Estrogen-related Receptor, hERR1, Modulates Estrogen Receptor-mediated Response of Human Lactoferrin Gene Promoter*

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We have shown previously that estrogen-stimulated transcription from the human lactoferrin gene in RL95-2 endometrium carcinoma cells is mediated through an imperfect estrogen response element (ERE) at the 5'-flanking region of the gene. Upstream from the ERE, a DNA sequence (−418 to −378, FP1) was selectively protected from DNase I digestion by nuclear extracts from endometrial and mammary gland cell lines. In this report, using the electrophoresis mobility shift assay, site-directed mutagenesis, and DNA methylation interference analyses, we show that three different nuclear proteins bind to the FP1 region (C1, C2, and C3 sites). The nuclear receptor, COUP-TF, binds to the C2 site. Mutations in the C1 binding region abolish C1 complex formation and reduce estrogen-dependent transcription from the lactoferrin ERE. When the imperfect ERE of the lactoferrin gene is converted to a perfect palindromic structure, the enhancing effect of the C1 binding element for estrogen responsiveness was abolished. We isolated a complementary DNA (cDNA) clone from an RL95-2 expression library that encodes the C1 site-binding protein. The encoded polypeptide maintains 99% amino acid identity with the previously described orphan nuclear receptor hERR1. A 2.2-kilobase mRNA was detected in RL95-2 cells by the newly isolated cDNA but not by the first 180 base pair of the cDNA sequence. The encoded polypeptide is a major 42-kDa protein that is detected in the RL95-2 nuclear extract by site-directed mutagenesis. When the imperfect ERE of the C1 binding region was expressed in the hormone-responsive gene, the regulation is mediated through steroid receptor binding to its respective hormone response element (HRE)1 (for review, see Evans (1988) and Beato (1989)). In many cases, other transcription factors bind near the HRE and interact with the steroid hormone receptor to modulate the hormonal responses (Bruggemeier et al., 1991; Danesch et al., 1987; Espinas et al., 1994; Wieland et al., 1991; Zhang and Young, 1991). For example, the direct participation of transcription factors, SP1 and AP1, were recently found to modulate estrogen-induced stimulation in several estrogen-responsive genes (Krishnan et al., 1994; Wu-Peng et al., 1992) lacking typical estrogen response elements (ERE) (Klein-Hitpass et al., 1988, 1989). Thus, the hormonal responsiveness of a particular gene is the result of a complicated interplay between steroid receptors and other transcription factors.

We have been studying lactoferrin, an estrogen-inducible gene product present in milk, tears, and saliva (Teng et al., 1990 and references therein). Lactoferrin has multiple functions that include modulating the immune response, promoting cell growth, and killing bacteria (Arnold et al., 1976; Broxmeyer et al., 1987; Esaguy et al., 1991; Legrand et al., 1992; Nichols et al., 1987; Sawatzki and Rich, 1989). Although the lactoferrin gene is expressed in many tissues, its expression in the mouse uterus is very sensitive to estrogen (Pentecost and Teng, 1987; Teng et al., 1989); estrogen injection into a 21-day-old mouse induces lactoferrin messenger RNA several hundred-fold (Pentecost and Teng, 1987). Accordingly, uterine lactoferrin protein and messenger RNA fluctuate with plasma estrogen levels during the estrus cycle (Newbold et al., 1992; Walmer et al., 1992). Expression of lactoferrin gene in human endometrium, however, is not nearly as robust as that in the mouse uterus (Teng et al., 1992; Walmer et al., 1995).

Comparisons of the promoter/enhancer region from human and mouse lactoferrin genes revealed a similar composite estrogen response element (Teng et al., 1992; Teng, 1994). The mouse lactoferrin ERE overlaps a functional COUP-TF binding element (Wang et al., 1989, 1991), generating a direct competition between these two transcription factors for binding to their overlapping regions of the element (COUP/ERE element) (Liu and Teng, 1992; Liu et al., 1993). We demonstrated that overexpression of COUP-TF in transfected uterine endometrial cells repressed estrogen stimulation (Liu et al., 1993). The human and mouse lactoferrin COUP/ERE elements are located at similar positions upstream from the start site and are well matched (18 of 22 nucleotides identical) (Liu and Teng, 1991; Teng et al., 1992; Teng, 1994). In contrast, COUP-TF binds DNA elements different from COUP/ERE in the human lactoferrin promoter (Teng et al., 1992; Yang and Teng, 1994).

Recently, we found a DNA sequence (−414 to −378, FP1) upstream from the COUP/ERE that was selectively protected from DNase I digestion by nuclear extracts of endometrial (RL95-2) and mammary gland (HB100) cell lines (Yang and Teng, 1994). We defined an extended steroid receptor half-site, TCAAGGTCA, within the FP1 that matches the consensus sequence (Yang and Teng, 1994). This sequence is flanked by two clusters of CCGT sequences, which are underlined in the FP1 sequence (Yang and Teng, 1994).

