Tissue and site-specific methylation correlates with expression of the mouse lactoferrin gene

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ABSTRACT

We have previously examined the regulatory region of the mouse lactoferrin gene and have identified sequences essential for basal and hormonally induced expression. In this study, we explore the relationship between the methylation state of the mouse lactoferrin gene promoter and its expression in selected mouse tissues. In a transient expression system, transcriptional activity was blocked after in vitro methylation of the regulatory region of the mouse lactoferrin gene. In addition, the in vivo methylation state of three promoter region sites was assessed using Southern blot analysis of DNA digested with methylation-insensitive and -sensitive restriction enzymes. The results showed that site −455, upstream of the mouse lactoferrin estrogen response module, was highly unmethylated in DNA from both hormone-treated and -untreated mouse lung, liver, and spleen tissues. Also, in both treated and untreated samples, the −54 site is uniquely highly unmethylated in liver DNA, while the −22 site is unmethylated in spleen DNA. Northern blot analysis showed lactoferrin expression in tissues that were unmethylated at a minimum of two sites. These results show that the alteration of the methylation status of the three sites are tissue-specific and are associated with constitutive expression of lactoferrin.

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INTRODUCTION

Lactoferrin was first characterized as a major iron-binding glycoprotein in milk (Masson & Heremans 1971). It has since been identified as a major gene product in estrogen-stimulated mouse uterine tissue (Pentecost & Teng 1987). Levels of lactoferrin mRNA increase significantly in immature mouse uterine tissue after treatment with the synthetic estrogen, diethylstilbestrol (DES). The molecular mechanisms by which estrogen acts on the mouse lactoferrin promoter is well characterized. An estrogen-responsive element (ERE) overlaps with a chicken ovalbumin upstream promoter (COUP) element to form the mouse lactoferrin estrogen response module (Liu & Teng 1992). Both the estrogen receptor and the COUP transcription factor bind to these elements to modulate lactoferrin expression in mouse uterine tissue. It has also been shown that lactoferrin expression is dependent upon serum estrogen levels during the mouse estrous cycle (Newbold et al. 1992, Walmer et al. 1992) and overexpression is frequently found in human endometrial adenocarcinomas (Walmer et al. 1995).

Previous work in our laboratory revealed differences in the degree of methylation of several restriction enzyme sites in the lactoferrin gene in genomic DNA isolated from leukemic cells of cancer patients, human breast cancer and leukemia cell lines (Panella et al. 1991). In addition, changes in lactoferrin expression were detected by immunocytochemical staining of these tissues. These data suggested that there may be a link between the methylation state of the lactoferrin gene and its expression.

In the present study, we explore the relationship between the methylation state of the mouse lactoferrin gene and its expression in selected mouse tissues taken from animals that were treated or not treated with DES. We tested the effect of in vitro methylation on reporter gene expression and used methylation-sensitive and -insensitive enzymes to determine the methylation status of three CCGG (MspI/HpaII) sites present in the promoter region of
the mouse lactoferrin gene. The results show that
in vitro methylation of the lactoferrin promoter
region decreases promoter activity and that the
differences in methylation of the MspI/HpaII sites is
tissue-specific and does not change with DES treat-
ment. With the exception of the uterus DNA
samples, we also found that lactoferrin expression
correlated with high levels of unmethylated DNA.
The results suggest an inverse correlation between
the degree of methylation and the degree of constitu-
tive expression in both experimental systems.

MATERIALS AND METHODS

Animals

Adult female CD-1 mice (Crl: CD-1 (ICR) BR)
(Charles River Laboratories, Raleigh, NC, USA)
together with 11-day-old female pups were housed
together in the animal facility until the pups were 18
days old. At that time, the pups were weaned and
they were injected with DES in corn oil (10 µg/kg
body weight) on days 18, 19, and 20. On day 21, the
pups were killed and tissues were collected on dry
ice for DNA samples or in liquid nitrogen for RNA
isolation. All tissue samples were stored in liquid
nitrogen cryotanks until further processing.

