4- Materials and Methodes

4.1- Material

4.1.1- Instruments

Microtom Leica VT 1000S

luminometer (CliniLumat LB 9502)

Ultra-Turrax T8 (IKA®-Werke, Frankfurt)

ultracentrifuge Beckmann XL-70

SW41 Ti rotor

Facscan Becton Dickinson with software CellQuest 3.3

Intensified Charged Coupled Photon Counting Device (ICCD)

Peltier Thermal Cycler (PTC-200) with heated lid (MJ Research, MA, USA)

4.1.2- Important chemicals, solutions and buffers

phenol liquefied washed in Tris buffer (Biomol, Germany cat. #58734)

TRIZOL®LS reagent (Life Technologies #10296)

Buffer S1 (50mM Tris/HCL, 10mM EDTA, 100µg RNase A/ml, pH 8.0)

Buffer S2 (200mM NaOH, 1% SDS)

Buffer S3 (2.8 M KAc, ph 5.1)

Buffer N1 (100mM Tris/H_3PO4, 15% ethanol, 400mM KCl, pH 6.3)

Buffer N2 (100mM Tris/H_3PO4, 15% ethanol, 900mM KCl, pH 6.3, 0,15% Triton X-100)

Buffer N3 (100mM Tris/H_3PO4, 15% ethanol, 1.15M KCl, pH 6.3)

Buffer N5 (100mM Tris/H_3Po4, 15% ethanol, 1M KCL, pH 8.5)

2x HEPES Buffered Saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, 50 mM HEPES, pH 7.5)

2 M Calcium Chloride: dissolve 14.7g $CaCl_2$ in 100 ml H_2O. Filter sterilize and autoclave.

Oligotex Suspension: 10%(w/v) suspension (=1mg/10µl) Oligotex particles in 10mM Tris/HCl pH 7,5; 500mM NaCl; 1mM EDTA; 0,1% SDS; 0,1% NaN_3

Wash buffer OW2: 10mM Tris/HCl pH 7,5; 150mM NaCl; 1mM EDTA

Elution Buffer OEB: 5mM Tris/HCl pH 7,5

Buffer OBB: 20mM Tris/HCl pH 7,5; 1mM NaCl; 2mM EDTA; 0,2% SDS

Psi broth medium (per Liter)

compound	amount
Bacto yeast extract	5 g
Bacto Tryptone	20 g
magnesium sulfate	5 g
pH 7.6 with potassium hydroxide	

TfbI buffer (per 200 ml)

compound	amount	final molarity/conc.
potassium acetate	0.588 g	30 mM
rubidium chloride	2.42 g	100 mM
calcium chloride	0.294 g	10 mM
manganese chloride	2.0 g	50 mM
Glycerol	30 ml	15% v/v
pH 5.8 with dilute acetic acid		

TfbII buffer (per 100 ml)

compound	amount	final molarity/conc.
MOPS	0.21 g	10 mM
calcium chloride	1.1 g	75 mM
rubidium chloride	0.121 g	10 mM
Glycerol	15 ml	15% v/v
pH 6.5 with dilute NaOH		

L-broth medium (for 1 liter)

compound	amount
2% Bactotryptone	20g
0.5% Bactoyeast extract	5g
2.5mM KCl	2.5ml of 1M KCl
10mM $MgCl_2$	10.0ml of 1M $MgCl_2$
20mM Glucose (‰ 0.2%)	10.0ml of 20% glucose
10mM $MgSO_4$	10.0ml of 1M $MgSO_4$
10mM NaCl	2.0ml of 5M NaCl

20x SSC buffer

3M NaCl	875g
0,3M Na3-citrate 2 H2O	440g
39% HCl	adjust to pH 7,0
double distelled water to	5 liter

Soak I buffer

1M NaCl	58,4g
0,5M NaOH	20g
double distelled water to	1000 ml

Soak II buffer

05M Tris pH 7,4	60,5g
3M NaCl	175,2g
39% HCl	adjust to pH 7,4
double distelled water to	1000 ml

Hybridization buffer

50x Denhardt	10 ml
20x SSC buffer	30 ml
10% SDS	5 ml
double distelled water to	100 ml

Fresh 10µg/ml Herring sperm DNA was added in buffer for use

10x E-Buffer

300 mM $NaH_2PO_4.H_2O$	41,39 g
50 mM EDTA	18,61 g
double distelled water/DEPC	1 liter

Formaldehyde gel

Agarose	1,5 g
10x E-Buffer / DEPC	15 ml
double distelled water/DEPC	105 ml

Luc-Lysis buffer

25mM Tris pH 7,8	5ml of 1M Tris/HCl
20mM DTT	616mg
2,5mM EDTA	1 ml of 0,5M pH8,0
5% Glycerin	10ml
1% Triton X100	2ml
double distelled water to	200 ml

Store at 4°C or at -20°C

Measure Luc buffer

15mM $MgSO_4$	1,85g
Glycylglycin $C_4H_8N_2O_3$	175,2g
39% HCl	Adjust to pH 7,5
double distelled water to	500 ml

Store at 4°C or at -20°C

4x TG buffer (500ml total)

1,5M Tris/HCl pH 8,8	90,75g
0,4% SDS	2g
0,01% NaN_3	0,5ml of 0,1% w/v Solution
double distelled water to	500 ml

4x SG buffer (250ml total)

0,5M Tris/HCl pH 6,8	30,25g
0,4% SDS	2g
0,01% NaN_3	0,25ml of 0,1% w/v Solution
double distelled water to	250 ml

Use Roth rotiphorese® Gel 30 (30%ige acrylamide, 0,8% bis-acrylamide)

5x Laemmi buffer

10% SDS	5g
25% 2-mercaptoethanol	12,5ml
20% Glycerol	10ml
20mM Tris/HCl pH 6,8	12,5ml of 1M Tris/HCl solution
Bromophenol blue	10g
double distelled water to	50 ml

10x Running buffer

Tris	75,7g
Glycin	360,3g
10% SDS	25ml
double distelled water to	2500 ml

Coomassie blue (2 liter)

Coomassie brilliant blue powder (BioRad)	2 g
methanol	1 liter
acetic acid	200 ml
double distelled water to	800 ml

Western Transfer buffer

Tris	5,82g
Glycin	2,93g
Methanol	200ml
10% SDS	3,75ml
double distelled water to	1000 ml

1x TBS buffer

1M Tris/HCl pH 8,8	10ml
5M NaCl	30ml
double distelled water to	1000 ml

4.1.3- Molecular weight markers

marker	fragment size
Lamda DNA (*HindIII* /*EcoRI*)	21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125 bp fragments

marker	fragment size
Smart DNA Ladder Eurogentec (#MW-1800-10)	10000, 8000, 6000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600 and 400 bp fragments
RNA Ladder GibcoBRL® (#15620-016)	9490, 7460, 4400, 2370, 1350 and 240 bp fragments
Rainbow Amersham Pharmacia Biotech (RPN 756)	

4.1.4- Cell types and characteristics

Cell-type	Origin/source	Peculiarity / characteristic
HuH7	Human Hepatoma cell line	human hepatoma cells harbouring mutant p53
AML12	Hepatocytes cell line	published by Wu J.C. et al., 1993. These cell line was provided by A. Israel (Institut Pasteur, Paris) thanks H. Sirma.
HeLa	Human carcinoma cell line	
HepG2	Human Hepatoma cell line	
X1/6 HeLa	Human carcinoma cell line	stably expressing luciferase under the tTA-responsive promoter (Gossen M. and Bujard H., 1992)
HR5-13S	Human carcinoma cell line	stably expressing transcriptional silencer element tTS^{Kid} (Freundlieb S. et al., 1999).
293	Human embryonic kidney cell line	stably expressed the E1 proteins of adenovirus genome (Granam F.L. et al., 1977).

