Identification of an Upstream Enhancer within a Functional Promoter of the Human Leukemia Inhibitory Factor Receptor Gene and Its Alternative Promoter Usage*  

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The leukemia inhibitory factor receptor (LIFR) is an integral component of gp130-LIFR signaling complex (1–4), which participates in signal transduction by members of the interleukin-6 (IL-6) cytokine family, currently including IL-6, leukemia inhibitory factor (LIF), interleukin-11 (IL-11), oncostatin M, ciliary neurotropic factor, and cardiotoxin (1, 5). Members of this family possess pleiotropic and redundant functions as assessed by in vitro assays and or gene knockout experiments (5–7). These include growth promotion and/or activation of hematopoietic, hepatic, osteogenic, and or neuronal cells (1, 2, 8, 9). On the other hand, these cytokines also exhibit respective specific actions. For example, adult female LIF knockout mice are unable to implant embryos, resulting in infertility (6), and LIF has also been found to synergize with corticotropin-releasing hormone to regulate pro-opiomelanocortin gene transcription (10–13); IL-6 appears to be a major mitogenic stimulant in liver regeneration and also a causative agent for postmenopausal osteoporosis (5, 14); and cardiotoxin expression is restricted to the heart at an early stage of mouse cardiogenesis (15). This common gp130-LIFR complex may explain some redundant functions of these cytokines, whereas existence of specific receptors for some cytokines and or their tissue-specific distribution and/or density of cytokines and their respective receptors may determine specific distal actions.

A 3.6-kb cDNA clone of the human LIFR was obtained in library screening, which contained an entire coding region of 1097 amino acid residues with a 44-residue signal sequence, a 789-residue extracellular domain, a 26-residue transmembrane domain, and a 238-residue cytoplasmatic domain, while identification of the 5’- and 3’-noncoding regions was incomplete (16). The murine LIFR cDNA sequence was also reported (16), and the existence of a soluble murine LIFR was predicted. HhLIFR is located on human chromosome 5p12-p13, and analysis of its protein structure shows that it belongs to a hematopoietic cytokine receptor family, which comprises receptors functioning in immune and hematopoietic systems such as IL-2R (β and γ chains), IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, erythropoietin receptor, granulocyte colony-stimulating factor receptor, granulocyte macrophage colony-stimulating factor (17–19), and other receptor proteins for factors that are believed normally to function outside the immune and hematopoietic systems, such as growth hormone, prolactin, ciliary neurotrophic factor, and leptin (17, 20, 21). This hematopoietic cytokine receptor family is characterized by an extracellular (–200 amino acid) portion composed of two folding domains,
usually with four conserved cysteine residues in the N-terminus, and a Trp-Ser-X-Trp-Ser motif in the C-terminal domain (17), while not possessing recognizable tyrosine kinase domains (18).

Knockout of the LIFR gene in mice demonstrates that LIFR is essential for animal survival (22, 23). Pleiotropic defects occurring in these mice (22) include disrupted placental architecture, leading to poor intrauterine nutrition; imbalanced bone development with both reduced bone volume and increased osteoclast numbers resulting in severe perinatal osteopenia; excessive fetal hepatic glycogen storage; and significant losses of neurons including reduced spinal cord and brain stem astrocytes, facial motor, spinal motor, and nucleus ambiguous neurons (23). Furthermore, these homozygote animals died within 24 h of birth (22, 23). These findings underlie the critical biological function of the LIFR gene. However, the molecular mechanism of the expression and regulation of this gene is unknown because no LIFR gene promoter has been identified in any species. As a major step to understand LIFR transcriptional regulation, we isolated a human LIFR promoter functional in placental tissue, identified a placenta-specific upstream enhancer, and demonstrated alternative promoter usage by the human LIFR gene.

EXPERIMENTAL PROCEDURES

Cell Culture—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) (Life Technologies, Inc.), another human choriocarcinoma JAR (ATCC HTB-144) and a human thyroid carcinoma TC1 (gift of Dr. Jerome Hershman) were maintained in RPMI 1640 with 10% FBS. A human osteogenic sarcoma cell line U-2 OS (ATCC HTB-96) was maintained in McCoy's 5A medium (Life Technologies, Inc.) with 10% FBS. A human hepatocellular carcinoma cell line Hep3B (ATCC HB-8064) and a human cervix carcinoma HeLa cell (ATCC CCL-2) were maintained in McCoy's 5A medium (Life Technologies, Inc.) supplemented with standard antibiotics, and cells were passaged twice weekly.

