# 4 Results

## 4.1 Isolation and characterisation of the MUC2 promoter

### *4.1.1 MUC2 5' region containing lambda clone*

For the analysis of the regulation of *MUC2* expression the regulatory sequences were recovered from the genomic library. Lambda Zap human genomic library (Clontech) was screened with PCR based method, using primers MUC2F15 and MUC2R13 which amplified 225bp fragment from the second exon of *MUC2*. Each round of screening results in 64 fold enrichment of the phage population with the clone which contained the second exon of *MUC2* and, presumably the promoter region. After 3 sequential rounds of PCR screening, obtained phage population was screened in the usual way on the agar plate. Several positive plaques were picked up, tested in PCR and used for the preparation of the high titer stock. One clone, containing *MUC2* sequence, named λMUC2G was selected for the further analysis.

Mapping of the obtained λMUC2G clone was done by restriction analysis with the following hybridisation. λMUC2G DNA was digested with following combinations of enzymes: EcoRI, BamHI, XhoI, EcoRI + BamHI, EcoRI + XhoI and BamHI + XhoI. Obtained digests were separated on 0.8% agarose (Fig. 5 A, C) and analysed by Southern blot with probes: 1-30 bp (Fig. 5 B) and 115-339 bp (amplimer obtained with primers MUC2F15 and MUC2R13) (Fig. 5 D). Hybridisation with the probe 1-30 bp revealed the fragment of about 900 bp in the case of digestion with BamHI, BamHI + EcoRI and BamHI + XhoI (Fig 5 B, Lanes 3, 4, 5), indicating that there are no restriction sites for

EcoRI and XhoI within 900 bp fragment flanked with BamHI sites. Digestion with EcoRI + XhoI and XhoI yielded fragments of 5 kb in size (Fig. 5 B, Lanes 2, 6), indicating that above mentioned BamHI fragment is located within 5 kb fragment flanked with XhoI restriction sites. Digestion with EcoRI only yielded the fragment of more than 15 kb (Fig 5 B, Lane 1). Hybridisation with the probe 115-339 bp showed the same fragments for XhoI and EcoRI digestion (Fig 5 D, Lanes 1, 6). Digestions with BamHI, BamHI + EcoRI, BamHI + XhoI and EcoRI + XhoI revealed fragments of 3.2 kb, 3.1 kb, 2.9 kb and 2.8 kb respectively (Fig. 5 D, Lanes 2, 3, 4, 5).

Taken all together data of restriction mapping and hybridisation lead to presented on the Figure 6 structure of the clone. λMUC2G clone contains 15 kb insert cloned between two XhoI sites in λEMBL3 vector. It contains about 5 kb of the promoter region of MUC2 gene. The analysed region of the transcribed sequences comprises first 3 exons with the sizes of about 100 bp, 600 bp and 200 bp. Exons were separated with introns with sizes of about 800 bp and 2.5 kb.

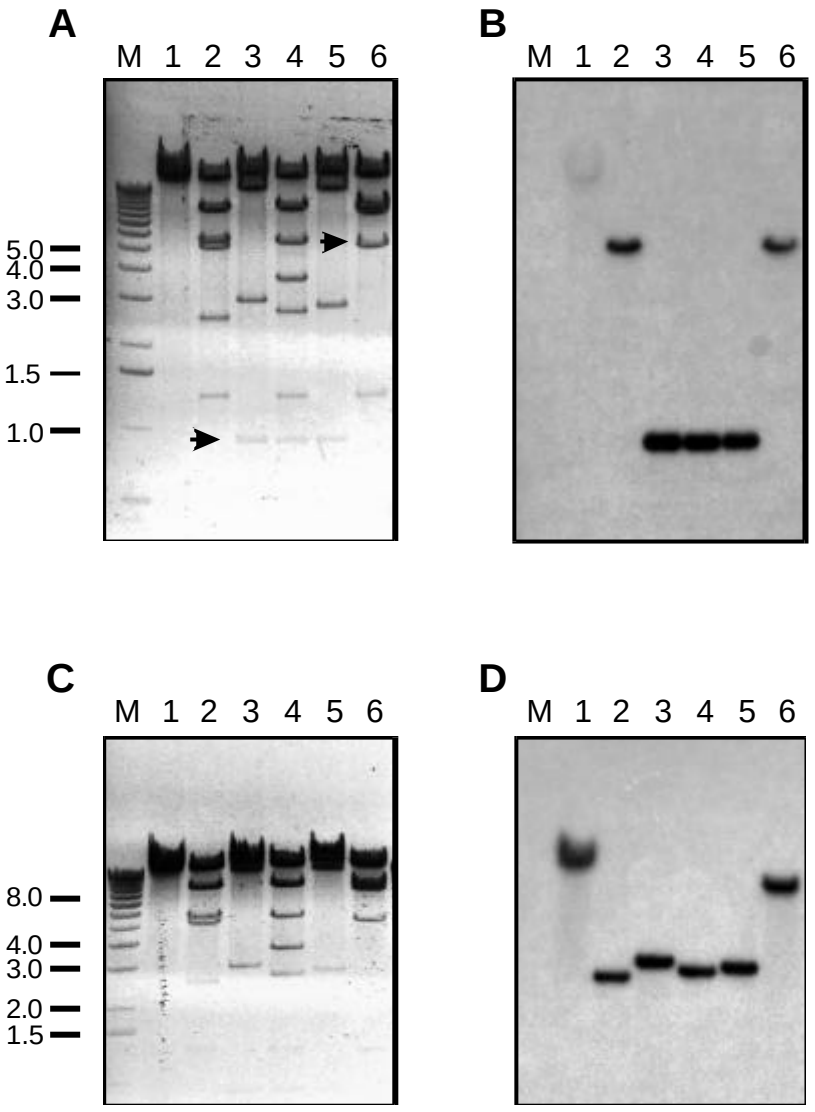


***Figure 5.*** *Restriction analysis of the λMUC2G clone. A: Ethidium bromide staining of the gel with separated fragments of λMUC2G clone digestion. B: Hybridisation of the membrane obtained after blotting of gel A with the probe 1-30 bp. C: Ethidium bromide staining of the gel with separated fragments of λMUC2G clone digestion. D: Hybridisation of the membrane obtained after blotting of gel A with the probe 115-339 bp. 1 – digestion with EcoRI, 2 – digestion with EcoRI and BamHI, 3 – digestion with BamHI, 4 – digestion with BamHI and XhoI, 5 – digestion with BamHI and EcoRI, 6 – digestion with XhoI.*

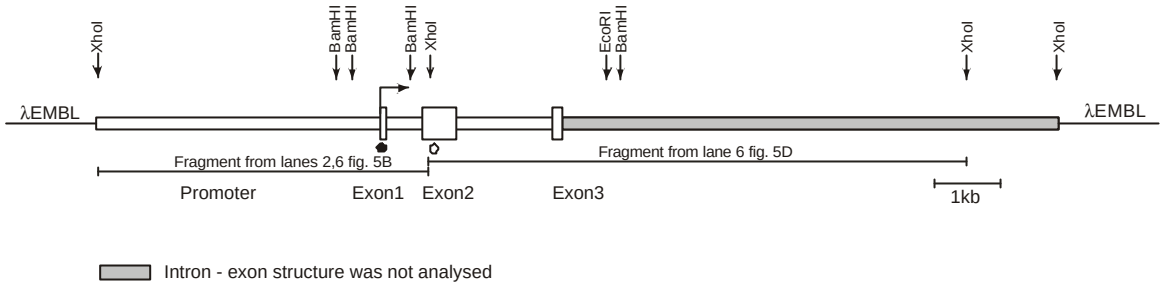


***Figure 6.*** *Structure of λMUC2G clone. Filled circle indicates the position of the probe used for hybridisation in figure 5B and opened circle indicates the position of the probe used for hybridisation in figure 5D.*

For further analysis of MUC2 regulatory sequences the resources of German Human Genome project were used. Basing on the fact that MUC2 gene is located on the chromosome 11 [100], the genomic library L4/FS11 specific for this chromosome was ordered. The library was supplied by the Resource Centre (Berlin, Germany) in the form of high density filter array, containing approximately 36864 independent clones. The obtained filter array was hybridised with the 900 bp fragment, containing the part of *MUC2* promoter and the first exon of the gene. Hybridisation revealed two strongly positive clones and several clones which were hybridised with much lower efficiency (Fig. 7A). Two strongest hybridised clones were ordered from the Resource Centre. The obtained clones were: ICRFc107D0463D1 and ICRFc107F0952D1. Analysis of the clones was done by restriction digestion with following hybridisation with MUC2 promoter probe. Cosmid DNA was isolated using the protocol for plasmid isolation. Obtained DNA (1µg) was digested with XhoI (Fig 7B, Lanes 1, 2), EcoRI (Fig 7B, Lanes 3, 4) and BamHI (Fig 7B, Lanes 5, 6) restriction enzymes. Digestion showed that the total

size of either insert is more than 50 kb. Therefore for the identification of MUC2 promoter containing fragment Southern blot hybridisation was used. Gel was blotted on the Nylon membrane and hybridised with MUC2 promoter probe. Hybridisation revealed 900 bp fragments after BamHI digestion in both clones (Fig 7C, Lanes 5, 6), and fragments with the size more then 10 kb after digestion with EcoRI or XhoI (Fig 7C, Lanes 1, 2, 3, 4). Since the data of λMUC2G analysis, indicated that there is a XhoI restriction site 1 kb upstream the transcription start, it can be concluded that the obtained clones contain at least 9 kb of the promoter region of MUC2 gene. However all further analysis was focused on the first 500 bp of the promoter region which contains all essential promoter elements according to the report of Gum et al [101].

