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During the last years, a direct link between DNA methylation and repressive chromatin structure has been established. This structural modification is mediated by histone deacetylases targeted to the methylated sequences by Methyl Binding Proteins (MBD). Human cancer cells exhibit both a global hypomethylation and some localized hypermethylations suggesting that the deregulation of the methylation machinery is a central event in tumorigenesis. Therefore, we have investigated in human tissues the expression of two major MBDs, *MeCP2* and *MBD2*, during the proliferation of normal breast and in benign and neoplasic breast tumors. Quantitation of the transcripts indicates that *MBD2* mRNAs are 20–30-fold more abundant than *MeCP2* transcripts in the adult and fetal human mammary gland. In pathological tissues samples *MBD2* mRNA levels are significantly higher ($P = 0.001$) in benign tumors compared with normal breast tissues, whereas *MeCP2* expression is not modified in these specimens. In neoplasic samples a deregulation of the expression of both genes was found. The amounts of *MBD2* and *MeCP2* transcripts vary greatly between samples in cancer cells compared to normal breast tissues or benign tumors, and in invasive ductal carcinomas the amount of *MBD2* mRNA is significantly ($P = 0.03$) associated with the tumor size. Taken together these data suggest that upregulation of *MBD2* might be associated with breast cell proliferation. In line with this hypothesis *MBD2* is also upregulated during the prenatal development of the human mammary gland, but in contrast to that observed in tumor cells, *MeCP2* is also coordinately upregulated in the fetal breast tissues, suggesting that deregulation of *MeCP2* and *MBD2* occurs in human breast cancers.

Keywords: breast cancer; prenatal developments; methyl binding proteins; RT–PCR

Introduction

The alterations of DNA methylation level and patterns are a common feature of human cancer cells. A global DNA hypomethylation has been observed in many cancers, without obvious sequence specificity. Indeed, repetitive and single copy sequences are equally affected (Ehrlich, 2000). Despite this reduced level of genomic methylation, some localized hypermethylation are also observed (Baylin *et al*., 1997; Ehrlich, 2000). Hypermethylation of CpG islands generally lead to the loss of expression of the genes possessing this structure at their 5′ end (Tate and Bird, 1993). The methylation status of tumor suppressor genes has been extensively investigated and such alterations have been reported in many human tumors (Robertson and Jones, 2000). Recently, an extensive study of about 1200 CpG islands has indicated that hypermethylated CpG islands are not randomly distributed and the patterns of the hypermethylation might be specific of subclasses of cancers (Costello *et al*., 2000).

Although the precise mechanisms involved in these phenomena remains to be determined, changes in DNA-methyltransferase activities have been suggested to play a role (Baylin *et al*., 1997). In cancer cells, significant variations of the different DNA methyltransferases (DNMT1, 2, 3A and 3B) expression have been observed. Moreover, mutations of the DNMT3B gene which are associated with DNA hypomethylation and chromosomal rearrangements in the recessive inherited ICF (immunodeficiency, centromeric region instability and facial abnormalities) syndrome (Xu *et al*., 1999) might be also involved in cancer-associated hypomethylations. At the opposite end of this process, the Methyl-CpG Binding Proteins (MBDs) are important constituent of the DNA methylation machinery, since they are directly involved in the mediation of the epigenetic signal (Bird and Wolffe, 1999). For example, it has been recently shown that, in colon cancer cell lines, the methyl-CpG binding protein MBD2 is associatd with the aberrantly methylated promoters of silent *p14*/*p16* genes and this methylation-dependent association seems to be responsible for their silencing (Magdinier and Wolffe, 2001).

The various mechanisms leading to the methylation-dependent down regulation of the transcription remain

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to be fully determined. However several lines of evidences indicate that, among them, the targeting of histone deacetylase (HDACs) complexes mediated by methyl-CpG binding proteins plays a major role (Bird and Wolffe, 1999).

The five MBD proteins identified to date share the functional Methyl Binding Domain (MBD domain) (Hendrich and Bird, 1998). Four of them are associatd with the transcriptional repression of methylated templates in vertebrates and bind methylated DNA without sequence specificity but exhibit several distinct features (Wade, 2001). MeCP2, the most studied member of the MBD protein family, is known as a transcriptional repressor for a long time (Nan *et al*., 1997) and it has been shown recently that MeCP2 interacts with Sin3A and recruits the HDAC1 complex (Nan *et al*., 1998; Jones *et al*., 1998). The role of MeCP2 in the formation of a transcriptionally repressive chromatin structure mediated by the HDAC complexes has been also demonstrated by the reversal effect of the inhibition of the deacetylase activities by the trichostatin A drug (TSA). However, *in vitro* transcription experiments indicate that MeCP2 may interact directly with the transcription machinery (Kaludov and Wolffe, 2000), suggesting that this protein might repress transcription, at least partially, independently of the HDAC pathway (Yu *et al*., 2000).

Three other members of the MBD protein family, MBD1, MBD2 and MBD3 are also associated with HDAC complexes. MBD3 is part of the Mi2/NuRD complex (Hendrich *et al*., 2001) which is targeted to methylated templates in the MeCP1 complex by MBD2 (Ng *et al*., 1999; Wade *et al*., 1999). Although MBD2 might repress some promoters by a mechanism independent of the HDAC activity (Ng *et al*., 1999), the repressive effect of MBD2 can be relieved by inhibition of the HDAC activities as observed for MeCP2. The other member of this protein family, MBD1, also mediates a methylation-dependent transcriptional repression. Although the HDAC complex associated with this protein is not yet identified it seems different from those associated with MeCP2 and MBD2 (Ng *et al*., 2000).

