4. Methods

In this work mostly basic or modified versions of molecular biological methods from *"Molecular Cloning"* written by Sambrook *et al.* (1989) and from *"Current Protocols in Molecular Biology"* by Ausubel *et al.* (1992) were used.

4.1. PREPARATION AND ANALYSIS OF DNA

4.1.0.1. Phenol/Chloroform Extraction and Ethanol Precipitation of Nucleic Acids

Phenol/Chloroform extraction serves the separation of nucleic acids and proteins. To an equal volume of sample (minimally 0,1ml) phenol/chloroform/isoamyl alcohol (chloroform : isoamyl alcohol - 24:1 for DNA extraction; 49:1 for RNA extraction; phenol : chloroform/isoamyl 1:1) was added and vortexed vigorously. After centrifugation (14.000rpm; 4min) the upper phase (containing the DNA) was transferred to a new Eppendorf tube. Chloroform/isoamyl alcohol (24:1 resp. 49:1) was added, vortexed and recentrifuged. The aqueous phase was transferred into a new Eppendorf tube with $^{1}/_{10}$ volume of 3M sodium acetate (pH 5,2), 2 to 2,5 volume of 100% ice-cold ethanol, vortexed and kept at -20°C for 20min. After centrifugation (14.000rpm; 20min; 4°C) the supernatant was removed, the remaining pellet washed with 1ml of 70% ethanol and centrifuged (14.000rpm, 2min). The supernatant was removed, the pellet was air-dried for 10min and resuspended in TE buffer.

4.1.1. Isolation of Genomic DNA

4.1.1.1. Isolation of Genomic DNA from Tissue

100mg of tissue or 1cm of a mouse tail were shaken for 4-5 hours at 56°C in 700µl of TENS lysis buffer until the tissue was completely dissolved. After two phenol/chloroform/isoamyl alcohol extractions and one chloroform/isoamyl alcohol (24:1) extraction the DNA was precipitated with isopropanol . The sample tube was centrifuged (14.000rpm; 30sec), the pellet was washed once with 70% ethanol, briefly spun down and air dried for 10min. It was dissolved in 100-150µl of TE buffer at 56°C for several hours.

4.1.1.2. Isolation of Genomic DNA from ES cells

The ES cell were cultivated in 24 well plates up to a confluent culture. Each well was treated separately when the culture reached confluence. Cells were washed with PBS and lysed in 500µl ES cell lysis buffer and left to incubate for up to one week while other clones reached confluence as well. Minimal time for the lysis is 3 hours. The DNA was precipitated with 500µl of isopropanol and washed with 70% ethanol. Using a pipette tip the DNA was transferred into 150µl TE buffer and dissolved at 56°C over night.

4.1.2. Isolation of Plasmid-DNA

4.1.2.1. DNA-Minipreparation - "Boiling Method"

A single colony was incubated over night in 3ml of LB medium with ampicillin (40mg/l) in a shaking incubator at 37°C. The bacterial suspension was pelleted and resuspended in freshly prepared STET-buffer with 4µl of lysozym (50mg/ml). After 5 min of incubation at room temperature the suspension was boiled for 45sec and centrifuged for 10min (13.000rpm). The pellet was removed with a toothpick. To the rest of the supernatant 1/10 volume of 3M sodium acetate was added. The DNA was precipitated with isopropanol and centrifuged (14.000rpm; 5min). Precipitated DNA was washed once with 70% ethanol, pelleted, air dried and resuspended in 50µl of TE buffer. For restriction enzyme analysis usually 1-5µl was used.

4.1.2.2. DNA-Maxipreparation - "Lithium Chloride Method"

500ml LB medium was inoculated with a single colony and shaken over night at 37°C. The saturated culture was centrifuged (7.500rpm; 10min), the supernatant discarded and the pellet resuspended in 20ml of Sol-I. After 10min of incubation at room temperature 40ml freshly prepared Sol-II was added and incubated on ice for 10min. The Sol-III was mildly mixed with the lysate, the proteins and chromosomal DNA were precipitated for more than 10min. The precipitate was centrifuged (7.500rpm; 10min), the supernatant was filtrated through a Whatman filter and precipitated with 0,6 volume of isopropanol for 30min at room temperature. After centrifugation (7.000rpm; 10min) the pellet was resuspended in 4ml of dest. H_2O and 4ml of 5M LiCl added to precipitate high molecular weight RNA. This suspension was incubated for 15 to 30min on ice and centrifuged (7.000rpm). To the supernatant 2,5 volumes of ethanol were added in a new tube and precipitated 30min at -20°C. After centrifugation (7.000rpm; 10min; 4°C) the pellet was resuspended in 500µl of

TE in an Eppendorf-tube, digested with 20µl of RNase A (10mg/ml) for 30min at 37°C and subsequently with proteinase K for 30min at 56°C. Then the sample was purified by phenol/chloroform extraction, precipitated with 1/2 volume of 7,5M ammonium acetate and 2 volumes of 100% ethanol. After centrifugation (14.000rpm; 15min; 4°C) the pellet was air dried and resuspended in 500µl.

4.1.2.3. Vector DNA-Maxipreparation on JETSTAR resin (Genomed)

This purification method of choice was used to isolate vectors of highest purity for electroporation. The JETSTAR anion exchange resin Maxi-preparation kit was used following the protocol as described by the manufacturer.

4.1.2.4. Isolation of Single Copy Vectors with Large Inserts

For the isolation of BAC plasmid DNA Qiagen MIDI preparation kits following the protocol for single-copy-number plasmids as described by the manufacturer were used.

4.1.3. Estimation of Concentration of Nucleic Acids

4.1.3.1. Measuring of Nucleic Acid Concentration by UV-Spectrophotometry

By UV-spectrophotometry the concentration and the purity of a sample is determined. The nucleic acids absorb UV light at 260nm. The OD_{260} = 1.0 is equal to 50µg/ml of dsDNA, 33µg/ml of ssDNA and 40µg/ml of RNA. To calculate a protein contamination of a sample the equation OD_{260} /$OD_{280\ is}$ used. It has to be 1,8 for a sample to be of good DNA quality.

4.1.3.2. Estimation of DNA Concentration by a "Dot-Plate"

To estimate very low concentrations (5-100ng/µl) from small-volume samples an agarose plate (1% agarose; 1µg/ml of ethidium bromide) was used. On this plate 1µl of the sample was dotted, incubated for 10min. The DNA concentration was estimated using a UV-light transilluminator at 312nm by comparing with standards on the agarose plate.

4.1.4. Resolution and Recovery of Large DNA fragments

4.1.4.1. Agarose Gel Electrophoresis

Standard agarose gels separate DNA fragments from 0,3 to 20kb. The gels were prepared with agarose concentration appropriate for the size of DNA fragments to be

separated (0,8 - 2,5%). Prior to pouring of the melted agarose ethidium bromide solution was added (0,5µg/ml). The mixture of the DNA sample and 10x GLB (gel loading buffer) were loaded into the samples wells and the gel was run at a voltage and for time that would achieve optimal separation in the 1x TAE buffer. DNA was visualized with UV-light (312nm) and directly photographed.

4.1.4.2. Isolation and Purification of Large DNA Fragments from Agarose Gel

After electrophoresis the agarose gel was illuminated with UV light (254nm). Using a scalpel the target band was cut out. For large DNA fragments a JET-sorb purification KIT was used according to manufacturers specifications.

4.1.5. Southern Blotting and Hybridization

DNA fragments were separated on an agarose gel and photographed with a ruler to be able later reproduce the proportion of separation between a size-marker and the DNA fragments. The fragments were denatured and transferred from the gel to a solid support (nylon membrane) in a transfer pyramid. There the gel was placed between a three Whatman paper bridge submersed in a reservoir with 0,4M NaOH. The nylon membrane was in direct contact with the top of the gel. The nylon membrane was covered by another three Whatman papers cut to the size of the gel. The pyramid was finished by a 5cm-thick stack of paper towels. A plate with a small weight to press the pyramid together was placed on top. After overnight blotting the sample wells were labeled on the membrane and the membrane was washed in 2x SSC.