Plasmid constructs and DNA probes

Promoter fusion constructs containing fragments of
the mouse lactoferrin promoter and the CAT
reporter gene were previously described (Liu
& Teng 1991). The 2·6 CAT construct contains
promoter sequences −2644 to −21; the 1·7 CAT
construct contains sequences −1739 to −21 and
0·6 mouse lactoferrin (mLF)-CAT contains se-
quences −589 to −21. The CAT control vector
(Promega, Madison, WI, USA) which contains
SV40 promoter and enhancer sequences was used as
a control. Two probes were used in the Southern
blot analysis of the methylation state of the
MspI/HpaII sites in the mouse lactoferrin pro-
moter. Probe 1, a 188 bp PCR fragment containing
promoter sequences −350 to −537 (Liu & Teng
1991), was used in determining the methylation of
the MspI/HpaII site at −455. Probe 2, a 600 bp
HincII/SmaI fragment containing promoter se-
quences −589 to −22 (Liu & Teng 1991), was used
in determining the methylation of MspI/HpaII
sites at −54 and −22. The lactoferrin cDNA,
T267 (Pentecost & Teng 1987), was used to analyze
the methylation state of the lactoferrin gene with
Southern blot and to assess lactoferrin expression
by Northern blot. Mouse PL-7 (ribosomal protein
PL7 cDNA, bp 249–629; Meyuhas & Klein 1990)
was used as a loading control for the Northern blots.

Cell culture, DNA transfection, and CAT
assay

Prior to DNA transfections, 10 µg plasmid DNA
were treated with 1 unit/µg AluI, HaeIII, HhaI,
HpaII, or SssI methylases (New England Biolabs,
Beverly, MA, USA). Complete methylation of
treated plasmids was confirmed by restriction
enzyme digestion for each methylase target site.
After methylation, the DNA was extracted with
phenol:chloroform/isomyl alcohol (1:1), ethanol
precipitated and then transfected into RL95–2
cells (CRL 1617; American Type Cell Culture,
Rockville, MD, USA). Cell culture, transfection
and CAT assay were performed as previously
described (Shi & Teng 1996).

Southern blot hybridization

Uterus, lung, liver, brain, kidney and spleen tissues
were dissected from 21-day-old female CD-1 mice
which were treated or not treated with DES. The
tissues were stored on dry ice following extraction
and subsequently stored in liquid nitrogen until use.
Tissues were pulverized and then homogenized in
digestion buffer (100 mM NaCl, 10 mM Tris–HCl,
PH 8, 25 mM EDTA, PH 8) at 1·2 ml per 100 mg
tissue. The DNA was extracted conventionally
(Ausubel et al. 1990), and digested with heat activat-
ed 1 µg/ml RNase (Ribonuclease A, Worthington
Biochemical, Lakewood, NJ, USA) in the presence
of 0·1% SDS at 37 °C for 1 h before use. DNA
samples (10 µg) were digested overnight (>16 h)
at 37 °C with MspI or HpaII to assess DNA
methylation. Subsequent additional restriction
digests were carried out as indicated (see figure
legends). Samples were separated by electrophoresis
on 1% agarose gels, transferred to nylon membranes
(Gene Screen, NEN Life Science Products,
Gaithersburg, MD, USA), and hybridized over-
night at 42 °C with 106 c.p.m./ml 32P-labeled DNA
probe conventional hybridization solution (Ausubel
et al. 1990). Membranes were washed and exposed
to X-ray film (Hyper Film MD, Amersham,
Arlington Heights, IL, USA) at −70 °C. The band
intensities on autoradiograms were quantified by
densitometric scanning with the IS-100 Digital
Imaging System (Alpine Inotech Corp., San
Leandro, CA, USA) to determine the levels of
methylation.

Northern blot hybridization

Total RNA was isolated from the uterus, lung,
liver, brain, kidney, and spleen of 21-day-old
CD-1 mice using TRizol Reagent (Gibco BRL,
Gaithersburg, MD, USA). After pulverization and
homogenization of the tissue, the homogenate was extracted with chloroform and then precipitated by isopropanol. Twenty micrograms RNA were electrophoresed through a 1·2% agarose gel containing 6% formaldehyde and then transferred by blotting to a nylon membrane (Gene Screen, NEN Life Science Products). The membrane was prehybridized and then hybridized overnight as described above.