Differences between these methylation-dependent repressors are also observed in their binding capacities. MeCP2 can recognize a single symmetrically methylated CpG (Meehan *et al*., 1992). MBD2 can bind, *in vitro*, to DNA sequences containing a few methylated CpGs (Hendrich and Bird, 1998), however, the MeCP1 complex containing this protein binds only to densely methylated DNA (Meehan *et al*., 1989). Invalidation of *MeCP2* in mice, indicates that MeCP1 cannot compensate for the absence of *MeCP2* (Guy *et al*., 2001; Chen *et al*., 2001) and conversely, invalidation of *MBD2* does not lead to the neurological disorders as observed in *MeCP2*-null mice mutants (Hendrich *et al*., 2001). The short survival time (2–3 months) of the *MeCP2*-null mice does not seem to be the result of developmental defect, but likely the consequence of the neuronal abnormalities due to *MeCP2* deficiency (Guy *et al*., 2001; Chen *et al*., 2001). The absence of compensation between these proteins, suggests that the MBDs may have specific roles in the cellular physiology. Furthermore, alterations of their expression patterns might be involved in the physiopathology of some human cancers, as described in digestive cancers, where variations of expression patterns and amounts of methylated CpGs may play a role in cancer progression (Saito *et al*., 2001; Kanai *et al*., 1999).

Although the expression patterns of the MBDs are not yet fully determined, it has been shown, in mouse and rat, that these genes are expressed at various levels depending on the cell type and the differentiation state. In mouse, *MeCP2* is expressed at a very low level in ES cells but *MeCP2* transcripts are detectable in differentiated embryoid bodies on day 10.5 of gestation (Tate *et al*., 1996). In adult somatic tissues, *MeCP2* expression level varies between tissues. For example the brain has the highest level and the testis the lowest level (Meehan *et al*., 1992). Mouse ES cells also exhibit a low level of *MBD2* RNAs and, in adult, tissue-specific expression patterns are observed as well (Hendrich and Bird, 1998). The reduced level, or the absence, of *MBD* RNAs in mouse ES cells is not very surprising, since invalidation of the DNMT genes, leading to a very low level of DNA methylation, does not affect ES cells viability (Li *et al*., 1992), suggesting that DNA methylation plays a minor role in the survival of these cells.

Taken together, these data indicate a profound modification/alteration of the DNA methylation machinery in human cancers, and variations of the expression of MBDs might be associated with methylation-dependent cancer progression. Indeed, cancer-linked DNA methylation disorders have been reported in very large numbers of tumor types (Baylin *et al*., 1997; Ehrlich, 2000) however little is known on the expression of these proteins in human cancers.

In order to get more insights on this question we have investigated the expression of *MeCP2* and *MBD2* in human breast carcinomas, a widespread disease where DNA methylation abnormalities have been frequently observed. *MBD* mRNA molecules have been quantitated in a series of human breast carcinomas and compared to their level in benign tumors and normal breast tissues using a competitive RT–PCR method. This method measures the absolute amount of a mRNA in a RNA sample and is not related to the amount of another 'control' mRNA, which may vary between samples. In addition the amounts of *MBD2* and *MeCP2* mRNAs were also monitored during normal mammary growth and differentiation in human fetal tissue samples.

Results

Validation of the RT–PCR method

The steady-state level of *MeCP2* and *MBD2* mRNAs was determined in cell lines and in human tissues using a competitive RT–PCR method allowing the determi-

nation of the number of mRNA molecules per μg of total RNA. For each competitor, amplification of serial dilution of the competitor RNAs was performed. The intensity of the corresponding bands was measured by densitometry and values obtained indicate that the signals were proportional to the log of the amount of the competitor RNAs within a wide range of concentrations (Figure 1a,c). Moreover the efficiency of the PCR amplification of cDNAs corresponding to *MeCP2* and *MBD2* RNA fragments and to the competitor RNAs were similar (data not shown). Independent determinations (2–5) from tissues and cell lines (17 samples) indicated that the variation per samples was inferior to 15%. Then, the mRNA level was determined after coamplification of decreasing amount of competitor RNAs and constant amount of total RNA from the samples. A representative assay is shown for *MBD2* (Figure 1b) and *MeCP2* (Figure 1d). The relative amounts of MBD2 proteins were evaluated in several human cell lines by immunoblotting using a polyclonal antibody (provided by Dr Paul Wade). Quantitation of the signals obtained indicated a ratio similar to that obtained in RT–PCR assays (Figure 2). Unfortunately, efforts for the determination

