V MATERIALS & METHODS

V.1 MATERIAL

V.1.1 Chemicals and reagents

The remaining chemicals and reagents were purchased at Roth (Karlsruhe), Merck (Darmstadt), Sigma (Seelze) or Fluka (Seelze).

Adenosintriphosphat (ATP)	Roch, Grenzach
Agarose	Promega, Mannheim
Anhydrotetracycline (AHT)	IBA, Goettingen
BSA (bovine serum albumin)	PAA, Austria
DAPI (4´, 6-Diamidino-2-phylindol)	Sigma, Seelze
Desthiobiotin	Sigma, Seelze
Dialysis membrane Spectra/Por	Roth, Karlsruhe
Didesoxynucleotides	Bioline, Luckenwalde
Digitonin	Sigma, Seelze
DOTAP	Roth, Karlsruhe
ExGen 500	Fermentas, St. Leon-Rot
Glass cover slides	Roth, Karlsruhe
HABA	IBA, Goettingen
Hybond-PVDF membrane	Amersham, Freiburg
Hyperfilm (ECL & MP)	Amersham, Freiburg
Proteinein A/G sepharose	Santa Cruz, USA
Rotiphoresegel 40	Roth, Karlsruhe
Steril filter units (0,45µm)	Braun, Melsungen
Trizol	Invitrogen, Karlsruhe
Whatman Paper	Whatman, UK

V.1.2 Devices

V.1.2.1 Gel electrophoresis

Horizontal electrophoresis systems GNA 100 and 200 with EPS 301 (Hoefer)	Amersham, Freiburg
Vertical electrophoresis systems SE 260 and 600 with EPS 301 (Hoefer)	Amersham, Freiburg
Semi-dry blotting chambers Semiphor and Multiphor II with EPS 301 (Hoefer)	Amersham, Freiburg

V.1.2.2 Centrifugation

Centrifuge 5415 R (refrigerated)	Eppendorf, Hamburg
Table centrifuge Biofuge fresco	Heraeus, Osterode
Minifuge 2	Heraeus, Osterode
Centrifugation tubes 50ml, 15ml	TPP, Trasadingen
Sorvall RC-5B refrigerated superspeed centrifuge with the rotors HB-4, GS-3, SS-34	Sorvall, Bad Homburg
Beckman L8-70 ultracentrifuge with the rotors SW-28 and SW-41	Beckman, USA
Centrifugation tubes for SW 35ml, 12ml	Beckman, USA
Centricon YM-10/30 (Amicon Bioseparations)	Millipore, Schwalbach
Spin-X steril filtration column 0,22µm (Costar)	Millipore, Schwalbach

V.1.2.3 Liquid chromatography

Äkta Explorer, Ethan LC, Purifier	Amersham, Freiburg
Gel filtration column HiTrap Desalting	Amersham, Freiburg
Strep-Tag Affinity chromatography column	IBA, Goettingen
StrepTactin sepharose	IBA, Goettingen

V.1.2.4 Microscopes

Light microscope Diavert	Leitz, Wetzlar
Fluorescence microscope Leitz DM RBE	Leitz, Wetzlar
CLSM (Axioplan) LSM 510	Zeiss, Jena
Electron microscope	Zeiss, Jena

V.1.2.5 Other devices

ABI Prism 7700 Sequence Detection System	Applied Biosystems
Film developer Curix 60	Aqua, Koeln
French Press (Emulsi Flex-C5)	Avestin, Canada
Homogenisator Sonoplus HD 2070	Bandelin, Berlin
Incubator	Heraeus, Osterode
pH meter 765 Calimatic	Knick, Berlin
Photometer Ultraspec 3300 pro	Amersham, Freiburg
Sartorius 1608 MP	Sartorius, Goettingen
Sartorius universal (analytical balances)	Sartorius, Goettingen
Shaker Unitron	HT-Infors, Switzerland
Sonificator Sonoplus HD 2070	Bandelin, Berlin
Sterile bench HeraSafe	Kendro, Langenselbold

Thermocycler PTC-100	MJ Research, USA
Thermomixer compact	Eppendorf, Hamburg
UV table UV-Biometra TB + camera BioToc	Biometra, Göttingen
Vacuum dryer (Slab Dryer Model 443)	BioRad, USA
Video Monitor WV-BM 900	Panasonic, Taiwan
Water bath GFL 1083	GFL, Burgwedel

V.1.3 Size standards

V.1.3.1 DNA standards.

peqGOLD 100bp DNA ladder	PeqLab, Erlangen
peqGOLD 1kb DNA ladder	PeqLab, Erlangen

V.1.3.2 Protein standards

Low molecular weight (LMW) marker	Amersham, Freiburg
High molecular weight (HMW) marker	Amersham, Freiburg

V.1.4 Kits

ABI PRISM ready reaction dye deoxy - terminator cycle sequencing kit	Perkin Elmer, Weiterstadt
BaculoGOLD Transfection Kit (Pharmingen)	BD Biosciences, USA
Bio-Rad Protein Assay	Bio-Rad, Munich
ECL Western Blotting Reagent	Amersham, Freiburg
HBs ELISA Enzygnost HBsAg 5.0	Dade Behring, Marburg
HBe ELISA Enzygnost habe monoclonal	Dade Behring, Marburg
High Pure Viral Nucleic Acid Kit	Roche, Mannheim
Qiaquick Gel Extraction Kit	Qiagen, Hilden
Qiaquick PCR Purification Kit	Qiagen, Hilden
Qiagen Plasmid Mini/Midi/Maxi Kit	Qiagen, Hilden
MTT cell culture tested	Sigma, Seelze

V.1.5 Enzymes

Alkaline phosphatase	Roche, Mannheim
BIOTAQ DNA Taq-polymerase (5 U/µl)	Bioline, Luckenwalde
Herculase Enhanced DNA polymerase (5 U/µl)	Stratagene, Netherlands
Hotstart Taq-polymerase (5 U/µl)	Bioline, Luckenwalde
Klenow DNA polymerase	Roche, Mannheim
Restriction endonucleases	Roche, Mannheim

T4 DNA ligase	Roche, Mannheim

V.1.6 Proteinase inhibitors

Aprotinin	PAA, Austria
Leupeptin	PAA, Austria
PMSF	PAA, Austria
Pepstatin	PAA, Austria

V.1.7 Cell culture

V.1.7.1 Cell lines

Huh7 cells	derived from a hepatocellular carcinoma of human origin [245]
Sf9 cells	derived from pupal ovarian tissue of *Spodoptera frugiperda* (DSMZ No. ACC 125)
PHH	Primary human hepatocytes isolated from human liver tissue samples, taken from patients undergoing partial hepatectomy for primary or secondary liver tumor.

PHH were kindly provided by Dr. Igor M. Sauer (Charité, Experimental surgery and regenerative medicine, Berlin).

V.1.7.2 Bacterial strains

DH5α	F-, endA1, hsdR17(rk-, mk+), supE44, thi1, lambda-, recA1, gyrA96, relA1, Ø80dlacZ M15 (GIBCO BRL, Eggenstein)

V.1.7.3 Reagents for cell culture

Sf9 insect cells

Fetal calf serum (FCS)	Invitrogen, Karlsruhe
L-glutamine	Invitrogen, Karlsruhe
HyQ Sfx insect medium	Perbio, Bonn
MTT cell culture tested	Sigma, Seelze
SeaPlaque low-melting temperature agarose	SMC/ Biozym, Hamburg
SfII900 medium	Invitrogen, Karlsruhe

HuH-7 cells

DMEM	Biochrom, Berlin
Fetal calf serum (FCS)	PAA, Austria
L-glutamine	PAA, Austria
Penicillin/ Streptomycin	PAA, Austria
Trypsin/ EDTA	PAA, Austria
Sodium Pyruvat	PAA, Austria

V.1.7.4 Tissue culture lab ware

Tissue culture flasks (T175 cm^2)	Nunc, Belgium
Tissue culture flasks (T75, T25 cm^2)	TPP, Trasadingen
Tissue scraper	TPP, Trasadingen
6 well, 12 well, 24 well, 96 well test plates	TPP, Trasadingen
Petri dishes (22,1 cm^2; 60,1 cm^2)	TPP, Trasadingen

V.1.8 Antibodies

Table 2: Primary antibodies

Diluted in PBS containing 10% BSA and 0,05% Tween20 (BSA-PBST). Abbreviations: WB = western blot, IF = Immunofluorescence, p = polyclonal, m = monoclonal