4.1.5.1. Screening of Bacterial Colonies by Hybridization

It is possible to screen hundreds of bacteria for a rare, particular clone. The bacteria were grown on an agar plate and parallel a replica of the plate was grown on a nylon membrane placed on another agar plate over night. Then, using a forceps, the nylon membrane was peeled off the plate and lain, colony side up, on a stack of three sheets of Whatman paper saturated with denaturation solution for 5min. The membrane was transferred to a Whatman paper saturated with neutralizing solution. After 5min the membrane was briefly washed in 2x SSC and placed between two dry Whatman papers. Finally the membrane was baked for 2hr at 80°C. The positive clones were detected by hybridization with radioactively labeled DNA probe.

4.1.5.2. Prehybridization of a Nylon Membrane (for Probes Longer than 300bp)

The nylon membrane was prehybridized in a hybridization tube for 10hr at 65°C in a hybridization oven. The prehybridization solution was always freshly prepared. Salmon sperm DNA used for the prehybridization was boiled for 5min, cooled on ice and added immediately before the start of the prehybridization.

4.1.5.3. Hybridization of a Nylon Membrane (with Probes Longer than 300bp)

The prehybridization solution was exchanged with hybridization solution. DNA from salmon testes (100µg/ml) and the radioactively labeled probe were boiled, cooled on ice and added immediately. The hybridization was incubated for 16hr the same way as for the prehybridization.

4.1.5.4. Washing of the Hybridized Nylon Membrane (with Probes Longer than 300bp)

After hybridization the labeled probe is also bound to the membrane unspecifically and has to be washed out. The stringency of the washing solutions depends on the homology between the targeted sequence and the probe. Washing stringency elevates with increasing temperature and decreasing salt concentration. Nevertheless the washing has to always be done gradually. Between changing of the stringency the membrane and the used solution has to be checked with a Geiger counter. Usually the membranes were washed with three different salt concentrations - 2x SSC/0,2% SDS; 1x SSC/0,2% SDS; 0,2x SSC/0,2% SDS) at 65°C twice for each solution for 20min.

4.1.5.5. Prehybridization of a Nylon Membrane (for Oligo-Probes)

The prehybridization solution was preheated to 50°C without the salmon sperm DNA. The nylon membrane was placed in the hybridization tube and prewetted with warm prehybridization solution. Then the salmon sperm DNA was preboiled for 5min and added to the warm prehybridization solution and the membrane. Prehybridization took 1-2hr at 42°C .

4.1.5.6. Hybridization of a Nylon Membrane (with Oligo-Probes)

The prehybridization solution was discarded and replaced by prewarmed hybridization solution with an appropriate amount of labeled oligonucleotide probe. Hybridization was incubated for 12hr at the temperature 5-10°C below T_m.

To calculate T_m the following formula was used:

$$T_m = \{(A + T) \times 2 + (G + C)\} \times 4$$

4.1.5.7. Washing of the Hybridized Nylon Membrane (with Oligo-Probes)

The washing of membranes hybridized with oligonucleotide probes must be carried out much more carefully than described above. Only one washing solution was used (6x SSC/0,1% SDS) at T_m -10°C. The stringency was increased only by slowly elevating the temperature if necessary.

4.1.5.8. Membrane Stripping Protocol for DNA and RNA Blots

The same nylon membrane could be used for several consecutive probings with different probes. For successful removal of probes, membranes must never be allowed to dry during or after hybridization and washing. A solution of 0,1% (w/v) SDS was boiled and pourred onto the membrane in a vessel. It was allowed to cool to room temperature under slight shaking. By a normal exposure on a phosphoimager it was checked that the probe had been removed. The membrane was then again prehybridized and hybridized with a new probe.

4.1.6. Resolution of Small DNA Fragments

4.1.6.1. Denaturing Polyacryamide Gel Electrophoresis

Denaturing polyacrylamide gels are used to resolve sizes from 2 - 1000 bases. The gel concentration depends on the size of separated nucleic acids fragments. In primer extension and in ribonuclease protection assays gel concentration ranged between 4 and 10%.

Denaturing acrylamide gel solution:

Reagent	Acrylamide concentration			
	4%	6%	8%	10%
Urea (ultrapure) (g)	25,2	25,2	25,2	25,2
38% acrylamide/2% bisacrylamide (ml)	6,0	9,0	12,0	15,0
10x TBE (ml)	6,0	6,0	6,0	6,0
H_2O (ml)	27,0	24,0	21,0	18,0
Total volume (ml)	60,0	60,0	60,0	60,0

A pair of glass-plates (40cm x 25cm) was meticulously washed and a silane solution was applied to smooth the plate surfaces with a Kleenex. The denaturing acrylamide gel

solution was degassed under vacuum for 10min. Immediately before pouring the gel 60µl of TEMED and 0,6ml 10%APS (ammonium persulfate) were added to initiate polymerization. Before loading the samples the gel was preheated by turning the power supplies to 45V/cm and 70W constant power for 30min. Gels were run vertically in 0,5x TBE buffer at power settings 45-70W. After the DNA separation the plates were pried apart, the gel was transferred to a Whatman paper and covered with plastic wrap. The gel on the paper was dried on a gel dryer for 1hr at 80°C and under the vacuum.

4.1.6.2. Exposure Analysis of Radioactive Material on Solid Support

The dry polyacrylamide gel on the Whatman paper or the nylon membrane after the washing were placed in an exposure cassette with an erased phosphor screen. The image was analyzed on Storm Imaging System according to the manufacturer instructions.

4.1.6.3. Sequence Analysis by Sequencing

Sequences were determined on both strands using the ABI Big Dye Terminator Cycle Sequencing ready reaction kit, and the products were resolved on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer/ Applied Biosystems).

4.1.7. Computer Analysis of DNA

The sequences were assembled and analyzed using the Heidelberg Unix Sequence Analysis Resources (HUSAR) software programs.

DNA Sequence Computer Search
http://www.ncbi.nlm.nih.gov/blast/blast.cgi

DNA Sequence Alignment
http://www.ebi.ac.uk/clustalw/

Computer Promoter Signal Analysis
http://bimas.dcrt.nih.gov/milbio/signal/
http://transfac.gbf-braunschweig.de/cgi-bin/patSearch/patsearch.pl

4.2. ENZYMATIC MANIPULATIONS OF NUCLEIC ACIDS

4.2.1. Polymerase Chain Reaction (PCR)

PCR is a cyclic, enzymatic amplification by Taq-polymerase of a specific segment of DNA determined by single stranded oligonucleotides - the primers. The first crucial parameter for successful PCR is the choice of the primers. Usually the length of primers varies between 20 and 30bp. The G/C content should not exceed 60%. Stretches of longer repetitive or self complementary sequences in the primer should be avoided. The melting temperature (T_m) has to be the same in both - *sense* and *antisense* - primers.
The reaction setting:

DNA	max. 10pM
Taq-Polymerase Buffer	1x
$MgCl_2$	1,5mM
sense primer	0,5µM
antisense primer	0,5µM
dNTP	200µM
Taq-Polymerase	2,5U
H_2O	to 50µl

The reaction is cycled between the annealing, extension and melting temperature. The length and temperature of each step of one cycle and as well the number of the cycles depends on the size, frequency of amplified fragment and on the T_m of the primers.

4.2.2. Restriction Digestion of DNA

4.2.2.1. Complete Digestion of Plasmid DNA with Restriction Endonucleases

The complete digestion of 1µg plasmid DNA with or without an insert is already achieved with 1 unit of a restriction endonuclease in one hour. The volume of the enzyme should not exceed 1/10 of the total volume of the reaction. Different enzymes might have their optimum of activity at different salt concentration. Thus attention has to be paid to the compatibility of used enzyme buffers, if two or more enzymes are used at the same time. Alternatively, the enzymes can be used in sequence with an intermediate step of phenol/chloroform extraction and redissolving the DNA in the buffer system for the next enzymatic reaction. The result of the digestion is analyzed by agarose gel separation with 10x GLB or/and could be used in an other DNA modifying enzymes reactions (usually prior those should be performed a phenol/chloroform extraction as well or purified through an agarose gel.

