2 Material and methods

2.1 Material

2.1.1 Equipment

Electrophoresis

Mini protean II camber (80 x 55 x 0.75 mm) (Bio-Rad, Munich); Mini-trans-blot apparatus (Bio-Rad, Munich); Power supply “Power Pac 300” (Bio-Rad, Munich); Sequence apparatus S2 (Gibco BRL, Eggenstein); Gel dryer 583 (Bio-Rad, Munich); Gel-Doc II (Biometra, Göttingen); Chamber “Hoefer SE 600 Standard Dual Cooled vertical unit” (180 x 160 x 0.75 mm) (Amersham Pharmacia, Freiburg)

Centrifuges

Centrifuge 5417 C (Eppendorf, Hamburg); Centrifuge 5417 R (Eppendorf, Hamburg); Centrifuge 5804 R (Eppendorf, Hamburg); Cell centrifuge RT 6000D (Sorvall/DuPont, Bad Homburg)

Bacteria and cell culture

Genpulser II/PulseControllerPlus/Capacitance Extender Plus (Bio-Rad, Munich); Incubator “HeraSafe” and “HeraCell” (Heraeus-Christ, Hanau)

Sequencing apparatus and thermocycler

ABI 377-DNA-sequencing apparatus (Applied Biosystems/PerkinElmer, Weiterstadt); DNA Thermal Cycler 480 (Applied Biosystems/Perkin Elmer, Weiterstadt); Trio Thermoblock (Biometra, Göttingen)

Others

UV-Photometer “Ultrospec 2000” (Amersham Pharmacia Biotech, Freiburg); Fluorescence microscope “Axioplan“ (Zeiss, Oberkochen); Liquid scintillation counter Wallac 1409 (PerkinElmer Wallac, Freiburg); Speed-Vac “Centrivac” (Heraeus-Christ, Hanau); Elisa-reader “Multiplate Reader MRX” (Dynatech, Guernsey, GB); Incubator shaker „G24 Environmental Shaker“ (New Brunswick Scientific/Labotec, Göttingen); Thermomixer comfort (Eppendorf, Hamburg); Luminometer “Micro Lumat Plus” (EG & G Berthold, Berthold Technologies, Bad Wildbad); Gel-Doc II (Biometra, Göttingen)

2.1.2 Consumables

- Reaction tubes; 0.5, 1.5, and 2 ml (Eppendorf, Hamburg)
- Dialyse microfilter; 0.025 µm pore size, type VS (Millipore, Eschborn)
- Sterile filter; 0.2 µm pore size, Minisart® (Sartorius, Göttingen)
- Nylon-transfer membrane; Hybond-N (Amersham Pharmacia Biotech, Freiburg)
- Whatman® 3MM filter paper (Whatman International Ltd., Maidstone, GB)
- ProbeQuant™ G-50 Micro Column (Amersham Pharmacia Biotech, Freiburg)
- Centri-Sep spin columns (Perkin Elmer/Applied Biosystems, Weiterstadt)

- X-ray film (Kodak, Stuttgart)
- Biomax (Kodak, Stuttgart)
- Electroporation cuvette; 0.2 cm (Bio-Rad, Munich)
- Cell culture dishes; 100 mm (Falcon/Becton-Dickinson, Heidelberg)
- Culture dishes; 6- and 24-wells (Falcon/Becton-Dickinson, Heidelberg)
- White microtiter plate; 96-well (flat bottom) (Corning and Costar, Wiesbaden)

All pipettes, screw cap-tubes, and dishes for bacteria or cells were from Falcon/Beckton-Dickinson (Heidelberg), Nunc (Wiesbaden) or Greiner (Nürtingen).

2.1.3 Reagents

- [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Freiburg)
- [α-^{35}S]-dATP (Amersham Pharmacia Biotech, Freiburg)
- 2-mercaptoethanol (Merck, Darmstadt)
- 8-hydroxyquinoline (Sigma, Munich)
- Acrylamide (29:1) and (19:1) (Bio-Rad, Munich)
- Acrylamide (Sigma, Munich)
- Acrylamide, rotiphoreseR Gel30, 30% acrylamide/0.8% bisacrylamide (Roth, Karlsruhe)
- Agar, select agar (Gibco BRL, Eggenstein)
- Agarose, „Low Melting“ (FMC Biozym, Hameln)
- Agarose, NA (Amersham Pharmacia Biotech, Freiburg)
- Alexa™ 488 goat anti-rabbit IgG conjugate (Molecular Probes)
- Ammonumpersulphat (Sigma, Munich)
- Ampicillin (Sigma, Munich)
- Bacto-Trypton (Dico, Detroit, USA)
- BCA-protein Assay Reagent (Pierce, Weisskirchen)
- Bisacrylamide (Sigma, Munich)
- Bovine serum albumin (Sigma, Munich)
- Bromphenol blue (Serva, Heidelberg)
- Chloroquine (Sigma, Munich)
- Coomassie brilliant blue R250 (Sigma, Munich)
- DEAE-dextran (Sigma, Munich)
- DMEM (Gibco BRL, Eggenstein)
- DMEM:HAM’S F-12 (1:1) (Biochrom, Berlin)

- DMSO (Serva, Heidelberg)
- DTT (Sigma, Munich)
- Ethidium bromide (Sigma, Munich)
- FCS (Gibco BRL, Eggenstein)
- Formamide (Sigma, Munich)
- Kanamycin sulphate (Gibco BRL, Eggenstein)
- LipofectAMINE 2000 Reagent (Life Technologies, Gibco BRL, Eggenstein)
- Low-fat milk powder (Nestlé, Frankfurt/M)
- NP-40 (Sigma, Munich)
- Nucleotidtriphosphate (ATP, CTP, GTP, and TTP) (Boehringer/Roche, Mannheim)
- PBS (Biochrom, Berlin)
- $PBS^{+/+}$ (Biochrom, Berlin)
- Penicillin (Biochrom, Berlin)
- Poly(deoxyinosinic-deoxycytidylic) acid sodium salt (Sigma, Munich)
- RNasin (MBI Fermentas, St. Leon-Rot)
- RPMI (Gibco BRL, Eggenstein)
- SDS (Serva, Heidelberg)
- Sephadex G-50 (Sigma, Munich)
- Streptomycin (Biochrom, Berlin)
- TEMED (Bio-Rad, Munich)
- TRIZOL reagent (Gibco BRL, Eggenstein)
- Trypsin (0.25%)/EDTA (0.02%) (Biochrom, Berlin)
- Tween 20 (Sigma, Munich)
- X-Gal (Gibco BRL, Eggenstein)

All other chemicals were from Merck (Darmstadt), Sigma (Munich), or Roth (Karlsruhe).

2.1.4 Buffers

PBS:
137 mM NaCl; 2.7 mM KCl; 1.4 mM KH_2PO_4; 4.3 mM $Na_2HPO_4 \cdot 7H_2O$; pH 7.4

$PBS^{+/+}$:
PBS with 0.1 g/l $MgCl_2 \cdot 6H_2O$ and 0.132 g/l $CaCl_2 \cdot 2H_2O$

HBS:
10 mM Hepes, pH 7.15; 137 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 0,7 mM Na_2HPO_4 (filtrated through a 0,22 µm Millipore filter)

PLP:
26 mM sodium phosphate pH 7.4, 94 mM lysine, 10 mM sodium iodate, 2% (v/v) paraformaldehyd

Elvanol:
PBS, pH 8.0; 16.6% (w/v) polyvinylalcohol; 30% (v/v) glycerin; 1mg/ml 1,2-phenylendiamine; 0.8% (v/v) 2-mercaptoethanol

TAE:
40 mM Tris acetic acid, pH 8.3; 1 mM EDTA

TBE:
100 M Tris/HCl, pH 8.3; 83 mM boric acid; 1 mM EDTA

TBS-T:
TBS with 0.1% (v/v) Tween-20

TE:
10 mM Tris/HCl, pH 8.0; 1 mM EDTA

TE^{-4}:
10 mM Tris/HCl, pH 8.0; 0.1 mM EDTA

DNA loading buffer:
100 mm EDTA; 4x TAE; 50% glycerin; 0.1% bromphenol blue

LB-medium:
1% (w/v) bactotrypton; 0.5% (w/v) yeast extract; 10 mM NaCl; pH 7.5

LB-agar:
LB-medium containing 1.5% (w/v) agar

SOC-medium:
2% (w/v) bactotrypton, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl; supplemented with 10 mM $MgCl_2$ and 20 mM glucose before use.

2.1.5 Enzymes

- PfuTurbo®DNA polymerase (Stratagene, Heidelberg)
- Superscript II-Reverse-Transcriptase (Gibco BRL, Eggenstein)
- T4 DNA ligase (New England Biolabs, Schwalbach/Taunus)
- T4 polynucleotide kinase (MBI Fermentas, St.Leon-Rot)
- Klenow fragment (NEB, Schwalbach/Taunus)
- Taq-DNA-polymerase (PerkinElmer, Weiterstadt)
- Calf intestine phosphatase (New England Biolabs, Schwalbach/Taunus)
- Mung-bean nuclease (New England Biolabs, Schwalbach/Taunus)
- Exonuclease III (New England Biolabs, Schwalbach/Taunus)

Restriction endonucleases were from New England Biolabs, Beverly, USA.