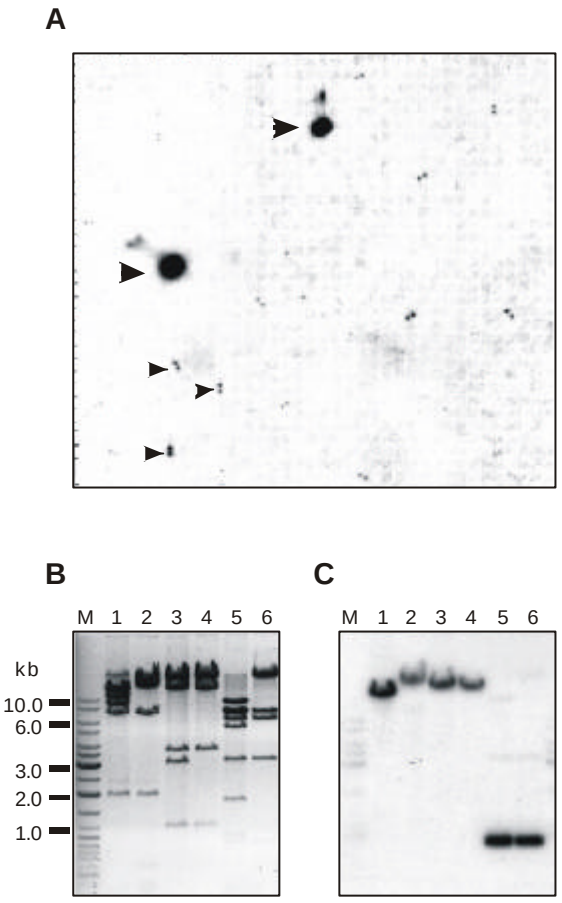


**Figure 7.** Screening of the library on the high density filter and analysis of the obtained clones. A: A fragment of high density filter, containing L4/FS11 library, hybridised with 900 bp MUC2 probe. Big arrowheads indicate true positive clones, small arrowheads indicate several false positive clones. B: Ethidium bromide staining of the gel with separated fragments of the obtained clones: 1 - XhoI digestion of ICRFc107D0463D1 clone, 2 – XhoI digestion of ICRFc107F0952D1 clone, 3 – EcoRI digestion of ICRFc107D0463D1 clone, 4 – EcoRI digestion of ICRFc107F0952D1 clone, 5 – BamHI digestion of ICRFc107D0463D1 clone, 6 – BamHI digestion of ICRFc107F0952D1 clone. C: Hybridisation of the filter obtained after blotting of gel, with MUC2 900 bp probe.

*4.1.2 Sequencing and analysis of the promoter region*

For detailed analysis and sequencing two fragments of λMUC2G insert were subcloned. λMUC2G was digested with BamHI and 900 bp fragment was subcloned in BamHI site in pBluescript KS vector and named pBSMUC2.1 (Fig 8), it contained about 500 bp of the promoter region sequence. Second fragment of 5 kb was cut out and cloned in pBluescript KS using XhoI enzyme, the obtained plasmid was named pBSMUC2.2 (Fig 8)**,** it contained about 4.5 kb of the promoter region sequence.

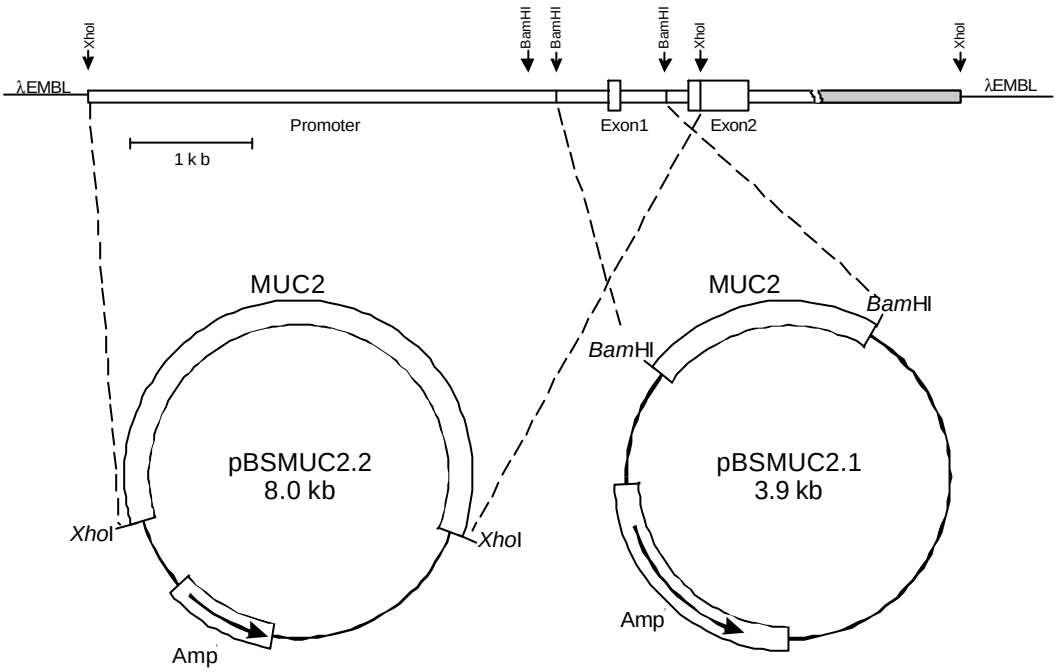


***Figure 8.*** *Subcloning of MUC2 promoter sequences in pBluescript vector. pBSMUC2.2: MUC2 DNA fragment was cut out of λMUC2G with XhoI restriction enzyme and was cloned within the same site in pBluescript SK vector; insert size is about 5 kb. pBSMUC2.1: MUC2 DNA fragment was cut out with BamHI and cloned within the same site in pBluescript SK vector; insert size is about 900 bp.*

The insert in pBSMUC2.1 was sequenced completely using dyedideoxynucleotide cycle sequencing procedure. Sequencing was performed at least two times in both directions. Obtained sequences were assembled in one contig using SEQ program group on the GENIUS server in EMBL (genius.embnet.embl-heidelberg.de) (Fig. 9) and produced a 930 bp sequence (Fig. 10).

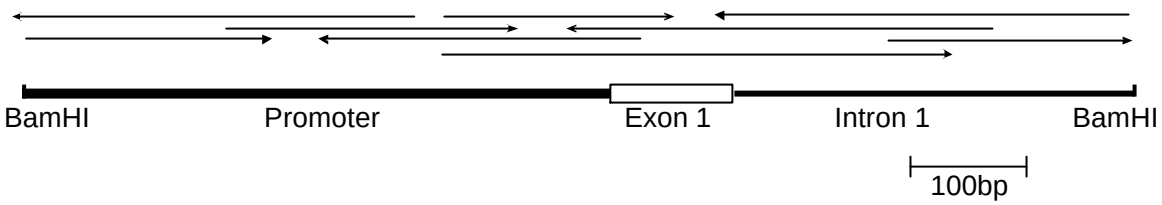


***Figure 9**. Sequencing of the pBSMUC2.1 clone*

Alignment of the obtained sequence with known cDNA sequence of MUC2 gene (Acc # L21998) showed that first exon is 103 bp long and the nucleotide 1 of cDNA corresponds to nucleotide 498 of the obtained sequence. At 3' end of the first exon standard intron donor junction sequence AGGTGAGA was found. In the cDNA sequence the intron junction sequence AGG was found in the position 102 - 104. Obtained promoter sequence showed 100% homology to simultaneously published sequences of *MUC2* promoter (Acc. # U67167 [101] and U68061 [102]). Computer analysis of the obtained sequence with the program MOTIF FINDER on GENIUS server revealed the presence of all typical promoter elements: TATA box (-29 - -24), CAAT element (-147 – 144), binding site for Sp1 (-349 –344), AP2 (-71 –64; -297 -290) and AP1 (-324 –321) as well as CACCC box described by Gum et al [101] (Fig. 10). Plasmid pBSMUC2.2 was

sequenced partially and revealed (ATCC)n microsatellite at the 5' end of the insert, as described also by Velcich et al [102].

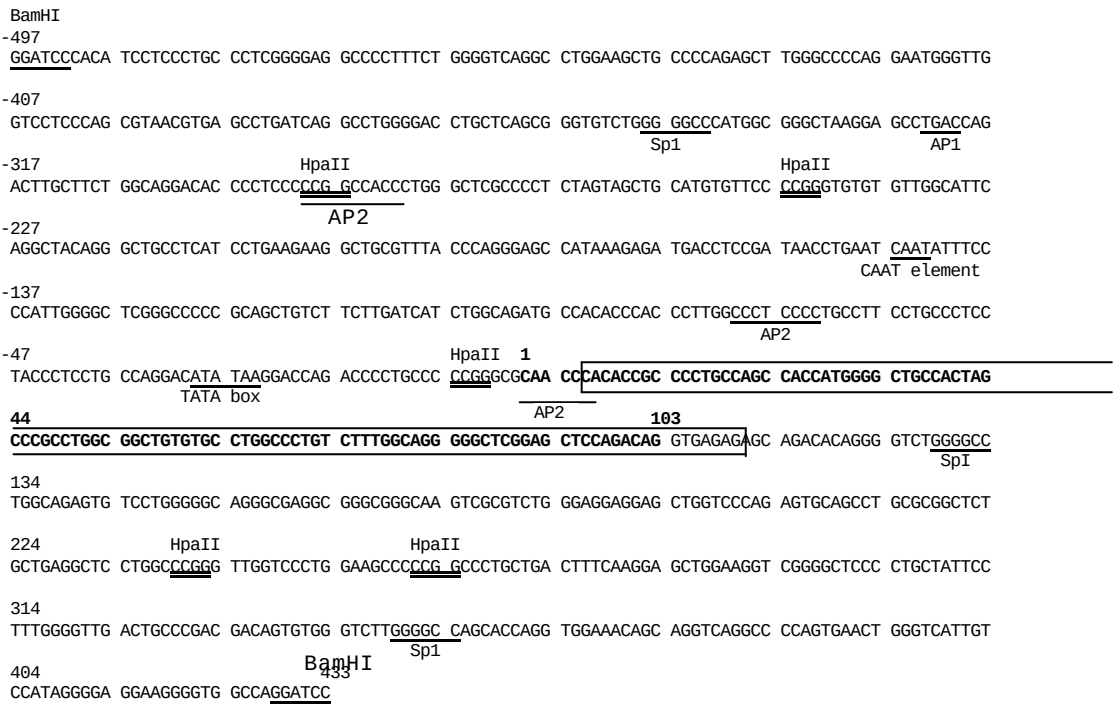


***Figure 10.*** *Sequence of the insert of pBSMUC2.1. Promoter elements and transcription factor binding sites are underlined. HpaII restriction sites are double underlined. Exon sequence is boxed.*

### *4.1.3 Analysis of the regulation of the MUC2 promoter*

To analyse the promoter activity in different cell lines luciferase reporter system was used. To generate the reporter plasmid MUC2 promoter sequence was amplified with primers MUC2F23 and MUC2R23 yielding 519 bp amplimer, comprising nucleotides from –493 to +26 of MUC2 genomic sequence. Obtained fragment contained BamHI restriction site on 5' end and HindIII restriction site on 3' end was introduced by PCR. Obtained amplimer was digested with BamHI and HindIII and cloned in pGL3basic vector upstream of the luciferase gene between BglII and Hind III sites (Fig. 11). During

the cloning BamHI site from the insert and BglII site from the vector were destroyed. The plasmid was named pGLMUC2p To check the size of the insert the digestion with restriction endonucleases HindIII and MluI was used resulting in cutting out of the promoter sequences. The absence of the mutations possibly introduced by Taq polymerase was confirmed by sequencing.