RESULTS

Hypermethylation by HpaII methylase decreases CAT

To establish the effect of the DNA methylation state on lactoferrin promoter function, constructs containing either 1·7 or 2·6 CAT promoter fragments (Liu & Teng 1991) were methylated in vitro with SssI methylase (recognizes CpG sites) and then transfected into RL95–2 cells. Figure 1A shows that hypermethylation with SssI methylase blocks digestion (no bands smaller than 2·3 kb) with methylation-sensitive HpaII (lanes 2, 4, and 6). The methylase treatment also abolishes the lactoferrin promoter activity (Fig. 1B, lanes 2 and 4), while activity of the control construct was unaffected (Fig. 1B, lane 6).

The effect of hypermethylation at specific restriction sites in the lactoferrin promoter on transcriptional activity was determined with a promoter fusion construct containing 0·6 kb of the promoter region (0·6 mLF-CAT). This promoter fragment contains the promoter and enhancer elements necessary for transcriptional activity and for estrogen responsiveness (Fig. 2A). It also includes the following restriction enzyme recognition sites: 3 AluI sites (AGCT), 5 HaeIII sites (GGCC), 1 HhaI site (GCGC) and 3 MspI/HpaII sites (CCGG). The 0·6 mLF-CAT plasmid was treated with AluI, HaeIII, HhaI, HpaII and SssI methylases that convert cytosine to 5-methylcytosine within each of their respective restriction enzyme recognition sequences and then transiently transfected into RL95–2 cells. Figure 2B shows a qualitative assessment of the transcriptional activity of this construct (0·6 mL14-CAT) and the control construct (CAT control) before and after treatment with the indicated methylase. Note that treatment with either HpaII or SssI methylase greatly decreases CAT activity (Fig. 2B, lanes 5 and 6) when compared with the no treatment control (lane 1). These results demonstrate that hypermethylation of MspI/HpaII sites in the mouse lactoferrin promoter strongly inhibits promoter activity in the transfected human endometrial carcinoma cell line. Other decreases in promoter activity are seen with AluI and HhaI methylase (Fig. 2B, lanes 2 and 4). The promoter activity of the CAT control plasmid (Fig. 2B, lanes 7–12) is mostly unchanged by each of the methylase treatments except for a slight decrease in activity by the HaeIII methylase (Fig. 2B, lane 9). These results strongly suggest that any in vivo methylation of MspI/HpaII sites within the lactoferrin promoter will affect lactoferrin gene expression.
The methylation state of the −455 MspI/HpaII site in the lactoferrin promoter is tissue-specific

The in vivo methylation state of the −455 site within the promoter region discussed above (0.6 mLF-CAT; Fig. 2A) was determined by Southern blot hybridization using methylation-insensitive MspI and -sensitive HpaII restriction enzymes. DNA samples isolated from mice that were treated or not treated with DES were used for the digests. Following digestion with MspI or HpaII, the samples were then digested with PstI to enable the production of fewer bands for analysis. Figure 3A shows the −455 MspI/HpaII site located 102 bp upstream from the COUP/ERE binding site and probe 1 which spans positions −537 to −350. After digestion with both MspI and PstI and hybridization to probe 1, bands of 216 bp and 454 bp are detected regardless of DES treatment (Fig. 3B, odd numbered lanes). By contrast, DNA digested with PstI and HpaII produced bands that were of higher molecular weight. Two large bands, 1299 bp and 1083 bp, appeared in addition to the band at 216 bp that was also produced with the MspI/PstI digest. Comparison of the hybridization signal of the 216 bp band between the MspI digests (Fig. 3, odd numbered lanes) and the HpaII digests (Fig. 3, even numbered lanes) reveals the demethylation state of the −455 site. As the methylation decreases at site −455, the 1083 bp band becomes more prominent as a result of digestion at that site by HpaII. Because the MspI/HpaII site at −909 was fully methylated in all mouse tissues, the 454 bp band that appeared in the MspI digests (Fig. 3, odd numbered lanes) is never present in the HpaII digests (Fig. 3, even numbered lanes).
even numbered lanes). The methylation state of the −1440 MspI site was not examined.