Eukaryotic gene promoters consist of multiple upstream regulatory elements that positively or negatively modulate transcriptional activity (for review, see Yamamoto (1985)). For the steroid hormone-responsive gene, the regulation is mediated through steroid receptor binding to its respective hormone response element (HRE)1 (for review, see Evans (1988) and Beato (1989)). In many cases, other transcription factors bind near the HRE and interact with the steroid hormone receptor to modulate the hormonal responses (Bruggemeier et al., 1991; Danesch et al., 1987; Espinas et al., 1994; Wieland et al., 1991; Zhang and Young, 1991). For example, the direct participation of transcription factors, SP1 and AP1, were recently found to modulate estrogen-induced stimulation in several estrogen-responsive genes (Krishnan et al., 1994; Wu-Peng et al., 1992) lacking typical estrogen response elements (ERE) (Klein-Hitpass et al., 1988, 1989). Thus, the hormonal responsiveness of a particular gene is the result of a complicated interplay between steroid receptors and other transcription factors.

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binding elements of SF-1/ELP (Ikedaa et al., 1993; Tsukiyama and Niwa, 1992). These tissue-specific transcription factors belong to a nuclear receptor subfamily that bind as monomers (Wilson et al., 1993; Ikeda et al., 1993; Tsukiyama and Niwa, 1992). Since different transcription factors may bind to identical response elements in various cell types, we sought the nuclear factors in RL95-2 cells that bind to this DNA element. In this study, we mapped the nuclear protein binding elements in the FP1 region and demonstrated that the TCAAGGTCACT element enhances estrogen responsiveness of the human lactoferrin gene. Subsequently, an RL95-2 expression library was used to isolate cDNA that encodes another binding protein for the extended steroid receptor half-site. The cDNA clone was used to isolate cDNA that encodes another binding protein for element enhances estrogen responsiveness of the human lactoferrin gene.

**MATERIALS AND METHODS**

Plasmids and Oligonucleotides—The pHL-414CAT plasmid was constructed as described previously (Y Liu and Teng, 1994). Location of the oligonucleotides used in EMSA is presented in Fig. 1A and mutated nucleotides are marked in Fig. 3B. The oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) and purified by column chromatography. The DNA sequences of oligonucleotides containing BglII BamHI linkers were as follows: oligonucleotide 418/378, S5-ACCTGGCCTAATCCTGCTAGAGCAGCACTCTAGCTGG; M1, 5-ACCTGGCCTAATCCTGCTAGACCTCTAGCTGG; GACCCCTCAAAGTTCCTGCTGG; M2, 5-ACCTGGCCTAATCCTGCTAGACCTCTAGCTGG; TACCCCTCAAGGTCACTCGTCTG3; M3, 5-ACCTGGCCTAATCCTGCTAGACCTCTAGCTGG; oligonucleotide 375/-340, 5-GAAAGATAGCTCTAGGCTAAGGCTAAGGCTAAGGCTAAGGCTAAGGCTAAGGCT; oligonucleotide 396/-382, 5-GACCCCTCAAAGTTCCTGCTGG; Complement strands of the above oligonucleotides were also synthesized and double-stranded DNA prepared (Liu and Teng, 1992). An oligonucleotide corresponding to the vitellogenin A2 promoter region was synthesized (Klein-Hitpass et al., 1987). The poly(A) + RNA was prepared from a standard oligo(dT) column library. The oligo(dT) expression library was constructed with a cDNA synthesis kit, according to the manufacturer’s specification (Life Technologies, Inc.). The cDNA library contains 1.5 × 106 individual recombinants.

Preparation of Probe for Expression cDNA Library Screening—The 46 base pairs of oligonucleotide (418/-378, the FP1, plus BamH I/BglII cloning sites) was hybridized with the DNA probe was isolated and designated as FP1.4. Double-stranded nucleotide sequencing of the FP1.4 was performed in our laboratory and by Lark Sequencing Co. (Houston, TX). Four additional positive clones, two from the RL95-2 expression cDNA library (FP1.4-2 and FP1.4-3) and two from the human hippocampus expression cDNA library (HP1 and HP2) (Stratagene, La Jolla, CA), were subjected to additional DNA sequencing at regions (from nucleotides 740 to 760 and 1190 to 1250) diverging from the published hERR1 (Giguere et al., 1991). Isolation and characterization of the protein-expressing clone interacting with the FP1.4 insert was performed as described previously (Liu and Teng, 1992).

