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 reported hERR1 sequence. By Western analysis, a major 42-kDa protein is detected in the RL95-2 nuclear extract with antibody generated against GST-hERR1 fusion protein. Finally, we show that the hERR1 interacts with the human estrogen receptor through protein-protein contacts.


**Estrogen-related Receptor, hERR1, Modulates Estrogen Receptor-mediated Response of Human Lactoferrin Gene Promoter*\(^1\)**

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† The abbreviations used are: HRE, hormone response element; ERE, estrogen response element; EMSA, electrophoresis mobility shift assay; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis.

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\(^{1}\) The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L38487 (Genome Sequence Data Base).

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binding elements of SF-1/ELP (Ikeda 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 sequenced and identified as hERR1 (Giguere et al., 1988). Furthermore, we showed that the hERR1 interacted with estrogen receptor through protein-protein contact, suggesting that ERR1 may participate in estrogen stimulation of human lactoferrin gene.

**Materials and Methods**

Plasmids and Oligonucleotides—The pH-414CAT plasmid was constructed as described previously (Yang and Teng, 1994). Location of the oligonucleotides used in EMSA are presented in Fig. 3A, 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, 5'-ACCTGCGCTAATGCCTAGTCGCACTAAGGTCTCTG-3'; m1, 5'-ACCTGCCAACATCTGCCTAGTAC-ACCTGCCCTAAGGTCATCTGCTG-3'; m2, 5'-ACCTGCCGATCTTCA-3'; m7, 5'-TCAAGGCGATCTTCA-3'; m6, 5'-CC CACCTTCAAGGTCATCTGCTG-3'; m5, 5'-CATCTGCTG-3'; m3; CC CACCTTCAAGGTCATCTGCTG-3'; m4, 5'-ACCTGCCCTAAGGTCATCTGCTG-3'; m8, 5'-GGACCTTCAAGGTCATCTGCTG-3'. The double-stranded oligonucleotides were gel-purified. The labeled third round of duplication, we obtained a plasmid, pSLFP32, containing four tandem repeats of the FP1 sequence was identified by sequencing and designated as pSLFP4. This plasmid was used for duplication of the insert. The third round of duplication, we obtained a plasmid, pSLFP32, containing 32 head to tail repeats of the FP1 sequence. This DNA fragment was isolated from the plasmid, pSLFP32, labeled with [gamma-32P]dCTP, and used as the probe for screening the expression cDNA library.

Construction and Screening of Expression cDNA Libraries—Total RNA from log phase of RL95 cell was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The poly(A)+ RNA was purified by a standard oligo(dT) column. The pGEX2 expression library was constructed with a cDNA synthesized, according to the manufacturer's specification (Life Technologies, Inc.). The cDNA library contains 1.5 x 10^6 individual recombinants.

The RL95-2 expression cDNA library was screened with labeled FP1 oligonucleotides (1.47 kilobase pairs of the pSLFP32 insert) as described (Vinson et al., 1988). The protein-expressing clone interacting with the FP1 DNA probe was isolated and designated as pEF1. 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 nucleotide 740 to 760 and 1190 to 1250) diverging from the published hERR1 (Giguere et al., 1988).

Generation of Prokaryotic Recombinant Protein—Large amounts of -DNA from hERR1 recombinant were prepared by standard methods (Maniatis et al., 1982). The cDNA insert was recovered by restriction enzyme digestion and then isolated to obtain the cDNA insert (using lambda DNA as a carrier). The coding region was amplified by PCR, using the primers 5'-GGAAATTCCTGACATGCTGACCTG-3' and 5'-GGAAATTCCTGACATGCTGACCTG-3', and the antisense primer, 5'-GGAAATTCCTGACATGCTGACCTG-3'. The PCR products were subcloned into EcoRI site of the pGEX-4t-3 expression vector (Pharmacia). The cloning site and the in-frame reading with the EcoRI linker were verified by dideoxy sequencing. This expression recombinant was designated as pGEX-HERR1. The pGEX-HERR1 was generated by PCR the HEO plasmid with the following primers containing the EcoRI linker: the sense primer, 5'-GGAAATTCCTGACATGCTGACCTG-3', and the antisense primer, 5'-GGAAATTCCTGACATGCTGACCTG-3'. The PCR products were subcloned into EcoRI site of the pGEX-4t-1.