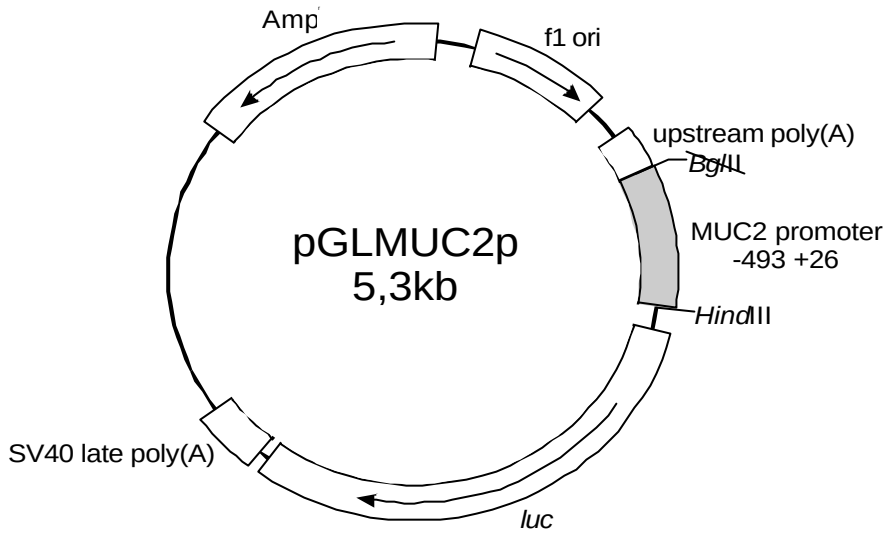


**Figure 11.** Map of the pGLMUC2p reporter plasmid**.**

Obtained plasmid was transfected into cell lines with different levels of MUC2 expression to analyse if these cell lines differ in the expression of any transcription factor, potentially responsible for the differences in the level of MUC2 expression. Two cell lines were selected: HT 29 – low MUC2 expressing cell line and LS 174T – a mucinous cell line with a high level of MUC2 expression. The difference of endogenous MUC2 expression in these two cell lines was previously determined by Northern blot and was found to be about 10 timed higher in LS 174T. The results of luciferase measurement were normalised to β-galactosidase activity. Surprisingly, the difference in promoter

activity in these two cell lines analysed in the reporter system appeared to be much lower, than the difference in the activity of the endogenous *MUC2* promoter, namely of about 2 fold **(Fig. 13)**.

These results suggested that the promoter sequence is not enough for the regulation of MUC2 gene expression, indicating the presence of the additional mechanisms, influencing the promoter activity. It was described previously, that tissue-specific gene expression may be regulated by tissue-specific enhancers. The property of the enhancer sequences is their ability to influence the activity of the promoter in position- and orientation-independent manner [103]. Therefore the possibility of the presence of enhancers within the exons of MUC2 gene was considered.

To test this hypothesis the first intron sequences were amplified with MUC2F20 and MUC2R15 primers from the first and second exons respectively. The obtained amplimer of about 1300 bp containing fragments of first and second exons and the complete first intron was cloned in pCRII cloning vector using TA cloning system and then subcloned in pGLMUC2p between *Bam*HI and *Sal*I restriction sites **(Fig. 12)**.

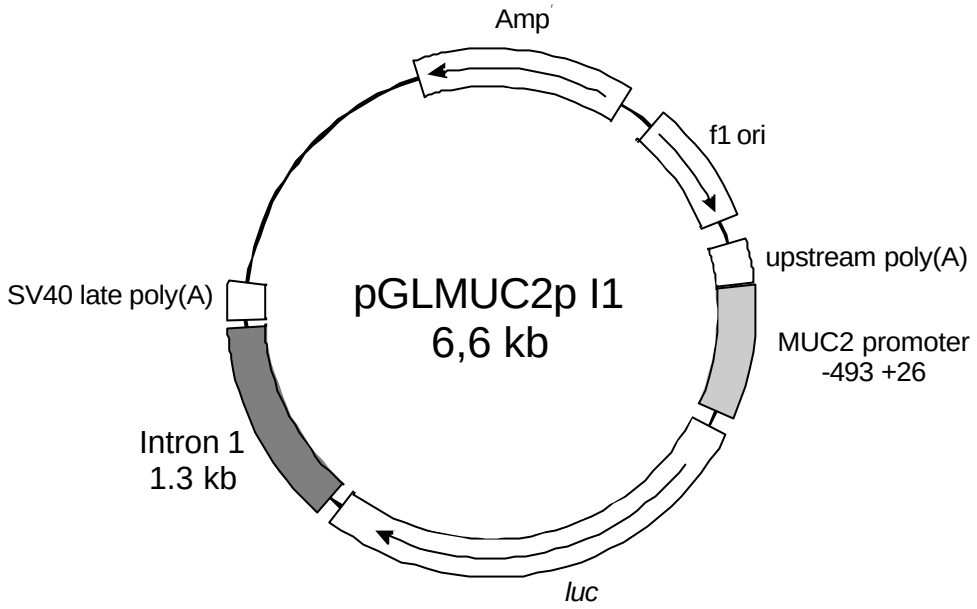


**Figure 12.** Map of the pGLMUC2pI1 plasmid**.**

The obtained plasmid named pGLMUC2pI1 was transfected into HT 29 and LS 174T cell lines. For the assessment of the transfection efficiency the pSVß-gal plasmid was cotransfected. The measurement of the luciferase activity showed that addition of the intron sequences in the reporter plasmid had only weak negative effect. The expression of the reporter gene from pGLMUC2pI1 was about 50% lower then from pGLMUC2p for both cell lines (**Fig 13)** indicating that the intron sequence contain no enhancers which can differently influence the activity of MUC2 promoter in different cell lines but may contain only a weak suppressor element.

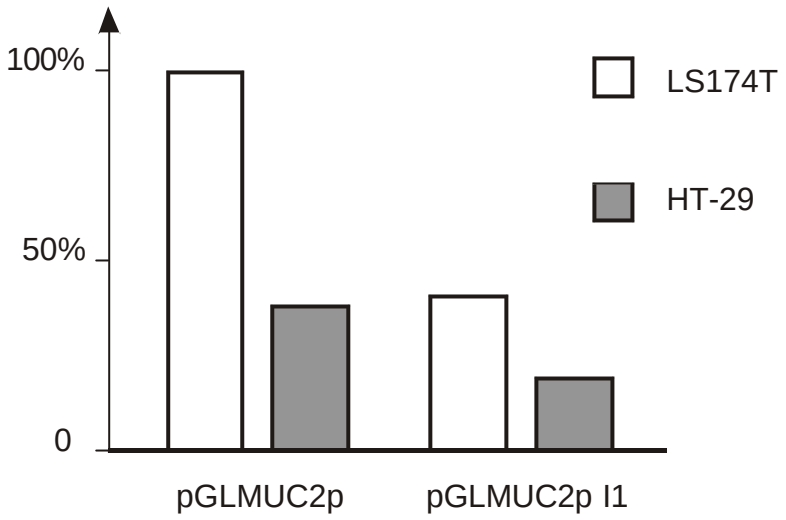


**Figure 13.** Activity of MUC2 promoter in LS 174T and HT 29 cell lines. The luciferase activity in LS174T transfected with üGLMUC2p was taken as 100%.

These results suggested that there must be an additional mechanism of MUC2 promoter regulation determining the difference in MUC2 expression in these cell lines. One of the possible mechanisms was DNA methylation. This chemical modification of DNA is involved in gene activity regulation [104] and also have been shown to participate in generation of tissue specific pattern of gene expression [105]. Taken together with the fact that 11p15 chromosomal loci is a hot spot for hypermethylation in human neoplasia [81] it was logical to analyse if the methylation of MUC2 promoter correlates with the expression of MUC2 gene.

## 4.2 Methylation of the promoter in different cell lines

### *4.2.1 MUC2 promoter methylation in MUC2 expressing and non expressing cell lines*

The methylation of MUC2 promoter in cell lines with different levels of MUC2 expression was analysed by Southern blot hybridisation, in part, by E.Riede. This method

is based on the property of some restriction enzymes to differentiate between methylated and nonmethylated DNA sequences. HpaII enzyme which can not digest DNA when the internal cytosine within the recognition site CCGG is methylated was used. Digestion with MspI was used to demonstrate the absence of mutations in the restriction sites. This enzyme has the same restriction site as HpaII but it digests methylated and nonmethylated DNA equally well.