Expression and Purification of Glutathione S-Transferase Fusion Protein—The expression and purification of glutathione S-transferase fusion protein were carried out with RediPack GST purification module according to the manufacturer’s specifications (Pharmacia). The fusion proteins, GST-HR1 and GST-HER, were prepared by batch method. Eluted proteins were analyzed on 10% SDS-PAGE and visualized by Coomassie staining. After the protein concentration was determined by precipitation. About 5 ng of labeled and methylated DNA (4 × 10 6 cpm) was incubated with 50 µg of nuclear protein extract in a 60-µl reaction similarly to the EMSA. Free and protein-DNA complexes were separated in a 3.5% nondenaturing gel. The individual complexes and the free DNA were excised from the gel, purified, and cleaved by 1 M piperidine at 90°C for 30 min. The samples were extracted with phenol/ chloroform/isoamyl alcohol and reprecipitated by ethanol, precipitated by 0.5 M lithium chloride, and separated on a 5% sequencing gel. The G + A chemical reaction for the same DNA fragment was included as a marker (Ausubel et al., 1990).

Cell Culture, DNA Transfection, and Chromaphenolic Acetyltransferase (CAT) Assay—Human endometrium carcinoma RL95-2 cells (ATCC CR1617) were grown in 1:1 mixture of Dulbecco’s minimal essential Ham’s F-22 supplemented with 10% fetal bovine serum, 5 µg of bovine insulin, and 100 units/ml penicillin/streptomycin under 5% CO2. Transient transfections were performed by the calcium phosphate method with a Cellfect transfection kit (Pharmacia LKB Biotech). The CAT assays were described previously (Liu and Teng, 1992), and the reaction products were analyzed with an ascending TLC following quantitation using the PhosphorImager System (Molecular Dynamic, Sunnyvale, CA). The cells were cotransfected with vector alone or with 5 µg of the reporter plasmid and 0.5 µg of the estrogen receptor expression plasmid (HEO). After transfection, the cells were cultured in 10% charcoal-stripped fetal bovine serum with or without hormone (diethylstilbestrol, 10-8 M) for 24 h. All experiments were repeated at least three times with duplicated samples. The results were calculated as mean ± S.D.

Preparation of Probe for Expression cDNA Library Screening—The 24 base pairs of oligonucleotide (378/-340, the FP1, plus BamH I/BglII cloning sites) was hybridized with the DNA probe was isolated and designated as FP1.4. Double-stranded DNA prepared (Liu and Teng, 1992). An oligonucleotide corresponding to the vitellogenin A2 promoter region was synthesized (Klein-Hitpass et al., 1987). The poly(A) + RNA was prepared from a standard oligo(dT) column library. The oligo(dT) expression library was constructed with a cDNA synthesis kit, according to the manufacturer’s specification (Life Technologies, Inc.). The cDNA library contains 1.5 × 106 individual recombinants.

The RL95-2 expression cDNA library was screened with labeled FP1 oligonucleotides (1.47 kilobase pairs of the pSLP-32 insert) as described (Vinson et al., 1988). The protein-expressing clone interacting with the DNA probe was isolated and designated as FP1.4. Double-stranded nucleotide sequencing of the FP1.4 was performed in our laboratory and by Lark Sequencing Co. (Houston, TX). Four additional positive clones, two from the RL95-2 expression cDNA library (FP1.4-2 and FP1.4-3) and two from the human hippocampus expression cDNA library (HP1 and HP2) (Stratagene, La Jolla, CA), were subjected to additional DNA sequencing at regions (from nucleotides 740 to 760 and 1190 to 1250) diverging from the published hERR1 (Giguere et al., 1991).

Progenitor Expression Combinatorial Reconstituent Chromatin—Large amounts of λ-DNA from HERR1 reconstituent were prepared by standard methods (Maniatis et al., 1982). The DNA insert was recovered by agarose gel digestion and then eluted from the gel with water and extracted into the appropriate restriction sites of the pGEX-4t-3 expression vector (Pharmacia). The cloning site and the in-frame reading with target 22A system were verified by dideoxy sequencing. This expression recombinant was designated pGEX-HERR1. The pGEX-HERR1 was generated by PCR the HEO plasmid with the following primers containing the EcoRI linker: the sense primer, 5′-GGGAATTCTCAGACTGTGGCAGGGAAACCCT-3′ and the antisense primer, 5′-GGGAATTCTCAGACTGTGGCAGGGAAACCCT-3′. The PCR products were subcloned into EcoRI site of the pGEX-4t-1
Bio-Rad protein reaction, the protein was aliquoted and stored at −70 °C until use.

Polyclonal Antibody Production, Affinity Purification, Western and Far-Western Analyses—A female New Zealand White rabbit was immunized with 500 μg of purified GST-hERR1. The IgG fraction was isolated by Sepharose chromatography and further purified by affinity chromatography of the GST-hERR1 coupled to the Affi-Gel 10 (Bio-Rad) column.