Rapid Amplification of 5'-cDNA End (5'-RACE) and 5'-RACE Product Cloning—The 5'-RACE took advantage of tobacco acid pyrophosphatase which was able to remove the "cap" structure from the 5'-terminus of eukaryotic mRNA (24, 25). In our study, JEG-3 or U-2 OS total RNA was first dephosphorylated with calf intestinal alkaline phosphatase (ICN Pharmaceuticals Inc.). All RNAs were subsequently ligated with ribo-oligonucleotide, and then tobacco acid phosphatase (Life Technologies Inc.) to prevent degraded RNA from being sequenced. The RNA was then treated with sequencing-grade reverse transcriptase (Life Technologies, Inc.) with a primer corresponding to the cytoplasmic region was amplified by RT-PCR from the genomic clone (see Fig. 1) inserted at KpnI site or SalI site (see Fig. 1) in pBSII/I- vector (Stratagene) to make pBSII-SE, then KpnI and SalI were used to recover an ~4-kb genomic fragment from pBSII-SE, which contained all the sequence upstream of the KpnI site (see Fig. 1) in pBSII-NoB, and this 4-kb fragment was cloned into pGL3-NP using KpnI and SalI sites. This new construct thus contained the entire ~4.8-kb sequence upstream of the detected transcription start site and a portion of the first exon and was designated pGL3-KP. Over 30 serial constructions with different deleted sizes using pGL3-KP as a starting template were obtained for promoter activity analysis. Deletions were made using either the exonuclease III and mung bean nuclease method (26) or unique restriction endonuclease sites. All deletions were verified by sequencing.

Construction of Plasmids for Enhancer Analysis—A 1.4-kb KpnI-SpoI fragment (~4876 to ~3452 nt) from pBSII-SE, containing the strongest transcriptional activation region, was subcloned into pGL3-Basic cut with KpnI and SmaI to make pGL3-KS. Since a XhoI site existed between the KpnI and Sall site in pBSII-SK(+)-vector, XhoI digestion on pGL3-KS released the entire 1.4-kb fragment, including the enhancer region. This region was then subcloned into the pGL3-Promoter (Promega) at either the XhoI site or SalI site, allowing the DNA region of interest to be placed upstream or downstream of the luciferase reporter gene in either orientation. These constructs were transfected into different cells for their functional analysis. The construct, designated pGL3PX-SK(+), with the enhancer insert upstream luciferase gene in the opposite orientation, was used further by deletion analysis to localize the minimal enhancer region.

Transient Cell Transfections—For promoter activity analysis, promoterless pGL3-Basic was used as a negative control. For enhancer activity analysis, pGL3-Promoter was used as negative control. Plasmid pCMV-β-gal (Life Technologies Inc.) was cotransfected as an internal control. Plasmid DNAs were prepared using MaxiPrep Kit (Qiagen). JEG-3, U-2 OS, JAR, AtT20, MCFT-7, EY, HeLa, and TC1 cells were transfected using the standard LipofectAMINE (Life Technologies Inc.) or calcium phosphate precipitation method. Testing plasmid DNA was mixed with pCMV-β-gal at a 5:1 ratio. Each plasmid sample was co-transfected with pCMV-β-gal in triplicate, and individual transfections were repeated at least twice. 48 h after transfection, cell lysates were prepared for measurement of luciferase and β-gal activities.
Mutagenesis in the Enhancer Region—Several site-directed mutageneses were performed according to the ExSite PCR method (Stratagene) targeting the Sp1 and NF-κB/Rel binding sites detected by DNase I footprinting. Only the mutagenic primers are portrayed here. For the Sp1-protected regions 1 (−4467 to −4481 nt) and 2 (−4495 to −4512 nt), the primers (lowercase represents mutated nucleotides) used were 1a, 5′-aggtTTACCTTTTACGACTGTTTTCTC-3′; 1b, 5′-aggtTTACCTTTTACGACTGTTTTCTC-3′; and 2, 5′-aggtTTACCTTTTACGACTGTTTTCTC-3′ respectively. For the NF-κB p50 protected regions 1 (−4424 to −4439 nt), 2 (−4460 to −4489 nt), and 3 (−4483 to −4508 nt), the primers used were 1a, 5′-ctgCCTTACTGGAGGATGAGGC-3′; 1b, 5′-ccggTGCCCTTACTGAGAGGATG-3′; and 2, 5′-ttTTCCCATCTAGGCGC-3′; 3, 5′-ttGGAATACGAGGGATAGTTCT-3′.