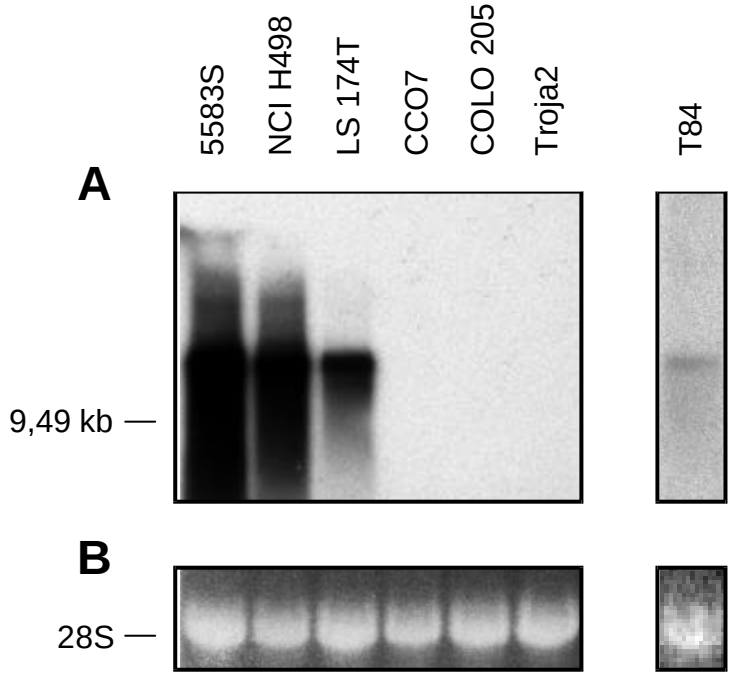


**Figure 14.** Analysis of the expression of MUC2 in colon carcinoma cell lines. A: Detection of MUC2 with SMUC41 probe. B: EtBr staining of the gel showing the equal amount of RNA in each lane. Northern blot carried out by U.Kobalz.

Seven cell lines were selected for the analysis: 3 mucinous cell lines 5583-S, NCI- H498, and LS 174T; 3 nonmucinous cell lines CCO7, COLO 205 Troja 2 and one intermediate expressing - T84. The expression of MUC2 was determined in these cell lines by U.Kobalz by Northern blot with SMUC42 probe (**fig 14)**.

Methylation was analysed by Southern blot within 495 bp of the promoter region and also within the first exon and part of the first intron. For this analysis genomic DNA isolated from cell lines was digested with BamHI (**Fig. 15 lane 1)**, to generate the 900bp fragment and consequently with HpaII (**Fig. 15 lane 2)** or MspI (**Fig. 15 lane 3)**. Obtained digests were separated on the 1.2% agarose gel and blotted on the nylon membrane. Hybridisation was carried out with the probe for 497 bp fragment of the promoter region – MUC2 promoter probe (**Fig. 15 C)** or 245 bp fragment, comprising first exon and part of the first intron sequences – MUC2 intron probe **(Fig. 15 C)**. The DNA from mucinous cell lines was found to be mostly nonmethylated in the promoter region. The faint band of about 700 bp in 5583-S detectable with MUC2 promoter probe (**Fig. 15A, lane 2)** and MUC2 intron probe (**Fig. 15B, lane 2)** indicate that there is some methylation in the sites #1 and #3. The resolution of the gel was not sufficient to distinguish between these two sites. In NCI-H498 cell line the band of 900 bp was observed after hybridisation with both probes (**Fig. 15 A,B, lanes 2**) indicating that in this cell line there is a subpopulation of cells in which the promoter region is completely methylated. In LS 174T cell line the band of 500 bp detectable with the MUC2 intron probe but not with MUC2 promoter probe indicated that there is partial methylation of CpG site #8 in this cell line. The DNA from nonmucinous cell lines appeared to be completely methylated in all cell lines. The band of about 700 bp detected with both probes after HpaII digestion in CCO7 and COLO 205 indicates that in these cell lines the methylation is incomplete only in CpG sites #21/22, but not within the promoter region. In Troja 2 cell line all sites investigated appeared to be methylated. The results obtained after the analysis of the cell line T84, moderately expressing MUC2, appeared to be more complicate. After the hybridisation with the promoter probe bands of all possible sizes are visible (**Fig. 15 A, lane 2**),

indicating the presence of all possible methylation patterns in the DNA isolated from this cell line. This can result from the fact that this cell line is not monoclonal and appears to be a mixture of cells with different methylation of the MUC2 promoter region.

The correlation between promoter methylation and level of MUC2 expression supports the hypothesis of the involvement of DNA methylation in the regulation of MUC2 gene. Further experiments were directed to additionally proof the hypothesis and to clarify the mechanisms of promoter activity regulation.

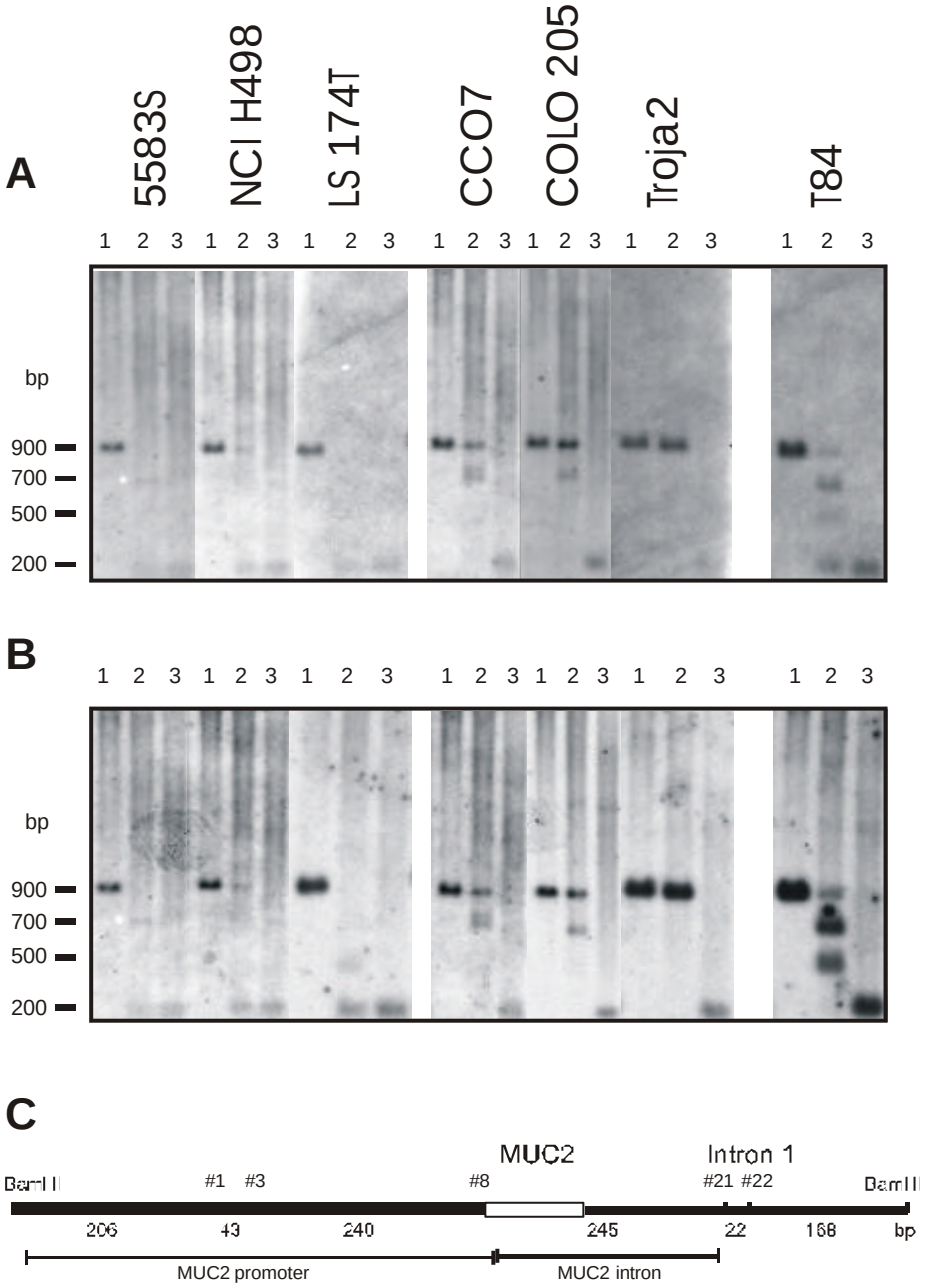


**Figure 15.** The analysis of MUC2 promoter methylation in Southern blot hybridisation. A: Hybridisation with MUC2 promoter probe. B: Hybridisation with MUC2 intron probe. C: Map of the analysed region, ## 1, 3, 8, 21, 22 are HpaII digestion sites, hybridisation probes are indicated. Southern blot was done, in part, by E.Riede.

### *4.2.2 Analysis of MUC2 promoter methylation by bisulphite sequencing*

Southern blot analysis can give only limited information about the methylation of the promoter region, because out of 14 CpG dinucleotides within the promoter region of MUC2 only 3 are located within the HpaII enzymes recognition sequences. Method for the precise analysis of methylation sites was invented by Clark et al in 1994 [106] and then modified by Olek et al [99]. The method is based on the treatment of DNA with sodium bisulphite. This treatment converts all nonmethylated cytosines in uracils where the methylated cytosines remain unchanged (Materials and methods 2.7.2). In the following PCR reaction thymins are incorporated into the DNA in place of uracils. The obtained PCR product can be either sequenced directly or cloned into a vector. Several plasmid clones are then analysed in purpose to obtain a statistically representative illustration of the DNA methylation status.

I used the second method of modified sequence analysis for examining the methylation in one mucinous cell line LS 174T and one nonmucinous cell line – COLO 205, previously analysed in Southern. DNA isolated from cell lines was treated with bisulphite and amplified in the nested PCR. First amplification was done with primers MUC2F41 and MUC2R45. The results of the first step of the PCR were not detectable on the gel due to very low amount of the PCR product. One µl of the first step reaction was used for the second step with primers MUC2F45 and MUC2R45. The obtained PCR product comprised 320 bp of the MUC2 promoter region and contained 9 CpG dinucleotides located within the promoter region and 1 in the position 11 in the first exon (**Fig. 16, A)**. Methylation sites were numbered. CpG sites ## 1, 3 and 8 corresponded to previously analysed in Southern blot sites. The amplimer was cloned into the pCRII vector, using TA cloning kit (Invitrogen). To obtain statistically evaluable data, 10 plasmid clones for each

DNA sample were analysed. Plasmids were isolated and sequenced. All cytosines within CpG dinucleotides which remained unchanged were considered as methylated in the original sequence.

