Functional Map of a Placenta-specific Enhancer of the Human Leukemia Inhibitory Factor Receptor Gene*

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We recently reported a placenta-specific enhancer in the human leukemia inhibitory factor receptor (LIFR) gene and now show detailed characterization of the 226-base pair enhancer (−4625/−4400 nucleotides). Four of twenty-two mutants in linker analysis showed reduced promoter activities to 45, 30, 10, and 10%, respectively. Specific binding of region A (−4617/−4602) with nuclear extract was competed by a known Oct-1 oligo and supershifted by Oct-1 antibody. Specific binding of region B (−4549/−4535) was competed by a GATA oligo, but could not be supershifted by four GATA antibodies. Nevertheless, mutagenesis showed that critical bases in region B were identical to the GATA core motif, indicating that region B may bind to a novel GATA family transcription factor. The other two adjacent regions designated as region C (−4464/−4445) showed no known consensus binding sites, and their specific placental JEG-3 nuclear extract binding was not evident in nonplacental nuclear extracts and was not competed by a trophoblast specific element (TSE), indicating that region C is a novel placenta-specific element (PSE, CATTCG/TAACTGTTT). Footprinting localized the binding boundary of PSE-binding protein (PSEB), and three Cs were found to be important for specific PSE binding. UV cross-linking showed that PSEB had a molecular mass of ~160 kDa, substituting the PSE with two previously reported placenta elements TSE or chorionic somatomammotropin enhancer factor 1 (CSEF-1) motifs resulted in markedly different promoter activities, indicating that PSEB is indeed different from TSE binding protein or CSEF-1. These results are the first demonstration that a novel PSE is the major element for placenta-specific enhancer activity in human LIFR gene.

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The abbreviations used are: LIFR, leukemia inhibitory factor receptor; IL, interleukin; LIF, leukemia inhibitory factor; PSE, placenta-specific element; PSEB, PSE-binding protein; TSE, trophoblast specific element; TSEB, TSE-binding protein; CSEF-1, chorionic somatomammotropin enhancer factor 1; CRE, cyclic AMP-response element; URE, upstream regulatory element; TEF, transcription enhancer factor; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; ADA, adenosine deaminase; CS-B, chorionic somatomammotropin B; hCS-B, human CS-B; bp, base pair(s); kb, kilobase pair(s).
utilizes alternative promoters to regulate its expression in placental and nonplacental tissues (25). Interestingly, a placenta-specific enhancer was identified −4.6 kb upstream to the placenta-specific promoter (25). The minimal enhancer, 226 bp, increases heterologous promoter activity 10–35-fold when placed upstream or downstream of the reporter gene in either orientation (25). Within this 226-bp region, several potential transcription factor binding sites were predicted, but site-directed mutagenesis failed to correlate two transcription factors, Sp1 and NF-κB, with enhancer activity (24). Here we report a detailed characterization of this minimal enhancer with three critical elements contributing to the enhancer activity, an Oct-1 binding site at −4617/−4602 site; a GATA-like element at −4549/−4535 site, and a novel placenta-specific element (PSE) at −4464/−4445.

**EXPERIMENTAL PROCEDURES**

Cell Lines, Plasmids, and Antibodies—A human choriocarcinoma cell line JEG-3 (ATCC HTB-36) was maintained in Dulbecco’s modified Eagle’s medium high glucose (Life Technologies Inc., supplemented with 10% fetal bovine serum (FBS); a human breast adenocarcinoma MCF-7 (ATCC HTB-22), a human cervix carcinoma cell HeLa (ATCC CCL-2), was maintained in McCoy’s 5A medium with 10% FBS, a human thyroid carcinoma TC1 (gift of Dr. Jerome Hershman) was maintained in RPMI 1640 with 10% FBS. A mouse pituitary tumor cell line AtT20 (ATCC CCL-89) and a rat pituitary tumor cell line GC were maintained in minimum essential medium with 10% FBS. Plasmids pGL3PX-(SK)1 (−4400/−4625 nt), pGL3-KP(-798), and pGL3-U2P were constructed as reported previously (25).