For deletion mutant lacking region −4625 to −4581 nt, the two primers used were 5′-CAATCTCCCTATCTATCCCTCC-3′ and 5′-GCTTGAGAGAATAGATTG-3′ (the deleted region corresponds to element A); for the deletion mutant lacking region −4418 to −4480 nt, the two primers used were 5′-CATTGGCTAGGAGGGATGAT-3′ and 5′-AGAAGGGATTCTACAGG-3′ (the deleted region corresponds to element B). All mutants were verified by sequencing.

Preparation of Nuclear Extract and Electrophoretic Mobility Shift Assay (EMSA)—Crude nuclear extract from JEG-3 cells was prepared according to the method of Dignam et al. (27) with modifications. About 1 × 10⁶ JEG-3 cells were used, and extraction buffer volumes were scaled down accordingly. The protein concentration of the nuclear extract was quantitated by Bio-Rad protein assay (Bio-Rad) and was usually between 5 and 7 μg/ml. For electrophoretic mobility shift assay, the oligonucleotide duplex corresponded to element A (−4625 to −4581 nt) and element B (−4418 to −4480 nt), respectively, in the enhancer region. One strand of the duplex was 5′-TTACTGGAGGATAGGAGGATCG-3′ for element A, and 5′-TTACTGGAGGATAGGAGGATCG-3′ for element B. Oligonucleotide sequences for Sp1, NF-κB, AP1, and cAMP response element-binding protein were used in the competition assay. The oligonucleotide duplex end-labeled with a [γ-32P]ATP, 20,000–30,000 cpm, probe was mixed with 5–7 μg of JEG-3 nuclear protein, 1 μg of poly(dI-dC) (Pharmacia) in 20 μl of reaction buffer (10 mM Tris-Cl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 8% glycerol) and incubated at room temperature for 15 min. For competition assays, 200-fold excess cold competitor oligonucleotide duplex was added in the reaction buffer 5 min after the addition of the labeled probe. Binding mixtures were resolved on 5% nondenaturing polyacrylamide gel electrophoresis gel.

RESULTS

Mapping the Placental Transcription Start Site of LIFR Gene—Reported human LIFR cDNA has a length of 3591 nt. However, Gearing et al. (16), at the time of cloning the human LIFR cDNA, found that the LIFR transcripts in human placental RNA corresponded to two major bands of −6 and −4.5 kb and a minor band of 5 kb. Later reports detected four hybridizing bands using a LIFR probe located within the cDNA cytoplasmic region in placental tissue (28), although no size estimation was provided. These previous data could not be used to define the transcription start site of this gene. Therefore, to accurately map the transcription start site, 5′-RACE and S1 mapping were performed. 5′-RACE is useful to capture extra 5′ sequences lost during conventional cDNA library construction. With LIFRSP2 as the reverse transcription primer and LIFR2AS as the gene-specific primer in the PCR, 5′-RACE using tobacco acid pyrophosphatase enzyme and T4 RNA ligase was successful in JEG-3 RNA samples (see Fig. 2a). However, cloning and sequencing of these 5′-RACE products showed discrepancies in the first 11 nucleotides in the largest 5′-RACE products as compared with the reported cDNA (Fig. 2b). Sequence from the 12th nucleotide in the largest 5′-RACE product is in agreement with the reported cDNA starting from its 25th nucleotide. Subsequently, we found that RT-PCR using a primer LIFRPRUS (5′-AAATCCCTACCCCTTCTCCAGC-3′) designed from the beginning of 5′-RACE product sequence as sense primer and LIFR2AS or LIFRDG1AS (5′-TTCCATCTCTGCAATGACGTC-3′, 186–164 nt in reported sequence) as antisense primer was successful, whereas RT-PCR using a primer designed from the beginning of the previously reported cDNA sequence (corresponding to its 13th to 35th nucleotide) and LIFR2AS or LIFRDG1AS primer was unsuccessful (data not shown), thus demonstrating that the first 11 nt sequence detected by 5′-RACE was correct and present in LIFR transcripts in JEG-3 cells.

