3 Materials and Methods

3.1 Tissue samples

Twelve paraffin-embedded colorectal carcinoma specimens were selected from tissue collections of the Institute of Pathology in the Benjamin Franklin Klinikum Steglitz, FU Berlin, Germany. Six were selected according to clear nonmucinous and six according to mucinous tumour phenotype.

3.1.1 Preparation of the sections for microdissection

Section of formalin-fixed and paraffin embedded tissue (5µm) were mounted for microdissection on a 1,3µm supporting membrane pre-treated with 0,1% Poly-L-Lysin for fixation of the tissue, deparaffinized in xylene and rehydrated in graded alcohols and water. After staining with hemalum the membrane with tissue was transferred in ethanol 100% to a 0,17mm thin object slide, fixed with nail polish and dried.

3.1.2 Microdissection

Microdissection was performed using the MOMeNT technique. Stained sections were examined under the microscope and areas of the possibly pure cell populations were selected. The selected group of cells was cut out with the laser beam until the small "bridge" connecting the selected cells with the section is left. After this the laser was adjusted to higher energy and one short impulse was made on the glass slide under the "bridge". By this procedure the bridge was destroyed and the piece of tissue was catapulted into an eppendorf tube cup moistured with mineral oil.

3.2 Cell lines

Cell lines LS174T, NCI-H498, Colo 205 and T84 were from ATCC, Rockville, MD, USA. 5583S is described in [94], CCO7 - [95], Troja 2 - [96].

3.3 Chemicals and products for molecular biology

All chemicals, if not mentioned particularly, were obtained from Merck (Darmstadt) and Sigma (Deisenhofen)

3.3.1 Buffers and mediums

TE 10mM Tris HCl pH 7.5; 1mM EDTA pH 8.0

TAE 40mM Tris-acetate; 1mM EDTA pH 8.0

TBE 40mM Tris-borate; 1mM EDTA pH 8.0

STE 0.1M NaCl; 10mM Tris HCl pH 8.0; 1mM EDTA pH 8.0

λ dilution buffer 10mM Tris HCl (pH 7.5); $MgSO_4$ 1mM

LB 1% tryptone; 0.5% yeast extract; 1% NaCl

6 x Loading buffer 30% glycerol; 0.25% bromphenol blue; 0.25% xylene cyanol; 10mM EDTA pH 8.0

Sequencing loading buffer 98% deionized formamide; 10mM EDTA pH 8.0; 0.025% bromphenol blue; 0.025% xylene cyanol

Radioactively labelled nucleotides

[α-^{23}P] dCTP (3000 Ci/mmol) (DuPont NEN, Bad Homburg)

[α-^{23}P] dTTP (3000 Ci/mmol) (DuPont NEN, Bad Homburg)

[α-^{23}P] dATP (3000 Ci/mmol) (DuPont NEN, Bad Homburg)

3.3.2 Kits

Geneclean II Kit	(Dianova, Hamburg)
QuiaEx II Gel extraction kit	(Quiagen, Hilden)
Quiagen Midi Plasmid kit	(Quiagen, Hilden)
Rapid Pure Minipreps kit	(Dianova, Hamburg)
TA cloning kit	(Invitrogen, De Schelp, Netherlands)
TOPO TA cloning kit	(Invitrogen, De Schelp, Netherlands)
MegaPrime DNA labelling kit	(Amersham, Braunschweig)
Sequenase 2.0 sequencing kit	(USB, Amersham, Braunschweig)
Nucleon DNA extraction kit	(Scotlabs, Coatbridge, Scotland)

3.3.3 Restriction enzymes

All restriction enzymes, if not mentioned particularly, were obtained from MBI Fermentas (St.Leon-Rot).

3.3.4 DNA modifying enzymes

DNase I, RNase free	(Boehringer, Mannheim)
MMLV Reverse transcriptase	(Gibco BRL, Karlsruhe)
RNase A	(Boehringer, Mannheim)
Proteinase K	(Boehringer, Mannheim)
Recombinant Taq polymerase	(MBI Fermentas, St.Leon-Rot)
T4- DNA Ligase	(MBI Fermentas, St.Leon-Rot)
Terminal transferase	(Boehringer, Mannheim)

Calf Intestinal Alkaline Phosphotase	(MBI Fermentas, St.Leon-Rot)
Klenow polymerase	(MBI Fermentas, St.Leon-Rot)
mHpaII methyltransferase	(MBI Fermentas, St.Leon-Rot)
mSssI methyltransferase	(NEB, Schwalbach)

3.3.5 Primers and oligonucleotides

All primers were synthesised in TibMolBiol (Berlin).