**Linker Analysis and Mutagenesis—**Previous transfection experiments demonstrated that the cloned human LIFR gene

| Exp. | Oligo | Sequence | Position |
|------|-------|----------|----------|
| I    | a     | AAG GTT TTA TCA TAG A | −4617/−4602 |
|      | b     | CTT ATC AGA CTT AAA   | −4548/−4535 |
|      | c     | CAT GCC CTG AAC TAG TTT TTT TT | −4644/−4445 |
|      | Oct-1 | TGG CGA ATG CAA ATC ACT AGA A | |
|      | GATA  | CAT GCC ATG AGC GTC CAG CCG GAA | |
|      | Sp1   | ATT CTC GAG GGC CGG GGC GAG C | |
|      | AP1   | AGC TGT ATG AGC GTC CAG CCG GAA | |
|      | TSE   | ACA AAA ATG ACC TAA GGG TTT AAA | |

**Expression Studies**

**Characterization of Human LIFR Placenta-specific Enhancer**

**Nuclear Extract Preparation, EMSA, and Supershift Assay—**Crude nuclear extracts from JEG-3, HeLa, TC1, MCF-7, U-2 OS, AtT20, and GC cells were prepared as previously reported (25). The protein concentrations of the nuclear extracts were quantitated by Bio-Rad assay (Bio-Rad). Equal amounts of nuclear extract from different cells were used in EMSA. Labeled oligonucleotide duplex (20,000–30,000 cpm) was mixed with −5 µg of nuclear extract, 1 µg of poly(dI-dC) in 25 µl of reaction buffer. For Oct-1 and GATA-like elements, the binding buffer was 10 mM Tris, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 8% glycerol. For the novel placental element, the binding buffer was 10 mM Hepes, pH 7.6, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol. For competition assay, 200-fold excess cold competitor oligos were added prior to the addition of labeled probe. For supershift assay, antibody was added after addition of labeled probe and incubated at room temperature for 1 h or 4 °C overnight. EMSA samples were resolved on 5% nondenaturing polyacrylamide gel electrophoresis gel.

Mutant oligos disrupting G residues and their ‘3’- neighboring bases in this region were also used as competitor oligos to test the contribution of Gs to the binding complex and are listed in Table 1. For location of the minimal region of PSE, nested oligos were synthesized as in Table I and used as competitor oligos.

**DNase I Footprinting—**DNase I footprinting was used to localize the binding boundary of placenta-specific element binding protein. The DNA probe was generated by a polymerase chain reaction in which only one primer was labeled with [γ-32P]ATP. Probes for both plus and minus strands were mixed with 30 µg of JEG-3 nuclear extract in the binding buffer as used in EMSA. After incubation on ice for 30 min, various amounts of DNase I were added and allowed to incubate at room temperature for 1 min. Stop buffer (200 mM NaCl, 2 mM EDTA, 1% SDS, 50 µg/ml RNA) was added to terminate the reaction. For the competition assays, 200-fold placenta-specific element characterized in EMSA was used as cold competitors. Samples were analyzed on 9% 15% urea-PAGE gels. A GA ladder reaction was performed according to standard methods (26).

**UV Cross-linking—**For UV cross-linking, 50,000 cpm oligo duplex probe was incubated with 10 µg of JEG-3 nuclear extract and resolved as for EMSA. The wet gel was then irradiated with a 312-nm UV transilluminator, and the corresponding gel slice was removed and autoradiographed.

**RESULTS**

**Linker Analysis Reveals Three Critical Regions within the LIFR Placenta-specific Enhancer**

## Table I

| Exp. | Oligo | Sequence | Position |
|------|-------|----------|----------|
| I    | a     | AAG GTT TTA TCA TAG A | −4617/−4602 |
|      | b     | CTT ATC AGA CTT AAA   | −4548/−4535 |
|      | c     | CAT GCC CTG AAC TAG TTT TTT TT | −4644/−4445 |
|      | Oct-1 | TGG CGA ATG CAA ATC ACT AGA A | |
|      | GATA  | CAT GCC ATG AGC GTC CAG CCG GAA | |
|      | Sp1   | ATT CTC GAG GGC CGG GGC GAG C | |
|      | AP1   | AGC TGT ATG AGC GTC CAG CCG GAA | |
|      | TSE   | ACA AAA ATG ACC TAA GGG TTT AAA | |

For linker analysis, all 22 mutant plasmids together with control pGL3PX-(SK)1 (−4400/−4625 nt) were transfected in the same experiment and repeated twice. 48 h after transfection, cell lysates were prepared for measurement of luciferase and β-galactosidase activities.