4.2.2.2. Complete Digestion of Genomic DNA with Restriction Endonucleases

The digestion reaction mix was calculated for a volume of 50µl. Usually 10 - 15µg of genomic DNA was used. For 1µg of genomic DNA 5-6 units of restriction endonuclease were used. Total digestion is completed after 5-6hr. Then the reaction mix was directly loaded onto an agarose gel in 10x GLB and run at maximum 70V.

4.2.2.3. Partial Digestion of Plasmid DNA with Restriction Endonucleases (Restriction Mapping)

The partial digestion of an insert within a plasmid allows to map the exact position of a restriction site of chosen restriction endonucleases. This is extremely important information for the structural analysis and for the next design of cloning steps in the cloning of a target vector. The insert was cut out of the vector by complete digestion with a restriction enzyme used for its cloning and purified by phenol/chloroform extraction. For each chosen restriction endonuclease that cleaves infrequently a separate set of reactions (0,05 enzyme unit/1µg of DNA) was done and stopped at different time points (0; 1; 2; 5; 10; 20, 40 and 90min) with EDTA (final concentration 10mM). This set of reactions, partially cleaved by an enzyme, was separated on an agarose gel. The resulting gel image was blotted onto a nylon membrane and hybridized and stripped with two different radioactively labeled oligonucleotide probes (oligo-probes) independently. These oligo-probes (usually 25mers) were chosen from the 3' and 5' extremities of the insert.

4.2.3. Radioactive Labeling of Nucleic Acids

4.2.3.1. T4 Polynucleotide Kinase - Labeling by the Forward Reaction

The forward reaction catalyzes the transfer of the terminal (γ) phosphate of ATP to the 5'-hydroxyl termini of DNA or RNA. This method was used for labeling oligonucleotides.

The usual reaction mix as follows:

Oligonucleotide	50pmol
Forward Buffer (10x)	2
γ^{32}P(ATP (3.000Ci/mmol)	3µl
T4 Polynucleotide Kinase (10U/µl)	1µl
H_2O	to 20µl

The reaction was stopped by adding of 1µl of 0,5M EDTA.

4.2.3.2. Labeling of DNA by Random Oligonucleotide-Primed Synthesis

Radioactive probes longer than 300bp were labeled with Klenow fragment (long fragment of Escherichia coli DNA polymerase I) using a labeling kit Rediprime. The protocol was followed as described by the manufacturer.

4.2.3.3. Spin-Column Procedure for Separating Radioactively Labeled DNA from Unincorporated dNTP precursors.

This method is more rapid then conventional chromatography but the removal of the dNTP is less efficient. Swelled column resin (Sephadex G-50 - 5g/100ml of TE buffer, autoclaved) was pipetted into a 1ml syringe, plugged with glass wool, and packed by centrifugation (1000rpm; 2min). The radioactive sample was applied to the column resin, the syringe was placed in the polypropylene tube and centrifuged (1.000rpm; 4min). The liquid at the bottom of tube contained the labeled DNA and was further used as a probe.

4.2.4. Construction of Hybrid DNA Molecules (Cloning)

Before insertion of a DNA fragment into a vector usually both DNA molecules must be modified on their 3' and 5' ends. Only in the case of two different restriction endonucleases modifying each end of the two DNA molecules (vector and insert), the ligation can be done directly.

4.2.4.1. Repairing of 3' and 5' Overhanging Ends to Generate Blunt Ends by Klenow fragment

The Klenow fragment retains DNA polymerase and weak 3' - 5' exonuclease activity of Escherichia coli DNA polymerase I.

The reaction setting:

DNA	max. 5µg
Klenow Buffer (10x)	5µl
Klenow fragment (5U/µl)	1µl
dNTP (each 100µM)	1µl
H_2O	to 50µl

The mix was incubated for 30min at 37°C. The DNA was purified by phenol/chloroform extraction and precipitated with 1/10 volume of 3M sodium acetate and 1/2 volume of 100% ethanol . The pellet was air dried and dissolved in water.

4.2.4.2. Repairing 3' and 5' Overhanging Ends to Generate Blunt Ends with T4 DNA Polymerase

T4 DNA polymerase posses a DNA-dependent DNA polymerase activity and a strong single and double stranded 3' - 5' exonuclease activity. At high concentration of dNTPs the polymerase predominates over the exonuclease activity. The single stranded 3' end is blunted up to the double strand in any case.

The reaction setting:

DNA	max. 4µg
T4 DNA Polymerase Buffer (10x)	5µl
T4 DNA Polymerase (5U/µl)	1µl
dNTP (each 100µM)	1µl
BSA (100mg/ml)	1µl
H_2O	to 50µl

The reaction was carried out at 11°C for 20min and stopped with 1µl of 0,5M EDTA.

4.2.4.3. Dephosphorylation of 5'ends by Calf Intestine Phosphatase (CIP)

To prevent religation of a vector CIP, that dephosphorylates 5' ends of an DNA substrate, was used. By 1U of CIP 1 to 2µg of a vector in 1x CIP-buffer with 50µg/ml of BSA could be dephosphorylated. The 30min reaction was terminated by phenol/chloroform extraction followed by a 100% ethanol precipitation.

4.2.4.4. Ligation

In ligation reactions the most important factor is the molar ratio between the vector and the insert. This ratio oscillates between 1:3 to 1:10 (vector : insert).

The reaction setting:

vector	20-25ng
DNA insert	3-10 times molar excess towards vector
Ligation Buffer (5x)	2µl
T4 DNA Ligase (2000/µl)	1µl
H_2O	to 10µl

This reaction mix was incubated in a PCR thermocycler for 150 cycles at:

30°C	30sec
16°C	10sec
10°C	10sec

The ligation was either used immediately for the transformation of competent cells or stored at -20°C.

4.2.4.5. Cloning of a PCR Fragment

Taq-polymerase used in the amplification of the DNA-fragment could interfere in the ligation reaction and must be degraded by proteinase K.

The reaction setting for 50µl of PCR reaction:

Proteinase K (10mg/ml)	1,5µl
SDS 3%	0,625µl
EDTA (0,5M)	1,25µl

The reaction was incubated for 30min at 37°C. The proteinase K was inactivated by phenol/chloroform extraction. The DNA pellet was dissolved in 37µl of H_2O.

The Taq-polymerase of the PCR reaction leaves single nucleotide overhangs of adenine on the PCR product. This has to be blunted by T4 DNA polymerase and in the same reaction mix phosphorylated by T4 polynucleotide kinase.

The reaction setting:

PCR product (max. 1µg)	37µl
T4 DNA Ligase Buffer (5x)	10µl
dNTP (10mM)	1µl
T4 DNA Polymerase (5U/µl)	1µl
T4 Polynucleotide Kinase (10U/µl)	1µl

After 1 hour of incubation at 37°C the reaction was stopped by heating to 68°C. Finally the product was purified on an agarose gel and used for ligation.

Alternatively a TA-cloning kit (Invitrogen) was used as recommended by the manufacturer.

4.2.5. Introduction of Plasmid DNA into Competent Cells

4.2.5.1. Preparation of Competent Cells Using the Calcium Chloride Method

A single colony was inoculated in 100ml of LB medium and cultured by shaking at 37°C over night. Next day 5ml of saturated culture was used to inoculate 500ml of LB medium and shaken until the OD_{595} of the culture reached 0,4 - 0,6 (ca. 3-4h). The suspension was centrifuged in prechilled, sterile polypropylene tubes (4.000rpm; 10min; 4°C). The pellet was resuspended in 200ml of ice cold 0,1M $CaCl_2$ solution, centrifuged (2.500rpm; 5min; 40°C), resuspended in the original volume of $CaCl_2$ solution, left on ice for 30min and centrifuged again (2.500rpm; 5min; 40°C). For last time the pellet was resuspended in 15ml $CaCl_2$ with 2,5ml ice cold glycerol and the 250µl aliquots were frozen immediately at -70°C. The transformation efficiency of the competent cells has to range from 10^6 to 10^8 transformants/µg of DNA.