3.3.5.1 Primers for PCR

3.3.5.1.1 MUC2 cDNA

MUC2F15

5‘-CGA TAC CAC GGC CGC AAC GTC TG-3‘ (bases 115 to 137)

MUC2R13

5‘-GTA GAT GGT GTC ATC CTT GAT GGT C-3‘ (bases 339 to 315)

3.3.5.1.2 MUC2 promoter region

MUC2F23

5‘-CGC GGA TCC CAC ATC CTC CCT GCC CTC GG-3‘ (bases –497 to -469)

MUC2R23

5‘-ATG GAA GCT TGC AGG GGC GGT GTG GGT TGC G-3‘ (bases 29 to -2)

3.3.5.1.3 MUC2 first intron region

MUC2F30

5'-GGA CAT ATA AGG ACC AGA CC-3' (bases –34 to –15)

MUC2R30

5'-GGA CAA TGA CCC AGT TCA CT-3' (bases 405 to 387)

3.3.5.1.4 Bisulphite modified MUC2 promoter sequence

MUC2mF41

5’-ACC TCG AGC TAA CCC CAA AAC CCA CAC T-3’ (bases –425 to –406)

MUC2mF45

5‘- ATT AGA TTT GTT TTT GGT AGG ATA T-3‘ (bases -327 to -297)

MUC2mR45

5‘- ACA ACC CCA TAA TAA CTA ACA AAA-3‘(bases 37 to 14)

MUC2mF113

5’-ATT TAG GGA GTT ATA AAG AGA TGA T-3’ (bases -188 to -164)

MUC2mR113

5’-AAT CTA ATC CTT ATA TAT CCT AAC AAA-3’ (bases -41 to -15)

MUC2 F124

5’-TTG ATT ATT TGG TAG ATG TTA TAT TTA T-3’ (bases -105 to -78)

MUC2 R127

5’-ATA AAT ATA ACA TCT ACC AAA TAA TCA A-3’ (bases -78 to -105)

MUC2 R128

5’-ATC ATC TCT TTA TAA CTC CCT AAA T-3’ (bases -164 to -188)

3.3.5.1.5 DNMT2 methyltransferase (Acc. Nr.: AF045888)

DNMT2 F129

5’-CTC GAA GAG TTT GAC AGA TTA TC-3’ (bases 190 to 212)

DNMT2 R129

5'-CTA TGT AGC TTC CAT ATC CTT TG-3' (bases 910 to 888)

3.3.5.1.6 Maintenance Methyltransferase DNMT1 (Acc. Nr.: X63692)

MTase F131

5'-AGT CGA TGA TAA CAT CCC AG-3' (bases 2349 to 2368)

MTase R131

5'-CAG AAG ATC TCT TTG ATC CG-3' (bases 3239 to 3220)

3.3.5.1.7 p21waf (Acc. Nr.: U03106)

p21 F54

5'-TTA AAC ACC TCC TCA TGT ACA TAC-3'(bases 682 to 705)

p21 R54

5'- CAC TAA GAA TCA TTT ATT GAG CAC-3'(bases 2097 to 2074)

3.3.5.1.8 PDH (Acc. Nr.: D90086)

Forward 5'- GGT ATG GAT GAG GAG CTG GA - 3' (bases 4294 to 4313)

Reverse 5'- CTT CCA CAG CCC TCG ACT AA –3' (bases 4478 to 4459)

3.3.5.2 Standard sequencing primers

T3

3'- CGC TTA GAA CTA GTA GTG GAT C – 5'

T7

3'- CGG GAT ATC ACT CAG CAT AAT G-3'

M13

3'-TGA CCG GCA GCA AAA TG-5'

3.3.5.3 Primers for Single Nucleotide Primer Extension (SNuPE)

Primer name	Primer sequence	Melting temperature	Analysed site
B1F148	5‘-TTG GTA GGA TAT TTT TTT TT-3‘	45°C	B1
B2F149	5‘-TTT TTY GGT TAT TTT GGG TT-3‘	51°C	B2
B3F150	5‘-TAG TAG TTG TAT GTG TTT TT-3‘	40°C	B3
B4F151	5‘-TTT TAT TTT GAA GAA GGT TG-3‘	46°C	B4
B5F111	5‘-AGT TAT AAA GAG ATG ATT TT-3‘	39°C	B3
B6F125	5‘-AAT ATT TTT TTA TTG GGG TT-3‘	47°C	B6
B7F126	5‘-TTT ATT GGG GTT YGG GTT TT-3‘	55°C	B7
B8F112	5‘-AAG GAT TAG ATT TTT GTT TT-3‘	45°C	B8
B9F152	5‘-ATT AGA TTT TTG TTT TYG GG-3‘	48°C	B9
B10F153	5‘-TTT TYG GGY GTA ATT TAT AT-3‘	43°C	B10

Y=C/T

3.3.6 Polymerase chain reaction (PCR)

PCR was used for screening of the genomic library as well as for the analysis of the gene expression and generation of probes for hybridisation. Typical PCR was set up in the volume of 25µl and contained: 1x PCR buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 0.5 U of Taq polymerase and 1.5 mM $MgCl_2$. Template DNA was added at the

amount of 200 – 500 ng, cDNA was added at the volume of 1-0.2 µl of the typical cDNA synthesis reaction. PCR samples were overlayed with one or two drops of mineral oil.