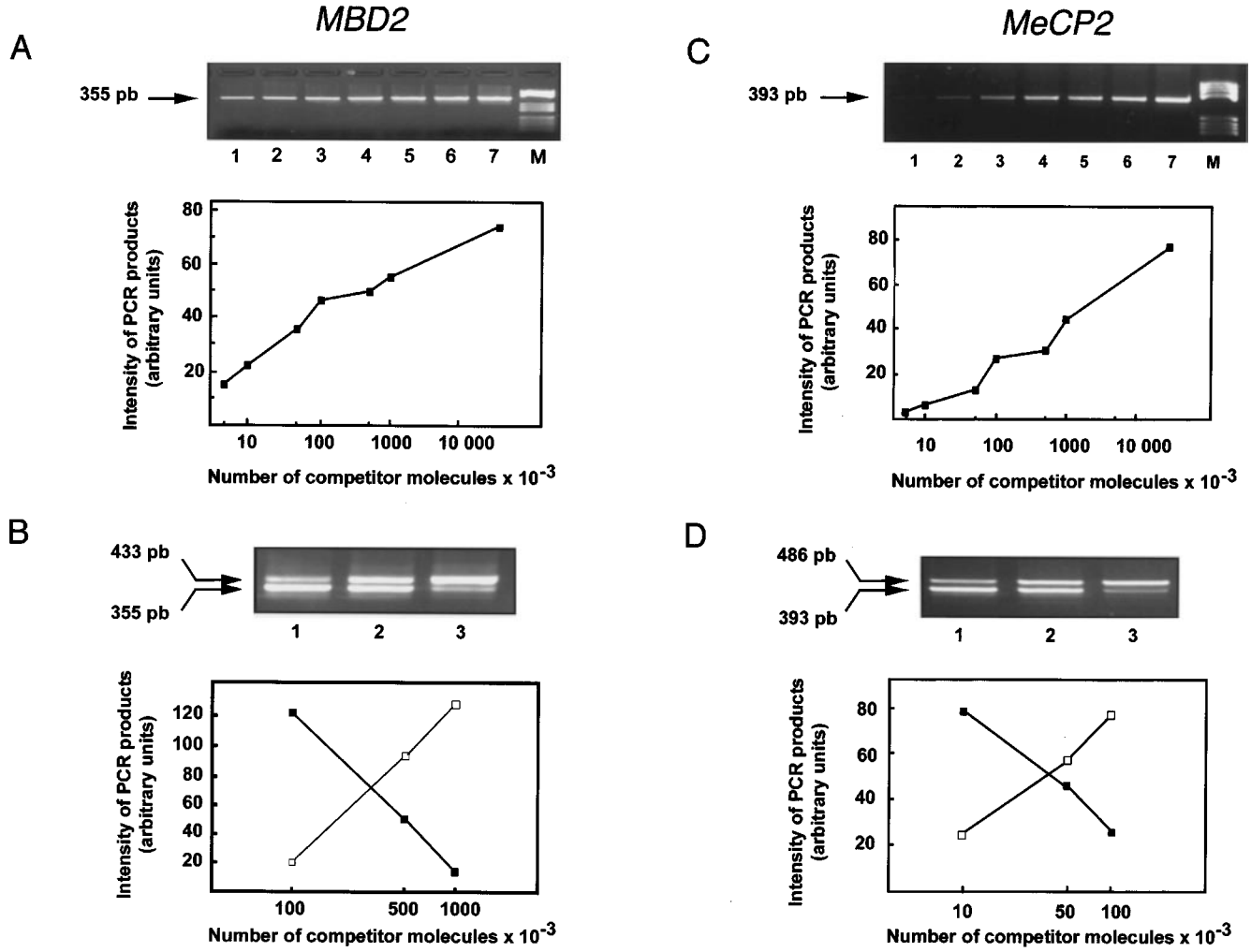

Figure 1 RT–PCR assay of *MBD* mRNAs. For each panel, 15 μl of the RT–PCR reaction (total volume 100 μl) were analysed on a 2% agarose gel containing ethidium bromide. The intensity of the bands corresponding to the PCR products was plotted against the initial number of competitor molecules. The diagrams of the intensity values are represented below the gels. (**a**) RT–PCR assay of *MBD2* mRNA was performed from serial dilution of competitor *MBD2*. The 355 pb band corresponds to the expected size of the PCR product. Initial concentrations of the competitor molecules are: lane 1, 5×10^3; lane 2, 10×10^3; lane 3, 50×10^3; lane 4, 100×10^3; lane 5, 500×10^3; lane 6, 1000×10^3; lane 7, $300\,000 \times 10^3$ molecules. (**b**) A quantitative RT–PCR analysis was performed from 0.1 μg of total RNA mixed with various amount of competitor *MBD2* RNA; solid square: 355-pb band, competitor *MBD2* RNA; open square: 433-pb band, wild type *MBD2* RNA. Amounts of competitor molecules, lane 1, 1000×10^3; lane 2, 500×10^3; lane 3, 100×10^3. (**c**) RT–PCR assay of *MeCP2* mRNA was performed from serial dilution of competitor *MeCP2* RNA. The 393 bp band corresponds to the expected size of the PCR products. Initial concentrations of the competitor molecules are: lane 1, 5×10^3; lane 2, 10×10^3; lane 3, 50×10^3; lane 4, 100×10^3; lane 5, 500×10^3; lane 6, 1000×10^3; lane 7, $300\,000 \times 10^3$ molecules. (**d**) RT–PCR reaction was performed from 0.3 μg of total RNA mixed with various amount of competitor *MeCP2* RNA. The 393 pb band (solid square) correspond to the competitor *MeCP2* RNA and the 486 pb band (open square) correspond to the wild type *MeCP2* RNA. The amounts of competitor molecules were: lane 1, 100×10^3; lane 2, 50×10^3; lane 3, 10×10^3