4.1.5- Bacteria Strains

Cell-type	Peculiarity / characteristic
DH5α *E. coli*	genotype: *sup*E44, *lac*U169, (O80 *lac*ZM15) *hsd*R17, *rec*A1, *end*A1, *gyr*A1, *gyr*A96, *thi*1, *rel*A1, (Tet^r)
XL1 Blue *E. coli*	genotype: *hsd*R17, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*(F´, *pro*AB, *lac*$I^qZ\Delta M15$, Tn10, (Tet^r)) (Stratagene)
BJ5183 *E.coli*	genotype: *end*A *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R (Str^r) (Hanahan D, 1983)
GM33 *E. coli*	genotype: F^- *Lam*$^-$ IN(*rrn*D^- *rrn*E)1 *dam*-3 *sup*-85 (Marinus MG. et al., 1973)

4.1.6- Mice types

	Peculiarity / characteristic
HBV1.3 fsX(-)3´5´	generated by transferring the 1.3 overlength HBV genome with frameshift in X gene (C. Kuhn and H. Schaller) into fertilized F2 (C57Bl/6) eggs (Weber O. et al., 2002)
LT1	$TA^{LAP}1$ (Kistner A. et al., 1996)
LT2	$TA^{LAP}2$ (Kistner A. et al., 1996)
SR-Lap	rTA^{LAP} (Schönig K. et al., 2002)
C57Bl/6	
MNRi	

4.1.7- Antibodies

H801	polyclonal rabbit antibody against HBV core protein (HBcAg) provided by C. Kuhn (ZMBH).
anti ß actin	monoclonal mouse antibody against human β actin
anti rabbit –OP	polyclonal sheep antibody against rabbit conjugated to peroxidase (PO), used as secondary antibody in Western blot analysis
anti rabitt –AP	polyclonal sheep antibody against rabbit conjugated to alkaline phosphatase (AP), used as secondary antibody in Western blot analysis
anti-hexon IMAGEN™	monoclonal antibody against hexon protein found in all serotypes of human adenovirus, conjugated to fluorescein isothiocyanate (FITC) (DAKO #K6100)

4.1.8- Oligonucleotids

Primer HBV 1745+AB	5´ GTT GCC CGT TTG TCC TCT AAT TC 3´
Primer HBV 1844-AB	5´ GGA GGG ATA CAT AGA GGT TCC TTG A 3´
Primer Luc PacISbis 40029	5´ TGT ATT TGA TCA AAG ACT TCA AG 3´
Primer LucPacIASbis 40030	5´ TGA AGT CTT TGA TCA AAT ACA AAG 3´
Primer pBi-2Luc 34797	5´ CGT GAC AAA ACA ATT GCA CT 3´
Primer SVpoly 34798	5´ CGG CTA GCC GGA TCA TAA TCA GCC AT 5´
Primer mIFNg(+)	5´ AGG CGT CGA CGA ATT CAA TGA ACG CTA CAC ACT 3´
Primer mIFNg(-)	5´ CCC AAG CTT CCC GGG TCA GCA GCG ACT CCT TT 3´
Primer mIFNalpha(+)	5´ TAA GGC GTC GAC GAA TTC CCA TGG CTA GGC TCT GTG 3´
Primer mIFNalpha(-)	5´ TAC CCA AGC TTC CCG GGG CTC AGG ACT CAC TCC TC 3´
Primer mIFNbeta(+)	5´ TAA GGC GTC GAC GAA TTC TCA TGA ACA ACA GGTGGA 3´
Primer mIFNbeta(-)	5´ TAC CCA AGC TTC CCG GGT TCA GTT TTG GAAGTTTCTG 3´

Primer srtTA(+)	5´ CCA TGT CTA GAC TGG ACA AGA 3´
Primer srtTA(-)	5´ CTC CAG GCC ACA TAT GAT TAG 3´
Primer muOAS 5.3	5´ ACGGAGCTCCAGCGGAACTTC 3´
Primer muOAS 3.3	5´ GCAACTCTAGGGCGTACTGTG 3´

4.1.9- DNA probes

HBV	3,3 kb fragment from digested pHG913091 plasmid *Sal I* / *Xma I* (provided by H. Schaller, ZMBH, Heidelberg).
mouse ß actin	741 bp fragment from digested pBSKS*II*-mβActin plasmid *XbaI* / *BamHI (*provided by S. Stahl, DKFZ, Heidelberg)
mouse GAPDH	814 bp fragment from digested pBSKS*II*-mGAPDH plasmid *XbaI* / *BamHI (*provided by S. Stahl, DKFZ, Heidelberg)
2´5´OAS	1,4 kb fragment from digested pMA25 plasmid *EcoRI* (provided by F.V. Chisari, The Scripts, USA)
rtTA	1,5 kb fragment from digested pUHD TTR 0.3/h-rtTA(FFF) *BstEII* / *Sal I* (provided by H. Bujard, ZMBH, Heidelberg)
mIFNγ	600 bp PCR product using primer mIFNg(+), primer mIFNg(-) and adenoviral genome of AdmIFNγ as template.

4.2- Methods

4.2.1- Methods for molecular analysis

4.2.1.1- Quick plasmid DNA mini-preparation

5 ml culture was seeded overnight and the bacteria were collected in 1,5ml tube. Following centrifugation at 5000 rpm for 5min, the pelled cells were resuspended in 100µl of buffer S1 (maxiprep Kit Nucleobond®). 200µl buffer S2 was added to lyse the cells for 5 min on ice. The lysis was stopped by addition of 150µl buffer S3. 5 min. later the cell debris were removed by centrifugation at 13000 rpm for 15min. The plasmid DNA in supernatant was precipited by addition of 1ml ice-cold 100% ethanol and centrifugation at 13000 rpm for 30 min. The pellet was washed with 70% ethanol and air- dried, before its elution in 50 µl double distilled water.

4.2.1.2- Preparation of large amounts of plasmid DNA

To prepare large quantity of plasmid DNA for transfection of cells, 200 ml of *E. coli* XL-1 blue or *E. coli* DH5α overnight culture was prepared by alkaline lysis method. This was done using buffers S1-S3 with the Nucleobond® ion exchange silica cartiges AX500 (Macherey-Nagel, Düren, Germany), washed with buffer N3 and eluted with buffer N5. Purified plasmid DNA was then precipitated with isopropanol and washed with 70% ethanol. Dried 500-800µg DNA was re-dissolved in TE buffer giving a total concentration of 1µg/µl that was used for further applications.

4.2.1.3- Ethanol and Isopropanol DNA precipitation

0,1 volume 3 M NaAc and 2 volumes absolute ethanol were added to a given amount of DNA containing solution and incubated for 10 min at –70°C, and then pelleted by centrifugation at 13000 rpm at 4°C for 30-40 min. The obtained pellet was washed with 70% ethanol, air dried and re-dissolved in a given volume of water or TE buffer.

For isopropanol precipitation 0,8 to 1,2 volume isopropanol was mixed with a given quantity of DNA depending on the salt concentration and then processed as described for ethanol precipitation.

4.2.1.4- Cleavage of DNA using restriction enzymes

Cleavage of plasmid DNA with restriction enzymes was done using standard methods according to the instructions of the manufacturer. The volume of the enzyme used was always less than 10% of the total volume of the digest to prevent inhibition of the reaction.

A diagnostic digest was done using 1 µg DNA (for example 5 µl miniprep DNA) in a total volume of 20 µl. For a double digestion, if no common buffer specific to both enzymes was available, the plasmid DNA was ethanol precipitated following the first digestion and re-dissolved in the appropriated buffer of the second enzyme.

4.2.1.5- Quick isolation or purification of DNA-fragments

The binding of DNA to the silica membrane in the Nucleospin Extract columns (Macherey-Nagel, Germany) is achieved by chaotropic salts in buffers NT1 and NT2. The buffer NT1 contains additional components in order to dissolve agarose gel pieces at 56°C. Contamination like salts and soluble macromolecular components are removed

by a simple washing step with ethanol containing buffer NT3. Pure DNA is finally eluted under low ionic strength conditions with 25-50µl slightly alkaline buffer NE (5mM Tris/HCl pH 8,5). Yield of larger fragments (5-10 kb) can be increased by using pre-warmed elution buffer NE (70°C) and incubate for 5 min. before collecting eluate by centrifugation.

4.2.1.6- Removal of the 5´end phosphate of DNA-fragment by alkaline phosphatase

Alkaline phosphatase catalyzes the removal of 5´phosphate groups from DNA, RNA and ribo- and deoxyribonucleotide triphosphates. The DNA was disolved in 10x CIP buffer and 0,5 units CIP (1U/µl) per µg vector DNA was added. After incubation for at least 60 min at 37°C, the DNA was purified using the Nucleospin column to remove alkaline phosphatase.

4.2.1.7- The polymerase chain reaction (PCR)

The PCR method results in the selective amplification of a chosen region of a DNA molecule in the presence of specific oligonucleotides (primers) complementary to the DNA molecule and heat stable polymerase. Each PCR was performed in a total volume of 50 µl composed of the following: 250nM dNTPs, 25ng template DNA, 20pmole each primer, 1 unit polymerase and PCR buffer (20mM Tris/HCl pH 8,2, 5mM KCl and 4mM $MgCl_2$).