3.3.6.1 PCR for MUC2 expression analysis

For the analysis of MUC2 expression by PCR, MUC2 sequences between 115 and 339 bases were amplified with primers MUC2F15, MUC2R13. The program for amplification was:

1 cycle	94°C	2 min
35 cycles	94°C 60°C 72°C	30 sec 1 min 1 min
1 cycle	72°C	5 min

3.3.6.2 MUC2 promoter region

MUC2 promoter region was amplified with primers MUC2F23 and MUC2R23 using 50 ng of λMUC2G as a template. The program was:

1 cycle	94°C	2 min
25 cycles	94°C 70°C	30 sec 2 min 30 sec
1 cycle	72°C	5 min

3.3.6.3 PCR of the first intron of MUC2

The first intron of MUC2 was amplified with primers MUC2F30 and MUC2R30, using λMUC2G as a template. The program was:

1 cycle	94°C	2 min
25 cycles	94°C 55°C 72°C	30 sec 1 min 2 min
1 cycle	72°C	5 min

3.3.6.4 PCR of bisulphite treated DNA isolated from cell lines

For the analysis of the methylation by bisulphite sequencing the fragment of MUC2 promoter was amplified in the seminested PCR, using bisulphite treated DNA as a template. The first step was performed with primers MUC2mF41 and MUC2mR45. The program was:

1 cycle	94°C	2 min
25 cycles	94°C 55°C 72°C	30 sec 1 min 1 min
1 cycle	72°C	5 min

The second step was performed with primers MUC2mF45 and MUC2mR45 with 1 µl of the first step reaction as a template. The program was:

1 cycle	94°C	2 min
35 cycles	94°C 50°C 72°C	30 sec 1 min 1 min
1 cycle	72°C	5 min

3.3.6.5 PCR of the bisulphite treated DNA from sections

To amplify bisulphite treated MUC2 promoter sequence from DNA from tissues 3 seminested PCR reactions were performed with the primers presented in the table 1:

Amplimer, containing sites:	I step primers	II step primers
B1-B4	MUC2mF45; MUC2mR127	MUC2mF45; MUC2mR128
B5-B7	MUC2mF113; MUC2mR45	MUC2mF113; MUC2mR113
B8-B10	MUC2mF113; MUC2mR45	MUC2mF124; MUC2mR45

The program for the first step amplification was:

1 cycle	94°C	2 min
5 cycles	94°C 51°C 72°C	30 sec 1 min 30 sec 1 min
35 cycles	94°C 46°C 72°C	30 sec 1 min 30 sec 1 min
1 cycle	72°C	5 min

The second step used 2 µl of the first step reaction as a template. The program was:

1 cycle	94°C	2 min
45 cycles	94°C 51°C 72°C	30 sec 1 min 1 min
1 cycle	72°C	5 min

3.3.6.6 PCR of DNMT2 methyltransferase

For the analysis of the expression of DNMT2 methyltransferase PCR with primers DNMT2 F129 and DNMT2 R129 was performed, using cDNA as a template. The program was:

1 cycle	94°C	2 min
35 cycles	94°C 60°C 72°C	30 sec 1 min 1 min 30 sec
1 cycle	72°C	5 min

3.3.6.7 PCR of DNMT1 methyltransferase

For the analysis of the DNMT1 methyltransferase PCR with primers MTase F131 and MTase R131 was performed, using cDNA as a template. The program was:

1 cycle	94°C	2 min
35 cycles	94°C 65°C 72°C	30 sec 1 min 1 min
1 cycle	72°C	5 min

3.3.6.8 PCR of bisulphite treated plasmid DNA

PCR of the pBluescript bisulphite treated DNA was performed with primers pBSmF101 and pBSmR101. The program was:

1 cycle	94°C	2 min
30 cycles	94°C 55°C 72°C	30 sec 1 min 1 min 30 sec
1 cycle	72°C	5 min

3.3.6.9 PCR for site directed mutagenesis in the promoter region

To introduce mutations in the MUC2 promoter sequence, a PCR splicing method was used. Overlapping fragments of the promoter were amplified with primers containing the mutation in the desired position and then used as a template for the amplification of the complete MUC2 promoter with primers MUC2F23 and MUC2R23.

3.3.7 Probes for Northern and Southern blots

3.3.7.1 Northern blot of MUC2

For analysis of the expression of MUC2 in Northern blot the probe from the repetitive region of MUC2 gene was used. DNA fragment of 600 bp was excised with EcoRI restriction enzyme from SMUC41 clone obtained from J.Gum [97], analysed on the gel and purified with QiaExII kit.

3.3.7.2 Southern blot of MUC2

For the analysis of MUC2 promoter methylation by Southern blot the probe was generated in PCR with primers MUC2F23 and MUC2R23. The probe for the first intron region of MUC2 was obtained in the PCR with the primers: MUC2F30 and MUC2R30. This amplimer (439 bp) was digested with Msp I and the longest obtained fragment (245 bp) was purified from the gel and used as a probe. Both probes were obtained with the

following program: denaturation 95°C 1 min, annealing and elongation 72°C 2.5 min, for 40 cycles.

3.3.7.3 Oligonucleotide of first 30 bp of MUC2 gene

Oligonucleotide of the first 30 bp of MUC2 cDNA was used for screening of genomic library and for determining the promoter containing fragments.

5'-CAT GGT GGC TGG CAG GGG CGG TGT GGG TTG-3'

3.3.7.4 18S rRNA

Oligonucleotide of 44bp of 18S rRNA was used for normalising the amount of RNA applied on the gel in Northern blot.

5'-ACG AAT GCC CCC GGC CGT CCC TCT TAA TCA TGG CCT CAG TTC CG-3'

3.3.7.5 p21 probe

Probe for the cyclin dependent kinase inhibitor p21 was generated in RT-PCR with primers p21 F54 and p21 R54. The program was: denaturing at 94° for 30 sec; annealing at 55°C for 1 min; extension at 72°C for 2 min. Resulting fragment of 1416 bp was analysed on the gel and purified with QiaExII Gel extraction kit.