Name	Species & clonality	Dilution for WB/IF	Manufacturer	Cat. #
anti-Actin	rabbit, p	WB 1:10000	Sigma, Seelze	A5316
anti-FLAG M2	mouse, m	WB 1:2000 IF 1:200	Sigma, Seelze	
anti-HBcAg aa 135-140 (clone 14E11)	mouse, m	WB 1:1000	Chemicon, Braunschweig	MAb16990
anti-HBcAg	rabbit, p	IF 1:150	Biomeda, USA	V2008
anti-HBcAg (code 2A22) MAb3120 [182]	mouse, m	IF 1:100	Institute of Immunology, Japan	
anti-HBsAg	rabbit, p	IF 1:150	Biomeda, USA	V2007

Table 3: Secondary antibodies
Diluted in PBS containing 10% BSA and 0,05% Tween20 (BSA-PBST). Abbreviations: WB = western blot, IF = Immunofluorescence, p = polyclonal, m = monoclonal

Name	Species & clonality	Dilution for WB / IF	Manufacturer	Cat. #
anti-Rabbit IgG, HRP-linked	donkey, p	WB 1:2000	Amersham, Freiburg	NA934
anti-Mouse IgG, HRP-linked	sheep, p	WB 1:2000	Amersham, Freiburg	NA931
anti-Mouse IgG, Cy3-linked	goat, p	IF 1:400	Dianova, Hamburg	115-165-003
anti-Rabbit IgG, Cy3-linked	goat, p	IF 1:400	Dianova, Hamburg	11-165-003
anti-Mouse IgG, Cy5-linked	goat, p	IF 1:400	Dianova, Hamburg	115-175-003
anti-Rabbit IgG, Cy5-linked	goat, p	IF 1:400	Dianova, Hamburg	11-175-003
anti-Mouse IgG, FITC-linked	horse, p	IF 1:400	Vector Labs, USA	
anti-Rabbit IgG, FITC-linked	horse, p	IF 1:400	Vector Labs, USA	

The secondary antibodies coupled with fluorescent reagent or horse-radish peroxidase (HRP) were dissolved in sterile 50% glycerine according to manufactures instructions.

V.1.9 High-Sensitive Stains

Nucleus/DNA:

Toto-3 iodid	(IF 1:100)	Molecular Probes, USA
SYBR-Green	(IF 1:400 000)	Molecular Probes, USA

F-Actin (cytoskeleton):

Phalloidin-FITC	(IF 1:400)	Sigma, Seelze
Phalloidin-TRITC	(IF 1:400)	Sigma, Seelze

V.1.10 Primers

All the oligonucleotides were purchased from TIB Molbiol (Berlin).

Table 4: Sequencing primer

Name	Sequence (5' to 3')
BacPak-f	aaccatctcgcaaataaata
BacPak-b	aacgcacagaatctagcgctt
pcDNA seq f	ttaatacgactcactataggg
pcDNA seq b	tagaaggcacagtcgaggc
pASK seq f	gagttattttaccactccct
pASK seq b	cgcagtagcggtaaacg

Table 5: Primer for cloning
Nomenclature of primers based on Galibert et al. [105]

Name	Description	Sequence (5' to 3')
f-1903-BamHI	BamHI-aa 1, HBc	aaaggatccatggactacgacccttataaagaa
f-TLM-2140-EcoRI	TLM spike insertion	tttggatccatgcccatatcgtcaatcttctcgaggattgg ggacggtacttgttcaagcctccaagctgtgcctt
b-2139-EcoRI	TLM spike insertion	aaagaattctggatcttccaaattaacacccaccca
b-2349-HindIII	aa 149 HBc	tttaagcttttaaacaacagtagtttccgg
b-2358-HindIII	aa 152 HBc	cccaagcttttatcgtcgtctaacaacagt
f-BamHI-Ts-1843	TLM-Streptag-aa 1843 HBc	cgggatccatgcccatatcgtcaatcttctcgaggattgg ggacccttggagccacccgcagttcgaaaaaatctcttg ttcatgtcct
f-BamHI-s-1843	Streptag-aa 183 HBc	cgggatccatgtggagccacccgcagttcgaaaaaatc tcttgttcatgtcct
f-BamHI-T2Is	2. inverse TLM- annealing at f-BamHI-s-1843	cgggatccatgcctgatggcatccgatcgtttatctcgag cttgccttctggttctcccttatcgtccaatc
f-BamHI-T2Rs	2. reverse TLM- annealing at f-BamHI-s-1843	cgggatccatgcctctgtcctccattttctcccgtatcggt gacccctctggttctcccatatcgtcaatcttc
f-NcoI-TLM2Is	2xTLM-inverse-Streptag	catgccatgggccctgatggcatccga
b-T2Is-EcoRI	2xTLM-inverse-Streptag	acgaattcctgcaggatatctttttcgaactgcg
b-SfWt-NotI	HBc aa 183 STOP	ataagaatgcggccgcctaacatggaggttcccg
f-SHBs-BglII	aa 1 SHBs	gggagatctatggagaacatcacatcaggattc
b-SHBs-BglII	SHBs STOP	cccagatctttaaatgtatacccaaagacaaaa
HBx TaqMan f	Real time PCR	attagcgtggcgtgcttttac
HBx TaqMan b	Real time PCR	ggtcaggctcctctttgc
HBx TaqMan Probe	Real time PCR	FAM-caaacacgcgcattaacgagagcacc-TAMRA
HBs TaqMan f	Real time PCR	ggacccctgctcgtgttaca
HBs TaqMan b	Real time PCR	gagagaagtccaccmcgagtctaga
HBs TaqMan Probe	Real time PCR	FAM-tgtzgaccaraatcctcaccataccrcaga-TAMRA

V.1.11 Plasmids

V.1.11.1 Commercially available plasmids

pASK-IBA7 (N-terminal Strep tag)	IBA, Göttingen
pcDNA 3.1. (-)	Invitrogen, Karlsruhe
pEGFP-N1	BD Biosciences/ Clontech, USA
pVL1393	BD Biosciences/ Pharmingen, USA
pBacPak9	BD Biosciences/ Clontech, USA
pVax1	Invitrogen, USA

V.1.11.2 Generated plasmids

Table 6: Generated plasmids
Abbreviations: S = Strep-tag, H = His-tag, R = Resistance, K = Kanamycin, A = Ampicilin

Name	Vector	Insert	Tag	Promoter	R
pVax1-eGFP	pVax1	eGFP from pEGFP-N1		CMV	K
pVax1-SHBs	pVax1	SHBs (ayw)		CMV	K
pASK7-wt_{149}	pASK-IBA7	wt HBc 149 aa	S	Tetracyclin	A
pASK7-wt_{152}	pASK-IBA7	wt HBc 152 aa	S	Tetracyclin	A
pASK7-$nTLM_{149}$	pASK-IBA7	N-terminal TLM HBc 149 aa	S	Tetracyclin	A
pASK7-$nTLM_{152}$	pASK-IBA7	N-terminal TLM HBc 152 aa	S	Tetracyclin	A
pASK7-$sTLM_{149}$	pASK-IBA7	spike tip TLM HBc 149 aa	S	Tetracyclin	A
pASK7-$sTLM_{152}$	pASK-IBA7	spike tip TLM HBc 152 aa	S	Tetracyclin	A
pBac-wt_{183}	pBacPak9	wt HBc 183 aa	S,H	Polyhedrin	A
pBac-TLM_{183}	pBacPak9	TLM HBc 183 aa	S,H	Polyhedrin	A
pBac-2xTLM_{183}I	pBacPak9	inverse 2xTLM HBc 183 aa	S,H	Polyhedrin	A
pBac-2xTLM_{183}R	pBacPak9	reverse 2xTLM HBc 183 aa	S,H	Polyhedrin	A
pBac-εHBV	pBacPak9	HBV genome (ayw) with N- & C-terminal epsilon-Signal		Polyhedrin	A
pBac-S-GFP-6(-)	pBacPak9	HBV genome (ayw) with 6 knocked-out ORF (pC,C,L,M,X,P)		Polyhedrin	A

V.1.12 Recombinant baculovirus

Table 7: Generated and used recombinant baculovirus

Name	Description of recombinant baculovirus
GFP	eGFP expressing positive control for inset cell infection
wt	wt HBc 183 aa + Strep-tag
TLM	N-terminal TLM HBc 183 aa + Strep-tag
T2Is	N-terminal inverse 2xTLM HBc 183 aa + Strep-tag
T2Rs	N-terminal reverse 2xTLM HBc 183 aa + Strep-tag
Pol	HBV ayw polymerase + Flag-tag
εHBV-SHBs	HBV ayw genome
εHBV-S-GFP (pC,C,L,M,X,P)$^-$	HBV ayw genome with 6 knocked-out ORF (pC,C,L,M,X,P)

The Pol_{Flag} baculovirus was kindly provided by Robert Landford [190].