Enzymes used for PCR were chosen according to the length of the fragment to be amplified. Short fragment PCR of less than 1 kb were performed with Taq polymerase. Long PCR fragments greater than 3 kb were generated with *Pfu* (*Pwo*) polymerase, which has a 3´-5´exonuclease proof-reading activity therefore minimizing the possibility of PCR based mutations introduced by *taq* polymerase. PCR was done on a Peltier Thermal Cycler (PTC-200) with heated lid (MJ Research, MA, USA) and general cycling conditions: at 95°C for 5 min; (at 95°C for 30sec; at 60°C for 30sec; at 72°C for 30sec) x 40 cycles; 72°C for 5 min . The amplified products were either directly purified by Nucleospin® DNA purification method or gel purification.

4.2.1.8- Real-time PCR by Light Cycler (Roche Molecular Biochemicals)

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the

endpoint detection by conventional quantitative PCR methods. The real-time PCR system is based on the detection and quantification of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The used alternative is the double-stranded DNA binding dye chemistry which determines quantitatively the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I). SYBR Green I is a minor groove binding dye. It does not bind to ssDNA. Fluorescence is greatly enhanced by binding. During the various stages of PCR, different intensities of fluorescence signals can be detected, depending on the amount of dsDNA that is present. After denaturing SYBR Green I dye will not bind and the intensity of fluorescence signals is low. During annealing the PCR primers hybridize to the target sequence, resulting in small parts of dsDNA to which SYBR Green I dye can bind, thereby increasing fluorescence intensity. The fluorescence is recorded at the end of the elongation phase (at 530nm) and increasing amounts of PCR product can be monitored from cycle to cycle. Real-time PCR mixture was performed as following:

2µl DNA extracted from 50µl serum or external standards

1,6µl 25mM $MgCl_2$

0,2µl each primers (10 pmol)

2µl Cyber Green (mixture of 60µl buffer 1a and 1b containing the polymerase);

14µl sterile water for PCR.

To determine the concentration of standard nucleic acid, the OD was measured at 260nm according to standard procedures. To calculate the copy number of the standard, the following mathematical correlation and formula was used as a guidelines for ds DNA:

1 mol = $6x10^{23}$ molecules or copies

Molecular weight = number of base pairs x 660 daltons/base pairs

amount (copies/µl) = $6x10^{23}$(copies/mol) x concentration (g/µl) / Molecular weight (g/mol)

4.2.1.9- Ligation of DNA fragments into vectors

Concentrations of vector and insert DNA were estimated by agarose gel electrophoresis with molecular weight markers of known concentration. Ligations were optimized by trying vector/insert ratios of 3:1, 1:1 and 1:3. The vector-insert DNA mixes were added to 2 µl 5x T4 ligase buffer (NEB), 1U T4-ligase and filled with double distilled water to a final volume of 20 µl. Ligation reactions were incubated at room temperature for 3 hours and then transformed into competent bacteria.

4.2.1.10- Generation of blunt ends in digested plasmid

To remove the 3´ overhang ends of double-strand DNA use the 3´→5´ exonuclease activity of T4 Polymerase or the large fragment of DNA Polymerase I (Klenow) to generate blunt ends. 100 µM of each dNTP was mixed and 1 to 3 units polymerase per µg DNA was added. The reaction was incubated at 12°C for 20 min. and then inactivated by heating at 75°C for 10 min. or by phenol/chloroform extraction.

4.2.1.11- Rubidium Chloride method for preparing transformation competent *E. coli*

1 ml overnight culture was innoculated in 100 ml Psi broth medium until an OD of 0.5-0,8 at 550 nm was attained. Then the culture was incubated on ice for 15 min and centrifuged at 3000-5000 xg for 5 min. The pellet was resuspended in 0.4 volume fresh prepared TfbI buffer on ice for 15 min and centrifuged at 3000 xg. The last step was the resuspension of the pellet in 0.04 volume with TfbII buffer on ice for 15 min. The competent bacteria were used immediately or quickly frozen at –70°C for storage. These were later thawed on ice just before using in a transformation experiment. To test the competence capacity, 50µl of bacteria were transformed with 0,1fmol pUC13 plasmid to obtain ~10^3 cfu/fmol pUC13 (or at least 10^8 cfu/µg DNA).

4.2.1.12- Bacteria transformation by heath stock

5 µl of the ligation reaction was added to 50µl of competent *E. coli* DH5α or XL-1 blue and then placed on ice for 5 min. Transformation efficiency was enhanced with a 2 min heat shock at 37^{o}C, followed by chilling bacteria on ice for a further 5 minutes. The bacteria were incubated with 1ml L-broth (LB) medium at 37^{o}C for 30 min. to 1 hour.

Cells were spun for 3 min at 3000 rpm. Most of the supernatant was removed and the pellet was resuspended with the remaining supernatant. The cells were spread on LB-agar/Ampicillin (100µg/ml) or LB-agar/Kanamycin (100µg/ml) plates. The plates were inverted and incubated overnight at 37^{o}C.

4.2.1.13- Production of transformation electro-competent bacteria

10ml (1/100 of the volume) of the overnight culture was seeded in 1 liter L-broth medium and was incubated until an OD at 600nm ranging from 0,5 to 1,0 was reached. The cells were centrifuged at 5000 rpm for 15 min. at 4°C and the pellet was washed with 1 liter ice-cold water. The bacteria were pelleted again and washed with 500ml ice-cold water. The bacteria were resuspended in 20ml ice-cold with 10% glycerol and centrifuged at 5000 rpm at 4°C. Finally the electro-competent bacteria were resuspended in 2-3ml 10% glycerol (‰ 10^{10}cells/ml) and frozen on dry ice for storage at –70°C.

4.2.1.14- Transformation of electro-competent bacteria

Chill cuvette (0.2cm gap) was used on ice. At least 40 µl plasmid sample was mixed with 40 µl aliquot of electro-competent cells in ice cold cuvette. The bacteria were electroporated at 200 ohms, 25 mfarads and 2.5 kV. The time constant was in the 3-5 ms range. Immediately, 1ml LB medium was added in transformed bacteria and incubated at 37°C for 30-60 min. The transformed bacteria were then plated on selection medium.

4.2.2- Biological methods

4.2.2.1- Cytotoxicity staining assay by crystal violet staining

The cells were fixed for 10 min by addition of 25 ml of 25% glutaraldehyde to each well. The medium containing the solution for cell fixation was discarded and the plates were washed with water. 100 µl of 0.4% crystal violet solution was added to each well of the plates, and the attached cells were stained for 30 min. The staining solution was discarded and the plates were washed with water. When the intracellular staining was eluated by addition of 10% aminoxid WS 35 for 15 min., the OD of solution was measured at 590 nm. The calculation of the cytotoxicity is following:

$$\text{Cytotoxicity (\%)} = (a-b)/(c-b) \times 100$$

a: OD_{590} values derived from wells treated with test samples

b: OD_{590} values derived from blank wells

c: OD_{590} values derived from control wells (i.e., added culture medium as a test sample).

4.2.2.2- *In vivo* monitoring of luciferase activity

The firefly luciferase can be used as a non-invasive bioluminescent reporter in living mice using an Intensified Charged Coupled Photon Counting Device (ICCD) (Contag *et al.*, 1997; Contag *et al.*, 1998, Sweeney *et al.*, 1999). Mice were anesthesized with avertin at dosis of 20 µl/g body weight (diluted 1/100 mixture: 1,0 g 2,2,2 tribromoethanol (Sigma) in 0,5g of 2-methyl-2-butanol (Sigma)) and were injected intraperitoneally with an aqueous solution of D-firefly luciferin / HCl (AppliChem, #2591-17-5) at dosis of 100 µg/g body weight and placed into a light-tight chamber. Anesthesia was maintained during imaging by delivery of avertin as necessary. Animal positioning was based on the back to localize signal in liver. The emitted light was acquired by a photon counting camera (2-stage ICCD C2400-47) fitted with a Nikon lens (35 mm/fl.2) and a computer with image analysis capabilities (Contag *et al.*, 1997). Body images were recorded as reference in daylight. Approximately 5 minutes after luciferin injection, the chamber was closed and the photon counting program was initiated for periods of 30 sec. to 2 min. depending on amount of light produced. Photons were collected over time, generated images converted to pseudocolor, digitized with the Argus 20 image processor (Hamamatsu) and stored in a computer.