3.3.7.6 Luciferase gene probe

The probe for determination of the transfection efficiency using dot blot hybridisation was obtained by excising of the luc gene out of pGL3basic plasmid with restriction enzymes HindIII and BamHI.

3.3.8 Plasmid vectors

pBluescript KS	(Stratagene)
pBluescript SK	(Stratagene)
pGL3basic	(Promega)

pGL3control (Promega)

pSVβ-gal (Promega)

pCRII (Invitrogen, De Schelp, Netherlands)

pCR TOPO II (Invitrogen, De Schelp, Netherlands)

3.3.9 Bacteria

TOP10F‘ (E.coli) (Invitrogen, De Schelp, Netherlands)

K802 (E.coli) (Clontech, Heidelberg)

3.3.10 Genomic library

Human genomic library in λEMBL3 SP6/T7 (Clontech, Heidelberg)

3.4 Methods of l phage analysis

3.4.1 Plating the phage

Lambda phage suspension (100µl) was mixed with 200µl of K802 bacterial overnight culture. After incubation for 15 min at 37°C in the water bath, 3 ml of molten LB agarose (0.7% agarose + LB + 10mM $MgSO_4$) at 47°C, were added. Obtained mixture was plated on the LB agarose plate (1.5% agarose + LB + 10mM $MgSO_4$) and dried for 10 min on the air. Plate was incubated overnight at 37°C

3.4.2 Titration of phage stock

Following dilutions of the λ phage stock were prepared: 1:10, 1:100; 1:1000; 1:10000 and 1:100000. One and 5 µl of obtained suspensions were mixed with 99 or 95µl of λ dilution buffer respectively. Phage was plated as described and obtained plaques counted. Titer = ($N_{plaques}$/volume) x dilution.

3.4.3 *PCR based screening of l DNA library*

λDNA library was screened using method described by Israel [98].

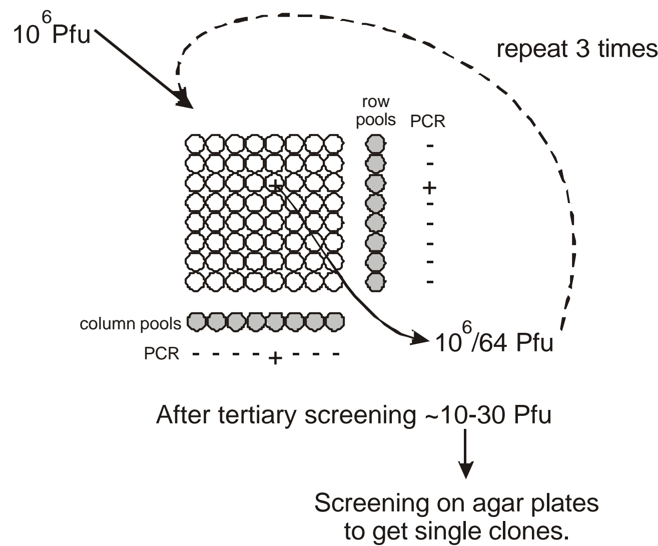
Minimal amount of phages necessary for obtaining the gene specific PCR product was determined in PCR.

N_{ti} - total number of phages, used for each step of the screening, i - step number (1, 2, 3)

N_{wi} - number of phages in one well for each step of the screening, i - step number (1, 2, 3).

I. Determination of the lowest number of plaque forming units (pfu) applicable:

- λ phage dilutions of 10^3, 10^4, 10^5 pfu/ml were prepared. One 1 and 5 μl of each



  dilution were used as a template for a PCR reaction to determine the minimal amount of phages required for obtaining the PCR product (N_{t1}).

II. Primary screening (Fig. 2)

Figure 2. *PCR based screening of genomic library*

- overnight culture of the bacteria host strain was prepared until OD_{600}=2.0.
- overnight culture (1 ml) was mixed with the equal volume of λ dilution buffer, containing $3xN_{t1}$ phages.

- After 15 min incubation in a water bath at 37°C, 18 ml of LB broth containing 10mM $MgSO_4$ was added.
- 6.4 ml of the suspension was distributed in 8x8 microtiter plate wells, 100 µl/well.
- plate was sealed with a sealing tape to prevent cross-contamination and incubated at 37°C for 5-6 hours, with shaking at 200-300 rpm.
- rows and columns were pooled by taking 20 µl from each well. Pooled phage suspensions were diluted with distilled water 1:1.
- PCR was performed on pooled phage suspensions, using 1 µl of each suspension as a template source.
- wells in the crossing of positive rows and columns were determined.
- to confirm positive wells PCR was performed with phages from individual wells.
- Phage titer was determined as described previously.
- **Note:** Sometimes it's useful to determine phage titer from the positive well, but usually titer are almost equal in all wells on the plate and is approximately 1-6x10^9 pfu/ml.
- phage dilution for the second step of the screening was: $N_{t2}=N_{w1}x2$
- **Note:** only during primary screening different positive wells on one plate may contain different phage clones. During secondary and tertiary screening all positive wells on one plate contain the same phage clones.

III. Secondary screening

- overnight culture (1 ml) was mixed with the equal volume of λ dilution buffer, containing 3xN_{t2} phages.
- After 15 min incubation in a water bath at 37°C 18 ml of LB broth containing 10mM $MgSO_4$ was added.
- 6.4 ml of the suspension was distributed in 8x8 microtiter plate wells, 100 µl/well.
- plate was sealed with a sealing tape to prevent cross-contamination and incubated at 37°C for 5-6 hours, with shaking at 200-300 rpm.

