Mouse Lactoferrin Gene: A Marker for Estrogen and Epidermal Growth Factor

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Lactoferrin mRNA in the 21-day-old mouse uterus can be increased several hundredfold by estrogen. The physiological role of lactoferrin in mouse uterus is unclear; however, it can be a useful marker for the estrogen action in the uterus. The structural organization and the chromosome localization of the lactoferrin gene are similar to members of the transferrin gene family. At the 5' flanking region of the lactoferrin gene, we have characterized two modules that respond to estrogen and growth factor stimulation. Each module is composed of either overlapping or multiple transcription factor-binding elements. The well-characterized estrogen and growth factor response modules in the mouse lactoferrin gene could serve as the foundation to understand the intricate molecular mechanisms of estrogen action and its relationship to growth factors. — Environ Health Perspect 103(Suppl 7):17–20 (1995)

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Introduction

Lactoferrin, an iron-binding glycoprotein, was originally found in milk 55 years ago (1). It was also present in neutrophilic leukocytes, saliva, tears, seminal plasma, uterine fluid, vaginal fluid, and wet surface mucosa (2–4). Lactoferrin, together with transferrin and melanoma antigen p97, belongs to a gene family that arose from ancient intragenic duplication 300 to 500 million years ago (5–7). The lactoferrin gene was mapped to human chromosome 3 and mouse chromosome 9 (8). Deduced mouse lactoferrin from the cDNA clone (9) exhibits internal homologies between N-terminal and C-terminal domains similar to those of the human lactoferrin protein (10,11). Like transferrin, lactoferrin has two metal-binding sites, each of which can bind a ferric (Fe³⁺) cation and a bicarbonate anion (10).

Lactoferrin protein has been reported to have multiple functions. It has a broad spectrum of antimicrobial properties (12–15); other functions, such as promoting DNA synthesis (16), modulating the immune system (17–23) and inhibiting tumor growth (24) have also been reported. The wide variety of biological functions highlights the importance of the regulation of this gene in individual tissue and cell types. Although many studies have been done on the distribution of the protein, relatively little is understood about the molecular mechanisms that regulate its expression. In the 10 years that our laboratory has been studying regulation of the lactoferrin gene in the mouse uterus, we have found that the gene is a sensitive marker for estrogen in the mouse uterus (9,25). Therefore, the expression of the lactoferrin gene in mouse uterus could be a parameter for detecting estrogenic chemicals in the environment. In this review, I will discuss the organization of the gene, the characterization of the estrogen and epidermal growth factor (EGF) response elements, and the gene's potential use in environmental study.

Organization of the Mouse Lactoferrin Gene

The mouse lactoferrin gene is organized into 17 exons interrupted by 16 introns. The size of the exons matches other members of the transferrin gene family and is conserved during evolution (26,27). There is strong homology between the pairs of exons and the splicing pattern (Tables 1 and 2), which explains the internal homology that exists between N- and C-terminal domains of the protein. These results strongly support the hypothesis that the transferrin gene family is originated by a duplication of a common ancestral gene half the size of transferrin (5–7).

| Exon | Exon size, nt | Amino acid interruption |
|------|--------------|------------------------|
| 2    | 161          | No                     |
| 9    | 155          | No                     |
| 3    | 109          | C/AG Glutamine         |
| 10   | 91           | A/AA Lysine            |
| 4    | 183          | G/CG Alanine           |
| 12   | 156          | A/AT Asparagine        |
| 5    | 148          | AG/G Arginine          |
| 13   | 142          | AG/G Arginine          |
| 6    | 56           | G/AG Glutamic acid     |
| 14   | 68           | G/GG Glycine           |
| 7    | 179          | No                     |
| 15   | 185          | No                     |
| 8    | 175          | A/AG Lysine            |
| 16   | 190          | C/CA Proline           |

nt, nucleotide. Compare the exon size of the 5' and 3' half of the gene.