4.2.5.2. Transformation of Competent Cells

150µl of fresh or cells thawed on ice were added to a new Eppendorf tube containing 5-10µl of ligation product and left to incubate on ice for 45min. After 90sec of heat shock at 42°C the cells were placed on ice for 2min, then 1ml of LB medium was added and incubated with shaking for 1hr at 37°C. Transformed cells were plated on agar plates containing appropriate antibiotics (or as well 300ml/l X-Gal) and incubated over night at 37°C.

4.3. PREPARATION AND ANALYSIS OF RNA

The most important factor in all RNA experiments is the isolation of intact RNA. Thus, it is important to optimize the isolation of RNA from given biological source and to prevent introduction of RNases and inhibitors of reverse transcriptase such as guanidinium salts, SDS and EDTA. To avoid RNases contamination, all solutions, should be treated with diethylpyrocarbonate (DEPC), apart from solutions containing Tris, because it inactivates DEPC. Glassware should be baked at 300°C for 4hr. Plastic ware, if used straight out of the package is usually free of contamination. Of course, gloves should be worn during all the preparations.

4.3.1. Isolation of Total RNA from Eukaryotic cells

4.3.1.1. TRIzol Reagent - Method

10^7 of cells or 50 to 100mg of tissue were placed in 1ml of TRIzol Reagent. Cell lysate was passed through a pipette several times or tissue samples were homogenized using a power homogenizer. The samples were incubated 5min at room temperature and then 0,2ml of chloroform per 1ml of TRIzol Reagent was added. The samples were shaken vigorously for 15sec and centrifuged at 14.000rpm at 4°C. Following centrifugation, the upper aqueous phase was transferred to a fresh tube with 500µl of isopropanol. The samples were, after 10min at room temperature, centrifuged with 14.000rpm at 4°C for 15min. The pelleted RNA was washed once with 1ml of 75% EtOH in DEPC-water, recentrifuged at 7.500rpm for 5min at 4°C. The supernatant was discarded, the pellet dried, resuspended in DEPC-water and incubated for 10min at 55° - 60°C to dissolve the RNA completely. After measuring the RNA concentration the RNA was stored at -70°C.

4.3.1.2. Acid Guanidium Thiocyanate-Phenol/Chloroform RNA Isolation (modified from Siebert and Chenchik (1993))

This method allows isolating very high quality of pure RNA although the yield is lower than by TRIzol Reagent method. Up to 10^8 of cells or up to 500mg of homogenized tissue can be used with 500µl of guanidium thiocyanate solution. To the viscous cell 50µl 2M sodium acetate pH 4,0, 500µl phenol, 100µl chloroform/isoamyl alcohol (49:1) lysate was added and vortexed for 10sec. After 15min of incubation on ice the samples were centrifuged for 20min (14.000rpm; 4°C) and the upper aqueous phase was transferred into new Eppendorf tube. RNA was precipitated with only $^1/_3$ of volume of 95% ethanol, incubation 5min on ice and centrifugation (15min; 14.000 rpm; 4°C). The pellet was dissolved in half the original volume of guanidium thiocyanate solution. The next precipitation was carried out at -20°C for 30min with 1ml of isopropanol and centrifugation (20min; 14.000rpm; 4°C). The pellet was resuspended in 300µl of guanidium thiocyanate solution and precipitated with 300µl of isopropanol (30min; -20°C) and centrifuged (20min; 14.000rpm; 4°C). At the end the pellet was washed with 70% ethanol (DEPC-treated water), briefly centrifuged and dry pellet resuspended in DEPC treated water.

4.3.2. Reverse Transcription (RT) - Synthesis of First Strand cDNA

Reverse transcriptase has the capacity to use a messenger RNA (mRNA) as template and 'overwrite' it into DNA code in 3' to 5' sense. The reactions were primed with oligo(dT) primer targeting the 3' poly(A) tail.

The reaction:

0,5 - 1µg	total RNA
10µg	Oligo(dT)
to 11µl	H_2O

after 10min at 72°C and cooling down on ice the rest of the reaction was pipetted in:

20mM	dNTPs
1x	Reverse Transcriptase Buffer
10mM	DTT
20U	Rnasin
100U	SuperScript II RNase H^-
to 50µl	H_2O

This was incubated for 1hr at 42°C and the reaction was stopped by denaturation at 95°C for 5min.

4.3.2.1. RT-PCR

RT-PCR is a PCR amplification of a product from the reverse transcription reaction. Usually max. 5µl of the RT product was used. The settings of the PCR reaction were already described in 4.2.1..

4.3.3 Rapid Amplification of cDNA Ends (RACE)

This method consists of amplification of nucleic acid sequences from a mRNA using one gene specific primer and second nested primer. It is used to enable amplification and subsequent characterization of unknown coding sequences.

For both - 5' and 3' RACE - a commercial system for rapid amplification of cDNA ends (Gibco BRL) was used as described by the manufacturer. Changes from the standard procedure are described below. The DNA sequences of the 5' RACE abridged anchor primer of the 3'RACE, the 3'-polyT+ adaptor primer and universal amplification primer were as described in the instructions of the kit.

3' RACE

It takes advantage of the natural poly(A) tails found in the majority of mRNAs as a generic priming site for PCR. For 3' RACE 1µg of poly(A) RNA was used and mRNAs were converted into cDNA using SuperScript II RNase H^- and an 3'-polyT+ adaptor primer. A specific cDNA was then amplified by semi-nested PCR. A gene-specific primer that anneals to a region of known part of the gene was used and an universal amplification primer that targets the 3'-polyT+ adaptor primer in poly(A) tail region. This permitted to capture the 3' mRNA sequence that lies between these two primers. The PCR condition greatly vary from the kit-manufacturer protocol, it was used as following:

5min	94°C		1min	94°C		1min	94°C	
10min	62°C	1x →→	2min	62°C	35x →→	2min	62°C	1x
20min	72°C		3min	72°C		5min	72°C	

5' RACE

An *antisense* gene specific primer was used for the synthesis of specific cDNA using SuperScript II RNase H^-. Prior to the semi-nested PCR, a terminal deoxynucleotidyltransferase (TdT) was applied to attach an adapter sequence to the unknown 5' end of the cDNA. Specific cDNA was then PCR amplified using a known gene

specific primer and the 5' RACE abridged anchor primer that targets the 5' terminus. The PCR conditions were used as described in 3' RACE.

4.3.4. Northern Blotting

Sample Preparation and Agarose-RNA-Gel Separation

The RNA concentration was determined by UV-spectrophotometer. For one sample 15-30µl of RNA was used. It was mixed with three times the volume of RNA-Sample Buffer, incubated for 10min at 70°C and cooled on ice. To this mixture $^{1}/_{10}$ volume RNA-Gel Loading Buffer was added and the sample loaded on a denaturing agarose RNA-gel. For the gel agarose was boiled in DEPC-water and let cool to 60°C. 0,1 total-volume of 10x MOPS, 0,15 total-volume of 37% formaldehyde and 1µg/ml ethidium bromide was added to it. RNA samples were loaded on the gel and run in 1x MOPS-Running Buffer for 3-5hr at ≅5V/cm, until the bromphenol blue is halfway down the gel.

4.3.4.1. Northern Electroblotting

After electrophoretic separation the RNA was visualized with UV light (312nm) and photographed with a ruler. Then, the gel was incubated in RNA -Transfer-Buffer (pH 7,0) for 10min and it was assembled into the electroblotting device, blotted overnight at 4°C onto a nylon membrane. Next day, the blot was disassembled and the gel-wells were labeled on the membrane. The membrane was baked for 2hr at 80°C to bond the RNA to the membrane. The Northern blot was prehybridized and then hybridized with a radioactive probe as described for Southern blot analysis.

4.3.5. Ribonuclease Protection Assay

The ribonuclease protection assay is a very sensitive method to detect and quantify gene expression on the mRNA level from cells. To the mRNA of a specific gene a RNA *antisense* probe synthesized with radioactive rNTP hybridizes. The mRNA strands which did not hybridize are enzymatically digested, while the double stranded RNA that stayed intact is analyzed on a polyacrylamide gel. The densitometric comparison reflects proportionally the level of synthesis of the investigated mRNA in specific cells. As a control in the reaction a β-actin RNA probe or other housekeeping gene is used to show the quantitative and qualitative property of the used mRNA.