2.1.6 Reaction kits

- QIAGEN Plasmid Maxi/Midi Kit (QIAGEN, Hilden)
- QIAquick Gel Extraction Kit (QIAGEN, Hilden)
- QIAquick PCR Purification Kit (QIAGEN, Hilden)
- QIAprep Spin Miniprep Kit (QIAGEN, Hilden)
- QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, Heidelberg)
- Marathon™ cDNA Amplification Kit (Clontech, Heidelberg)
- T7-sequencing kit (Amersham Pharmacia Biotech, Freiburg)
- TOPO TA Cloning® Kit (Invitrogen, Groningen, Holland)
- Dual-Luciferase Reporter Assay System (Promega, Mannheim)
- ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems/PerkinElmer, Weiterstadt)
- ECL-Detection Kit (Amersham Pharmacia Biotech, Freiburg)

2.1.7 Antibodies

Primary antibodies

- Polyclonal anti-β-catenin antibody (Sigma, Munich)
- Polyclonal anti-α-catenin antibody (Sigma, Munich)
- Monoclonal anti-E-cadherin antibody (Transduction Laboratories, Dianova, Hamburg)
- Polyclonal anti-LI-cadherin antibody (Angres et al., 2001)
- Monoclonal anti-Sp1-antibody (Sigma, Munich)
- Polyclonal anti-Ap1 antibody (c-jun, human) (Promega, Mannheim)
- Monoclonal anti-Cdx2 antibody (Biogenetics; DCS Innovative Diagnostik-Systeme, Hamburg)
- Polyclonal anti-HNF-1 antibody (Santa Cruz Biotechnology, Heidelberg)

Secondary antibodies

- Goat anti rabbit-IgG (GAR), Alexa™ 488 conjugate (Molecular Probes, Leiden, Holland)
- Swine anti rabbit-IgG (SAR), HRP-conjugated (Dakopatts, Hamburg)
- Rabbit anti mouse-IgG (RAM), HRP-conjugated (Dakopatts, Hamburg)

2.1.8 Oligonucleotides

All oligonucleotides used in this study were synthesized by TIB Molbiol (Berlin).

2.1.9 Molecular size and weight standards

50 bp DNA-ladder (Gibco BRL, Eggenstein):
Fragment sizes (bp): 50, 100, 150, 200, 250, 300, 350, 450, 600, 800, >2000

100 bp DNA-ladder (Gibco BRL, Eggenstein):
Fragment sizes (bp): 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 2072

1 kb DNA-ladder (Gibco BRL, Eggenstein):
Fragment sizes (bp): 75, 134, 154, 201, 220, 298, 344, 396, 506, 517, 1018, 1636, 2036, 3054, 4072, 5090, 6108, 7126, 8144, 9162, 10180, 11198, 12216

SDS-7B molecular weight standard (Sigma, Munich):
Molecular masses (kD): 26, 36, 48, 58, 84, 116, 180

2.1.10 DNA plasmids

pCR®2.1-TOPO (Invitrogen, Groningen, Holland)

pRL-SV40 (Promega, Mannheim)

pGL3-basic (Promega, Mannheim)

pGL3-control (Promega, Mannheim)

pEGFP-1 (Clontech, Heidelberg)

pEGFP-C1 (Clontech, Heidelberg)

pBluescript®S/K(+) (Stratagene, Heidelberg)

2.1.11 Bacteria

E. coli DH5α; Genotype: supE44, Δlac, U169, (ø80, lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, RelA1

E. coli TOP10F‘ (Invitrogen, Groningen, Holland); Genotype: [F’lacIq, Tn10(tetR)], mcrA D(mrr-hsdRMS-mcrBC), ø80, lacZΔM15, ΔlacX74, deoR, recA1, araD139Δ(ara-leu)7679, galU, galK, rpsL(Str^R), endA1, nupG

E. coli XL1-Blue (Stratagene, Heidelberg); Genotype: recA1, endA1, gyrA96, thi-1, λ^-, hsdR17, (rk^-, mk^+), supE44, relA1, lac^-, [F’, proAB, $lacI^qZ\Delta M15$, $Tn10(tet^R)$]

2.1.12 Cell lines

STC-1:
Mouse neuroendocrine cells from intestine (Rindi et al., 1990); AG Prof.Wiedemann, Klinik für Innere Medizin, Institut für Gastroenterologie und Hepatologie der HU Berlin

CMT-93:
Mouse colon carcinoma cell line (ATCC nr. CCL-223; Rockville, USA)

Caco2:
Human colon carcinoma cell line (ATCC nr. HTB-37; Rockville, USA)

B16F0:
Mouse skin melanoma cell line (ATCC nr. CRL-6322; Rockville, USA)

IEC-6:
Rat normal small intestine cell line (DSMZ nr. ACC 111; Braunschweig)

293:
Human embryonal kidney cell line (DSMZ nr. ACC 305; Braunschweig)

CHO:
Chinese hamster ovary cell line (DSMZ nr. ACC 110; Braunschweig)

AGS:
Human gastric adenocarcinoma cell line (ATCC nr. CRL-1739; Rockville, USA)

HepG2:
Human hepatocellular carcinoma cell line (ATCC nr. HB-8065; Rockville, USA)

23132/87:
Human gastric adenocarcinoma cell line (DSMZ nr. ACC 201; Braunschweig)

A549:
Human lung carcinoma cell line (ATCC nr. CCL-185; Rockville, USA)

HT-29:
Human colon adenocarcinoma cell line (ATCC nr. HTB-38; Rockville, USA)

2.2 Methods: Molecular biology

2.2.1 Purification of DNA

Ethanol precipitation

To the solution of the DNA, 0.1 volume of 3 M sodium acetate (pH 5.2) was added. After vortexing, 2.5 volume of 100% ethanol was added. The DNA was precipitated at –20°C for at least 30 min and pelleted by centrifugation at 20,000 x g at 4°C for 30 min. The supernatant was removed and the pellet was washed with ice-cold 70% ethanol. After a second centrifugation at 20000 x g for 10 min, the supernatant was removed, and the precipitated DNA was air-dried for 5-10 min. The dried pellet was resuspended in a volume of water or TE^{-4} (10 mM Tris/HCl pH 8.0; 0.1 mM EDTA) to a final concentration of 1 µg/µl.

Isopropanol precipitation

An equal volume of 100% isopropanol was given to the DNA solution. The DNA was precipitated without any incubation step and further prepared in the same way as described in the procedure for ethanol precipitation.

Phenol extraction

Protein contaminants were removed from aqueous DNA solutions by extraction with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The phenol was saturated with TE-buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA). 8-hydroxyquinolin was added as an antioxidation agent to a concentration of 1 mg/ml. An equal volume of phenol:chloroform:isoamyl alcohol was added to the DNA solution and mixed until an emulsion formed. After centrifuging the mixture at 6800 x g for 5 min, the aqueous phase was transferred to a fresh tube. A subsequent extraction with an equal volume of chloroform removed traces of phenol from the DNA preparation. After further centrifugation, the aqueous phase was transferred to a fresh tube and the DNA was recovered by precipitation with ethanol as described.

Purification of PCR-products

PCR products were purified from contaminating oligonucleotides and enzymes with use of the QIAquick PCR Purification Kit (QIAGEN, Hilden). The appropriate buffers were added according to the protocol. DNA was absorbed by a silica-membrane in the presence of high salt while contaminants passed through the mini column. Impurities were washed away, and the DNA was eluted with TE^{-4} or dH_2O.

2.2.2 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 (QIAGEN, Hilden). The appropriate buffers were added according to the manufacturer's protocol. The purification of the DNA was accomplished by the use of the silica-membrane, as described in section 2.2.1.

2.2.3 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. An absorbance of 1 at 260 nm corresponds to approximately 50 µg/ml for double-stranded DNA and 40 µg/ml for RNA (Sambrook and Fritsch, 1989). The ratio between the readings at 260 and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have A_{260}/A_{280} values between 1.5 and 2. A diluted solution of DNA was added to a 500 µl quartz cuvette and measured in a UV-photometer and the DNA concentration was calculated.

For sparse DNA quantities (concentration less than 100 ng/µl), or to analyze the integrity of isolated RNA, an aliquot of the DNA or RNA solution was loaded on an agarose gel parallel with a DNA ladder with defined masses (defined DNA-bands between 10-120 ng; Gibco BRL, Eggenstein). After staining with ethidium bromide, an estimation of the DNA concentration was made by comparing the sample of interest with the intensity of the stained DNA ladder.

2.2.4 PCR amplification

The polymerase chain reaction, PCR, (Mullis and Faloona, 1987) 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 the following steps:

Denaturation: Separation of the double-stranded DNA

Annealing: Hybridisation of the primers to their complementary sequences

Elongation: Synthesis of the complementary strand with the use of a heat-stable *Taq* polymerase.