Table 2. Identical splicing pattern of the corresponding exons in the mouse lactoferrin gene.

| Exons | Splicing pattern |
|-------|------------------|
| 1,3,10⁸ (4,12) (6,14) (8,16), 11 | X⁰ [GT AG²] Y² |
| (5,13) | X⁰ [GT AG²] Z² |
| (2,9) (7,15) | X² [GT AG²] |

*The homologous exons are in parentheses. The nucleotides of the last and first codons of the adjacent exons. The intron interrupting the coding sequence XYZ starts with GT and ends with AG.
introns, however, differ greatly among the gene family, which contributes to the varying sizes of the genes. The mouse lactoferrin gene is encoded by a single copy gene of 30 kilobases (kb) that includes the 7.5 kb promoter/enhancer and 23 kb of the gene (27–29).

**Estrogen Regulation of the Lactoferrin Gene**

The expression of the lactoferrin gene in mouse uterus is especially sensitive to estrogen (4,9,25). Four hours after a 21-day-old mouse was injected with estrogen, the level of lactoferrin messenger RNA (mRNA) increased (Figure 1); with three injections, the level reached up to 300-fold (9). Furthermore, lactoferrin protein and mRNA in the uterus and the estrogen levels in the plasma fluctuate during the estrous cycle of a mouse (30). At diestrus the lactoferrin mRNA was low and immunostaining of the lactoferrin in uterine epithelial cells was weak. There was no detectable lactoferrin in the uterine fluid. At proestrus, the mRNA and protein increased and the lactoferrin was readily detectable in the uterine fluid. Both lactoferrin mRNA and protein reached the highest level at estrus and then decreased as the cycle entered metestrus. Fluctuation of lactoferrin and its mRNA was the result of both production and the number of cells that were involved. Oviductal and vaginal epithelial cells also produce lactoferrin under the influence of estrogen; however, production was not as dramatic as in the uterus. There was no evidence that the lactoferrin gene was regulated by estrogen in other tissues or cells other than the female reproductive tract.

**The Estrogen Response Module**

Analyzing the 5′ flanking region of the lactoferrin gene, we found an imperfect estrogen response element (ERE) that overlapped with a chicken ovalbumin upstream promoter (COUP) (28,31). This composite estrogen response module (mERM) was located at position -349 to -329 from the transcription initiation site. The ERE element differed from the consensus ERE sequence (32) by one nucleotide at the second position of the 3′ half of the element (G to A); the COUP element differed by one nucleotide from the chicken COUP element (33). The ERE element and COUP element, either alone or together (mERM), were cloned in a reporter CAT plasmid and then transiently transfected into human endometrium carcinoma RL95-2 cells to assess hormone responsiveness. We found that the ERE element conferred estrogen action to both homologous and heterologous promoters (Figure 2)(30).

Results of the electromobility shift assay (EMSA) showed that the estrogen receptor and COUP transcription factor (COUP-TF) were present in mouse uterine tissue and interacted with the COUP/ERE element specifically. In addition, specific antibodies to the estrogen receptor and the COUP-TF could correspondingly supershift the band. The unusual organization of the ERE in the mouse lactoferrin gene provided an opportunity to study the functional relationship between the two members of the steroid hormone receptor superfamily. Mutation and deletion of the COUP element or reduction of the endogenous COUP-TF increased mERM estrogen responsiveness. Likewise, overexpression of the COUP-TF expression vector blocked the estrogen-stimulated response of mERM in transfected cells. The molecular mechanism of this repression is due to the competition between COUP-TF and estrogen receptor to bind at identical contact sites in the overlapping region of the mERM (34).

