colors®User Manual, 2001.
EGFP expressing cells/tissues	FACS analysis	Hadjantonakis and Nagy, 2000
EGFP expressing cell type	Histology	Kondoh et al., 1999; Walter et al., 2000.

2.4 The use of Firefly Luciferase as reporter gene in transgenic mice

2.4.1 Structure and characteristics of firefly luciferase

The enzyme firefly luciferase was isolated form the North American Firefly by deLuca and McElroy (1978). The monomeric 61 kDa protein (Wood et al., 1985) catalyzes luciferin oxidation using ATP●Mg^{2+} as a cosubstrate (Figure 6).

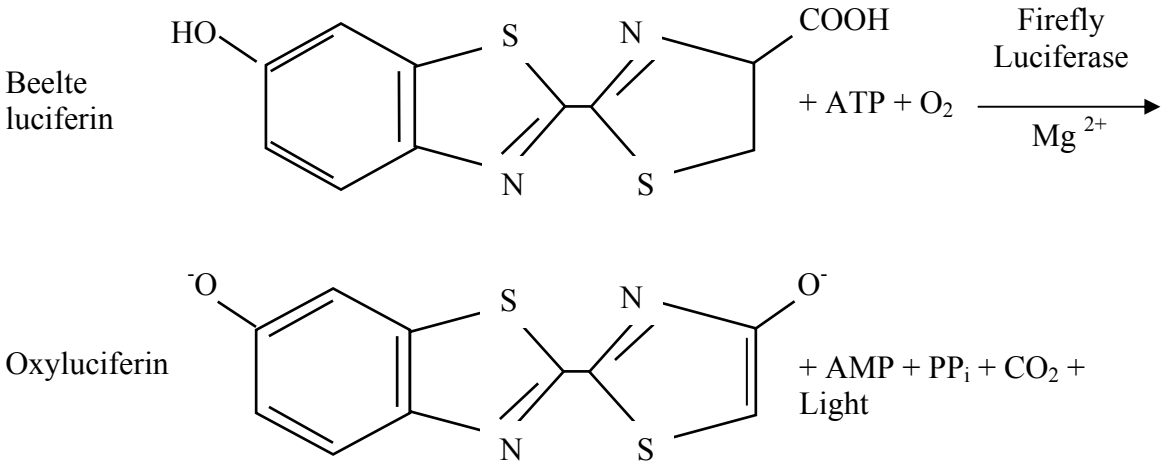

Figure 6: Bioluminescent reaction catalyzed by firefly luciferase

The reaction emits yellow-green light for approx. 60 sec at pH 7.5 to 8.5 with the peak emission at 560 nm. The light intensity can be measured using a luciferase assay: less than 10^{-20} moles of luciferase have been detected under optimal conditions. The nucleotide sequence was determined from the analysis of firefly cDNA and genomic clones (de Wet et al., 1985; de Wet et al., 1987). The gene containes 6 introns. The complete cDNA sequence (2,387 bp) is deposited at GenBank accession number M15077. Luciferase was first expressed in mammalian cells in 1987 (de Wet et al., 1987). The authors found luciferase to be a highly sensitive mean to monitor promoter activity. In addition Luciferin showed to diffuse across mammalian cytoplasmatic membranes, which allowed the detection of luciferase in intact cells. The vector utilized in this study, pB-Luciferase (pBL), contains the *Photinus pyralis* luciferase cDNA, fused

to the SV 40 poly (A) signal derived from pBLCAT (Luckow and Schütz, 1987) and cloned into the polylinker of Bluescript KSM13+ (Stratagene, Heidelberg, Germany) (Hoppe-Seyler et al., 1991).

2.4.2 Qualitative and quantitative analysis of firefly luciferase in transgenic mice

The first firefly luciferase transgenic mouse was created by Shockett et al. (1995). Since then, numerous approaches have been made to examine integration and expression of luciferase in mouse tissue. DNA integration can routinely be investigated using PCR and Southern blot.

Wu et al. (2002) screened mouse genomic DNA, isolated according to laboratory manuals (Sambrok and Russel, 2001), for luciferase transgenesis, utilizing 5´-GCAGATCTCAGAATACACTCAGA-´3 sense and 5´-ATAAATAACGCGCCCAAC AC-3` antisense primers to amplify a sequence from the luciferase gene (pGL3, Promega) in 30 cycles at 55°C annealing temperature. Southern blot can be performed for quantification of transgene copy number (Chen et al., 1996; Kistner et al., 1996). As standards for the determination of copy numbers, as well as for positive control, 10 µg of genomic DNA from a wildtype mouse spiked with an appropriate amount of linearized plasmid were used. Northern blot analysis is described in detail by Wu et al. (2002).

Expression of luciferase protein in different organs can exactly be quantified by Luciferase assay systems (Promega, Madison, Wi). Mouse tissue has to be processed prior to subjecting it to luciferase assay. An overview of selected methods is given on Table 5. The protein content of the tissue extract has to be measured by protein assays (e.g. Pierce, Rockford, IL). Luciferase activity is specified as relative light units (RLU) per mg protein in a specific time (10 sec) after subtraction of the lysis buffer background. Luciferase activity in non-transgenic organs ranges from 0 (heart) to 617 (lymphnodes). Firefly luciferase protein standard (Promega, Madison, Wi) can be added to extracts of wildtype mouse tissue as positive control (Shockett et al., 1995).

Table 5: Extraction of luciferase protein from frozen mouse tissue (-80°C)

Lysis buffer	**Procedure**	**Reference**
Passive Lysis Buffer (PLB) (Promega, Madison, Wi)	Tissue pulverization by mortar and pestle (-80°C) Addition of Promega lysis buffer + 15 min vortexing 3x freeze-thaw cycles (liquid N_2 to 37°C water bath) Centrifugation (3 min at 10,000 *g*) Removal of supernatant A New Promega lysis buffer added to pellet + 15 min vortexing Centrifugation (3 min at 10,000 *g*) Combination of the supernatant A with B + Measurement	Manthorpe et al., 1993; Promega Technical Bulletin No. 281.
Luciferase Cell Culture Lysis Reagent 5x (Promega, Madison, Wi)	Tissue pulverization by mortar and pestle (-80°C) Addition of CCLR and incubation 15 min RT Centrifugation 10 sec at 14,000 rpm Measurement of the supernatant (or storage at –80°C)	Shockett et al., 1995
25 mM Tris phosphate pH 7.8, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100, 2 mM diaminocyclohexanetetraacetic acid	Homogenization on ice in lysis buffer with Ultraturrax 1x freeze thaw (liquid N_2 to 37°C water bath) Centrifugation 5 min at 15,000 g Measurement of the supernatant (or storage at –80°C)	Kistner et al., 1996
100 mM Tris (hydroxymethyl) aminomethane pH 7.8, 1mM EDTA, 1mM dithiothreitol, 2 µg/ml aprotinin, 5 µg/ml leupeptin, 170 µg/ml phenylmethylsulfonyl 0.7 mg/ml pepstatin	Homogenisation in lysis buffer Centrifugation 15 min 10,000 *g* at 4°C Measurement of the supernatant (or storage at –80°C)	Swoap, 1998

Localization of luciferase protein expression can not only be obtained by assay, immunoprecipitation or Western blot analysis of organs (Shockett et al., 1995; Swoap, 1998), but also by histological methods and imaging of bioluminescence in living animals (Wu et al., 2002).

Imaging of bioluminescence in living animals was achieved in MMP-13-luciferase transgenic mice. The MMP-13 gene is known to play a role in cutaneous wound healing. Anesthetized wounded mice were imaged as follows: luciferin/DMSO solution was applied topically onto wounds and unwounded skin. After 5 minutes animals were placed in a light-tight chamber. A gray-scale image and a pseudocolor image were taken using a intensified charged coupled device (ICCD) camera. Pictures were superimposed by image processing software. Background values from unwounded skin were subtracted from wound signals (Wu et al., 2002). This method of monitoring biolumeniscence can also be applied to other mouse organs *in vivo*. Luciferin can be administered systemically (126 mg/kg body weight) in mice and rats (Honigman et al., 2001). Luminescence is measured between 5 and 20 min after injection of luciferin, depending on the target organ. *In vitro* imaging of bioluminescence in 440 µm thick tissue slices was reported by Geusz et al. (1997).

In situ hybridization analysis of mouse skin can be achieved by digoxigenin-labeled riboprobes as described by Hoff et al. (1999). Mouse skin was fixed in 4% paraformaldehyde, embedded in paraffin, deparaffinized with xylenes and rehydrated to PBS. Tissue was fixed in paraformaldehyde before and after treatment with proteinase K. Following acetylation with 0.5% acetic anhydride, tissue was dehydrated through graded ethanols and air-dried prior to hybridisation. Tissue sections were hybridized at 55°C with digoxigenin-labeled riboprobes (antisense and sense) in a humid chamber. After washing of the slides, the digoxigenin detection procedure was carried out using the genius TM3kit (Boehringer, Mannheim, Germany).

Detection of luciferase protein by *immunohistochemistry* has so far only been described for rat muscle tissue (Ashley and Russell, 2000). Cryosections (10 µm thick) were fixed with 4% paraformaldehyde and incubated with the primary anti-luciferase antibody (Promega, Madison, WI) and a secondary, fluorescein-labeled antibody. The sections were analyzed for luciferase protein expression under the microscope.

3 ANIMALS, MATERIALS AND METHODS

3.1 Animals

3.1.1 Mice

Female NMRI outbred mice at the age of 8 weeks to 6 months were chosen as recipient animals. Vasectomized NMRI males were kept for synchronisation of the recipients. B6D2F1 hybrid mice (C57BL/6 female x DBA/2 male) were utilized for production of fertilized oocytes. Transgenic founders were backcrossed to C57BL/6 inbred strain background. Mice were purchased at Charles River Laboratories (Sulzfeld, Germany).

3.1.2 Housing and husbandry

Mice were maintained in the facilities of the Gene Center under non-barrier conditions (Table 6). Acidified water (6 mM HCl) was provided ad libitum in bottles. Pregnant mice recieved breeding diet (Ssniff®M-Z extruded pellets) ad libitum. Maintenance diet (Ssniff®R/M-H pellets) was fed ad libitum to the others. Fodder pellets, as well as any other solid objects entering the mouse facility, were autoclaved. Mice were kept, separated by sex, in standard macrolon cages with grid lids on softwood fibre, paper and hay. Enrichment was provided in all cages of sufficient size with aspen wood pieces and activity wheels.

Table 6: Environmental conditions in the mouse facility

Parameter	Description
Type of facility	Conventional facility (non-barrier)
Temperature	21°C (+/- 1°C)
Relative humidity	60 % (+/- 5%)
Ventilation rate	15 air changes/hour
Air pressure	2 pascal positive pressure
Lighting regimen	12 hours light/12 hours dark

3.1.3 Breeding system

Founders obtained from DNA-microinjection were bred from the age of 8 weeks to 6 months (females) or up to 12 months (males) with C57BL/6 inbred mice to generate transgenic lines. Female and male mice were caged together until the appearance of a vaginal plug. Males were thereafter caged singly.

3.2 Preparation of constructs for DNA-microinjection

The plasmids pBL-HERV-L and pEGFP-HERV-H-H6 were kindly donated by Dr. Schoen and PD Dr. Leib-Mösch, GSF, Neuherberg. Transformed plasmids were propagated using competent bacteria. Plasmid DNA was isolated in minipreps. The gene construct was released from the vector backbone by restriction enzyme digest and eluted in injection buffer.

3.2.1 The pBL-HERV-L construct

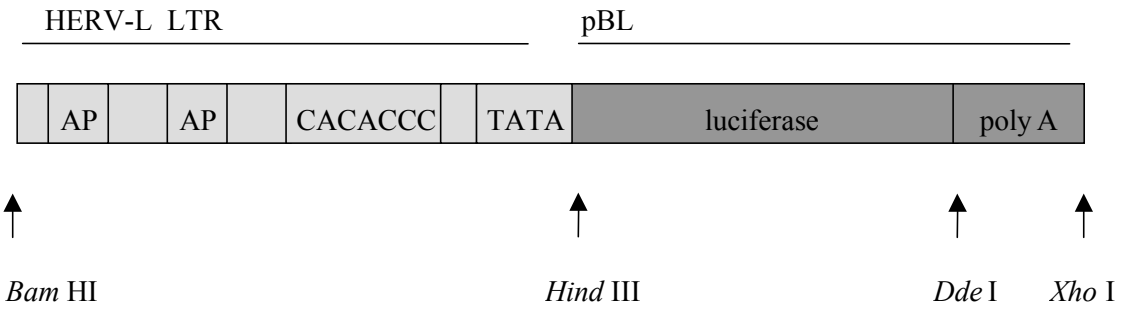

Figure 7: Schematic representation of gene construct pBL-HERV-L (3,123 bp). HERV-L LTR: Human Endogenous Retrovirus Long Terminal Repeat from family L (430 bp); CACACCC: characteristic motive of the keratinocyte-specific EBV ED-L2 promoter and other keratinocyte-specific promoters. Two of them have been identified in the HERV-L LTR (Leib-Mösch, 2000); AP: AP1 is a characteristic motive for the keratinocyte specific expression of the human profiaggrin gene (Maurer et al., 1991); TATA-box: element of eukaryotic and yeast promoters; pBL: expression vector cloned by Hoppe-Seyler et al. (1991); luciferase: *P. pyralis* luciferase complementary deoxyribonucleic acid (1714 bp); poly A: SV 40 polyadenylation signal (979 bp).

3.2.2 The pEGFP-HERV-H-H6 construct

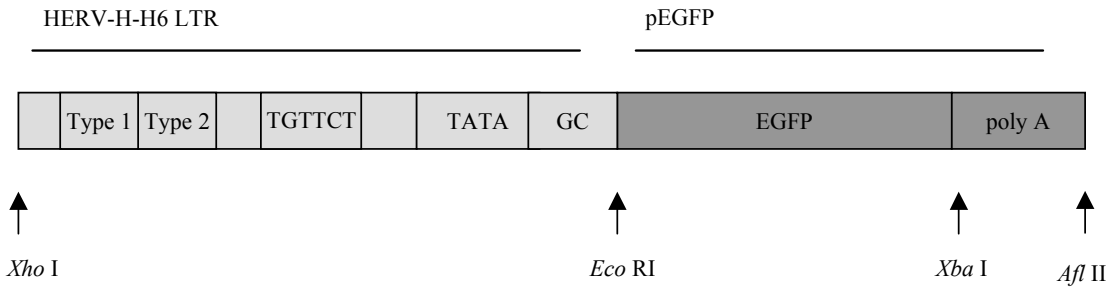

Figure 8: Schematic representation of gene construct pEGFP-HERV-H-H6 (1,455 bp). HERV-H-H6 LTR: Human Endogenous Retroviral Long Terminal Repeat from family H (438 bp); Type 1: type one repeat; Type 2: type two repeat; TGTTCT: steroid-regulatory sequence (Kasumata et al., 1998); TATA-box: element of eukaryotic and yeast promoters; GC: GC-rich region containing binding sites for transcription factors Sp1 and Sp 3, which may be involved in the specific expression pattern of HERV-H elements (Nelson et al., 1996; Sjottem et al.,1996); EGFP: coding region for the Enhanced Green Fluorescent Protein (784 bp); poly A: SV 40 polyadenylation signal (233 bp).

3.2.3 Preparation of competent bacteria

E. coli bacteria strain TOP 10 were grown in 300 ml Luria-Bertani Medium (LB) at 37°C with vigorous shaking to an OD_{600} of 0,7 to 0,8. After chilling on ice, bacteria were recovered by centrifugation at 4,000 rpm (rounds per minute) and 4°C for 10 minutes (min) in a centrifuge (Sorval, RC5C Plus) using the SS-34 rotor.

LB medium:	*Bacto-tryptone*	*10 g*
	Bacto-yeast extract	*5 g*
	NaCl	*10 g*
	Bidistilled water	*ad 1 l*

The solution was adjusted to pH 7.0 with 5 M NaOH and autoclaved.

After removal of the medium, the pellet was carefully resuspended in 225 ml of ice-cold Tfb I buffer and stored for 10 min on ice. Bacteria were centrifuged and the supernatant was removed. As from now, centrifugation was carried out in an Eppendorf centrifuge (5417 R) using the F 45-30-11 rotor.

Tfb I buffer:	*1 M KOAc*	*9 ml*
	3 M $MnCl_2$	*5 ml*
	1 M $CaCl_2$	*30 ml*
	Glycerol	*45 ml*
	Bidistilled water	*211 ml*

$MnCl_2$ was sterilized by filtration and added to the autoclaved components.

The pellet was resuspended in 9 ml ice-cold Tfb II buffer. Competent bacteria were aliquoted (200 µl) in microfuge tubes and stored at –80°C.

Tfb II buffer:	*1 M MOPS*	*0.3 ml*
	1 M KCl	*3 ml*
	1 M $CaCl_2$	*3.2 ml*
	Glycerol	*4.5 ml*
	Bidistilled water	*19 ml*

3.2.4 Transformation of bacteria

Competent bacteria were thawed on ice. A volume of 40 µl was transferred to a sterile centrifuge tube and 1 µl of plasmid (10 ng/µl) was added. The tubes were stored on ice for 30 min, followed by 30 s incubation at 37°C. The tubes were rapidly returned to ice, allowing the cells to chill for 5 min. 800 µl of SOC medium were added to each tube and cultures were incubated for 1 hour (h) at 37°C with vigorous shaking.

SOC medium:	*Bacto-tryptone peptone*	*20 g*
	Bacto-yeast extract	*5 g*
	NaCl	*0.5 g*
	Bidistilled water	*up to 950 ml*

10 ml of a 0.25 M KCl solution were added. The pH was adjusted to 7.0 and the solution was autoclaved. Finally 100 mM $MgCl_2$ and 20 mM glucose were added.

The appropriate volume of transformed competent cells was transferred onto four agarose-LB plates with the corresponding antibiotic (Table 7). Using a sterile glass rod, the transformed cells were gently spread over the surface of the agar plate. Plates were inverted and incubated at 37°C. Colonies appeared within 16 h.

Agarose LB: *LB medium* *1 l*
Agar, granulated *15 g*

After autoclaving, the solution was swirled gently to distribute the melted agar evenly. When the temperature reached 50°C, the antibiotic was added and 35 ml medium were poured directly from the flask in 90 mm Petri dishes. After the medium had hardened completely, plates were inverted and stored at 4°C.

Table 7: Different quantities of competent cells were applied to the agar plates. After incubation at 37°C, single bacteria colonies were picked.

Plate	Plasmid	Antibiotic	Volume of competent cells
A	pBL-HERV-L	Ampicillin 100 µg/ml	5 µl
B	pBL-HERV-L	Ampicillin 100 µg/ml	50 µl
C	pEGFP-HERV-H-H6	Kanamycin 30 µg/ml	5 µl
D	pEGFP-HERV-H-H6	Kanamycin 30 µg/ml	50 µl

3.2.5 Preparation of plasmid DNA (Miniprep)

Plasmid DNA was isolated from *E. coli* using the QIA prep Spin Miniprep Kit® (Quiagen, Hilden, Germany) according to the manufacturer´s instructions. Colonies were picked with a sterile tooth-pick, immersed in 4 ml LB medium with the appropriate antibiotic (100 µg/ml Ampicillin or 30 µg/ml Kanamycin) and incubated for 12 h at 37°C with vigorous shaking. 2 ml of the bacterial culture were transferred to a centrifuge tube and centrifuged at 13,000 rpm for 30 s at 4°C. The supernatant was discarded and the bacterial pellet resuspended in 250 µl P1 buffer. Tubes were placed on ice.