The method was used as an analytical test for identification of DNA molecules, to amplify DNA sequences to be ligated into the TOPO-TA vector, to generate site-specific mutations or to delete one or more base pairs in reporter gene constructs (section 2.2.16). In analytical reactions and for amplification of DNA fragments to be ligated (table 2.3-2.10), the standard PCR reaction mixture and conditions used in this study are shown in table 2.1 and 2.2.

All the oligonucleotides were purchased from TIB Molbiol (Berlin). The Taq-DNA-polymerase from Perkin Elmer (Weiterstadt) was used when nothing else is mentioned.

Table 2.1: Standard PCR reaction mixture.

Component	**Volume**
dH_2O	15.5 µl
DNA template	5 µl
Reaction buffer (10x) with 15 mM $MgCl_2$	2.5 µl
Nucleotides (10 mM; 2.5 mM each)	0.5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
Taq-DNA-polymerase (5 U/µl)	0.5 µl
Total reaction volume	25 µl

Table 2.2: Standard reaction conditions.

Pre-cycle	Denaturation	3 min	94°
	Denaturation	30 sec	94°C
Cycle	Annealing	20 sec	A temp
	Elongation	E time	72°C
Post-cycle	Final elongation	7 min	72°C
	Cooling	Unlimited	8°C

A temp = annealing temperature, E time = elongation time

Table 2.3: Murine LI-cadherin, 5′-RACE-PCR.

Template	Adapter-ligated cDNA from mouse intestine diluted 1:100
Forward primer	AP1 (adapter primer) 5′-CCATCCTAATACGACTCACTATAGGGC-3′
Reverse primer	P 400 R (mLI-cadherin-cDNA) 5′-GATGAACCGCTCTTGTTTCTCTGTCCA-3′
Reaction conditions	35 cycles; A temp =65°C and E time =1 min

Table 2.4: Murine LI-cadherin, cDNA control PCR.

Template	Adapter-ligated cDNA from murine intestine diluted 1:100
Forward primer	In situ 1 (mLI-cadherin-cDNA) 5′-GGCCATGAGCTCATGGTGTCTGCCCAGCTTCACTTC CTG-3′
Reverse primer	P 400 R (mLI-cadherin-cDNA) 5′-GATGAACCGCTCTTGTTTCTCTGTCCA-3′
Reaction conditions	35 cycles; A temp =65°C and E time =1 min

Table 2.5: Colony-PCR of *E.coli* clones containing 5′-RACE-PCR-products.

Template	10 µl dH_2O, inoculated with bacteria colony
Forward primer	AP1 (adapter primer) 5′-CCATCCTAATACGACTCACTATAGGGC-3′

Reverse primer	Ex1R (mLI-cadherin-cDNA) 5′-CAGTAAGTAAGAAATGCTGC-3′
Reaction conditions	35 cycles; A temp =55°C and E time =15 sec

Table 2.6: Cloning of 1.8 kb of the LI-cadherin 5′-flanking region.

Template	QIAGEN-purified cosmid cLI-1-DNA (1 ng/µl)
Forward primer	Pro-1795 F (mLI-cadherin-genomicDNA) 5′-AACTCAGCGGTATCTACCCTGACCCATG-3′
Reverse primer	P.E.III (mLI-cadherin-cDNA, 5´UTR 5′-TGTCGTCCATTCAGCCGTGGAGAC-3′
Reaction conditions	34 cycles; A temp =65°C and E time =4 min

Table 2.7: Cloning of 2.8 kb of the LI-cadherin 5′-flanking region.

Template	QIAGEN-purified cosmid cLI-1-DNA (1 ng/µl)
Forward primer	Pro-2780 F (mLI-cadherin-genomicDNA) 5′-GCCTCTGACTCTATGAGTTTCCCAACCTTG-3′
Reverse primer	P.E.III (mLI-cadherin-cDNA, 5´UTR) 5´-TGTCGTCCATTCAGCCGTGGAGAC-3′
Reaction conditions	34 cycles; A temp =65°C and E time =4 min

Table 2.8: Cloning of 55 bp of the LI-cadherin 5′-flanking region.

Template	QIAGEN-Maxiprep pGL3-basic/2.8 kb (0.6 ng/µl)
Forward primer	mLI Pro-55 (mLI-cadherin-genomicDNA) 5´-GTGGTTAATGTTTGACTGG-3′
Reverse primer	P.E.III (mLI-cadherin-cDNA, 5´UTR) 5´-TGTCGTCCATTCAGCCGTGGAGAC -3′
Reaction conditions	34 cycles; A temp =60°C and E time =15 sec

Table 2.9: Cloning of 33 bp of the LI-cadherin 5′-flanking region.

Template	QIAGEN-Maxiprep pGL3-basic/2.8 kb (0.6 ng/µl)
Forward primer	mLI Pro-33 (mLI-cadherin-genomicDNA) 5´-GGTGGGTGAAGGGAGAGGCT-3′
Reverse primer	P.E.III (mLI-cadherin-cDNA, 5´UTR) 5´-TGTCGTCCATTCAGCCGTGGAGAC-3′
Reaction conditions	34 cycles; A temp =60°C and E time =15 sec

Table 2.10: Cloning of 22 bp of the LI-cadherin 5′-flanking region.

Template	QIAGEN-Maxiprep pGL3-basic/2.8 kb (0.6 ng/µl)
Forward primer	mLI Pro-22 (mLI-cadherin-genomicDNA) 5´-GGAGAGGCTAGAGGAGCAGA-3′
Reverse primer	P.E.III (mLI-cadherin-cDNA, 5´UTR) 5´-TGTCGTCCATTCAGCCGTGGAGAC-3′
Reaction conditions	34 cycles; A temp =60°C and E time =15 sec

2.2.5 Nucleic acid gel electrophoresis

Agarose gel electrophoresis

Agarose was added to a volume of electrophoresis buffer (TAE) to prepare the gel for the size of DNA fragments to be separated. After melting the agarose in a microwave oven it was poured into the gel casting platform and the gel comb was inserted. Before loading the samples into the gel wells, 0.25 volume loading buffer (100 mM EDTA, 4 x TAE, 50% glycerin, 0.1% bromphenol blue) was added to the DNA samples. The gel was run at a voltage of 10 V/cm and for a time period that would achieve optimal separation. The gel was then stained with ethidium bromide (25 µg/ml in TAE) for 10 min and visualized directly by illumination with UV light.

Denaturing gel electrophoresis for sequencing

A 6% denaturing polyacrylamide gel was used for the separation of single-stranded DNA with a resolution of 1 bp. The electrophoresis was performed in the S2-sequencing gel apparatus with TBE buffer. For each gel, a premixed gel solution (Acryl-a-Mix 6; Promega, Mannheim) was used to which 400 µl 10% APS were added to polymerize the gel. After a 2 h polymerization, the gel was placed in the chamber and pre-run at 20 W for 30 min. Denaturation of the samples followed at 95°C for 5 min before they were cooled on ice. They were then loaded on the gel and separated by electrophoresis at 60 W for about 1 h. The gel was placed on a sheet of Whatman® 3MM filter paper (Whatman International, Maidstone, GB) and covered with a plastic wrap. Finally, the gel was dried 1-1.5 h on a gel dryer (Gel Dryer 583, Bio-Rad, Munich) and exposed to an X-ray film (Kodak, Stuttgart) for several days at –80°C.

Sequencing gels for automated DNA-sequencing

The acrylamide sequencing gels used for the automated DNA-sequencing on the ABI 377-DNA-sequencing apparatus were made according to the manufacturer's recommendations (ABI tips, October 1998). The following gel system was used: 48 cm WTR, 6% polyacrylamide gel/7 M urea. Composition of the gel: 21 g urea, 7.1 ml 30% acrylamide (29:1 acrylamide:bisacrylamide), 6 ml 10 x TBE buffer, 21 ml dH_2O, 15 µl TEMED, and 350 µl 10% APS.

2.2.6 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. 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. The enzyme was either inactivated by heating or by adding a stop solution (100 mm EDTA, 4 x TAE, 50% glycerin, 0.1% bromphenol blue), which also served as a loading buffer for agarose gel electrophoresis.

2.2.7 Dephosphorylation of DNA

Hydrolysis of 5′-phosphatase residues by calf intestine phosphatase (CIP) was used to prevent self-ligation of vector termini by ligation. The dephosphorylated product possesses a 5′-hydroxyl group, which can subsequently be labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. A DNA-fragment (1-5 µg) generated by PCR or by digestion with restriction enzymes were dephosphorylated with 5 units CIP using the recommended buffer conditions. The reaction mixture was incubated at 37°C for 1 h. Inactivation of the enzyme was performed by heating to 75°C for 10 min in the presence of 5 mM EDTA.