4.2.2.3- Preparation of avertin narcose solution

To prepare concentrated avertin solution, 1,0 g of 2,2,2 tribromoethanol (Sigma #T4,840-2) was suspended in 0,5g of 2-methyl-2-butanol (Sigma # 24,048-6) and filtered for storage at 4°C protected from light. 120 µl of concentrated solution was resolved in 10 ml of 154 mM NaCl solution, filtrated and could be used as i.p. injection of 20µl per g weight of mouse.

4.2.2.4- Calcium phosphate transfection of plasmid in cell culture

50-60% confluent cells were washed in PBS and were incubated in fresh medium 2 hours before the transfection. 10-20 µg of DNA per $3x10^6$ cells was ethanol precipitated and resolved in 225 µl sterile double water. 50 µl of 2M $CaCl_2$ was added. The DNA solution was added slowly in 250 µl 2x HEPES-buffered saline and the mixture was vortexed for 5 sec. To form a precipitate containing the DNA, the mixture was incubated at least 20 min. in room temperature and then transferred on the cells. After 12-16 hours, the cells were washed and incubated in fresh medium.

4.2.3- Methods for biochemical analysis

4.2.3.1- Electrophoretic separation of DNA on agarose gel

A non-denaturing electrophoretic separation of DNA fragments was performed on a 0,8-1% agarose horizontal gels with 2 µg/ml EtBr in TAE buffer at a voltage of 6-8 V/cm. Before dispensing the samples on the gel they were mixed with 0,2 volumes 5x loading buffer. A commercially available 10 kb marker Smart Ladder (Eurogentec, Belgium) was used to determine fragment sizes. DNA fragments were made visible by fluorescence of the intercalating EtBr with a UV-transilluminator (254nm) and the results documented by a video detection system.

4.2.3.2- DNA extraction with cell lysate or tissue by phenol/chloroform extraction

The sample was adjust to 100 µl with 1xTE Buffer (pH 8.0) and will be added to 100 µl phenol liquefied (Biomol, Germany cat. #58734) followed by vortexing and centrifugation at 13000 rpm for 15 min at 4°C. The top aqueous layer was transferred into a new tube and 100 µl Chloroform was added. After vortexing and centrifugation at 13000 rpm for 15 min at 4°C, the top aqueous layer was collected for ethanol precipitation. Precipitated DNA was dissolved in 10-50 µl sterile 1xTE buffer (pH 8.0) and stored at -20°C.

4.2.3.3- DNA extraction from the tail of transgenic mouse for PCR screening

1mm mouse tail was incubated in 600 µl DNA lysis buffer and 10 µl proteinase K (20mg/ml) was added for each sample at 37°C overnight or at 56°C for at least 4 hours. The tissue debris was removed by centrifugation and the DNA was precipitated from

supernatant by isopropanol. After washing with 70% ethanol, the extracted DNA was dissolved in 50µl sterile distilled water. 1-2 µl of the extracted DNA was used in PCR screening method.

4.2.3.4- DNA extraction from blood or serum of mouse for real-time PCR

Using the QIAamp® Mini Blood Kit (250), a determined volume of serum (50 µl) was added with appropriated volume of PBS to 200 µl. 20 µl QIAGEN proteinase was added to 200 µl AL buffer. After vortexing, the sample was incubated at 56°C for 10 min. 200µl of 100% ethanol was added and the mixture was applied to the QIAamp spin column. The column was washed with 500 µl of AW1 buffer and in the second step with 500 µl AW2 buffer. To elute the DNA, 200 µl of AE buffer or distilled water was added in the column and by centrifugation at 8000 rpm for 1 min the isolated DNA was collected for real-time PCR procedure.

4.2.3.5- Preparation of nuclease-free water

Diethylpyrocarbonate (DEPC) (Sigma) was added to deionized water to a final concentration of 0.1%. Following incubation at 37^{o}C for 30 minutes the DEPC treated water was autoclaved.

4.2.3.6- Extraction of total RNA from cell lysate or tissue by TRIZOL®LS methods

TRIZOL®LS reagent (Life Technologies #10296) is a ready-to-use reagent for the isolation of total RNA from liquid samples or homogenize tissue or cell samples. For homogenization, 5x10^{6} cells were lysed or 50-100 mg tissue were homogenized using the Ultra-Turrax T8 (IKA®-Werke, Frankfurt) in 750µl TRIZOL®LS reagent. To permit the complete dissociation of nucleoprotein complexes, the homogenized samples were incubated for 5 min. at 15°C before adding 200 µl chloroform. After vortexing and incubation at 15°C for 2 to 5 min, the samples were centrifuged at 13000 rpm for 30-40 min. at 4°C. The aqueous phase containing RNA was transferred into a new tube. The RNA was precipitated by addition of 500µl of isopropyl alcohol and centrifugation at 13000 rpm for 30-40 min. at 4°C. The invisible RNA pellet was washed in 1 ml 75% ethanol and centrifuged at 13000 rpm for 20 min. at 4°C. The pelleted RNA was resuspended in 200µl DEPC treated distilled water.

4.2.3.7- Extraction of mRNA from total RNA by Oligotex™ kit (Quiagen)

Before starting the extracting procedure, Oligotex suspension has to be heated to 37°C and mixed. Although the buffer OEB was incubated at 70°C. 200 µg total RNA was used in a volume with RNase-free water to 250 µl. 250 µl OBB binding buffer and 15 µl Oligotex suspension was added before heating of the samples at 70°C for 5 min and cooled at room temperature for 10 min to allow hybridization between the oligotex and the polyA tail of the mRNA. The Oligotex:mRNA was centrifuged at 13000 rpm for 2 min and the pellet was resuspended into 400 µl wash buffer OW2. The washing step was repeated two time before the mRNA elution with 50 µl hot buffer OEB. To ensure maximal yield, the elution step was repeated.

4.2.3.8- Western Blot analysis

Preparation of PAGE gels: glass plates were cleaned with ethanol and assembled. Solutions for separation gel were mixed. Poured into the plates leaving about 2 cm at the top to allow insertion of the comb. The separation gel was then carefully overlaid with isopropanol. The mixed solutions for the stacking gel were then overlaid on the separation gel and the comb. About 10–25 µl of samples could be loaded. The upper chamber was filled with 1x SDS running buffer. The gels were run at 35mA in upper gel and at 55mA for 1 hour until dye front was at the bottom of gel. See below for Western transfers. Gels were stained for 20 min with Coomassie blue solution, then destainned in strong destain solution (50% methanol, 10% acetic acid) for 20 min and left in 10% acetic acid.

Western transfer using a semi-dry apparatus: large (13cm x 15cm) pieces 3MM paper were soaked in transfer buffer. The polyvinyl Fluorid transfer membrane BioTrace™ PVDF 0,45 µm (Pall, MI, USA) (10cm x 14cm) was washed in methanol and then in water and in transfer buffer. The transfer apparatus was assembled according to CTI manual with 3 large 3MM, filter, equilibrated gel with upper gel removed, 3 small 3MM on the "base" of the transfer apparatus. Time for transfer was calculated as following: 1.2 mA/cm² x area of gel for 1 hour or 2.5 mA/cm² x area of gel for 45 min. One small gel = 62.5mA/hour. 3MM paper were removed and the bands corresponding to Rainbow marker RPN 756 were marked on the filter. The filter was incubated in 50 ml

TBS/0,2% Tween buffer and 2,5 g milk powder was added for blocking for 30 min. Coomassie staining was used to determine, if all the proteins had been transferred.

Antibody western: The filter was incubated in 50ml TBS/Tween + milk powder with 5 µl antisera at appropriate dilution (1:200 - 1:2000) like H801 overnight at 4°C. The filter was washed 3 times with TBS/0,2% Tween solution for 10 min before to get the goat anti-rabbit (as second antibody to H801) or anti-mouse (as second antibody to anti-ß actine) coupled to HRP or alkaline phosphatase at 1:1000 - 1:2000 in TBS/Tween + milk powder for 1-2 hours at room temperature. The filter was washed 3 times with TBS/Tween solution for 10 min and then it was washed with TBS solution for 10 min. For detection of protein the complex with ECL reagent (Amersham #RPN 2106) or with EFL reagent (Amersham #RPN 5785) respectively was applied on the filter.