P1 buffer: *Tris/HCl pH 8,5* *50 mM*
EDTA *10 mM*
RNase A *100 µg /ml*

250 µl of buffer P2 were added and the tube was inverted gently 6 times to mix.

P2 buffer: *NaOH* *200 mM*
SDS *1 % (w/v)*

350 µl of buffer N3 was added and the tubes were again inverted gently 6 times.

N3 buffer: *contains potassium acetate and a chaotrope salt*

Tubes were centrifuged for 10 min, at 4°C and 13,000 rpm. The supernatants were decanted to the provided spin columns. Columns were centrifuged 60 s at 13,000 rpm and the flow-through was discarded. Columns were washed by adding 750 µl of buffer PE and centrifugation (13,000 rpm) for 60 s.

PE buffer: *80 % Ethanol*

After removal of the flowthrough, the columns were centrifuged for an additional min to remove residual wash buffer. The columns were placed into new 1.5 ml microfuge tubes. DNA was eluted after application of 50 µl TE buffer (10 mM Tris-HCl; pH 8) to the center of the column, incubation for 2 min at RT and centrifugation (13,000 rpm) for 1 min.

3.2.6 Restriction enzyme digestion

Restriction enzyme digests from plasmid DNA were performed to release the gene constructs from the vector backbone for microinjection (Table 8). All buffers and enzymes except for 10x buffer H and *Sca* I (Roche, Mannheim, Germany) were purchased from MBI fermentas, St Leon-Rot, Germany.

Table 8: Conditions for restriction enzyme digestion

pBL-HERV-L		
20 µl	plasmid DNA	400 ng/µl
112 µl	H_2O	
16 µl	10x buffer H	50 mM Tris-HCl; 10 mM $MgCl_2$; 100 mM NaCl; 1 mM DTE.
4 µl	*Sca* I	10 U/µl
4 µl	*Not* I	10 U/µl
4 µl	*Xho* I	10 U/µl

pEGFP-HERV-H-H6		
20 µl	plasmid DNA	400 ng/µl
96 µl	H_2O	
32 µl	2x buffer Y+/ Tango 10x	33 mM Tris-acetate pH 8.5 at 37°C ; 10 mM Mg-acetate ; 66 mM potassium acetate ; 0,1 mg/ml BSA.
4 µl	*Bsp* TI (*Afl* II)	10 U/µl
4 µl	*Xho* I	10 U/µl

Both digests were performed in an incubator at 37°C over night. 32 µl 6x loading dye (0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, 60 mM EDTA) was added to each sample. Two 1% TAE agarose gels were prepared.

50 x TAE pH 8.5: *Tris* *242 g/l*
glacial acetic acid *5.71% (v/v)*
Na_2 EDTA-dihydrate *37.2 g/l*

Each construct was distributed into 4 slots of one gel. Plasmid DNA fragments were separated by electrophoresis. One half of each gel, containing the DNA length standard and one plasmid slot, was dyed in an ethidiumbromide bath (0.5 µg EtBr/ml TAE) for 45 min at RT. The DNA fragment of the appropriate size was detected by ultraviolet illumination and marked by incision. Stained and unstained parts of the gel were

thereafter put together and gel slices were cut out of the unstained half using the gel incision as mark.

3.2.7 Extraction of DNA fragments from agarose gels

The DNA was extracted from the gel slices using the Jetquick Spin Column Technique® (Genomed, Bad Oeyenhausen, Germany) according to the manufacturer´s instructions. Agarose gel slices were weighed and distributed into microfuge tubes. For each 100 mg gel slice 300 µl of solution L1 (contains concentrated $NaClO_4$, sodium acetate and TBE-solubilizer) was added. After 20 min incubation at 50°C, the samples were loaded into the provided spin column. Centrifugation was carried out at RT and 14,000 rpm for 1 min. The flowthrough was discarded. Another 500 µl of solution L1 were added to the columns, incubated for 1 min and centrifuged at 14,000 rpm. The flowthrough was discarded and 500 µl of solution L2 (contains 80 % Ethanol, NaCl, EDTA and Tris/HCl) was loaded into the column. After 5 min of incubation, the columns were centrifuged at 14,000 rpm for 1 min. The flowthrough was discarded and the centrifugation repeated. Jetquick Columns were placed into a new microfuge tube and 30 µl injection buffer (5 mM Tris, 0.1 mM EDTA, pH 7.38) preheated to 70°C were added. After an incubation time of 1 min, centrifugation was carried out with 14,000 rpm for 1 min. The DNA concentration was determined using a spectrophotometer. As control 2 µl of the DNA solution were electrophoretically separated on a agarose gel and the concentration was estimated using the lambda DNA/*Eco*RI + *Hind* III marker as reference (MBI fermentas, St Leon-Rot, Germany). The eluted fragments for DNA microinjection were stored at –20°C.

3.3 Production of transgenic mice

3.3.1 Superovulation and isolation of fertilized oocytes

At noon of day one, B6D2F1 mice were injected intraperitoneally with 8 international units (U) PMSG (pregnant mares serum gonadotropin). At noon of day three, donor mice were injected intraperitoneally with 7 U HCG (human chorionic gonadotropin) and thereafter caged with stud males overnight. In the morning of day four, successful mating was checked by looking for the presence of vaginal plugs. Positive mice were sacrificed by cervical dislocation. Ovaries, oviduct and uterus were collected and embryos were flushed from the ampulla of the oviduct into M2 medium.

M2_medium:	*NaCl*	*94.66 mM*
	KCl	*4.78 mM*
	$CaCl_2.H_2O$	*1.71 mM*
	KH_2PO_4	*1.19 mM*
	$MgSO_4.7H_2O$	*1.19 mM*
	$NaHCO_3$	*4.15 mM*
	HEPES	*20.85 mM*
	Sodium lactate	*23.24 mM*
	Sodium pyravate	*0.33 mM*
	Glucose	*5.56 mM*

3.3.2 Microinjection

On a depression slide in a flat drop of M2 medium overlayed with equilibrated paraffin oil, only fertilized and intact oocytes were selected under the microscope for injection of 1-2 pl DNA solution (3-5 ng DNA/µl injection buffer). Injected oocytes were stored in M2 medium until transfer to recipients.

3.3.3 Transfer of embryos into the oviduct of synchronized recipients

Transfer of injected embryos was performed on the same day into the oviducts of synchronized NMRI mice. Foster mothers had been mated with vasectomized NMRI males over night. A positive vaginal plug indicated successful synchronisation.

Recipients were anesthetized with neuroleptanalgesia (ketamine hydrochloride 100 mg/kg body weight + xylazine 10 mg/kg body weight). Through paravertrebral incisions in the lumbar region, the reproductive tract was exposed and embryos inserted into the infundibulum of the oviducts. Ovary, oviduct and ends of the uterus were put back in place. Incisions were clamped. To observe the development of pregnancy, recipient mice were weighed every second day. In case of weight loss they were dissected to check for signs of abortion.

3.4 Identification of transgenic mice

3.4.1 Identification using the Polymerase Chain Reaction (PCR)

3.4.1.1 Preparation of genomic DNA from mouse tails

To collect tail samples, mice were put to ether anesthesia, marked by ear punches and clipped at the age of 3 weeks. The amputated tail tip was treated with histoacryl. Tail clips were directly frozen on dry ice and stored at –80°C. Fragments of 3-5 mm were cut and incubated for 16-24 h at 56°C in a 1.5 ml centrifuge tube containing 12 µl Proteinase K (20 mg/ml in bidistilled water) and 200 µl of Kawasaki buffer. After the digest, the samples were heated to 95°C for 15 min to inactivate proteinase K and centrifuged at 14,000 rpm, 4°C for 1 min. 3 µl of the supernatant were used as template in PCR.

Kawasaki buffer:	*Tris-HCl, pH 8.3*	*20 mM*
	$MgCl_2$	*1.5 mM*
	KCl	*25 mM*
	Tween 20	*0.5% (v/v)*

3.4.1.2 PCR conditions

To confirm the integrity of the DNA, a sequence of the mouse ß-actin gene was amplified. For detection of transgenesis, a sequence of the corresponding reporter gene was amplified. PCR reactions with a total volume of 20 µl were prepared on ice, mixed and directly transferred to Biometra Uno II thermocyclers (Biometra, Göttingen, Germany). PCR reagents derived from Taq DNA polymerase® (Quiagen, Hilden, Germany).

ß-Actin PCR

reagents	concentration	volume (µl)
reaction buffer	10 x	2
dNTPs	1 mM	2
primer sense	2 µM	2
primer antisense	2 µM	2
$MgCl_2$	50 mM	1.25
Q Solution (contains DMSO)		4
H_2O bidest.		3.7
Taq polymerase	5 U/µl	0.1
DNA templete		3

ß-actin primer sense: **5**`GGC ATC GTG ATG GAC TCC **3**`

ß-actin primer antisense. **5**`GTC GGA AGG TGG ACA GGG **3**`

amplification steps		temperature	time
1	denaturation	94°C	4 min
2	denaturation	94°C	1 min
3	annealing	60°C	1 min
4	extension	72°C	2 min
5	extension	72°C	10 min
6	cooling	4°C	∝

Step 2 to 4 were repeated 30 times before progressing to step 5 and 6.

EGFP PCR

reagents	concentration	volume (µl)
reaction buffer	10 x	2
dNTPs	1 mM	2
primer sense	2 µM	1
primer antisense	2 µM	1
$MgCl_2$	50 mM	1.25
Q Solution (contains DMSO)		4
H_2O bidest.		5.7
Taq polymerase	5 U/µl	0.1
DNA Templete		3

eGFP-primer sense: **5`** TCG AGC TGG ACG GCG ACG TAA A **3`**

eGFP-primer antisense: **5`** TAG TGG TTG TCG GGC AGC AGC A **3`**

amplification steps		temperature	time
1	denaturation	94°C	4 min
2	denaturation	94°C	1 min
3	annealing	62°C	1 min
4	extension	72°C	1 min
5	extension	72°C	10 min
6	cooling	4°C	∝

Step 2 to 4 were repeated 30 times before progressing to step 5 and 6.

pBL PCR

reagent	concentration	volume (µl)
reaction buffer	10 x	2
d NTPs	1 mM	2
primer sense	2 µM	2
primer antisense	2 µM	2
$MgCl_2$	50 mM	1.25
Q Solution (contains DMSO)		4
H_2O bidest.		3.7
Taq polymerase	5 U/µl	0.1
DNA Templete		3

luciferase primer sense: **5`** CCG CTG GAG AGC AAC TGC AT **3`**

luciferase primer antisense: **5** `TCT ATG CGG AAG GGC CAC AC **3** `

amplification steps		temperature	time
1	denaturation	94°C	4 min
2	denaturation	94°C	1 min
3	annealing	60°C	1 min
4	extension	72°C	2 min
5	extension	72°C	10 min
6	cooling	4°C	∝

Step 2 to 4 were repeated 30 times before progessing to step 5 and 6.

3 µl bidistilled H_2O as well as 3 µl Kawasaki buffer served as negative control in each PCR. 100 ng mouse genomic DNA served as positive control in each ß-actin PCR. As positive control for pBL-HERV-L and pEGFP-HERV-H-H6 PCRs 100 ng of the appropriate plasmid were utilized.

3.4.1.3 Agarose gel electrophoresis

After cycling, the PCR reactions were mixed with 6 x loading dye and separated on 2% TAE agarose gels containing 0.5 µg/ml ethidiumbromide. pUC Mix Marker 8 (MBI fermentas, St Leon-Rot, Germany) was employed as DNA length marker displaying a range from 67 bp to 1,116 bp.

3.4.2 Southern blot analysis

3.4.2.1 Extraction of genomic DNA and determination of concentration

Genomic DNA was isolated from mouse tissue using the Wizard genomic DNA purification kit® (Promega, Wisconsin, USA) according to the manufacturer´s instructions. Pieces of mouse liver (3-5 mm^2) were incubated for 16 h at 55°C with gentle shaking in 116 µl 0.5 M EDTA solution pH 8, 484 µl Nuclei Lysis solution and 17.5 µl of proteinase K solution (20 mg/ml). After confirming the complete digestion of samples, 3 µl of RNase solution (4 mg/ml) were added. The mixtures were incubated for 15 min at

37°C. Samples were then cooled to RT, mixed with 200 µl Protein Precipitation solution and vortexed vigorously. After 5 min of incubation on ice and 4 min centrifugation at 14,000 rpm, the supernatants were transferred to new tubes. 600 µl isopropanol (RT) were added. Mixtures were gently inverted until thread-like strands of DNA formed a visible mass. DNA pellets were recovered by centrifugation (14,000 rpm, 2 min), washed with 600 µl 70% ethanol, allowed to air-dry for 10 min and resuspended in 50 µl bidistilled water. To assure that the DNA was completely dissolved, it was stored at 4°C for at least 24 h prior to further manipulation. DNA concentration was determined by measuring the optical density (OD) of 100 µl of a 1:50 dilution of the samples at 260 nm and 280 nm in a spectrophotometer. DNA concentration was calculated using the following equation:

$$\textit{DNA concentration (µg/ml)} = \textit{dilution factor} \times OD_{260} \times 50 \textit{ (unit factor)}$$

DNA purity was assessed on behalf of the ratio OD_{260}/OD_{280}. Ratios between 1.8 and 2.0 were considered to be appropriately pure.

3.4.2.2 Digestion of genomic DNA

Genomic DNA of pEGFP-HERV-H-H6 transgenic mice was seperately digested with *Hind* III and *Eco* R1 (MBI fermentas, St Leon-Rot, Germany) for 16 h at 37°C in an incubator.

Hind III digest		*Eco* R1 digest	
Hind III (50 U/µl)	2 µl	*Eco* R1 (50 U/µl)	2 µl
Buffer R + (10 x)	5 µl	Buffer O + (10 x)	5 µl
Genomic DNA	x µl (15 µg)	Genomic DNA	x µl (15 µg)
Bidistilled water	ad 50 µl	Bidistilled water	ad 50 µl

Plasmid DNA of the pEGFP-HERV-H-H6 plasmid was digested for 2 h at 37°C.

Hind III digest	
Hind III (0.2 U/µl)	1 µl
Buffer R + (10 x)	1.5 µl
Plasmid DNA (10 ng/µl)	1.5 µl
Bidistilled water	11 µl

10 µl of 6 x loading buffer were added to the genomic digests and 15 µl H_2O plus 6 µl 6x loading buffer was added to the plasmid digest. The cleaved DNA was separated on a 0.8% TAE agarose gel in 4 h at 80 V (voltage constant). Gel as well as running buffer (1 x TAE) contained 8 µl EtBr/l. The gel was then photographed with a ruler, marked at the bottom right-hand corner and trimmed.

3.4.2.3 Transfer of the DNA

Depurination of the DNA in the gel was accomplished by soaking the gel in several volumes of 0.2 N HCl for 30 min. The gel was rinsed several times with deionized H_2O. Thereafter, it was soaked in 10 gel volumes of denaturation solution for 45 at RT with gentle agitation.

Denaturation solution:	*NaCl*	*1.5M*
	NaOH	*0.5M*

After rinsing the gel briefly in deionized H_2O, the gel was soaked twice in neutralization buffer at RT (1 x 30 min, 1 x 15 min).

Neutralization buffer:	*Tris (pH 7.4)*	*1M*
	NaCl	*1.5 M*

Meanwhile the membrane was prepared for transfer by soaking it 5 min in 10 x SSC.

10 x SSC: *87.65 g/l NaCl*
44.1 g/l Sodium citrate
The pH was adjusted to 7.0 with a few drops of 10 N NaOH solution before autoclaving.

The DNA was transferred from the gel to a Biodyne® nylon membrane (Pall, NY, USA) by neutral blotting. 10 x SSC was used as neutral transfer buffer. The assembly of the transfer apparatus is displayed in Figure 9. Blotting papers, paper towels and the nylon membrane were cut to fit the gel size. Blotting papers were wetted and piled onto one another. At each layer, air bubbles were removed with the help of a glass pipette. Capillary transfer took place at RT for 36 h.

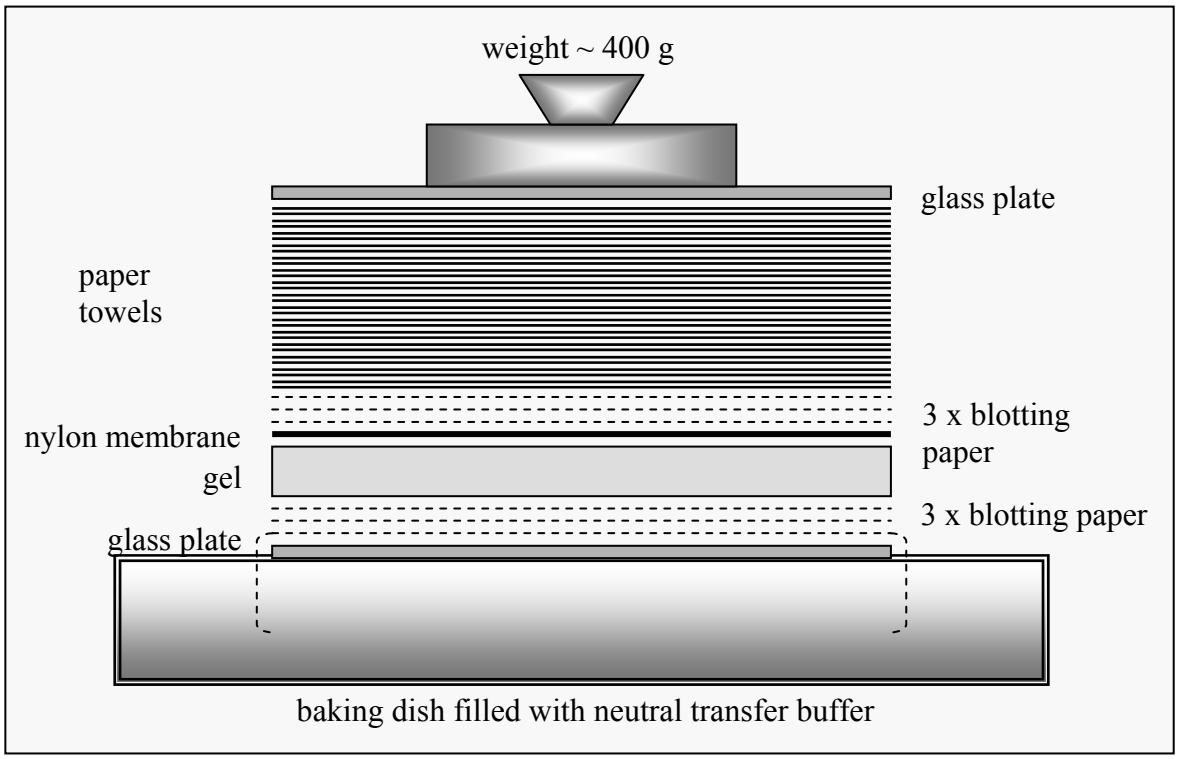

Figure 9: Assembly of the Southern blot transfer apparatus

After DNA transfer, the membrane was rinsed 5 min in 6 x SSC at RT and air-dried for 30 min. To fix the DNA, the membrane was baked at 70°C for 1 h and thereafter exposed to UV light (0.12 Joule for 136.5 cm²) to crosslink nucleic acids.