2.2.8 Ligation of DNA

Ligation of restriction fragments

Vector-DNA (20-50 ng) and a 10-fold molar excess of the DNA-fragment to be ligated were mixed and incubated with 400 units of the T4 DNA ligase at 16°C for 4-16 h (cohesive-end ligation) or at 20°C for 16 h (blunt-end ligation). The reaction was performed in a total volume of 10 µl. Reaction conditions: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA).

TOPO-TA cloning of PCR-products

The TOPO-TA Cloning Kit was used to insert a PCR-product into a plasmid. TOPO-TA cloning provides a 5 min, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. Taq polymerase has a template-independent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′-ends of PCR products. The linearized vector pCR®2.1-TOPO contains single, overhanging 3′-deoxythymidine (T) residues on both ends that allow the PCR insert to ligate efficiently with the vector. The plasmid vector is provided as a complex with the enzyme topoisomerase. After addition of the PCR product, the ligation takes place within 5 min at room temperature. The components of the kit were added as described in the manufacturer's protocol.

2.2.9 Transformation of *E.coli*

Heat shock transformation

Competent *E.coli*-bacteria (50-100 µl) were incubated for 30 min on ice with 5-10 µl of the ligation product or with 10 ng of undigested plasmid. Subsequent to 2 min incubation at 42°C, 500 µl of SOC-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.

Electroporation

Electroporation with high voltage is currently the most efficient method for transforming *E.coli*-bacteria with plasmid DNA. Subjecting bacteria to an electrical pulse results in formation of pores that are large enough to allow the plasmid to enter the bacteria. If the DNA to be introduced into the bacteria is a ligation product, the ligation sample has to be dialyzed to remove disturbing salts prior to the transformation.

The ligation product was added to a microfilter with 0.025 µm pore size and dialyzed against dH_2O for at least 2 h. 2 µl of the dialyzed sample or 100 pg plasmid-DNA were transferred to a tube with 40 µl electrocompetent *E.coli* DH5α thawed on ice. The DNA and the cells were

put in a cold 0.2 cm electroporation cuvette. The cuvette was connected to a power supply and the cells were subjected to a high-voltage electrical pulse of defined magnitude and length. The optimal field strength for the transformation of *E.coli*-bacteria was between 16-19 kV/cm, which gave the following settings for a 0.2 cm cuvette by the apparatus used (Genpulser II; Bio-Rad, Munich): 2.8 kV voltage, 25 µF capacity, and 200 Ω resistance. The transfected bacteria were diluted in a 450 µl SOC-medium and then shaken for 1 h at 37°C. 100 µl aliquots were plated on LB-agar dishes containing the proper antibiotic for selection and incubated for 16 h at 37°C.

Single clones were picked with a sterile toothpick and 2 ml LB-medium (with the proper antibiotic) were inoculated and incubated for 12-16 h at 37°C in an incubator shaker at 230 rpm. Positive clones were either identified by analyzing purified plasmid DNA by minipreparation (method described below) or by direct PCR-analysis of single clones identified on the agar plate.

2.2.10 Plasmid-DNA preparation

Minipreparation

Plasmid DNA from a 2 ml overnight bacterial culture in LB-medium was prepared using the QIAprep Spin Miniprep Kit. This procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto a silica gel matrix in the presence of high salt concentration. After a wash step, the DNA is eluted from the column in 50 µl dH_2O. The appropriate buffers were added as recommended by the manufacturer.

Maxipreparation

Large scale purification of plasmid DNA was performed with the QIAGEN Plasmid-Maxi-Kit. As recommended by the manufacturer, 100 ml of an overnight culture of transformed *E.coli*-bacteria were lysed with the appropriate buffer supplied with the kit, followed by binding of the plasmid DNA onto an anion-exchange-resin column under the recommended low salt and pH conditions. The DNA was eluted in a high salt buffer and concentrated and desalted by isopropanol precipitation.

2.2.11 DNA sequencing

The sequencing of double-stranded DNA was performed by the dideoxy chain-termination method (Sanger et al., 1992).

Radioactive sequencing with T7 DNA polymerase

Radioactive sequencing with T7 DNA polymerase was used in order to generate a 1 bp-ladder. With the T7 sequencing kit (Amersham Pharmacia Biotech, Freiburg), the sequencing was performed according to the manufacturer's protocol. The DNA was labeled with α-[^{35}S]-dATP and chain terminated fragments were generated by adding the respective dideoxy/deoxy mixture to the reaction („short mix"). After termination the loading buffer (98% deionized formamide, 10 mM EDTA pH 8, 0.025% xylen cyanol FF, 0.025% bromphenol blue) was added to the sequencing samples. After heat denaturation, the sequencing products were separated on a sequencing gel (section 2.2.5).

Sequencing with fluorescence labeled dideoxynucleotides

Nonradioactive sequencing was performed on a fluorescence sequencing machine (ABI 377-DNA sequencing machine) using the „Big Dye“ and the „dRhodamin-Dye Terminator Cycle Sequencing Ready Reaction Kit“ (Applied Biosystems/PerkinElmer, Weiterstadt). 4 µl purified plasmid DNA (ca. 0.8 µg) were mixed with 0.5 µl 10 mM sequencing primer, 4 µl Big Dye Ready Reaction Mix, and dH_2O to a total volume of 10 µl. The reaction conditions for the cycle sequencing were: 2 min 94°C; 25 x (30 sec 94°C; 20 sec 50°C; 4 min 60°C); cooling at 4°C. The thermal-cycler TC480 (Perkin Elmer/Applied Biosystems, Weiterstadt) was used for the cycle sequencing.

Removal of unincorporated fluorescence nucleotides and primers from the sequencing product was performed by chromatography through a sephadex G-50 spin column. For each sample to be purified, 55 mg sephadex G-50 (Sigma, Munich) were made to swell in 700 µl dH_2O; 700 µl were pipetted on a 1 ml spin column (Princeton Separations; Applied Biosystems/PerkinElmer, Weiterstadt) and centrifuged at 1000 x g for 2 min to remove the water. The sample was adjusted to 20 µl with water and loaded onto the column. After centrifuging at 1000 x g for 2 min, the purified sequencing product was dried in a vacuum centrifuge and analyzed with the ABI 377-DNA-sequencing machine on a urea polyacrylamide sequencing gel (section 2.2.5). Analysis of raw data was performed with the software “Sequencing Analysis” (Applied Biosystem/PerkinElmer, Weiterstadt). The nucleotide sequences received were further analyzed with the Tetra-Analyze/Complign Software (MacMolly, Berlin).

2.2.12 RNA isolation

Isolation of total RNA from tissue

The isolation of RNA from animal tissues was performed with the trizol reagent (Gibco BRL, Eggenstein) in accordance with the manufacturer’s recommendations. The RNA isolation is based on a one-step isolation including an extraction with acidic guanidinium-thiocyanate-phenol-chloroform. This is an optimization of the method developed by Chomczynski and Sacchi, 1987. For the isolation of total RNA from mouse intestine, the intestine was rinsed with PBS and minced in trizol (optimum: 50 mg tissue/ml) with a ultra-thurax-homogenizer for 3-5 min. After ethanol precipitation of the isolated RNA, the RNA was resuspended in DEPC-treated dH_2O and stored at –80°C. For longer storage periods exceeding 2 weeks, the DNA was kept as an ethanol precipitate.

Isolation of poly(A)$^+$-RNA

The isolation of poly(A)$^+$-RNA from total RNA was performed with the QIAGEN Oligotex Suspension. RNA molecules containing poly(dA) sequences were hybridized with oligo(dT) bound to a latex particle. The separation of the latex particle from the rest of the solution was performed with spin columns as described by the manufacturer. The eluted RNA was precipitated with ethanol and stored under the same conditions as total RNA.

2.1.13 Rapid amplification of cDNA ends (RACE)

In order to amplify unidentified cDNA-ends, the Marathon™ cDNA Amplification Kit (Clontech, Heidelberg) was used according to the manufacturer's specifications. Starting with 1 µg poly(A)$^+$-RNA from the appropriate tissue (section 2.2.12), adapter-ligated double-stranded-cDNA-libraries were synthesized. With these libraries, the cDNA region of interest was amplified using appropriate gene specific primers in 5′-RACE reactions.

2.1.14 Primer extension

Primer extension was used to map and quantify the 5′-termini of RNA. The RNA was hybridized with an excess of a single-stranded DNA primer radioactively labeled at its 5′-terminus. Reverse transcriptase was then used to extend this primer to produce cDNA complementary to the RNA template. The length of the resulting end-labeled nucleotide, as measured by electrophoresis through a polyacrylamide gel under denaturing conditions (section 2.2.5), reflects the distance between the end-labeled nucleotide of the primer and the 5′-terminus of the RNA.