V.2 METHODS

V.2.1 Molecular biology techniques

Apart from the techniques described in the following part of this section, standard procedures for molecular cloning, sequencing and targeting vector construction were carried out according to "Molecular Cloning" [246] or manufacturer's instructions in the case of kits.

V.2.1.1 Polymerase chain reaction (PCR)

The PCR [247] was used to amplify specific DNA sequences by simultaneous primer extension of complementary strands of DNA. The reaction requires deoxynucleotides, DNA polymerase, primers, a template, and a buffer containing magnesium. DNA accumulates exponentially by cyclic repetitions of denaturation (separation of DNA strains), annealing (hybridisation of the primers to their complementary sequences) and elongation (synthesis of the complementary strand with the use of a heat-stable *Taq* polymerase).
For analytical PCRs, the DNA polymerase of *Thermus aquaticus* was used (BIOTAQ DNA polymerase). It displays a temperature optimum of 72°C. However, it does not show any proofreading activity, leading to an error rate of 1 in 10^5 bases. For preparative PCRs, a DNA polymerase with proofreading activity was used (Herculase Enhanced DNA polymerase). It displays a temperature optimum of 68°C. As a consequence of the proofreading activity, the error rate is much lower (1 in 10^6 bases). PCR was performed according to the following protocol:

PCR reaction mixture	10-100 ng	DNA template (100 ng)
	2 µM	Forward primer
	2 µM	Backward primer
	1 mM each	Deoxynucleotides (dATP/dCTP/dGTP/dTTP)
	2 mM	$MgCl_2$
	1 U	BIOTAQ DNA polymerase
	in 1x PCR reaction buffer to total reaction volume of 25-50 µl	

PCR reaction conditions

Initial denaturation	3 min	94°C	
Denaturation	30 sec	94°C	x cycles
Annealing	30 sec	54-60°C	
Primer extension	E time	72°C (Biotaq) / 68°C (Herculase)	
Final extension	10 min	72°C (Biotaq) / 68°C (Herculase)	
Standby	∞ min	4°C	

For optimal results the conditions for PCR has to be optimized. The elongation (E) time correlated with the length of the expected DNA fragments (amplicon). For generation of a 1 kb encompassing PCR amplicon the DNA polymerase needs 1 minute. Between the denaturation- and primer extension-step, the reaction was repeated 24-30 times (cycles).

V.2.1.2 TaqMan real time PCR

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 TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR. (I) An target sequence specific oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5´ end and a quencher dye on the 3´ end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. (II) If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of Taq DNA polymerase as this primer is extended. (III) This cleavage of the probe: separates the reporter dye from the quencher dye, increasing the reporter dye signal and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. (IV) Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

Real time PCR mixture	2 µl	DNA
	4,5 µl	50mM $MgCl_2$
	2,5 µl	fw primer (10 µM)
	2,5 µl	bw primer (10 µM)
	0,5 µl	Probe (FAM-HBx/HBs-TAMRA)
	0,8 µl	TE buffer pH 8.0
	0,4 µl	dNTPs (25 mM)
	0,25 µl	Hotstart Taq DNA polymerase (10 µM)
	in a total volume of 50 µl ddH_2O	
TE buffer	10 mM	Tris-HCl pH 8.0
	1 mM	EDTA
Real time PCR reaction condition	2 min	50°C
	10 min	94°C
	30 sec	94°C (45 cycles)
	1 min	60°C
	∞ min	4°C

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 guideline 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)

V.2.1.3 PERT assay

The high sensitive and specific *Product Enhanced Reverse Transcriptase Assay* (PERT) uses PCR for the selective enhancement of the MS2 cDNA synthesized by reverse transcription (RT) activity of a viral polymerase. In this procedure it was critical to guarantee that the reagents used did not contain amplifiable DNA, that such DNA was not introduced with the sample, and that no cDNA was produced from the RNA template by endogenous RT activity associated with the assay reagents themselves.
The isolated HBV polymerase was preincubated with RNase and DNase. After a final purification step the PERT assay was performed with genomic RNA of bacteriophage MS2 RNA (Roche) which serves as template as described by Pyra et al. [248]. Alternatively, MnCl2 was used instead of MgCl2 at a comparable concentration. The product amplification was performed in two steps (95 °C/ 20 sec, 64 °C/1 min) over 45 PCR cycles using the ABI PRISM 7700 (Applied Biosystems). For real time quantitation

we used an oligo-nucleic probe (5'-6FAM-TCT TTA GCG AGA CGC TAC CAT GGC TA-TAMRA-Tp), which was labeled at the 5'-terminus with the reporter dye 6-carboxy-fluorescein phosphoramidite (6FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3'-terminus as the quenching dye. The HBV polymerase activity was estimated by comparison with a standard dilution series of Superscript reverse transcriptase (Invitrogen, Karlsruhe).

V.2.1.4 Extraction of plasmid DNA

E. coli cells containing plasmid DNA were usually grown, in autoclaved sterilized LB-medium with an appropriate antibiotic, ampicillin (100 µg/ml) or kanamycin (30 µg/ml), over-night at 37°C. Small-scale preparations (mini-preps) were performed by the alkaline lysis method [249]. Medium-25ml culture and large -250ml culture scale preparations of plasmid DNA were carried out by means of the respective Plasmid Midi- and Maxi-Kit from Qiagen, according to the manufacturer's protocol. It is based on the lysis of bacteria under alkaline conditions in the presence of SDS. Under these conditions, both chromosomal DNA and plasmid DNA are denatured. However, upon neutralization, plasmid DNA is selectively renatured whereas chromosomal DNA remains insoluble. Soluble (plasmid) DNA is subsequently purified by the means of an anion exchange chromatography and eluted under high salt conditions. The final purification of the DNA involves the precipitation by the means of isopropanol. The precipitate is washed and air-dryed before being dissolved in ddH_2O.

LB Medium	10 g	Bacto-tryptone
	5 g	Yeast extract
	10 g	NaCl
	in 1l H_20, pH 7.0	

V.2.1.5 Agarose gel electrophoresis

Linearized DNA fragments can be separated by agarose gel electrophoresis according to their size. Agarose is resuspended in TAE buffer at the appropriate concentration (0.5-2 % agarose). After melting the agarose in a microwave oven ethidium bromide was added (50 ng/ml). The completely dissolved agarose is cooled to 50-60°C and poured into a horizontal gel chamber that is stuffed with a comb. Before loading the samples into the gel wells, 0.25 volume loading buffer was added to the DNA samples. The gel was run using TAE buffer as the running buffer at a voltage of 10 V/cm and for a time period that would achieve optimal separation. The DNA is detected by the means of ethidium bromide, a dye that intercalates into the

DNA double helix. DNA with ethidium bromide was visualized directly by the means of UV light irradiation. The size of linearized DNA fragments can be determined by specific size standards running on the same gel.

Loading buffer (5x)	100 mM	NaCl
	100 mM	EDTA
	10 mM	Tris-HCl pH 8.0
	50 %	Glycerin
	0.25 %	Bromphenol blue
TAE buffer	40 mM	Tris/HCl, pH 8.0
	40 mM	Sodium acetate
	1 mM	EDTA

V.2.1.6 Isolation of DNA from agarose gel

The DNA fragment was excised from the agarose gel with a clean scalpel under illumination with UV-light and extracted from the agarose with use of the QIAquick Gel Extraction Kit [250] according to the manufacturer's instruction. In principle, the DNA is solubilized from the gel slice by the means of chaotropic salts. The solubilized DNA is bound to a silica gel matrix under high-salt conditions. After a washing step, the DNA is eluted under low-salt conditions with ddH_2O.