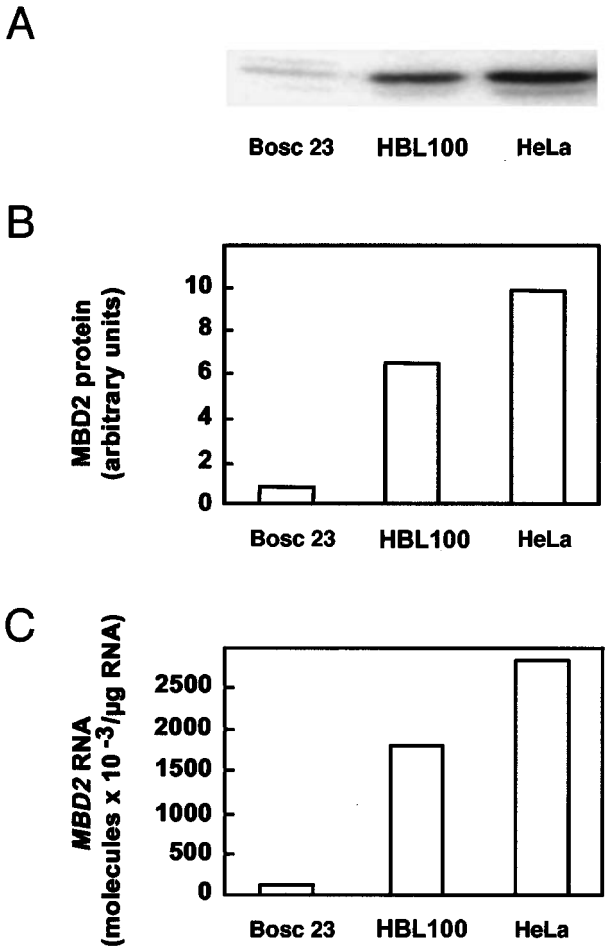

Figure 2 *MBD2* expression in human cell lines. (**a**) Immunoblot analysis of MBD2 in cell lines. Whole cell extracts (40 μg) from Bosc 23, HBL100 and HeLa cells were separated on a 10% SDS–polyacrylamide gel and transferred onto immobilon-PVDF membrane (Millipore, Saint Quentin, France). MBD2 proteins were probed using a rabbit polyclonal antibody against MBD2 protein (provided by Dr P Wade). (**b**) Amount of MBD2 protein. (**c**) Number of *MBD2* RNA molecules per μg of total RNA

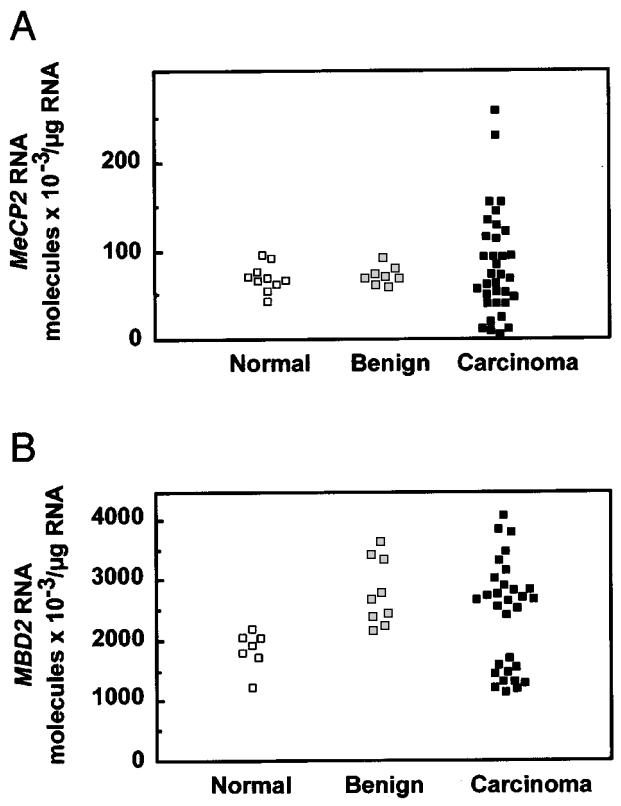

Figure 3 *MBD2* and *MeCP2* mRNA in normal, benign and neoplasic human breast tissues. (**a**) *MeCP2* mRNA; open squares: normal breast tissues; tinted squares: benign breast tumor tissues; solid squares: neoplasic breast tissues. Differences between normal ($72.71 \pm 15.26 \times 10^3$ copies/μg RNA), benign ($71.56 \pm 9.75 \times 10^3$ copies/μg RNA) and neoplasic ($82.68 \pm 58.47 \times 10^3$ copies/μg RNA) were not statistically significant. (**b**) *MBD2* mRNA. Open squares normal breast tissues; tinted squares benign breast tumor tissues; solid squares neoplasic breast tissues. The difference between benign tumors ($2706.75 \pm 524.49 \times 10^3$ copies/μg RNA) and normal breast tissues ($2055 \pm 387 \times 10^3$) is significant (Kruskal-Wallis test, $P = 0.01$). The breast carcinoma tissues samples fall in two groups statistically different ($P = 0.0001$), one group expressing *MBD2* at a high level ($2985.6 \pm 505.07 \times 10^3$ copies/μg RNA), and the other group at a low level ($1403 \pm 173.4 \times 10^3$ copies/μg RNA)

of the amounts of MeCP2 proteins were unsuccessful, the low level of the signals obtained in our immunoblotting experiments prevented an accurate determination of the relative amounts of these proteins in the samples analysed.

