

3 Materials

3.1 Cell lines

HuH-7 cells	derived from a hepatocellular carcinoma of human origin (Nakabayashi et al., 1982)
Sf9 cells	derived from pupal ovarian tissue of <i>Spodoptera frugiperda</i> (DSMZ No. ACC 125)
Sf21 cells	derived from pupal ovarian tissue of <i>Spodoptera frugiperda</i> (DSMZ No. ACC 119)

HuH-7 I₃₇₇/NS3-3[`]/wt/9-13 and HuH-7 I₃₈₉/NS3-3[`]/LucUbiNeo-ET were kindly provided by Dr. Ralf Bartenschlager (University of Heidelberg). They represent clonal HuH-7 cell lines that contain autonomously replicating HCV replicons (Frese et al., 2003; Lohmann et al., 1999).

3.2 Bacterial strains

<i>E. coli</i> K12 DH5 α	GIBCO BRL, Eggenstein
(F ⁻ , <i>thi-1</i> , <i>gyrA96</i> , <i>supE44</i> , <i>hsdR17</i> (r _k ⁻ , m _k ⁺), <i>recA1</i> , <i>endA1</i> , <i>relA1</i> , Φ 80d, <i>lacZ</i> Δ M15)	

3.3 Antibodies

Table 3.1 Antibodies used for Western blotting

Antibody	Species	Clonality	Dilution	Manufacturer
anti-V5	Mouse	monoclonal	1:2000	Invitrogen
anti-c-Raf1 (C-12)	Rabbit	polyclonal	1:1000	Santa Cruz, USA
anti-c-Raf	Mouse	monoclonal	1:1000	BD Transduction Laboratories, Canada
anti-NS5A	Rabbit	polyclonal	1:1000	Generated as described in chapter 5.1
anti-phospho-MEK	Rabbit	polyclonal	1:2000	Cell Signaling Technology, USA
anti-active-MAPK	Rabbit	polyclonal	1:2000	Promega, Mannheim

Antibody	Species	Clonality	Dilution	Manufacturer
anti-ERK2 (C-14)	Rabbit	polyclonal	1:500	Santa Cruz, USA
anti-Actin	Mouse	monoclonal	1:10000	Sigma, Seelze
anti-Rabbit IgG, HRP-linked	donkey	Polyclonal	1:2000	Amersham Biosciences, Freiburg
anti-Mouse IgG, HRP-linked	sheep	Polyclonal	1:2000	Amersham Biosciences, Freiburg

Table 3.2 Antibodies used for immunofluorescence analysis

Antibody	Species	Clonality	Dilution	Manufacturer
anti-NS5A	Rabbit	polyclonal	1:1000	generated as described in chapter 5.1
anti-HA (Y-11)	Rabbit	polyclonal	1:200	Santa Cruz, USA
anti-c-Raf	Mouse	monoclonal	1:200	BD Transduction Laboratories, Canada
anti-V5	Rabbit	polyclonal	1:200	Sigma, Seelze
anti-NS5B	Mouse	monoclonal	1:2	Dr. Darius Moradpour (University of Freiburg)
anti-Mouse IgG, Cy3-linked	Goat	polyclonal	1:400	Dianova, Hamburg
anti-Rabbit IgG, Cy3-linked	Goat	polyclonal	1:400	Dianova, Hamburg
anti-Mouse IgG, FITC-linked	Horse	polyclonal	1:300	Vector Labs, USA
anti-Rabbit IgG, FITC-linked	Horse	polyclonal	1:300	Vector Labs, USA

The NS5B-specific antibody was kindly provided by Dr. Darius Moradpour (University of Freiburg) (Moradpour et al., 2002).

3.4 Kits

ABI PRISM ready reaction dye deoxy terminator cycle sequencing kit	Perkin Elmer, Weiterstadt
BaculoGOLD Transfection Kit	BD Biosciences/Pharmingen, USA
Bio-Rad Protein Assay	Bio-Rad, Munich
Dual-Luciferase Reporter Assay System	Promega, Mannheim
ECL Western Blotting Reagent	Amersham, Freiburg
Qiaquick Gel Extraction Kit	Qiagen, Hilden
Qiaquick PCR Purification Kit	Qiagen, Hilden
Qiagen Plasmid Maxi Kit	Qiagen, Hilden

3.5 Enzymes

AMV reverse transcriptase	Promega, Mannheim
Alkaline phosphatase	Roche, Mannheim
BIOTAQ DNA polymerase	Bioline, Luckenwalde
Casein kinase II	Merck/ Calbiochem, Darmstadt
Herculase Enhanced DNA polymerase	Stratagene, Netherlands
Klenow DNA polymerase	Roche, Mannheim
MEK-1	Santa Cruz, USA
Restriction endonucleases	Roche, Mannheim
T4 DNA ligase	Roche, Mannheim