Since methylation state is linked to gene expression, and it is well established that lactoferrin expression in mouse uterine tissue is significantly induced by DES (Pentecost & Teng 1987), it is logical to hypothesize that the methylation state of the lactoferrin promoter in the DNA from uterine tissue would be affected by DES treatment. However, we found that the methylation state differed between tissues rather than with hormone treatment. The results show that the −455 site appears unmethylated in the lung, liver, and spleen samples when observing the 216 bp and 1083 bp bands (Fig. 3, lanes 6, 8, 10, 12, 22, and 24). However, treatment with DES did not noticeably increase the demethylation state of site −455 in these tissues. The demethylation state of the −455 site is greatest in the spleen than in other tissues examined when considering the intensity of the band at 216 bp and the increase in intensity of the band at 1083 bp (Fig. 3, lanes 21–24). Surprising were the levels of methylated DNA in the uterine samples. It was anticipated that the demethylation state would be higher especially with the DES-treated samples since lactoferrin expression in the uterus is significantly increased in the presence of DES. However the −455 site is highly methylated in the uterus. These results suggest that methylation state of the −455 MspI/HpaII site varies with tissue and does not change with hormone treatment.

Figure 3. Methylation state of the lactoferrin promoter −455 MspI/HpaII site. (A) Diagram of the mouse lactoferrin promoter showing the restriction sites and the expected set of fragments hybridizing to the probe resulting from complete or partial digestion at MspI/HpaII sites. The probe used for hybridization is indicated. (B) Southern blot analysis of DNA from 21-day-old CD-1 mice that were treated (DES) or not treated (Cont) with DES. Genomic DNA (10 µg) from mouse tissues was digested with MspI (M) or HpaII (H) and then PstI before electrophoresis in a 1% agarose gel.
The methylation state of the −54 and −22 MspI sites in the lactoferrin promoter is tissue-specific

The −54 and −22 MspI sites are on either side of the ATAAA sequence in the lactoferrin promoter (Fig. 4A). To determine the methylation state of the MspI −54 and −22 sites, Southern blot hybridization was performed using MspI and HpaII restriction enzymes. DNA samples were isolated from mice treated or not treated with DES. Following digestion with MspI or HpaII, the samples were then digested with XbaI to enable the production of fewer bands for analysis. Figure 4A shows the HincII/SmaI fragment, probe 2, that spans −589 to −21. The probe also includes the sequences found in probe 1 (Fig. 4A). After digestion with MspI and XbaI, three distinct DNA bands (including two doublets) of sizes 454/437 bp, 273/241 bp, and 160 bp are detected by probe 2 regardless of DES treatment (Fig. 4A and B, odd numbered lanes). The 273/241 bp bands result from cleavage at XbaI −295 and MspI −22/−54 respectively, and are not resolved by the gel system. In the other doublet, the 437 bp band includes coordinates −54 to +383 (MspI to XbaI site; Fig. 4A) and the 454 bp band has been described previously (see Fig. 3 and text). By contrast, DNA digested with HpaII and XbaI produces a higher molecular weight band. The band at 678 bp appears which consists of the 273/241 bp, 437 bp, and putative 405 bp fragment. Probe 2 does not hybridize the 405 bp fragment so that band never appears (Fig. 4A). Subsequently, if the −54 site is unmethylated a strong band appears at 437 bp and at the 273/241 bp doublet; and if the −22 site is unmethylated a strong band appears only at 273/241 bp since the accompanying 405 bp
fragment is not hybridized by probe 2. The fragment at 678 bp disappears in both cases. Comparison of the hybridization signal of the 273/241 bp band and the 437 bp band between the MspI digests (Fig. 4B, odd numbered lanes) and the HpaII digests (Fig. 4B, even numbered lanes) reveals the methylation state of the −54 and −22 sites.

The results show that the −54 site is highly unmethylated in liver DNA samples and that hormone treatment does not have an effect (Fig. 4, lanes 10 and 12). HpaII digestion at −54 will produce a prominent 273/241 bp band as well as a 437 bp fragment. The 437 bp band is not present in the remaining HpaII digest lanes of most of the remaining tissue samples so the 273/241 bp doublet in those samples is composed of only the 273 bp fragment. Notably, the lung and the uterus DES samples showed residual amounts of the 437 band (Fig. 4, lanes 4, 6, and 8). These results further show that the demethylated state of the −54 site is very dominant only in the liver tissue.