**Oligos**—Oligos used for electrophoretic mobility shift assay (EMSA) assays are listed in Table I.

**Linker Analysis and Mutagenesis—**A total of 22 10-bp replacement (AGCTTAAAGC) mutants in linker analysis were generated by ExSite (Stratagene) or Kunkel’s method (28). Site-directed mutagenesis disrupting Oct-1 binding site or GATA binding site and substitution mutagenesis with TSE or chorionic somatomamotropin enhancer factor 1 (CSEF-1) binding sites at PSE position were also performed. The mutagenic primer sequences are available upon request. All mutants were verified by sequencing.

**Transient Cell Transfection—**All plasmids were prepared using Maxi-prep kit (Qiagen). pCMV β-galactosidase was co-transfected as an internal control. JEG-3 were transfected using standard LipofectAMINE method (Life Technologies Inc.). Transfections were performed in triplicate. For linker analysis, all 22 mutant plasmids together with control pGL3PX-(SK)1 (−4400/−4625 nt) were transfected in the same experiment and repeated twice. 48 h after transfection, cell lysates were prepared for measurement of luciferase and β-galactosidase activities.

**Differential footprinting**—DNase I footprinting was used to localize the binding boundary of placenta-specific element binding protein. The DNA probe was generated by a polymerase chain reaction in which only one primer was labeled with [γ-32P]ATP. Probes for both plus and minus strands were mixed with 30 µg of JEG-3 nuclear extract in the binding buffer as used in EMSA. After incubation on ice for 30 min, various amounts of DNase I were added and allowed to incubate at room temperature for 1 min. Stop buffer (200 mM NaCl, 2 mM EDTA, 1% SDS, 50 µg/ml RNA) was added to terminate the reaction. For the competition assays, 200-fold placenta-specific element characterized in EMSA was used as cold competitors. Samples were analyzed on 9% 15% urea-PAGE gels. A GA ladder reaction was performed according to standard methods (26).

**UV Cross-linking**—For UV cross-linking, 50,000 cpm oligo duplex probe was incubated with 10 µg of JEG-3 nuclear extract and resolved as for EMSA. The wet gel was then irradiated with a 312-nm UV transilluminator, and the corresponding gel slice was removed and autoradiographed.

**RESULTS**

**Linker Analysis Reveals Three Critical Regions within the LIFR Placenta-specific Enhancer**—Previous transfection experiments demonstrated that the cloned human LIFR gene
Characterization of Human LIFR Placenta-specific Enhancer

The specificity of cloned LIFR enhancer and promoter in placental and pituitary cells. Activity of the cloned placenta-specific enhancer (A), placenta-specific promoter and alternative promoter (B) in placental and pituitary cells was measured in triplicate transfection assays. Luciferase activity was normalized to β-galactosidase activity and for each cell line the normalized pGL3-promoter or pGL3-basic control readings were set as 1, respectively. pGL3PX-(SK)1 (~4400/~4625) was used to test for enhancer specificity with pGL3-promoter and alternative promoter (25) is strongly active in these pituitary cells. Also, the LIFR gene enhancer, active in placental cell lines, is not active in the three pituitary cell lines tested, further demonstrating its stringent tissue specificity.

Data base searching predicted several potential transcription factor binding sites within this minimal enhancer region (25), including cyclic AMP-response element (CRE), Sp1, GATA, and NF-kB. Notably, three NF-κB binding sites (27–29). We therefore chose several available pituitary cell lines, namely ACTH-producing AtT20 and growth hormone secreting GH4 and GC cell lines to further test the specificity of the cloned LIFR gene enhancer and two LIFR promoters. The transfection results shown in Fig. 1 depict the inactive placenta-specific LIFR promoter in pituitary cells, while the alternative LIFR promoter (25) is strongly active in these pituitary cells. Also, the LIFR gene enhancer, active in placental cell lines, is not active in the three pituitary cell lines tested, further demonstrating its stringent tissue specificity.