Variations of MeCP2 *expression are associated with malignancies and variations of* MBD2 *expression with proliferation, in adult human mammary tissues*

MeCP2 transcripts were quantitated in normal and pathological human breast tissues using the competitive RT–PCR method described above. In the tumoral samples analysed the mean value for *MeCP2* expression was not statistically different from those observed in benign tumors or normal breast tissue samples (Figure 3a). However, in benign tumors and normal tissues, the amounts of *MeCP2* transcripts are relatively homogenous, ranging from 61 to 93×10^3 molecules (coefficient of variation = 14) and from 54 to 96×10^3 molecules (CV = 19) respectively, whereas in neoplasic samples these values are scattered over a wide range, from 7 to 258×10^3 molecules (CV = 71), suggesting a deregulation of *MeCP2* expression in some breast cancer samples (Figure 3a). Although the variations of *MeCP2* expression are not correlated with the prognostic factors, it should be noted that the low level of *MeCP2* mRNAs was found in the histologic types containing differentiated or very differentiated cells corresponding to a favorable prognosis. Although this observation was done from a small number of samples ($n = 4$), the difference is statistically significant ($P = 0.02$) when compared to the benign or normal samples.

Alterations of MBD expression are not restricted to *MeCP2*. In benign breast tumors *MBD2* mRNA level is higher ($P = 0.001$) than that observed in normal

breast tissues (Figure 3b). Moreover, breast carcinomas also exhibit alterations of *MBD2* expression. When classified according to their *MBD2* expression level, the breast carcinoma samples can be divided in two groups (Figure 3b) statistically different ($P=0.0001$) when compared to normal breast tissue, one group expressing *MBD2* at a high level (2985.6×10^3 copies/μg RNA, $P=0.0001$) and the other at a low level (1403×10^3 copies/μg RNA, $P=0.003$).

The alterations of *MeCP2* and *MBD2* expression in pathological breast tissues prompted us to investigate the potential association between *MBD*s expression and the biological and clinical parameters of the invasive breast carcinoma not otherwise specified (NOS), the most common histological type of breast cancer (Table 1). When breast carcinomas are classified according to their size, a significant ($P=0.03$) association between the size of the tumor and level of *MBD2* expression was observed, large tumors expressing *MBD2* at a higher level. Therefore, these data might suggest that *MBD2* expression could be associated with the proliferation rate of the tumoral cells. Although some subclasses correspond to a very limited number of samples, no obvious association was detected between the expression of *MeCP2* and *MBD2* and the other prognostic factors. However, the amount of *MBD2* transcripts is higher in estrogen receptor positive tumors than in estrogen receptor negative tumors ($P=0.06$). In contrast *MBD2* expression level is not associated with the expression of progesterone receptors, which is a marker of fully functional estrogen receptors in breast tissues, suggesting that the estrogen receptors are not directly involved in the control of *MBD2* expression.

Since up-regulation of *MBD2* is observed in benign tumors and is also associated with the size of the tumors in the samples analysed, these data suggest that the up-regulation of *MBD2* is associated with breast tissue proliferation.

Histone deacetylase activity in human breast tumor samples

The repressive effect of MeCP2 and MBD2 on gene transcription is mainly mediated by the recruitment of HDAC complexes to methylated DNA. We have therefore investigated, in a limited number of samples exhibiting various levels of MBD2 and MeCP2 expression, the HDAC activities using a synthetic substrate (Zhou *et al*., 2001). The deacetylation of the acetylated lysine of Fluor de Lys, which comprises an acetylated lysine side chain, was proportional to the amount of total protein from nuclear extract over a wide range of concentrations (0.3 – 3 μg/assay) and fully inhibited by TSA (final concentration: 5 nM), an inhibitor of HDAC activities (data not shown). In the panel of breast tumor samples analysed there is no meaningful association between HDAC activity level and *MBD2* or *MeCP2* expression levels (Figure 4), suggesting that the deregulation of *MBD2* and *MeCP2* is not a consequence of an alteration of HDAC activity in human breast tumors.

Up-regulation of MeCP2 *and* MBD2 *during human fetal mammary gland development*

During fetal life, development of the human mammary gland is a relatively late event, the mammary bud becomes well visible between the 6th and 7th week. The last stage, between the 20th and 33rd weeks of embryonic life, corresponds to the canalization by desquamation and lysis of the central epithelial cells of these structures (for review, see Russo and Russo, 1987). After the canalization stage, the mammary gland follows the general growth of the body and remains unmodified until the approach of the puberty (Tanner, 1962). The terminal differentiation is attained during the adult life at the term of pregnancy.

Table 1 Prognostic factors and *MBD2* mRNA level in patients with invasive breast ductal carcinoma NOS

	Patients No	MBD2 *mRNA*	P	MeCP2 *mRNA*	P
Tumor Size (cm)			0.03		0.69
$0<3$	11	2218 ± 654		92.1 ± 67	
$\geqslant3$	5	2985 ± 825		73.5 ± 53	
Nodal status			0.89		0.88
0	9	2384 ± 711		95.3 ± 73	
1 – 3	4	1933 ± 669		87.7 ± 77	
>3	2	3096 ± 89		84.4 ± 66	
Histological grade of Scarff, Bloom and Richardson			0.81		0.9
I	1	1715		55	
II	11	2488 ± 760		101.64 ± 57	
III	4	2240 ± 1163		110.2 ± 80	
Estrogen receptor status			0.06		0.56
<10 fmol/mg protein	7	1815 ± 869		86.6 ± 78	
$\geqslant10$ fmol/mg protein	7	2872 ± 1676		103.1 ± 80	
Progesterone receptor status			0.17		0.84
<10 fmol/mg protein	7	2201 ± 845		100 ± 91	
$\geqslant10$ fmol/mg protein	7	2788 ± 892		86.9 ± 60	