3.6 Primers

Table 3.3 PCR primers

Name	Description	Sequence (5' to 3')
tb001	fw-BglII-NS5A	ggaagatctatgggctccggctcgtgg
tb004	bw-BglII-V5	ggaagatctcgtagaatcgagacc
tb011	fw-BglII-H6-NS5A	ggaagatctatgcaccatcaccatcaccatggctccggctcgtgg
tb012	bw-V5-NS5A	cgacatcgtctcgtcggtaagcctatccctaac
tb013	fw-V5-polyA	ggtaagcctatccctaacctctcctcggctcgtcgttctacgtgaccgctgatcagcctcgac
tb014	bw-BglII-polyA	cctagatctgccatagagcccaccgc
tb019	fw-Strep-NS5A	gatggatccatgtggagccaccgcagttcgaaaaacccggccatggggctccggctcg tggctaagg
tb020	bw-His6-V5	cgtagatctttagtgatggatggatggatggctctagaattccgtagaatcgagaccgag
tb021	fw-BamHI-NS5A	ggaggatccggctccggctcgtgg
tb023	bw-HindIII-V5-NS5A	ctcaagctttcacgtagaatcgagaccgaggagaggggttagggataggcttaccggtagccg cagcagacgatgctc
tb032	fw-Strep-NS5A(105)	gatggatccatgtggagccaccgcagttcgaaaaacccggccatggccgaacta ttccagggcg
tb033	fw-Strep-NS5A(211)	gatggatccatgtggagccaccgcagttcgaaaaacccggccatggacagcaga gacggctaaac
tb037	bw-NS5A	gcagcagacgatgctgc
tb039	fw-NS5A	ttccaggtcgggctcaaccaatac
tb059	fw-beta-Glob	cgtgctggttattgtgctgtctc
tb060	bw-NS5A	attggttgagcccacctggaatg
tb061	fw-tubulin	tcactgtgctgaacttacc
tb062	bw-tubulin	ggaacatagccgtaaaactgc
	fw-albumin	gctacggcacagtgttg
	bw-albumin	caggattgcagacagatagtc
	BacPak-fw	aaccatctcgaaataaata
	BacPak-bw	aacgcacagaatctagcgctt
	pcDNA seq fw	ttaatcagactcactataggg
	pcDNA seq bw	tagaaggcacagtcgaggc
	pASK seq fw	gagttatcttaccactcct
	pASK seq bw	cgtagtagcgtaaacg

3.7 Plasmids

3.7.1 Commercially available plasmids

pAP-1-Luc	Stratagene, Netherlands
pASK-IBA7 (N-terminal <i>Strep</i> tag)	IBA, Göttingen
pcDNA 3.1. (-)	Invitrogen, Karlsruhe
pEGFP-N1	BD Biosciences/ Clontech, USA
pNF- κ B-Luc	Stratagene, Netherlands
pSRE-Luc	Stratagene, Netherlands
pVL1393	BD Biosciences/ Pharmingen, USA

3.7.2 Other plasmids

Table 3.4 Other plasmids

Name	Resistance	Description
pASK-IBA7-NS5A-V5	Ampicillin	Prokaryotic expression plasmid that codes for Strep-NS5A-V5
pBacPak9-Strep-NS5A(211-449)-V5	Ampicillin	Transfer vector for the generation of recombinant baculoviruses that codes for Strep-NS5A(211-449)-V5
pVL1393-Strep-NS5A-V5	Ampicillin	Transfer vector for the generation of recombinant baculoviruses that codes for Strep-NS5A-V5
pcDNA-NS5A	Ampicillin	Eukaryotic expression plasmid that codes for H6-NS5A-V5
pcDNA-NS5A <i>inverse</i>	Ampicillin	Eukaryotic expression plasmid that contains the NS5A ORF in inverse orientation
pcDNA-HA-NS5A-V5	Ampicillin	Eukaryotic expression plasmid that codes for HA-NS5A-V5
pcDNA-Strep-NS5A(105-449)-V5	Ampicillin	Eukaryotic expression plasmid that codes for Strep-NS5A(105-449)-V5
pcDNA-Strep-NS5A(211-449)-V5	Ampicillin	Eukaryotic expression plasmid that codes for Strep-NS5A(211-449)-V5

Name	Resistance	Description
pRK5-Raf C4	Ampicillin	Eukaryotic expression plasmid that codes for a dominant-negative mutant of c-Raf1
pSTAT3-TA-Luc	Ampicillin	Reporter plasmid that codes for luciferase under the control of a STAT-3-dependent promoter

pRK5-Raf C4 was a kind donation of Dr. Ulf Rapp (University of Würzburg) (Bruder et al., 1992). pSTAT3-TA-Luc was kindly provided by Dr. Yung Wong (Hong Kong University of Science and Technology).