3.4.2.4 Radioactive probe labeling

The probe (776 bp) was released by restriction enzyme digestion from the pEGFP-HERV-H-H6 plasmid, separated on a 1% TAE agarose gel by electrophoresis and extracted using the Jetquick Spin Column® Technique as described in 3.2.7.

Not I and *Eco* RI double digest	
DNA (2 µg/µl)	3 µl
Not I (10 U/µl)	2 µl
Eco RI (10 U/µl)	2 µl
H_2O	38 µl
Buffer 0 + (10 x)	4 µl

The digest was incubated at 37°C for 2 h.

The probe was labeled with the Hexa label DNA labeling kit® (MBI fermentas, St Leon-Rot, Germany) and α^{32}P-dCTP as follows: 5 µl of DNA (20 ng/µl), 10 µl hexamers in 5 x buffer and 28 µl deionized H_2O were boiled at 95°C for 10 min. The mixture was thereafter chilled on ice and the following reagents were added: 3 µl Mix C, 3 µl α^{32}P-dCTP and 1 µl Klenow polymerase (5 U/µl, exonuclease$^-$). After incubation for 15 min at 37°C, 4 µl dNTPs (0.25 mM) were added and the mixture was put back to 37°C for another 5 min. To stop the reaction, 1 µl of EDTA (0.5 M, pH 8.0) was added. Unicorporated nucleotides were removed by purification with Amersham Microspin columns type S-300 (Amersham Pharmacia, Freiburg, Germany) according to the manufacturer`s instructions. 5 µl of a 1:100 dilution in water were put into a scintillation vial and the number of counts per minute (cpm) were determined in a multi-purpose scintillation counter. The final cpm value was obtained employing a formula to correct the lack of scintillation fluid in the measurement:

radioactivity/µl = cpm x 20 (dilution) x 1.55

The probe was stored on ice to be used at the same day.

3.4.2.5 Hybridization, washing and signal detection

The crosslinked membrane was placed into a glass tube and prehybridized with 10 ml Rapid-Hyb buffer (Amersham Pharmacia, Freiburg, Germany) at 65°C for 2 h. Meanwhile the labeled probe was boiled for 10 min at 95°C, directly put back on ice and then added completely to the prehybridization reaction. The membrane was hybridized at 65°C for 12 h. The following washing steps were performed to remove non-specificly bound probes from the membrane:

- 20 min RT 2 x SSC, 0.1% SDS;
- 15 min 65°C 1 x SSC, 0.1% SDS;
- 15 min 65°C 0.1 x SSC, 0.5% SDS;
- 20 min 42°C 0.4 M NaOH;
- 30 min 42°C 0.1 x SSC, 0.1% SDS.

Only the first three washes were put in the radioactive waste. The membrane was sealed in a plastic bag and exposed to a Storage Phosphor screen. The membrane was not allowed to dry at any time.

3.5 Evaluation of gene expression at RNA level

3.5.1 Reverse transcription PCR (RT-PCR)

To avoid degradation by RNases, following rules were observed while working with ribonucleic acids:

- *gloves were worn all the time and changed frequently*
- *all equipment was autoclaved*
- *glassware, magnet stirrers and dissection instruments were baked at 180°C for at least 4 h*
- *benches were cleaned with 100% ethanol*
- *to all solutions, except those containing Tris, 0.1% DEPC (diethylpyrocarbonate) was added*
- *all procedures were carried out on ice*

3.5.1.1 Extraction of RNA from mouse tissue

Tissue samples (10-100 mg) stored at –80°C were cut on blocks of dry ice and added frozen to 1 ml of TriPure® Isolation Reagent (Roche, Mannheim, Germany).

TriPure® Isolation Reagent:	*phenol*	*40%*
	guanidiniumthiocyanate	*24%*

Homogenization was carried out immediately in a polytron blender at 26,000 rpm (position E for skin) or at 23,500 rpm (position D for muscle, heart, liver, kidney, mammary gland, lung, brain and gonads) for 1 min. After each homogenization step, the homogenizer was cleaned with bidistilled water and 0.2 M NaOH. Homogenates were stored on ice until further processing. Skin homogenate was liberated from hair by centrifugation and transfer of the supernatant to a new tube. RNA was isolated from the homogenates by extraction with chloroform, precipitation with isopropanol and washing with ethanol according to the manufacturer´s instructions. RNA pellets were dried and resuspended in 10-40 µl DEPC-H_2O depending on the size of the pellet. RNA concentration and purity was determined using a spectrophotometer. 10 µg total RNA were digested with 10 U Rnase-free DNase I (Roche, Mannheim, Germany) in a total volume of 20 µl 1 x DNase I digestion buffer.

10 x DNase I digestion buffer:	*Tris (pH 7.4)*	*10 mM*
	$MgCl_2$	*10 mM*

DNase digest was carried out at 37°C for 60 min. The enzyme was inactivated at 75°C for 10 min. RNA was stored at –20°C.

3.5.1.2 Reverse transcription of mRNA and PCR from cDNA

2.5 µg DNase I digested RNA were reverse transcribed to cDNA for 60 min at 37°C.

volume	reagent	concentration
5.3 µl	H_2O-DEPC	0.1% diethylpyrocarbonate
1.6 µl	Oligo dT (15 T)	500 µg/ml
4 µl	5x first strand buffer	250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$
2 µl	DTT	0.1 M
0.1 µl	M-MLV RTase	200 U/µl
5 µl	total RNA	0.5 µg/µl

M-MLV RTase was inactivated at 95°C for 10 min. cDNA was stored at –20°C.

PCR from cDNA was performed under the same conditions as PCR from DNA described in 3.4.1.2. ß-Actin PCR was performed from DNase I digested RNA as well as from cDNA to detect genomic DNA contamination or the loss of integrity of the cDNA.

3.6 Evaluation of gene expression at the protein level

3.6.1 Western Blot

3.6.1.1 Extraction of protein from tissue samples

Tissue samples stored at –80°C were weighed, transferred to 10 ml roundbottom tubes containing extraction buffer and homogenized in a polytron blender at at 26,000 rpm (position E for skin) or at 23,500 rpm (position D for muscle, heart, liver, kidney, mamma, lung, brain and gonads) for 1-2 min. For each 20 mg tissue, 500 µl extraction buffer were used.

Protein extraction buffer:	*5 x Laemmli buffer*	*20%*
	Tris (pH 7.4)	*20 mM*
	Triton X-100	*2%*

The homogenizer was cleaned after each sample with distilled water and PBS.

Phosphate-buffered saline (PBS):	*NaCl*	*8 g/l*
	KCl	*0.2 g/l*
	Na_2HPO_4	*1.44 g/l*
	KH_2PO_4	*0.24 g/l*
	The pH was adjusted to 7.4.	

Samples were transferred to 1.5 microfuge tubes, boiled at 95°C for 5 min and chilled on ice. In an end-over-end shaker, samples were incubated over night at 4°C. Samples were thereafter boiled again for 5 min at 95°C, chilled on ice and centrifuged at 12,000 rpm, at 4°C for 5 min. An aliquot was removed for determination of protein concentration. Samples were stored at –20°C.

3.6.1.2 Determination of protein concentration

Protein concentration was measured using the bicinchoninic acid (BCA) protein assay. A set of protein standards of known concentration was prepared by serially diluting a bovine serum albumin (BSA) stock solution (4 mg/ml) in PBS. 50 µl of the standards and of the samples (diluted 1:10 in PBS) were pipetted into a 96-well plate. 200 µl bicinchoninic acid solution containing 4% $CuSO_4$ were added to each well. The plate was incubated at 37°C for 30 min. The absorbance at 562 nm (absorbance maximum for BCA-Cu^{1+} complexes generated as consequence of reduction of Cu^{2+} to Cu^{1+} by proteins) was measured in a spectrophotometer. A standard curve was prepared by plotting the absorbance of standards versus protein concentration. Using the standard curve, the protein concentration of the samples was determined.

3.6.1.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight on SDS-polyacrylamide gels in the Mini Protean II System (Bio-Rad, Munich, Germany). First, the separating gel was prepared in an Erlenmeyer flask under continuous agitation and poured into the gap

between the glass plates of the system, leaving enough space for the stacking gel. The separating gel was immediately overlaid with bidistilled water.

Separating gel (12%):	*Bidistilled water*	*3.35 ml*
(for 2 gels)	*1.5 M Tris pH 8.8*	*2.5 ml*
	30% Acrylamide	*4 ml*
	10% SDS	*100 µl*
	Temed	*5 µl*
	10% APS	*50 µl*

After 45 min polymerisation at RT, the water was discarded. The stacking gel was prepared in the same way and loaded on the top of the separating gel. The comb was inserted without trapping air bubbles.

Stacking gel (5%):	*bidistilled water*	*7.0 ml*
(for 4 gels)	*0.5 M Tris pH 6.8*	*1.25 ml*
	30% Acrylamide	*1.5 ml*
	10% SDS	*100 µl*
	Temed	*5 µl*
	10% APS	*100 µl*

After 30 min polymerisation at RT, the gels were mounted in the electrophoresis chamber which was then filled with electrophoresis buffer. Combs were removed carefully and wells were washed immediately with a pasteur pipette containing electrophoresis buffer. Air bubbles at the bottom of the gels were flushed away with a syringe.

Electrophoresis buffer:	*Tris*	*30.3 g/l*
	Glycine	*144 g/l*
	SDS	*10 g/l*

Gel slots were loaded with 5-30 µl of the protein extract corresponding to 50-80 µg of protein. A low range protein marker covering molecular weights between 6.5 and 66 kD was used for estimation of protein size. Electrophoresis was carried out at 100 V for 10 min and then at 160 V until the bromophenol blue reached the bottom of the separating gel.

3.6.1.4 Electroblotting

The separated proteins were transferred from the gel to a polyvinylidendiflouride (PVDF) membrane (Immobilon-P®, Millipore, MA, USA) by semidry electroblotting. The membrane was cut to the size of the gel, preincubated for 10 min in absolute methanol and subsequently incubated in transfer buffer for 30 min at RT.

Transfer buffer:	*Methanol*	*20%*
	Tris	*5.82 g/l*
	Glycine	*2.92 g/l*
	SDS	*0.37 g/l*

12 pieces Whatman blotting paper were cut to the size of the membrane and soaked in transfer buffer. 6 pieces were stacked, one on top of the other, on the anode of a graphite electroblotter (Millipore, Ma, USA). PVDF membrane and gel were added and another 6 pieces of wet blotting paper were stacked exactly on top. Layers were repeatedly squeezed with a pipette to remove air bubbles. The cathode was placed on top. Transfer took place for 90 min at 1 mA/cm^2 gel plane. After the transfer, the membrane was stained with Ponceau red for 2 min, rinsed with bidistilled water and dried. Molecular weight markers were labeled with a pen. Membranes were stored at 4°C.

Ponceau red:	*Ponceau S*	*2 g/l*
	Acetic acid	*30%*

3.6.1.5 Protein detection by peroxidase-labeled antibodies

Membranes were incubated in a hybridization oven with blocking solution for 1 h at RT.

Blocking buffer:	*Spray dried skimmed milk*	*3%*
	Tween 20 (polyoxyethylene sorbitol-monolaurate)	*0.1%*
	TBS (Tris-buffered saline)	

After blocking, the membrane was washed 3 times (1 x 15 min; 2 x 5 min) with Tris-buffered saline containing 0.1% Tween 20 (TBS-T 0.1%) at RT in an incubation oven.

TBS-T 0.1%:	*Tris (pH 7.4)*	*3 g/l*
(Washing buffer)	*NaCl*	*8 g/l*
	Tween 20	*0.1%*

The membrane was incubated with the primary antibody (rabbit anti-EGFP antibody, purified IgG fraction, Molecular probes) diluted 1:1,000 in blocking buffer for 2 h at RT. Three washing steps with TBS-T 0.1% were subsequently performed as described above. Incubation with the second antibody (peroxidase-conjugated, affinity-purified goat anti-rabbit antibody, Jackson Immuno Research distributed by Dianova, Hamburg, Germany) diluted 1:5,000 in blocking buffer was performed for 1.5 h at RT. Finally, membranes were washed 5 times (1 x 15 min, 4 x 5 min) at RT. Detection of peroxidase activity was performed by incubating the membrane with 3 ml of ECL Western blotting detection reagent (Amersham Pharmacia, Freiburg, Germany). The membrane was mounted on a glass plate, sealed with Saran wrap and exposed to an ECL film (Amersham Pharmacia, Freiburg, Germany).

3.6.2 Histology

3.6.2.1 Collection and fixation of tissues

At the age of 10 to 12 weeks, mice were put to ether anesthesia, killed by cervical dislocation and dissected. Pieces of muscle, gonads, liver, lung and brain were collected and directly processed for histology. Histology was carried out in cooperation with the Institute of Histology of the VU (Veterinärmedizinische Universität) Vienna. As positive control in histology, the institute provided EGFP expressing tumor tissue (precWAPeGFP transfected MCF7-tumor xenografts).

Organs were fixed in 4% buffered formaldehyde for 6 h at RT and embedded in paraffin. Embedding times and solvents were as follows:

Solvent	Time	Temperature
Ethanol 70%	1 hour	55°C
Ethanol 80%	1 hour	55°C
Ethanol 96%	1 hour	55°C
Ethanol 96%	1 hour	55°C
Ethanol 100%	1 hour	55°C
Ethanol 100%	1 hour	55°C
Xylene	1 hour	55°C
Xylene	1 hour	55°C
Paraffin (melting point 50-52°C)	Over night	50°C

Paraffin (melting point 56°C) and specimen were thereafter blocked in a mold.

3.6.2.2 Preparation of slides

Glass slides for immunohistochemical staining were pretreated with poly-L-lysine to improve adhesion of tissue on glass. These slides were incubated for 5 min at RT in a 10% poly-L-lysine solution (Sigma, Taufkirchen, Germany). Glass slides for haematoxylin & eosin, mowiol and propidium iodide staining were not pretreated. From each organ, five 5 µm thick sections were cut. The paraffin ribbons were floated on deionized water (45°C) and mounted on the appropriate glass slides. Sections were dried over night at 55°C. All slides were deparaffinized and rehydrated as follows: 2 x 5 min xylene, 2 x 2 min 100% ethanol, 2 min 96% ethanol, 2 min 70% ethanol.

Sections for mowiol and propidium iodide staining were furthermore incubated for 2 min in bidistilled water.

3.6.2.3 Haematoxylin & eosin staining

One deparaffinated and rehydrated section of every organ was incubated for 3 min in Mayer´s hemalum (Merck, Wertheim, Germany), rinsed in tap water for 5 min, incubated for 3 min in Eosin Y solution (Merck, Wertheim, Germany) and for 1 min in 0.1% acetic acid. Sections were dehydrated in an ascending series of ethanol (3 min ethanol 70%, 3 min ethanol 96% and 3 min ethanol 100%), incubated in xylene for 2 min and mounted with Entellan® (Merck, Wertheim, Germany). Slides were stored in the dark at +4°C until histological investigation.

3.6.2.4 Mowiol and propidium iodide staining

One deparaffinized and rehydrated section of every organ was mounted in anti-fading medium (mowiol solution). Another section of every organ was treated with propidium iodide to stain cell nuclei, at 1:10,000 dilution in bidistilled water for 2 min at RT and thereafter mounted with 1-2 drops of mowiol solution. Slides were stored in the dark at +4°C until histological investigation.

Mowiol solution: *Mowiol 4-88 29 g*
in 80 ml PBS
The solution was stirred for 12 h at RT before adding 40 ml glycerol. After another 12 h of stirring at RT and centrifugation for 1 h at 15,000 rpm, the supernatant was recovered, supplemented with NaN_3 and stored at +4°C.

3.6.2.5 Immunohistochemical staining

Deparaffinized sections were pretreated with 0.6% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase and rinsed with tap water. Slides were then put into an immunostaining centre where all the following steps were accomplished. Slides were incubated with 1.5% normal goat serum for 30 min at RT to minimize unspecific background staining. The primary antibody, rabbit anti-GFP (Molecular probes, Leiden, Niederlande), was diluted in phosphate-buffered saline (PBS) to a final concentration of

1:4,000. Slides were incubated with the primary antibody overnight at +4°C. On the next day, slides were washed with PBS and covered with EnVision+™ solution, containing a goat anti-rabbit antibody conjugated to peroxidase, (DAKO, CA, US) for 30 min at RT. After that, sections were washed with PBS and slides developed for 10 min at RT in 0.1% 3.3´-diaminobenzidine (DAB) hydrochloride containing 0.03% hydrogen peroxide. The reaction was stopped in bidistilled water for 5 min. Finally, sections were counterstained with Mayer´s hemalumn, dehydrated and mounted in DPX medium.

DPX: *contains distyrene*
plasticizer (tricresyl phosphate)
xylene

Slides were stored in the dark at +4°C until histological investigation.

3.6.2.6 Histological investigation of tissues

Histological investigation of tissues was carried out under a Zeiss axiovert 200-N microscope (Zeiss, Jena, Germany). Pictures were taken with an Axiocam (Zeiss, Jena, Germany). Sections stained with haematoxylin & eosin and immunohistochemistry were evaluated by bright field microscopy at 400x and 1000x magnification. All sections mounted with mowiol, including those counterstained with propidium iodide, were investigated by fluorescence microscopy at 400x magnification to detect native EGFP fluorescence. EGFP fluorophores were excited with ultraviolet light passing through a 450-490 nm filter (FT 510/LP520).

3.7 Phenotypic consequences of transgene expression

3.7.1 Analysis of body weight, body length and organ weights

At the age of 10 to 12 weeks, mice were put to ether anesthesia, sacrificed by cervical dislocation and weighed entirely. The length from the distal end of the nose to the proximal end of the tail was measured (nose-rump length). The following organs were removed quickly and directly put on dry ice to freeze to –80°C: tail, skin from the back,

tissue from all 12 mammary complexes, the musculus quadriceps, the gonads, spleen, both kidneys, liver, heart, lung and brain. Partly, complete organs were weighed after removing surplus blood by softly pressing pieces to a kimwipe (gonads, pancreas, spleen, both kidneys, liver, lobus thoracicus dexter and sinister of the thymus, heart, lung, brain). Additionally, pieces from muscle, liver, gonads, lung and brain were fixed in 4 % PBS-buffered formaldehyde (pH 7) for histological examination.

3.8 Statistics

The data was analyzed for significance of differences using a two tailed *Student´s t-test*. A difference was considered to be statistically significant at $p< 0.05$.