- rows and columns were pooled by taking 20 µl from each well. Pooled phage suspensions were diluted with distilled water 1:1.
- PCR was performed on pooled phage suspensions, using 1 µl of each suspension as a template source.
- wells in the crossing of positive rows and columns were determined.
- to determine positive wells PCR was performed with phages from individual wells.
- One positive well was selected and phage titer was determined as described previously.
- *optional:* nested PCR or hybridisation of PCR product was performed to ensure the correctness of the PCR product.
- phage dilution for the next step of the screening was: $N_{t3}=N_{w2}x2$

IV. Tertiary screening.

- overnight culture (1 ml) was mixed with the equal volume of λ dilution buffer, containing $3xN_{t3}$ phages.
- After 15 min incubation in a water bath at 37°C 18 ml of LB broth containing 10mM $MgSO_4$ was added.
- 6.4 ml of the suspension was distributed in 8x8 microtiter plate wells, 100 ml/well.
- plate was sealed with a sealing tape to prevent cross-contamination and incubated at 37°C for 5-6 hours, with shaking at 200-300 rpm.
- rows and columns were pooled by taking 20 µl from each well. Pooled phage suspensions were diluted with distilled water 1:1.
- PCR was performed on pooled phage suspensions, using 1 µl of each suspension as a template source.
- wells in the crossing of positive rows and columns were determined.
- to determine positive wells PCR was performed with phages from individual wells.
- One positive well was selected and phage titer was determined as described previously.

V. Screening on agar plate.

- 100 pfu was plated on 90 mm plate and incubated overnight at 37°C
- plaques were picked using Pasteur pipette (maximum twice more than was inoculated in single well)
- picked plaques were transferred to the Eppendorf tube, containing 200 µl l dilution buffer and a drop of chloroform, vortexed briefly.
- phage was eluted at 37°C with shaking for 4-6 hr.
- debris was removed by centrifuge at 10.000 x rpm for 2 min.
- positive plaques were determined by PCR.

3.4.4 High titer stock preparation

For preparation of the high titer stock 1000 to 10000 pfu were plated as described in 2.4.1 and incubated at 37°C until the complete confluence of plaques achieved. Three ml of λ dilution buffer was added on the plate and incubated 2h at RT with shaking at 100 rpm. Obtained phage suspension was transferred in the sterile tube, containing 200µl of chloroform. Titer was determined as described in 2.4.2. Obtained stock was stored at 4°C for up to 6 months.

3.4.5 Large scale phage DNA preparation

Preheated to 37°C LB medium (400 ml) was inoculated with 2 ml of overnight culture of the host bacterial strain K802 and incubated for 2 h at 37°C with shaking at 200 rpm. Obtained culture was infected with 10^{10} pfu of the phage and incubated at 37°C with shaking at 200rpm. After 4 hours incubation culture was checked for lysis every 15 min until the complete lysis achieved.

To purify the phage 24 g of solid NaCl was added to the obtained phage suspension, dissolved and incubated on ice for 1 h. Debris was removed by centrifugation at 11000 x g for 10 min at 4°C (13000 rpm in JA21 rotor). PEG 6000 was added to supernatant to final concentration of 10% and dissolved on the magnetic stirrer. Obtained solution was incubated overnight at 4°C. Precipitate was removed by centrifugation for 5 min at 11000

g at 4°C. To obtained supernatant 0.5 g of solid CsCl per 1 ml was added and dissolved. Obtained phage suspension was overlayed on the gradient of CsCl solutions: (2 ml 1.7 g/ml, 1 ml 1.5 g/ml, 1ml 1.45 g/ml) and centrifuged in swing out rotor 50000 x g for 2h at 4°C (22000 rpm in SW41 rotor). Phage particles were collected from the interphase between second and third steps of the gradient. Phage suspension was dialysed twice 1h against 1l of 10 mM NaCl, 50 mM Tris HCl pH8.0, 10 mM $MgCl_2$.

Phage DNA was extracted by adding to phage suspension EDTA pH 8.0 to final concentration of 20 mM, Proteinase K, final concentration 50 µg/ml and SDS to 0.5%. Mixture was incubated 1 h at 56°C and extracted twice with equal volume of phenol:chlorophorm:isoamyl alcohol 25:24:1. After additional extraction with chloroform, sodium acetate was added to 0.3 M and DNA was precipitated by adding of 2 volumes of 100% ethanol. DNA was taken out using a Pasteur pipette, washed in 1ml of 70% ethanol, dried on the air for 15 min and dissolved in 50-100 µl of TE buffer pH 8.0. Concentration was determined spectrophotometrically.

3.5 Plasmids preparations

3.5.1 Transformation of E.coli

Vial of competent cells TOP10F' (Invitrogen) was thawed on ice and 2 µl of 0.5 M β-mercaptoethanol was added and mixed gently. The ligation mixture (2-5 µl) or supercoiled plasmid (10-20 ng) was added to competent cells, mixed gently and incubated on ice for 30 min. After incubation, heat shock was done at 42°C for 30 sec without shaking and vial was again transferred on ice for 2 min. To cooled cells 250 µl of SOC medium was added and vials were incubated for 30 min at 37°C with shaking to develop the antibiotic resistance. Bacteria were plated on agar plates with appropriate antibiotic and grown overnight.