MBD2 mRNA and *MeCP2* mRNA levels are given as the number of mRNA molecules $\times10^{-3}$ per μg of total RNA. Results are expressed as the mean $\pm$ s.d. Significance of differences between each subgroup was assessed by the Kruskal-Wallis test

The analysis of breast tissue samples indicated that the expression of *MeCP2* and *MBD2* is altered during the pathological proliferation of breast tissue suggesting an involvement of the MBDs in the proliferation of the mammary epithelium. In order to investigate the steady-state level of *MeCP2* and *MBD2* mRNA level during normal cellular proliferation, the level of expression of both genes was determined in human fetal breast samples spanning the different stages of prenatal breast development between the 20th and the 30th week of gestation.

MeCP2 and *MBD2* are expressed at a low level during the first steps analysed (20th–22nd week of gestation). At the 20th week of gestation, the amount of *MeCP2* transcripts is sixfold lower than the mean value observed in adult tissue. Then the amount increases progressively during the mammary gland development (Figure 5) and reaches the value observed in the adult breast tissue at the 31st week of gestation.

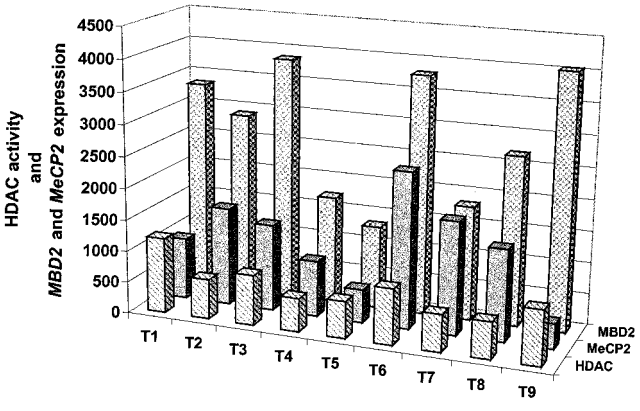

Figure 4 HDAC activity assays in human breast tumor tissues. Hatched bars: HDAC enzymatic assays were performed using nuclear extracts from frozen breast tumor tissues, and normalized to the total amount of protein used in the assay. For each tumor samples analysed (T1 to T9); tinted bars: the number $\times 10^2$ of *MeCP2* mRNA molecules per μg of total RNA; and chequered bars: the number $\times 10^3$ of MBD2 mRNA molecules per μg of total RNA are indicated

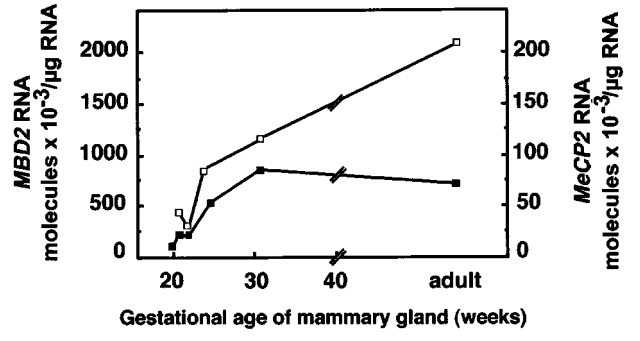

Figure 5 RT–PCR assays of *MBD2* and *MeCP2* transcripts during human mammary gland development. Solid squares: *MeCP2* RNA molecules per μg of total RNA; open squares: *MBD2* RNA molecules per μg of total RNA. Data obtained for each point (2–4 independent assays) indicated that the variations were inferior to 15%

In the case of *MBD2*, a strong enhancement (4–5-fold) of its expression is also observed, but the level of *MBD2* transcripts remains, in the various fetal stages, lower than the value observed in the adult mammary gland.

Taken together these data indicate that both MBDs are up-regulated during human fetal mammary gland development, with some differences between *MeCP2* and *MBD2*. At the end of the mammary gland morphogenesis (33rd week of gestation) the amount of *MeCP2* transcripts is equivalent to the amount found in the adult mammary gland, while *MBD2* is expressed at a level lower than that observed in the adult, suggesting that *MBD2* expression might be enhanced during the subsequent steps of the growth of the mammary gland.

Discussion

The determination of the absolute number of specific mRNA molecules allows both a direct comparison between samples and a comparison of the steady-state level of expression for different genes. Data obtained using this method indicate that *MBD2* transcripts are 20–30-fold more abundant than *MeCP2* mRNAs in the human adult mammary gland (29 ± 11, C V = 39). This difference is also observed during the first steps of the fetal development of the gland despite a low level of *MeCP2* and *MBD2* expression, suggesting that this ratio might be important in the mammary gland physiology. In line with this hypothesis, it should also be noted that this ratio (51 ± 49, C V = 97) is profoundly altered during the pathological growth of the gland.