V.2.1.7 Quantification of nucleic acids

Quantification of nucleic acids was performed by UV-spectrometry. The absorption of RNA or DNA in water was measured at wavelengths of 260 and 280 nm, which are the absorbance maximums for DNA and proteins. At this wavelength, an extinction of 1.0 corresponds to the concentration of approximately 50 µg/ml of double-stranded DNA, 40 µg/ml of RNA or 30 µg/ml of oligonucleotide [246]. The ratio between OD_{260} and OD_{280} provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have a ratio values between 1.5 and 2. To analyze the integrity of isolated nucleic acid, an aliquot of the DNA or RNA solution was loaded on an agarose gel parallel with a DNA ladder with defined masses.

V.2.1.8 Digestion of DNA with restriction endonucleases

Restriction endonucleases recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequences (4-8 bp) that are oftentimes palindromic. Digestion of double-stranded DNA with restriction endonucleases either produces blunt ends or

sticky ends, depending on the nature of the enzyme. The different digestions were performed under the conditions recommended by the respective manufacturer. Analytical digestions were performed in a volume of 15 µl; preparative digestions were done with a DNA concentration of 0.1 µg/µl. To cleave 1 µg of DNA, 1 unit enzyme was added and the reaction mixture was incubated 1-16 h at the recommended temperature. If it was necessary, the enzyme was either inactivated by heating or by adding a stop solution, which also served as a loading buffer for agarose gel electrophoresis.

V.2.1.9 Dephosphorylation of DNA

Linearized vectors that are prepared for ligation are usually dephosphorylated at the 5' end in order to prevent self ligation (relegation) of the vector. Dephosphorylation is catalyzed by the enzyme alkaline phosphatase. A DNA-fragment (1-5 µg) generated by PCR or by digestion with restriction enzymes was dephosphorylated with 5 units of alkaline phosphatase using the recommended buffer conditions. The reaction mixture was incubated at 37°C for 1 h. If necessary the inactivation of the enzyme was performed by heating to 75°C for 10 min in the presence of 5 mM EDTA.

V.2.1.10 Ligation of DNA

DNA ligase is an enzyme that catalyzes the formation of a phosphodiester bond between the 5' phosphate group and the 3' hydroxyl group of a DNA molecule. This is frequently used during cloning to insert a gene of interest into an expression vector. The reaction was performed in a total volume of 10 µl. Ligation of linearized Vector-DNA (20-50 ng) and a 10-fold molar excess of the Insert-DNA to be ligated were mixed and incubated with the appropriate amount of T4 DNA ligase at 16°C for 4-16 h (cohesive-end ligation) or at 20°C for 16 h (blunt-end ligation). The ligation product is immediately used for the transformation of *E. coli*.

V.2.1.11 Generation of chemically competent *E. coli*

By treating the bacteria with divalent metal ions, they are rendered competent to take up foreign DNA. In order to generate chemically competent bacteria, 800 ml of LB medium are inoculated with 10 ml of an overnight-culture of the respective *E. coli* strain. After grown to an optical density of OD_{600}=0.5, the bacteria were pelleted by centrifugation using

sterile centrifuge tubes. The bacteria pellets were subsequently resuspended in buffer TFB1. After incubating the bacteria on ice for 90 min, the procedure was repeated and the pellet was subsequently resuspended in 15-20 ml of buffer TFB2. The suspension of chemically competent bacteria is aliquoted (100-500 µl), immediately frozen in liquid nitrogen and stored at -80°C.

Buffer TFB1	30 mM	Potassium acetate, pH 5.8
	100 mM	$RbCl_2$
	50 mM	$MnCl_2$
	10 mM	$CaCl_2$
	15 %	Glycerol
Buffer TFB2	10 mM	MOPS, pH 8.0
	10 mM	$RbCl_2$
	75 mM	$CaCl_2$
	15 %	Glycerol

V.2.1.12 Heat shock transformation of *E. coli*

Chemically competent *E. coli*-bacteria (50-100 µl) were incubated for 30 min on ice with 5-10 µl of the ligation product or with the linearized and dephosphorylated vector (serves as an negative control) plasmid. Subsequent to 90 seconds incubation at 42°C, 500 µl of cold LM-medium were added. The bacteria were then allowed to recuperate for 1 h at 37°C. 100 µl were plated on LB-agar dishes containing the proper antibiotic (ampicillin 100 µg/ml or kanamycin 25 µg/ml) for selection and incubated for 16 h at 37°C to allow for the growth of bacterial colonies.

V.2.1.13 DNA sequencing

DNA sequencing is based on the method of chain termination that was originally developed by Sanger [251]. Sequencing was performed at a central facility in the Robert Koch-Institut on a fluorescence sequencing machine (ABI 377-DNA sequencing machine).The 'Big Dye' and the 'dRhodamin-Dye Terminator Cycle Sequencing Ready Reaction Kit' (Applied Biosystems, Weiterstadt) was used.

sequencing PCR reaction mixture	0,5-1µg	purified plasmid DNA
	0.5 µl	10 mM sequencing primer
	4 µl	Big Dye Ready Reaction Mix
	to a total volume of 10 µl ddH_2O	

Sequencing PCR reaction conditions	2 min	94°C	
	30 sec	94°C	┐
	20 sec	50°C	25 cycles
	4 min	60°C	┘
	∞ min	4°C	

V.2.2 Cell culture

V.2.2.1 Cultivation of HuH7 cells

HuH-7 cells were grown in complete DMEM at 37°C. Cells were cultivated in adherent cell culture flasks (175 cm^2) at 37°C, 6 % CO2 and >90 % humidity. As soon as the cells reached confluency, they were washed with PBS and detached by addition of trypsin/EDTA. Activity of trypsin was terminated by addition of complete DMEM. Cells were seeded at different dilutions (1:3 to 1:5) in order to ascertain optimal growth. To check for viability in the spinner culture, the cells were counted using a Neubauer chamber, after treatment with a solution of Trypan blue to a final concentration of 0.3 %.

Complete DMEM	10 %	FCS 1 U/ml penicillin
	1 %	Sodium pyruvate
	1 U/ml	Streptomycin
	2 mM	Glutamine

PBS	40 g	NaCl
	1 g	KCl
	5,75 g	$Na_2HPO_4 \times 2H_2O$
	1 g	KH_2PO_4
	ad 5 l H_2O	

V.2.2.2 Transfection of HuH7 cells

HuH-7 cells were transfected using ExGen500 according to the manufacturer's instructions. One day before transfection, cells were plated in normal growth media so that they were 70 % confluent at the time of transfection. For transfection the appropriate amount of plasmid DNA (as well as a GFP positive control plasmid) and ExGen500 were prepared (diluted in NaCl). After shaking (vortex) and incubation at room temperature for 10 min, the mix was applied to cells (pre-washed first with

PBS, and fresh DMEM). The solution was spread evenly over the plate by gently shaking or by rocking back and forth. The plates were incubated at 37°C in a CO_2 incubator over night. Control cells were analyzed by immunofluorescence 48 h post transfection.

V.2.2.3 Isolation and cultivation of primary human hepatocytes

All liver samples were taken from patients undergoing partial hepatectomy for primary or secondary liver tumors. The use of samples of the liver specimen for the scientific purposes of this study had been authorized by the local ethics committee of the Charité - Campus Virchow. Informed consent was obtained from the donors.

Generally, the liver tissue (3-5 g) was collected in transfer medium (DMEM supplemented with penicillin and streptomycin) following surgical resection from pathologically not affected liver. Hepatocytes were isolated using a two-step perfusion technique based on Collagenase-P digestion as previously described [252]. Briefly, liver tissue was perfused extensively with perfusion solution A. Cells were then dissociated by a 25-30 min perfusion at 37°C with perfusion solution B. The tissue capsule was dissected the cells were scraped into a culture dish and filtered through sterile gauze. To remove death and nonparenchymal cells Hepatocytes were then purified by differential centrifugation at 50 × g for 4 min at 4°C. Viability of the initial cell suspension (trepan blue) ranged between 85 % and 95 %. The cells were seeded into collagen coated 24-well plates (2 × 10^5 cells/well), and removed prior to plating. in hepatocyte media at 37°C and cultivated as described previously [253].