3.5.2 Minipreps

For minipreparation of plasmid DNA 5 ml of LB medium with appropriate antibiotic in 13 ml tube were inoculated with a single colony obtained on the plate after transformation.

Bacteria were propagated overnight at 37°C with shacking at 150 to 200 rpm.

Minipreps were isolated from 1.5 ml of overnight bacterial culture with RPM kit (Bio101) according to the protocol of the manufacturer. For the restriction analysis 5 µl of the obtained plasmid solution was used.

3.5.3 Midipreps

For isolation of big amounts of plasmid DNA 100 ml of LB medium with appropriate antibiotic in 500 ml flask were inoculated with 100 µl of overnight culture obtained in 2.5.2.

Bacteria were propagated overnight at 37°C with shacking at 150 to 200 rpm.

Plasmid DNA was isolated with Qiagen MIDI kit according to the protocol of the manufacturer. Obtained DNA pellet was dissolved in 100 µl of H_2O and concentration was determined spectrophotometrically. For the plasmid used for transfection the last washing step was performed under sterile conditions.

3.5.4 Isolation of DNA fragments from the agarose gel

DNA fragments were isolated from the agarose gel with GeneCleanII or QiaexII kit according to the recommendations of the manufacturer.

3.5.5 Cloning of the DNA fragment into the plasmid vector

Plasmid vector was prepared for ligation by digestion with appropriate restriction enzyme and dephosphorylated with Calf Intestinal Alkaline Phosphotase (CIAP) to prevent the religation of the vector. Obtained DNA was purified from the agarose with QiaexII kit.

Fragment for insertion was prepared by digestion with appropriate restriction enzyme and purified from the agarose with QiaexII kit.

Purified vector and insert were analysed on the same agarose gel to estimate the DNA concentration.

For ligation reaction 200 ng of the vector DNA and equimolar amount of the insert were used. Reaction was carried out in 10 µl reaction mixture, containing 1x T4 DNA ligase buffer and 10 U of T4 DNA ligase. After 1 h incubation at room temperature reaction was stopped by incubation at 65°C for 10 min. Obtained ligation reaction was used directly for transformation of competent *E.coli*.

3.5.6 Cloning of the PCR product in TOPO TA Cloning vector

For cloning of the PCR product obtained after amplification with Taq polymerase TOPO TA cloning system was used. Two µl of typical PCR sample were mixed with 2 µl of sterile water and 1 µl of pCR®-TOPO II vector.

After 5 min incubation at RT tube was transferred on ice and 2 µl of the ligation mixture were used for transformation of *E.coli* according to 2.5.1.

3.6 Analysis of genes expression

3.6.1 Total RNA isolation

Total RNA was isolated from cultured cells with RNAclean (AGS GmbH) according to recommendations of the manufacturer.

3.6.2 Northern blot

3.6.2.1 Preparation of the gel

For Northern blot total RNA (25 µg) and 5 µg of RNA marker (Gibco, #15620-016) were denatured in the following reaction for 1 h at 50° C:

5.4 µl	RNA (25 µg total RNA)
5.4 µl	40% Glyoxal (deionized, pH 5.0)
16 µl	DMSO
3 µl	0.1 M Sodium phosphate buffer, pH 7.0

After incubation, the samples were placed on ice and 4 µl of loading buffer were added. Gel electrophoresis was performed in 1% agarose gel at 3-4 V/cm for 3-4 h. The gel was then photographed on the UV transilluminator together with a ruler.

3.6.2.2 Transferring the RNA on the membrane

RNA was blotted using the capillary blotting method with 20xSSC on the Nylon membrane (NY 13 N, Nytran, Schleicher & Schuell, #414596).

3.6.2.3 Labelling of the probe

Labelling of the probe for Northern and Southern hybridisation was performed with MegaPrime DNA labelling kit (Amersham) according to the recommendations of the manufacturer. Obtained probe was purified from nonincorporated nucleotides using gel filtration chromatography in Sephadex G50.

3.6.2.4 Hybridisation of the membrane

Hybridisation of the membrane was carried out in QuickHyb solution (Stratagene).

Membrane was placed in glass tube and prehybridised in 10-15 ml of QuickHyb solution for 15 min at 68°C in rotating oven. After prehybridisation labelled probe was added ($2x10^6$ cpm/ml). Hybridisation was carried out for 1 to 3 h at 68°C with rotation. Hybridised membrane was washed in following solutions:

2xSSC; 0.1% SDS	15 min	65°C
0.2xSSC; 0.1% SDS	15 min	65°C
0.1xSSC; 0.1% SDS	15 min	65°C

During the last washing step the radioactivity on the membrane was checked every 5 minutes until the background became sufficiently low. The membrane was wrapped in SaranWrap film and exposed with Kodak BioMax MR film at –70°C.

3.6.3 RT-PCR

3.6.3.1 cDNA synthesis

To eliminate possible contamination with genomic DNA, RNA samples were digested with DNase before cDNA synthesis. Total RNA (20 µg) was incubated with 200 U of RNase free DNase I in following buffer conditions: 50 mM Tris-HCl, pH 6.5, 10 mM $MgCl_2$, 10 mM DTT in 50 µl reaction buffer in presence of 20 U of RNase inhibitor. Digestion was carried out for 2 h at 37°C. After the reaction DNase was inactivated by incubation at 100°C for 10 min. To DNase digested RNA sample (6.25 µl) 2.5 µl of random hexanucleotide primers were added. Volume was adjusted to 22 µl with DEPC-H_20 and primer annealing reaction was carried out for 10 min at 70° C.