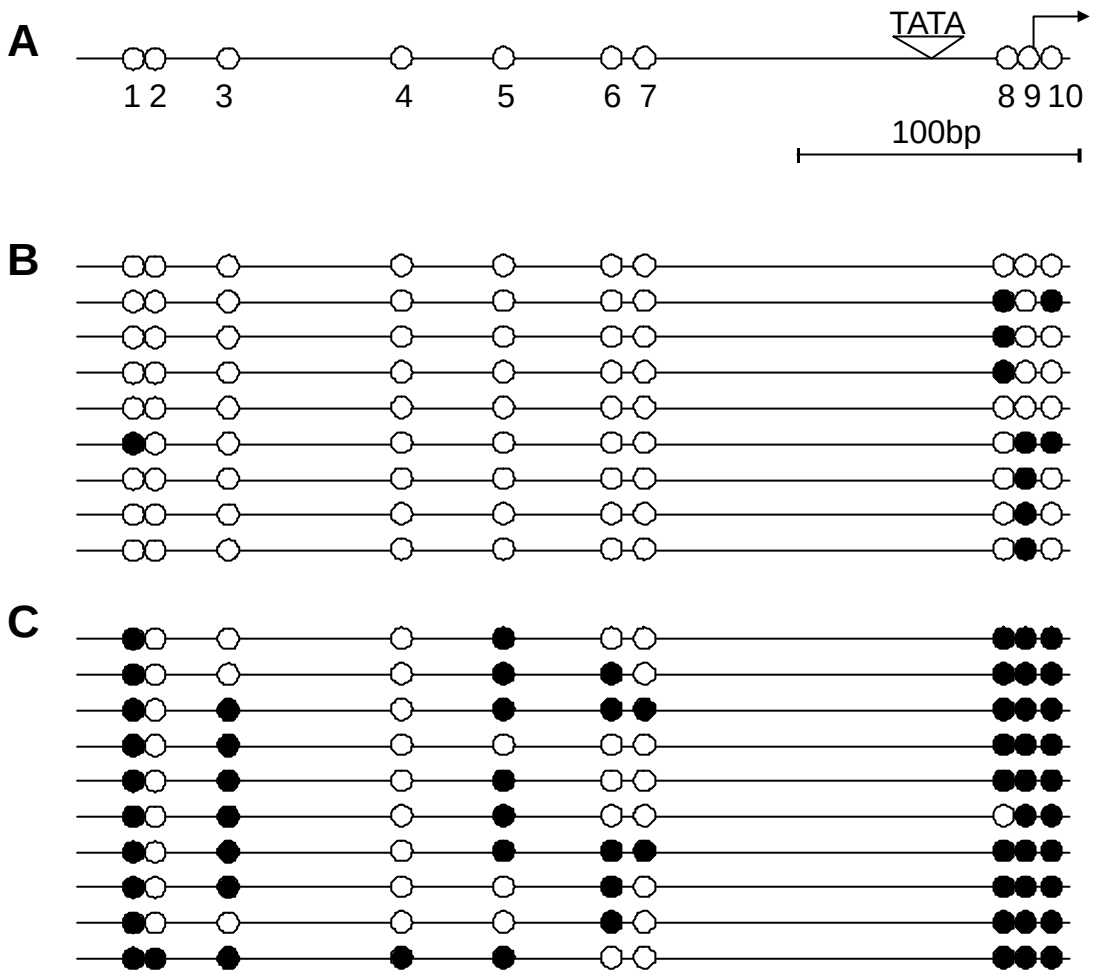


**Figure 16.** Analysis of methylation by bisulphite sequencing. A: Map of 320 bp of the promoter region, circles are placed in CpG positions. B: bisulphite sequencing results for LS 174T cell line. C: bisulphite sequencing results for COLO 205 cell line. Filled circles indicate methylated cytosines, opened circles indicate nonmethylated cytosines.

Both cell lines COLO 205 and LS174T exhibit the heterogeneous pattern of methylation, indicating the polyclonality of cell lines (**Fig. 16**). The overall methylation in COLO 205

was 62% and in LS174T – 10%. The analysis of particular sites methylation confirmed the results of the Southern blot. CpG site #8 is partially methylated in LS 174T. CpG sites # 1, 3 and 8 are almost completely methylated in COLO 205. The analysis of methylation of particular methylation sites showed, that CpG sites #2, #4 and #7 are very low methylated in COLO 205 and nonmethylated in LS 174T suggesting that these sites may not play a significant role in the regulation of the expression of MUC2 gene. All other sites were much higher methylated in COLO 205 than in LS 174T. All remaining sites were highly methylated in COLO 205 in comparison to LS 174T. Therefore it was concluded, that the site-specific methylation as well as overall may be important for the regulation of the expression of MUC2.

### *4.2.3 Activation of MUC2 expression by inhibition of methylation*

To additionally proof the hypothesis of methylation dependent regulation of MUC2 promoter activity the attempt of activation of MUC2 expression by inhibition of methylation was undertaken by E.Riede and M.-L.Hanski. For this experiment T84 cell line was selected. This cell line has low, but detectable in Northern blot expression of MUC2 (**Fig. 14**), and, therefore, it was easy to monitor the changes of the expression during the experiment. MUC2 expression was analysed in Northern blot with SMUC41 probe (**Fig. 17 A**). To normalise the amount of RNA on the membrane hybridisation with 18S rRNA probe was done (**Fig. 17 A**). The obtained autoradiogramms were analysed densitometrically. The levels of MUC2 expression on the different stages of the treatment are presented on the diagram (**Fig. 17 B**). After 24h of the treatment the expression of MUC2 was already increased 2.7 fold. The following treatment did not have such a strong effect, however after 72 h the expression was increased up to 3 fold in comparison to non-treated cells.

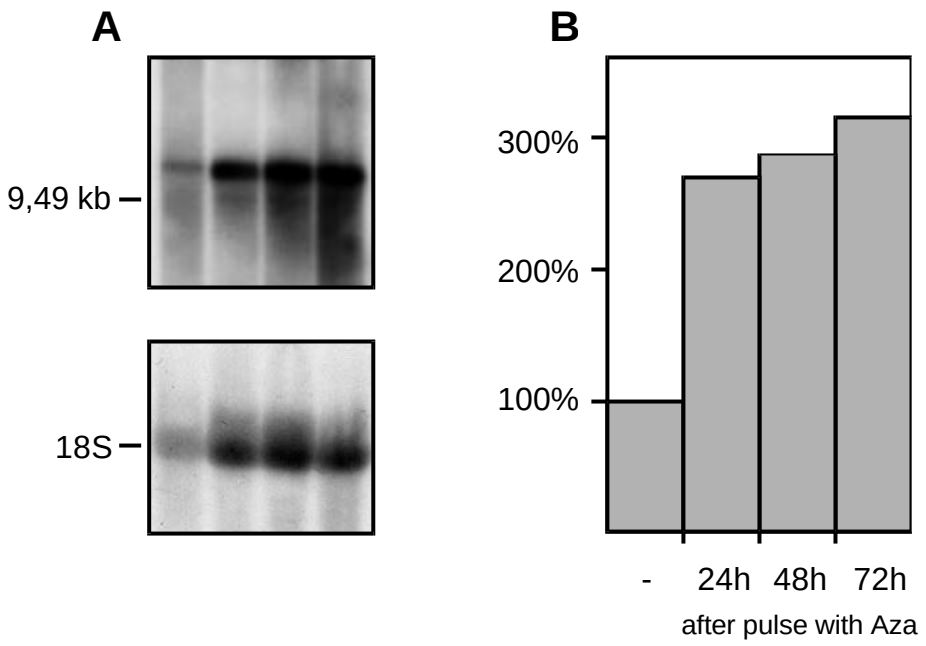


**Figure 17.** Activation of the expression of MUC2 gene by inhibition of methylation. A: Analysis of MUC2 expression in T84 after 5-aza-2'-deoxycytidine treatment, upper panel – hybridisation with SMUC41 probe; lower panel – hybridisation with 18S probe. B: Level of the expression of MUC2 gene after normalising of the results of hybridisation with SMUC41 probe to the intensity of 18S band. Experiment carried out by E.Riede.

This experiment directly shows that the inhibition of methylation can enhance the expression of MUC2 gene, providing additional support to the hypothesis that methylation is involved in the regulation of MUC2 expression. However the disadvantage of the cell line used is its polyclonality. For detailed analysis of methylation inhibition on the MUC2 expression the experimental system which allows to overcome the problem of polyclonality was developed.

## 4.3 MUC2 promoter methylation in cloned cells

### *4.3.1 Inhibition of methylation and selection of clones*

For detailed analysis of methylation influence on MUC2 expression in cell lines the following experiment was carried out (**Fig. 18**). MUC2 non expressing cell line COLO 205 was treated with 5-aza-2'-deoxycytidine until the expression of MUC2 became detectable in RT-PCR; then cells were cloned and several clones with different levels of MUC2 expression were selected; obtained clones were analysed for MUC2 expression by RT-PCR and Northern blot, and for the promoter methylation by Southern blot and bisulphite sequencing.

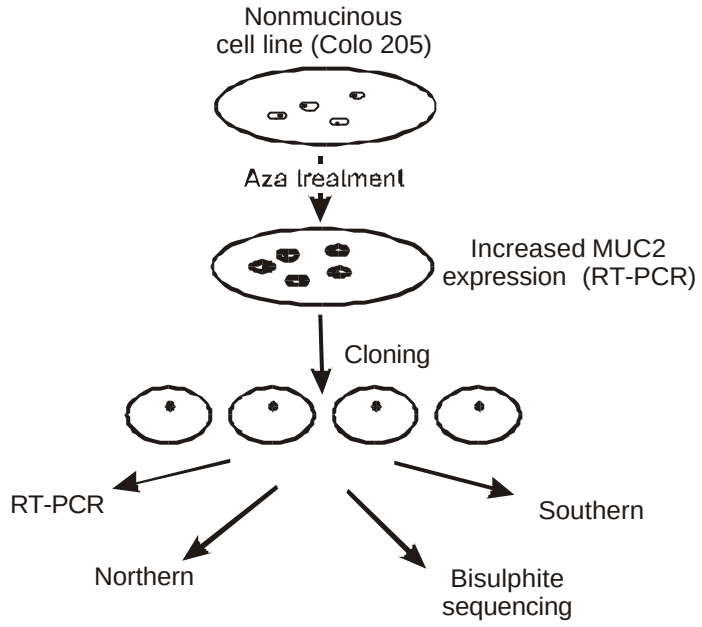


**Figure 18**. The scheme of the experiment.

Treatment with 5-aza-2'-deoxycytidine for the inhibition of methylation was done by M.-L.Hanski in 1 week cycles with 24 h treatment and following 6 days recovery in non supplemented medium. The expression of MUC2 was monitored starting from the 4th week with RT-PCR. MUC2 expression became detectable after 6 week of treatment.