4.2.3.9- Southern Blot analysis

The cellular DNA extracted from tissue or cell lysate was digested into several pieces of different sizes with *Hind III* overnight. 10-20 µg digested DNA was poured into an agarose gel and an electrical charge of 65V was applied to the gel. The pieces of DNA will be attracted towards the bottom of the gel; the smaller pieces, however, will be able

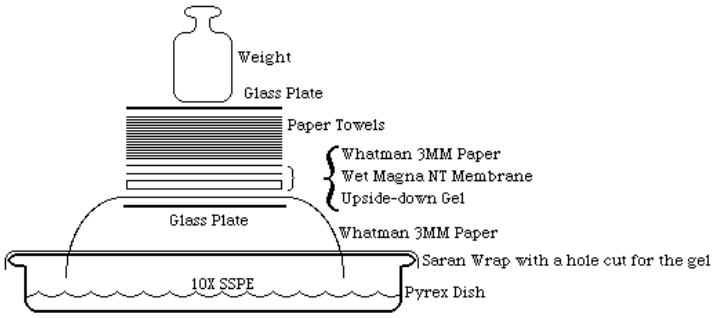

Fig. A: Blotting apparatus

to move more quickly and thus further towards the bottom than the larger pieces. The gel was washed with 0,25M HCl solution (23 ml 39% HCl solution filled to 1 liter) for 10 min and then with Soak I buffer for 20 min for the denaturation of DNA. The gel was neutralized by incubating in Soak II buffer at least 30 min. The DNA was blotted by capillary action with a high salt solution (20x SSC buffer). For assembly of the blotting apparatus see fig.A. A nylon membrane (Biodyne® B Membrane 0,45µm, Pall Germany) was employed.

Neutralization and Cross-linking: the hybridized membrane was washed carefully with 2x SSC buffer, and the DNA was fixed on the nylon membrane by UV cross-linking.

Hybridization: An $\alpha^{32}P$ labeled DNA probe specific for the gene in question was heated at 90°C for 10 min in hybridization buffer and then incubated with the blot at 65°C overnight. The blot was washed 2 times for 10 min. each step at 65°C to remove non-specifically bound probe with wash buffer. The blot was exposed to film or a screen for phospho-imaging.

4.2.3.10- Dot Blot analysis

A Dot Blot analysis is similar to Southern Blot, except that gelelectrophoresis separation and blotting by capillary action is not necessary. A concentration of the DNA occurred on nylon membrane by filtration. The DNA was denatured by alkaline treatment in Soak II buffer for 2x 1,5 min and then was neutralized by incubating in Soak II buffer for 4x 1 min. The DNA was fixed onto the nylon membrane by UV cross-linking.

4.2.3.11- Northern Blot analysis

A northern blot analysis is similar to Southern blot except that RNA was separated instead DNA. Total RNA extracted from 50 mg liver tissue was isolated according to the TRIZOL™ method and eluted in 100 µl double distilled water treated with DEPC. mRNA was then isolated from 250 µg of total RNA using the Oligotex® mRNA Mini Kit (Qiagen) to prevent any non-specific binding of the probe. The mRNA was loaded onto a gel for electrophoresis in a formaldehyde gel (see loading mix). The first lane has size standard RNA Ladder and the other lane corresponds to about 5 µg extracted mRNA.

The gel was washed for 10 min in 20x SSC to remove the formaldehyde. The mRNA was then blotted onto a nylon membrane (Biodyne® B Membrane 0,45µm, Pall Germany) using a capillary blot (see fig.A). To immobilize the RNA, the nylon membrane was subsequently UV crosslinked two times. For the hybridization, 50 ml of hybridization buffer (see Southern Blot, section 4.2.3.12)- to which the $\alpha^{32}P$ labeled DNA probes had been added, was used. The hybridization proceeded at 55°C for 16 h or overnight. The membrane was then washed for 2x 10 min in 2x SSC/0.1% SDS at 55°C. The blot was exposed to film or a screen for phospho-imaging.

Loading mix

Sample RNA	10 µl
10x E-Buffer /DEPC	2 µl
Formaldehyd	3 µl
Deionised Formamid	8 µl

Heat to 65°C and then cold at ice. Add 2,5 µl 10x loading buffer for gel

4.2.3.12- Luciferase activity assay

The luciferase enzyme used for reporter gene expression is derived from the coding sequence of the *luc* gene cloned from the firefly *Photinus pynalis* (Wood, K.V., 1995). The firefly luciferase protein has a shorter half life in transfected mammalian cells (Pazzigli, M. et al., 1992). The luciferase enzyme catalyzes a reaction using D-luciferin and ATP in presence of oxygen and Mg^{2+} resulting in light emission. The luciferase reaction is quantified using a luminometer (CliniLumat LB 9502) which measures light output in 10 s.

Before the lysis of the cultured cells, the medium was removed and the cells were washed with 1xPBS following by the lysis in 100 µl Luc-lysis buffer. The tissue samples were prepared in 500 µl Luc-lysis buffer by homogenizing using the Ultra-Turrax T8 (IKA®-Werke, Frankfurt). The homogenate was centrifuged at 13000 rpm for 5 min. and the supernatant was collected in new tube for the firefly Luciferase reporter assay (Promega). 250 µl Measure Luc buffer supplemented with 5mM ATP were transferred into 1 ml PS tube (Neolab, #E-1646). The luminometer was programmed to perform a 2 sec. pre-measurement delay followed by a 10s measurement period for each assay. 10 µl supernate of cell lysate or tissue homogenate were transferred into the reaction tube. 105 µl D-luciferin solution (125 µM from Promega #E1015) were injected into the tube and the light emission was read.

4.2.3.13- Protein determination of Bradford methods

The bradford dye-binding assay is a colorimetric assay for measuring total protein concentration. It involves the binding of Coomassie Brilliant blue to protein. There is no interference from cations nor from carbohydrates such as sucrose. However, detergents such as sodium dodecyl sulfate and triton X100 can interfere with the assay, as well as strongly alkaline solutions. Standards with BSA (0,1-2,0 mg/ml) were prepared. 10 µl of standard or sample were added to 1 ml of diluted Bradford Reagent. Dilution of samples provided from liver tissue should be diluted. The reaction was incubated at

room temperature for 5-30 min. (in study only 25 min.) The OD was measured in standards and samples at 595nm. Using the standard curve with diluted BSA protein, the unknown protein concentration was determined.

4.2.3.14- Murine IFNg specific ELISA (PharMingen, San Diego, CA,USA)

The first step corresponds to coat the antibody anti-mIFNγ R46A2 (Hybridoma) on the surface of a microplate (Nunc Maxisorb; Cat #2442404) in 100 µl coating solution (50 mM sodium carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; or 10 mM PBS, pH 7.2) at room temperature for 4 hours. The coated plate was washed with blocking solution (1xPBS, 1% BSA) to prevent non-specific binding. 100 µl standard mIFNγ (10µg/ml diluted from 100 ng to 10 pg per ml) was added and incubated at 4°C overnight. The plate was 3 times washed with wash solution (1xPBS, 10% (v/v) Tween 20). 100 µl of the secondary antibody biotinylated anti-mIFNγ AN18.24 (0,5-2,0 µg/ml dilution 1:500 in 1x PBS/ 0,1% BSA) solution was added to each well and incubated for one hour at room temperature. Following washing steps, 100 µl of 1:1000 diluted POX (Avidin Horseradish Peroxidase) solution was added to each well and incubated for 30 min. at room temperature. The plate was washed carefully 3 times and 100 µl substrate 2´2´-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (0,6 mg/ml in 0,1M anhydrous citric acid, pH 4,3) was added with 3% H_2O_2 (30% w/v) into each well to induce the reaction. After sufficient color development at room temperature (for 30 min), the plate was read at 405nm and the concentration of mIFNγ in sample was determined using the established standard curve.

4.2.3.15- HBsAg and HBeAg specific ELISA (AXSYN®)

Determination of HBsAg and HBeAg levels in medium or sera of mice were performed using the commercial ELISA test HBsAg V2 and HBeAg 2.0 of Abbott Laboratories, Wiesbaden.

4.2.3.16- Titration of alanine transaminase (ALT or GTP) (Roche®)

The ALT levels in fresh serum of mouse was determined using specific bioreaction strip from Roche and the corresponding reader, Reflovex.

4.2.4- Cell culture

4.2.4.1- Cell culture medium

Adherent cells were grown in Dulbecco´s modified Eagle´s medium with 4,5 g/l glucose (DMEM, Gibco #41965-039) supplemented with 10% (v/v) fetal bovine serum (Bio Whittaker Europe #DE14-801F), 4 mM glutamine (Gibco #25030-024l) and 10^4 I.U./ml penicillin and 10^4 µg/ml streptomycin (Gibco #15070-022) at 37°C in a 5% CO_2 incubator. The HuH7, HepG2 and AML12 cells were grown in DMEM including the same above supplements added with 1% (v/v) 100 mM sodium pyruvate (Gibco #11360-039) and 1% (v/v) MEM solution of non-essential amino acids (Sigma #31K2342). A tetracycline-free FCS (Clontech # 8630-1) was employed to compose the medium for experiments, which expected the tetracycline-inducible gene expression.