The Western blots were probed by antiserum either to GST-hERR1 or to ER (H222, Abbott, Chicago, IL) with an ECL kit (Amersham Corp.) according to the manufacturer’s specification. The far-Western technique was performed as described (Kaelin et al., 1992) with 32P-labeled GST-hERR1. To label the protein, the Sepharose-bound GST-hERR1 was incubated with [γ-32P]ATP and cAMP-dependent protein kinase (Sigma) in HMK buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM MgCl2) containing 1 mM dithiothreitol for 30 min. After washing, the 32P-GST-hERR1 was eluted from the Sepharose beads by reduced glutathione buffer (10 μM reduced glutathione in 50 mM Tris-HCl, pH 8.0).

Northern Blot Analysis—Total RNA was extracted (Chomczynski and Sacchi, 1987). The RNA samples were denatured in formaldehyde, electrophoresed through a 1.2% agarose gel, and transferred to a Hybond-N+ membrane (Amersham). An oligonucleotide specific for the 5′ end of the published hERR1 sequence (180R, from nucleotide 1 to 180; Giguere et al., 1988) was synthesized by PCR. The 5′ oligonucleotide probe from hERR1 (185B, from nucleotides 1 to 185, Fig. 1A) was obtained by cutting the cDNA in clone FP1.4 with SalI/BstXI. The cDNA probes, β-actin (Oncor, Gaithersburg, MD), 180R, and 185B were radiolabeled to a specific activity of approximately 109 counts/min/μg with PRIME-IT 2 random primer labeling kit (Stratagene, La Jolla, CA). Hybridization of the blots were described previously (Liu and Teng, 1994).

RESULTS

Mapping Nuclear Protein Binding Sites in FP1 Region of Human Lactoferrin Promoter—Fig. 1A shows the locations of oligonucleotides and the footprint areas of the human lactoferrin gene. We examined the interactions of RL95-2 nuclear proteins with the FP1 region (−418 to −378) by EMSA, methylation interference, and mutagenesis. Nuclear extracts from RL95-2 cells mixed with radiolabeled FP1 oligonucleotide produced three more slowly migrating complexes in a band shift assay and were designated C1, C2, and C3 (Fig. 1B, lane 1). The competition experiment demonstrated specific interactions for all three complexes (lane 2). In order to identify the DNA contacts for these proteins, labeled FP1 was partially methylated before EMSA. The individual bands were excised from the gel, and DNA methylation interference analyses were performed. DNA contacts in the bottom strand of DNA for all three complexes were obvious (Fig. 2A, lane 8, 9, and 10); however, only C1 and C3 contacts in the top strand could be gleaned (Fig. 2A, lanes 5 and 3, respectively). The G contacts by the nuclear protein in all three complexes were indicated in Fig. 2B.

To further examine the binding sites for the proteins that interacted with FP1, we performed EMSA using mutated oligonucleotides (Fig. 3). In agreement with the methylation interference findings, mutations (G to C) at the C1 and C3 contacts (m1 and m3, respectively) abolished the protein binding at these regions (Fig. 3A, lanes 2 and 12, respectively), whereas a mutation at the noncontact Gs (m2) did not interfere with protein-DNA interaction (Fig. 3A, lane 7).

The competition experiments demonstrated that the oligonucleotide −418/−378 containing the entire FP1 region (Fig. 1) competed for binding with all three complexes (Fig. 3A, lanes 4, 9, and 14). Oligonucleotide −375/−340, however, containing the COUP-TF binding element and the imperfect ERE competed for C2 (lanes 5, 10, and 15). The oligonucleotide −418/−394, which covered the 5′ half of the FP1 region, competed with C3 (lane 6 and 11). As expected, mutation at the C1 contact sites (m1) weakened the C2 binding (compare lanes 1 and 2), since this was also the C2 binding region. Unexpectedly, under this condition, oligonucleotide −418/−394 could compete with C2, but not if the C1 contacts were intact (compare the intensity of C2 in lane 6 to lanes 11 and 16). Similarly, C3 binding was influenced by mutation at C1 contact sites (compare the intensity of C3 in lanes 2 and 5). C2 binding was not affected by mutations at other locations such as m2 and m3 (compare the intensity of C2 in lanes 7, 11, 12, and 16 with lane 1). By using specific COUP-TF antibody in the EMSA, the C2 complex was supershifted (Fig. 3C, lane 2 and 4). Although the C2 binding was substantially reduced with m1 oligonucleotide as the probe, COUP-TF antibody interacted with the C2 complex. This observation confirmed the competition experiments (Fig. 3A) that COUP-TF is present in the C2 complex.