3.9 Equipment and reagents

Company	Reagent/ device	Catalogue number
Air liquide Muenchen, Germany	Dry ice slices	400101
Amersham Pharmacia Freiburg, Germany	Rapid-Hyb hybridisation buffer	RPN 1636
	ECL western blotting reagent	RPN 2109
	ECL X-ray film	RPN 2103 K
	Micro Spin S-300 columms	27-5/30-01
ART Labortechnik Mülheim, Germany	Tissue homogenizer	
Bachhofer Reutlingen, Germany	Hybridization oven (Mini 38)	
Beckmann CA, US	Spectrophotometer DU640	
	Scintillation counter	
Bio-Rad Munich, Germany	Mini Protean System	
	30% acrylamide	161-0156
	ammonium persulfate	161-0700
	Temed	161-0800
	POWER PACK 300	165-5050

Company	Reagent/ device	Catalogue number
Biometra **Göttingen, Germany**	UNO II thermocycler	
DAKO corporation **CA, US**	EnVision+ antibody	
DIFCO **Le Pont de Claix, France**	Tryptone peptone Yeast extract Agar, granulated	123-17-0 0127-17-9 0145-17-0
Dow chemicals Company **MI, USA**	Saran Wrap	
Electron Microscopy Sc. **PA, US**	DPX mountant	13510
Eppendorf **Hamburg, Germany**	1.5 centrifuge tubes 2.0 centrifuge tubes thermomixer type 5436 centrifuge type 5417R	0030 120.086 0030120.094
Genomed **Bad Oeyenhausen,** **Germany**	Jetsorb kit	110150
Greiner **Frickenhausen, Germany**	Petri dishes 90 mm, sterile	663161
Invitrogen **Paisley, Scotland**	MLV-Reverse Transcriptase	28025013
Kisker **Steinfurt, Germany**	Quali-PCR tubes	G002-A
MBI fermentas **St Leon-Rot, Germany**	PUC Mix Marker 6x loading dye 1 kb ladder *Eco* RI (HC) *Bam* HI (HC) *Not* I *Xho* I *Sca* I	SM 0301 R 0611 SM 0312 ER 0273 ER 0053 ER 0591 ER 0691 ER 0432

Company	Reagent/ device	Catalogue number
MBI fermentas	*Hind* III	ER 0503
St Leon-Rot, Germany	Klenow fragment exo$^-$	EP0422
	Hexa Label DNA labeling kit	K 0612
Merck	Glycerol	4094
Wertheim, Germany	Mayer`s hemalum	1.09249
	Bomophenol blue	8122
	Natrium chloride	1.06404
	Natrium hydroxide	1.06498
	Dodecyl sulfate sodium salt	1.13760
	2-propanol (Isopropanol)	1.09634
	Formaldehyde solution (37%)	1.04003
	Chloroform	1.02445
	Copper II sulfate	2791
	Ethidiumbromide 1%	1.11608.0030
	Eosin Y solution	1.09844
	Entellan	1.07961
Millipore	Milliblot-Graphite	MBBDGE 001
MA, USA	Electroblotter	IPVH 00010
	Immobilon-P (PVDF)	
Molecular probes	Rabbit anti-GFP	
OR, US		
Pall	Biodyne (nylon membrane)	P/N 60113
NY, USA		
Perkin Elmer	α^{32}-P-dCTP	PC 3919-0101
MA, USA		
Polysciences	Mowiol 4-88	17951
Eppelheim, Germany		
Promega	Wizard genomic DNA	A 1120
WI, USA	purification kit	
Quiagen	*Taq* DNA polymerase	201203
Hilden, Germany	QIAprep Minispin Kit	27106
Ratiolab	Semperguard nitril gloves	9900043
Buchschlag, Germany	Safe skin latex gloves	220

Company	Reagent/ device	Catalogue number
Roche	DNase I (RNase free)	776 785
Mannheim, Germany	TriPure Isolation Reagent	1667165
	Sure cut buffer H	1417991
Carl Roth	Diethylpyrocarbonate (DEPC)	K 028.2
Karlsruhe, Germany	Tris	4855.2
	Glacial acetic acid	3783.1
	Ethylenediamine-tetraacetic	8043.2
	acid	4627.1
	Methanol	3908.2
	Glycine	832.1
	Kanamycin	
Sigma	Propidium Iodide	P-4170
Taufkirchen, Germany	Tween 20	P-1379
	Potassium chloride (KCl)	P-4504
	Calcium chloride ($CaCl_2$)	C-7902
	Proteinase K	P-6556
	Triton X-100	X-100
	Protein LR Marker	M-3913
	Bovine serum albumin	A-4503
Sigma	Ponceau S	P-3504
Taufkirchen, Germany	3.3´-Diamonibenzidine	D 5637
	Poly-L-Lysine	P 8920
	Bicinchoninic acid solution	B 9643
	Hepes	H-4034
Schleicher& Schuell	Gel blotting paper GB 002	10426694
Dassel, Germany		
Schubert and Weiss	Nunc cryo tubes 1.5 ml	375418
Muenchen, Germany		
Stratagene	Eagle Eye II	
Heidelberg, Germany		
Vogel	HistoComp	
Gießen, Germany		
Zeiss	Axiovert microscope	
Jena, Germany	200 – N	

4 RESULTS

4.1 Purification of gene constructs for DNA microinjection

Purification of the gene constructs pBL-HERV-L as well as pEGFP-HERV-H-H6 from agarose gels yielded high DNA quality for microinjection (Figure 10).

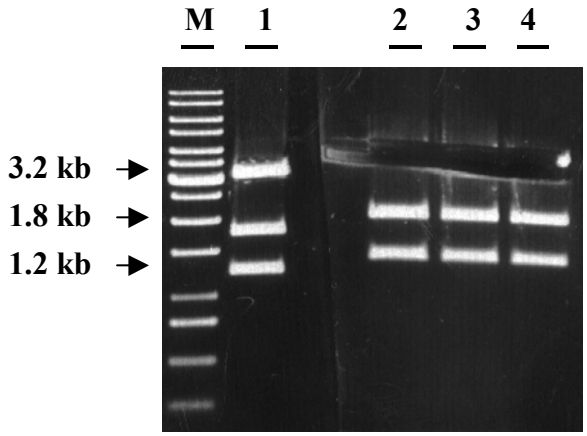

Figure 10: Agarose gel electrophoresis of the restiction enzyme digested plasmid pBL-HERV-L. M: molecular weight marker; 1, 2, 3, 4: pBL-HERV-L plasmid DNA; The 3.2 kb fragment of lane 2, 3 and 4 was cut out and and subsequently purified.

4.2 Generation of transgenic animals and breeding of transgenic lines

Transgenic animals were identified by PCR (Figure 11 and 12) from genomic DNA obtained from tail samples. The efficiency of gene transfer is displayed in Table 9.

Table 9: Efficiency of gene transfer in mouse for the constructs pBL-HERV-L and pEGFP-HERV-H-H6. [1]recipients with litter/number of embryo tranfers; [2] number of transgenic animals after weaning/total number of animals after weaning.

Gene construct	Injected embryos	Embryo transfers	Pregnancy rate [1]	Integration rate [2]	Founder animals
pEGFP-HERV-H-H6	238	11	82%	19%	10
pBL-HERV-L	374	18	67%	26%	9

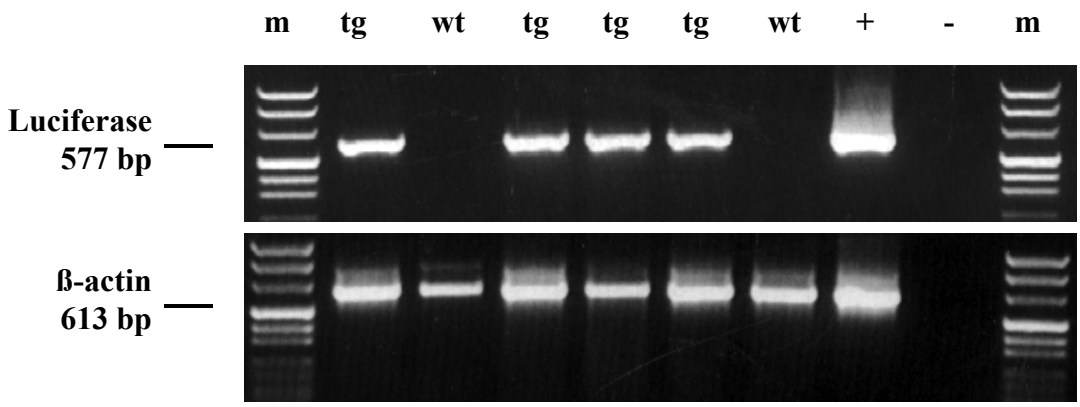

Figure 11: Screening of pBL-HERV-L mice by PCR. Upper picture: Luciferase (reporter gene) PCR; Lower picture: ß-actin (housekeeping gene) PCR. M: DNA length marker; tg: transgenic mouse (line #6); wt: wildtype mouse; +: positive control; -: negative control.

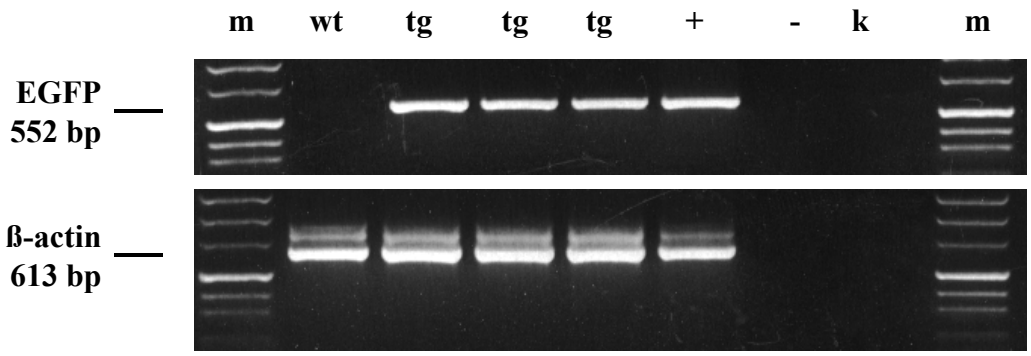

Figure 12: Screening of pEGFP-HERV-H-H6 mice by PCR. Upper picture: EGFP (reporter gene) PCR; Lower picture: ß-actin (housekeeping gene) PCR. M: DNA length marker; wt: wildtype mouse; tg: transgenic mouse (line #1, #5, #10); +: positive control; -: negative control; k: kawasaki buffer.

Founder mice were mated with C57BL/6 mice to generate transgenic lines. Four out of ten pEGFP-HERV-H-H6 transgenic founders showed germline transmission. From these founders, four transgenic lines were established (line #1, #5, #9, #10) (Table 10). One pBL-HERV-L founder animal transmitted the gene construct to its offspring, resulting in one pBL-HERV-L transgenic line (line #6) (Table 11).

Table 10: Generation of pEGFP-HERV-H-H6 transgenic lines

Founder No.	Mated	Number of litters	Tg offspring	Line #
610-9	+	2	+	1
610-12	+	1	-	2
609-5	+	0	-	3
609-6	+	1	-	4
708-50	+	2	+	5
610-14	+	1	-	6
610-16	+	1	-	7
704-21	+	2	-	8
704-22	+	1	+	9
706-26	+	1	+	10

Table 11: Generation of pBL-HERV-L transgenic lines

Founder No.	Mated	Number of litters	Tg offspring	Line #
614-1	+	2	-	1
614-5	+	2	-	2
614-6	+	3	-	3
614-11	+	1	-	4
614-3	+	1	-	5
716-8	+	2	+	6
716-11	+	2	-	7
716-15	+	1	-	8
717-16	+	2	-	9

Transgenic mice were bred with C57BL/6 mice to generation F2 to obtain 87.5% C57BL/6 genetic background. In all 4 pEGFP-HERV-H-H6 transgenic lines, the proportion of transgenic F1 animals from total F1 animals was below 50%. Maximum 57% transgenic pEGFP-HERV-H-H6 mice were born in generation F2. The proportion of pBL-HERV-L transgenic mice from total mice was below 50% in both generation F1 and F2 (Table 12 and 13).

Table 12: Transmission rates of transgenes in generation F1

gene construct	line #	founder	total mice in generation F1	transgenic mice (%) in generation F1
pEGFP-HERV-H-H6	1	610-9	11	4 (36%)
pEGFP-HERV-H-H6	5	708-50	12	4 (33%)
pEGFP-HERV-H-H6	9	704-22	11	3 (27%)
pEGFP-HERV-H-H6	10	706-26	11	2 (18%)
pBL-HERV-L	6	716-8	14	5 (35%)

Table 13: Transmission rates of transgenes in generation F2

gene construct	line #	F1 mouse	total mice in generation F2	transgenic mice (%) in generation F2
pEGFP-HERV-H-H6	1	610-9-1	7	4 (57%)
pEGFP-HERV-H-H6	5	708-50-7	9	4 (44%)
pEGFP-HERV-H-H6	9	704-22-10	11	2 (18%)
pEGFP-HERV-H-H6	10	706-26-10	8	3 (37%)
pBL-HERV-L	6	716-8-4 / -5	18	8 (44%)

The breeding data obtained in generation F1 and F2 indicates one integration site of the pEGFP-HERV-H-H6 and the pBL-HERV-L construct.

Further characterization of transgene integration was performed in pEGFP-HERV-H-H6 mice by Southern blot analysis of DNA isolated from liver tissue (Figure 14). Genomic DNA of transgenic and wildtype mice was digested using *Hind* III and *Eco* RI. The positive control, pEGFP-HERV-H-H6 plasmid DNA (4,151 bp), was linearized with *Hind* III. The radioactively labelled probe (720 bp) hybridized with the EGFP reporter gene (Figure 13).

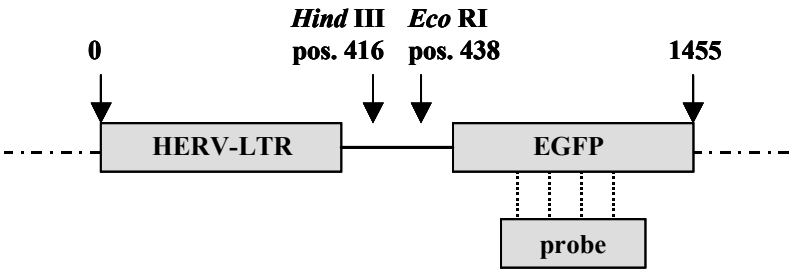

Figure 13: Restriction enzyme sites in the pEGFP-HERV-H-H6 gene construct and alignment of the radioactively labelled probe with the transgene in Southern blot analysis.

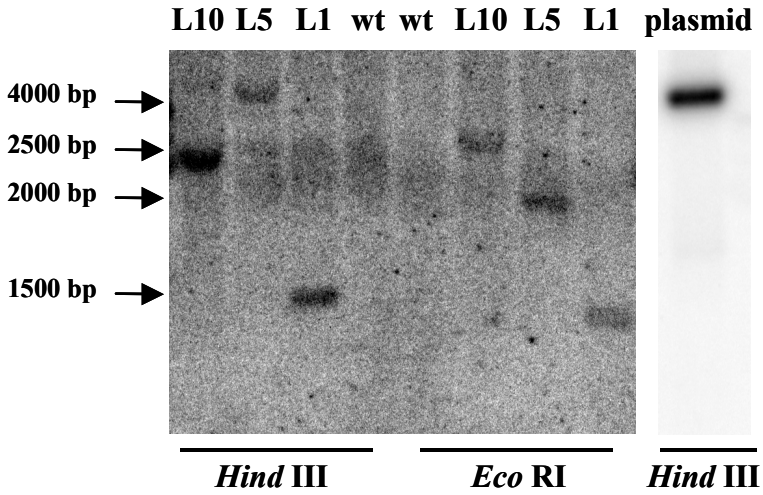

Figure 14: Southern blot analysis of genomic DNA from pEGFP-HERV-H-H6 mice.

By displaying single bands, Southern blot analysis of genomic DNA indicated in all tested transgenic lines of pEGFP-HERV-H-H6 mice a single integration site of the transgene.

4.3 Expression studies

Expression studies at mRNA level were performed in pBL-HERV-L and pEGFP-HERV-H-H6 mice of both sexes at the age of 10 to 12 weeks. The following organs were used for isolation of RNA: muscle, heart, liver, kidney, spleen, skin, mammary gland, lung, brain (without pituitary gland) and gonads. Absolute body weight, nose rump length (NRL) and organ weights were determined (Table 14 and 15). These phenotypic traits of transgenic and wildtype mice were subjected to statistical analysis. No significant differences were observed between transgenic mice and their non-transgenic littermates. Therefore, no indication for insertional mutagenesis was provided.

Table 14: Body weight, nose rump length (NRL) and organ weights of pEGFP-HERV-H-H6 transgenic mice and their control littermates at the age of 10 to 12 weeks. The values represent the mean ± standard deviation (SD).

Parameter		male tg (n=6)	male wt (n=3)	female tg (n=6)	female wt (n=3)
Body weight	[g]	24.52 ± 1.5	26.06 ± 1.1	20.70 ± 1.5	20.95 ± 2.4
NRL	[cm]	9.36 ± 0.3	9.70 ± 0.2	8.60 ± 0.1	8.90 ± 0.4
Gonads	[mg]	199.30 ± 14.6	191.30 ± 10.0	20.00 ± 2.7	21.67 ± 2.8
Pancreas	[mg]	141.33 ± 17.6	155.33 ± 13.2	128.16 ± 10.3	146.00 ± 5.1
Spleen	[mg]	68.83 ± 9.2	79.66 ± 9.6	75.33 ± 9.8	80.66 ± 22.7
Liver	[g]	1.46 ± 0.1	1.48 ± 0.3	1.12 ± 0.1	1.18 ± 0.22
Thymus	[mg]	48.00 ± 7.4	42.5 ± 3.5	59.83 ± 19.7	57.33 ± 6.4
Heart	[mg]	164.33 ± 33.7	171.67 ± 19.8	147.00 ± 32.1	140.00 ± 9.0
Lung	[mg]	155.30 ± 12.6	157.00 ± 12.1	139.80 ± 18.2	161.3 ± 3 5.9
Brain	[mg]	373.33 ± 44.0	398.33 ± 53.4	400.67 ± 34.5	373.33 ± 31.2
Kidney*	[mg]	176.00 ± 16.4	188.33 ± 10.4	137.00 ± 8.7	152.00 ± 9.8

* Arithmetic mean of both kidney weights

Table 15: Body weight and organ weights of pBL-HERV-L transgenic mice and their control littermates at the age of 10 to 12 weeks. The values represent the mean ± standard deviation (SD)

Parameter		male tg (n=4)	male wt (n=3)	female tg (n=3)	female wt (n=4)
Body weight	[g]	27.15 ± 0.9	26.06 ± 1.1	19.82 ± 2.5	21.65 ± 2.4
Spleen	[mg]	78.77 ± 12.7	79.66 ± 9.6	75.5 ± 9.8	82.77 ± 19.0
Liver	[g]	1.34 ± 59.3	1.48 ± 0.3	1.09 ± 0.0	1.18 ± 0.2
Heart	[mg]	129.8 ± 11.0	171.67 ± 19.8	104.53 ± 3.0	133.45 ± 15.0
Kidney*	[mg]	169.15 ± 2.9	188.33 ± 10.4	121.16 ± 5.9	147.93 ± 9.9

* Arithmetic mean of both kidney weights

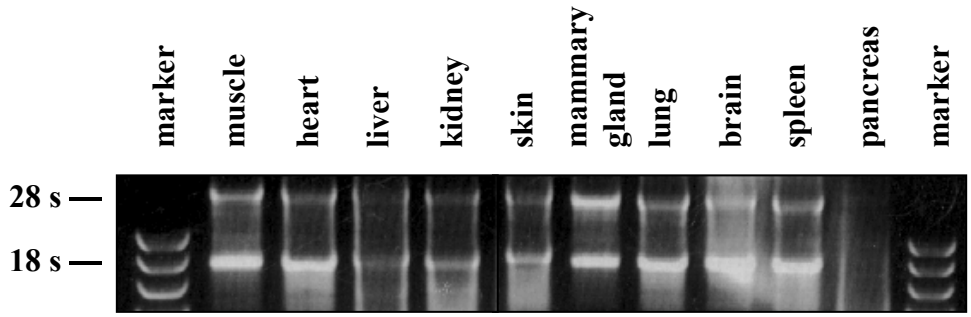

Figure 15: Total RNA isolated from mouse tissue

RNA was extracted from all tissues (Figure 15) and subjected to RT-PCR. pEGFP-HERV-H-H6 transgenic mice of lines #1, #5 and #10 showed expression of mRNA in muscle, liver, gonads, brain and lung (Table 16). In line #5 mRNA was additionally found in heart, kidney, mammary gland and spleen (Figure 16). In line #9 no transcription of EGFP was present. pBL-HERV-L transgenic mice of line #6 did not display pBL-RNA in any tissue (Figure 17).