4.2.4.2- Passage of cells

Adherent cells were washed with 1x PBS from the plate surface, treated with 0,05% Trypsine/0,02 (w/v) EDTA (Biochrom AG #L2143) for 1 to 5 min. depending of the cells (293, HeLa cells or HuH7, HepG2, AML12 cells respectively) and then further cultivated in fresh medium.

4.2.4.3- Infection of cells with adenovirus

90% confluent cells were infected with adenovirus at moi ranging from 0,5 to 200 i.u. per cell in reduced volume of fresh medium. The titer of adenovirus was determined in the 293 cells in this study. The infection time was ranged from 2 hours to overnight at 37°C in a 5% CO_2 incubator. Supplementation of 1,7% (v/v) DMSO (Merck #2950.0500) in medium was performed with mouse cells. The infected cells were washed carefully with 1xPBS and cultivated in fresh medium.

volume of medium during adenoviral infection

	account of HeLa cells	min. volume of medium
mini flask 25cm²	$2,5x10^6$	1,5 ml
flask 75cm²	$8x10^6$	5 ml
big flask 150cm²	$2x10^7$	13 ml
dish 10 cm diameter	$9x10^6$	2 ml
24-wells plate	$2x10^5$	100 µl
12-wells plate	$4x10^5$	300 µl
6-wells plate	$1,5x10^6$	500 µl

4.2.4.4- Preparation of primary mouse hepatocytes

Injected i.p. of mouse 100 µl Heparin solution (10^4 Unit) and wait half hour before the second injection i.p. with avertin (0,02 ml avertin solution per g body weight) for general anaesthetic of mouse. Then open the mouse to perfuse via the portal vein with perfusion buffer. After 1 min perfusion cut the heart of animal to evacuate liquids like blood. After 100 ml perfusion buffer continue to perfuse with collagenase medium (17 mg in 100 ml medium). Before the tissue structure disrupt, collect the perfused liver and suspend it in wash medium. Centrifuge carefully at 1000 rpm and wash 3 times with wash buffer the cells suspension. Resuspend the homogenous liver cell in MM medium and determine the cell account. Seed at a density of $5x10^5$ cells/cm² onto collagen type I (Sigma Aldrich, Irvine, CA, USA) -coated tissue culture plates in supplemented Williams E medium (MM medium). 2 hours later change the MM medium. Primary cells were maintained at 37°C and 5% CO_2.

Wash medium

Spinners MEM	500 ml		Gibco #31380-025
Glutamine	5,6 ml	200mM	Gibco #25030-024l
Glucose	6,0 ml	5%	Serva #22720
HEPES pH 7,4	11,5 ml	1M	Sigma #H9136
Insulin	0,16 ml		Serva #26350
Sodium-pyruvate	28 ml	100mM	Gibco #11360-039
Penicillin/Streptamycin	5,6 ml	5000i.U./ml	Gibco #15070-022

Perfusion medium

Wash medium			
EGTA	1:200	100mM	Serva #11290
Heparin	1:1000	104 U/ml	Sigma #H3149

Collagenase medium

Williams Med.E	500 ml		Gibco #22551-022
$CaCl_2$	1,8 ml	1M	
Glutamine	5,6 ml	200mM	Gibco #25030-024l
Glucose	6,0 ml	5%	Serva #22720
HEPES pH 7,4	11,5 ml	1M	Sigma #H9136
Insulin	0,16 ml		Serva #26350
Sodium-pyruvate	30 ml	100mM	Gibco #11360-039
Penicillin/Streptamycin	5,6 ml	5000i.U./ml	Gibco #15070-022

For collagenase perfusion, dissolve collagenase in Williams Med. E (5mg/ml) and sterilize the solution by filtration through an 0,45 µm filter.

Maintainance Medium (MM)

Williams Med.E	500 ml		Gibco #22551-022
Gentamycine	0,55 ml	50 mg/ml	Sigma #G1397
Glutamine	5,6 ml	200mM	Gibco #25030-024l
Glucose	6,0 ml	5%	Serva #22720
HEPES pH 7,4	11,5 ml	1M	Sigma #H9136
Hydrocortison	0,5 ml		Sigma #H4881
Inosine	2,8 ml	2,5 mg/ml	Serva #26250
Insulin	1,4 ml		Serva #26350
Penicillin/Streptamycin	5,6 ml	5000 i.U./ml	Gibco #15070-022
DMSO	8,7 ml	100%	Merck #2950.0500

EGTA: 0,1M in bidest. Water pH 8,0 with 10M NaOH

Glucagon: 2 mg dissolved in 28,5 ml 1x Earle´s salt solution with 1% FCS and 10mM HEPES pH 7,4

Hydrocortisone: 100 mg dissolved in 20,6 ml of 1x Earle´s salt solution with 10 mM HEPES pH 7,4

Insulin: 25 mg dissolved in approx. 0,5 ml 0,1M HCl with 1% FCS. Dilute with the same solvent to 8,3 ml, 0,45µm filtration and store in 150µl.

4.2.6- Adenovirus generation and production

4.2.6.1- Constructing recombinant Shuttle plasmid

The gene of interest was cloned into the multiple cloning site (MCS) of the pShuttle plasmid via the appropiriate restriction enzymes (*HindIII* and *Sal I*). The gene of interest contain neither a *Pac I* nor *Pme I* restriction site since they are essential for adenovirus generation. Protein expression was tested by transfecting HuH7 cells with the recombinant Shuttle vector and was verified by Western blotting or ELISA analysis.

4.2.6.2- Generation of recombinant adenoviral DNA

The cassette containing the transgene and a promoter was inserted from the shuttle plasmid into the adenoviral backbone (pAdEasy1) by homologous recombination in electrocompetent bacteria. 100 fmol of *Pme I* digested shuttle plasmid was co-transformed with 50 fmol adenoviral backbone pAdEasy1 into highly competent BJ5183 *E.coli* (10^4 cfu per fmol of pUC13 plasmid) using electroporation or chemical standard methods see fig.B step1). To reduce nonspecific recombination, the

transformed cells were incubated in 1 ml LB medium at 37°C for maximal 15 min., and were plated on LB plate with 50 µg/ml kanamycin. After 16-20 hours incubation at 37°C the smallest Kan-resistant colonies were picked for DNA analysis with *Hind III* and *Pac I* digestion. Candidate clones usually yield a large fragment (over 30 kb) plus a smaller fragment of 3,0 or 4,5 kb by *Pac I* digestion. The selected adenoviral DNA plasmid was amplified in XL-1 blue or DH5α *E. coli* strain to prepare greater quantity of the recombinant adenoviral DNA vector.

4.2.6.3- Production of recombinant Adenovirus

Before packaging of adenoviral DNA, 10 µg of the recombinant adenoviral plasmid have to be digested with *Pac I*. The plasmid contains two *Pac I* restriction sites, which are located at both ends of the viral genome, 3´and 5´to the inverted terminal repeats (ITR) (see fig.B step 2). Because ITRs contain the origins of adenovirus DNA replication, they are positioned at the termini of the linear adenoviral DNA molecule to support the formation of the replication complex (Tamanoi & Stillman, 1982).

The digested adenoviral plasmid was transfected into 293 cells (50% confluency) in 25cm² flasks by calcium phosphate precipitation. The transfection efficiency could be estimated by intracellular GFP expression 24 hours post-transfection if pAdTrack or pAdTrack-CMV were used as shuttle plasmid. Viral production could also be monitored by GFP expression in cells after spread of virus to neighboring cells 6 to 7 days post transfection. The 293 cells were collected without trypsine treatment and were lysed by three consecutive freeze-thaw cycles. After the third cycle, the cell debris was remove by centrifugation at 5000 rpm at 4°C for 10 min. The supernatant containing recombinant adenovirus was used to infect 293 cells (80% confluency) plated in 25cm² flask. Residual supernatant was stored at -80°C in 10% glyceride. Following the cytopathic effect on infected 293 cells (day 5 p.i.), cells were collected and lysed as above. A titer of 10^7 i.u. of recombinant adenovirus per ml was obtained after the second infection on 293 cells plated. The successive passage on 293 cells increased the adenoviral titer 10-fold per infection. Thereby titers up to $5x10^8$ i.u./ml of recombinant adenovirus could be produced.