During the incubation, the first strand mixture was prepared:

5x reverse transcriptase buffer	10 µl
dNTP mix (2.5 mM each)	10 µl
0.1 M DTT	5 µl
RNase inhibitor	0.5 µl
Superscript Reverse transcriptase (500U)	2.5 µl
Total volume	28 µl

For control of DNase digestion RNA sample a cDNA synthesis reaction without reverse transcriptase was prepared. After primer annealing, the probe was incubated on ice and the first stand mixture added (28 µl, final volume 50 µl). cDNA synthesis was performed for 1 h at 37° C. The reaction was stopped by incubation for 10 min at 100°C.

3.7 Analysis of DNA methylation

3.7.1 Southern blot

Genomic DNA was isolated from cultured cells with Nucleon kit (Scotlabs) according to recommendations of the manufacturer. DNA (20 µg) was digested with 160 U of BamHI, then subsequently with 80 U of HpaII or MspI and separated on 1.2% agarose gel at 4 V/cm. Gel was incubated for 10 min in 0.5 M HCl, denatured for 40 min in 0.5M NaOH, 1.5M NaCl and neutralised for 40 min in 0.5M Tris HCl pH 7.5, 1.5M NaCl. DNA was capillary blotted on the GeneScreen+ membrane as described in 2.6.2.2. DNA was fixed on the membrane by incubating for 2 h at 80°C and hybridised with appropriate probe as described in 2.6.2.4.

3.7.2 *Analysis of methylation by bisulphite sequencing*

Methylation analysis by bisulphite sequencing was performed according to Olek et al [99].

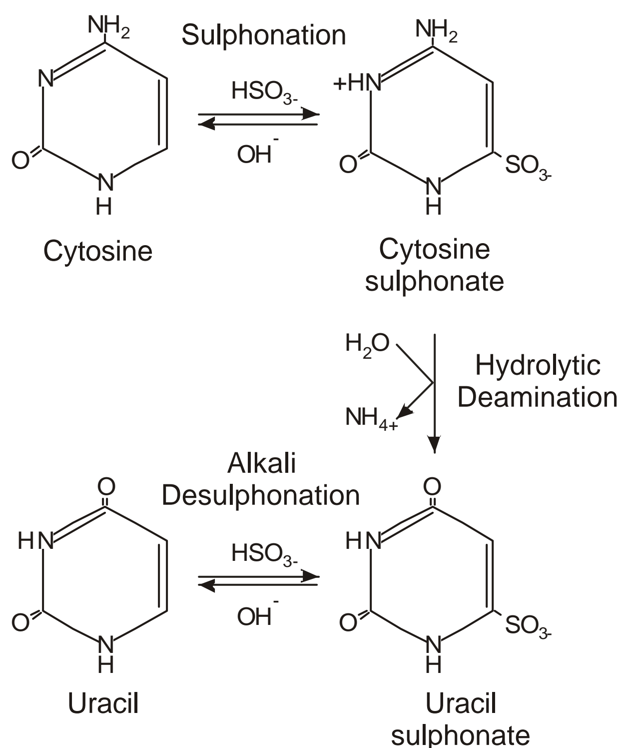


***Figure 3**. Conversion of C to U by bisulphite treatment*

Genomic DNA (50 to 200 ng) was digested with EcoRI. Reaction was stopped by boiling for 5 min. DNA was denatured in 0.3 N NaOH at 37°C for 15 min. Agarose beads were formed by pipetting of 2x volume of 2% low melting agarose in the DNA solution and pipetting of 10 µl aliquots of DNA/agarose mixture in cold mineral oil. Treatment was carried on in 5 M Na_2SO_4 solution for 4 h at 50°C. After treatment agarose beads were washed with TE buffer pH8.0 6 times for 15 min. Modification was completed by incubation of the agarose beads 2 times 15 min in 0.2 N NaOH (Fig.3). After this beads were washed 3 times for 15 min in double distilled H_2O (ddH_2O). One µl of the obtained DNA was used for PCR.

3.7.3 *Analysis of methylation by SNuPE*

Bisulphite-treated DNA was amplified in semi nested PCR as described in 2.3.6.5. Obtained amplimer was purified with GeneClean. Single nucleotide primer extension was

performed in 1x PCR buffer, containing 1pM of primer corresponding to the site of analysis according to 2.3.5.3 and 1µCi of dCTP or dTTP.

PCR products were analysed on agarose gel and specific product was excised. After purification of amplimers with QiaEx II kit 2 µl were analysed on the gel. Only well purified good visible samples were used for the further analysis.

Master mix was prepared as following:

Primer	2µl (20 pM)
10xPCR buffer with 15mM Mg^{++}	2 µl
Amplimer	2 µl
Water	10 µl
Total	18 µl

Master mix was distributed in 2 eppendorf tubes (8µl in each) and stored on ice, tubes were labelled "T" and "C". Dilution of Taq polymerase 0.5 U/µl (2.1µl for each tube) in PCR buffer was prepared and stored on ice.