In contrast, the −22 site is highly unmethylated in spleen DNA samples and the methylation state is not dependent on hormone treatment (Fig. 4, lanes 22 and 24). Moreover, because the −54 site is highly methylated in spleen DNA, no 437 bp band appears. The fragment at the 273/241 bp band is 273 bp. Other tissues such as the uterus and lung DNA samples showed a much higher level of methylation at the −22 site (Fig. 4, lanes 2, 4, 6, and 8). Because the 437 bp and 241 bp bands are so prominent in liver samples, the level of HpaII digestion at the −22 site in the liver samples cannot be determined qualitatively from the gel. By subtracting the intensity of the 437 band from the doublet (assuming that the 241 bp band intensity is equal), we estimated that the 273 bp fragment may be about 20% of the doublet. The methylation state still does not vary with hormone treatment. The lung control and DES samples were very similar in band intensities through many Southern hybridizations (Fig. 4, lanes 6 and 8) but lung control sample anomalies produced the apparent differences in the present blot. These results underscore the tissue-specific methylation status of MspI/HpaII sites −54 and −22.

Methylation state of the mouse lactoferrin gene in various tissues

Because there were differences in the methylation state of MspI/HpaII sites within the lactoferrin gene promoter, we investigated if those differences existed within the coding region of the gene. Figure 5 shows an analysis of the methylation state of the lactoferrin gene coding region. MspI- or HpaII-digested DNA from four mouse tissues was hybridized with the lactoferrin cDNA. Even though the liver, spleen and lung DNA samples had unmethylated MspI/HpaII sites within the promoter region (Figs 3B and 4B), only the spleen samples showed numerous distinct bands decreasing in size through 1·2 kb from HpaII digestion (Fig. 5, lanes 14 and 16) while the other samples consistently showed bands that were 4·0 kb or larger. In sharp contrast to the promoter region, the coding region of the lactoferrin gene appears to be heavily methylated in DNA from liver. Again, hormone treatment appears to have no influence on methylation state as the DNA samples from control and DES-treated spleen show about the same degree of demethylation (the difference in band intensity is due to loading). These results further support the tissue-specific variations in the methylation state of MspI/HpaII sites and that unmethylated sites are associated more with the 5’ flanking region than the coding region of the mLF gene.

Lactoferrin gene expression in tissues from untreated and DES-treated mice

It has been well established that the expression of the lactoferrin gene in the mouse uterus is sensitive to estrogen (Pentecost & Teng 1987, Liu & Teng 1992). To determine if lactoferrin expression in the six mouse tissues was correlated to the variation in methylation at MspI/HpaII sites observed in the
lactoferrin promoter and gene, we measured the level of lactoferrin mRNA in 21-day-old CD-1 mice. Total RNA (20 µg) was electrophoresed in a 6% formaldehyde, 1·2% agarose gel, blotted, and hybridized to mouse lactoferrin or mouse PL-7 cDNA. (A) Ethidium bromide staining. (B) Lactoferrin mRNA detected in lung, liver, brain, kidney, spleen, and uterus tissue RNA from control and DES-treated mice. (C) PL-7 loading control. The design of this experiment takes into account potential differences in the inducibility in different mouse tissues, since the autoradiogram of the DES-treated uterine sample was produced after a 3-h exposure but the autoradiogram of all other tissue samples was exposed for 3·5 days.

Methylation state of sites −455, −54, and −22 correlates with lactoferrin expression

Table 1 summarizes the relationship of the methylation state of the three MspI sites in the lactoferrin promoter and in the gene to the lactoferrin mRNA level in the six mouse tissues. Three of the samples that show lactoferrin expression have at least two unmethylated MspI/HpaII sites. The spleen sample has unmethylated −455 and −22 sites. Both the lung and liver have all three sites unmethylated although those in the lung are at a much lower level than the sites in the liver. Unmethylated DNA in brain and kidney samples was below residual levels and there was virtually no lactoferrin expression. In sharp contrast is the correlation between unmethylated sites in the uterus DNA samples and lactoferrin expression. While there is no dominant unmethylated MspI/HpaII site, there is low expression of lactoferrin in untreated uterine samples and extremely high expression in DES-treated samples. The spleen, which has unmethylated sites at −455 and −22 as well as unmethylated MspI/HpaII sites within the coding region, showed induced lactoferrin expression without a change in methylation status due to hormone treatment. The liver and spleen have dominant unmethylated sites and the highest lactoferrin expression in untreated samples. Taken together, the results, with the exception of the uterus samples, show that samples having unmethylated MspI/HpaII sites at −455, −54, and/or −22 express lactoferrin at some level.

