MeCP2 and MBD2 expression during normal and pathological growth of the human mammary gland
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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 neoplastic 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 neoplastic 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, and 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.

**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 associated 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...
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 associated
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 M2/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 promotors 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 tran-
scriptional 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 methyl-
ated CpG (Meehan et al., 1992). MBD2 can bind, in vitro,
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 expres-
sion 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 differen-
tiated 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 ma-
achinery in human cancers, and variations of the
expression of MBDs might be associated with methyla-
tion-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 differen-
tiation 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 MBD2 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 × 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 were: 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 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 × 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.
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 × 10³ molecules (coefficient of variation = 14) and from 54 to 96 × 10³ molecules (CV = 19) respectively, whereas in neoplastic samples these values are scattered over a wide range, from 7 to 258 × 10³ 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 samples (P = 0.001), one group expressing MBD2 at a high level (2985.6 ± 505.07 × 10³ copies/μg RNA), and the other group at a low level (1403 ± 173.4 × 10³ copies/μg RNA).
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 \(\text{MBD2 at a high level (2985.6} \times 10^3\text{ copies/\mu g RNA, } P = 0.0001\text{) and the other at a low level (1403} \times 10^3\text{ copies/\mu g RNA, } P = 0.003\).

The alterations of MeCP2 and MBD2 expression in pathological breast tissues prompted us to investigate the potential association between MBDs 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 \(\text{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 subclases 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 \(\text{MBD2 transcripts is higher in estrogen receptor positive tumors than in estrogen receptor negative tumors } (P = 0.06).\) In contrast \(\text{MBD2 expression level is not associated with the expression of progestereone receptors, which is a marker of fully functional estrogen receptors in breast tissues, suggesting that the estrogen receptors are not directly involved in the control of MBD2 expression.\)

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

### 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 \((0.3–3 \mu g/assay)\) 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 \(\text{MBD2 and MeCP2 is not a consequence of an alteration of HDAC activity in human breast tumors.}\)

### 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

<table>
<thead>
<tr>
<th>Patients No</th>
<th>MBD2 mRNA</th>
<th>P</th>
<th>MeCP2 mRNA</th>
<th>P</th>
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<tr>
<td>Tumor Size (cm)</td>
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<td></td>
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<tr>
<td>0&lt;3</td>
<td>11</td>
<td>2218±654</td>
<td>0.03</td>
<td>92.1±67</td>
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<td>≥3</td>
<td>5</td>
<td>2985±825</td>
<td>73.5±53</td>
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<tr>
<td>Nodal status</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>0</td>
<td>9</td>
<td>2384±711</td>
<td>0.89</td>
<td>95.3±73</td>
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<tr>
<td>1–3</td>
<td>4</td>
<td>1933±669</td>
<td>87.7±77</td>
<td></td>
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<tr>
<td>≥3</td>
<td>2</td>
<td>3096±89</td>
<td>84.4±66</td>
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</tr>
<tr>
<td>Histological grade of</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Scarff, Bloom and Richardson</td>
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<tr>
<td>I</td>
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<td>1715</td>
<td>0.81</td>
<td>55</td>
</tr>
<tr>
<td>II</td>
<td>11</td>
<td>2488±760</td>
<td>101.6±57</td>
<td>110.2±80</td>
</tr>
<tr>
<td>III</td>
<td>4</td>
<td>2240±1163</td>
<td>0.06</td>
<td>86.6±78</td>
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<td>Estrogen receptor status</td>
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<tr>
<td>&lt;10 fmol/mg protein</td>
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<td>1815±869</td>
<td>0.17</td>
<td>100.9±80</td>
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<td>103.1±80</td>
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<tr>
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<td>7</td>
<td>2788±892</td>
<td>86.9±60</td>
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</table>

\(\text{MBD2 mRNA and MeCP2 mRNA levels are given as the number of mRNA molecules} \times 10^{-3} \text{ per } \mu g \text{ of total RNA. Results are expressed as the mean} \pm \text{ s.d. Significance of differences between each subgroup was assessed by the Kruskal-Wallis test.}\)
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.

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 MBDs expression is a relatively common event in human cancers. However, the MBDs expression patterns might be specific of the cancers analysed. In breast carcinomas, up- and down-regulation are observed for both MBDs, 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 MBDs 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 MBDs 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% CO2 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 neoplastic 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 neoplastic 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 mammoplasty surgery.

RNA extraction
RNA was extracted from frozen samples and cell lines with the RNeasy kit (Qiagen, Courtaboeuf, France). After
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’-TTTGATGTAGCTGACTGACT-3’, reverse 5’-CGCAAATACCCTCCTTAG-3’ and forward 5’-TCAGTCAAGCAGCCCTAGTG-3’, reverse 5’-CAGAGCCTTTG-TGCAAGAAGCA-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 HindIII and a 78–pb long deletion within MBD2 using Sau3AI. 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, Courtabœuf, France)), these competitor RNAs were 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).

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 assays (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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