Oligonucleotides used by the primer extension:

Primer 1 (LI P.E.IIIrev): 5′-TGTCGTCCATTCAGCCGTGGAGAC

Primer 2 (LI 5′UTR Ex1R): 5′-CAGTAAGTAAGAAATGCTGC

9 µl RNA (10 µg/µl DEPC-treated dH_2O) were mixed with 3 µl 5 x reaction buffer (Gibco BRL, Eggenstein), 2 µl [^{32}P]-primer (ca. 4500 cpm; equivalent to about 20 fmol), 1 µl 0.1 M DTT, and 0.5 µl RNasin (20 u/µl, MBI Fermentas, St. Leon-Rot). The mixture was incubated for 10 min at 65°C, followed by an incubation at 50°C for 3 min. For each sample, the following components were mixed in a second reaction tube: 1 µl 5 x reaction buffer, 1 µl DTT, 0.5 µl RNasin, 1.6 µl 2.5 mM dNTP (0.2 mM end concentration), and 1 µl Superscript II (Gibco BRL, Eggenstein). 5 µl were added to the RNA-containing hybridizing mixture to a total volume of 20.5 µl. The reaction was further incubated for 30 min at 42°C. 0.1 volume of 3 M sodium acetate (pH 5.3) was added to the DNA/RNA-hybrid mixture, followed by a phenol/chloroform extraction (section 2.2.1). The DNA/RNA hybrid in the aqueous phase was precipitated with ethanol (section 2.2.1) and resuspended in 1.5 µl 10 mM Tris/HCl (pH 8) including 20 µg/ml RNase A (QIAGEN, Hilden). 4 µl DNA loading buffer were added before denaturation, and the product was analyzed on a 6% acrylamide 8 M urea sequencing gel (section 2.2.5). 2 µl of a radioactively labeled sequencing product with known sequence were loaded in parallel as a 1 bp-ladder, as well as 1 µl of a [^{32}P]-labeled 50 bp-ladder.

2.1.15 Generation of nested sets of deletions with Exonuclease III

The enzyme exonuclease III was used to generate a series of murine LI-cadherin-promoter gene mutants progressively lacking more nucleotides from the 5′-end. The enzyme catalyses the subsequent removal of 5′-mononucleotides from a recessed or blunt 3′-hydroxyl terminus of double-stranded DNA (Weiss, 1976). To create unidirectional deletion mutants (Henikoff, 1987), the double stranded DNA was digested by two restriction enzymes. One enzyme cleaved near to the target sequences and generated a blunt end or a recessed 3′-terminus, and the other enzyme generated a 3- or 4-nucleotide protruding 3′-terminus. Digestion with

exonuclease III proceeded unidirectionally from the cleavage site with the recessed 3′-end into the target DNA sequences. The exposed single strands were removed by digestion with nuclease S1 and by the 3′ → 5′-exonuclease activity of the Klenow fragment of *E.coli* DNA polymerase I. The linear DNA was then recirculated and sequenced with a universal primer.

20 µg of the plasmid pGL3-basic containing 2.8 kb of the mouse LI-cadherin promoter were digested with KpnI (40 U) and PmlI (40 U) over night at 37°C in a total volume of 200 µl. The DNA was purified by extraction with phenol (section 2.2.1), and after precipitation with ethanol (section 2.2.1) dissolved in 100 µl 1 x exonuclease III buffer and stored on ice. 25 aliquots of 7.5 µl mung-bean reaction mixture (1 x mung-bean buffer, 60 U mung-bean nuclease) were placed in 0.5 ml microfuge tubes. The DNA/exonuclease III mixture was incubated for 5 min at 37°C and 4 µl were transferred to the first tube. 3 µl (300 U) exonuclease III were added to the remainder of the DNA solution and at 1 min intervals, samples of 4 µl were removed and placed in successive tubes. After all the samples had been transferred, the microfuge tubes were incubated for 30 min at 30°C and then 1 µl stop solution (0.3 M Tris-base; 50 mM EDTA, pH 8.0) was added to each tube. The tubes were incubated for 10 min at 70°C and transferred to ice. 2 µl of the aliquots were analyzed on a 0.6% agarose gel. 1 µl of a Klenow mixture (200 mM $MgCl_2$; 10 mM Tris-HCl pH 7.6; 6 U Klenow fragment) was added to the samples containing the DNA of desired size. Then, the samples were incubated for 5 min at 37°C. 0.5 µl 10 mM dNTPs were added to each sample and the incubation was continued for 15 min at room temperature. The DNA in each sample was ligated into closed circles with 800 units T4 ligase and incubated for 2 h at room temperature under the appropriate buffer conditions (section 2.2.8). *E.coli* DH5α cells were transformed with the plasmids using the heat shock method (section 2.2.9). After plasmid preparation (section 2.2.10) from ten colonies of each transformation, the DNA was digested with EcoRI and NotI (section 2.2.6) and analyzed on a 1.2% agarose gel. The clones of an appropriate size were chosen for sequencing.

2.1.16 Site-directed mutagenesis

The QuickChange site-directed mutagenesis method uses either miniprep plasmid DNA or cesium-chloride-purified DNA. The basic procedure starts with a supercoiled, dsDNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by PfuTurbo®DNA polymerase*. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks is generated. After temperature cycling, the product is treated with DpnI. The DpnI is used to digest the parental DNA template and select the synthesized DNA containing mutations. Since DNA isolated from most *E. coli* strains is dam methylated, it is susceptible to DpnI digestion, which is specific for methylated DNA. The nicked vector DNA incorporating the desired mutations is then transformed into XL1-Blue cells. The small amount of starting DNA template required to perform QuickChange mutagenesis, the high fidelity of the PfuTurbo®DNA polymerase, and the low cycle number all contribute to high mutation efficiency and decreased potential for random mutations.

The QuickChange™ Site-Directed Mutagenesis kit was used to generate site-specific mutations in double-stranded plasmids. Two synthetic oligonucleotide primers containing the desired mutation, each complementary to opposite strands of the vector, were extended during temperature cycling by PfuTurbo®DNA polymerase*. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated.

A 25 µl reaction mixture containing the primers, the plasmid to be mutated, and PfuTurbo®DNA polymerase was prepared (table 2.11). By temperature cycling, the plasmid was denatured and the primers were annealed, extended and incorporated (table 2.12). The parental DNA was removed by digestion with 0.5 µl DpnI added directly to the reaction mixture. After incubation for 1 h at 37°C, the resulting double-stranded, nicked DNA molecules were transformed into competent *E.coli*-DH5α (section 2.2.9). The nicks in the plasmids were repaired in the bacteria after transformation.

*PfuTurbo®DNA polymerase permits template DNA replication with an error rate that is significantly lower than conventional thermostable enzymes, thereby minimizing undesirable second-site mutations.

Table 2.11: Standard reaction mixture.

Component	**Volume**
dH_2O	15.5 µl
DNA template (4 ng/µl)	5.0 µl
10 x cloned *Pfu DNA polymerase* reaction buffer	2.5 µl
Nucleotides (10 mM; 2.5 mM each)	0.5 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
PfuTurbo®DNA polymerase (2.5 u/µl)	0.5 µl
Total reaction volume	25 µl

Table 2.12. Standard reaction conditions.

Pre-cycle	Denaturation	1 min	94°C
	Denaturation	30 sec	94°C
Cycle	Annealing	1 min	A temp
	Elongation	E time	72°C
Post-cycle	Cooling	Unlimited	8°C

A temp: Annealing temperature, E time: Elongation time

These standard reaction conditions were used by the generation of the mutations or deletions shown in table 2.13-2.21.

Table 2.13: Deletion of 181 bp in the LI-cadherin 5′-flanking region.

Template	pLI(1794)-luc and pLI(2774)-luc
Forward primer	mLIProDel 181F 5′-TAAAACAACACCACCAGGTTGAGGAAGTGTCTCTT-3′
Reverse primer	mLIProDel 181R 5′-ACACTTCCTCAACCTGGTGGTGTTGTTTTAAATAT-3′
Reaction conditions	16 cycles; A temp=55°C and E time=12 min

Table 2.14. Mutation 1 introduced into the GC-box (mGC1).

Template	pLI(1802)-luc, pLI(91)-luc, and pLI(55)-luc
Forward primer	GCMut1F 5′-ATGTTTGACTGGGATAGTGGGTGAAGGGAG-3′
Reverse primer	GCMut1R 5′-CTCCCTTCACCCACTATCCCAGTCAAACAT-3′
Reaction conditions	16 cycles; A temp=55°C and E time=12 min

Table 2.15: Mutation 2 introduced into the GC-box m(GC2).

Template	pLI(1802)-luc, pLI(91)-luc, and pLI(55)-luc
Forward primer	GCMut2F 5′-ATGTTTGACTGGGGGGATAGGTGAAGGGAG-3′
Reverse primer	GCMut2R 5′-CTCCCTTCACCTATCCCCCCAGTCAAACAT-3′
Reaction conditions	16 cycles; A temp=55°C and E time=12 min

Table 2.16: Mutation introduced into the Cdx2-a binding site (mCdx2-a).

Template	pLI(1802)-luc
Forward primer	mCdxA-F 5′-GACTCTCAGATATCAAAAAAAATGTTGTGG-3′
Reverse primer	mCdxA-R 5′-CCACAACATTTTTTTTGATATCTGAGAGTC-3′
Reaction conditions	18 cycles; A temp=55°C and E time=12 min

Table 2.17: Mutation introduced into the Cdx2-b binding site (mCdx2-b).