Perfusion solution A	142 mM	NaCl
	6,7 mM	KCl
	100 mM	HEPES pH 7.4
	2,5 mM	EGTA
Perfusion solution B	67 mM	NaCl
	6.7 mM	KCl
	4.8 mM	$CaCl_2$
	100 mM	HEPES
	0.5 %	BSA
	0.025 %	Collagenase type P (Roche)
Collagen solution	0,5 %	Collagen Corporation, USA
		for 30 min

PHH medium	10^{-6} M	Insulin
(William's medium E)	1.4×10^{-6} M	Hydrocortisone
	1 U/ml	Penicillin
	1 U/ml	Streptomycin
	20 mM	HEPES
	100 mM	Sodium pyruvate
	10 %	Low endotoxin calf serum

V.2.2.4 Cytosol preparation

HuH7 cells were harvested and washed five times with cold PBS to remove excess protein. All solutions were kept on ice at all times and the centrifugation steps were carried out at 4°C. For cytosol preparation the cells were lysed by homogenization using a Potter Elvjehm (Berlin, Germany) homogenisor. Unbroken cells, lysosomes and nuclei were removed by centrifugation at 10000 x g for 20 min. For preparation of pure cytosolic fraction the supernatant was centrifuged in a Kontron centrifuge at 400000 x g for 18 min. The cytosol fractions of the cells were used for the protein-protein interaction assay.

V.2.2.5 Nucleus preparation

HuH7 cells were harvested and washed five times with cold PBS to remove excess protein. All solutions were kept on ice at all times and the centrifugation steps were carried out at 4°C. After centrifugation at 250 x g for 10 min, the cells were washed with PBS and centrifuged a second time. The cell pellet was resuspended in five cell pellet volumes of NP buffer A. After incubation on ice for 10 min the cells were centrifuged at 250 x g for 10 min. The supernatant was discarded and the cell pellet was resuspended in three cell pellet volumes of NP buffer B. The sample was homogenized with twenty strokes of a tight-fitting Dounce homogenizer to release the nuclei. The nuclei were washed (in PBS), pelleted by centrifuging at 200 x g for 10 min and finally resuspended in PBS supplemented with 0,5 mM PMSF.

NP buffer A	10 mM	Hepes, pH 7.9
	1,5 mM	$MgCl_2$
	10 mM	KCl
	0,5 mM	DTT
	0,5 mM	PMSF

NP buffer B	10 mM	Hepes, pH 7.9
	26 %	Glycerin
	1,5 mM	$MgCl_2$
	300 mM	NaCl
	0,2 mM	EDTA
	0,5 mM	DTT
	0,5 mM	PMSF

V.2.2.6 Translocation assay with isolated nuclei

Freshly isolated HuH7 nuclei were pelleted by centrifuging at 200 x g for 10 min and resuspended in complete DMEM. The integrity of isolated nuclei was constantly monitored by phase-contrast light microscopy. The resuspended intact nuclei were incubated with 20 nM nucleocapsids in complete DMEM at 37°C on a shaker. After the incubation nuclei were washed twice with cold PBS (200 x g for 10) and were fixed with 4 % paraformaldehyde (diluted in 50 mM Hepes, pH 7.2) for 30 min at room temperature followed by 10 min with EtOH on ice. The nuclei were pelleted by centrifugation at 250 x g for 5 min and resuspended in PBS-T to permeabilize the fixed nuclear membrane. After three washing steps in PBS-T the nuclei were blocked according to the immunofluorescence protocol. To change the different solutions centrifugation steps at 250 x g for 5 minutes were performed. Finally the stained nuclei were transferred to a microscope slide.

V.2.2.7 Translocation assay with digitonin-permeabilized cells

Permeabilization was performed as described [94], based on the protocol of Adam et al. [201]. In brief, 70 % confluent HuH7 cells grown on cover slides in 24 well plates were washed with 1 ml serum-free DMEM and permeabilized with 80 µg/ml digitonin (Sigma) in DMEM (without FCS) for 10 min at 37°C. They were washed twice for 10 min at 4°C in washing buffer followed by a 10 min incubation at 37°C in a humidified box using washing buffer. Permeabilized cells were incubated with 20 nM nucleocapsids in transport buffer containing 30 µg/µl rabbit reticulocyte lysate (RRL; Promega), proteinase inhibitors and an ATP-generating system (1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase; Sigma) for 20 – 120 min at 37°C. The cells were than fixed and prepared for immunofluorescence analysis.

Washing buffer	= transport buffer	+	1%	BSA
			10%	goat serum (Dianova)

Transport buffer	2 mM	Mg-acetate
	20 mM	Hepes, pH 7.3
	110 mM	K-acetate
	1 mM	EGTA
	5 mM	Na-acetate
	1 mM	DTT

V.2.2.8 Cultivation of Sf9 cells

Sf9 cells were grown in 100ml suspension cultures (SfII-900 serum free medium) at 28°C and 135 rpm. As soon as the cells reached a density of $1x10^7$ cells/ml, they were diluted 1:10 with fresh medium (usually twice a week).

V.2.2.9 Generation of recombinant baculovirus

The baculovirus expression system is a convenient and versatile eukaryotic system for heterologous gene expression. Baculovirus expression provides correct folding of recombinant protein as well as disulfide bond formation, oligomerization and other important post-translational modifications. Consequently the overexpressed protein exhibits the proper biological activity and function. The baculovirus expression system is based on the introduction of a foreign gene into a nonessential region of the modified *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome via homologous recombination with a transfer vector containing the cloned gene; an event that occurs in the co-transfected insect cells (**Figure 46**). The production of foreign protein is then achieved by infection of additional insect cell cultures with the resultant recombinant virus. The gene of interest was inserted into the transfer vector pBacPak9 or pVL1393. Correct insertion was verified by DNA sequencing. Recombinant baculoviruses were generated using the BaculoGOLD transfection kit according to the manufacturer's instructions. In brief, $4x10^6$ Sf9 cells were seeded in a 25 cm^2 flask. Cells were transfected with the transfer vector containing the gene of interest together with linearized baculovirus DNA. Recombinant baculoviruses were generated by homologous recombination of the transfer vector with the linearized baculovirus DNA. The supernatant (4 ml) containing the first recombinant virus was harvested 6 days post transfection (passage 1). In order to amplify baculovirus, 0,6 ml of virus containing supernatant were used to infect new 80% confluent adherent Sf9 cells (passage 2). The supernatant was harvested by centrifugation (150 x g for 10 min.). Resulting high virus titers were analyzed by a plaque assay and immunofluorescence.