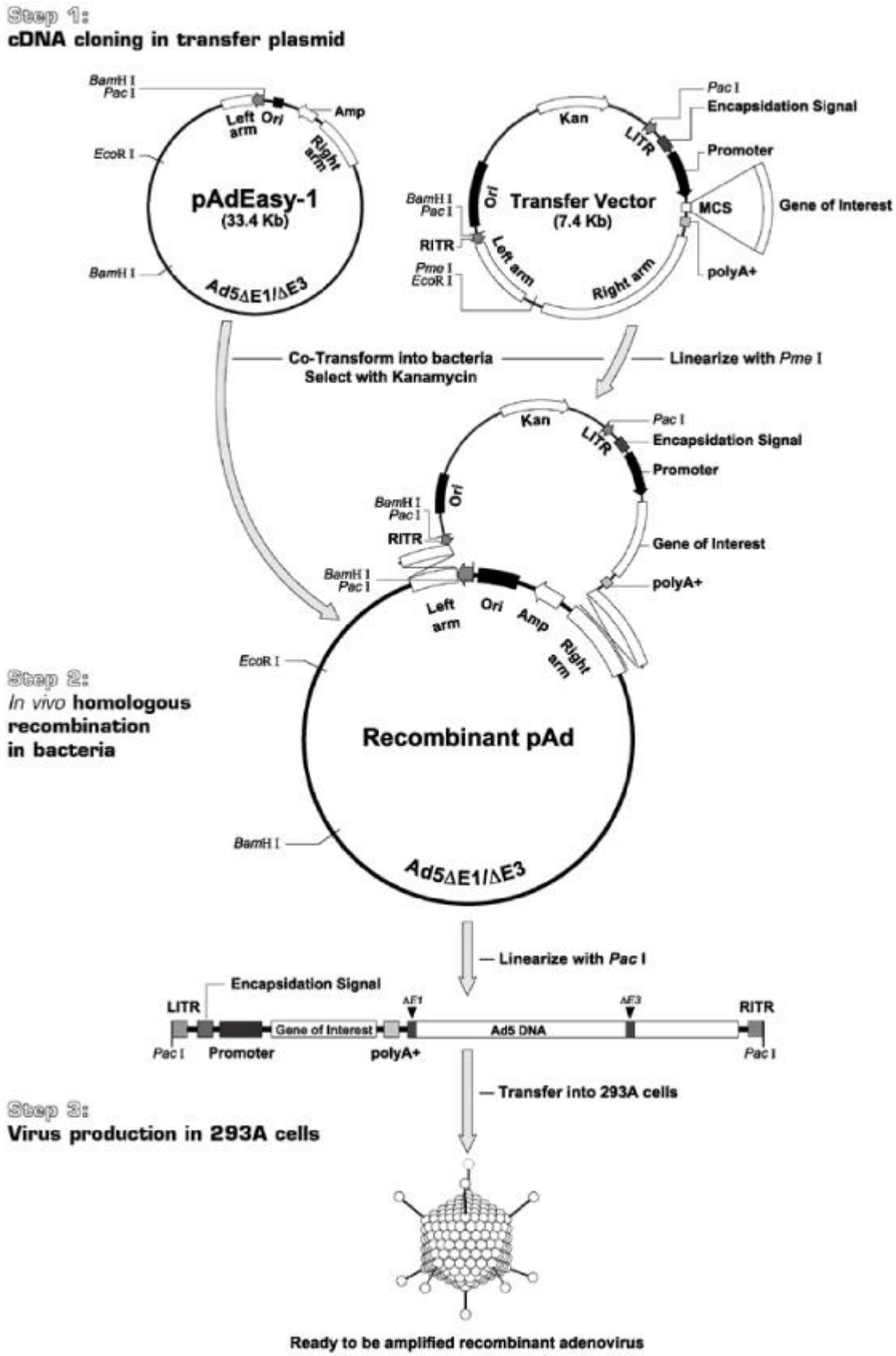

Fig. B: schematic presentation of the AdEasy method for the generation of adenoviral plasmid and for the production of recombinant adenovirus Ad5DE1/E3 (figure was provided by www.adenovirus.com).

4.2.6.4- Production of high titer recombinant adenovirus stocks

$2x10^8$ 293 cells, corresponding to eight 150cm² flasks, were infected with the recombinant adenovirus at a moi of 5 i.u. per cell. At day 3 to 4 p.i., the infected cells were collected and resuspended in 2 ml of 0,1M Tris/HCl pH 8.0 buffer per 150cm² flask. The cells were lysed by three cycles of freeze/thaw/vortex. Following the removing of cell debris, 8 ml of clear supernatant containing recombinant adenovirus was mixed with 4,4 g of pure CsCl (density of 1,35 g/ml), overlayed with mineral oil and centrifuged at 33000 rpm at 10°C for 18 hours. Following collection of the virus containing fraction, a second CsCl gradient was performed at the same conditions. The virus containing fraction was dialysed with the 10,000 MWCO Slide-A-Lyzer dialysis cassette in storage Buffer (137mM NaCl, 5mM KCl, 10mM Tris pH 7.4, 1mM $MgCl_2$ for 4 to 6 hours. The virus containing solution was stored in 10% glyceride at -80°C. Thereby titers of about 10^{11} i.u./ml could be obtained.

4.2.6.5- Titration of adenovirus stocks by GFP expression and/or cytopathic effect

Confluent 293 cells in 12 well plate were infected with a dilution series of the adenovirus preparation. 48 hours p.i. the percentage of GFP-expressing cell was determined in each dilution using inverted fluorescence microscope. Statistically one GFP-expressing cell was infected with 3 adenoviral particles. If the adenovirus do not express GFP, the cytopathic effect (CPE) of 293 cells, which is characterized by rounded cells and plaque formation, has to be estimated. One cell showing the phenotype of CPE was infected with 3 viral particles.

4.2.6.6- Titration of adenovirus stocks by immunofluorescence staining

Immunofluorescence staining has the advantage to be able to determine the titer of non GFP-expressing adenoviruses. The titration is determined by comparison to other adenoviruses that express GFP. infected 293 cells were fixed in ice-cold methanol at -20°C for 20 min and then washed carefully with 1x PBS/0,25% Triton X100. 4 µl of anti-hexon IMAGEN™ monoclonal antibody (DAKO K6100) were added to 100 µl blocking buffer (2% BSA; 0,25% Triton X100 in 1x PBS). Following incubation in the dark for 2 hours at room temperature, the cells were washed with 1x PBS and the percentage of green stained cells was estimated relative to reference of diluted GFP-expressing adenovirus.

4.2.6.7- Titration of adenovirus stocks by OD measurement at 260nm

The blank solution consisted of 7,5 µl 1,35 g/ml CsCl; 7,5 µl storage buffer and 100 µl 1xTE/0,1% SDS. 15µl of the high titer adenovirus stock were mixed with 100 µl 1xTE/0,1% SDS and vortexed for 30 sec.. The debris were centrifuged at 13000 rpm for 5 min and the OD was measured at 260nm. One OD unit at 260nm corresponds to $1,1x10^{12}$ viral particles (v.p.) per µl (Mittereder D.A. et al., 1996). Only 1 of 1000 viral particles obtained by $CsCl_2$ gradient centrifugation was infectious. Usually a stock of 10^{13} viral particles per ml was produced, which corresponds to 10^{11} i.u. per ml after two $CsCl_2$ gradients.

4.3- Cloning and generation of adenoviral vectors

Recombinant adenoviral vector of the first generation (Ad5 genome with deletion of E1A/E1B and E3-region) were generated using the first published version of the established AdEasy system (He et al., PNAS 1998). The plasmids pShuttle, pAdTrack-CMV, pAdTrack and pAdEasy1 were kindly provided by B. Vogelstein (Howard Hugues Medical Institut, Balitmore, MD, USA).

4.3.1- AdShuttle: empty adenovirus as control vector

The plasmid pShuttle was linearized with *Pme I* and co-transformed into BJ 5183 *E. coli* (Hanahan D.,1993) for homologous recombination with pAdEasy1 to create the adenoviral plasmid pAdShuttle. pAdShuttle was digested with *Pac I* and transfected into 293 cells to generate the adenovirus AdShuttle.

4.3.2- AdmIFNg or AdvIL10: Adenovirus coding for mouse interferon gamma or viral analogue interleukin 10 driven by a CMV promoter

AdmIFNγ and AdvIL10 were provided by T. Ritter (Charité, Berlin, Germany). These adenoviruses were produced by co-transfection of shuttle vector and adenoviral backbone plasmid into 293 cells and then screened by plaque purification. No reporter gene was expressed.