Template	pLI(1802)-luc
Forward primer	mCdxB-F 5′-TGTAATGATACCGCGCGCGATACCCAGTGA-3′
Reverse primer	mCdxB-F 5′-TCACTGGGTATCGCGCGCGGTATCATTACA-3′
Reaction conditions	18 cycles; A temp=55°C and E time=12 min

Table 2.18: Mutation introduced into the HNF-1 binding site (mHNF-1).

Template	pLI(1802)-luc
Forward primer	mHNF-1-F 5′-TAAAAATGTTGTGGTTCTGACTTGACTGGG-3′
Reverse primer	mHNF-1-R 5′-CCCAGTCAAGTCAGAACCACAACATTTTTA-3′
Reaction conditions	18 cycles; A temp=55°C and E time=12 min

Table 2.19: Deletion of the GAGA-element in the human LI-cadherin 5′-flanking region.

Template	pGL3-hLI Pro
Forward primer	hLIPro169/188F 5′-GGGAGGCAAAGCAGGGAAGA-3′
Reverse primer	hLIPro137/156R 5′-CAGCTTCAGTCAAACATTAA-3′
Reaction conditions	18 cycles; A temp=50°C and E time=12 min

Table 2.20: Deletion of region -86 to -58 in the human LI-cadherin 5′-flanking region.

Template	pGL3-hLI Pro
Forward primer	hLIPro-57/-38F 5′-AAAACTTTTTCCACTCTAAC-3′
Reverse primer	hLIPro-106/-87R 5′-GTTCATGACATCAAAGACTT-3′
Reaction conditions	18 cycles; A temp=50°C and E time=12 min

Table 2.21: Deletion of 181 bp in the human LI-cadherin promoter.

Template	pGL3-hLI Pro
Forward primer	hLI Del-3/168F 5′-CATCTACATTGAAAAGGGAGGCAAAGCAGGGAAGA-3′
Reverse primer	hLI Del-3/168R 5′-CCTGCTTTGCCTCCCTTTTCAATGTAGATGAGTCA-3′
Reaction conditions	18 cycles; A temp=65°C and E time=12 min

2.1.17 Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (EMSA) provides a method for detecting sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The assay was performed by incubating a purified protein or a complex mixture of proteins with a ^{32}P end-labeled DNA fragment containing the putative protein binding site. The reaction products were then analyzed on a non-denaturing polyacrylamide gel. Specificity of the DNA-binding protein for the putative binding site was established by competitive experiments using oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

^{32}P-labeling of oligonucleotides

Double-stranded 30-mer oligonucleotides covering the region within position -180 to +1 in the mouse LI-cadherin promoter were labeled with [^{32}P]-γ-ATP (3000 Ci/mmol, 10 mCi/ml). The synthetically produced complementary oligonucleotides were mixed and diluted to a concentration of 0.5 µM for each oligonucleotide. 6 µl of the oligonucleotide mixture, 7 µl dH_2O, 2 µl 10 x T4-kinase-forward buffer (A), 3 µl [^{32}P]-γ-ATP, and 2 µl T4-polynucleotide kinase were mixed and incubated at 37°C for 45 min. The reaction was inhibited by adding 2 µl 20 mM EDTA. Removal of unincorporated nucleotides from the DNA probe was performed by purification through a ProbeQuant^{TM} G-50 Micro Column (Amersham Pharmacia Biotech, Freiburg) as described by the manufacturer. 8 µl 3 M NaCl were added to stabilize the hybridizing of the double-stranded oligonucleotide. The activity of 1 µl of the labeled sample was diluted in 49 µl dH_2O and measured in a scintillation counter (measurement in P^{32}-canal; Cerenkov-counts; Wallac 1409). The cpm obtained represented the cpm/µl of purified labeled DNA. The two complementary oligonucleotides were then hybridized by first heating at 94°C for 10 min, followed by annealing at 60°C for 10 min and incubation at 37°C for 10 min. The labeled DNA probes were stored at –20°C.

Binding reaction

A master reaction mixture with the reagents listed in table 2.22 was prepared in a 1.5 ml Eppendorf tube without the nuclear extract and the probe, and splitted into the number of appropriate reaction tubes. The nuclear extract was thawed on ice and added to each tube. If a specific competitor was added, it was supplied before addition of the probe. For a super shift assay, the antibody was added before the probe as well, and incubated 30-45 min on ice. Finally, the probe was diluted in dH_2O to 50000 cpm/µl, added to the binding reaction, and incubated on ice for 30 min. The samples were loaded on a 4% nondenaturing polyacrylamide

gel (table 2.23) without loading buffer, since bromphenol blue disturb the protein-DNA binding. In a separate lane, loading buffer containing bromphenol blue was added to follow the run, and the gel was run at 4°C in 0.5% TBE buffer at 165 V for 2 h 50 min by a gel length of 16 cm.

All oligonucleotides used in EMSA are listed in table 2.24.

Table 2.22: Binding reaction.

Final concentration	**Stock solution**	**25 µl**
20 mM HEPES, pH 7.9	100 mM HEPES, pH 7.9	5 µl
4% Glycerol	50% Glycerol	2 µl
Poly[dI-dC] (2 µg/25 µl)*	PolydI-dC (0.5 µg/µl)	4 µl
1 mM $MgCl_2$	100 mM $MgCl_2$	0.25 µl
1 mM EDTA	100 mM EDTA	0.25 µl
0.5 mM DTT	100 mM DTT	0.125 µl
PMSF (0.1 mg/ml)	PMSF (10 mg/ml)	0.25 µl
Nuclear extract (5-10 µg/25 µl)	Nuclei extract (x µg/µl)	2 µl
Unlabeled oligonucleotide	Unlabeled oligonucleotide (10 µM)	2 µl
Labeled oligonucleotide (50000 cpm/25 µl)	Labeled oligonucleotide	1 µl
Antibody (1-2 µg/25 µl)	Antibody (1-2 µg/µl)	2 µl
26 mM KCl**	200 mM KCl	3.25 µl
dH_2O	dH_2O	2.875 µl

* poly[dI-dC] is a synthetic, double-stranded DNA without any specific sequence, which is added to bind sequence-unspecific DNA-binding proteins.

** The nuclear extracts contain 300 mM NaCl. While the total concentration of monovalent cations should not exceed 50 mM, not more than 4-5 µl of the protein preparation was added to each binding reaction. If the nuclear extract was concentrated higher, and less than 4 µl were added, the binding reaction was supplemented with 200 mM KCl to an end concentration of 50 mM monovalent cations.

Table 2.23: Formulation of gel (20 ml); gel size 110 x 90 x 0.75 mm.

Component	**Volume**
TBE 10 x buffer	2 ml
40% acrylamide:bisacrylamide (39:1)	2 ml
dH_2O	16 ml
TEMED	20 µl
10% APS	60 µl

The gel is placed on a sheet of Whatman® 3MM filter paper and covered with a plastic wrap and finally dried on a gel dryer. The gel is then exposed to an X-ray film overnight at –80°C.

Table 2.24: Oligonucleotides used by EMSA.

Oligonuclotide	**Sequence**
Pro180/150	5′-ACAACTCAGCAACCAGTTTGAAAGCATGTT-3′ 3′-TGTTGAGTCGTTGGTCAAACTTTCGTACAA-5′
Pro100/71	5′-ACCTTTTATGATACCCAGTGACTCTCAGAT-3′ 3′-TGGAAAATACTATGGGTCACTGAGAGTCTA-5′
Pro70/41	5′-ATCAATAAAAATGTTGTGGTTAATGTTTGA-3′ 3′-TCAAACATTAACCACAACATTTTTATTGAT-5′
Pro50/21	5′-TAATGTTTGACTGGGGGGGTGGGTGAAGGG-3′ 3′-ATTACAAACTGACCCCCCCACCCACTTCCC-5′
Exon3/21	5′-GGGAAGTTCAGCGGTCCCCTGAAGCCCATG-3′ 3′-CCCTTCAAGTCGCCAGGGGACTTCGGGTAC-5′
Consensus Cdx2	5′-ACCTTTTATGATACC-3′ 3′-TGGAAAATACTATGG-5′
Consensus HNF-1	5′-GGTTAATAATTAACG-3′ 3′-CCAATTATTAATTGC-5′
MutGC1	5′-ATGTTTGACTGGGATAGTGGGTGAAGGGAG-3′ 3′-TACAAACTGACCCTATCACCCACTTCCCTC-5′
mutCdxA	5′-ATCAAAAAAAAATGTTGTGGTTAATGTTTGA-3′ 3′-TAGTTTTTTTTTACAACACCAATTACAAACT-5′
mutCdxB	5′-ACCGCGCGCGATACCCAGTGACTCTCAGAT-3′ 3′-TGGCGCGCGCTATGGGTCACTGAGAGTCTA-5′
mutHNF-1	5′-ATCAATAAAAATGTTGTGGTGCTGATCCGA-3′ 3′-TAGTTATTTTTACAACACCACGACTAGGCT-5′
mutCCAAT	5′-ACCTTTTATGATACCATAAGACTCTCAGAT-3′ 3′-TGGAAAATACTATGGTATTCTGAGAGTCTA-5′
MutAP-1a	5′-TAATGTTTAAAAAGGGGGGTGGGTGAAGGG-3′ 3′-ATTACAAATTTTTCCCCCCACCCACTTCCC-5′
MutAP-1b	5′-ACCTTTTATGATACCCAGATCTGATCAGAT-3′ 3′-TGGAAAATACTATGGGTCTAGACTAGTCTA-5′

2.3 Methods: Cell biology

2.3.1 Cultivation of eukaryotic cells

The cell lines were cultivated in 100 mm cell culture dishes in an atmosphere of 5% CO_2 and 95% air at 37°C. The composition of the cell culture medium for the different cell lines is listed in table 2.25. Reaching 80-90% confluence, adherent cells were routinely sub-cultured. First, the cell sheet was rinsed with PBS, then a solution of 0.25% trypsin/0.02% EDTA was added at room temperature and removed after a short time. When the cells started to lose contact with the dish, 10 ml of the appropriate medium were added and the cells were dispersed through repeated pipetting.