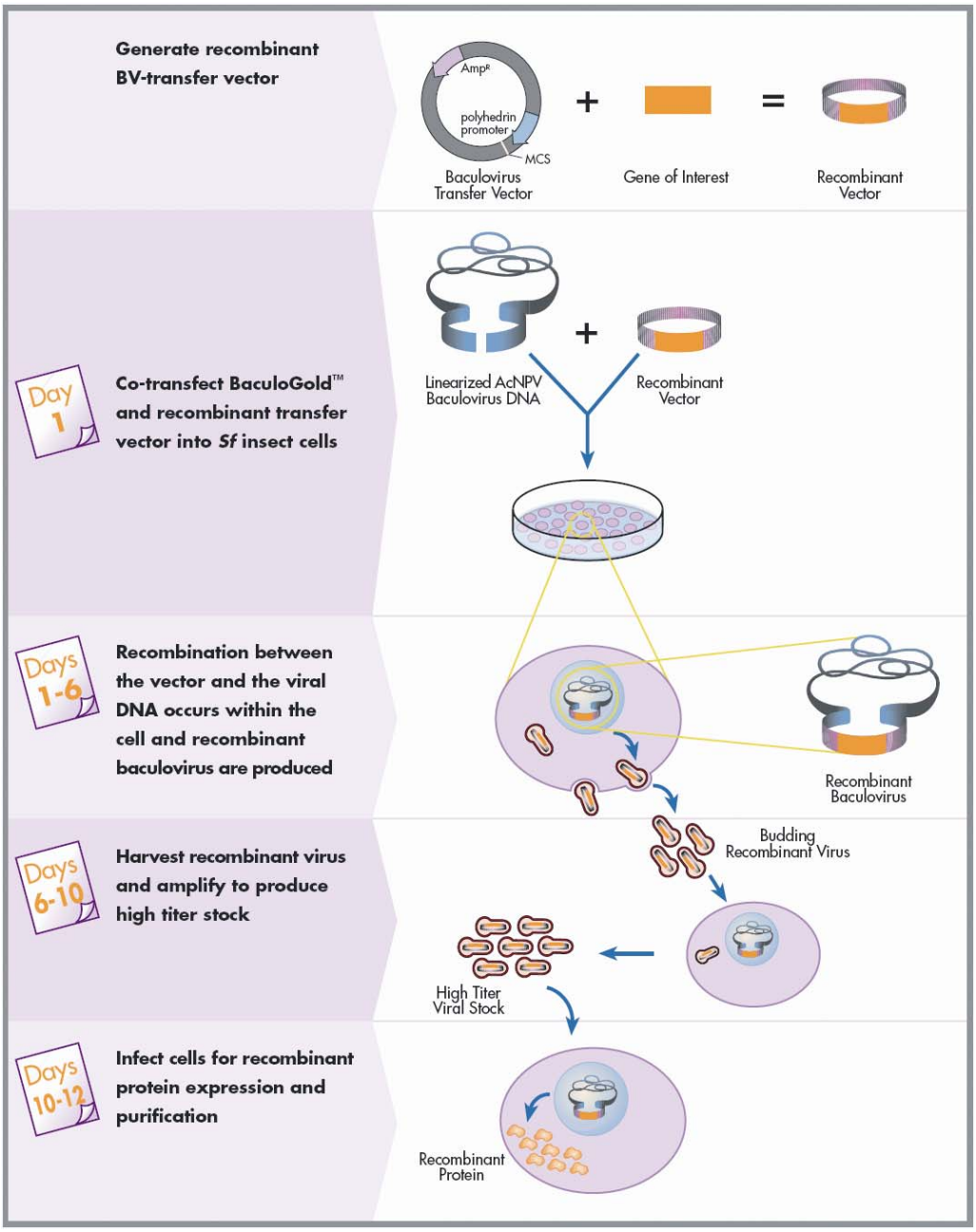

Figure 46: Baculovirus expression system [254]

V.2.2.10 Quantification of recombinant baculovirus

Plaque assay

The infectious potency of a stock of baculovirus is determined by examining and counting plaque formations in an immobilized monolayer culture. Sf9 cells were seeded in 6-well plates at confluency. Cells were infected with serial dilutions of the recombinant baculovirus (5 dilutions per virus) for 2 h on a shaker. Meanwhile, low-melting temperature agarose was diluted in HyQ SfX insect medium (4 % w/v). The solution was boiled to melt the

agarose and kept at 41°C in a water bath. Once the infection was completed, the agarose solution (4 %) was diluted with an equal volume of fresh pre-warmed (37°C) HyQ SfX insect medium. The virus was aspired from the 6-well plates and the cells were overlaid with the agarose solution (2 %) to prevent free diffusion of baculovirus. Once the agarose had become solid, it was overlaid with 1ml of HyQ SfX insect medium to prevent the overlay from drying out. The infected cells were incubated at 28°C for 7 days. To score for production of viral plaques, the cells were stained with MTT according to the manufacturer's instructions. The MTT assay is a viability assay that allows the discrimination between alive (uninfected) and dead (infected cells). Infected cell colonies were counted to calculate the viral titer.

Immunofluorescence assay

The only information which can be received by using the plaque assay is the number of infectious baculoviruses in the supernatant. In order to proof the expression rate of the gene of interest as well as the cellular localisation of the recombinant protein as well as to compare different viral stocks immunofluorescence was performed. Therefore adherent Sf9 cells were infected with serial dilutions of the recombinant baculovirus (with known titer) for 2 h. After 3 days the cells were fixed, blocked, stained with appropriate antibodies and analysed by a confocal laser scanning microscope (see immunofluorescence).

V.2.3 Protein biochemistry

V.2.3.1 Expression and Purification of recombinant proteins

E. coli cells harboring expression plasmids were induced at OD_{595} = 0,5 with 200 ng/ml AHT, grown for additional 3 h and harvested by low speed centrifugation.

Sf9 cells were infected in 100ml suspension cultures at a density of 2-3 x 10^6 cells/ml. For infection, cells were cultivated in serum free HyQ SfX medium at 27°C and 135 rpm. 72h post infection, infected cells were harvested by centrifugation (150 x g for 10min.). Single infections were initiated by adding the appropriate recombinant baculovirus at a multiplicity of infection (MOI) of 2.5 to 5. For generation of nucleocapsids, cells were triple infected with core-protein-, polymerase-protein- and the packaging-plasmid encoding baculoviruses. The infected cells were harvested 3 days post infection.

E. coli or Sf9 cells (40 g wet weight) were resuspended in a physiological buffer (W) including proteinase inhibitors and lysed in a French press (1000 psi). The cellular debris was removed by centrifugation (18000 × g, 30 min at 4°C). Strep-tagged recombinant proteins of the steril filtered (0.45 µm) supernatant were and the supernatant was applied to StrepTactin Sepharose column (affinity choromatography system [255] used according to the instruction of the manufacturer's (IBA)) to allow Strep-tagged capsids to bind to the column. Next, the column was washed with buffer W. Strep-tagged capsids were eluted from the column in 6 fractions (500 µl each) by addition of buffer E.

Properly assembled recombinant capsids (from *E. coli*) or nucleocapsids (from Sf9 cells) were separated from HBcAg- oligomers or monomers by size exclusion using a superose 6 column on an Ettan system (Amersham-Pharmacia). The fractions containing complete particles were pooled and concentrated by YM-30 centricons (Millipore) to a protein concentration of 1 mg/ml. Protein purity was analyzed by silver-stained 15% SDS PAGE as well as by Western blotting using a HBcAg-specific antiserum (MAb16990, Chemicon).

Buffer W	100 mM	Tris/HCl, pH 8.0
	150 mM	NaCl
	1 mM	EDTA
	+ Proteinase inhibitors	
Buffer E	100 mM	Tris/HCl, pH 8.0
	150 mM	NaCl
	1 mM	EDTA
	5 mM	Desthiobiotin

V.2.3.2 Determination of protein concentration

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

V.2.3.3 Size exclusion chromatography

To separate properly assembled recombinant capsids or nucleocapsids HBcAg- oligomers or monomers size exclusion chromatography was performed. The fully assembled HBV capsids with a molecular mass of approximately 5000 kDa ca be separated from HBcAg monomers (21 kDa), dimers (42 kDa) or oligomers by using a calibrated superose 6 column (Amersham Biosciences, **Figure 47**) on an Ettan system (Amersham Biosciences) . The analytical or preparative size exclusion columns were equilibrated in PBS (pH 8.0) with a appropriate flow rate according to the instruction of the manufacturer's.

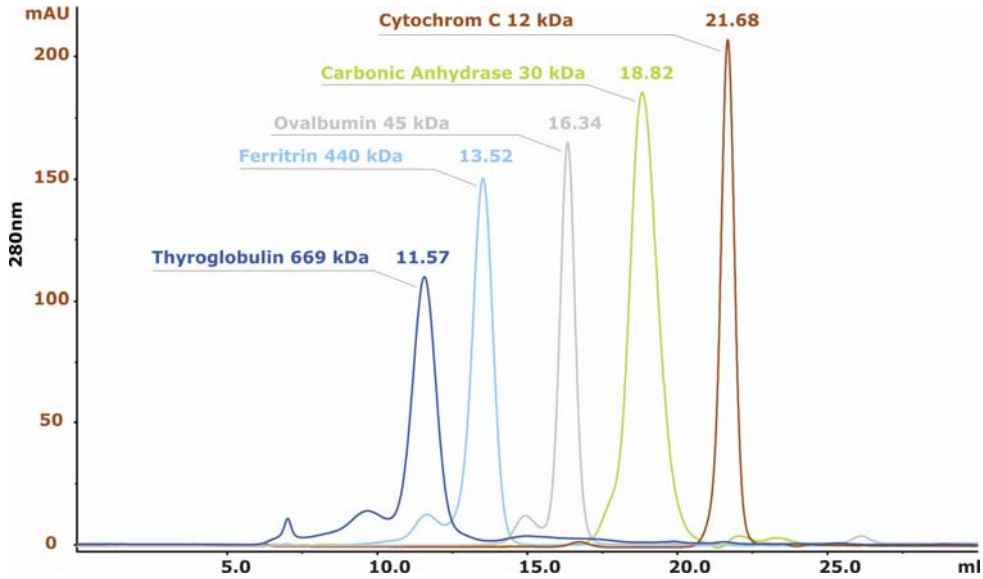

Figure 47: Calibrated size exclusion column (preparative superose 6)

V.2.3.4 SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) is used to separate heterogenous protein mixtures [256]. The polyacrylamide gel matrix is formed by the copolymerization of two monomers: acrylamide and bis-acrylamide. The porosity of the matrix can be altered by changing the percentage of acrylamide in the mixture. In order to most accurately separate proteins according to their sizes, the proteins must be denatured. The anionic detergent sodium dodecyl sulfate (SDS) is used as the denaturing agent The hydrophobic portion of the molecule coats the protein, disrupting the secondary, tertiary, and quaternary structures and coated the protein with negative charges. Therefore, in an electric field, they migrate toward the positive pole separated by the size of the proteins. Denatured protein samples migrate quickly though the large pores of the stacking gel (5 % acrylamide) before entering the narrow pores of the running gel