Table 16: RT-PCR results indicating expression of EGFP mRNA in different organs of pEGFP-HERV-H-H6 transgenic mice. +: presence of EGFP cDNA amplikon; -: absence of EGFP cDNA amplikon.*ß-actin RT-PCR negative

pEGFP-HERV-H-H6 transgenic mice	muscle	heart	liver	kidney	spleen	skin	mammary gland	lung	brain	gonads
Line #1	+	-	+	-	-	-	-	-	+	+
Line #5	+	+	+	+	+	-*	+	+	+	+
Line #9	-	-	-	-	-	-	-	-	-	-
Line #10	+	-	+	-	-	-	-	+	+	+

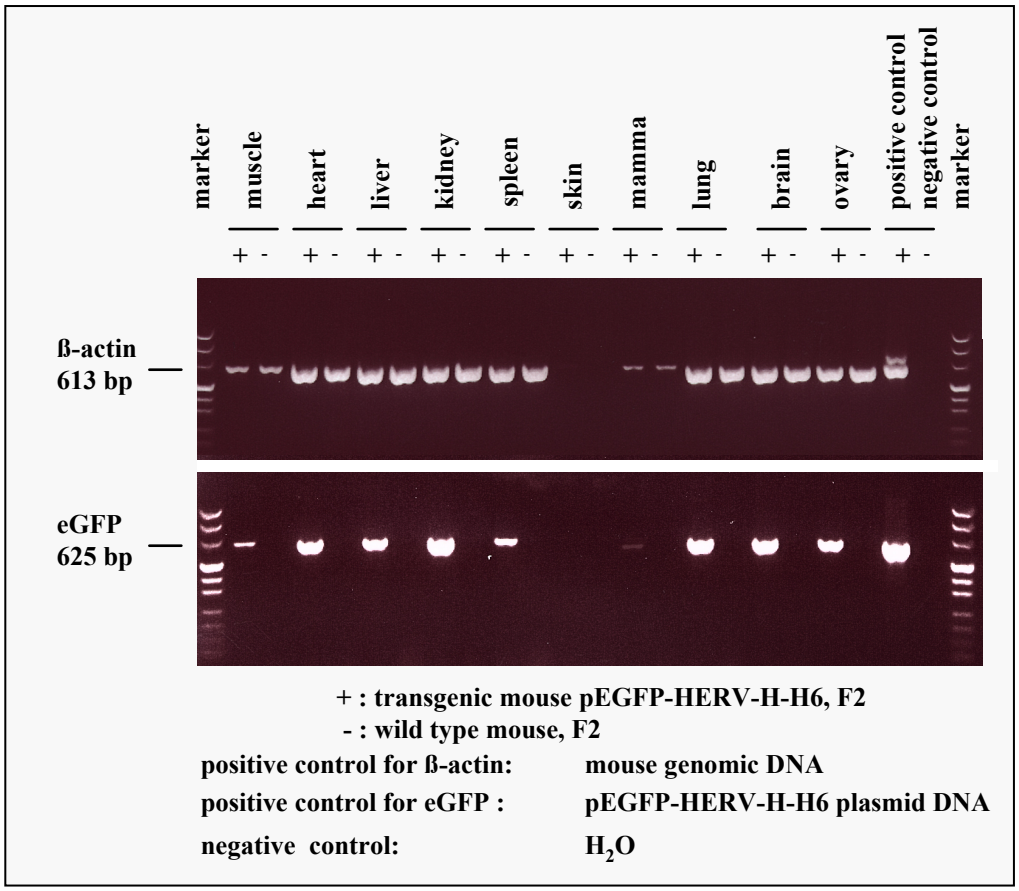

Figure 16: RT-PCR from a wildtype and a pEGFP-HERV-H-H6 transgenic mouse of line #5

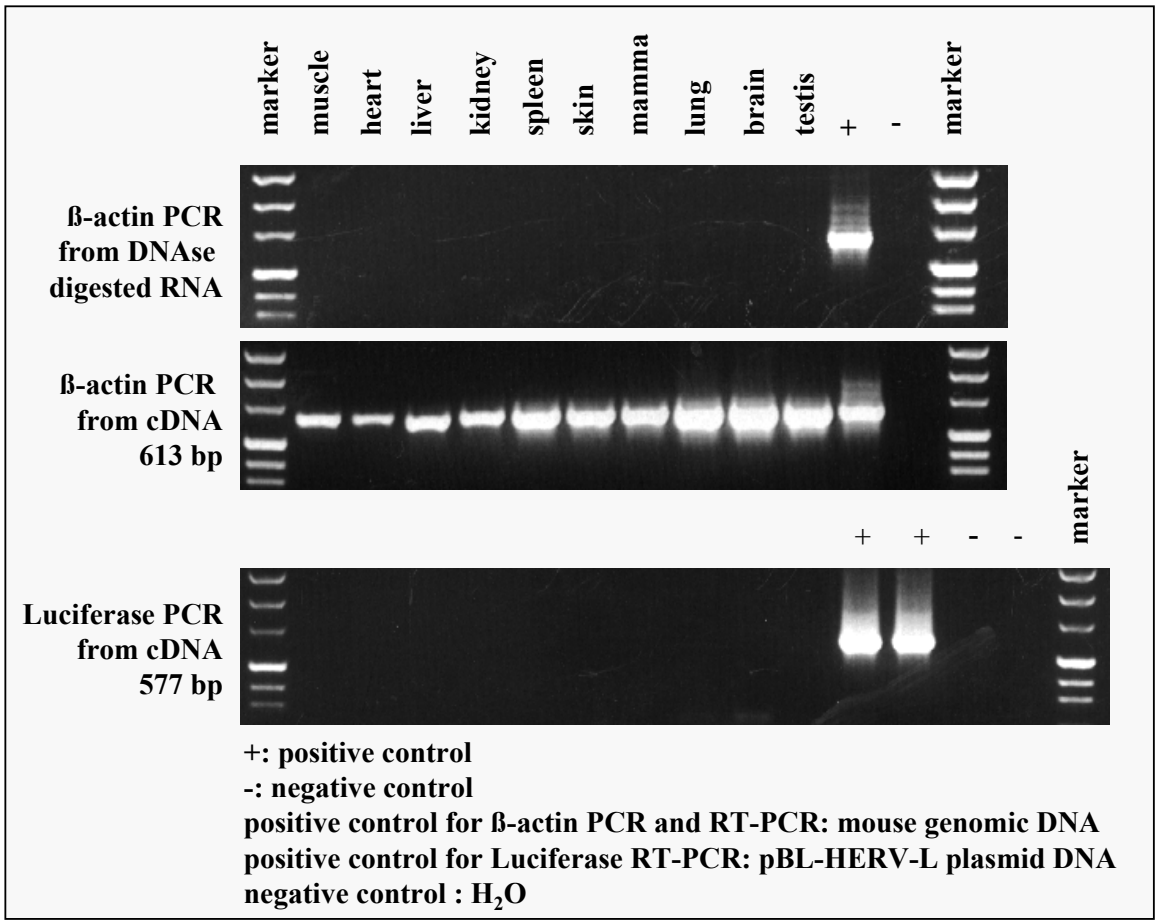

Figure 17: RT-PCR from pBL-HERV-L transgenic mice

Expression studies at the protein level were carried out in pEGFP-HERV-H-H6 mice of lines #1, #5 and #10. RNA-positive organs such as muscle, liver, gonads, brain and lung were examined for EGFP protein expression in F2 mice of both sexes at the age of 10 to 12 weeks. Western blot and histology were performed in two transgenic animals of each transgenic line and two wildtype mice. Expression of EGFP protein was detectable by Western blot in testis tissue of line #5 and #1 mice (Figure 18).

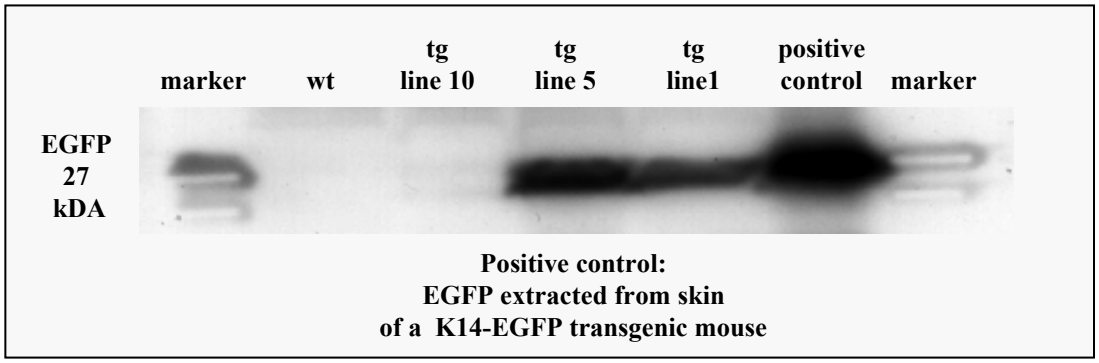

Figure 18: Western blot of testis of pEGFP-HERV-H-H6 transgenic mice

Histological sections from muscle, liver, gonads, brain and lung were prepared according to Walter et al. (2001). Four stainings of each organ were evaluated under the microscope:

- Native section mounted in mowiol
- Native section counterstained with propidium iodide and mounted in mowiol
- Immunohistochemistry
- Haematoxylin & eosin staining.

In the testis of line #5 pEGFP-HERV-H-H6 transgenic mice, the Leydig cells (Figure 19) appeared EGFP positive in mowiol and propidium iodide/mowiol treated sections (Figures 20 and 21). This could not be confirmed by immunohistochemistry (Figure 22).

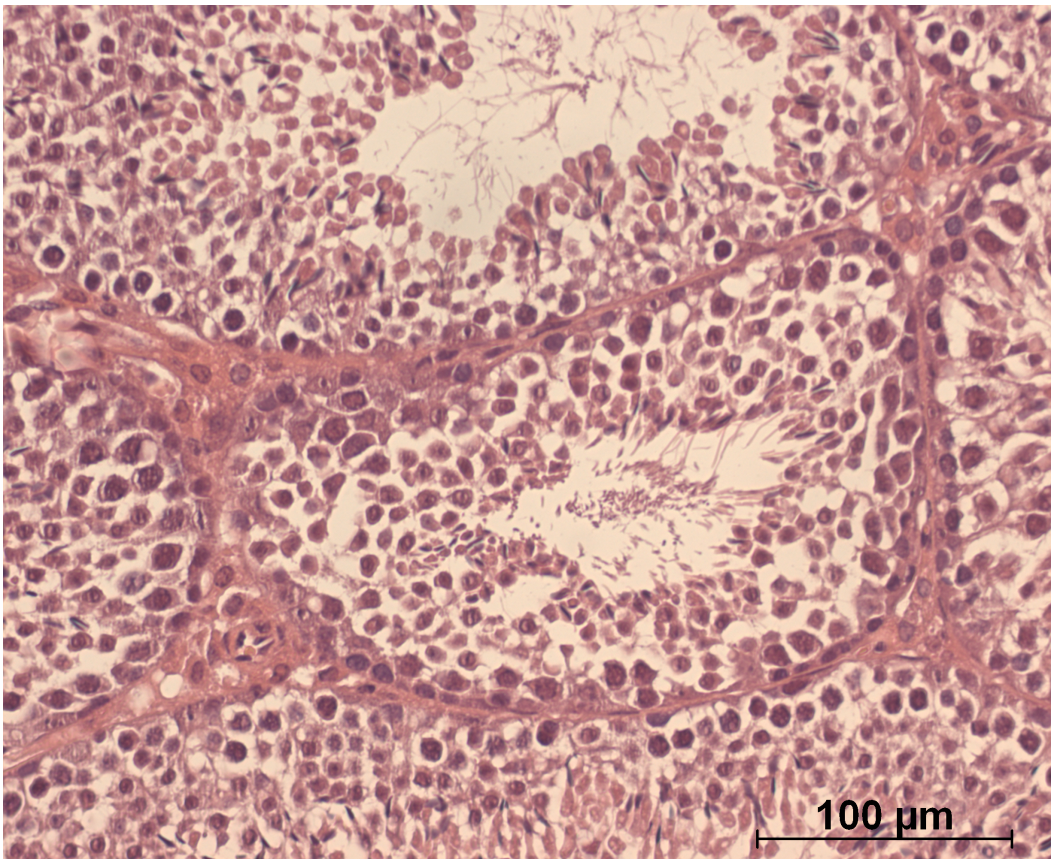

Figure 19: Haematoxylin & eosin staining of seminiferous tubules and intertubular tissue in mouse testis. The intertubular tissue contains clusters of Leydig cells, blood vessels, a lymphatic system, macrophages and fibroblasts within a loosely arranged matrix. The seminiferous tubules contain different types of germ cells.

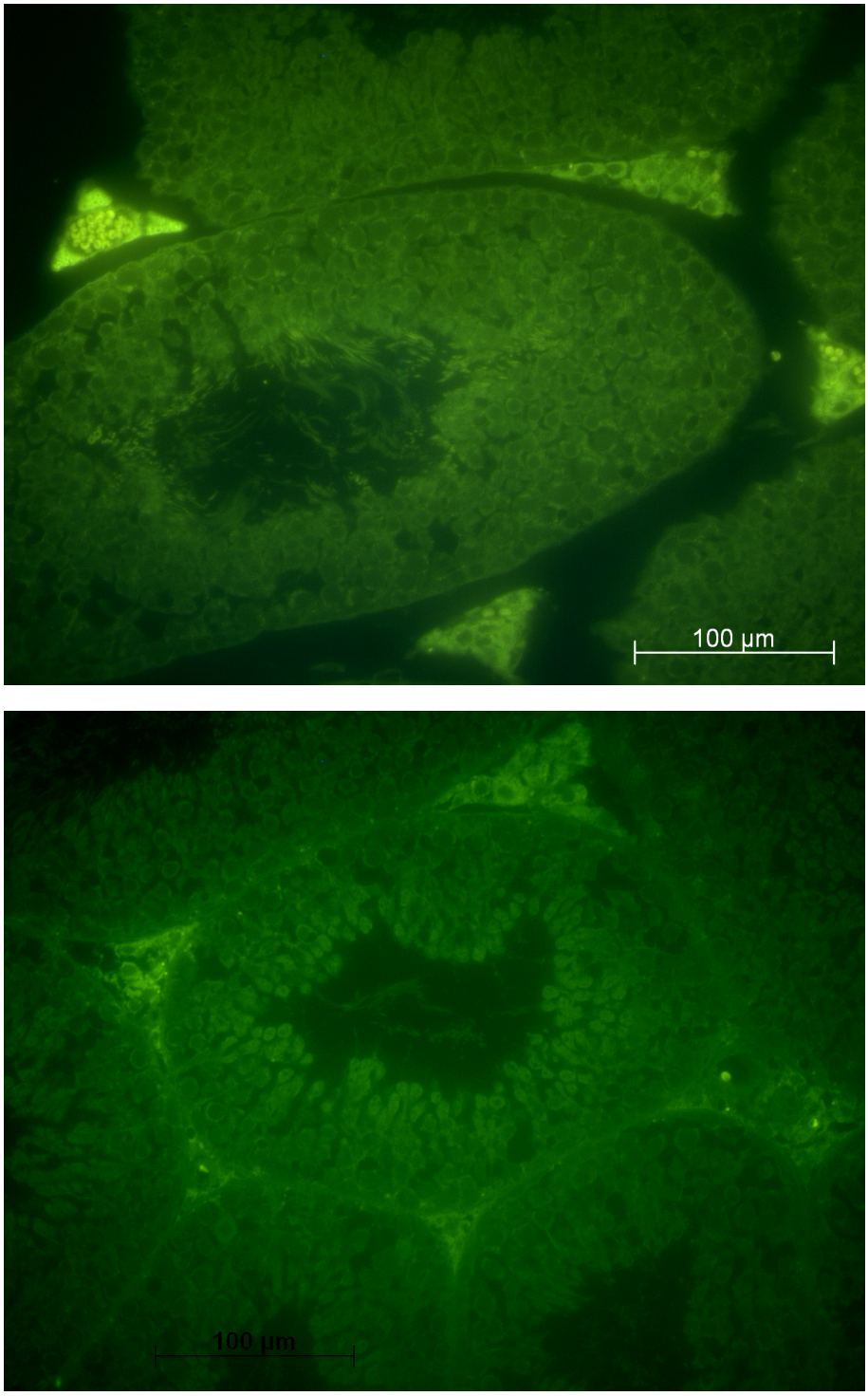

Figure 20: Native section of testis mounted in mowiol. The wildtype mouse (bottom picture) displays less fluorescence than the EGFP-HERV-H-H6 transgenic mouse of line #5 (top picture).

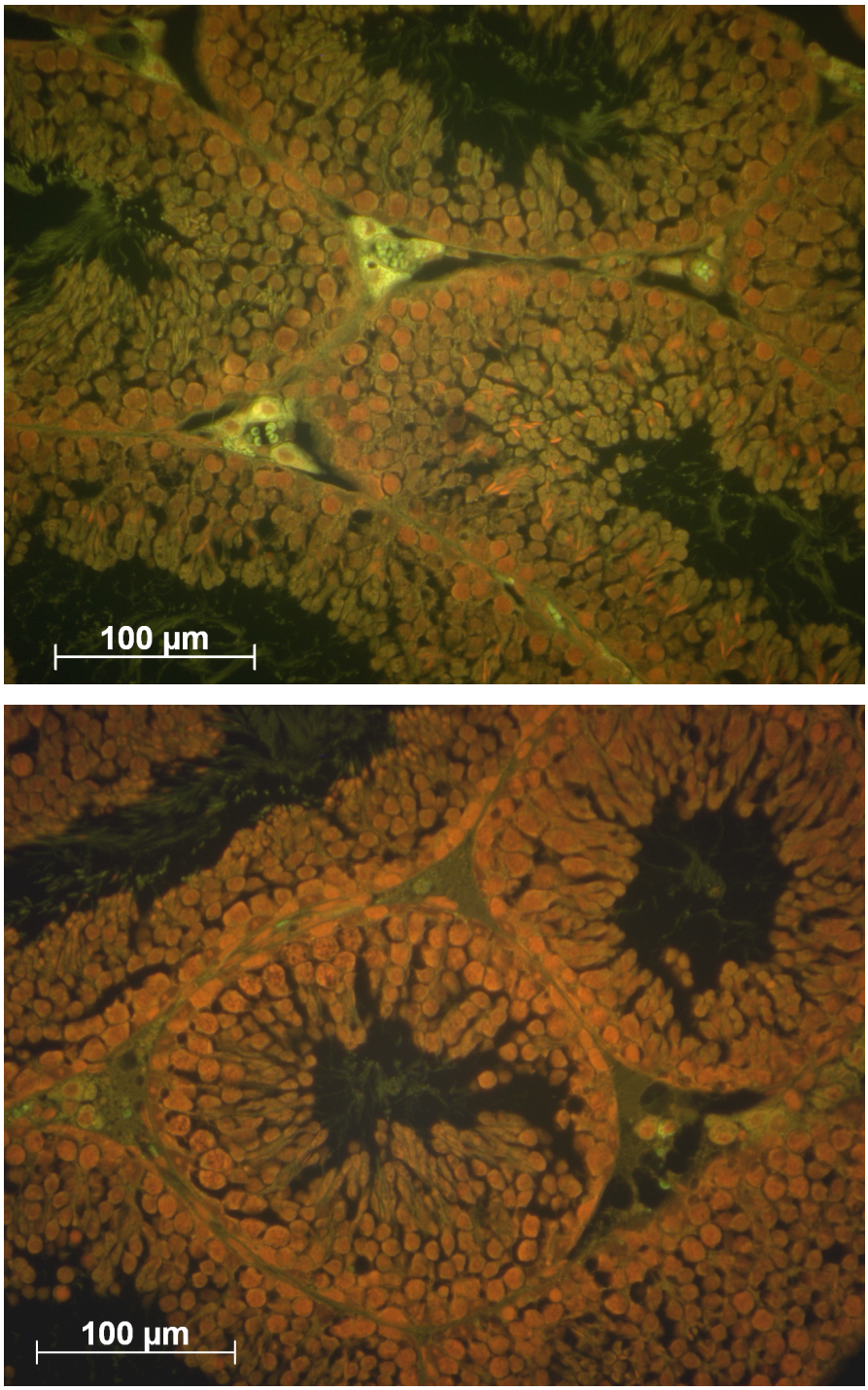

Figure 21: Native section of testis mounted with mowiol. Nuclei are stained with propidium iodide. The wildtype mouse (bottom) displays no fluorescence, whereas the EGFP-HERV-H-H6 transgenic mouse of line #5 (top) exhibits green intertubular structures such as Leydig cells.