**The Mitogen Response Module**

Searching the lactoferrin gene nucleotide sequence for DNA elements through which second messenger signals are transduced,

![Figure 1](image1.png)

**Figure 1.** Estrogen effect on lactoferrin gene expression in the mouse uterus. Twenty-one-day-old female mice were intraperitoneally injected with DES (20 μg/kg) for 4 and 8 hr or three injections of DES (20 μg/kg/day for 3 days) before sacrifice. Total uterine RNA was prepared and analyzed by Northern blotting. Mouse lactoferrin (mLF) cDNA (T267) and histone H2B were used as probes (9). The sizes of the mRNA are indicated.

![Figure 2](image2.png)

**Figure 2.** The imperfect ERE of the mouse lactoferrin gene conferred estrogen responses through a heterologous promoter. Synthetic oligonucleotides containing the mouse lactoferrin imperfect estrogen response element were cloned into the reporter gene with simian virus 40 promoter (SV). The various CAT constructs are schematically presented at the left. The orientation and number of the insert(s) relative to the start site is indicated by the arrows. Human endometrium carcinoma RL95-2 cells were transiently cotransfected with chimeric CAT reporter plasmid (5 μg per well), human estrogen response expression vector (HEO, 0.5 μg per well), and β-galactosidase expression vector (pCH110, 0.25 μg per well). The transfected cells were treated with 10−6 M DES for 16 hr before harvest. CAT enzyme activity from the whole-cell extract was measured (30). Relative CAT activity was represented after normalization for β-galactosidase activity, and the results presented are the averages from at least four independent experiments performed in duplicate. Control, no treatment.
we found a DNA sequence (5'-GGGCA ATAGGGTGCCGCACTCCGGT GAGGTACCAGCA-3') within 100 bp upstream from the transcription initiation site that is capable of conferring EGF/TGF-α, cAMP, forskolin, and TPA action (35). The nucleotide sequence, 5'-GTTGAGGTC ACC-3' (mLF-CRE), which housed the elements resembling both AP1 and CRE, was responsible for cAMP and TPA stimulation. Indeed, mobility shift assays supported the binding of AP1 and CRE to this sequence. Cotransfection experiments demonstrated the involvement of both PKC and PKA pathways in the activation of the lactoferrin gene promoter through the mLF-CRE element. Mutation experiments showed that the mLF-CRE plays a dual role in basal and inducible expression. We were able to delineate the functional role that confers EGF/TGF-α and cAMP/TPA-induced regulation in the mitogen response unit into two separate regions in the mouse lactoferrin gene. We named the region that was responsible for the EGF/TGF-α stimulation EGFR (5'-GGGCAATAGGGTGGGGCC-3').

The in vivo study showed that expression of the lactoferrin gene in mouse uterus was regulated by EGF (36). It was proposed that EGF-regulated lactoferrin expression was via an estrogen receptor route (36,37). The presence of EGF response elements in the mouse lactoferrin gene provided an alternative route of activation. In addition, the organization of the estrogen response element and EGF response elements in the mouse gene (Figure 3) provides an interesting model system to study the cross communication between the steroid/thyroid and the second messenger signaling pathways at the gene level.

**Marker for Environmental Chemicals**

Many environmental chemicals can mimic estrogenic effects in animals, but the prevailing concern is whether these estrogenic chemicals pose any threat to human health. Before addressing the risk to human health, several factors should be considered. The level, time, and duration of exposure to these chemicals are critical elements in establishing any biological significance. Therefore, a test is needed to determine these factors; currently in mammalian systems, there is no established marker to monitor the effect of estrogenic chemicals. Based on its sensitivity to estrogen, the lactoferrin gene in the mouse uterus could be a useful marker to measure the effect of estrogenic chemicals. It is possible to use the lactoferrin mRNA and protein as the biochemical markers to determine the dose and duration of the chemicals that mimic the estrogenic effect. The unique organization of the estrogen and mitogen response module in the mouse lactoferrin promoter region serves as a model system to study the cross communication between the steroid receptor and protein kinase pathways.

**Figure 3.** Schematic representation of the putative and characterized regulatory elements in the 5'-flanking region of the mouse lactoferrin gene promoter.

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