(10-15 % acrylamide). An additional focusing effect is obtained by shifting the pH from 6.8 in the stacking gel to 8.8 in the running gel: Glycine, which is the main constituent of the running buffer, has an isoelectric point of ~6.0. This means that, once glycine enters the stacking gel, it assumes its zwitterionic form and is essentially uncharged. The resulting deficiency of charge carriers is synonymous with an increased electrical resistance R and – according to Ohm's law (E=RI) - and an increased electric field E in the stacking gel. In response to this increased field, proteins are accelerated in the stacking gel relative to the running gel.
SDS-polyacrylamide gels were poured as a block of 10 minigels (9x6cm).
Samples were resuspended in sample buffer and boiled for 5 minutes at 95°C. The gels were run on a Biorad gel electrophoresis apparatus in electrophoresis buffer at ~100 - 150 V. After the run, the gel was either further processed for immunoblotting or stained with coomassie blue stain for 20 minutes and destained to reveal proteins directly on the gel.

SDS sample buffer	250 mM	Tris-HCl, pH 6.8
	8 %	SDS
	50 %	Glycerin
	0,5 %	bromphenol blue
	2 mM	EDTA
	20 mM	Dithiothreitol (DDT)
Electrophoresis buffer (10X)	250 mM	Tris base
	1,92 M	Glycine
	1 %	SDS
Stacking gel buffer (4X)	0,5 M	Tris/HCl, pH 6.8
	0,4 %	SDS
Running gel buffer (4X)	1.5 M	Tris/HCl, pH 8.8
	0.4 %	SDS
Composition of stacking gel (5 %)	5 ml	Rotiphoresegel 40
	10 ml	Stacking gel buffer (4X)
	25 ml	H_2O
	40 µl	TEMED
	200 µl	Ammonium peroxo disulfate (10%)
Composition of running gel (15 %)	30 ml	Rotiphoresegel 40
	20 ml	Running gel buffer (4X)
	30 ml	H_2O
	60 µl	TEMED
	200 µl	Ammonium peroxo disulfate (10%)

Coomassie blue stain	0,25 %	Coomassie blue R-250 (w/v)
	45 %	Ethanol
	10 %	acetic acid
Coomassie blue destain	45 %	Ethanol
	7,5 %	Acetic acid

V.2.3.5 Silver staining of SDS-polyacrylamide gels

Silver staining of proteins in SDS-polyacrylamide gels is a very sensitive method that allows the detection in lower nanogram range (~10 ng). Under the buffer conditions used, redox-active groups in the electrophoresed proteins catalyze the selective reduction of silver ions in the presence of formaldehyde. The resulting silver crystal seeds can be visualized by addition of the developer.

Once the electrophoresis is completed, the SDS-polyacrylamide gel is soaked in fixative for 30 min. Next, the gel is transferred to the conditioner for 20 min. Once conditioning is completed, the gel is rinsed at least three times (5 min each) in ddH_2O. Then, the gel is soaked with silver nitrate solution for 20 min before it is developed by addition of the developer. Development can be stopped by addition of acetic acid.

Fixative	25 %	Ethanol
	10 %	Acetic acid
Conditioner	0,4 M	Sodium acetate, pH6.0
	30 %	Ethanol
	4,4 mM	Sodium thiosulfate
	1 %	Glutaraldehyde
Silver nitrate solution	0,1 %	Silver nitrate
	0.01 %	Formaldehyde
Developer	2.5 %	Sodium carbonate
	0.015 %	Formaldehyde

V.2.3.6 Western blot

Proteins separated in an SDS-PAGE can be transferred to a solid membrane for western blot analysis. For this procedure, an electric voltage is applied to the gel, so that the separated proteins migrate through the gel onto a PVDF membrane in the same pattern as they were separated on the SDS-PAGE. The PVDF-membrane was submerged in methanol prior to soaking in the Anode buffer II. The transfer was performed under semi-dry conditions

using a discontinuous buffer system (1-1,5 mA/cm^2 for 50 min). The blot was set up as follows:

Cathode (-)
6 Whatman papers soaked with cathode buffer
SDS-polyacrylamide gel
PVDF membrane
2 Whatman papers soaked with anode buffer II
4 Whatman papers soaked with anode buffer I
Anode (+)

Anode buffer I	300 mM	Tris base
	20 %	Ethanol
Anode buffer II	25 mM	Tris base
	20 %	Ethanol
Cathode buffer	40 mM	Isocaproic acid
	20 %	Ethanol

Once the transfer to the PVDF membrane was completed, the PVDF membrane was rinsed in water and stained with a Ponseau S solution. Excess of Ponseau S dye was washed away with water until protein marker bands appeared on the membrane and their positions could be marked on the membrane.

To block unspecific binding sites the membrane was incubating with blocking solution at room temperature or overnight at 4°C on a shaker. The blot was rinsed for 1 min in PBS to remove BSA and incubated with the diluted primary antibodies (in 1-10 % BSA or slim milk powder PBS-T) for 1 h at room temperature on a shaker, followed by washing 3 times with PBS-T for 15 min. The blot was incubated with the HRP-conjugated secondary antibodies (1:2000 diluted in blocking solution A or B) for 1 h at room temperature on a shaker. The blot was washed 3 times with PBS-T for 15 min and developed using ECL western blotting detection system (Amersham Bioscience) according to the manufacturer's instructions.

PBS-T	0.05 %	Tween 20 in PBS
Blocking solution A	2 %	Skim milk powder in PBS-T
Blocking solution B	10 %	bovine serum albumin (BSA) in PBS-T

V.2.3.7 In vitro packaging of plasmid DNA into *E. coli* derived capsids

In vitro assembly of hepatitis B virus capsid protein is highly dependent on protein and NaCl concentration. Also micromolar concentrations of Zn^{2+} are sufficient to initiate assembly of capsid protein, whereas other mono- and divalent cations elicited assembly only at millimolar concentrations, similar to those required for NaCl-induced assembly [257].

HBV capsids were bacterial expressed and purified as described above. They were dialyzed against buffer A resulting in decrease of the NaCl concentration to below 30 mM. The capsids were then disassembled by dialysis over night at 4°C against buffer D. To get rid of the denaturing urea the solution containing dissociated capsids was dialyzed against storage buffer (unused HBcAg was stored at -80°C). After a treatment with RNaseA (200 µg/ml) for 15 min at 30°C HBcAg dimers were concentrated via ultrafiltration using YM 10 Centricons (Amicon). Capsids were assembled at 20°C by mixing a stock of dimers (0,8 - 2,0 mg/ml) with the more concentrated reassembly buffer R. (typically a 1:1 dilution). For packaging of DNA into the capsids the reassembly process occurs in the presence of plasmid DNA (0,1 µg pVax1-SHBs). Reconstituted nucleocapsids were treated with DNase (200 µg/ml for 20 min at 30°C). The detection of DNA after DNase treatment is proof of its protection by packaging into reassembled VLPs.

Buffer A	50 mM	Tris HCl, pH 7.5
	1 mM	EDTA
	5 mM	DTT
Disassembly buffer D	8 M	Urea
	100 mM	Tris HCl, pH 9.5
	2 mM	DTT
Storage buffer	50 mM	Tris HCl, pH 7.5
	1 mM	EDTA
	2 mM	DTT
	+ Proteinase Inhibitors	
Reassembly buffer R (2x)	150 mM	Hepes, pH 7.5
	1,4 M	NaCl
	0,05 mM	ZnCl

V.2.3.8 Affinity chromatography approach to identify potential binding partners HBV capsid

To analyse direct or indirect interaction of HBV capsids with cytosolic partners, wt and TLM nucleocapsids were subjected to a new protein-protein interaction assay (

Figure 48). High pure fully assembled Strep-tagged HBV capsids were applied to StrepTactin sepharose column (1 ml bed size) to allow the capsids to bind to the column. After washing the column with 15 ml of buffer W, it was incubated with the cytosolic fraction derived from human hepatoma cells (HuH-7) to allow potential interaction partners to bind to the immobilized HBV capsids. After another washing step with buffer W (5 ml), Strep-tagged HBV capsids and potential interaction partner were eluted in 6 fractions (0.5 ml each) by addition of buffer E. Finally the eluate fractions E1-E6 was analyzed by SDS-page and Western blot analysis.