After the 7-th week the treatment was stopped. The analysis of methylation of MUC2 promoter in this mixed population by Southern blot did not show any detectable methylation (data not shown). Cloning by limiting dilution yielded several clones with different levels of MUC2 expression, determined in Northern blot with SMUC41 probe (**Fig. 19 A**). RT-PCR revealed MUC2 expression in all clones investigated and also in COLO 205 cell line (**Fig. 19 C**). Clones 4 and 5 did not show any detectable expression, clones 7, 15 and 16 expressed MUC2 on the low but detectable level, and in clone 14 the expression was significantly higher than in all other clones. Differences in expression obtained in Northern blot were not quantified. The clones were separated in three major groups: non expressing - clones 4 and 5; weakly expressing - clones 7, 15 and 16; and strongly expressing - clone 14.

To control if the observed difference in MUC2 expression is not a result of an overexpression of a methylation dependent transcription factor, at least one clone from every group was transfected with the luciferase reporter plasmid pGLMUC2p, containing nonmethylated MUC2 promoter. The expression of the reporter did not correlate with the expression of MUC2 in corresponding clones, indicating that the differences in MUC2 expression did not result from a transcription factor expression alteration, but rather reflected the effect of methylation changes in the promoter region of MUC2 gene (data not shown).

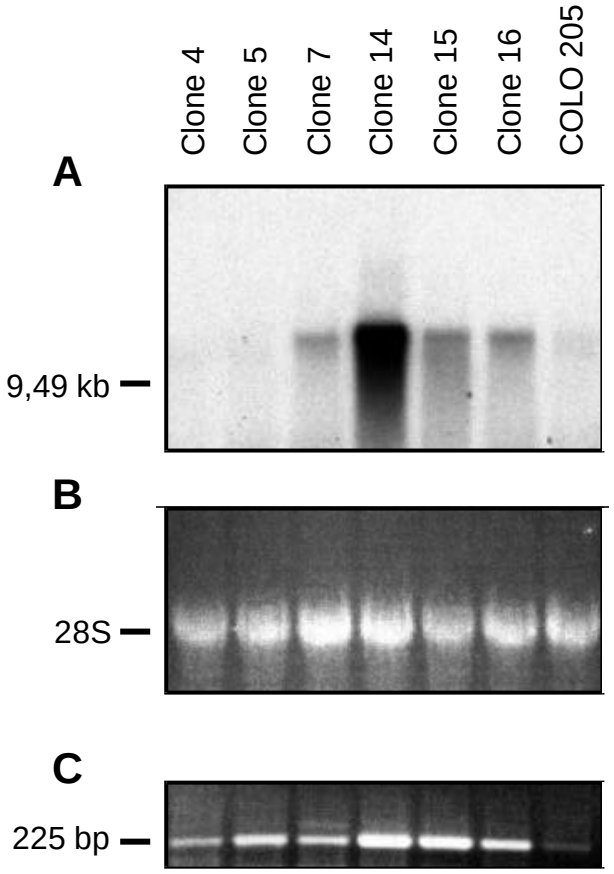


**Figure 19.** Analysis of the expression of MUC2 in the clones. A: Northern blot with SMUC41 probe. B: EtBr staining of the gel showing the equal amount of RNA applied. C: RT-PCR detection of MUC2 expression with primers MUC2F15 MUC2R13. Nothern blot was done by U.Kobalz.

### *4.3.2 Methylation of the MUC2 promoter in obtained clones*

To test if MUC2 promoter methylation was changed in 4 expressing clones and if this change correlates with the level of MUC2 expression Southern blot was used (**Fig. 20**). DNA was digested with BamHI and HpaII, blotted and hybridised with MUC2 promoter probe. All clones analysed showed different methylation pattern. Clone 4 showed the presence of all possible methylation patterns within the promoter region, including

completely methylated promoter (band of 900bp) and completely nonmethylated promoter (band of 200 bp), indicating, that the methylation was restored during cloning. Analysis of the promoter methylation in clones 5, 14 and 15 did not reveal 900bp band indicating that in all these clones at least one methylation site within the promoter region of MUC2 is not methylated. The most remarkable was the clone 14 which had the highest MUC2 expression (**Fig. 19**). It showed only two bands: the major band of 200 bp corresponding to completely nonmethylated promoter region and a minor band of 500 bp which can result from methylation of one single site within the investigated region.

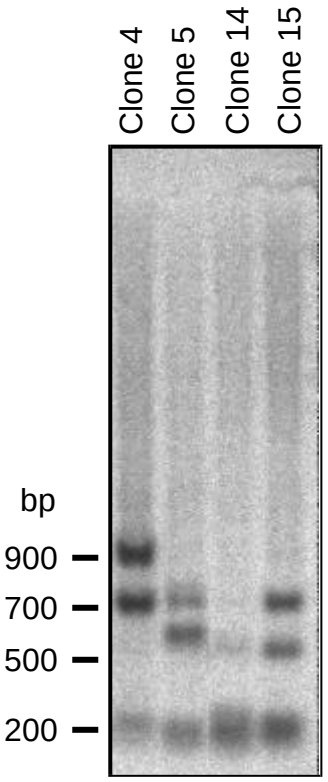


**Figure 20**. Analysis of MUC2 promoter methylation by Southern blot. Hybridisation with MUC2 promoter probe.

For detailed analysis of MUC2 promoter region methylation in clones bisulphite sequencing was used.

Results of the sequencing were evaluated in percents and are presented in the **Table 1**. The clone 14 had the lowest total methylation in comparison to other clones. For example non expressing clone 5 had lower total methylation (47%) than low expressing clone 16 (59%). Statistical analysis made by Dr. Mannsman showed, that the CpG sites #5 and #8 are exceptionally low methylated in clone 14 in comparison to all other with $P_{MIN}$= 0.94 and 0.995 respectively, calculated as a posterior probability in Bayesian fixed effect ranking model [107] (**Table 1 row $P_{MIN}$**). These findings suggested the particular importance of these sites for the regulation of MUC2 expression.

| Site | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | MED |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Colo 4 | 92 | 25 | 50 | 33 | 92 | 0 | 50 | 100 | 58 | 100 | 60 |
| Colo 5 | 0 | 70 | 40 | 30 | 70 | 40 | 0 | 80 | 50 | 90 | 47 |
| Colo 7 | 0 | 30 | 0 | 50 | 80 | 0 | 0 | 50 | 50 | 90 | 35 |
| **Colo 14** | **0** | **0** | **0** | **58** | **33** | **8** | **0** | **0** | **25** | **83** | **20** |
| Colo 15 | 50 | 33 | 0 | 33 | 75 | 17 | 0 | 75 | 17 | 75 | 37 |
| Colo 16 | 50 | 40 | 40 | 70 | 90 | 30 | 40 | 100 | 60 | 70 | 59 |
| $P_{MIN}$ | 0,355 | 0,889 | 0,348 | 0,023 | **0,940** | 0,112 | 0,253 | **0,995** | 0,309 | 0,209 | |

**Table 1.** Methylation of CpG sites in the promoter region of MUC2 gene in clones obtained after bisulphite treatment. $P_{MIN}$ – posterior probability determines in Bayesian fixed effect ranking model. MED – overall methylation in the promoter region of MUC2. Statistically significant differences are shown in bold.

### *4.3.3 Gene expression alterations in clones*

Difference in methylation in the clones obtained after 5-aza-2'-deoxycytidine treatment indicated, that demethylation or remethylation occurred differently in these clones. To understand the reason of this finding the analysis of methylation-related genes expression was done. The expression of following genes was analysed: DNMT1, DNMT2 and p21. The expression of DNMT1 was analysed in semiquantative RT-PCR in clones 4, 5, 7, 14, 15 and 16. The results of amplification are presented on the **Figure 21A**. The expression DNMT1 did not correspond to the level of MUC2 promoter methylation. Therefore it can be concluded, that DNMT1 did not determine the level of methylation within the promoter region of MUC2 in the investigated clones. RT-PCR analysis of DNMT2 showed the expected band of 720 bp in all clones (**Fig. 21B**). The observed levels of DNMT2 did not correlate with the methylation level of MUC2 promoter. Therefore the expression of DNMT2 appeared to be irrelevant to the level of methylation of MUC2 promoter, as in the case of DNMT1.

The third protein reported to be involved in the regulation of methylation maintenance is p21. It was analysed in Northern blot with the p21 probe. The level of the expression of p21 does not show strong difference between clones **(Fig. 21C)**.