4.3.5.1. Synthesis of Radioactive Antisense RNA Probe

The vector (pGEM) containing a 3'-5'sense cloned 250-300bp cDNA fragment of an analyzed gene was linearized and the *antisense* RNA probe was synthesized under radioactive conditions:

	Analyzed Gene	β-Actin
Sp6 or T7 RNA Polymerase Buffer (5x)	6µl	6µl
DTT (100mM)	2µ	2µl
RNasin (40U/µl)	1µl	1µl
ATP (10mM)	3µl	3µl
CTP (10mM)	3µl	3µl
UTP (10mM)	3µl	3µl
GTP (200µM)	-	3µl
α^{32}P-GTP (400Ci/mmol)	5µl	1µl
Linearized Template-DNA	0,2-1µg	0,2-1µg
Sp6 or T7-RNA Polymerase	1µl	1µl
H_2O	to 30µl	to 30µl

The reaction was incubated for 1 hour at 37°C and for another hour after addition of another 1µl of Sp6 respective T7 polymerase. The template DNA was digested for 30min with 10U of RQ-DNase at 30°C, the reaction was brought to 100µl and phenol/chloroform extracted followed by the addition of 50µl of 7,5M ammonium acetate and ethanol precipitated. The pellet was solved in 100µl of water. The phenol/chloroform extraction with the precipitation was repeated twice. The final pellet was dissolved in 100µl of TE and the incorporation of radioactivity into probe was measured on a scintillation counter.

4.3.5.2. Hybridization of the RNA Probe

5-30µg of the investigated total RNA was mixed with 200.000cpm of the *antisense* RNA probe, with 100.000cpm *antisense* β-Actin RNA and filled with water to 300µl. This was precipitated by addition of 30µl of 3M sodium acetate (pH 5,2), 750µl of ethanol and immediately centrifuged (13.000rpm; 15min, 4°C). The pellet was dissolved in hybridization buffer by shaking for 20min at 40°C. The RNA was denatured at 90°C for 2min and hybridized over night at 45°C.

4.3.5.3. RNA Digestion and Resolution of Products on Polyacrylamide Gel

The next day the single stranded RNA that did not hybridize was digested at 35°C by addition of 300µl RNase Buffer for 1 hour. The reaction was then incubated with 15µl of SDS (10%) and 4µl of proteinase K (10mg/ml) at 45°C for 30min. This was

phenol/chloroform extracted, precipitated with 5µg of tRNA, 850µl of ethanol and centrifuged (13.000rpm; 15min; 4°C). The dry pellet was resuspended in 15µl of deionized formamide (90%) in 1x TBE-running buffer (1% Bromphenol Blue; 1% Xylencyanol Blue in TBE). This probe was then separated at 250-350V on 6% denatured polyacrylamide gel.

4.3.6. Primer Extension

Primer extension analysis was used to map the 5'-end of the mRNA and to determine the exact start site(s) for transcription. A specific end-labeled *antisense* primer was used to synthesize a cDNA by reverse transcriptase using the RNA as template. The resulting cDNA was analyzed on a denaturing polyacrylamide gel. The length of the cDNA shows the number of bases between the labeled nucleotide of the primer and the 5' end of the mRNA.

4.3.6.1. Hybridization of the Primer

To 50µg of total RNA 100fmols of labeled primer was added. This was precipitated by 0,1 Vol. of 3M sodium acetate (pH 5,2; in DEPC water) and 2,5 Vol. of 100% ethanol for 30min at -20°C. The RNA with the primer was pelleted by centrifugation (14.000rpm; 10min; 4°C) and washed with 70% ethanol (DEPC water). The dry pellet was dissolved by pipetting in 30µl of hybridization solution. This mixture was incubated at 85°C for 10min and then 50°C over night. The next day the RNA x primer hybrids were precipitated. The volume was filled to 200µl. The product was precipitated adding 400µl of 100% ethanol, incubating at 4°C for 1hr, centrifuged (14.000rpm; 15min, 4°C), washed with 70% ethanol, briefly centrifuged and air dried. The presence of the radioactive primer was checked at each step with a Geiger counter.

4.3.6.2. Primer Extension by Reverse Transcription

The RNA x primer hybrids were redissolved in 20µl of reverse transcriptase buffer (5x) and the reaction was performed in 20µl reaction volume. Reverse transcription was stopped by 1µl 0,5M EDTA.

4.3.6.3. RNA Digestion and Resolution of Primer Extension Product on Polyacrylamide Gel

1µl of RNase A (5µg/ml) was incubated with the RT for 30min at 37°C. To the reaction mixture 135µl TE (pH 7,6) and 15µl NaCl (1M) was added. The product was

phenol/chloroform extracted and the aqueous phase precipitated in a fresh tube with 100% ethanol for 1hr at 4°C. After centrifugation (14.000rpm; 15min; 4°C) the pellet was washed with 70% ethanol, pelleted and air dried. The dry pellet was dissolved in 4µl of TE (pH 7,4) and mixed with 6µl of formamide loading buffer 80%. The mixture was heated for 5min to 95°C, cooled down on ice and loaded on the gel. The marker was diluted 100 times and as well heated prior the loading. The run of denaturing 8% polyacrylamide gel was described already earlier.

4.4. ANALYSIS OF PROTEINS

4.4.1. Protein Extraction

4.4.1.1. Isolation of the Stomach Epithelium by "Scraping" for Protein Extract

The stomachs were excised and cut along the greater curvature and washed ten times in ice cold PBS. Finally they were transferred to 10cm dish filled with 5ml of PBS. By using blunt tweezers the stomach mucosa was scraped off the musculature of the stomach. Then the mucosa was centrifuged, the supernatant was discarded and once washed again with 10ml of PBS. The pellet was used for total protein extract or for RNA preparation.

4.4.1.2. Total Cell Protein Extracts from Cells and Organs of Mice

The tissue (≅300mg) was cleared of contents (colon, stomach), cut into small pieces and washed several times in an excess of ice cold PBS or the cells - 3 x 10^7- were washed twice with 10ml of ice cold PBS. The sample was transferred to an Eppendorf tube, the PBS was replaced by 150µl of RIPA buffer and the tissue respective the cells were resuspended in it. This was incubated on ice for 20min with occasional, very brief sonications. Then the lysat was centrifuged (14.000rpm, 10min, 4°C), the supernatant was pipetted into new tubes in 40µl aliquots, immediately frozen in liquid nitrogen and stored at -70°C.

4.4.1.3. Total Protein Cell Extract from Cell Lines by Triton X-100 Method

This method of choice for preparation of total protein cell extract from the cell lines. It was employed prior to purification of carbonic anhydrases. For this purpose it is important to eliminate the presence of strong detergents as in the method where used RIPA buffer. Strong detergents intervene with adsorption to sulfonamide agarose. All steps of purification were carried out at 0 - 5°C, pH 7,2 and at physiological concentration of salts. Special care

was taken to keep physiological concentrations of halide ions. The halide ions show a strong affinity to carbonic anhydrases and alter their binding to sulfonamide agarose.

The 10cm dishes with a dense cell culture were washed with PBS and on 0,5ml of 1% Triton X-100 with Complete Proteinase Inhibitor Cocktail® was applied. This cell extract was diluted 1 : 6 with PBS and centrifuged on a high performance centrifuge (14.000rpm; 1hr; 4°C). The supernatant was used directly for purification in a single cycle of adsorption on the chromatography column.

4.4.1.4. Quantification of Proteins by the Bradford Assay

The Bradford method is a colorimetric, comparative method measuring binding of Coomassie brilliant blue to unknown protein and compared with a protein bovine serum albumin (BSA) standard. In a cuvette 1ml of Bradford reagents was mixed with 1µl of a protein sample, incubated at room temperature for 2min and measured by UV-spectrophotometry at A_{595}. The protein concentration of a sample is deduced from the standard BSA curve.

4.4.2. Western Blotting

Denaturing Discontinuous Polyacrylamide Gel Electrophoresis of Proteins (SDS-PAGE).

In SDS-PAGE the proteins first enter into a short "stacking gel" and then the "separating gel" separates the proteins based on their molecular size. The concentration of the separating gel depends on the size of protein of interest.