Steroid Receptor “Half-site” in FP1 Region Modulates Estrogen Responsiveness of the Human Lactoferrin Gene—To examine whether the steroid receptor half-site in the FP1 region plays any role in estrogen responsiveness, we constructed a CAT reporter plasmid, pHL-414CAT, which contains 414 base pairs of the human lactoferrin promoter/enhancer region. Both the imperfect ERE and the FP1 were present. The wild type and mutated FP1 plasmids were transfected together with estrogen receptor expression vectors into human endometrial carcinoma RL95-2 cells. Fig. 4A shows that the pHL-414CAT responded to estrogen stimulation with an 18-fold increase of CAT activity (Fig. 4A, wt). Mutations made at all three locations in the FP1 region reduced the basal CAT activity, hence the estrogen-stimulated activities accordingly (Fig. 4A, m1, m2, and m3). Nevertheless, the folds of estrogen stimulation were
When the double Gs in the conserved steroid receptor half-site (C1 binding region) were changed to Cs in m1-CAT reporter constructs (Fig. 3B), the estrogen stimulation was reduced significantly (Fig. 4A, compare wt and m1). Although the basal activity of m1 was slightly lower than wild type, the estrogen-stimulated CAT activity was affected more by mutation at this region. By using different lots of RL95-2 cells we found variations in both basal and estrogen-stimulated CAT activities (compare wt in Fig. 4, A–C). Despite this variation, mutation at C1 binding region consistently showed 2-fold reduction in estrogen responsiveness (compare wt and m1 in Fig. 4, A and B). It was interesting to find that destruction of ER binding to the ERE (m6) did not attenuate estrogen-stimulated activity completely unless C1 binding was also destroyed at the same time (m1/m6). These results suggest that both C1 and the imperfect ERE in the human lactoferrin gene are required for maximum strength of estrogen induction.

The question arises as to whether the C1 dependence could be abolished by a palindromic ERE, which is a stronger enhancer than the imperfect ERE in the lactoferrin gene. To test this possibility, we converted the imperfect ERE to a palindromic ERE (m7) in the pH41CAT reporter construct containing an intact or a mutated C1 (m1). These reporter constructs were transfected into RL95-2 cells, and the estrogen responses were examined (Fig. 4C). When the imperfect ERE was converted into a perfect ERE, the strength of estrogen action was doubled (Fig. 4C, compare wt and m7). Destroying the C1 has no effect on estrogen-stimulated activity (compare m7 to m1/m7). Therefore, only the weak imperfect ERE needs extra help from C1 to confer ER-mediated activity, whereas a strong ERE can function independently.

Identifying the Critical Nucleotides in C1 Binding—To examine which nucleotides were involved in C1 binding, we used wild type and mutated oligonucleotides (−396 to −362) to
compete for binding in EMSA. The COUP-TF binding element and the steroid receptor half-site were included in the 20-mer double-stranded oligonucleotides. Fig. 5 shows that every nucleotide tested was important for C1 binding. Mutants c and d could partially compete for binding at the C1 region (lanes 9–11). Mutation of C to A in mutant d did not affect COUP-TF binding, since this nucleotide is at the center of the palindromic COUP-TF binding element. Therefore, mutant d could compete with C2 complex (lanes 10 and 11) efficiently. Nucleotides beyond the 3' end of the steroid receptor half-site were also needed for C1 binding (data not shown). Thus, the nucleotide sequences at both ends of the steroid receptor half-site were important for establishing the C1 complex. Results from these studies suggest that the minimum C1 binding element is TCAAGGTCATC. Since m1 mutation prevents C1 complex formation and hampers C2 binding (Fig. 3A, lane 2), it is necessary to confirm that the protein in the C1 complex is actually responsible for the enhanced estrogen-stimulated activity. We tested mutant d (Fig. 5A) that binds C2 but not C1 in the transfection assay. The results showed a reduction of estrogen responsiveness similar to the m1 reporter construct (compare fold of stimulation between m1 in Fig. 4 and mutant d in Fig. 5B).

Isolation and Identification of the cDNA Clone That Binds to C1 Region—We screened an expression library made from poly(A) RNA of the RL95-2 cells with a concatenated FP1 sequence in order to isolate the nuclear protein that binds to the C1 region in human endometrial cells. Among the 1.5 × 10^6 clones screened, we identified six positive clones, and the longest, FP1.4, was completely sequenced. From the nucleotide sequence of the cDNA and the deduced amino acid sequence, we found that the FP1.4 was nearly identical to hERR1 (Giguere et al., 1988). The major differences between our cDNA and published hERR1 sequence occurred at the 5' end and few
Identification of hERR1.

A, nucleotide sequence and deduced amino acids of hERR1. The nucleotide sequence and the longest open reading frame of hERR1 were presented. The different nucleotides from published hERR1 were indicated on top of the sequence and the amino acids on the bottom. The two zinc-fingers were boxed.