DISCUSSION

In previous work we elucidated the structure and function of the lactoferrin gene and promoter, and determined its expression in various tissues. In this study we have examined in detail the methylation state of three MspI/HpaII (CCGG) sites in the lactoferrin gene promoter in mouse tissues, and investigated the relationship of their methylation state and that of the coding region of the gene to expression of lactoferrin in vivo.

The importance of the methylation state of the lactoferrin promoter for expression was demonstrated using a transient transfection assay (Figs 1 and 2). Our results clearly show that methylation of CCpGG sequences by HpaII methylase in lactoferrin promoter-CAT reporter constructs inhibits CAT gene expression in transiently transfected RL95–2 cells. Other studies have also demonstrated that treatment of DNA with HpaII methylase drastically reduces transcriptional activity of CAT constructs containing the leukosialin promoter (Kuda & Fukuda 1995). In addition, SssI methylase (CpG) treatment of the galectin-I gene promoter was found to reduce transcriptional activity (Benvenuto et al. 1996). In the present work with the mouse lactoferrin promoter, AluI methylase
(AGCT) treatments also decreased CAT-reporter gene activity (Fig. 2). It is interesting to note that the AluI and HaeIII (GGCC) sites are located near one another in the 600 bp mouse lactoferrin, yet methylation at the AluI sites has a stronger effect than methylation at the HaeIII sites. One of the AluI sites is next to the COUP/ERE sequence of the mouse lactoferrin gene (Liu & Teng 1992). It is possible that methylation of this AluI site, as well as methylation of the MspI sites at coordinates −455, −54 and −22, are more critical than other restriction sites studied to the basal activity of the lactoferrin promoter in the RL95–2 cells. Interestingly, methylation of the HaeIII sites within the −234 to −21 region of the lactoferrin promoter does not markedly decrease transcriptional activity (Fig. 2B).

Hormonal treatment with DES did not change the methylation state of the MspI/HpaII sites in the mouse lactoferrin promoter (Figs 3 and 4). Since DES increases lactoferrin expression in the mouse uterus, an altered methylation pattern in the lactoferrin promoter or gene in DNA from DES-treated mouse uterine tissue was anticipated. Unexpectedly, DES treatment has no effect on the methylation state of MspI/HpaII sites within the lactoferrin promoter from uterine tissue, as well as little effect in other tissues. This finding does not eliminate the possibility that the methylation state at other CpG sites in the lactoferrin promoter changes following DES treatment. In fact, the CpG site at −464 was found to be abnormally demethylated in the mature mouse uterus following neonatal DES treatment (Li et al. 1997).

In contrast, this study demonstrates striking tissue-specific differences between the methylation states of the −54 MspI/HpaII site in liver tissue, and of the −22 MspI/HpaII site in spleen tissue (Fig. 4 and Table 1). It is intriguing that the −54 MspI/HpaII site is predominantly unmethylated in DNA from liver but not in DNA from other tissues. This site is located adjacent to the critical nucleotides in the forskolin and cAMP response element (CRE) of the lactoferrin promoter (Shi & Teng 1994, 1996). In previous work, mutagenesis at this MspI/HpaII site (CCGG) indicated that mutation at either of the two C residues of the MspI site reduced but did not block the forskolin response, and that mutation at the G next to the C blocked the response completely (Shi & Teng 1994). In addition, the CRE was found to be an integral part of the mouse lactoferrin basic promoter (Shi & Teng 1996). Therefore, the methylation state of the −54 MspI/HpaII site may have an important effect on the constitutive expression of the gene (Liu & Teng 1991). The −22 MspI/HpaII site is located 6 nucleotides downstream from the noncanonical TATA of the mouse lactoferrin gene. Its methylation state could affect assembly of the transcription initiation complex at this site. In view of the location of the −54/−22 MspI sites, it is likely that both of them may play a critical role in the constitutive expression of the gene.

The expression pattern of lactoferrin in mouse tissue shows a relationship to the methylation state of the three MspI sites in the promoter (Figs 3–6 and Table 1). The tissues which showed high methylation levels for at least 2 of the MspI/HpaII sites, such as the brain and kidney, show only trace

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+ , Unmethylated residual levels; ±, very trace; ++, medium; ++++, high; ++++, very high; − methylated.