Separating Gel

	Final acrylamide concentration in the separating gel		
	6%	8%	10%
30% acrylamide/0,8% bisacrylamide	3,00ml	4,00ml	5,00ml
4x Tris.Cl/SDS, pH 8,8	3,75ml	3,75ml	3,75ml
H_2O	8,25ml	7,25ml	6,25ml
10% ammonium persulfate	0,05ml	0,05ml	0,05ml
TEMED	0,01ml	0,01ml	0,01ml

Stacking gel

In a flask 0,65ml of 30% acrylamide/0,8% bisacrylamide, 1,25ml of 4x Tris.HCl/SDS, pH 6,8 and 3,05ml H_2O was mixed. Prior to pouring 25µl of 10% ammonium persulfate and 5µl of TEMED was added.

The protein samples (20-100µg) were diluted 1:4 with 4x SDS/sample buffer (max. total volume 20µl) and heated 5min at 100°C. The electrophoresis was run in 1x protein

running buffer at 200V and 10mA constant current until the bromphenol blue entered the separating gel. Than the current was increased to 15mA.

4.4.2.1. Western Electroblotting

After separation of the proteins, the gel and the nylon PVDF membrane were prewetted in transfer buffer with fresh 20% methanol for 20min. Then the gel was laid in between the prewetted nylon membrane with three prewetted Whatman papers underneath and three prewetted Whatman papers on the gel. The power supply of the electroblotting apparatus was set at 0,8mA/cm^2 for 45min.

Shaking at room temperature, the membrane was blocked for non-specific binding of antibody with 50ml of blocking buffer (5% dry, low fat milk in 1x PBS) for 1 hour. The primary, specific antibody was diluted in 1% dry, low fat milk in 1x PBS and incubated with the membrane for an additional 1 hour. After intensive washing in 1x PBS - three times; 20min with shaking, the second peroxidase conjugated antibody was applied for 30min. Then the membrane was washed 3 times 10min in 1x PBS. The membrane was placed on a plastic, transparent foil, the ECL-kit was applied and covered with a second foil. X-ray film was exposed to the chemiluminescent signal.

4.4.3. Affinity Chromatography of Carbonic Anhydrases (Falkbring, 1972)

Using this method, modified for our purpose by Zavada *et al.* (2000), it is possible to purify active carbonic anhydrases. The advantage is taken from their capacity of binding to sulfonamide (p-aminomethylbenzene) that had been insolubilized on cross-linked 4% beaded agarose.

The protein extract was prepared with 1% Triton X-100 as described in 4.4.1.3.. Once the cell debris had been separated from the supernatant by centrifugation, it was applied to a Bio-Rad Biological LP Chromatography system with a flow-rate 0,44ml/min. After loading the sample the chromatography column was run with a flow-rate of 0,44ml/min with PBS over night for washing. During elution with 0.1mM acetazolamide (flow-rate 0.14 ml/min) 0,5ml aliquots were collected. All steps of purification were monitored by dot-blots. The aliquots that contained the highest amounts of MN/CA IX were joined and the protein purity was confirmed on 8% acrylamide gel stained with Coomasie Blue.

4.4.4. Staining SDS-Polyacrylamide Gels with Coomassie Blue

The gel was immersed in about 5 volumes of Coomassie Blue staining solution and let rock at room temperature for 4 hours. To remove the excessive stain a destaining solution for Coomassie Blue was used. The gel was soaked in this solution for 4-8 hours while slowly rocking.

4.4.5. Protein Sequence Analysis

The protein analysis was carried out by a fragment analysis of tryptic digested polypeptides by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS). From the resulting fragment masses a protein matching the MN/CA IX protein sequence was confirmed. Spectra were recorded at the FMP (Department of peptide chemistry).

4.4.6. Computer Analysis of Protein Sequence

Protein Sequence Analysis
http://protein.toulouse.inra.fr/prodom/doc/blast
http://www.expasy.ch

4.5. CELL CULTURE METHODS, ES CELLS HANDLING AND USE

All tissue culture procedures described were carried out under sterile conditions using a laminar flow cabinet, sterile plasticware and detergent-free glassware. The media and solutions were prepared from directly sterile, autoclaved or sterile filtrated components. Before the cells were placed in the media, solutions had to be warmed to 37°C. A DNA that is put in contact with cells has to be precipitated by 100% ethanol, washed twice with 70% ethanol, air dry under a laminar flow cabinet and resuspended in sterile PBS prior to use.

4.5.1. Embryonic Feeder Cells

4.5.1.1. Preparation of Embryonic Fibroblast Feederlayers

To maintain the pluripotency ES cells were cultured in the presence of leukemia-inhibitory factor (LIF) on a layer of mitotically inactive feeder cells, derived from embryonic fibroblasts. The breeding pairs of a male homozygous transgenic animal with 3-5 wild type females was set up and checked every morning for vaginal plugs (day 1). On day

13 (or 14), the pregnant mice were sacrificed, rinsed with 70% ethanol, the uterus was isolated and transferred into sterile PBS. After several washings in new PBS the uterus was opened, the embryos were decapitated, fetal liver and heart were removed and the rest was cut in small pieces. That prepared embryo was transferred together with glass-beads (5mm) and 50ml 1x trypsin/EDTA into an Erlenmeyer flask and stirred gently at 37°C for 30min. The feeder medium was added to inactivate trypsin and the cell suspension was centrifuged in 50ml tubes for 5min at 1.000rpm. The cell pellet was resuspended in feeder medium, centrifuged and the cells were plated in density 10cm plates/10ml feeder medium/1x10^6cells. The next day the medium was changed and in four days the confluent cells were trypsinized and frozen in aliquots.

4.5.1.2. Embryonic Fibroblast Cell Culture

Thawed or freshly isolated embryonic cells (5x10^6) were plated onto five 10cm dishes with 10ml of feeder medium. In 3-4 days, when the cells reached confluence (ca. 4-5x10^6 cells/plate), the cells were split 1:3 or 4 for additional expansion. For splitting, the cell were twice washed with PBS, each plate was incubated with 2ml of 1x trypsin/EDTA at 37°C for 5min. Once the cells detached, they were gently pipetted up and down to break aggregates. Trypsinized cells were added to 6ml of feeder medium in a conical tube and centrifuged at 1.000rpm for 5min. The supernatant was discarded, the cell pellet resuspended in feeder medium and plated. Generally, embryonal fibroblasts were not passed more than 3 times, because they are not anymore suitable for ES-cell culture.

4.5.1.3. Inactivation of Embryonic Fibroblast "Feederlayer" by Mitomycin C

A confluent plate of embryonic fibroblasts was washed with PBS and incubated for 2-4 hours with 4ml of mitomycin C solution (10µg/ml) in feeder medium. Then the cells were washed three times with PBS, incubated with 2ml of 1x trypsin/EDTA at 37°C for 5min, resuspended and centrifuged in feeder medium. The cell pellet was brought to a concentration 2-3x10^5 cells/ml of feeder medium and plated on gelatinized plates as following:

Plate	Surface (cm^2)	Relative Size	Cell-Suspension (ml)	Number of Plates
10cm	78,5	1	10	1
6cm	28	1 : 2,8	3,3	3
6 well	9,6	1 : 8	1,25 / well	8 wells
24 well	1,8	1 : 44	0,20 / well	2
96 well	0,38	1 : 100	0,05 / well	2

4.5.2. Cell Culture of ES-cells

Undifferentiated ES-cells form, under optimal cell culturing conditions, give colonies with clear-border definition. The individual cells in a colony are hard to distinguish from each other, they are conspicuous through a characteristically large, pigmented nucleus.

ES cells were cultivated at 37°C and 7,5% CO_2 on gelatinized plates with a feederlayer for two to three days. To postpone differentiation ES media contained a murine leukemia inhibitory factor (LIF) (Smith *et al.* 1988). For splitting, the cells were twice washed with DMEM, incubated with 2ml of 1x trypsin/EDTA at 37°C for 5min, disintegrated and centrifuged with 2 volumes of feeder medium. The supernatant was removed, the cell-pellet resuspended in ES medium and plated.