In benign breast tumors, *MeCP2* is expressed at a level similar to that observed in non-pathological samples, whereas *MBD2* is up-regulated. This absence of coordinated alterations is also observed in cancer breast tissue samples. Furthermore, the histologic types corresponding to a more favorable prognosis which contain differentiated or very differentiated cells, exhibit a low level of *MeCP2* transcripts, while the amount of *MBD2* mRNAs is distributed in these samples over a wide range of values. When compared to normal breast, a high level of *MBD2* expression is observed in large-diameter tumors and in ER-rich tumors, whereas *MeCP2* is not associated with these clinical parameters. These data suggest that high level of *MBD2* expression might be associated with abnormal cellular proliferation.

Alterations of MBDs expression have been also observed in other human cancers using a semi-quantitative RT–PCR method in which the expression of the gene was normalized to the *GAPDH* (Kanai *et al*., 1999; Saito *et al*., 2001). These authors have reported a reduced level of *MBD2* transcripts in colorectal and stomach cancers (Kanai *et al*., 1999) and during hepatocarcinogenesis (Saito *et al*., 2001). In addition the relative level of *MeCP2* expression seems

to be associated with some subclasses of human hepatocellular carcinoma (Saito *et al*., 2001).

Taken together these data suggest that the alteration of *MBD*s expression is a relatively common event in human cancers. However, the *MBD*s expression patterns might be specific of the cancers analysed. In breast carcinomas, up- and down-regulation are observed for both *MBD*s, and some tumors exhibit a normal level of *MeCP2* transcripts. In contrast coordinated up-regulation of *MeCP2* and *MBD2* occurs during human prenatal mammary gland development, suggesting that the alterations of the *MeCP2* and *MBD2* expression, observed in breast tumors, are not only the result of the growth of the mammary gland but might be associated with some of the pathological events leading to abnormal cellular proliferation.

Other alterations of the DNA methylation machinery have been already observed in breast cancers but, as described in this study for the expression of the MBDs, no correlation between the global DNA methylation status and clinical or biological parameters has been observed (Bernardino *et al*., 1997). As suggested for the involvement of DNA hypomethylation in breast cancers (Bernardino *et al*., 1997), the variation of *MBD*s expression level might represent an independent parameter of tumor progression. More recently, an overall deregulation of the DNA methylation control, during esophageal carcinogenesis, has also been suggested from the analysis of a large number of CpG islands (Eads *et al*., 2001). Thus, the alteration of *MeCP2* and *MBD2* expression, which are a consistent characteristic of the breast tumors analysed, might indicate that the alteration of the DNA methylation is not limited to the distribution of the methyl-groups but might extend to some of the proteins mediating the methylation signal.

During the last decade, the emphasis in studies of DNA methylation has focused mainly on the distribution of methylated CpG throughout the genome associated with either hypermethylation and silencing of tumor suppressor genes or hypomethylation and genomic instability. However, the epigenetic control of gene regulation is also driven by numerous proteins such as the DNA methyltransferases and the methyl-binding proteins and a potential cause of abnormal methylation in cancer could be an inappropriate expression or targeting of the different proteins.

The physiological consequence of a deregulation of *MeCP2* and *MBD2* expression remains to be determined. In mouse homozygous invalidations of *MeCP2* or *MBD2* result in neurological disorders, suggesting that these ubiquitously expressed genes have a prominent role in cerebral functions and a minor role in other tissues or organs (Guy *et al*., 2001; Chen *et al*., 2001; Hendrich *et al*., 2001). However, it should be mentioned that the alteration of *MeCP2* and *MBD2* expression is associated with aberrant changes of the methylation patterns in cancers.

In addition it has been shown that the Mi2/NuRD corepressor complex can be targeted to methylated DNA by other proteins than MBD2 (Hendrich *et al*., 2001) not yet fully characterized. The presence of other 'MeCP1' complexes, might explain why misexpression of endogenous methylated genes was not detected in *MeCP2* and *MBD2* deficient mice, since it had been suggested that independent repressors might cooperate to repress methylated genes (Hendrich *et al*., 2001). The full understanding of the physiological consequences of the alterations of *MBD*s expression awaits, therefore, a complete description of the methylation-dependent gene silencing mechanisms.

Nevertheless the alterations of expression of the *MBD* genes observed in human cancers, and the differences between the type of cancers and the *MBD* analysed, suggest that the expression patterns of genes coding for protein mediating the methylation signal might be an important parameter in human cancers.

Materials and methods

Cell lines

Human breast cell lines (HBL100), cervix cell line (HeLa) or kidney cell line (Bosc 23) were obtained from ATCC (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (Sigma, L'Isle Dabeau, France) supplemented with 5% fetal calf serum. All cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Sample preparation

For both normal and pathological samples, specimens were fixed in formalin, paraffin embedded, sectioned and stained with hemaluneosin-safran. Breast tumors were classified according to the WHO classification of breast tumors (Poulsen *et al*., 1975). Thirty-four neoplasic lesions were examined for their *MeCP2* mRNA content, including invasive ductal carcinomas NOS ($n=26$), apocrine carcinoma ($n=1$), lobular carcinomas *in situ* ($n=1$), medullary carcinomas ($n=1$), invasive lobular carcinomas ($n=3$), invasive mucinous carcinomas ($n=1$) and invasive ductal carcinomas with predominantly *in situ* component ($n=1$). Thirty neoplasic lesions were examined for *MBD2* expression: invasive ductal carcinomas NOS ($n=24$), apocrine carcinoma ($n=1$), medullary carcinomas ($n=1$), invasive lobular carcinomas ($n=3$) and invasive ductal carcinomas with predominantly *in situ* component ($n=1$). In the Table 1 only invasive ductal carcinomas NOS samples, in which at least four of five parameters (tumor size, nodal status, grade and hormonal receptor status) were available, are described. Benign tumors ($n=8$ for *MeCP2* and $n=9$ for MBD2) are fibroadenoma samples. Human mammary glands from 10 normal aborted female fetuses, ranging in gestational age from 20 to 33 weeks were snap frozen immediately after removal and stored in liquid nitrogen until used. Normal breast tissue was obtained from patients undergoing mammoplastic surgery.