B, northern blot analysis of hERR1 mRNA in RL95-2 cells and mouse tissues. A, 125 ng poly(A)RNA from RL95 cells and from mouse kidney tissue were analyzed. The 5'-specific probes were described under "Materials and Methods." Lanes 1 and 2 were probed with 180R of hERR1 (Giguere et al., 1988). Lanes 3 and 4 were probed with 185B of hERR1 (Fig. 1). The same blots were reprobed with β-actin after stripping. The position of 28 S, 18 S, hERR1 mRNA are indicated.

C, detection of hERR1 by Western blotting. Proteins from nuclear extract of the RL95-2, HBL100, HeLa, and Comma-D cells were separated on a 10% SDS-PAGE and blotted onto the nitrocellulose. A major 42-kD protein and a minor 53-kD protein (arrow) were detected by antiserum to hERR1. The molecular markers are indicated.

Fig. 6. Identification of hERR1.
internal deletions. To verify the cDNA sequence, we submitted the FP1.4 clone to commercial sequencing (Lark Sequencing Co.) and sequenced two additional clones isolated from the expression cDNA library generated from our laboratory (RL95-2 cell), and two clones obtained from the commercial expression cDNA library (human hippocampus) at the regions in question (nucleotides 740–750 and 1190–1250) and confirmed the FP1.4 sequence. Among the 10 cDNA clones isolated from both libraries, the FP1.4 was the longest, yet still 176 base pairs less than the published hERR1 sequence at the 5' end.

Comparison of the FP1.4 to the hERR1 sequence indicated that there were seven deletions and one mutation in the coding region and two deletions and one addition at the 3' non-coding region of the clone. Deletions occurring in the coding region caused frameshift mutations and generated three areas of amino acid discrepancy from the published hERR1. The differences in amino acids were marked at the bottom of the sequence (Fig. 6A).

Presence of Truncated hERR1 mRNA and Protein in the RL95-2 Cells—By using 5' probes from the published hERR1 sequence (180R) and FP1.4 (185B), we examined the presence of hERR1 mRNA in RL95–2 cells and mouse kidney tissue. Fig. 6B shows that the 180R probe generated according to the published hERR1 sequence (nucleotides 1–180; Giguere et al., 1988) did not hybridize to any mRNA from RL95-2 cells (lane 1) and mouse kidney tissue (lane 2), whereas the 185B probe from the FP1.4 clone (nucleotides 1–185 in Fig. 6A) detected a prominent hERR1 mRNA at the 2.2-kilobase region from both samples (RL95-2 cells and mouse kidney tissue at lane 3 and 4, respectively). The same Northern blots were reprobed with β-actin as a positive control (Fig. 6B, lower panel).

From the Western blot analysis, antibody produced against the hERR1 fusion protein detected a 42-kDa protein from human uterine and mammary gland cell lines (Fig. 6C). A minor protein band at the 53-kDa region was also detected in these cell lines. In HeLa cells, there were equal amount of 53- and 42-kDa protein. The predominant protein expressed by mammary gland cells from both human (HBL100) and mouse (comma-D) was the 42-kDa protein.

Identification of the hERR1 as the C1-binding Protein—The hERR1 fusion protein bound specifically to the FP1 oligonucleotide and produced a protein-DNA complex in EMSA (Fig. 7A, lane 1). The polyclonal antibody to hERR1 disrupted the protein-DNA complex (lane 3), whereas preimmune serum did not (lane 2). By methylation interference analysis (Fig. 7B), we showed GST-hERR1 fusion protein expressed in a bacterial system interacted with the TCAAGGTCATC element in FP1 region (lanes 3 and 8). To confirm that the hERR1 in the RL95-2 nuclear protein indeed formed the C1 complex with FP1 in EMSA, we incubated the hERR1 antibody with the RL95-2 nuclear protein prior to the binding reaction. Fig. 8 shows the three complexes generated by the RL95-2 nuclear protein and FP1 interaction (lane 1). The hERR1 antibody, but not the preimmune serum, supershifted only the C1 complex (lanes 2–4). To rule out the possibility that the estrogen receptor also binds to the C1 region, we used vitellogenin A2 (vit-A2) ERE in competition experiments. Even with the inclusion of 100-fold molar excess of double-stranded vit-A2 ERE, the oligonucleotides were unable to compete with C1 complex (Fig. 8, intensity of C1 in lanes 5–8). This result was in agreement with our previous observations that the estrogen receptor antibody (H222) did not interact with any of the complexes (Yang and Teng, 1994).

hERR1 Interacts with ER through Direct Protein-Protein Contacts—To examine whether hERR1 could interact with estrogen receptor in vitro, we performed far Western analysis. Human ER was expressed in the bacteria system as the GST fusion protein and verified by Western blotting (data not shown). Fig. 9 shows 32P-labeled hERR1 interacted with GST-ER fusion protein only (lanes 12 and 13). GST protein by itself (lane 11) nor with other GST fusion protein that were expressed similarly interacted with the hERR1 (lanes 14–16).