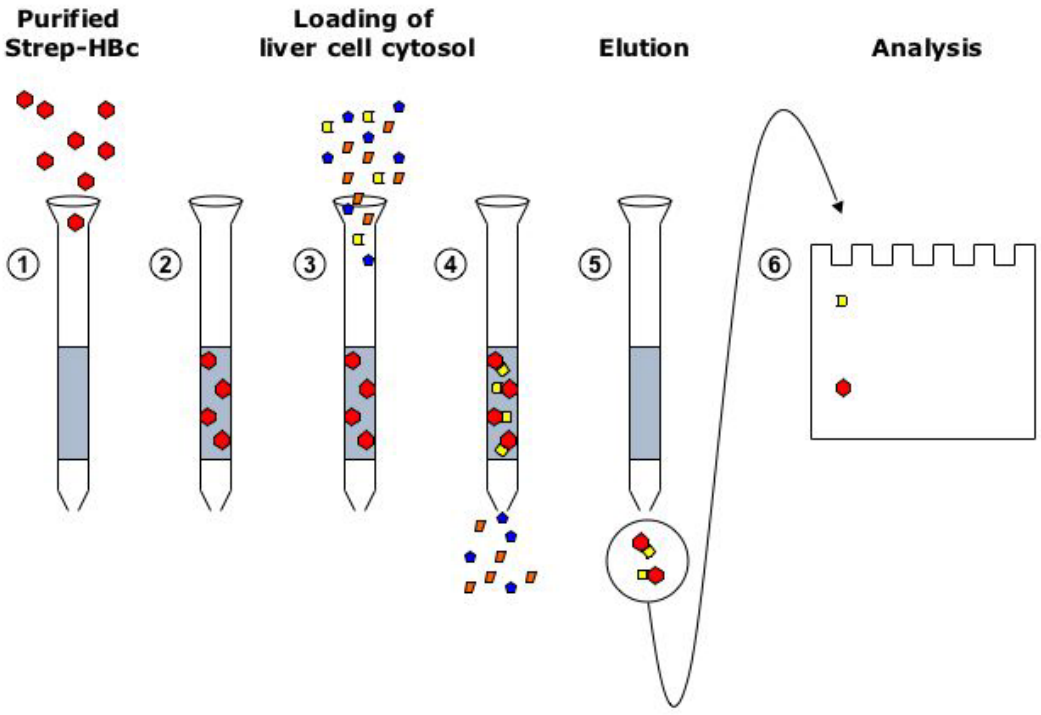

Figure 48: Schematic representation of the protein-protein interaction assay

1. Purified fully assembled Strep-tagged HBV capsids were applied to the StrepTactin affinity column.
2. Strep-tagged HBV capsids were immobilized on a StrepTactin affinity column.
3. The column was incubated with hepatoma cell cytosol fraction (HuH-7).
4. Interaction partners of HBV capsid (yellow) that are present in the hepatoma cell cytosol bind to the immobilized capsid.
5. Strep-tagged HBV capsid is eluted from the column. Potential interaction partners are coeluted.
6. Eluates are analyzed by SDS-PAGE and Western blotting.

V.2.3.9 FITC labelling of core protein

Based on a method of protein labeling by Schreiber et al. [258], the covalent labeling protocol was adapted to our requirements. Briefly, aqueous capsid containing solution (10 nM) was adjusted at pH 7,2. The resulting fluorescent dye (Fluos-NHS, Fluka) was dissolved in DMSO at a concentration of 1 mg/ml. A 100 µl amount of the dye solution was added to the capsid solution and stirred for 48 hour at 6°C. The reaction was stopped by adding 20 µl ethanolamine. Free FITC was removed by size exclusion chromatography using a superose 6 column.

V.2.3.10 ELISA Quantification

After having incubated the HuH7 and PHH cells with cell permeable nucleocapsids to perform a gene transfer, the supernatant was collected and stored imminently at -80°C. To quantify the amount of secreted HBsAg in the supernatant of cells a ELISA assay was performed. Therefore the commercial 'HBs ELISA Enzygnost HBsAg 5.0' was used according to the instruction of the manufacturer's (Dade Behring).

V.2.4 Microscopy

V.2.4.1 Confocal laser scanning microscopy

Huh7 cells or PHHs were grown on cover slides in 24 well plates and incubated with purified wildtype or TLM (nucleo)capsids (10-20 nM) diluted in DMEM. Incubation time (20 – 60 min) was followed by two brief washes (warm PBS) and addition of fresh DMEM. Cells were first fixed at the indicated time points with 4 % formaldehyde/PBS (30 min at 25°C) and second by ice-cold ethanol (10 min). After three washing steps with PBS-T the cells were blocked with 10 % BSA/PBST (45 min at 25°C). For visualization of the F-actin filaments the cells were incubated with FITC-or TRITC-labeled Phalloidin (Sigma, 1.5 mM). The nucleus was stained by Toto-3 (final 2 µM, Molecular Probes) or SYBR Green (1:100.000 dilution, Molecular Probes). Reacting primary antibodies were visualized with adequate FITC-, Cy5- or Cy3-conjugated secondary antibodies. All stainings were performed in 10 % BSA/PBST for 60 min followed by washing steps with PBS-T (tree times 5 min). Confocal immunofluorescence microscopy was performed using the Zeiss LSM 510 microscope (20 × and 63 water × objectives).

To eliminate background staining of cells and to adjust the CLSM, cells only incubated with PBS were used. Sections presented were taken

approximately at the mid-height level of the cells. Z-stacks presented were taken from the bottom (glass slide) to the top of the cells. The imaging was performed in sequential mode in order to avoid bleed through between the photomultiplier tubes. Photomultiplier gain and laser power were identical within each experiment. The pinhole setting in every experiment was ≤ 1. The resulting thickness of cell slices was ~ 1,5 µm. Measurements were made by using the overlay function of the LSM 510 software.

V.2.4.2 Transmission electron microscopy

Highly purified core particles were collected and spotted on pioloform-carbon coated copper grids and then negatively stained with saturated uranyl acetate (2 %).
For detection of fully assembled nucleocapsids inside the cytoplasm of cells, HuH7 cells were incubated for 45 min with highly purified nucleocapsids (20 nM). After several washes with culture medium and PBS the cells were fixed for 30 min at 4°C in PBS containing 4 % paraformaldehyde and 0,1 % glutaraldehyd. For ultra-thin sections, cells were stained *en bloc* with 2 % aqueous uranyl acetate and leadcitrate (2 %), followed by ethanol dehydration. The dishes were embedded in Epon 812. Sections were cut using an ultramicrotome. Transmission electron microscopy was performed at a central facility in the Robert Koch-Institut (Dr. Hans Gelderblom and coworkers). The specimens were analyzed with a transmission electron microscope (EM10A, Zeiss).

V.2.5 Molecular computing

V.2.5.1 Protein charge plot

The European Molecular Biology Open Source Software Suite (EMBOSS) offers a comprehensive package of integrated sequence analysis applications released under the open source model on UNIX platforms [259]. The EMBOSS charge program reads a protein sequence and writes a file (or plots a graph) of the charges of the amino acids within a window of specified length as the window is moved along the sequence. The Algorithm of the charge program uses the column "charge" from the datafile Eamino.dat. It gives the residues 'D' and 'E' a charge of -1, 'K' and 'R' a charge of +1, and the residue 'H' a charge of +0.5. Then it calculates the mean charge across -window (default is 5).

V.2.5.2 Molecular modeling

Molecular Modeling was performed with Hyper Chem 7.5, Amber 99 (Hypercube). Visualization was done with DS Viewer Pro 5 (Accelrys) Structural data of the Human hepatitis B Viral Capsid (HBcAg) can be accessed from the RCSB Protein Data Bank (file 1QGT [111]). The calculation of single and double TLM peptides structure was performed under water conditions in a cube of X = 40 Å, Y = 26 Å and Z = 42 Å. The total root-mean-square gradient (RMS) is calculated by using the Polak-Ribiere geometry optimization algorithm (in kcal/mol x Å). Further conditions for the molecular dynamics were:

Heat time	0.1 ps
Run time	1 ps
Cool time	0.1 ps.
Start Temperature	0 K
Simulations Temp	310 K (37°C)
Final Temp	0 K
Temp Step	31 K