Table 1. Correlation of methylation of MspI sites and expression of the lactoferrin (LF) gene
amounts of lactoferrin expression. In other work, human brain and kidney DNA samples were methylated at MspI/HpaII sites associated with the expression of the leukosialin gene (Kuda & Fukuda 1995). There is no expression of the leukosialin gene in those tissues. DNA from spleen showed by far the greatest amount of unmethylated DNA at MspI/HpaII − 455, − 22 sites and throughout the gene (Fig. 3B, lanes 22 and 24; Fig. 4B, lanes 22 and 24; Fig. 5, lanes 14 and 16). That may be one reason why that tissue has the highest level of lactoferrin expression of the untreated samples. With the exception of uterine tissue, decreased methylation of the − 455 and − 22 MspI/HpaII sites appears sufficient for lactoferrin expression. While the DNA from uterine tissue is heavily methylated, the expression level in uterine tissue is only exceeded by that for spleen and liver. The − 455 MspI/HpaII site is most unmethylated in the spleen followed by the liver, lung, brain and kidney. This pattern correlates inversely with the relative level of expression of lactoferrin mRNA in these tissues. A correlation between tissue-specific expression and methylation state was also found for other genes (Kuda & Fukuda 1995, Benvenuto et al. 1996). Immunohistochemical studies have also demonstrated that lactoferrin is expressed in specific cell subtypes in mouse tissues. For example, different expression levels are observed in bronchus epithelium within the lung, proximinal tubular cells of the kidney, and within the red pulp cells of the spleen (Shigeta et al. 1996). Lactoferrin is found in high concentrations in the secondary granules of neutrophils and also in the luminal and glandular epithelial cells in the uterus during the estrous cycle (Walmer et al. 1992). Thus, when the methylation state of the lactoferrin gene is determined in tissue specimens that include multiple cell types, the possibility should be considered that the results are a combination of differing values for different subfractions of the cell population. Methylation levels for specific cell types may be diluted. The results described here do not preclude the existence of additional regulatory mechanisms that affect lactoferrin gene expression. For example, the dramatic increase of lactoferrin mRNA in mouse uterine tissue exposed to estrogen reflects a complex signaling pathway in which the methylation state of a few CpG sites in the promoter region may not be crucial. It is also possible that significant tissue-specific or hormone-induced demethylation occurs at CpG sites not examined in this study.

The methylation state of the MspI sites in the lactoferrin promoter and gene could result from the process of tissue differentiation during mouse development. Lactoferrin mRNA and protein are present in the liver of the neonate mouse, and then decrease in amount with age (Shigeta et al. 1996). Lactoferrin expression was not detected in the adult mouse liver (Pentecost & Teng 1987). Experiments need to be carried out to determine if the methylation state of the lactoferrin gene in liver and other tissues is correlated with its expression during the course of development. DNA demethylation has also been linked to chromatin modification and subsequent transcriptional activation (Cooper et al. 1993); conversely, methylation of a promoter leads to transcriptional silencing (Singal et al. 1997). An hypothesis consistent with these results is that demethylation at a transcription factor binding element facilitates the binding of specific proteins for tissue-specific expression (Yokomori et al. 1995a,b, Short et al. 1996). Therefore, understanding the chromatin structure of the lactoferrin gene and its promoter may be important in elucidating the mechanism(s) of the estrogen-stimulated response in immature mice. Previous studies have shown that DNA from uterine tissue is hypomethylated in samples taken from neonatal mice which were treated with DES in utero but not with untreated mice (C T Teng, unpublished results). Studies comparing methylation, expression, and DNase I hypersensitivity of the lactoferrin promoter in uterine tissue from developing and immature mice might shed light on the mechanism(s) by which tissue-specific and hormone-inducible lactoferrin expression is achieved in developing and mature mouse tissue.

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REFERENCES


Cooper GE, Bishop PL & Turk er MS 1993 Demethylation is sufficient for chromatin relaxation and transcriptional activation of methylated APRT gene in mouse P19 embryonal carcinoma cell line. Somatic Cellular Molecular Genetics 19 221–229.


Pentecost BT & Teng CT 1987 Lactotransferrin is the major estrogen inducible protein of mouse uterine secretions. *Journal of Biological Chemistry* 262 10134–10139.


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