**DISCUSSION**

We mapped the C1, C2, and C3 proteins binding sites (Figs. 1–3) through a series of experiments to characterize the proteins that bind FP1 region of the human lactoferrin gene. We confirmed our previous finding that COUP-TF binds C2 (Yang and Teng, 1994). The C3 protein and its DNA binding element were investigated but not characterized in this study. The EMSA, transient transfection, and site-directed mutagenesis studies showed a correlation between C1 binding to the DNA element, TCAAGGTCATC, at the 3' end of the FP1 region and up-regulating the estrogen response of the human lactoferrin gene. The functional studies were conducted in transiently transfected human endometrial carcinoma cells. An inherent problem of transient transfection experiments is the changing basal promoter strength in mutant constructs (Fig. 4). An aberrant initiation of transcription or lost binding of the positive or negative transcription factors that are part of the basal promoter machinery might contribute to the variable basal promoter activities. In addition, transfection experiments carried out with cells in various passages and different lot numbers could have inconsistent basal activities. Obviously, these changes will also affect estrogen-stimulated activities (Fig. 4A). Despite these variables, the estrogen responsiveness of the human lactoferrin promoter is unchanged in reporter constructs having mutations outside the C1 binding site (compare fold of stimulation in wt to m2 and m3). Mutations within the C1 binding site have significant effect on estrogen responsiveness, regardless the basal promoter strength (compare fold of stimulation in wt to m1 in Fig. 4, A and B). Mutant d exclusively prevents formation of the C1 complex showed reduced estrogen response in transient transfection experiments (Fig. 5). These results provided further support for an important role of the C1 protein. Collectively, information from the EMSA and transfection experiments strongly suggests that C1 binding is important in maximizing estrogen stimulation.

We isolated the cDNA that encodes C1-binding protein, and by sequencing, we verified that it is hERR1 (Fig. 6A). Several internal deletions in the hERR1 coding region predicted an amino acid deletion at nucleotide 746 and a frameshift at 1208–1236, which caused 10 mismatched amino acids in the potential ligand binding region. These differences may be significant in terms of ligand binding. The polypeptide encoded by hERR1 was tested for steroid binding capability, but none were found (Giguere et al., 1988). Changes of amino acid sequence in the potential ligand binding domain of the hERR1 could render
ligand binding. The apparent differences between the published and our hERR1 sequences lies at the 5' end. 2.2-Kilobase hERR1 mRNA in RL95-2 cells and mouse kidney, detected by the 5' probe of our sequence (nucleotides 1–185, Fig. 6A), but not by the 5' probe of published sequence (nucleotides 1–180; Giguere et al., 1988), suggests a truncated hERR1 mRNA in these cell and tissue. Examining the published hERR1 cDNA sequence, nucleotides 1–178 originated from \( \lambda hKE4 \) and nucleotides 179–2,430 from \( \lambda hKA1 \). It is possible that the RL95-2 cell and mouse kidney express hERR1 mRNA with nucleotide sequence similar to the hKA1. Recent evidence showed that multiple isoforms could be generated by members from the steroid/thyroid receptor superfamily through different promoter usage and alternative RNA splicing (Ikeda et al., 1993; Giguere et al., 1994; Guiramand et al., 1995). The same mechanism could be used to produce different forms of hERR1 in various cell types or tissues. We cannot exclude the possibility that hKE4 sequence was present in a minor portion of the hERR1 mRNA in RL95-2 cells and mouse kidney, however, undetectable by the limited sensitivity of Northern blot analysis. Consistent with the short hERR1 mRNA in the RL95-2

**Fig. 7.** Identification of the contact sites of GST-hERR1 fusion protein at the FP1 of the human lactoferrin gene. A, specific binding of GST-hERR1 to FP1 oligonucleotides in EMSA. One µg of GST-hERR1 fusion protein was interacted with preimmune serum (PI) or hERR1 antibody (ERR1) before incubation with the labeled FP1 (−418/−378) in 10 µl of reaction mixture. B, methylation interference analysis. Five ng of labeled and partially methylated DNA fragment (FP1) was incubated with 10 µg of GST-hERR1 fusion protein in 60 µl of reaction mixture. The locations of GST-hERR1 contacts on both DNA strands are indicated. The solid symbols represent strong contacts, and the open symbols represent weak contacts. The sequence is presented at the bottom.