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-HERR1 and GST-HEO, 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

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The preparation of protein extracts and the analysis of protein expression were performed as described previously (Liu et al., 1993). Briefly, a single-stranded FP1 oligonucleotide (~418 to ~378 plus BamHI/BglII cloning site; Yang and Teng, 1994) was end-labeled with T4 kinase and [[gamma-32P]ATP, then annealed to the unlabeled complementary strand. The double-stranded oligonucleotides were gel-purified. The labeled DNA was incubated in a 200-µ1 reaction mixture containing 50 mM dimethyl sulfoxide, 50 mM sodium cacodylate, and 1 mM EDTA, pH 8.0, at room temperature for 3 min. DNA was purified by repeated ethanol precipitation. About 5 ng of labeled and methylated DNA (4 x 10^3 cpm) was incubated with 50 µg of nuclear protein extract in a 60-µ1 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 analyzed on an 8% 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 Chioromphenicol Acetyltransferase (CAT) Assay—Human endometrium carcinoma RL95-2 cells (ATCC CR1617) were grown in 1:1 mixture of Dulbecco's minimal essential medium/Ham's F-12 supplemented with 10% fetal bovine serum, 5 µg/mL of bovine insulin, and 100 units/mL penicillin/streptomycin under 5% CO2. Transient transfections were performed by the calcium phosphate method with a Cellphex transfection kit (Pharmacia LKB Biotech). The CAT assays were performed previously (Liu and Teng, 1992), and the reaction products were analyzed with an ascending TLC followed by quantitation using the PhosphorImager System (Molecular Dynamic, Sunnyvale, CA). The cells were cotransfected with vector alone or with 5 µg/mL 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 presented as means ± SD.

Preparation of Probe for Expression cDNA Library Screening—The 46 base pairs of oligonucleotide (~418/-378, the FP1, plus BamHI/BglII cloning sites) was immobilized on nylon membranes with modifications in a direct head to tail orientation according to Rosenfeld and Kelly (1986). After the ligation reaction, the larger DNA fragments were isolated from 1.2% agarose gel and subsequently subcloned into the BamHI/BglII sites of pSL1180 vector (Pharmacia). A plasmid containing four tandem repeats of the FP1 sequence was identified by sequencing and designated as pSLFP4. This plasmid was used for duplication of the insert. The third round of duplication, we obtained a plasmid, pSLFP32, containing 32 head to tail repeats of the FP1 sequence. This DNA fragment was purified, labeled with [gamma-32P]dCTP, and used as the probe for screening the expression cDNA library.

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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 mM 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 formamide/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 DNA in lane FP1.4 with EcoRI/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 (Li 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 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 levels 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 pHL414CAT 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).
Fig. 6. 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. 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-kDa protein and a minor 53-kDa protein (arrow) were detected by antiserum to hERR1. The molecular markers are indicated.
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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 loss 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 aberrant initiation of transcription or lost binding of the positive or negative transcription factors that are part of the basal promoter machinery. 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 λ hKE4 and nucleotides 179–2,430 from λ 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...
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 predicated 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 predicated 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 dendrogram analysis of the hERR1 to other steroid receptors 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 AGGGTCA 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 AGGGTCA 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., 1991; Lavorgna et al., 1991; Tsukiyama and Niwa, 1992; Ikeda et al., 1993; Giguere et al., 1994) that bind to the extended steroid receptor half-site as a monomer (Wilson et al., 1991; Giguere et al., 1992; Tsukiyama and Niwa, 1992; Ikeda, Y., Lala, L. D., Luo, X., Kim, E., Moisan, M., and Parker, K. L. (1993) Mol. Endocrinol. 7, 853–860).

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., 1990; Wu-Peng et al., 1991; Huang et al., 1992; Teng, C. T., Liu, Y. H., and Panella, T. (1992) Mol. Endocrinol. 6, 1969–1981; Teng, C. T. (1994b) in Lactoferrin: Structure and Function (Hutchens, T. W., ed) pp. a7.5.1–a7.5.7, John Wiley & Sons, New York). Posttranslational modification and degradation might produce a protein larger or smaller than predicated 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.

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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., 1991; Krishnan et al., 1992; 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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Nengyu Yang, Hiroyuki Shigeta, Huiping Shi and Christina T. Teng

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