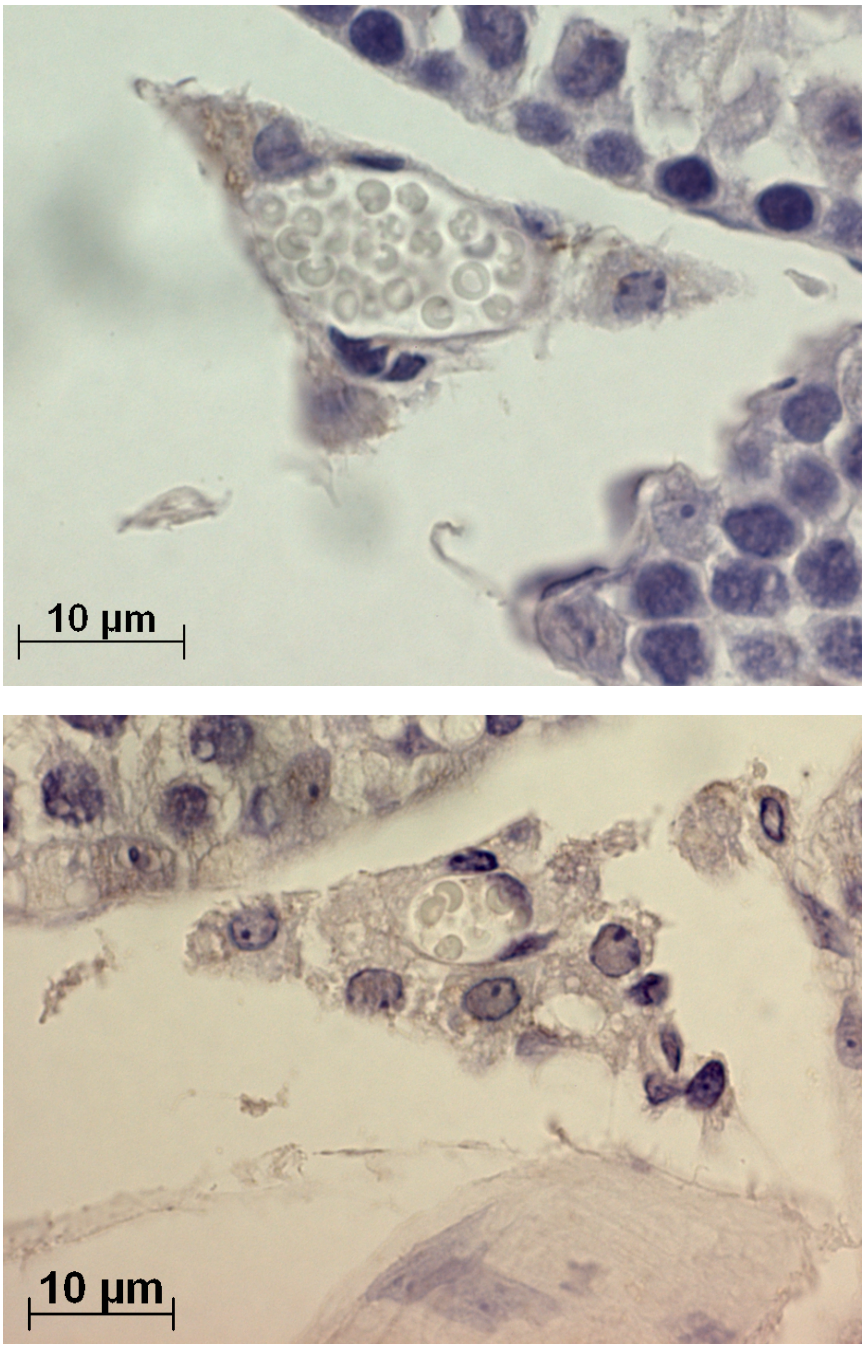

Figure 21: Immunohistochemistry revealed no remarkable difference in color between Leydig cells of wildtype mice (bottom) and Leydig cells of pEGFP-HERV-H-H6 transgenic mice of line #5 (top). EGFP positive cells are marked by peroxidase labeled antibodies. Oxidation of the substrate, 0.1% 3,3`-diaminobenzidine (DAB) hydrochloride, would result in brown color of the labelled cell.

5 DISCUSSION

5.1 Analysis of transgene integration

If a non-mosaic founder with one integration site of the transgene is mated to a wildtype animal, the expected number of transgenic animals in the F1 and in subsequent generations is 50% (Gannon et al., 1990). The proportion expected in the case of a non-mosaic founder carrying two integration sites is 75%. As the proportion of transgenic F1 animals obtained from founders with germline transmission did not differ significantly from 50%, one integration site is probable in each pEGFP-HERV-H-H6 transgenic line and in the pBL-HERV-L transgenic line. For the pEGFP-HERV-H-H6 transgenic lines #1, #5 and #10, Southern blot analysis confirmed the conclusion drawn from the breeding data by displaying single bands.

5.2 Expression level and pattern in pBL-HERV-L transgenic mice

From 9 transgenic founder mice, one transgenic line was bred to generation F2. Animals of generation F2 did not show expression of luciferase RNA in 10 investigated organs. The establishment of a single transgenic line allows only very limited conclusions on the activity of the HERV-L promoter. The only conclusion that can be drawn is that the promoter is not active in the investigated transgenic line. However, this might be due to position effects. As promoters and enhancers in the vicinity of the integration site can influence transgene expression (Hammes and Schedl, 2000), several lines have to be bred and evaluated to assess the activity and specificity of a gene construct. Although this possibility was not given, examination of mRNA expression in the available animals could be performed easily by RT-PCR, which yielded negative results. Further investigation of pBL-HERV-L animals was not carried out.

Nevertheless, the idea to investigate the HERV-L promoter activity in conjunction with the luciferase reporter gene in the mouse remains very promising. In the transient transfection pBLuciferase assay published by Schoen et al. (2001) the HERV-L promoter

displayed high and specific activity in human keratinocytes (HaCaT cell line). This makes the HERV-L-LTR especially interesting for the regulation of therapeutic genes in the skin. The fact that gene expression is to be monitored with pBLuciferase comprises additional advantages:

- The *in vitro* studies on promoter activity of the HERV-L-LTR have been carried out in exactly the same expression vector (pBL);
- Luciferase is a very sensitive reporter gene, allowing to exactly quantify even small amounts of protein;
- Imaging of bioluminescence in the skin of living mice should easily be achieved by systemic administration of luciferin (Honigman et al., 2001) and subsequent measurement of luminescence.

As a consequence of these considerations, we have continued to generate pBL-HERV-L transgenic mice. New lines of pBL-HERV-L transgenic mice will be examined in the near future to gain certainty about the promoter qualities of the HERV-L-LTR *in vivo*. To recognize position effects, more lines have to be established.

5.3 Expression level and pattern in pEGFP-HERV-H6 transgenic mice

From 10 transgenic founders, 4 transgenic lines were bred (#1, #5, #9, #10). mRNA expression was found in muscle, liver, gonads, brain and lung of transgenic mice from lines #1, #5 and #10. Animals of line #9 did not express EGFP-mRNA. They were not further investigated. F2 animals of both sexes of lines #1, #5 and #10 were examined for EGFP by Western blot and histology. In Western blot analysis only mice of the lines #1 and #5 showed expression of EGF-protein in testis. In mowiol and propidium iodide/ mowiol stained histological sections of testis, the Leydig cells appeared EGFP positive in line #5. This could not be confirmed by immunohistochemistry.

The fact that animals of line #9 did not express EGFP could be due to position effects. However, more factors can influence the level of transgene expression. The most important factor appears to be the integration site (position effect), but also copy number and DNA methylation play a decisive role. In some reviews, the number of inserted copies of the transgene is reported to have no influence on transgene expression (Gannon et al., 1990). This has been disproved by Garrick et al. (1998). Using the lox/Cre system of site-specific recombination, they generated transgenic mouse lines in which different numbers of a transgene were present at the same chromosomal location. Reduction in copy number resulted in a marked increase in expression of the transgene. Expression of the transgene can also be influenced by DNA methylation (Reik et al., 1987). This epigenetic modification, which is associated with the phenomenon of genetic imprinting, can completely repress the expression of a gene construct (Jaenisch, 1997).

A clear conclusion can be drawn from the positive Western blot results: EGFP is present in testis of line #1 and #5 mice. Looking only at the Western blot result, activity of the HERV-H-H6 LTR in testis could be explained by the fact that the LTR showed high activity *in vitro* in a germ cell tumor cell line (human teratocarcinoma cell line: NTera2D1; Feuchter and Mager, 1990). However, as histology indicates, the LTR is active in Leydig cells and not in germ cells. The fact that the HERV-H-H6 LTR drives expression in Leydig cells has to be explained differently. The steroid regulatory sequence TGTTCT is present in the HERV-H-H6 LTR as well as in HERV-R LTR. HERV-R is strongly expressed in human placenta and human adrenal gland. It is discussed in relation with differentiation and steroid hormone production by adrenocortical cells (Katsumata et al., 1998). Leydig cells are responsible for the production of the steroid hormone testosterone in the male mouse. The promoter activity of the HERV-H-H6 LTR could be linked to steroid hormone production. As a consequence, the HERV-H-H6 LTR should be active in the cortex of the adrenal gland (produces steroid hormones like aldosteron and cortisol), the ovary (produces the steroid hormone estrogen) and the placenta (produces estrogen), too. Why has no EGFP expression been reported in these organs? Unfortunately, neither adrenal gland nor placenta have been examined. Why was no EGFP expression detected by Western blot in

protein extracts of ovaries? This could simply be a quantity problem. As one ovary from each mouse was embedded for histology, there was only one (approx. 10 mg) ovary left for extraction of both RNA and protein. Detection of EGFP protein by Western blot is recommended (Living Colors® User Manual) for 25-75 µg of protein per polyacrylamid gel lane. The amount of ovary protein (80 ng/lane to 144 ng/lane) subjected to SDS-PAGE was much smaller than the amount of testis protein (60 µg/lane to 80 µg/lane). In addition, theca cells represent only a small fraction of the total ovary cells.

The histological methods to visualize EGFP in mouse tissue (Walter et al., 2000) have to be discussed critically. Paraffin embedded sections are known to increase background fluorescence (Ikawa et al., 1999) and GFP is unlikely to withstand the complete dehydration required for paraffin embedding (Living Colors® User Manual, 2001). Nevertheless, Walter et al. (2000) published the detection of the reporter gene in paraffin sections form organs of CMV-EGFP transgenic mice. Native tissue sections mounted in mowiol and propidium iodide/mowiol showed EGFP fluorescence in the same pattern as visualized by immunohistochemistry. Only in weakly expressing tissue such as nerves, ganglia and submandibular gland, immunohistochemistry indicated EGFP expression while no fluorescence was detected by fluorescence microscopy. The authors suggested that the very low amount of EGFP, expressed in these tissues, could only be detected by the more sensitive immunohistochemical staining. A very impressive aspect of this publication surely is the broad spectrum of investigated organs (25 organs). The intention to produce paraffin sections, exhibiting excellent morphological preservation of tissue for better localization of EGFP expressing cells, is also understandable. However, the fact that EGFP was driven by the strong CMV promoter restricts the applicability of the published method. Investigators of EGFP transgenic mice with weaker promoters than CMV (and thus weaker EGFP expression) could easily be troubled with the distinction between the strong autofluorescence, due to paraffin embedding, and the weak EGFP fluorescence. Moreover, the announced conformity of EGFP fluorescence and immunohistochemistry is surely impressive, but no further investigations have been carried out. No Western blot or EGFP fluorometry results are available to confirm and quantify the merely optical impressions of microscopy. The relation of histology to

molecular biological investigation would have been especially interesting with regard to the pEGFP-HERV-H-H6 mice.

Were the appropriate measures taken to investigate the promoter activity of the HERV-H-H6 LTR? Generally the measures that were taken to investigate the expression pattern of EGFP-HERV-H-H6 transgenic mice have produced valuable hints about the activity of the HERV-H-H6 LTR promoter: RT-PCR and Western blot results indicated tissue specificity and histology indicated the corresponding cell type. Before, mice had been screened for transgenesis by PCR and Southern blot and bred to generation F2 to obtain an inbred strain dominated genetic background (87.5% C57BL/6). During dissection, transgenic mice were checked for phenotypic effects of insertional mutagenesis by determination of specific organ weight, body weight and body length. It is questionable to perform the labour intensive weighing of mouse organs during dissection, as most phenotypic effects only occur in homozygous, transgenic mice (Wagner et al., 1983). To round off the obtained results, it would be interesting to investigate a completed organ spectrum, including the cortex of the adrenal gland and the placenta, and to quantify the available EGFP by fluorometry.

The original intention to measure the promoter activity of the HERV-H-H6 LTR in the transgenic mouse model with EGFP as reporter gene is actually conflicting. The available cell culture data on the promoter activity of the HERV-H-H6 LTR (Schoen et al., 2001) was obtained with the HERV-H-H6 LTR linked to another reporter gene than EGFP: to pBL. Influences of reporter genes on the promoters can never be excluded. The activity of the HERV-H-H6 LTR in different cell lines (Schoen et al., 2001) ranged from 20 % to 100 % relative promoter activity. This is rather weak in comparison to the HERV-L LTR with 270 % relative promoter activity. The conjunction of a weak promoter to a EGFP reporter gene is critical. As EGFP is not an enzyme but a protein containing at most one chromophore, no amplification of signals helps to detect small amounts of protein. EGFP detection by fluorescence microscopy is limited by autofluorescence. Distributed in the cell cytoplasm, 4,000 molecules per cell are detectable (CLONTECHniques, 1997). When targeted to a defined subcompartment of the cell, already 300 molecules of EGFP

are visible (Shelby et al., 1996). Histological analysis of pEGFP-HERV-H-H6 transgenic mice can thus be problematic. Similar problems can be encountered with all discussed methods for detection of EGFP in the mouse: Whole body optical imaging (Yang et al., 2000), fluorometry (Ikawa et al., 1995) and FACS analysis (Hadjantonakis and Nagy, 2000) are all methods described for strongly expressing tissue. To exactly quantify fluorescence of pEGFP-HERV-H-H6 tissue, it might be necessary to first sort out the EGFP expressing cells from tissue by FACS analysis and to subsequently perform fluorometry from the concentrated, expressing cells. After all, the intention to evaluate the expression patterns of two HERV LTRs *in vivo* in two different reporter gene systems is not advantageous. Apart from the fact that the establishment of two different detection techniques is time consuming, different influences of the reporter genes on the promoter genes cannot be denied. A direct comparison of expression levels and patterns between HERV-H-H6 and HERV-L transgenic mice can thus not be performed.

5.4 Final considerations

Investigation of promoter activity and specificity of the HERV-L and the HERV-H-H6 LTR in view to using them as control elements of therapeutic genes remains interesting. Now that the complete human genome is readily at hand, functional analysis of the indentified sequences is imperative. 1.8% of the human genome are retroviral LTRs.

In gene therapy, the use of retroviral vectors as vehicles for the delivery of therapeutic genes is a promising alternative. The ability to target the delivery and expression of therapeutic genes in retroviral vectors *in vivo* is a prerequisite for their widespread and routine usage. However heterologous promoters, inserted into retroviral vectors for safety reasons, may interact with the retroviral LTRs and influence the expression of adjacent genes negatively (Wu et al., 1996). Localization of tissue specific regulatory elements in retroviral LTRs offer the possibility to replace the complete LTR of the gene vehicle by a tissue specific and highly active heterologous LTR (Leib-Mösch, 2000). As HERV LTRs contain all regulatory elements necessary to drive targeted gene expression, they are especially suitable for the use in retroviral vectors for gene therapy.

So far, only one HERV LTR transgenic mouse model has been published (Ting et al., 1992). The *in vivo* models presented in this piece of work, offer further perspectives in functional analysis of the HERV-LTRs. It may be interesting to know that pBL-HERV-L and pEGFP-HERV-H-H6 transgenic rabbits have been produced (Agrobiogen GmbH, Hilgertshausen, Germany) to also consider species specific aspects of transgene expression. This work will be displayed in the dissertation of Zoltan Hubbes at the Department of Molecular Animal Breeding/LMU Munich. The gene construct pBL-HERV-L will be investigated in additional transgenic mouse lines. Indication that the HERV-H-H6 LTR regulates gene expression in a tissue-specific manner renders this LTR especially interesting for further studies.

6 SUMMARY

Generation of Transgenic Mice to Evaluate Promoter Activity and Specificity of two Human Endogenous Retrovirus Long Terminal Repeats

Human Endogenous Retrovirus Long Terminal Repeats (HERV-LTRs) comprise 1.8% of the human genome (52.7 Mb). These sequences contain all the signal structures necessary for the regulation of gene transcription, such as promoters, enhancers and transcription factor binding sites. There is evidence that HERV-LTRs regulate gene expression in tissue-specific manner. This potential could be used to drive the expression of therapeutic genes, delivered by retroviral vector systems, in a safe and efficient manner.

The HERV-H-H6 LTR and the HERV-L LTR were chosen for the generation of transgenic mice. Their promoter activity and specificity had prior been tested in a luciferase expression vector *in vitro* (Schoen et al., 2001). HERV-L was cloned into a luciferase expression vector and HERV-H-H6 was inserted into a enhanced green fluorescent protein (EGFP) expression vector. Transgenic mice were generated by DNA-microinjection into pronuclei of zygotes. One pBL-HERV-L transgenic line and four pEGFP-HERV-H-H6 transgenic lines were established and analyzed. While the HERV-L promoter was not active in transgenic animals, pEGFP-HERV-H-H6 was expressed in gonads of mice of two transgenic lines. As only a single, non-expressing transgenic line was available, HERV-L promoter activity and specificity could not be evaluated. Additional transgenic lines have to be established. Expression level and pattern of the HERV-H-H6 promoter indicate specificity for gonad tissue. Whether the HERV-H-H6 promoter activity is linked to steroid production in cells remains to be clarified.