**Fig. 8.** Immunodepletion of the C1 complex by antibody produced against the GST-hERR1 fusion protein. Three µg of nuclear protein from RL95-2 cells was incubated with labeled FP1 in an EMSA. Preimmune serum (PI), affinity-purified hERR1 antibody (ERR1), Vit-A2 ERE, oligonucleotide of vitellogenin A2 estrogen response element and free probe F are indicated.

**Fig. 9.** Direct interaction of hERR1 and ER. Bacteria expressed various fusion proteins (2 µg, lanes 3, 4, 6–8 and 11, 12, 14–16; 5 µg, lanes 5 and 13) were separated on a 10% SDS-PAGE, Western blotted, and hybridized with 32P-labeled GST-hERR1 as described under “Materials and Methods.” GST-HAV (viral protein); GST-NS (mouse DNA-binding protein PO-GA, gift from T. Sueyoshi); GST-EST (mouse testis estrogen sulfotransferase, gift from W. C. Song); GST, protein alone; Alb, bovine serum albumin; ST, standard; and GST-ER (human estrogen receptor). Lanes 1–8, Coomassie Blue stain. Lanes 9–16, radiogram.
cells, the major nuclear protein detected by hERR1 antibody in Western blot was 42 kDa. Therefore, hERR1 in the RL95-2 cells might be translated from the Met at nucleotide 177 (Fig. 6A), which predecated a 47-kDa protein. A minor 53-kDa protein was also detected by the hERR1 antibody in the nuclear extract of RL95-2 and HeLa cells (Fig. 6C). Posttranslational modification and degradation might produce a protein larger or smaller than predecated size from its amino acid sequence. It has been reported that hERR1 was copurified with COUP-TF as 53 kDa (Wang et al., 1991) and with a cellular transcriptional repressor of the SV40 major late promoter as 55 kDa (Wiley et al., 1993) protein from HeLa cell nuclear extract. Whether these hERR1 proteins were encoded by the same hERR1 mRNA in the RL95-2 cells is unknown. Reverse transcriptase PCR of various human tissue and cell line RNAs with specific hERR1 primers might reveal different forms of hERR1 mRNA. Alternatively, different hERR1 proteins could be detected by antibodies generated to specific peptides at different regions of the hERR1.

By using hERR1 as a probe, we isolated several cDNA clones from mouse brain and kidney cDNA libraries.2 Sequence comparison between human and mouse ERR1 revealed that the homologies are 90% in nucleotides and 98% in amino acid. This finding suggests that the hERR1 is evolutionary conserved. Protein alignment and dendogram analysis of the hERR1 to other steroid receptor show a close relationship to ER, particularly the DNA binding domain. There is 68% homology at this region and the nine cystine residues constituting the zinc-fingers are conserved (Green et al., 1986). This is paradoxical, since the hERR1 binds an extended AGGTCA motif and ER binds palindromic AGGTCA as dimer (see review by Glass (1994) and references therein). The mutagenesis and EMSA competition experiments (Fig. 5) suggest that the nucleotides surrounding the AGGTCA are important in order for hERR1 to bind. It is likely that the hERR1 belongs to the new subclass of orphan receptors (Ueda and Hirose, 1990; Wilson et al., 1992; Lavorgna et al., 1991; Tsukiyama and Niwa, 1992; Ikedai et al., 1993; Giguevre et al., 1994) that bind to the extended steroid receptor half-site as a monomer (Wilson et al., 1993).

Examining the distance between the hERR1 and ER binding sites (center to center) in the lactoferrin promoter, we found that there are three DNA helical turns between them (Teng et al., 1992). It is possible that hERR1 and ER both bind to their DNA element on the same side of the helix and interact with each other through a direct protein-protein contact. Indeed, by far-Western analysis, we were able to demonstrate protein-protein contact between hERR1 and ER (Fig. 9). Interaction between ER and other nuclear proteins has been found. Several lines of evidence suggest that AP-1-binding proteins, such as Fos and Jun and the ubiquitous transcription factor SP1, are involved in ER-mediated transactivation of estrogen-responsive genes that do not process the typical ERE (Gaub et al., 1990; Wu-Peng et al., 1992; Krishnan et al., 1994; Umayahara et al., 1994). Our preliminary data suggest that the hERR1 binding element of the human lactoferrin gene did not bind AP1 or SP1 (data not shown). At present, there is no evidence of ER heterodimer with other receptors or transcription factors. Nonetheless, several ER-associated proteins were recently identified (Halachmi et al., 1994; Cavailles et al., 1994, 1995). These proteins bind to the estradiol-activated ER, but not to the inactive ER. Whether hERR1 could interact with these ER-associated proteins needs to be examined. It was interesting to find that hERR1 has no effect on a strong palindromic ERE (Fig. 4C). Therefore, hERR1 may not be a required coactivator for estrogen action, but could be an integral part of estrogen response module specifically for human lactoferrin gene.

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