4.3.3- AdGmIFNb: Adenovirus coding for mouse interferon beta under the control of the CMV promoter with GFP as reporter

The insert containing the mIFNβ gene was obtained from digestion of the plasmid pMPSVHEmuIFNβ (provided by GBF, #154, Braunschweig, Germany) with *Eco RI*, filled up by klenow reaction and then digested with *Hind III*. The separated fragment of 750 bp was cloned into pAdTrack-CMV digested with *Eco RV* and *Hind III*. The obtained plasmid, called pAdTmuIFNβ (10 kb), was linearized with *Pme I* and co-transformed into BJ5183 *E. coli* with pAdEasy1 to generate the adenoviral plasmid pAdAdGmIFNβ. pAdAdGmIFNβ digested with *Pac I* was then transfected into 293 cells to produce the adenovirus AdGmIFNβ.

4.3.4- AdGFP: Adenovirus coding for EGFP under the control of the CMV promoter

Reporter adenovirus coding for EGFP was generated by M. Nassal (Protzer et al.,1999) by co-transfection into 293 cells and selected by plaque purification.

4.3.5- AdTTR-rtTA: Adenovirus coding for the reverse tet-transactivator under control of the CMV promoter

The 1,5kb insert containing the reverse-tet-transactivator rtTA2^{S}-S2 (Urlinger et al., 2000) under controle of the liver specific promoter for transthyretin P_{TTR} was isolated from the plasmid pUHD.TTR.0.3/h-rtTA(FFF). It has been cut out with *Bst EII*, filled up by the klenow reaction, and then digested with *Sal I*. The fragment was cloned into pShuttle digested with *Sal I* and *Eco RV*. The vector pAdshuttle-TTR-rtTA was constructed. Following homologous recombination in BJ5183 *E. coli* the adenoviral plasmid pAdTTR-rtTA was digested with *Pac I* and transfected into 293 cells to produce the adenovirus AdTTR-rtTA. Production of high titers of the virus was achieved after six passages in 293 cells.

4.3.6- Adbiluc and Adbiluc2: Adenovirus mediated tet-inducible luciferase expression

The plasmid pAdbiluc was composed from the cassette containing the tet-transactivator responsive bidirectional promoter P_{bi-1} (Baron U. et al., 1995) fused to the firefly luciferase gene and at opposite site of P_{bi-1} a multiple cloning site (MCS), and from the

adenoviral shuttle vector pShuttle (He et al., 1998). The cassette containing P_{bi-1} was provided from the modified plasmid pBi-2 (Baron U. et al., 1995). The recognition site of *Pac I* in the luciferase gene was removed by double PCR mutagenesis (primer 40029; 40030; 34797; 34797). No decrease of luciferase activity was detected in transfected HuH7 cells with the modified pBi-2 plasmid. The multiple cloning side of pBi-2 was exchanged by a double digestion with *Mlu I* and *Eco RV* to a new MCS containing *Mlu I, Sal I, Hind III, Xho I* and *Not I* sites. Thus, the plasmid pBI-2MCS_2ΔPacI was designed. Following transformation of pBI-2MCS_2ΔPacI into GM33 *E. coli* (Marinus MG. et al., 1973), the 3,8 kb fragment luc-P_{bi-1}-MCS_2 was obtained by digestion with *Bsa BI* and *Ase I* and was filled to create blunt ends. The MCS of pShuttle was exchanged from *Kpn I / Bgl II* to a shorter adapter (5´GATCTGATATCGCGCGTAC 3´ and 5´GCGCGATCA 3´) including the unique site of *Eco RV* .The *Kpn I* site was destroyed. The plasmid pShuttle2 was generated. The 3.8 kb insert luc-P_{bi-1}-MCS_2 was cloned into pShuttle2 linearized with *Eco RV*. The orientation of the insert was determined by restriction analysis with *Hind III* and *Xmn I* or with *Stu I* : the construct with luciferase gene downstream of the left terminus of adenoviral type 5 (1-440) was called pAdbiluc. The clone with the insert in opposite orientation was called pAdbiluc2. Following homogenous recombination in BJ5183 *E. coli* both adenoviral plasmids were generated: pAdAdbiluc or pAdAdbiluc2. Their corresponding adenoviruses were produced by transfection into 293 cells: Adbiluc and Adbiluc2.

4.3.7- Adbiluc-GFP: Adenovirus mediated tet-inducible luciferase and GFP expression

The 810 bp fragment containing the GFP gene was extracted from the pCD16S-GFPh plasmid digested at first with *BstE II* , filled to blunt end, and then digested with *Xho I* . This insert was cloned into pAdbiluc digested at first with *Sca I,* filled to blunt end and then digested with *Xho I.* Following the homologue recombination in BJ 5183 E. coli, the adenoviral plasmid pAdAdbiluc-GFP was digested with *Pac I* and transfected into 293 cells to generate the adenovirus Adbiluc-GFP.

4.3.8- Adbiluc-mIFNg: Adenovirus mediated tet-inducible luciferase and mIFNg expression

The 600 bp fragment coding for mIFNγ was generated by PCR using primer mIFNg(+) and primer mIFNg(-) thereby introducing the site of *Sal I* at the 5´end and the site of *Hind III* at the 3´end. The adenoviral DNA extracted from AdmIFNγ served as template for the PCR reaction. This fragment was cloned into the pAdbiluc plasmid digested with *Sal I* and *Hind III* . Functional test for secreted mIFNy was performed by ELISA analysis (data no shown). After homologue recombination in BJ5183 *E. coli* the adenoviral vector pAdAdbiluc-mIFNγ was digested with *Pac I* and transfected into 293 cells to produce the adenovirus Adbiluc-mIFNγ.

4.3.9- Adbiluc-mIFNa: Adenovirus mediated tet-inducible luciferase and mIFNa expression

The 590 bp fragment coding for mIFNα was generated by PCR using primer mIFNalpha(+) and primer mIFNalpha(-) introducing the site of *Sal I* at the 5´end and the site of *Hind III* at the 3´end. As template pORF-mIFNαv.11 (InvivoGen, San Diego, CA, USA) was used for PCR reaction. The obtained fragment was cloned into pAdbiluc digested with *Sal I* and *Hind III* . Secreted mIFNα from transfected cells induced the 2´5´OAS transcription as detected by Northern Blot analysis (data no shown). After homologue recombination in BJ5183 *E. coli*, the adenoviral vector pAdAdbiluc-mIFNα was digested with *Pac I* and transfected into 293 cells to produce the adenovirus Adbiluc-mIFNα.

4.3.10- Adbiluc-mIFNb: Adenovirus mediated tet-inducible luciferase and mIFNb expression

The 460 bp fragment coding mIFNβ was generated by PCR using primer mIFNbeta(+) and primer mIFNbeta(-) introducing the site of *Sal I* at 5´end and the site of *Hind III* at the 3´end. The plasmid pMPSVHEmuIFNβ (provided by GBF, #154, Braunschweig, Germany) was used as template in the PCR reaction. The obtained product was cloned into pAdbiluc digested with *Sal I* and *Hind III*. Secreted mIFNβ from transfected AML12 cells with pAdbiluc-mINβ plasmid induced 2´5´OAS transcription as detected by Northern Blot analysis (data not shown). After homologue recombination in BJ5183

E. coli, the adenoviral vector pAdAdbiluc-mIFNβ was digested with *Pac I* and transfected into 293 cells to produce the adenovirus Adbiluc-mIFNβ.

4.3.11- Adbiluc-rtTA or Adbiluc2-rtTA: One component adenovirus mediated tet-inducible luciferase expression

The fragment of 776 bp coding for reverse tet-transactivator rtTA2^{S}-S2 was isolated from plasmid pUHD TTR 0.3/h-rtTA(FFF) by digestion with *Bam HI*, was filled up to blunt ends, and digested with *Hind III*. The rtTA fragment was cloned into pAdbiluc and pAdbiluc2 digested at first with *Not I*, filled to blunt ends, and then digested with *Hind III*. The pAdbiluc-rtTA or pAdbiluc2-rtTA were used as shuttle vectors to generate the adenoviral plasmids pAdAdbiluc-rtTA or pAdAdbiluc2-rtTA by homologue recombination in BJ5183 *E. coli*. Both adenoviral plasmids were digested with *Pac I* and transfected into 293 cells to produce the corresponding adenoviruses Adbiluc-rtTA and Adbiluc2-rtTA.