Evaluating promoter activity in transgenic mice in two different expression vectors is not exclusively about the promoters, but also involves knowledge about the reporter genes. Advantages and limits of current applications of both luciferase and EGFP (with focus on the EGFP gene) are described in REVIEW OF THE LITERATURE. The conjunction of EGFP with the HERV-H-H6 promoter is to be seen critically, as all published methods

for detection of EGFP in mice are described with EGFP linked to strong promoters. Problems like autofluorescence in fluorescence microscopy might be encountered when weaker promoters, such as HERV-LTRs, drive EGFP expression.

7 ZUSAMMENFASSUNG

Untersuchungen zur Promotor-Aktivität und –Spezifität von zwei Long Terminal Repeats humaner endogener Retroviren in transgenen Mäusen

1.8% des humanen Genoms bestehen aus Long Terminal Repeats Humaner Endogener Retroviren (HERV-LTRs). Solche Sequenzen enthalten alle Strukturen, die für die Regulierung von Transkription benötigt werden: Promotoren, Enhancer and Bindungsstellen für Transkriptionsfaktoren. Es gibt Hinweise, daß HERV-LTRs die Expression von Genen gewebespezifisch regulieren können. Eingebaut in retrovirale Genfähren, könnten HERV-LTRs therapeutische Gene sicher und effizient aktivieren.

Zur Generierung transgener Mäuse wurden der HERV-H-H6 LTR und der HERV-L LTR ausgewählt. Deren Promoter Eigenschaften, wie Aktivität und Gewebespezifität, waren bereits *in vitro* untersucht worden (Schoen et al., 2001). Der HERV-L LTR wurde in einen Luciferase Expressionsvektor und der HERV-H-H6 LTR in einen Enhanced Green Fluorescent Protein (EGFP) Expressionsvektor kloniert. Transgene Mäuse enstanden durch DNA-Mikroinjektion in den Vorkern von Zygoten. Eine pBL-HERV-L transgene Linie und vier pEGFP-HERV-H-H6 transgene Linien wurden gezüchtet und auf Integration sowie Expression der Genkonstrukte untersucht. Während der HERV-L Promoter keine Aktivität zeigte, war Expression von pEGFP-HERV-H-H6 in Keimdrüsen von Mäusen aus zwei transgenen Linien nachweisbar. Da für das Genkonstrukt pBL-HERV-L nur eine einzige, nicht-exprimierende transgene Linie aufgebaut werden konnte, können keine Aussagen über die Aktivität und Gewebespezifität des HERV-L Promoters getroffen werden. Zu diesem Zwecke müssten weitere pBL-HERV-L transgene Linien untersucht werden. Das Expressionsmuster des pEGFP-HERV-H-H6 Genkonstruktes, weißt auf eine mögliche Gewebespezifität für Keimdrüsen hin. Eine eventuelle Verknüpfung der Aktivität des HERV-H-H6 LTRs mit der Produktion von Steroidhormonen müsste weitergehend geklärt werden.

Da in dieser Arbeit zwei unterschiedliche Reportergen Systeme in der Maus angewandt wurden, sind im Literaturteil Vorteile und Einschränkungen von aktuellen Nachweisverfahren beider Reportergene, mit Schwerpunkt EGFP, in Mausgewebe zusammengefasst. Die Verbindung von EGFP mit dem HERV-H-H6 Promoter ist als kritisch zu beurteilen: Alle beschriebenen Nachweisverfahren für EGFP in der Maus gründen auf Mausmodellen, in denen das EGFP von einem starken Promoter kontrolliert wurde. Bei potenziell schwächeren Promotoren, wie HERV-LTRs, können Probleme auftreten, wie z.B. Autofluoreszenz bei der Fluoreszenzmikroskopie.

8 BIBLIOGRAPHY

Abrink M, Larsson E, Hellman L (1998), Demethylation of ERV3, an endogenous retrovirus regulating the Kruppel- related zinc finger gene H-plk, in several human cell lines arrested during early monocyte development, *DNA Cell Biol.* 17: 27-37.

Anagnostopoulos AV, Mobraaten LE, Sharp JJ, Davisson MT (2001), Transgenic and knockout databases: behavioral profiles of mouse mutants, *Physiol Behav.* 73: 675-689.

Anderson MT, Tjioe IM, Lorincz MC, Parks DR, Herzenberg LA, Nolan GP, Herzenberg LA (1996), Simultaneous fluorescence-activated cell sorter analysis of two distinct transcriptional elements within a single cell using engineered green fluorescent proteins, *Proc.Natl.Acad.Sci.U.S.A* 93: 8508-8511.

Anderssen S, Sjottem E, Svineng G, Johansen T (1997), Comparative analyses of LTRs of the ERV-H family of primate-specific retrovirus-like elements isolated from marmoset, African green monkey, and man, *Virology* 234: 14-30.

Ashley WW, Jr., Russell B (2000), Tenotomy decreases reporter protein synthesis via the 3'-untranslated region of the beta-myosin heavy chain mRNA, *Am.J.Physiol Cell Physiol* 279: C257-C265.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y (1999), Production of goats by somatic cell nuclear transfer, *Nat.Biotechnol.* 17: 456-461.

Baust C, Seifarth W, Schon U, Hehlmann R, Leib-Mosch C (2001), Functional activity of HERV-K-T47D-related long terminal repeats, *Virology* 283: 262-272.

BD Biosciences Clontech (2002) Living Colors User Manual; *PT* 2040-1.

Beardmore JA. (2002) Transgenics, autotransgenics and allotransgenics. *Transgenic Research* 6, 107-108.

Benit L, De Parseval N, Casella JF, Callebaut I, Cordonnier A, Heidmann T (1997), Cloning of a new murine endogenous retrovirus, MuERV-L, with strong similarity to the human HERV-L element and with a gag coding sequence closely related to the Fv1 restriction gene, *J.Virol.* 71: 5652-5657.

Benit L, Lallemand JB, Casella JF, Philippe H, Heidmann T (1999), ERV-L elements: a family of endogenous retrovirus-like elements active throughout the evolution of mammals, *J.Virol.* 73: 3301-3308.

Benit L, Dessen P, Heidmann T (2001), Identification, phylogeny, and evolution of retroviral elements based on their envelope genes, *J.Virol.* 75: 11709-11719.

Blond JL, Lavillette D, Cheynet V, Bouton O, Oriol G, Chapel-Fernandes S, Mandrand B, Mallet F, Cosset FL (2000), An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor, *J.Virol.* 74: 3321-3329.

Boese A, Sauter M, Galli U, Best B, Herbst H, Mayer J, Kremmer E, Roemer K, Mueller-Lantzsch N (2000), Human endogenous retrovirus protein cORF supports cell transformation and associates with the promyelocytic leukemia zinc finger protein, *Oncogene* 19: 4328-4336.

Brem G, Brening B, Goodman HM, Selden RC, Graf F, Kruff B, Springman K, Hondele J, Meyer J, Winnacker EL, Kräusslich H (1985) Production of transgenic mice, rabbits and pigs by microinjection into pronuclei, *Zuchthygiene* 20: 251-252.

Brinster RL, Chen HY, Trumbauer ME, Yagle MK, Palmiter RD (1985), Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs, *Proc.Natl.Acad.Sci.U.S.A* 82: 4438-4442.

Brosius J (1999), RNAs from all categories generate retrosequences that may be exapted as novel genes or regulatory elements, *Gene* 238: 115-134.

Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C (1992), Normal development and behaviour of mice lacking the neuronal cell- surface PrP protein, *Nature* 356: 577-582.

Cabot RA, Kuhholzer B, Chan AW, Lai L, Park KW, Chong KY, Schatten G, Murphy CN, Abeydeera LR, Day BN, Prather RS (2001), Transgenic pigs produced using in vitro matured oocytes infected with a retroviral vector, *Anim Biotechnol.* 12: 205-214.

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996), Sheep cloned by nuclear transfer from a cultured cell line, *Nature* 380: 64-66.

Chada K, Magram J, Raphael K, Radice G, Lacy E, Costantini F (1985), Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice, *Nature* 314: 377-380.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994), Green fluorescent protein as a marker for gene expression, *Science* 263: 802-805.

Chan AW, Homan EJ, Ballou LU, Burns JC, Bremel RD (1998), Transgenic cattle produced by reverse-transcribed gene transfer in oocytes, *Proc.Natl.Acad.Sci.U.S.A* 95: 14028-14033.

Chen J, Thomas HF, Jin H, Jiang H, Sodek J (1996), Expression of rat bone sialoprotein promoter in transgenic mice, *J.Bone Miner.Res.* 11: 654-664.

Chen TT, Vrolijk NH, Lu JK, Lin CM, Reimschuessel R, Dunham RA (1996), Transgenic fish and its application in basic and applied research, *Biotechnol.Annu.Rev.* 2: 205-236.

Chevillard C, Ozaki J, Herring CD, Riblet R (2002), A three-megabase yeast artificial chromosome contig spanning the C57BL mouse Igh locus, *J.Immunol.* 168: 5659-5666.

Choi T, Huang M, Gorman C, Jaenisch R (1991), A generic intron increases gene expression in transgenic mice, *Mol.Cell Biol.* 11: 3070-3074.

Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW (1993), Chemical structure of the hexapeptide chromophore of the Aequorea green- fluorescent protein, *Biochemistry* 32: 1212-1218.

Cordonnier A, Casella JF, Heidmann T (1995), Isolation of novel human endogenous retrovirus-like elements with foamy virus-related pol sequence, *J.Virol.* 69: 5890-5897.

Cormack BP, Valdivia RH, Falkow S (1996), FACS-optimized mutants of the green fluorescent protein (GFP), *Gene* 173: 33-38.

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995), Understanding, improving and using green fluorescent proteins, *Trends Biochem.Sci.* 20: 448-455.

Custer RP, Bosma GC, Bosma MJ (1985), Severe combined immunodeficiency (SCID) in the mouse. Pathology, reconstitution, neoplasms, *Am.J.Pathol.* 120: 464-477.

De Parseval N, Casella J, Gressin L, Heidmann T (2001), Characterization of the three HERV-H proviruses with an open envelope reading frame encompassing the immunosuppressive domain and evolutionary history in primates, *Virology* 279: 558-569.

de Wet JR, Wood KV, Helinski DR, DeLuca M (1985), Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli, *Proc.Natl.Acad.Sci.U.S.A* 82: 7870-7873.

de Wet JR, Wood KV, DeLuca M, Helinski DR, Subramani S (1987), Firefly luciferase gene: structure and expression in mammalian cells, *Mol.Cell Biol.* 7: 725-737.

Elowitz MB, Surette MG, Wolf PE, Stock J, Leibler S (1997), Photoactivation turns green fluorescent protein red, *Curr.Biol.* 7: 809-812.

Feuchter-Murthy AE, Freeman JD, Mager DL (1993), Splicing of a human endogenous retrovirus to a novel phospholipase A2 related gene, *Nucleic Acids Res.* 21: 135-143.

Feuchter A, Mager D (1990), Functional heterogeneity of a large family of human LTR-like promoters and enhancers, *Nucleic Acids Res.* 18: 1261-1270.

Funahashi H, Ideta A, Konishi M, Urakawa M, Uruno K, Aoyagi Y, Okabe M, Niwa K (2001), Nuclear Transfer of Blastomeres Expressing EGFP-Reporter Gene May Improve the Efficiency of Transgenic Cattle, *Cloning Stem Cells* 3: 183-190.

Gannon F, Powell R, Barry T, McEvoy TG, Sreenan JM (1990), Transgenic farm animals, *J.Biotechnol.* 16: 155-170.

Garrick D, Fiering S, Martin DI, Whitelaw E (1998), Repeat-induced gene silencing in mammals, *Nat.Genet.* 18: 56-59.

Gervaix A, West D, Leoni LM, Richman DD, Wong-Staal F, Corbeil J (1997), A new reporter cell line to monitor HIV infection and drug susceptibility in vitro, *Proc.Natl.Acad.Sci.U.S.A* 94: 4653-4658.

Geusz ME, Fletcher C, Block GD, Straume M, Copeland NG, Jenkins NA, Kay SA, Day RN (1997), Long-term monitoring of circadian rhythms in c-fos gene expression from suprachiasmatic nucleus cultures, *Curr.Biol.* 7: 758-766.

Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980), Genetic transformation of mouse embryos by microinjection of purified DNA, *Proc.Natl.Acad.Sci.U.S.A* 77: 7380-7384.

Gordon JW, Ruddle FH (1981), Integration and stable germ line transmission of genes injected into mouse pronuclei, *Science* 214: 1244-1246.

Gordon JW, Ruddle FH (1982), Germ line transmission in transgenic mice, *Prog.Clin.Biol.Res.* 85 Pt B: 111-124.

Gordon JW (1993), Production of transgenic mice, *Methods Enzymol.* 225: 747-771.

Gordon JW (1997), Transgenic Technology and Laboratory Animal Science, *ILAR.J.* 38: 32-41.

Gossen M, Bujard H (1992), Tight control of gene expression in mammalian cells by tetracycline- responsive promoters, *Proc.Natl.Acad.Sci.U.S.A* 89: 5547-5551.

Green MC (1981) Genetic Variants and Strains of the Laboratory Mouse. Stuttgart, Germany, Fischer.

Hadjantonakis AK, Nagy A (2000), FACS for the isolation of individual cells from transgenic mice harboring a fluorescent protein reporter, *Genesis.* 27: 95-98.

Hagenfeldt-Johansson KA, Herrera PL, Wang H, Gjinovci A, Ishihara H, Wollheim CB (2001), Beta-cell-targeted expression of a dominant-negative hepatocyte nuclear

factor-1 alpha induces a maturity-onset diabetes of the young (MODY)3- like phenotype in transgenic mice, *Endocrinology* 142: 5311-5320

Hammes A, Schedl A. (2000), Generation of transgenic mice from plasmids, BACs and YACs.In: *Mouse genetics and Transgenesis A Practical Approach*. 217-245. Oxford, Oxford University Press.

Hanahan D (1989), Transgenic mice as probes into complex systems, *Science* 246: 1265-1275.

Heim R, Cubitt AB, Tsien RY (1995), Improved green fluorescence, *Nature* 373: 663-664.

Herbst H, Sauter M, Kuhler-Obbarius C, Loning T, Mueller-Lantzsch N (1998), Human endogenous retrovirus (HERV)-K transcripts in germ cell and trophoblastic tumours, *APMIS* 106: 216-220.

Hirose Y, Takamatsu M, Harada F (1993), Presence of env genes in members of the RTVL-H family of human endogenous retrovirus-like elements, *Virology* 192: 52-61.

Hishikawa T, Ogasawara H, Kaneko H, Shirasawa T, Matsuura Y, Sekigawa I, Takasaki Y, Hashimoto H, Hirose S, Handa S, Nagasawa R, Maruyama N (1997), Detection of antibodies to a recombinant gag protein derived from human endogenous retrovirus clone 4-1 in autoimmune diseases, *Viral Immunol.* 10: 137-147.

Hoff CR, Perkins DR, Davidson JM (1999), Elastin gene expression is upregulated during pulmonary fibrosis, *Connect.Tissue Res.* 40: 145-153.

Hogan B, Beddington R, Constantini FLE (1994) Manipulating the mouse embryo: A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Hong YH, Moon YK, Jeong DK, Han JY (1998), Improved transfection efficiency of chicken gonadal primordial germ cells for the production of transgenic poultry, *Transgenic Res.* 7: 247-252.

Honigman A, Zeira E, Ohana P, Abramovitz R, Tavor E, Bar I, Zilberman Y, Rabinovsky R, Gazit D, Joseph A, Panet A, Shai E, Palmon A, Laster M, Galun E (2001), Imaging transgene expression in live animals, *Mol.Ther.* 4: 239-249.

Hoppe-Seyler F, Butz K (1991), Repression of the human papillomavirus type 18 enhancer by the cellular transcription factor Oct-1, *J.Virol.* 65: 5613-5618.

Hrabe dA, Balling R (1998), Large scale ENU screens in the mouse: genetics meets genomics, *Mutat.Res.* 400: 25-32.

Ikawa M, Kominami K, Yoshimura Y, Tanaka K, Nishimune Y, Okabe M (1995) Green fluorescent protein as a marker in transgenic mice. *Develop.Growth Differ.* 37: 455-459.

Ikawa M, Yamada S, Nakanishi T, Okabe M (1999), Green fluorescent protein (GFP) as a vital marker in mammals, *Curr.Top.Dev.Biol.* 44: 1-20.

Inouye S, Tsuji FI (1994), Evidence for redox forms of the Aequorea green fluorescent protein, *FEBS Lett.* 351: 211-214.

Inouye S, Tsuji FI (1994), Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein, *FEBS Lett.* 341: 277-280.

Jaenisch R (1997), DNA methylation and imprinting: why bother?, *Trends Genet.* 13: 323-329.

Johnson FH, Shimomura O, Saiga Y, Gershman LC, Reynolds GT, Waters JR (1962) Quantum Efficiency of *Cypridina* luminescence, with a note on that of *Aequorea*. *J.Cell.Comp.Physiol*. 60: 85-104.

Johnston JB, Silva C, Holden J, Warren KG, Clark AW, Power C (2001), Monocyte activation and differentiation augment human endogenous retrovirus expression: implications for inflammatory brain diseases, *Ann.Neurol.* 50: 434-442.

Jonas AM (1984), The mouse in biomedical research, *Physiologist* 27: 330-346.

Kaneko S, Onodera M, Fujiki Y, Nagasawa T, Nakauchi H (2001), Simplified retroviral vector gcsap with murine stem cell virus long terminal repeat allows high and continued expression of enhanced green fluorescent protein by human hematopoietic progenitors engrafted in nonobese diabetic/severe combined immunodeficient mice, *Hum.Gene Ther.* 12: 35-44.

Karlsson H, Bachmann S, Schroder J, McArthur J, Torrey EF, Yolken RH (2001), Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia, *Proc.Natl.Acad.Sci.U.S.A* 98: 4634-4639.

Kato M, Yamanouchi K, Ikawa M, Okabe M, Naito K, Tojo H (1999), Efficient selection of transgenic mouse embryos using EGFP as a marker gene, *Mol.Reprod.Dev.* 54: 43-48.

Kato N, Shimotohno K, Van Leeuwen D, Cohen M (1990), Human proviral mRNAs down regulated in choriocarcinoma encode a zinc finger protein related to Kruppel, *Mol.Cell Biol.* 10: 4401-4405.

Katsuki M, Sato M, Kimura M, Yokoyama M, Kobayashi K, Nomura T (1988), Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice, *Science* 241: 593-595.

Kelleher CA, Wilkinson DA, Freeman JD, Mager DL, Gelfand EW (1996), Expression of novel-transposon-containing mRNAs in human T cells, *J.Gen.Virol.* 77 (Pt 5): 1101-1110.

Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lubbert H, Bujard H (1996), Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice, *Proc.Natl.Acad.Sci.U.S.A* 93: 10933-10938.

Klein D, Bugl B, Gunzburg WH, Salmons B (2000), Accurate estimation of transduction efficiency necessitates a multiplex real-time PCR, *Gene Ther.* 7: 458-463.

Kondoh G, Gao XH, Nakano Y, Koike H, Yamada S, Okabe M, Takeda J (1999), Tissue-inherent fate of GPI revealed by GPI-anchored GFP transgenesis, *FEBS Lett.* 458: 299-303.

Kowalski PE, Mager DL (1998), A human endogenous retrovirus suppresses translation of an associated fusion transcript, PLA2L, *J.Virol.* 72: 6164-6168.