Plasmid pCRII-NoB was partially sequenced around the primer LIFRPR15 site, and a HindIII site (Fig. 1) was detected at −183 of the first nucleotide obtained in the 5′-RACE product. For the S1 protection assay, the positive genomic DNA clone was therefore digested with HindIII, and primer LIFR2AS was used to synthesize a single-stranded DNA probe. S1 mapping showed a single protected fragment of 143 nt (Fig. 2c), which agrees with the transcription start site as determined by 5′-RACE. Interestingly, the U-2 OS sample did not yield the corresponding signal, whereas both JEG-3 and placental samples gave strong positive protected signals.

At the same time, partial sequencing of pCRII-NoB plasmid revealed discontinuity of the cDNA sequence at the 159-nt position, indicating the existence of intron(s). This information was later utilized for plasmid constructions in the promoter activity analysis.

Promoter Activity of Human LIFR Gene 5′-Flanking Sequence Upstream to the Identified Placental Transcription Start Site—Over 30 serial nested deletion subclones generated from pGL3-KP, which contained the 4.8-kb region upstream of the identified transcription start site and 52 nt of the first exon, were sequence-verified and used for promoter activity assay. The 4876-nt 5′-flanking fragment sequence has been deposited

**Fig. 1.** Restriction map of the 18-kb human LIFR genomic clone. The restriction map was determined as follows. Several restriction enzymes were used to partially digest the 18-kb NotI insert from a DNA, which were blotted and hybridized separately with two end-labeled primers specific for the T3 or T7 promoter flanking region besides the NotI sites. Comparisons of the hybridizing band sizes were used to create the restriction map. More detailed restriction map of the 5-kb HindIII region is derived from sequencing data from both pCRII-NoB and pGL3-KP (see “Experimental Procedures”). The 4.8-kb sequence upstream plasmid transcription start site has been deposited in GenBank™ under accession no. U78104. *Numbers in this line correspond to those in the previously reported human LIFR cDNA sequence.
Alternative Promoter Usage of the Human LIFR Gene—An enhancer in a functional HLIFR promoter (29), was also chosen for thorough analysis along with an alternative promoter for LIFR transcription. As previously stated, in the positive genomic clone containing the functional promoter described above, we detected the presence of an intron situated 145 nt behind the transcription start site, so we decided to use this first exon (1–145 nt) present in JEG-3 cells as the probe to rehybridize to the same blot shown in Fig. 4a. The result is shown in Fig. 4b. Not surprisingly, this unique exon indeed failed to detect a signal in U-2 OS cells, providing further evidence that an alternative promoter is utilized by the LIFR gene in U-2 OS cells.

Since the original primers used in 5'-RACE and S1 mapping for JEG-3 and U-2 OS RNA samples are in fact all located within the first exon in LIFR transcripts in JEG-3 cells, novel primers were designed from within the translated region of the LIFR (outside the first LIFR exon in JEG-3 cells) to repeat 5'-RACE with the U-2 OS sample, and the new 5'-RACE product revealed a different, high GC content fragment of 29 nt at the 5'-end (Fig. 5, a and b). Using a primer derived from this 29-nt sequence, a PCR-based genomic walking was performed, and a 676-bp genomic fragment upstream of the 29-nt sequence was cloned into pGL3-Basic, designated as pGL3-U2P, and found to have promoter activity in U-2 OS cells and other nonplacental cells tested including Hep3B, MCF-7, AT20 (Fig. 5c), HeLa, EY, and TC1 cells, but not in JEG-3 (Fig. 5c) or JAR cells. This 676-bp genomic sequence has been deposited in GenBank™ under accession no. AF018079.