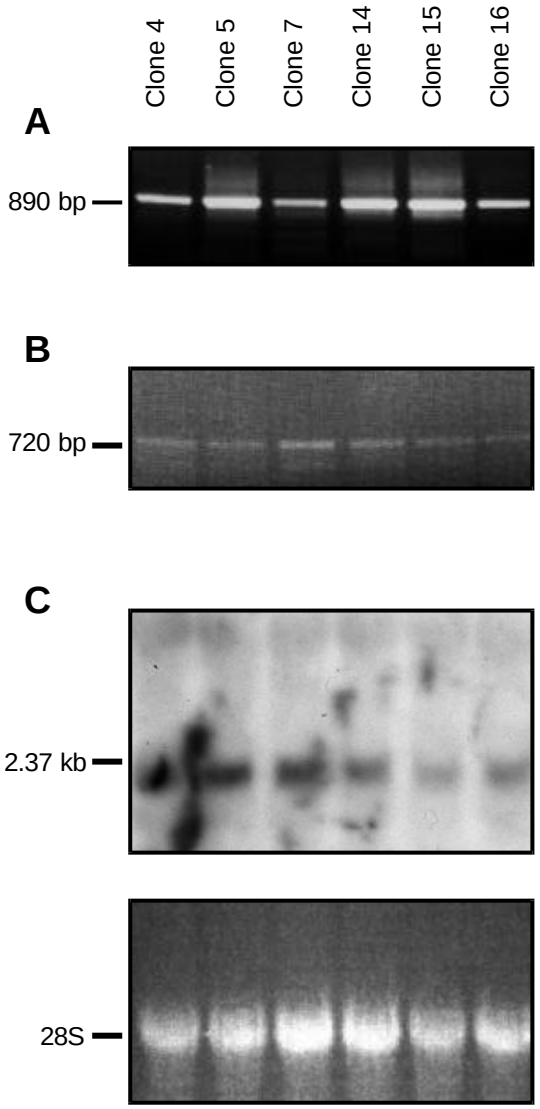


**Figure 21.** Analysis of the expression of methylation related genes in clones. A: RT-PCR analysis of DNMT1 expression. B: RT-PCR analysis of DNMT2 expression. C: upper panel – Northern blot analysis of p21-mRNA expression in clones, lower panel – EtBr staining of the gel showing similar amounts of RNA applied to each lane. (Membrane prepared by U.Kobalz).

## 4.4 Influence of the site-specific methylation influence on the promoter activity

### *4.4.1 Influence of methylation on the promoter activity in different cell lines*

For *in vitro* analysis of the influence of methylation on the MUC2 promoter activity luciferase reporter system was used. The plasmid was obtained by incomplete digesting of pGLMUC2prom with *Sma*I enzyme and religation of the fragment, containing first 245 bp of the promoter region (**Fig. 22**). Obtained plasmid was methylated with mHpaII or mSssI methyltransferase. For transfection three cell lines were selected: COLO 205; T84 and LS 174T. The obtained results are presented on the **figure 23**. In all 3 cell lines methylation with only mHpaII suppress the activity of the promoter to 60% in COLO 205, to 30% in T84 and to 10% in LS 174T cell lines. Further methylation of the promoter caused even stronger reduction of the promoter activity in COLO 205 and T84 (to about 10%), but did not in LS 174T, where the activity stayed at the level of 10%. These results indicate, that methylation with mHpaII methyltransferase is already enough for strong suppression of the promoter activity. Interestingly, this enzyme methylates only one cytosine within the promoter region, namely the CpG site #8 which was shown to be less methylated in MUC2 expressing cells in comparison to non expressing. The vector sequence which has the methylation sites can also influence the activity of the reporter plasmid. Therefore the role of the methylation of vector sequences on the activity of the reporter plasmid was analysed, to clarify the role of CpG #8 methylation in the suppression of MUC2 promoter.

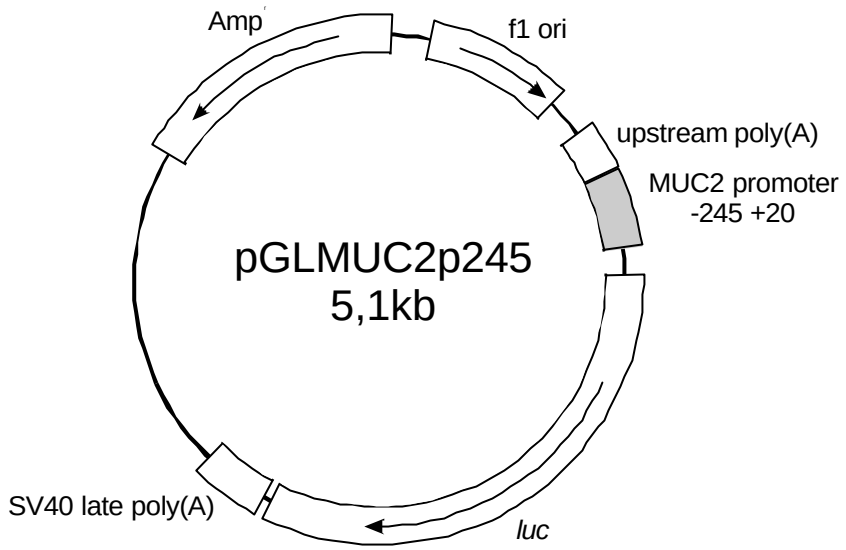


**Figure 22.** Map of pGLMUC2p245 plasmid**.**

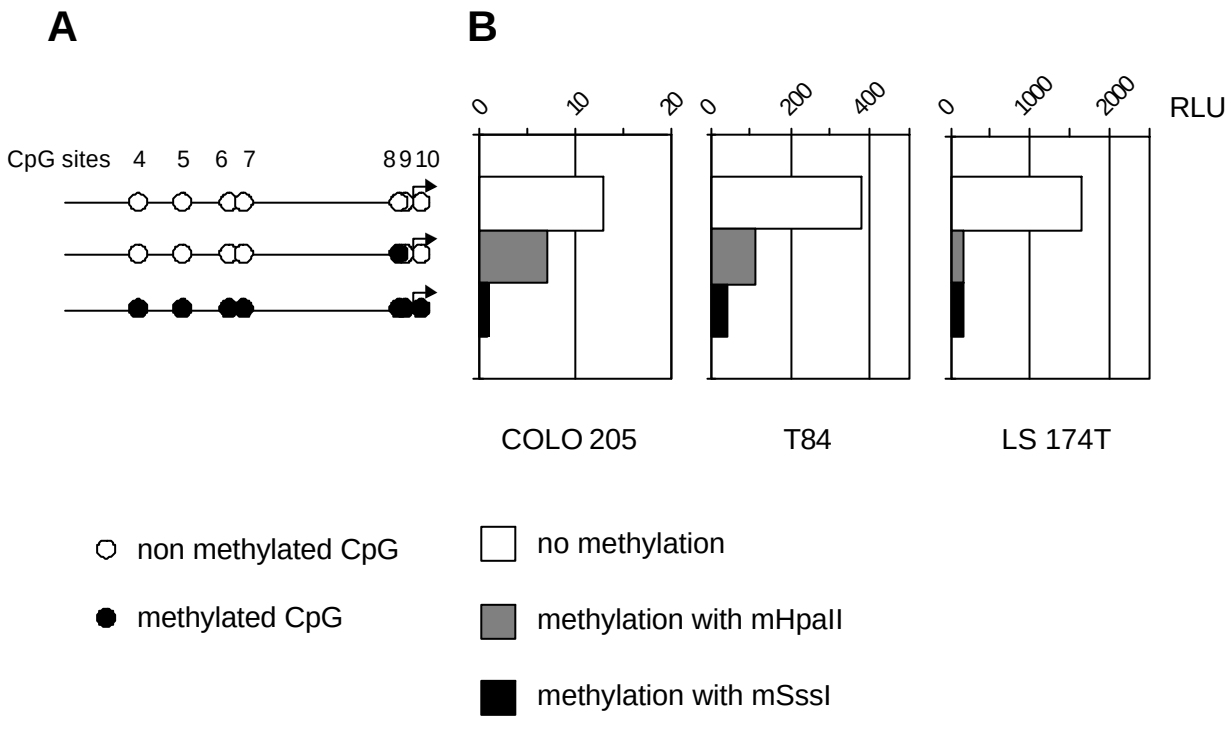


**Figure 23. Transcriptinal activity of the differently methylated promoter in different cell lines.** A: Methyl group insertion within the promoter region after treatment of the

plasmid with different methyltransferases. B: Luciferase activity of differently methylated plasmid in COLO 205, T84 and LS 174T cell lines. RLU – relative luciferase units.

### *4.4.2 Analysis of the effect of mutations at CpG sites #5 and #8*

To analyse the effect of methylation only at the CpG #8 on the promoter activity this site was mutated by substituting C within CpG dinucleotide with T. The mutated sequences were obtained by PCR as described in methods and inserted into the luciferase reporter plasmid. The expression of the reporter from the nonmutated promoter when the plasmid was methylated with mHpaII was reduced in comparison to non methylated one to about 25% (**Fig. 24, b**). For the plasmid with C->T mutation in CpG site #8 this reduction was only to 60% (**Fig. 24, c**). Therefore it was concluded that the role of CpG#8 methylation in the suppression of the reporter gene is about 35%.

To analyse the contribution of methylation of CpG site #5 in the regulation of MUC2 expression, plasmid, containing mutations in both CpG sites #5 and #8 was generated and ligated into the luciferase reporter plasmid. Methylation of all CpG sites caused the reduction of the promoter activity to 10% of the activity of nonmethylated one (**Fig. 24 d**). Mutation of CpG #8 reduced this effect. After methylation of all CpG sites with the exception of mutated CpG #8 the promoter activity was reduced only to 30% (**Fig. 24 e**). The addition of the mutation in CpG #5 site resulted in the same effect, i.e. the expression from the plasmid methylated with mSssI was 30% in comparison to non mutated one (**Fig. 24 f**). This result indicates that the methylation of CpG #5 is not involved in the regulation of MUC2 promoter activity.