RNA extraction

RNA was extracted from frozen samples and cell lines with the RNeasy kit (Qiagen, Courtaboeuf, France). After

extraction, the integrity of total RNA was examined on a 1.2% agarose gel containing 1 μg/ml ethidium bromide and quantified by densitometry using the fluorimeter Fluor's and the Quantity One software (Biorad, Ivry, France) in comparison with serial dilutions of a standard RNA (Roche Molecular Biochemicals, Meylan, France).

RT–PCR assay

cDNA fragments of *MeCP2* (from position 9 to position 495, Gen-Bank™ accession number X99686) and *MBD2* (from position 789 to 1210, Gen-Bank™ accession number AF120989) were amplified by RT–PCR using, respectively: forward 5′-TTTGATGTGACCTGTGACTC-3′, reverse 5′-CGCAATCAACTCCACTTTAG-3′ and forward 5′-TCAGAAGCAAGCCTCAGTTG-3′, reverse 5′-CAGAGCTTGTGTGCAAAGCA-3′. After cloning the PCR products into a pGEM-T easy vector (Promega, Lyon, France), a 92-pb long deletion was performed within the *MeCP2* cDNA using *Hind*III and a 78-pb long deletion within *MBD2* using *Sau3*AI. The two competitors RNAs were synthesized using the *Sp6 RNA* polymerase according to the instructions of the manufacturer (Promega, Lyon France). After purification (*Dnase*I digestion and RNeasy purification (Qiagen, Courtaboeuf, France)), these competitor RNAs were quantified by densitometry using the Fluorimeter Fluor's and the Quantity One software (Biorad, Ivry, France), and then diluted in the presence of yeast tRNA as a carrier (40 ng/μl).

Equal amounts of total RNA samples (0.3 μg for *MeCP2* assay and 0.1 μg for *MBD2* assay) were coamplified with increasing amounts of competitor RNAs in a final volume of 100 μl containing 10 mM Tris-HCl, 3 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml Gelatin, 200 μM each of the four deoxynucleoside triphosphates and 0.25 μM of each primers. After an initial denaturation at 94°C for 2 min, 6 units of Expand Reverse Transcriptase (Roche Molecular Biochemicals, Meylan, France) were added to the reaction mixture and incubated for 35 min at 42°C. Reverse transcriptase was inactivated by heating at 94°C for 4 min, and then cooling to 0°C. PCR amplification of the cDNA was accomplished by adding 0.6 unit of *Taq* DNA polymerase (Roche Molecular Biochemicals, Meylan, France). The PCR amplification was accomplished after 35 cycles in a thermocycler under the following conditions: 1 min denaturation at 94°C, 2 min annealing at 55°C for *MeCP2* cDNA amplification and 62°C for *MBD2* cDNA amplification and 3 min extension at 72°C. In addition, control experiments for each competitor RNA were performed by omitting the Reverse Transcriptase to ensure that the signal was the result of RNA and not DNA amplification. PCR products were analysed on a 2% agarose gel containing 1 μg/ml ethidium bromide and quantitated by densitometry as described.

Then, the normalized signals corresponding to the target mRNA and the competitor were plotted against the initial number of competitor molecules added to the test tubes. The abscissa of the intersection of the curves represents an estimation of the equivalence point between the initial amount of the competitor molecules and the number of copies of the mRNA assayed (Ribieras *et al*., 1997).

Protein extraction and Western blot analysis

Whole cell lysates were prepared and analysed by Western blot (Magdinier *et al*., 1999). After quantitation of total proteins by Bradford coloration (Biorad, Ivry, France), 40 μg of total protein for each cell line were electrophoresed through a SDS–polyacrylamide gel, transferred onto a PVDF membrane (Immobilon-P, Millipore, Saint Quentin, France) and blocked for 1 h with Tris-buffered saline Tween 20 solution (0.5%) containing 5% non-fat milk powder. The equivalence of gel loading and the quality of transfer were controlled by Ponceau Red S staining. For the detection of the MBD2 protein, a rabbit polyclonal antibody against MBD2 protein (provided by Dr P Wade) was diluted 1/2000. The secondary anti rabbit-HRP conjugate antibody were diluted 1/2500. The immunocomplexes were detected using the ECL system (Amersham Pharmacia Biotech, Saclay, France). To quantify the relative changes in the protein levels, autoradiograms were scanned and the intensity of each band was quantified by densitometry using the fluorimeter Fluor's with the Quantity One software (Biorad, Ivry, France).

HDAC activity assay

Nuclear extracts were prepared from frozen breast tumor samples (Arnaud *et al*., 1985) and assays were performed using, the HDAC fluorescent activity assay kit according to manufacturer's instructions (Biomol, Tebu, Paris, France) and a CytoFluor microplate spectrofluorometer (PerSeptive Biosystems) with excitation at 360/340 nm and emission at 460/444 nm.

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