### Table 1: Potential transcription factor binding sites for functional regions in the LIFR placenta-specific enhancer.

| Region | Site 1 | Site 2 | Site 3 | Site 4 | Site 5 | Site 6 |
|--------|--------|--------|--------|--------|--------|--------|
| oligo a | - | + | - | - | - | - |
| oligo Sp-1 | - | + | - | - | - | - |
| oligo Oct-1 | - | - | + | - | - | - |
| NI serum | - | - | - | + | - | - |
| Oct-1 Ab | - | - | - | - | + | - |

Fig. 3. Potential transcription factor binding sites for functional regions in the LIFR placenta-specific enhancer. Three functional regions A, B, and C are shown in boldface with putative factors shown below; the consensus binding cores for corresponding transcription factors are double underlined.

Fig. 4. Region A is an Oct-1 binding site. Competition EMSA and supershift assay of oligo a and its binding protein. The probe used was from ~4617 to ~4602 nt (Table I). Lane 1, oligo a only, without any competitor; in lanes 2–4, cold competitor oligos as indicated were added at 290-fold excess concentrations; lane 5 was the same as lane 1 except that an Oct-1 antibody was also added in the mixture.
formed site-directed mutagenesis to disrupt critical bases within the corresponding Sp1 or NF-κB consensus regions within this enhancer, but failed to observe loss of enhancer activity (25). Thus, it appears that the predicted Sp1 and NF-κB/c-Rel binding sites are irrelevant to enhancer activity in LIFR gene.

To characterize this placenta-specific enhancer from a functional point of view, we performed a comprehensive linker analysis with the introduction of 10 bp of replacement mutation covering the entire enhancer. Twenty-two mutants were thus obtained, and their sequences were confirmed. These mutants together with an enhancer control were transfected into JEG-3 cells, and the result is as depicted in Fig. 2. Four mutants demonstrated consistent loss of enhancer activity. Mutants 2, 9, 17, and 18 have base substitutions from −4614 to −4605 nt, −4544 to −4535 nt, −4464 to −4455 nt, and −4454 to −4445 nt, respectively, and their corresponding enhancer activities are reduced to 45, 30, 10, and 10%, respectively. Also, mutant 17 and mutant 18 have adjacent base substitutions. Taken together, these results indicate that three elements in this enhancer, namely A (−4614/−4605 nt), B (−4544/−4535 nt), and C (−4464/−4445 nt) are critical to the full enhancer activity. Subsequently, a new data base search was performed specifically for these three elements, and the result is as shown in Fig. 3. Potential transcription factors capable of binding these regions were identified including Oct-1 and GATA.

Region A Is an Oct-1 Binding Site—Region A (−4614/−4605 nt) was found to have considerable homology to a known tran-
scription factor Oct-1 consensus binding site (30), although a previous data base search using higher stringency failed to predict a potential binding site in this locus. To test whether Oct-1 does in fact bind to region A, both competition EMSA and Oct-1 antibody supershift assay were performed. An oligo a (−4617/−4602 nt) covering region A was used as a probe to perform these assay (Fig. 4). This oligo produced a specific binding complex as determined by competition assay, including using itself as a cold competitor (lanes 1–3). Specific binding of the complex was also dissipated when a known Oct-1 oligo was added separately as competitor; moreover, this binding complex was supershifted with the addition of an Oct-1 antibody (lane 5). These results demonstrate that the transcription factor Oct-1 binds to element A. Also, cotransfection of Oct-1 expression vector into JEG-3 cells slightly increased enhancer activity, similar to previous observations that although TEF-5 is required for hCS-B enhancer activity and binds to two elements necessary for hCS-B enhancer activity, overexpression of TEF-5 does not activate hCS-B enhancer activity (31).

Region B Is a GATA-like Element—Region B (−4544/−4535 nt) exhibited high homology to the GATA family member consensus binding site (WGATAR), as demonstrated both in a previous search (25) and a current search after linker analysis. Since GATA family members are implicated in the placenta-specific regulation of glycoprotein hormone a subunit and adenosine deaminase (ADA) (32, 33), it was important to determine whether region B is GATA-related.