3.8 Recombinant baculoviruses

Table 3.5 Recombinant baculoviruses

Name	Description
AcNPV Strep-NS5A(211-449)-V5	Recombinant baculovirus coding for Strep-NS5A(211-449)-V5
AcNPV Strep-NS5A-V5-H6	Recombinant baculovirus coding for Strep-NS5A-V5-H6
AcNPV GST-Raf	Recombinant baculovirus coding for GST-Raf
AcNPV GST-Raf K375W	Recombinant baculovirus coding for GST-Raf K375W
AcNPV Strep-HBc	Recombinant baculovirus coding for Strep-HBc (Hepatitis B virus core)

AcNPV GST-Raf and AcNPV GST-Raf K375W were kindly provided by Dr. Walter Köhler (University of Glasgow, UK). AcNPV Strep-HBc was kindly donated by Böttcher Brandenburg (Robert-Koch-Institut, Berlin).

3.9 siRNAs

Silencer validated siRNA: c-Raf1

Ambion, UK

Silencer negative control 1

Ambion, UK

3.10 Size standards

3.10.1 DNA standards.

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

3.10.2 Protein standards

Low molecular weight (LMW) marker	Amersham, Freiburg
SeeBlue Plus 2	Invitrogen, Karlsruhe

3.11 Reagents for cell culture

3.11.1 Sf9 and Sf21 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

3.11.2 HuH-7 cells.

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

3.12 Mice

Inbred FVB mice (FVB/N CrIbR) were obtained from Charles River Laboratories, Sulzfeld.

3.13 Radioactive chemicals

[γ -³²P]ATP

Hartmann Analytics, Braunschweig

3.14 Chemicals and reagents

Agarose

Promega, Mannheim

Anhydrotetracycline (AHT)

IBA, Göttingen

BAY43-9006 (Raf kinase inhibitor II)

Merck/ Calbiochem, Darmstadt

BSA (bovine serum albumin)

PAA, Austria

CitiFluor AF-1

Citifluor LTD., England

Desthiobiotin

Sigma, Seelze

Dialysis membrane Spectra/Por MWCO 2000

Roth, Karlsruhe

Didesoxynucleotides

Bioline, Luckenwalde

DOTAP

Roth, Karlsruhe

Ethanol

RKI, Berlin

ExGen 500

Fermentas, St. Leon-Rot

G418 sulfate

PAA, Austria

Glutathione Sepharose

Amersham, Freiburg

HABA

IBA, Göttingen

Hybond-P membrane

Amersham, Freiburg

Hyperfilm (ECL & MP)

Amersham, Freiburg

D-Luciferin

Roche, Mannheim

NHS Sepharose

Amersham, Freiburg

PD98059

Merck/ Calbiochem, Darmstadt

Phalloidin-FITC

Sigma, Seelze

Protein A/G Sepharose

Santa Cruz, USA

PMA (Phorbol 12-myristate 13-acetate)

Fluka, Seelze

RNAasin

Promega, Mannheim

Rotiphoresegel 40 (Acrylamide/ bisacrylamide)

Roth, Karlsruhe

siPORT Lipid transfection agent

Ambion, UK

*Strep*Tactin Sepharose

IBA, Göttingen

Trizol	Invitrogen, Karlsruhe
U0126	Merck/ Calbiochem, Darmstadt
Whatman Paper	Whatman, UK

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

3.15 Devices

3.15.1 Gel electrophoresis

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

3.15.2 Centrifugation

Centrifuge 5415 R (refrigerated)	Eppendorf, Hamburg
Table centrifuge Biofuge fresco	Heraeus, Osterode
Minifuge 2	Heraeus, Osterode
Sorvall RC-5B refrigerated superspeed centrifuge with the rotors HB-4, GS-3, SS-34	Sorvall Instruments, Bad Homburg
Beckman L8-70 ultracentrifuge with the rotors SW-28 and SW-41	Beckman, USA

3.15.3 Liquid chromatography

Äkta Explorer Amersham, Freiburg

3.15.4 Microscopes

Light microscope Diavert Leitz, Wetzlar

Fluorescence microscope Leitz DM RBE Leitz, Wetzlar

Confocal laser scanning microscope (Axioplan) Zeiss, Jena

3.15.5 Other devices

Cryotome Frigocut 2800 (Reichert-Jung) Leica, Bensheim

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, Göttingen

Sartorius universal (analytical balances) Sartorius, Göttingen

Shaker Unitron HT-Infors, Switzerland

Sterile bench HeraSafe Kendro, Langenselbold

Thermocycler PTC-100 MJ Research, USA

Thermomixer compact Eppendorf, Hamburg

UV table UV-Biometra TB Biometra, Göttingen

with the camera BioToc

Vacuum dryer (Slab Dryer Model 443) BioRad, USA

Video Monitor WV-BM 900 Panasonic, Taiwan

Water bath GFL 1083 GFL, Burgwedel