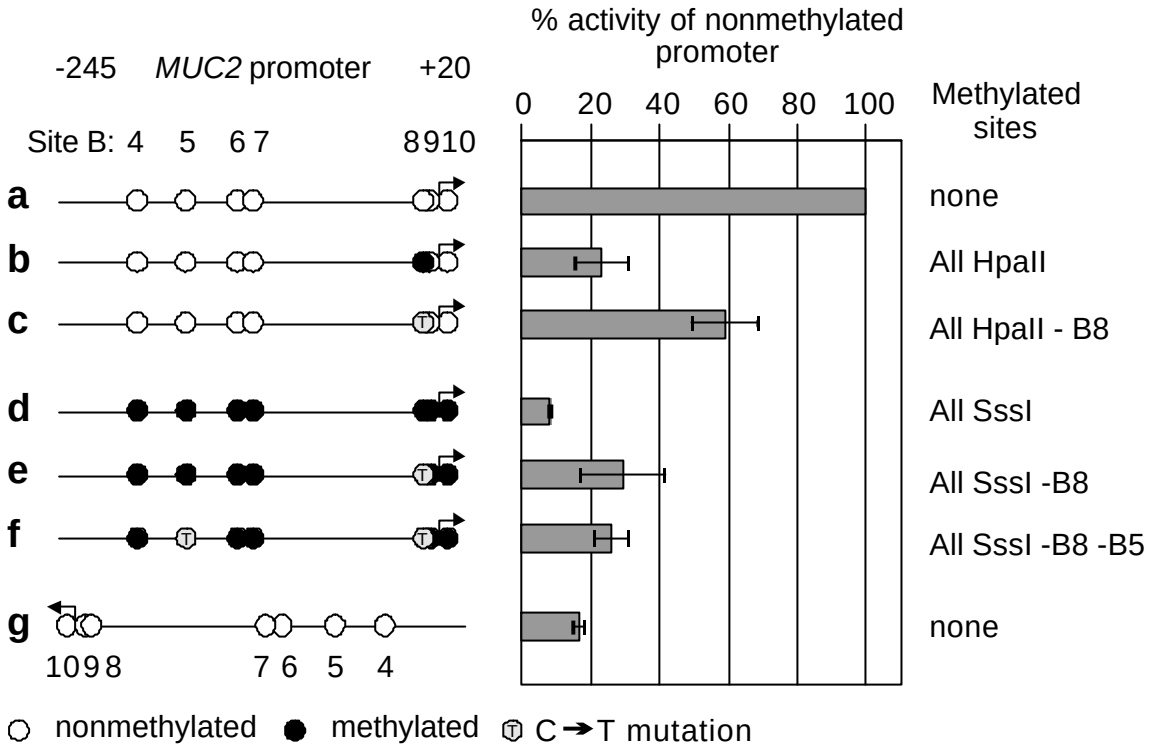


**Figure 24**. Analysis of the role of CpG #5 and #8 in the regulation of the activity of MUC2 promoter. Standard deviations were obtained after evaluation of at least three independent experiments. Luciferase assays carried out by Dr.A.Siedow. Values were normalised to plasmid amount in the transfected cells.

### *4.4.3 Analysis of possible de novo methylation or demethylation of the transfected plasmid*

The results of the reporter analysis supported the hypothesis that the promoter of MUC2 gene is regulated by methylation. However it is necessary to check if the results of reporter experiments are not influenced by the change of plasmid methylation after transfection. For this experiment pGLMUC2p was used. Nonmethylated and mSssI or mHpaII methylated plasmids were transfected in LS 174T and COLO 205 cell lines. Methylation analysis by bisulphite sequencing showed, that no methylation alterations in the plasmids obtained after 48 h transfection. Neither *de novo* methylation of the non

methylated nor loss of methylation in the methylated plasmid was observed (data not shown).

## 4.5 MUC2 promoter methylation in tissues

### *4.5.1 Application of microdissection*

Analysis of methylation in tissue samples was done initially using Southern blot hybridisation of the DNA isolated from normal and tumour tissue samples. The hybridisation revealed the heterogeneous pattern of methylation within the promoter region of MUC2 gene (data not shown). The presence of all possible methylation patterns was explained by the mixed population of cells in the tissue samples which were used for the isolation of DNA. Therefore laser microdissection was selected as a method of purification of tumour and normal cells for the analysis of methylation in colon carcinomas and normal colonic tissues.

For this experiment paraffin sections of mucinous, nonmucinous and normal tissues were selected. Microdissection procedure was done at least 2, but not more than 4 times for each section. The collected samples of normal tissues comprised 2 to 4 normal crypts, and samples of tumour tissues were represented by the 2 to 4 pieces of sections, containing cells with clear mucinous or nonmucinous morphology. This method allowed to analyse the methylation changes in relatively pure population of cells with minimised contamination with the cells of other origins.

*4.5.2 Analysis of MUC2 promoter methylation in tissue*

The microdisseced tissue was subjected to methylation analysis by bisulphite treatment. Due to the DNA degradation it was impossible to amplify the complete promoter region of MUC2. Therefore three nested PCR procedures were performed.

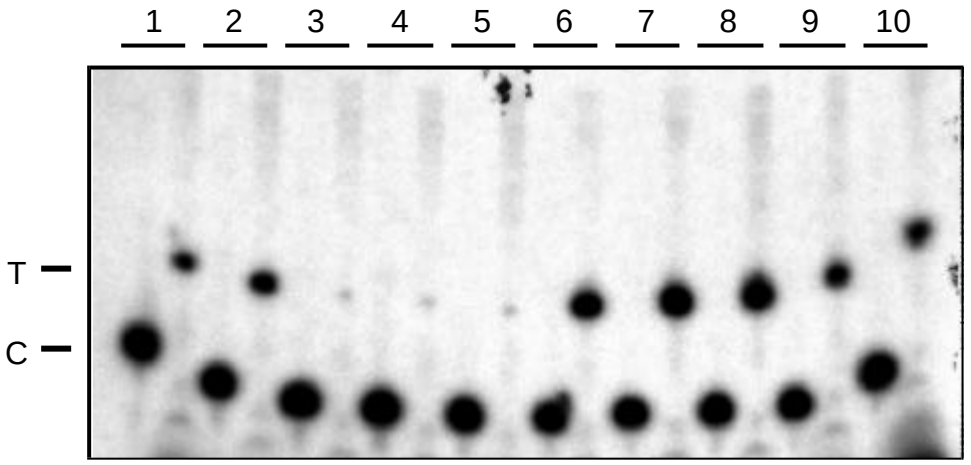


**Figure 26**. Typical autoradiogramm of a SNuPE reaction. Numbers denote investigated CpG sites.

The obtained fragments were used for Single Nucleotide Primer Extension (SNuPE) as described. The typical result of SNuPE is presented on **Figure 26**. Samples labelled with dCTP were loaded first and the samples labelled with dTTP ten minutes later to simplify quantification (**Fig. 26**). In every lane the only one major spot corresponding to labelled primer is visible. Every sample analysed is presented as two lines: first – primer labelled with dCTP and second – primer labelled with dTTP. The ratio of the amount of the radioactivity in C spot related to the amount of the radioactivity in T and C spots exhibit the % of methylated cytosines in this sample. For example in samples 3, 4 and 5 the methylation was almost 100%, and in samples 6, 7 and 8 – about 50%.

For this analysis 3 mucinous and 3 non mucinous tumours were selected (6 patients). From each patient 2 samples of tumour tissue and 1 sample of normal tissue were analysed. Each sample was analyses in SNuPE once, however to control the reproducibility of the results 3 samples were analysed twice and showed that the difference in measurement does not exceed 5% - the typical instrumental mistake of the experiment.

The results of the analysis of methylation in the promoter region with SNuPE are presented on the **figure 27**. The level of methylation in the normal crypt was 81±8%. The pure population of nonmucinous carcinoma cells showed the methylation of 91±7%, consistent with the fact that there is no MUC2-expressing cells in these tumours. In the mucinous tumour cells methylation of the promoter region was reduced in comparison to normal tissue. Taken together these data indicate that there is alteration of methylation in tumour cells in comparison to normal tissue. For non-mucinous tumours hypermethylation was observed while there was hypomethylation in the case of mucinous tumours, relative to the normal tissue.

### *4.5.3 MUC2 promoter methylation in isolated goblet cells*

For the analysis of the methylation in normal cells of goblet phenotype the fresh human colon was used as a source of epithelial cells. Epithelial cell suspension was stained with anti-MUC2 antibody CCP58 and MUC2 positive cells were manually collected. The total number of 75 cells was used for each reaction. Bisulphite treatment was done the same way as for tissue sections. In contrast, single cells were expected to contain non fragmented DNA, therefore the PCR amplification of the whole promoter region was done in the first PCR. Amplimers obtained after the nested-PCR were used for SNuPE.

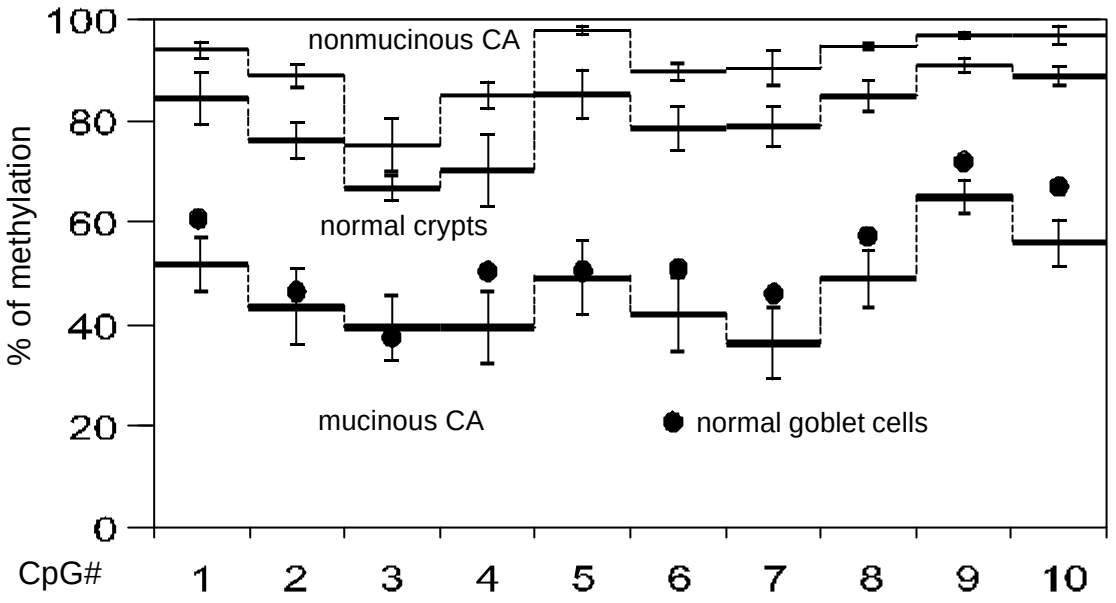


**Figure 27.** Methylation of MUC2 promoter in clinical samples and selected goblet cells.

The evaluated results of SNuPE analysis are presented on the **figure 27**. Methylation of CpG sites in goblet cells was similar to the methylation of the same sites in samples from mucinous carcinomas.