Kowalski PE, Freeman JD, Mager DL (1999), Intergenic splicing between a HERV-H endogenous retrovirus and two adjacent human genes, *Genomics* 57: 371-379.

Kozak M (1987), An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs, *Nucleic Acids Res.* 15: 8125-8148.

Kozak M (1989), Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems, *Mol.Cell Biol.* 9: 5073-5080.

Krovel AV, Olsen LC (2002), Expression of a vas::EGFP transgene in primordial germ cells of the zebrafish, *Mech.Dev.* 116: 141-150.

Kubisch HM, Larson MA, Eichen PA, Wilson JM, Roberts RM (1997), Adenovirus-mediated gene transfer by perivitelline microinjection of mouse, rat, and cow embryos, *Biol.Reprod.* 56: 119-124.

Lahti PP, Shariatmadari R, Penttinen JK, Drevet JR, Haendler B, Vierula M, Parvinen M, Huhtaniemi IT, Poutanen M (2001), Evaluation of the 5'-flanking regions of murine glutathione peroxidase five and cysteine-rich secretory protein-1 genes for directing transgene expression in mouse epididymis, *Biol.Reprod.* 64: 1115-1121.

Lai L, Park KW, Cheong HT, Kuhholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS (2002), Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells, *Mol.Reprod.Dev.* 62: 300-306.

Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H (1992), Targeted oncogene activation by site-specific recombination in transgenic mice, *Proc.Natl.Acad.Sci.U.S.A* 89: 6232-6236.

Lala DS, Rice DA, Parker KL (1992), Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I, *Mol.Endocrinol.* 6: 1249-1258.

Leib-Mösch C (2000), Herstellung brustkrebs-spezifischer Vektoren. In: *Forschungsverbund Grundlagen Gentechnischer Verfahren.* Zwischenbericht 2000: 17-27.

Leib-Mösch C, Schön U (2001), Herstellung Brustkrebs-Spezifischer Vektoren. In: *Forschungsverbund Grundlagen Gentechnischer Verfahren*. Zwischenbericht 2001: 15-20.

Lindeskog M, Medstrand P, Blomberg J (1993), Sequence variation of human endogenous retrovirus ERV9-related elements in an env region corresponding to an immunosuppressive peptide: transcription in normal and neoplastic cells, *J.Virol.* 67: 1122-1126.

Ling V, Wu PW, Finnerty HF, Agostino MJ, Graham JR, Chen S, Jussiff JM, Fisk GJ, Miller CP, Collins M (2001), Assembly and annotation of human chromosome 2q33 sequence containing the CD28, CTLA4, and ICOS gene cluster: analysis by computational, comparative, and microarray approaches, *Genomics* 78: 155-168.

Lo CW, Coulling M, Kirby C (1987), Tracking of mouse cell lineage using microinjected DNA sequences: analyses using genomic Southern blotting and tissue-section in situ hybridizations, *Differentiation* 35: 37-44.

Lohmann W, Paul E (1989), Native fluorescence of unstained cryo-sections of the skin with melanomas and nevi, *Naturwissenschaften* 76: 424-426.

Lower R, Boller K, Hasenmaier B, Korbmacher C, Muller-Lantzsch N, Lower J, Kurth R (1993), Identification of human endogenous retroviruses with complex mRNA expression and particle formation, *Proc.Natl.Acad.Sci.U.S.A* 90: 4480-4484.

Lower R, Lower J, Kurth R (1996), The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences, *Proc.Natl.Acad.Sci.U.S.A* 93: 5177-5184.

Luckow B, Schutz G (1987), CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements, *Nucleic Acids Res.* 15: 5490.

Mager DL, Henthorn PS (1984), Identification of a retrovirus-like repetitive element in human DNA, *Proc.Natl.Acad.Sci.U.S.A* 81: 7510-7514.

Mager DL, Freeman JD (1995), HERV-H endogenous retroviruses: presence in the New World branch but amplification in the Old World primate lineage, *Virology* 213: 395-404.

Majors J (1990), The structure and function of retroviral long terminal repeats, *Curr.Top.Microbiol.Immunol.* 157: 49-92.

Mangeney M, De Parseval N, Thomas G, Heidmann T (2001), The full-length envelope of an HERV-H human endogenous retrovirus has immunosuppressive properties, *J.Gen.Virol.* 82: 2515-2518.

Manthorpe M, Cornefert-Jensen F, Hartikka J, Felgner J, Rundell A, Margalith M, Dwarki V (1993), Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice, *Hum.Gene Ther.* 4: 419-431.

Maruyama J, Nakajima H, Kitamoto K (2001), Visualization of nuclei in Aspergillus oryzae with EGFP and analysis of the number of nuclei in each conidium by FACS, *Biosci.Biotechnol.Biochem.* 65: 1504-1510.

Maurer B, Serfling E, Rethwilm A (1991), Transcription factor AP-1 modulates the activity of the human foamy virus long terminal repeat, *J.Virol.* 65: 6353-6357.

McGeoch DJ (1990), Protein sequence comparisons show that the 'pseudoproteases' encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family, *Nucleic Acids Res.* 18: 4105-4110.

McKnight RA, Spencer M, Wall RJ, Hennighausen L (1996), Severe position effects imposed on a 1 kb mouse whey acidic protein gene promoter are overcome by heterologous matrix attachment regions, *Mol.Reprod.Dev.* 44: 179-184.

Medstrand P, Lindeskog M, Blomberg J (1992), Expression of human endogenous retroviral sequences in peripheral blood mononuclear cells of healthy individuals, *J.Gen.Virol.* 73 (Pt 9): 2463-2466.

Mepham TB, Combes RD, Balls M, Barbieri O, Blokhuis HJ, Costa P, Crilly RE, de Cock BT, Delpire VC, O'Hare MJ, Houdebine LM, van Kreijl CF, van der MM, Reinhardt CA, Wolf E, van Zeller AM (1998), The Use of Transgenic Animals in the European Union: The Report and Recommendations of ECVAM Workshop 28, *Altern.Lab Anim* 26: 21-43.

Metzger D, Clifford J, Chiba H, Chambon P (1995), Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase, *Proc.Natl.Acad.Sci.U.S.A* 92: 6991-6995.

Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC, Jr., McCoy JM (2000), Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis, *Nature* 403: 785-789.

Miserey-Lenkei S, Lenkei Z, Parnot C, Corvol P, Clauser E (2001), A functional enhanced green fluorescent protein (EGFP)-tagged angiotensin II at(1a) receptor recruits the endogenous Galphaq/11 protein to the membrane and induces its specific internalization independently of receptor-g protein coupling in HEK-293 cells, *Mol.Endocrinol.* 15: 294-307.

Mold DE, Wu TC, Askin F, Huang RC (1997), Four Classes of HERV-K Long Terminal Repeats and Their Relative Promoter Strengths for Transcription, *J.Biomed.Sci.* 4: 78-82.

Moore R, Dixon M, Smith R, Peters G, Dickson C (1987), Complete nucleotide sequence of a milk-transmitted mouse mammary tumor virus: two frameshift suppression events are required for translation of gag and pol, *J.Virol.* 61: 480-490.

Morin JG, Hastings JW (1971), Biochemistry of the bioluminescence of colonial hydroids and other coelenterates, *J.Cell Physiol* 77: 305-312.

Morise H, Shimomura O, Johnson FH, Winant J (1974), Intermolecular energy transfer in the bioluminescent system of Aequorea, *Biochemistry* 13: 2656-2662.

Mrochen S, Klein D, Nikol S, Smith JR, Salmons B, Gunzburg WH (1997), Inducible expression of p21WAF-1/CIP-1/SDI-1 from a promoter conversion retroviral vector, *J.Mol.Med.* 75: 820-828.

Nelson DT, Goodchild NL, Mager DL (1996), Gain of Sp1 sites and loss of repressor sequences associated with a young, transcriptionally active subset of HERV-H endogenous long terminal repeats, *Virology* 220: 213-218.

Niswender KD, Blackman SM, Rohde L, Magnuson MA, Piston DW (1995), Quantitative imaging of green fluorescent protein in cultured cells: comparison of microscopic techniques, use in fusion proteins and detection limits, *J.Microsc.* 180 (Pt 2): 109-116.

No D, Yao TP, Evans RM (1996), Ecdysone-inducible gene expression in mammalian cells and transgenic mice, *Proc.Natl.Acad.Sci.U.S.A* 93: 3346-3351.

Nolte C, Matyash M, Pivneva T, Schipke CG, Ohlemeyer C, Hanisch UK, Kirchhoff F, Kettenmann H (2001), GFAP promoter-controlled EGFP-expressing transgenic mice: a tool to visualize astrocytes and astrogliosis in living brain tissue, *Glia* 33: 72-86.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997), 'Green mice' as a source of ubiquitous green cells, *FEBS Lett.* 407: 313-319.

Ono M, Kawakami M, Ushikubo H (1987), Stimulation of expression of the human endogenous retrovirus genome by female steroid hormones in human breast cancer cell line T47D, *J.Virol.* 61: 2059-2062.

Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ (1996), Crystal structure of the Aequorea victoria green fluorescent protein, *Science* 273: 1392-1395.

Paces J, Pavlicek A, Paces V (2002), HERVd: database of human endogenous retroviruses, *Nucleic Acids Res.* 30: 205-206.

Paigen K (2002), Understanding the human condition: experimental strategies in Mammalian genetics, *ILAR.J.* 43: 123-135.

Palmiter RD, Sandgren EP, Avarbock MR, Allen DD, Brinster RL (1991), Heterologous introns can enhance expression of transgenes in mice, *Proc.Natl.Acad.Sci.U.S.A* 88: 478-482.

Pascual M, Martin J, Nieto A, Giphart MJ, van der Slik AR, de Vries RR, Zanelli E (2001), Distribution of HERV-LTR elements in the 5'-flanking region of HLA-DQB1 and association with autoimmunity, *Immunogenetics* 53: 114-118.

Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW (1997), Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy, *Biophys.J.* 73: 2782-2790.

Pepin MC, Pothier F, Barden N (1992), Impaired type II glucocorticoid-receptor function in mice bearing antisense RNA transgene, *Nature* 355: 725-728.

Perera OP, Harrell II RA, Handler AM (2002), Germ-line transformation of the South American malaria vector, Anopheles albimanus, with a piggyBac/EGFP transposon vector is routine and highly efficient, *Insect Mol.Biol.* 11: 291-297.

Pfeifer A, Verma IM (2001), Gene therapy: promises and problems, *Annu.Rev.Genomics Hum.Genet.* 2: 177-211.

Pfeifer A, Kessler T, Yang M, Baranov E, Kootstra N, Cheresh DA, Hoffman RM, Verma IM (2001), Transduction of liver cells by lentiviral vectors: analysis in living animals by fluorescence imaging, *Mol.Ther.* 3: 319-322.

Pfeifer A, Ikawa M, Dayn Y, Verma IM (2002), Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos, *Proc.Natl.Acad.Sci.U.S.A* 99: 2140-2145.

Phillips GN, Jr. (1997), Structure and dynamics of green fluorescent protein, *Curr.Opin.Struct.Biol.* 7: 821-827.

Pinkert CA (1994), Transgenic Animal Technology. Academic Press.

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ (1992), Primary structure of the Aequorea victoria green-fluorescent protein, *Gene* 111: 229-233.

Rathkolb B, Fuchs E, Kolb HJ, Renner-Muller I, Krebs O, Balling R, Hrabe dA, Wolf E (2000), Large-scale N-ethyl-N-nitrosourea mutagenesis of mice--from phenotypes to genes, *Exp.Physiol* 85: 635-644.

Reik W, Collick A, Norris ML, Barton SC, Surani MA (1987), Genomic imprinting determines methylation of parental alleles in transgenic mice, *Nature* 328: 248-251.

Russell WL, Kelly EM, Hunsicker PR, Bangham JW, Maddux SC, Phipps EL (1979), Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse, *Proc.Natl.Acad.Sci.U.S.A* 76: 5818-5819.

Ryding AD, Sharp MG, Mullins JJ (2001), Conditional transgenic technologies, *J.Endocrinol.* 171: 1-14.

Saez E, Nelson MC, Eshelman B, Banayo E, Koder A, Cho GJ, Evans RM (2000), Identification of ligands and coligands for the ecdysone-regulated gene switch, *Proc.Natl.Acad.Sci.U.S.A* 97: 14512-14517.

Saller RM, Ozturk F, Salmons B, Gunzburg WH (1998), Construction and characterization of a hybrid mouse mammary tumor virus/murine leukemia virus-based retroviral vector, *J.Virol.* 72: 1699-1703.

Salmons B, Gunzburg WH (1993), Targeting of retroviral vectors for gene therapy, *Hum.Gene Ther.* 4: 129-141.

Sambrok J, Fritsch EF, Maniatis T (2001), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.

Samuelson LC, Wiebauer K, Gumucio DL, Meisler MH (1988), Expression of the human amylase genes: recent origin of a salivary amylase promoter from an actin pseudogene, *Nucleic Acids Res.* 16: 8261-8276.

Sano H, Nakamura A, Kobayashi S (2002), Identification of a transcriptional regulatory region for germline- specific expression of vasa gene in Drosophila melanogaster, *Mech.Dev.* 112: 129-139.

Schon U, Seifarth W, Baust C, Hohenadl C, Erfle V, Leib-Mosch C (2001), Cell type-specific expression and promoter activity of human endogenous retroviral long terminal repeats, *Virology* 279: 280-291.

Seifarth W, Skladny H, Krieg-Schneider F, Reichert A, Hehlmann R, Leib-Mosch C (1995), Retrovirus-like particles released from the human breast cancer cell line T47-D display ty, *J.Virol.* 69: 6408-6416.

Seifarth W, Baust C, Murr A, Skladny H, Krieg-Schneider F, Blusch J, Werner T, Hehlmann R, Leib-Mosch C (1998), Proviral structure, chromosomal location, and expression of HERV-K- T47D, a novel human endogenous retrovirus derived from T47D particles, *J.Virol.* 72: 8384-8391.

Seifarth W, Baust C, Schon U, Reichert A, Hehlmann R, Leib-Mosch C (2000), HERV-IP-T47D, a novel type C-related human endogenous retroviral sequence derived from T47D particles, *AIDS Res.Hum.Retroviruses* 16: 471-480.

Shelby RD, Hahn KM, Sullivan KF (1996), Dynamic elastic behavior of alpha-satellite DNA domains visualized in situ in living human cells, *J.Cell Biol.* 135: 545-557.

Shimomura O, Johnson FH, Saiga Y (1962), Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J.Cell.Comp.Physiol.* 59: 223-239.

Shockett P, Difilippantonio M, Hellman N, Schatz DG (1995), A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice, *Proc.Natl.Acad.Sci.U.S.A* 92: 6522-6526.

Siegel MS, Isacoff EY (1997), A genetically encoded optical probe of membrane voltage, *Neuron* 19: 735-741.

Silver LM (1995), Mouse Genetics, Oxford, Oxford University Press.

Smit AF (1999), Interspersed repeats and other mementos of transposable elements in mammalian genomes, *Curr.Opin.Genet.Dev.* 9: 657-663.

Suzuki H, Hosokawa Y, Toda H, Nishikimi M, Ozawa T (1990), Common protein-binding sites in the 5'-flanking regions of human genes for cytochrome c1 and ubiquinone-binding protein, *J.Biol.Chem.* 265: 8159-8163.

Swoap SJ (1998), In vivo analysis of the myosin heavy chain IIB promoter region, *Am.J.Physiol* 274: C681-C687.

Tavare JM, Fletcher LM, Welsh GI (2001), Using green fluorescent protein to study intracellular signalling, *J.Endocrinol.* 170: 297-306.

Ting CN, Rosenberg MP, Snow CM, Samuelson LC, Meisler MH (1992), Endogenous retroviral sequences are required for tissue-specific expression of a human salivary amylase gene, *Genes Dev.* 6: 1457-1465.

Tsien R, Prasher D (1998), Molecular Biology and Mutation of Green Fluorescent Protein. In: *Green Fluorescent Protein. Properties, Applications, and Protocols.* 97-120.

Tsien RY (1998), The green fluorescent protein, *Annu.Rev.Biochem.* 67: 509-544.

Vinogradova T, Volik S, Lebedev Y, Shevchenko Y, Lavrentyeva I, Khil P, Grzeschik KH, Ashworth LK, Sverdlov E (1997), Positioning of 72 potentially full size LTRs of human endogenous retroviruses HERV-K on the human chromosome 19 map. Occurrences of the LTRs in human gene sites, *Gene* 199: 255-264.

Wagner EF, Covarrubias L, Stewart TA, Mintz B (1983), Prenatal lethalities in mice homozygous for human growth hormone gene sequences integrated in the germ line, *Cell* 35: 647-655.

Ward WW, Cormier MJ (1979), An energy transfer protein in coelenterate bioluminescence. Characterization of the Renilla green-fluorescent protein, *J.Biol.Chem.* 254: 781-788.

Ward WW, Prentice HJ, Roth AF, Cody CW, Reeves SC (1982), Spectral pertubations of the *Aequorea* green-fluorescent protein. *Photochem.Photobiol.* 35: 803-808.

Weissmann C (2002), Molecular genetics of transmissible spongiform encephalopathies: an introduction, *J.Toxicol.Sci.* 27: 69-77.

Wilkinson DA, Freeman JD, Goodchild NL, Kelleher CA, Mager DL (1990), Autonomous expression of RTVL-H endogenous retroviruslike elements in human cells, *J.Virol.* 64: 2157-2167.

Wilkinson DA, Goodchild NL, Saxton TM, Wood S, Mager DL (1993), Evidence for a functional subclass of the RTVL-H family of human endogenous retrovirus-like sequences, *J.Virol.* 67: 2981-2989.

Wilkinson DA, Mager DL, Leong JC (1994), Endogenous Retroviruses. In: *The Retroviridae*. 3: 465-535.

Wolf E, Schernthaner W, Zakhartchenko V, Prelle K, Stojkovic M, Brem G (2000), Transgenic technology in farm animals--progress and perspectives, *Exp.Physiol* 85: 615-625.

Wood KV, de Wet JR, Dewji N, DeLuca M (1984), Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns, *Biochem.Biophys.Res.Commun.* 124: 592-596.

Woychik RP, Wassom JS, Kingsbury D, Jacobson DA (1993), TBASE: a computerized database for transgenic animals and targeted mutations, *Nature* 363: 375-376.

Wu N, Opalenik S, Liu J, Duco JE, Gabriella GM, Davidson JM (2002), Real-time visualization of MMP-13 promoter activity in transgenic mice, *Matrix Biol.* 21: 149-161.

Wu X, Holschen J, Kennedy SC, Ponder KP (1996), Retroviral vector sequences may interact with some internal promoters and influence expression, *Hum.Gene Ther.* 7: 159-171.

Yang F, Moss LG, Phillips GN, Jr. (1996), The molecular structure of green fluorescent protein, *Nat.Biotechnol.* 14: 1246-1251.

Yang M, Baranov E, Jiang P, Sun FX, Li XM, Li L, Hasegawa S, Bouvet M, Al Tuwaijri M, Chishima T, Shimada H, Moossa AR, Penman S, Hoffman RM (2000), Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases, *Proc.Natl.Acad.Sci.U.S.A* 97: 1206-1211.

Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF, Gilboa E (1986), Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells, *Proc.Natl.Acad.Sci.U.S.A* 83: 3194-3198.

Zakhartchenko V, Alberio R, Stojkovic M, Prelle K, Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R, Duchler M, Steinborn R, Mueller M, Brem G, Wolf E (1999), Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures, *Mol.Reprod.Dev.* 54: 264-272.

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