Competition experiments with EMSA showed that the specific binding complex formed by an oligo b (−4549/−4535 nt) covering region B and JEG-3 nuclear extract was competed by a known GATA oligo at 200-fold excess concentration (Fig. 5A, lanes 1–4), while specific complexes formed by the known GATA oligo probe and JEG-3 nuclear extract could only partially be competed by the test oligo b at 200-fold excess concentration (Fig. 5A, lanes 11–14), suggesting that region B is a GATA-like element; GATA oligo efficiently binds to the same proteins that this element can bind, but the element cannot efficiently bind to all proteins to which the GATA oligo binds. This hypothesis was supported by supershift assays using antibodies to GATA-1, -2, -3, and -4 as shown in Fig. 5A, lanes 5–8 and lanes 15–18. Specific complexes between the known GATA oligo with JEG-3 nuclear extract were supershifted by antibody to GATA-2, -3, or -4, indicating the capability of the known GATA oligo to bind to GATA-2, -3, or -4 proteins, which are known to be present in JEG-3 cells (32, 34). In contrast, specific complexes between the oligo b and JEG-3 nuclear extract could not be supershifted by antibody to GATA-2, -3, or -4, indicating that these specific complexes involved a protein other than GATA-2, -3, or -4 recognizing region B.

Previous observations revealed that mutation of two bases within the GATA consensus binding site (GA → CT in WGATAR) abolishes binding with GATA family members. To test whether these two bases are also critical within this GATA-like element, we performed site-directed mutagenesis to change GA in region B to CT, and in transient transfection assays (Fig. 5B) showed that this new mutant lost enhancer activity to a similar extent as the 10-bp replacement mutant 9, demonstrating that this GA is a critical contact site between region B and its corresponding binding protein.

The tissue distribution pattern of this corresponding protein was examined by EMSA using nuclear extracts from cell lines of different tissue origin (Fig. 5C). As depicted, this protein is also present in all nuclear extracts tested, showing that it is not placenta-specific; however, it seems to be more abundant in placental JEG-3 cells than other nonplacental cells.
**Fig. 7. Analysis of the PSE.** **A.** DNase I footprinting on the plus strand of LIFR placenta-specific enhancer. A single end-labeled probe was used; in lane 1, no nuclear extract was added, and 0.1 unit of DNase I was added; in lanes 2–5, 40 μg of JEG-3 nuclear extract were added, with DNase I at 0.2, 0.5, 0.8, and 1 unit, respectively; in lane 6, 40 μg of JEG-3 nuclear extract were added, with 0.5 unit of DNase I and 200-fold excess cold oligo c competitor included. Sequences of the protected region are indicated by the break in the brackets. **B.** DNase I footprinting on the minus
added competitor, suggesting that mutant oligos cM2, cM3, and cM6 used in lanes 5, 6, and 9 contained mutations at critical bases, as underlined in CATTTCCTGAACTAGTTTTT. To determine the minimal region of PSE required for specific binding to this protein, we designed a set of nested oligos and used them as competitor oligos to test their ability to compete the specific binding. A typical competition assay to determine this minimal region is shown in Fig. 7C (lanes 10–12). The 14-bp oligo from 24459 to 24446 nt (lane 11) competed specific binding, while a 12-bp oligo from 24458 to 24447 nt (lane 12) did not compete. Thus the 14-bp element (24459/24446 nt) was defined as the minimal functioning element for region C.

Two previously reported DNA binding proteins, TSE binding protein (TSEB) and chorionic somatomammotropin enhancer factor 1 (CSEF-1), are implied to play important roles in regulating several placenta-specific transcripts and appear to be detected mainly in placental cell lines. In order to see whether this PSE bind protein (PSEB) is indeed different from TSEB or CSEF-1 and whether these PSE, TSE, and CSEF-1 binding sites are functionally interchangeable, we made two mutants with substitutions at the PSE position in the enhancer to the TSE and CSEF-1 binding sites, respectively. Transfection into placental JEG-3 cells and nonplacental U-2 OS cells were performed, and the results are as shown in Fig. 8A. As depicted, substitution of PSE with TSE or CSEF-1 binding sites resulted in 1/5- and 4-fold difference of promoter activity, respectively, as compared with the wild-type construct. And all three constructs showed no promoter induction in U-2 OS cells, demonstrating the inactiveness of these constructs in nonplacental cells. We also performed UV cross-linking to determine the molecular mass of this PSEB. As shown in Fig. 8B, the free probe recovered from EMSA showed no signal in SDS-PAGE after UV cross-linking, while the placenta-specific binding complex recovered from EMSA revealed a molecular mass band of approximately 160 kDa in SDS-PAGE after UV cross-linking.
kDa, respectively (36, 37), it is therefore apparent that PSEB is a novel protein rather than TSEB or CSEF-1.