Enhancer Activity Analysis and Tissue Specificity of the Promoter and Enhancer—The XhoI fragment (4876 to −3452 nt) in pGL3-KS, which contained the entire region I, was subcloned into pGL3-Promoter vector at different sites or orientation to test for its effect on SV40 promoter activity. Subsequently, this 1.4-kb fragment is referred to as KpnI-SapI (KS) fragment from −4876 to −3452 nt (see Fig. 1). Eight different subclones were obtained and were designated pGL3PX-(KS)1, (KS)2, (SK)1, and (SK)2 for insertion at XhoI site upstream the SV40 promoter according to orientation and tandem repeat occurrence, and PGL3PS-(KS)1, (KS)2, (SK)1, and (SK)2 for insertion at SalI site downstream of the luciferase reporter gene according to the orientation and tandem repeat occurrence. Luciferase activity of these constructs is shown in Fig. 6. One copy of the 1.4-kb KS fragment, when placed either upstream or downstream to the reporter gene in either orientation, increased promoter activity by 10–35-fold, with PGL3PX-(SK)1 (KS fragment placed upstream the SV40 promoter in opposite orientation to luciferase reporter) having the largest induction (35-fold), thus confirming that this 1.4-kb fragment indeed contained an enhancer region. Tandem repeats of the 1.4-kb KS fragment placed at those positions increased promoter activity 24–74-fold, showing cumulative enhancer activity of this KS fragment.

As a further step to study this newly found functional promoter and enhancer, we transfected pGL3-KP, pGL3-KP(-798), and pGL3PX-(SK)1 in comparison with control pGL3-Basic or pGL3-Promoter vectors into two placental cell lines and seven nonplacental cell lines to test the tissue specificity of the promoter and enhancer. Results of two placental and three nonplacental cell transfections are shown in Fig. 7. As portrayed, this promoter and enhancer were only functional in placental cell lines such as JEG-3 and JAR, while remaining inactive in hepatocarcinoma Hep3B, lung cancer EY, pituitary tumor AT20 cells, and other cells including sarcoma U-2 OS, cervix carcinoma HeLa, breast cancer MCF-7, and thyroid carcinoma TC1 (data not shown).

Analysis of the Enhancer Region—PGL3PX-(SK)1 was then
used for deletions in the KS fragment insert from both ends to determine the minimal enhancer region. Subclones with about 300 bp intact (−4705 to −4400 nt, BstXI to XmnI fragment) retained full enhancer activity, and this 300-bp fragment was used for subsequent studies.

As shown in Fig. 8, the minimal enhancer can be further localized as a 226-nt fragment from −4625 to −4400 nt, and predicted potential transcription factor binding sites for this region are shown in Fig. 9. Notably, two Sp1 and three NF-κB/c-Rel binding sites with moderate binding strength were predicted. To determine whether these Sp1 or NF-κB/c-Rel binding sites are functional in binding corresponding proteins and contributing to enhancer activity, both DNase I footprinting and site-directed mutagenesis were performed. Although two Sp1 and three NF-κB/c-Rel binding sites bound to Sp1 and NF-κB p50 correspondingly, only site-directed mutagenesis at...
one c-Rel site caused about 30% loss of enhancer activity, while other mutants retained full enhancer activity. These results demonstrate that the predicted Sp1 or NF-κB/c-Rel binding sites, albeit capable of binding to corresponding transcription factors, do not play major roles in enhancer function. However, as shown in Fig. 8, two deletion mutants losing either end of this fragment, namely D2 without the −4625 to −4583 nt region and D6 without the −4453 to −4400 nt region, were found to be completely inactive, suggesting that −4625 to −4583 nt and −4453 to −4400 nt are two important regions for enhancer activity. Since one NF-xB binding site (−4439 to −4424 nt) was within the −4453-nt to [minus]4400 nt region, another deletion mutant was made to retain the NF-xB binding site while losing the fragment from −4418 to −4400 nt. Transient transfection showed that this new deletion mutant, D7 as shown in Fig. 8, did not possess enhancer activity. Thus, two elements, A (−4625 to −4583 nt) and B (−4418 to −4400 nt) have been found to be indispensable for enhancer activity.