Prior to use for gene targeting experiments, the germline competence of ES-cell line has to be tested by the production and test breeding of chimeras. It is important to keep the cells under appropriate conditions that allow, even after a longer culture, to retain their capacity to participate in germline development. The medium has to be exchanged daily or even twice a day, if culturing at higher concentration. The density has to be regulated by frequent cell splitting (every 2-3 days) to avoid cell differentiation. When trypsinizing ES cells it is important to fully disintegrate cell aggregates, because larger aggregates tend to start differentiating. The max. ES cell concentration is $1{,}5x10^7$ cell/10cm plate. The fetal calf serum used for ES cell media must be tested for its plating efficiency and colony morphology. ES cells should not be passaged more than absolutely necessary as longer maintenance in cell culture might cause mutations preventing germline transmission. Also, because an ES cell line is a heterogeneous population of cell, the fraction of cells which have lost pluripotency may increase with the passage number. Any cells treated under suboptimal conditions have to be discarded.

4.5.2.1. Electroporation of ES Cells

ES cell were plated at a concentration of $1x10^6$ cells/10cm plate and cultivated for two to three days as long as the culture contained sufficient cells for electroporation but still was in the optimal growth phase. A few hours before transfection ES medium was exchanged to increase the number of cells in S-phase in order to enhance efficiency of gene targeting. Cells were washed twice with PBS. 2ml of 1x trypsin/EDTA was added and then incubated at 37°C (~ 5min). The cells were resuspended with a pipette so that no aggregates were left and centrifuged in 6ml of feeder medium. The supernatant was discarded, the cell pellet was

resuspended in transfection buffer and centrifuged at 4°C. This washing step was repeated twice. After the last centrifugation 10^7 cells were resuspended in 800µl of transfection buffer and mixed with 25µl of target vector in PBS (1µg/µl).

The target plasmid must be prepared in ultrapure quality. Prior to transfection the construct must be linearized, phenol/chloroform extracted, precipitated with 100% ethanol, twice washed with 70% ethanol, air dry under a laminar flow cabinet and resuspended in sterile PBS.

The cells within the transfection buffer and with 25µg of target vector were pipetted into an ice cold electroporation cuvette (1 cm) and placed on ice. After 10min of incubation on ice the cells were electroporated at 400V and with the electroporator capacitance set at 250µF. The cuvettes with the cells were transferred back onto ice for 10min. The cells were plated on gelatinized plates with a feederlayer in ES medium at a concentration of $2x10^6$ survived cells/10cm plate.

4.5.2.2. Selection of Resistant Clones

After electroporation the cells grew undisturbed for 48 hours (the medium was changed after 24 hours) before the selection was initiated. ES medium with G418 (200µg/ml of active G418) was used and the selection continued until the clones became macroscopically visible (8-12 days). Once selection was complete the resistant clones must be isolated. Colonies should be large but still maintain a discrete border. Clones were picked individually under a microscope with 200µl tips and transferred into a U-bottom 96 well plate with 30µl of Trypsin/EDTA. Once the plate was full it was incubated 5min at 37°C. Then the clones were disintegrated with 200µl of ES medium and transferred into a new flat-96 well plate with a feederlayer. After one to three days the ES medium was discarded, the wells were washed with DMEM medium and trypsinized with 30µl of Trypsin/EDTA for 5min in an incubator. The cells were disintegrated in 200µl of ES medium and with a 8-canal pipette divided into two 96 well plates – a master plate, that was frozen and a replica plate, that served to expand the cells to isolate genomic DNA. Two days later the cells from then 96 well plate were transferred into a gelatinized 24 well plate to continue their growth for several days. When the cells reached confluence in the 24 well plate the genomic DNA was isolated for Southern or PCR analysis.

4.5.2.3. Freezing and Thawing of ES Cells

Cells must be frozen by decreasing the temperature with rate 1°C/min. That can be achieved by freezing cells in Cryo-freezing container containing isopropanol or in a cardboard freezing box placed in –80°C freezer.

To freeze larger amounts of cells in separated tubes the cells were washed with PBS, trypsinized, resuspended with medium and centrifuged. The pellet was dispersed in a freezing medium (10% DMSO, 50% FCS in medium) and tubes were placed with Cryo-freezing container in a –80°C freezer. For longer storage the cells were transferred into liquid nitrogen.

For freezing a 96well plate the cells were trypsinized, resuspended in a freezing medium and placed in a cardboard freezing box in a –80°C freezer.

When thawing cells it is important to separate the cells from the toxic DMSO as fast as possible and thaw quickly. The cells were thawed in a water bath and the content of the tube respective well was transferred into a 15ml tube with prewarmed medium and centrifuged. The pellet was resuspended in medium and plated on an appropriate sized cell culture plate.

4.5.2.4. Gelatinization of Plates prior to Embryonic Stem (ES)-Cell Culture

The culture plates were covered with a solution of 0,1% gelatine in PBS and incubated for >5min at 4°C. Before use the gelatine solution was discarded.

4.5.3. Cultivation of Established Cell Lines

The cells were seeded out at about 1 x 10^6 cells/ml of complete medium. They were cultured at 37°C in humidified atmosphere containing 5% CO_2. To maintain the culture, when the cells reached cofluent density they were split (as described for ES cells) 1:3 or 1:5, dependent on their growth features. The medium was changed every second day.

Harvesting and freezing of the cell lines was proceeded exactly as described for the ES cell culture (4.5.2.).

4.5.4. Generation of ES Chimera Mouse

4.5.4.1. "Superovulation" and "Foster Mother" Preparation

By superovulation the regulation of ovulation in mice with exogenous hormones by i.p. injections is meant. This way the number of isolated blastocytes could be effectively enhanced.

For the mouse strain C57/BL6J the following strategy was used:

At noon the day - 0 - sexually immature females were injected with 10U pregnant mare serum gonadotropin (PMSG that mimics follicle stimulating hormone); at day - 2 - 46-48 hours later 10U human chorionic gonadotropin (HCG that mimics luteinizing hormone) was injected and the injected females housed with males overnight. Next day, day - 3 - the females were checked for plugs and caged separately. In the morning of day 5 the blastocysts were isolated.

Usually six to eight animals were used to isolate enough usable blastocysts (20 - 40). About 18-20 blastocysts were required for one transfer into a foster mother. The yield of blastocytes depends very much on the age of mice. In the case of too young mice the hormonal stimulation is not yet able to induce ovulation and in the case of older mice, the natural level of its hormones might be already difficult to disestablish. Older animals (7-8 weeks) could be used for production of blastocyts by natural breeding. In this case 30-40 females were set up and checked the next day for vaginal plug. Of course, the quality of blastocysts, prepared this way is better than by superovulation although the quantity is significantly lower (three to four blastocysts per mouse could be isolated and only 20-25% of females are positive for vaginal plug).

In parallel to ovulation stimulation the breeding of foster mothers was set up. For this purpose F1 female mice 8 – 16 weeks old were used. They reproduce fairly well and exhibit good maternal instincts. Thirty BALB/c females were bred to vasectomized males and next day they were checked for a vaginal plug. Foster mothers are used as host for the injected blastocyst on day 2,5 days p.c.

4.5.4.2. Isolation of Blastocysts

At day 3,5 days p.c. the females were sacrificed, their abdomen was disinfected with 70% ethanol and the abdomen was open by gripping the skin anterior and posterior to the incision, the skin was torn by pulling it back to the front and hind limbs. The peritoneum was opened. The uterus was lifted, the cervix was cut across and another cut through the utero-tubular junction on each side, freeing the entire tract. All the fat pad and the supportive tissue of the uterus was cut away on a dry paper towel. The so cleaned uteri were transferred into a 6cm dish filled with M2 medium. The uteri were rinsed with a 26 gauge needle that is cut off and bent 45 degrees 10-20mm from the end. This modified injection needle was put onto a syringe filled with M2 medium. Under the microscope the needle was inserted into the vaginal end of the uterus. The uterus distended upon filling it with medium and the

entrance to the fallopian tube became clearly identifiable. The junction was cut there whereupon the solution in the uterus and the blastocytes run into the medium. Once all uteri were rinsed this way, the blastocytes are allowed to settle for 5 minutes and are collected with a pipette from the medium. The blastocytes were rinsed with M16 medium and stored in aggregate drops with preincubated M16 medium under embryo tested paraffin at 37°C and 5% CO_2 until injection.