Table 2.25: Composition of the cell culture medium for the different cell lines.

Cell line	Species	Cell type	FCS	Medium
STC-1	Mouse	Gut neuroendocrine cells	20%	DMEM
CMT93	Mouse	Colorectal carcinoma	10%	DMEM
Caco2	Human	Colorectal adenocarcinoma	10%	DMEM
HT29	Human	Colorectal adenocarcinoma	10%	DMEM
IEC-6	Rat	Normal small intestine	5%	DMEM + insulin 10 µg/ml
HepG2	Human	Hepatocellular carcinoma	10%	DMEM + insulin (1 µg/ml)
HuH7	Human	Hepatocellular carcinoma	10%	DMEM
293	Human	Normal embryonic kidney	10%	DMEM
CHO	Hamster	Ovary cells	10%	DMEM
A549	Human	Lung carcinoma	10%	DMEM
AGS	Human	Gastric adenocarcinoma	10%	DMEM
23132/87	Human	Gastric adenocarcinoma	10%	DMEM:HAM'S F-12
BON	Human	Pancreas; neuroendocrine	10%	RPMI
B16F0	Mouse	Skin melanoma	10%	RPMI

2.3.2 Transfection of DNA into cultured eukaryotic cells

In order to investigate promoter activity, cells were transiently transfected with different reporter gene constructs containing the promoter under analysis. The control plasmid pRL-SV40 was co-transfected to normalize the variation in transfection efficiency. The promoter-reporter gene construct and the pRL-SV40 were added in a ratio of 10:1 molecules regardless of the transfection method and the cell line used. The methods used for transfection of each cell line are summarized in table 2.26, as well as the cell numbers seeded per well. The optimal cell density was 70-80% confluence on the day of transfection.

Table 2.26: Transfection conditions used for the different cell line.

Cell line	Transfection method	Cell number/well	Culture dish
293	Calcium phosphate	3×10^5	6-well
CHO	Calcium phosphate	3×10^5	6-well
CMT93	DEAE-dextran	5×10^5	6-well
B16f(0)	DEAE-dextran	5×10^5	6-well
HT29	DEAE-dextran	5×10^5	24-well
IEC-6	DEAE-dextran	5×10^5	24-well
HepG2	DEAE-dextran	5×10^5	24-well
HuH7	Lipofectamine™ 2000 Reagent	1×10^5	24-well
BON	Lipofectamine™ 2000 Reagent	1×10^5	24-well
23132/87	Lipofectamine™ 2000 Reagent	1×10^5	24-well
AGS	Lipofectamine™ 2000 Reagent	1×10^5	24-well
A549	Lipofectamine™ 2000 Reagent	1×10^5	24-well
STC-1	DEAE-dextran Lipofectamine™ 2000 Reagent	1×10^6 1×10^5	6-well 24-well
Caco2	DEAE-dextran Lipofectamine™ 2000 Reagent	5×10^5 1×10^5	6-well 24-well

DEAE-dextran

Cultured cells were incubated in medium containing plasmid DNA and DEAE-dextran. The positively charged polymer DEAE-dextran forms complexes with negatively charged DNA molecules, enabling the DNA to bind to the cell surface. Chloroquine is included to optimize endocytic intake of DNA. The DNA-DEAE complex is delivered into the cell by osmotic shock using DMSO (Kawai and Nishizawa, 1984).

The appropriate number of cells (table 2.26) were seeded 24 h before transfection in a 6-well culture dish in 2 ml of their normal growth medium with antibiotic. $1x10^{11}$ molecules of the promoter-reporter gene plasmid and $1x10^{10}$ copies of the control plasmid pRL-SV40 were mixed with $PBS^{+/+}$ to a total volume of 142.5 µl. The cells were rinsed once with $PBS^{+/+}$ and then 7.5 µl DEAE-dextran (10 mg/ml) were added to the DNA-PBS mixture. The $PBS^{+/+}$ was removed from the cells, and the „transfection cocktail“ was added onto the cell monolayer. The cells were incubated for 30 min at 37°C/5% CO_2; and shaken every 10 min to avoid drying up. 1 ml growth medium containing 80 µM chloroquine was added and the cells were further incubated for 2.5 h at 37°C. The medium was removed and the cells were incubated with 0.5 ml medium containing 10% DMSO for 2.5 min. The DMSO-medium was completely removed and replaced with 2 ml regular medium. The cells were finally incubated at 37°C for 48 h.

Cationic lipid transfection reagent

The cationic lipid reagents function by spontaneous electrostatic interaction of their positive charges with the negative charges in the phosphate backbone of DNA, condensing the extended macromolecules to a compact structure. These aggregates are allowed to interact with and cross the negatively charged and hydrophobic cell membrane and enter the target

cells (Felgner et al., 1987). The LipofectAMINE 2000 (LF2000) reagent (Invitrogen, Groningen, Holland) was used in this study.

The day before transfection, the appropriate number of cells (table 2.26) were seeded in a 24-well culture dish in 0.5 ml growth medium containing serum and antibiotic so that they were 70-80% confluent on the day of transfection. For each well of cells to be transfected, $1x10^{11}$ molecules of the promoter-reporter gene plasmid and $1x10^{10}$ copies of the control plasmid pRL-SV40 were diluted into 50 µl of DMEM medium without serum and antibiotic. For each well, 2 µl of LF2000 Reagent were diluted into 50 µl DMEM-medium and incubated for 5 min at room temperature. The DNA solution was added to the diluted LF2000 Reagent and incubated at room temperature for 20 min to allow DNA-LF2000 reagent complexes to form. The DNA-LF2000 reagent complexes (100 µl) were added directly to each well and mixed gently by rocking the plate back and forth. Finally, the cells were incubated at 37°C for 48 h.

Calcium phosphate

The mixing of calcium chloride, DNA, and phosphate buffer precipitates small, insoluble particles of calcium phosphate containing condensed DNA (Graham et al., 1973). The calcium phosphate-DNA complexes adhere to cell membranes and enter the cytoplasm of the target cell through a yet undetermined mechanism. Glycerol or dimethyl sulfoxide shock increases the amount of DNA absorbed to some cell types.

The appropriate number of cells was seeded in a 6-well culture dish 24 h before transfection. $1x10^{12}$ molecules of the promoter reporter gene plasmid and $1x10^{11}$ copies of the control plasmid pRL-SV40 were added to 250 µl HBS (per dish). 16 µl of 2 M $CaCl_2$ were added, and the solution was mixed by vigorous shaking before incubating for 5-10 min at room temperature to form a fine co-precipitate of calcium phosphate and DNA. The precipitate was added directly to the medium on the culture dishes, and the dishes were incubated for 4-6 h at 37°C. The transfection medium was removed from the dishes and replaced with 2 ml normal growth medium before incubating for 48 h at 37°C.

2.3.3 Fluorescence microscopy

Cells transfected with an EGFP-reporter gene construct were incubated 48 h at 37°C and analyzed for GFP-expression with a fluorescence microscope. At a wavelength of 488 nm, cells expressing GFP showed a green fluorescence with an emission maximum at 507 nm.

2.3.4 Determination of promoter activity

In order to examine promoter activity, the Dual-Luciferase®Reporter Assay System kit (Promega, Mannheim) was used. The term „dual reporter“ refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. The „experimental“ reporter (firefly luciferase) is correlated with the effect of specific experimental conditions, while the activity of the co-transfected „control“ reporter (*Renilla* luciferase) provides an internal control which serves as the baseline response. Normalizing the activity of firefly luciferase to the activity of *Renilla* luciferase minimizes experimental variability caused by differences in cell viability or transfection efficiency. Firefly luciferase functions as a genetic reporter immediately upon translation. Photon emission is achieved

through oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+}, and O_2. The luminescent reaction catalyzed by *Renilla* luciferase utilizes O_2 and coelenterate-luciferin. Because of the dissimilar enzyme structures and substrate requirements for firefly and *Renilla* luciferase, it is possible to selectively discriminate between their respective bioluminescent reactions. The luminescence from the firefly luciferase reaction may be quenched while simultaneously activating the luminescent reaction of Renilla luciferase.