Diluted Taq polymerase was split in 2 eppendorf tubes and 0.5 µCi of dCTP was pipetted in one. Two µl of the obtained mixture was pipetted in the tube labelled "C". The same was repeated for the "T" sample, using dTTP instead of dCTP. dTTP and dCTP of the same specific activity were used. Tubes were loaded in the thermocycler and the following program run: denaturing 95°C, 1 min; annealing at the Tm of the primer (from the list in 2.3.5.3), 2 min; extension 72°C, 2 min. Then samples were transferred on ice and 4µl of sequencing loading buffer was added to each sample (Fig. 4).

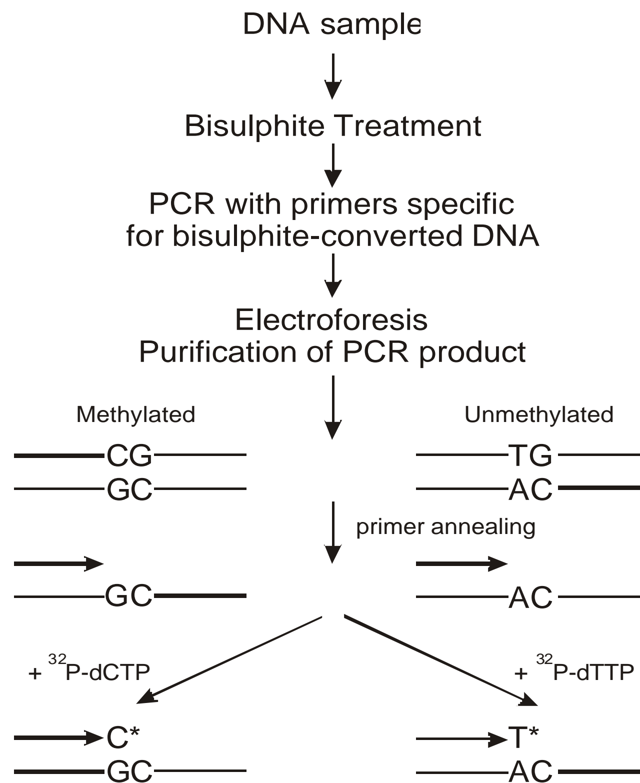


Figure 4. SNuPE procedure

Samples were denatured for 2 min at 95°C and loaded on preliminary prerun 10% acrylamide gel containing 1xTBE and 7 M urea. Electrophoresis was performed at 40-45 mW/cm^2 for 30-40 min. The gel was dried on the vacuum dryer (BioRad) for 2 h at 80°C. Autoradiography was performed in Kodak X-Omat cassette with intensifying screens for 10 min to 2 h at room temperature.

Autoradiogramms were analysed, aligned to the gel and pieces of the gel corresponding to the bands were excised. The amount of radioactivity incorporated was measured in the scintillation counter (Wallac). The percentage of methylation was determined as a ratio of the sample C activity (as c.p.m.) to the total (C+T) incorporation (as c.p.m.).

3.7.4 *Inhibition of methylation with 5-aza-2'-deoxycytidine*

Inhibition of methylation was done by treatment of cells with 5-aza-2'-deoxycitidine. The treatment was done with 5 µM concentration of the drug for 24 h, and then cells were grown in non supplemented medium for 48h. Samples were taken every 24 h until the expression become detectable.

3.8 Reporter analysis of the promoter activity

3.8.1 *In vitro methylation*

For *in vitro* methylation 5µg of the plasmid of DNA was mixed with 20U of mHpaII or mSssI in the presence of S-adenosylmethionine at the concentrations of 80 µM and 160 µM respectively. Reaction was carried out at 37°C overnight and was followed by purification of the DNA with GeneClean II kit in sterile conditions.

3.8.2 *Transfection of cells*

Transfection was performed with Lipofectin reagent (GIBCO/BRL) according to the recommendations of the manufacturer. In each experiment pSVβ-gal vector (Promega) was contransfected to allow assessment of transfection efficiency. Cells transfected in parallel with pGL3promoter vector (Promega) were used as a positive control. The data were normalised to β-galactosidase activity determined in parallel as a measure of transfection efficiency in each experiment.

3.8.3 *Luciferase and **b**-galactosidase assays*

Luciferase Assay System with reporter lysis buffer (Promega) and Galacto-Light chemiluminescent reporter assay (Tropix, Bedford, MA, USA) were used to determine the reporter activity using Lumat LB 9501 luminometer (Berthold, Wildbad, Germany), according to the recommendations of the manufacturer.

3.8.4 Normalising the transfection with DOT blot hybridisation

For normalising the transfection efficiency ½ volume of the cell suspension obtained after harvesting of the transfected cells was treated with proteinase K at the concentration of 0.4 µg/µl 2-12 h at 56°C. To the obtained mixture 200 µl of phenol/chloroform 1/1 mixture was added, vortexed for 1 min and centrifuged for 1 min at 13000 rpm. Aqueous phase was transferred into a new tube and extracted for the second time with 200 µl of chloroform. To 40 µl of the obtained lysate 160 µl of 0.5N NaOH was added. After denaturing for 10 min at 80°C samples were applied on the membrane using slot blotting instrument (BioRad). The obtained membrane was neutralised in 2xSSC, dried on air and backed 1 h at 80°C. Membrane was hybridised with luciferase or β-galactosidase probe as described.