4.5.4.3. Injection, Holding and Transfer Capillaries

The quality of the injection capillaries is crucial for the success of the injection of ES cells. The aperture of the capillary should be large enough so that the cell is not deformed during aspiration. On the other hand a too large opening leads to more damage of the blastocytes. The tip of the capillary should be smooth and angled at 60 degrees.
Capillaries were made with a pipette puller and broken at an appropriate position under the microscope. The tips were polished. They were melted and angled 30 degrees in the opposite direction of the tip angle. The capillary was finally rinsed with ethanol and dried in a dessicator.

Holding capillaries were pulled manually, broken at a suitable position with a micro furnace and polished. The capillary can be angled 30 degrees in order to create the same angle in the injection chamber. Transfer capillaries were also pulled manually, broken at a suitable position and polished with the furnace.

4.5.4.4. Injection of ES Cells into Blastocytes

ES cells were detrypsinated and suspended in ES or M2 medium. The single cell suspensions were kept on ice. Two or three blastocytes were put in the injection chamber together with several hundred ES cells. 15 ES cells were aspirated into the injection capillary. It is important that the cells are lined up. Blastocytes were oriented so that the inner cell mass (ICM) comes to lie at the opening of the capillary or are 90 degrees offset. The blastocyte is fixated by aspiration that the cell delineation of the trophectoderm comes to lie in the focal plane between the holding and injection capillary. The injection capillary is approximated that the zona pelicuda is in contact. The injection between cells of the trophectoderm facilitates the penetration, minimizes damage to the blastocytes and decreases blocking of the injection capillary. The injection was done fast enough to avoid the blastocyte collapsing but avoiding damage to the ICM.

Once the injection capillary has penetrated, the cells were deposited on the ICM (7-15

cells). The injection capillary is extracted and the blastocyte released. Injected blastocytes initially collapse but recover after 3 hours if they are kept in culture medium if the procedure is successful. The injected cells were visible in the blastozole.

4.5.4.5. Anesthesia and Embryo Transfer

For transfer six to nine blastocytes in M2 medium were aspirated into a transfer pipet so that they were lined up and separated by air gaps. Oil that might be on the outside of the capillary was rinsed away with M2 medium in order not to aggravate the uterus. Generally the transfer of embryos is done under anesthesia with Avertin (2.5 g of 2,2,2-tribromoethanol (Aldrich) dissolved at 50°C in 5ml of 2-methyl-2-butanol). To this solution 200ml of water were added under stirring until the butanol is distributed evenly. The ready anesthesia solution was stored in the dark at 4°C in aliquots of 10ml. For narcosis 0.2 - 0.35ml/g body weight was injected peritoneally. Anesthesia keeps for 30-50min.

The fur on the back of anesthetized animals was sterilized with ethanol. A 2mm cut was made along the spine on the level of the hind legs. The cut was fixated with tweezers and the fallopian tubes localized. The uterus was pulled out of the ventrical cavity. A seraphin clip was attached to the fatty tissue around the uterus. The uterus was punctured with a syringe. The transfer capillary was entered into the opening and the blastocytes injected. The air gaps serve as optical aids. Should the transfer capillary be blocked by debris, it can be deblocked by a short aspiration. After injection the uterus was gently pushed back into the ventrical cavity. The procedure was repeated for the second fallopian tube.

4.5.5. Animal Housing and Breeding

4.5.5.1. Hygiene

Strict regulations govern the precautions for the protection of animals from mouse pathogens. Animals that have left the housing facility are never reintroduced. The health of animals in respect to mouse pathogens is regularly checked with detailed tests.

4.5.5.2. Test of Chimeric Mice for Germline Transmission

In order to check chimeric mice for germline transmission they are crossed with wildtype animals from which the blastocytes were taken. The ES cells were isolated from 129ola, a mouse strain with cream colored fur. If the ES cells have partaken in the transmission, the offspring of crossed animals can be distinguished by the color of their coat.

4.5.5.3. Backcrossing

In order to generate a pure genetic background from a mixed strain 10 generations of backcrosses to the inbred mouse strain have to be done.

For this reason an animal with a mixed background is crossed with an animal of the desired strain. Offspring of this crossing is again crossed with the desired strain. This is continued for 10 generations. If it is desirable to introduce a defined genetic defect into the offspring, animals are tested for mutations and heterozygous animals used for further backcrosses.

4.6. ANALYSIS OF PHENOTYPIC CHANGES IN THE MICE

4.6.1. Analysis of Blood Gases, Electrolytes, Serum Gastrin and Serum Albumin

Analysis of blood gases, serum gastrin and albumin were done in the Campus Virchow-klinikum, Berlin (Germany).

Awake mice were gently warmed for 10-15 min on a heating pad to increase peripheral blood circulation. Blood (1,5 - 5,5ml) from the tail vein was collected from several animals in heparin-, EDTA- treated tubes or in not-treated tubes and analyzed for gases, electrolytes and pH using automated laboratory analyzers (Boehringer Mannheim, Germany) according to manufacturers recommendations. The gastrin levels were measured using the specific antibody raised against human gastrin 17, according to the procedure described in Hocker *et al.* (1996).

4.6.2. Histochemical and Immunohistochemical Analysis

The histochemical and immunohistochemical analysis were done in the Department of Anatomy of the University of Oulu (Finland).

The mice were fed with standard diet and were sacrificed at desired ages. Tissue specimens were cut from stomach, duodenum, jejunum, ileum, colon, liver, pancreas, and kidney. The specimens were fixed in Carnoy's fluid (absolute ethanol + chloroform + glacial acetic acid 6:3:1) for 24 hr at 4^{o}C or in 4% neutral-buffered formaldehyde 24-48 hr at room temperature. The samples were then dehydrated, embedded in paraffin wax in a vacuum oven at 58^{o}C, and sections of 5µm were placed on gelatin-coated microscope slides.

The immunostaining of tissue sections was performed using the biotin-streptavidin complex method, employing the following steps: *(1)* Pre-treatment of the sections with undiluted cow colostral whey for 40 min and rinsing in phosphate-buffered saline (PBS). *(2)*

Incubation for 1hr with the first antibody (5µg IgG/100µl) in PBS containing 1% bovine serum albumin (BSA). *(3)* Treatment with cow colostral whey for 40min and rinsing in PBS. *(4)* Incubation for 1hr with biotinylated swine anti-rabbit IgG (Dakopatts) diluted 1:300 in 1% BSA-PBS. *(5)* Incubation for 30min with peroxidase-conjugated streptavidin (Dakopatts) diluted 1:500 in PBS. *(6)* Incubation for 2min in DAB solution containing 9mg 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15ml PBS + 5µl 30% H_2O_2. The sections were washed three times for 10min in PBS after incubation steps 2, 4, and 5. All the incubations and washings were carried out at room temperature and the sections were finally mounted in Permount (Fisher Scientific, Fair Lawn, NJ). The stained sections were examined and photographed with a Nikon Eclipse E600 (Tokyo, Japan) microscope.

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)

Sections of 4 m in thickness were floated onto glass slides coated with poly-L-lysine (Sigma, St. Louis, MO) and dried at 37 °C overnight. The 3-end labeling of apoptotic cell DNA was performed using an ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) with minor modifications as described (Tormanen *et al.*, 1995). Before the procedure, the specimens were heated at 70°C for 10 minutes. After deparaffinization in xylene and rehydration through graded ethanol series, the sections were incubated with 20 g/ml proteinase K (Boehringer Mannheim Gmbh, Mannheim, Germany) at room temperature for 15 minutes. Endogenous peroxidase activity was quenched in 2% hydrogen peroxide in phosphate-buffered saline (PBS). Digoxigenin-labeled nucleotides were catalytically added to the 3-OH ends of the fragmented DNA by using terminal transferase enzyme. Thereafter, peroxidase-conjugated antidigoxigenin antibody was applied on the slides. Diaminobenzidine-hydrogen peroxide was used to develop the color reaction. Finally, specimens were counterstained lightly with hematoxylin.