The activities of firefly (*Photinud pyralis*) and *Renilla (Renilla reniformis*) luciferase were measured sequentially from a single sample. A reporter gene plasmid containing firefly luciferase as reporter gene and the mLI- or the hLI-promoter was co-transfected with the control plasmid pRL-SV40 containing the SV40-promoter upstream of the coding region of *Renilla* luciferase. After 48 h the cells were rinsed once with PBS and lysed by adding 100 µl (6-well culture dishes) or 250 µl (24-well culture dishes) passive lysis buffer (PLB; Promega, Mannheim). After shaking the cells horizontally for 15 min at room temperature, 20 µl of the supernatant were transferred on a white 96-well plate. First, the firefly luciferase reporter was measured by adding 100 µl Luciferase Assay Reagent II (LAR II) to generate a signal. After measuring the firefly luminescence, 100 µl Stop & Glo® Reagent were added which simultaneously quenched the reaction and initiated the *Renilla* luciferase reaction. The measurements were performed automatically in the microplate luminometer EG&G Berthold MicrolumatPlus.

2.3.5 Indirect immunofluorescence of murine colon carcinoma cells

3-4 sterile cover slips were placed in a 100 mm culture dish, and STC-1 cells were cultured as described in section 2.3.1. When the cells were growing confluent, each cover slip was washed three times with $PBS^{+/+}$ and fixed for 20 min at room temperature in 500 µl PLP each. To saturate the fixative, each cover slip was subsequently washed three times with 500 µl $PBS^{+/+}$ /0.1 M glycine, followed by blocking in 500 µl $PBS^{+/+}$ and 1% (w/v) BSA/1% (v/v) FCS. Polyclonal rabbit serum (Angres et al., 2001) was used to detect murine LI-cadherin. Incubation with 400 µl primary antibody solution (diluted 1:500 in blocking solution) followed for 1-1.5 h at room temperature. Unbound primary antibodies were removed through subsequent washing (three times) with $PBS^{+/+}$/0.1% (v/v) Tween 20. The anti-mouse LI-cadherin rabbit-antibody was then detected by a secondary HRP-conjugated antibody (Alexa™ 488 goat anti-rabbit IgG conjugate, 2 mg/ml). The secondary antibody was diluted 1:1000 and incubated for 30 min at 37°C. Subsequently, the preparations were rinsed three times with $PBS^{+/+}$/0.1% (v/v) Tween 20 for 5 min and once with dH_2O. The cover slip was dried in a dark chamber. 30 µl of preheated elvanol were added on an object slide, and the cover slip with the cells face down was put on the elvanol. Conservation of the preparation followed in a dry chamber at 4°C overnight. In this form, the conserved preparation could be stored for several months at 4°C or –20°C. Detection of LI-cadherin followed using a fluorescence microscope with a 488 nm filter.

2.3.6 Preparation of nuclear extracts from cell cultures

Protein extracts were prepared from isolated nuclei from confluent cells. All solutions were kept on ice at all times and the centrifugation steps were carried out at 4°C. The cells were

washed with PBS and trypsinized as described (section 2.3.1). After centrifugation at 208 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 buffer A. After incubation on ice for 10 min a second centrifugation step at 208 g for 10 min was performed. The supernatant was discarded and the cell pellet was resuspended in three cell pellet volumes of buffer A. Nonidet P-40 was added to a final concentration of 0.05%. The sample was homogenized with twenty strokes of a tight-fitting Dounce homogenizer to release the nuclei. The nuclei were pelleted by centrifuging at 208 x g for 10 min and resuspended in 1 ml buffer C. The total volume was measured and 3 M NaCl was added to a final concentration of 300 mM. After centrifuging at 24000 x g for 20 min, the supernatant was allocated to twenty samples of 50 µl and snap-frozen in liquid nitrogen. The nuclear extract was stored at –80°C.

Buffer A:
10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl. 0.5 mM DTT, 0.5 mM PMSF

Buffer B:
5 mM HEPES, pH 7.9, 26% glycerol, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA,
0.5 mM DTT, 0.5 mM PMSF

2.4 Methods: Protein chemistry

2.4.1 Preparation of protein lysates from cell cultures

Protein samples were prepared from different murine and human intestinal cell lines. For sample preparation, the culture medium was decanted from the petri dishes, and the cells were rinsed with PBS and removed from the base of the petri dishes in 0.5 ml PBS by using a disposable cell scraper. The cells were homogenized by sonificating 3 x 5 sec and stored at –20°C.

2.4.2 Determination of protein concentration

Protein concentrations were determined according to the bicinchoninic acid (BCA) method (Smith et al., 1985). The BCA-protein-assay reagent (Pierce, Weisskirchen) was used in the reaction. A standard curve was prepared in sample buffer using several duplicate serial dilutions of bovine serum albumin (BSA) ranging from 0.2-1.4 mg/ml. Standard samples and appropriately diluted samples were added in duplicate into the wells of a 96-well microtiter plate (10 µl/well). 200 µl of the detection reagent were added to each well, and the samples were incubated at 37°C for 30 min. The absorbance of the samples was measured with an ELISA-reader at 570 nm. Sample protein concentrations were estimated from the standard curve.

2.4.3 SDS-polyacrylamide gel electrophoresis

Protein samples from lysed cells were electrophoresed on a denaturing SDS-polyacrylamide gel system under discontinuous conditions according to the method of Laemmli (Laemmli et al., 1970). The protein samples were dissolved in a 4 x concentrated Laemmli buffer and heated at 95°C for 5 min prior to separation on a 7.5% polyacrylamide gel (table 2.27). The

7B-marker ranging from 180 kD to 25.5 kD was used as a molecular weight standard. The gel was run in the Mini protean II chamber at 15 mA until the bromphenol blue tracking dye entered the separating gel. The current was then increased to 25-30 mA.

Table 2.27: Separating and stacking polyacrylamide gel.

Solution	**Stacking gel 3%**	**Separating gel 7.5%**
30% acrylamide/0,8% bisacrylamide	1.2 ml	3.45 ml
1.5 M Tris/HCl, pH 8.7	-	3.75 ml
1.5 M Tris/HCl, pH 6.8	625 µl	-
10% SDS	50 µl	50 µl
dH_2O	3.15 ml	2.7 ml
TEMED	12.5 µl	8.5 µl
10% APS	25 µl	35 µl

Laemmli sample buffer:
62.5 mM Tris/HCl (pH 6.8), 3% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10% (w/v) glycerine, 25 µg/ml bromphenol blue

Electrophoresis buffer:
0.3% (w/v) Tris base, 1.44% (w/v) glycine, 0.1% (w/v) SDS

2.4.4 Staining of protein gels

The polyacrylamide gel was placed in the staining solution and shaken horizontally for 30 min at room temperature. To eliminate background staining the gel was destained in a solution by shaking.

Staining solution:
50% (v/v) methanol, 10% acetic acid (v/v), 0.025% coomassie brilliant blue R250

Destaining solution:
10% (v/v) methanol, 10% (v/v) acetic acid

2.4.5 Western blot analysis

Western blot was used to detect a protein of interest by the indirect visualization of the protein by an enzyme-linked antibody reaction.

Cells were lysed and electrophoresed on a 7.5% sodium dodecyl sulphate (SDS) polyacrylamide gel. Prior to transferring the proteins to a nylon-transfer membrane, the gel was equilibrated in transfer buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 10% (v/v) methanol) for 5-15 min. The gel-membrane transfer sandwich was prepared as follows: A buffer-soaked fibre pad, three layers of buffer-soaked Whatman® 3MM filter paper, the equilibrated gel, the nylon-transfer membrane, three layers of buffer-soaked Whatman® 3MM filter paper, and a fibre pad on the top. The transfer took place in the mini-trans-blot apparatus for 2 h at 400 mA on ice.

Following the protein transfer, the membrane was rinsed in TBS-T (0.1% (v/v) Tween-20 in TBS) and incubated in blocking solution (5% (w/v) low-fat milk powder in TBS-T) for at least 1 h at room temperature. Incubation with the primary antibody was performed in 4 ml primary antibody solution in a heat-sealable plastic bag for 1 h at room temperature with agitation on an orbital shaker. The membrane was removed from the plastic bag and washed three times by agitating with TBS-T, 10 min each time. Incubation with the horse HRP-conjugated secondary antibody was performed for 1 h at room temperature. The membrane was again rinsed three times with TBS-T. To detect horse HRP-activity, the membrane was bathed in 4-8 ml ECL solution (according to size) for 1 min and then exposed to a Biomax-film.

2.5 Statistics

The *t* test was employed in all statistical analyses using the StatView software.