**DISCUSSION**

Placenta-specific transcriptional controls fall into two broad categories: first, they may share the same promoter as other tissues, but utilize placenta-specific enhancers or upstream elements as observed for glycoprotein hormone α subunit, CS-B, adenosine deaminase (ADA), and leptin genes (28, 29, 32, 38, 39); second, they may utilize both placenta-specific promoters and enhancers, as previously shown for the aromatase gene (33) and as we now show for the *LIFR* gene.

A composite enhancer (~180/111 bp) of the glycoprotein hormone α subunit gene promoter is responsible for its placental expression rather than pituitary gonadotropic or thyrotropic expression (29). This enhancer was composed of two CREs and an upstream regulatory element (URE) (40, 41), whereas URE could be further subdivided into three overlapping sites, α activator element, TSE, and URE1 (42). It was also found that the α activator element binds to hGATA-2 and hGATA-3. TSE/URE1 forms an overlapping element that may bind two functionally interchangeable proteins, TSEB and CSEF-1.

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The proximal promoter for *hCS-B* is active in rat pituitary cells (44), however, an enhancer at 2.2 kb 3′ of the *hCS-B* gene mediates its placenta-specific expression (45). Within this 138-bp enhancer, DNase I footprinting revealed four binding sites (DF-1, -2, -3, and -4). DF-3 and DF-4 appeared to be placenta-specific (46) and DF-4 may bind with the transcription enhancer factor 1 (TEF-1) (46). More recently, hCS enhancer function was shown to be mediated by cooperative binding of TEF-1 and a 30-kDa placenta-specific factor, CSEF-1 to multiple, low affinity binding sites within the region (37).

The ADA gene promoter is active in several cells, but a 770-bp enhancer located ~5.4 kb upstream of the murine ADA gene conferred its placenta-specific expression. Within this enhancer, two motifs similar to TSE were found in a strong placenta-specific footprint (FP1) (33). Two GATA motifs, two CREs, and five basic helix-loop-helix sequence motif (CANNTG) were predicted to be involved in enhancer activity (33).

For the leptin gene, although the same promoter is used for adipose and placental transcription, an upstream enhancer (~1561/1546 nt) functions in JEG-3 and JAR choriocarcinoma cells but not in adipocytes or HeLa cells (39). Three elements were suggested to participate in DNA-protein interaction within the enhancer by DNase I footprinting, including two motifs PLE1 (~1948/1913) and PLE3 (~1909/1874), which appeared to be placenta-specific (39).

In the second group of placenta-specific gene transcripts, aromatase expression is under placenta-specific control for its placental transcripts, albeit mature aromatase protein is present in multiple tissues. DNA sequences (~301/115 nt) upstream to placenta-specific exon I increased reporter expression 20-fold in its natural orientation, and two important elements in this region contained a TSE consensus sequence and are able to bind to TSEB (47).

Thus far identified regulatory elements participating in placenta-specific transcription include CRE (TGACGTCA core), GATA (WGATAR core), basic helix-loop-helix (CANNTG core), TEF-1 binding site (TGGAATG core), and placenta-specific elements such as TSE (AACAATATCAGTCAGGCAGT), CSEF-1 binding site (TAGACCTGGAATGTGGCCAG), and the less characterized URE1 (GGGTTGAAACAGATAA), PLE1 (~1948/1913 CAGTACCTCAGGTCTAATGGTTGTTGA AAAACTC), PLE3 (~1909/1874 CCTGGTAAAATTTGTGGTCCAAGACAGTTTCTGCTCT). Corresponding binding proteins include CREB, GATA-2, GATA-3, TEF-1, basic helix-loop-helix binding protein, UREB, TSEB, and CSEF-1, among which CREB, GATA-2, GATA-3, and TEF-1 are ubiquitous transcription factors; while TSEB and CSEF-1 are two proteins preferentially expressed in the placenta.