Several internal deletion mutants possessing intact ends, but disrupting the integrity of the enhancer fragment, were also found to lose their activity, as shown in Fig. 8. To test whether specific proteins can bind to these two regions, EMSA was performed using an oligonucleotide duplex corresponding to these two elements as probes, and the results are shown in Fig. 10. Both elements can give rise to specific protein binding bands, with element A showing two major bands (arrowed) and two minor bands, and element B showing one band (arrowed). However, several known transcription factor consensus binding sequences could also not compete with the specific bindings, and the two elements could also not compete with each other. Further analysis of elements A and B using nuclear extracts from U-2 OS and HeLa showed that all three arrowed shifted complexes shown in Fig. 10 can also be detected in these two nonplacental sources, implying that the corresponding proteins may not be unique to placental tissue (data not shown).

**DISCUSSION**

LIFR is a widely expressed transcript, supporting the observed diverse functions of LIF in different tissues. However, in the human cell lines that we used for transfection studies, RT-PCR using LIFRS and LIFRAS were all able to generate the expected PCR product, while RT-PCR using primer LIFRR1S found in the first exon in JEG-3 cell and a primer downstream ATG initiation codon LIFR1AS (5′-CTTTTATTGTCACCATCCAG-3′, 234–214 nt in reported sequence) could generate only the expected PCR products in two placental cells, JEG-3 and JAR cells, and in normal placental tissue. These RT-PCR results, together with transfection studies on different cells, indicate that the cloned functional LIFR promoter and enhancer is probably placental-specific. Furthermore, detection of an alternative promoter and the utilization of this alternative promoter by the LIFR gene also supports the

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**FIG. 6.** Identification of the cloned enhancer. Effect of KS fragment(s) in different location or orientation on the SV40 promoter activity were measured. Transfections were performed in JEG-3 cells as stated under “Experimental Procedures.” Relative promoter activity of each construct is shown as fold-increase over a SV40 promoter luciferase vector pGL3-Promoter control, whose activity is taken as 1, after normalizing to β-gal activity. Values represent mean ± S.E. of triplicate determinations.

**FIG. 7.** Tissue specificity study of the cloned promoter and enhancer. Activity of the cloned enhancer (a) and promoter (b) in different cell lines was measured in triplicate transfection assays, and representative results from two placental and three nonplacental cell lines are presented. Luciferase activity was normalized to β-gal activity and for each cell line the normalized pGL3-Promoter or pGL3-Basic control readings were set as 1, respectively. pGL3-PX-(SK)2 was used to test for enhancer tissue specificity with pGL3-Promoter as a control; pGL3-KP and pGL3-KP(−798) were used to test for promoter tissue specificity with pGL3-Basic as a control. 1, pGL3-Promoter; 2, pGL3PX-(SK)2; 3, pGL3-Basic; 4, pGL3-KP(−798); 5, pGL3-KP.

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placenta specificity of the characterized promoter and enhancer, whereas the alternative promoter appears to possess broader tissue activity.

Transcription factor Sp1 and NF-κB have broad tissue distribution and have been found to be involved in many enhancer elements. For example, Sp1 contributes to the enhancer activity in fibroblast growth factor-4 gene (30), human adenosine deaminase gene (31), and insulin-like growth factor-II/manose 6-phosphate receptor gene (32), while members of the NF-κB family transcription factor are necessary for the enhancer activity of 3-9IgH enhancer (33), HIV-1 enhancer (34), and CD28RE enhancer in the IL-2 gene (35). In some cases, both Sp1 and NF-κB participate in activating enhancers; constitutively bound Sp1 and induced heterodimeric NF-κB give rise to maximal activity of the cytokine-inducible enhancer of the cytokine-inducible enhancer of vascular cell adhesion molecule-1 gene (36), and Sp1 and NF-κB interact with HIV-1 enhancer activator (37). Specifically for placental tissue, a Sp1-like element was found to participate in the regulation of several pregnancy-specific glycoprotein (PSG) genes including PSG-3, PSG-5, and PSG-6 (38).

However, in the LIFR enhancer described here, although the enhancer does contain two Sp1 and three NF-κB/c-Rel binding sites (EMSA was also performed with JEG-3 crude extract and specific binding were observed for both Sp1 and NF-κB binding sequences, data not shown), these transcription factors do not play major roles in enhancer activity.