Although *LIFR* placenta-specific transcription appears characteristic of the same group as aromatase, *LIFR* gene enhancer utilizes a different set of DNA elements and binding proteins for its placenta-specific function. An Oct-1 binding site, a GATA-like element, and a novel PSE were found to be critical. Nevertheless, these elements share some resemblance to other placenta-specific elements. Although GATA-2, -3, and -4 antibody produced supershifts, it is clear that these proteins do not account for all the specific binding complexes containing GATA oligo; on the other hand, the binding protein for he GATA-like element was competed by the known GATA oligo, suggesting this novel GATA family member also has the potential to bind with previously detected GATA motifs in other placenta-specific enhancers. For the Oct-1 binding site, an interesting report has shown that TEF-1 binds to an Oct binding site (CAAAGGACAT) in the SV40 enhancer (30) and also an Oct element overlaps with one of the TEF-1 binding sites in the HPV-16 enhancer (30). In our Oct-1 supershift assay, the specific binding complex was only partially supershifted, implying that the remaining binding complex might be derived from the Oct-1 binding site with another transcription factor such as TEF-1 (48) or TEF-5 (31). The core sequence of the novel placenta-specific element is CATGGCCTGAACTAGTTTTT. It does not share homology to TSE, CSEF binding site, PLE1, or PLE3, and thus this might be a new mechanism for regulation of placenta-specific gene expression.

One intriguing observation in studying placenta-specific transcription of these genes is that no hormone response element has been shown to be involved in their placenta-specific transcription, given the fact that placenta is the major excretory organ that can produce hormones and also an organ under the influence of several hormones during pregnancy. For the human *LIFR* placenta-specific enhancer and promoter, this also seems to be the case; although some glucocorticoid response element, progesterone response element, retinoic acid response element, and estrogen response element were predicted in the data base search, neither dexamethasone, retinoic acid, progesterone, nor 17β-estradiol treatment of transfected JEG-3 cells showed significant effects on *LIFR* promoter activity (data not shown). Nor did the Northern analysis of JEG-3 cells treated with these steroids reveal any effect on *LIFR* mRNA levels (data not shown). These observations appear to be in constrast to the observation that human *LIFR* mRNA levels increase during the first trimester and term placenta. A possible explanation is that *JEG-3* cells, as a differentiated cell line secreting high level of human chorionic gonadotropin hormone, may not represent a suitable placental cell type which is responsive to hormone treatment.

Since TSE was found to be involved in several placenta-specific enhancers such as α subunit, *hCS-B*, aromatase, and ADA, it was felt that TSE might be a master switch in placenta cell differentiation that simultaneously regulates a range of placenta-specific gene expression (36). However, *LIFR* is not under the same control because no TSE motif was detected. Leptin gene transcription also does not appear to be under TSE control. These observations suggest that the placenta may not possess a universal master switch for all placenta-specific gene expression, although TSE is involved in placenta-specific expression of several genes.

Several knockout mice targeting placenta-specific genes and transcription factors involved in their regulations have been
produced. Glycoprotein hormone α subunit homozygous knock- 
out mice did not exhibit placental abnormality, although they 
are hypogonadal and exhibit profound hypothryoidism and 
dwarfism (49). Mice lacking a functional leptin gene not only 
became massively obese (50), but also are infertile (51), but no 
placental abnormality was reported. ADA-deficient fetuses 
lacking ADA in their adjoining placenta die during late fetal 
development (52), while genetically restoring ADA to placentas 
of ADA-deficient fetuses rescued them from perinatal lethality 
(53). While GATA-3 knockout mice have severe abnormalities 
in the nervous system and fetal liver hematopoiesis (54), and 
GATA-2 knockout mice are defective in early hematopoietic cell 
proliferation and mast cell formation (55), both GATA-2 and 
GATA-3 knockout mice did not show placental abnormalities, 
thus supporting the presence of other GATA family members 
functioning in the placenta. TEF-1 null mice show defects only 
in the heart, but not in other tissues, including the placenta 
(56). Notably, these knockout results, except those of the ADA 
knockout, showing no murine placental abnormalities, are in 
marked contrast to the LIFR knockout mice which demonstra-
ted severe placental dysfunction (13).

We previously hypothesized that in placenta and/or during 
pregnancy, placenta-specific transcription factor(s) are active 
and interact with the LIFR gene placenta-specific enhancer 
and promoter, leading to enhanced transcription of LIFR 
mRNA restricted to the placenta. Identification of this novel 
PSE in LIFR gene enhancer strongly supports this hypothesis, 
and the cDNA cloning of the 160-kDa PSEB will further elucidate 
the specific placental transcriptional control of the LIFR.

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