Regulatory elements mediating enhanced expression of some placental genes have been reported. For the human chorionic gonadotropin α subunit gene, a composite enhancer was found located within the first 435 bp of the promoter for placenta-specific expression. This enhancer contains upstream regulatory element (URE), two tandem CREs, and CCAAT box (39), in which URE is actually a composite of α ACT, trophoblast-specific element (TSE), and URE1 (40), among which α ACT can bind to hGATA-2 and hGATA-3 (41), TSE/URE1 can bind to TSEB and UREB. For the human chorionic somatomammotropin gene, a placental-specific enhancer chorionic somatomammotropin, located about 3 kb downstream of the hCS-2 gene, contains multiple DNA elements observed in footprinting with corresponding proteins including TEF-1 interacting cooperatively (42), and more recently, human transcription factor TEF-5 has been shown to be important to regulate the hCS-B gene enhancer (43). Also, CRE, GATA, basic helix loop helix, and TSE have been found indispensable for the function of a 770-bp enhancer located 25.4 kb upstream of the murine adenosine deaminase gene in trophoblast cells of chorioallantoic placenta (44). As shown in Fig. 7, potential transcription factor binding sites in this LIFR enhancer region include CRE, GATA, HNF-3, Nkx25, Sp1, c-Rel, Oct-1, Sp1 NF-κB, NF-1, and E47. Other potential elements are shown in italics with corresponding factors shown underneath.

An Enhancer in a Functional HLIFR Promoter

- [Image 207x537 to 558x729]
- [Image 55x378 to 355x484]
- [Image 55x346]
other or with co-factors and allow the protein-protein interaction separately. Rather, they probably cooperate with each other. Elements in this enhancer region do not contribute to enhancer activity. In this context, it is reasonable to conclude that binding proteins confirmed their binding specificity. Furthermore, elements A and B could not compete with each other, showing that they are binding to different proteins. Those two major shifted complexes for element A and one shifted complex for element B have also been found to be detectable in U-2 and HeLa nuclear extracts (data not shown), implying that those corresponding proteins may not be unique to placenta. Considering that deletion of either element A or element B resulted in the total loss of enhancer activity, and disruption of the enhancer integrity by internal deletion also rendered the enhancer inactive, it is reasonable to conclude that binding proteins in this enhancer region do not contribute to enhancer activity separately. Rather, they probably cooperate with each other or with co-factors and allow the protein-protein interaction to permit enhancer activity.

On the other hand, in this 226-bp enhancer region there is another 162 bp between element A and element B, in which two Sp1 and three NF-xB/c-Rel binding sites mentioned above were located. Although those Sp1 or NF-xB/c-Rel binding sites do not contribute to enhancer activity, internal deletion in this region disrupted the enhancer activity, possibly other transcription factor binding sites in this 162-bp region are necessary for enhancer activity.

Interestingly, transfections have shown that this enhancer could also be placental specific because when placed upstream of an SV40 promoter, this enhancer was not active in several nonplacental cell lines tested including breast cancer, pituitary tumor, osteogenic sarcoma, cervix carcinoma, liver carcinoma, lung cancer, and thyroid carcinoma. The presence of functional placental-specific transcription factor(s) binding to this enhancer region will be a good hypothesis to explain the placental enhancer specificity.

LIFR knockout mice have severe defects in placental organization (22), showing the importance of LIFR in normal placental development. In mice, mLIFR mRNA expression level was shown to increase in placenta during pregnancy (45). In humans, LIFR mRNA also increases during the first trimester in placenta (46). No explanation has yet been provided for these LIFR mRNA increases being restricted to placenta and or during pregnancy. With the discovery of functional placentaspecific LIFR enhancer described here, we hypothesize that in placenta and/or during pregnancy, placentaspecific transcription factor(s) are active and able to bind to the placentaspecific LIFR promoter and enhancer, leading to increased transcription of LIFR mRNA restricted to the placenta. An alternative LIFR gene promoter is active in a wide variety of nonplacental tissues, allowing nonplacental expression of LIFR and also supports the notion that LIFR possesses specific regulatory signals to regulate its expression and special functions in placenta. Cloning of this functional human LIFR promoter and enhancer has provided the basis to study the regulation of LIFR expression, and detailed analysis in the enhancer and promoter region will reveal further mechanisms for their demonstrated tissue specificity.

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