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THE NUCLEAR FACTOR NF-IL6 ACTIVATES HUMAN PLACENTAL LACTOGEN GENE EXPRESSION

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Transient transfection studies using deletion mutants of the hPL promoter indicate that the DNA elements for NF-IL6 responsiveness are located between -2.3 to - 1.1 kb. Subsequent transfection studies using a hPL promoter fragment containing the region between -1376 to -1088 bp ligated to a heterologous SV40 CAT vector (NF-IL6/hPL-CAT) demonstrated that the NF-IL6/hPL-CAT construct is responsive to NF-IL6. Mobility shift assays using nuclear extracts from BeWo choriocarcinoma cells overexpressing NF-IL6 demonstrated specific binding of the extracts to a labeled oligonucleotide probe to this region of the hPL promoter. These studies therefore strongly suggest that the effect of IL-6 on hPL gene expression is mediated, at least in part, by the binding of NF-IL6 to a region of the hPL promoter that contains three NF-IL6 responsive elements.

Interleukin-6 (IL-6) was originally characterized as a cytokine produced by immune cells that regulates the inflammatory response (1). Recent studies have demonstrated that IL-6 is also produced by non-immune cells and has a broad range of biological activities. IL-6 binds to a high-affinity membrane receptor that consists of the ligand-binding subunit gp8O and a signal-transducing protein, gp130 (3). The gp130 has also been associated with several other cytokine receptors, including interleukin- II (IL-11), leukemia inhibitory factor (LIF) and oncastatin M (OSM), suggesting that all these cytokines may use similar signaling pathways (4). Recently it has been reported that gp130 is also associated with protein tyrosine kinases of the JAK family (5). Furthermore, JAKs may also interact with another group of protein tyrosine kinases of the MAPK family (6).

The effect of IL-6 on gene transcription has been reported to occur by activation of two types of IL-6 response elements (IL-6REs), designated type 1 and type 2. Type 1 IL-6REs contain sequences (TT/GNNGNAAT/G) that bind the nuclear factors NF-IL6 (C/EBPβ) and NF-IL6β

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(C/EBPγ), members of the C/EBP family of transcription factors (7). NF-IL6 has recently been shown to be phosphorylated by a Ras dependent MAPK cascade (8). In addition, NF-IL6 is activated by a variety of external signals that activate Ras, such as interleukin-I (IL-1), tumor necrosis factor-α (TNFα) and lipopolysaccharide (LPS)(9). In contrast, type 2 IL-6REs contain sequences (CTGGGAA) that bind to another transcription factor, acute-phase response factor (APRF), that becomes rapidly activated by phosphorylation via the JAK cascade upon IL-6 activation (10). The two types of IL-6REs may also be further distinguished since one pathway is mediated via a Ras-dependent cascade (Type 1 IL-6RE) and the other by a Ras-independent pathway (Type 2 IL-6RE)(11).

The finding that trophoblast cells synthesize and express several cytokines implicate a role for cytokines in placental function. IL-6 has been demonstrated to stimulate trophoblast cells to secrete human chorionic gonadotropin (hCG) and human placental lactogen (hPL)(12). In addition, IL-1 stimulates the release of hCG and hPL from trophoblast cells (13). Addition of specific anti-serum to the IL-6 receptor abolishes the effect of IL-1 on hCG secretion, suggesting that the effect of IL-1 is mediated by the release of endogenous IL-6 by trophoblast cells (13). IL-6 has also been shown to activate the hPL gene in transient transfection experiments in choriocarcinoma cells using a 2.3 kb fragment of the hPL promoter coupled to a chloramphenicol acetyltransferase (CAT) reporter gene (14). Computer analysis of the hPL promoter fragment reveals several regions containing putative NF-IL6 binding sites, especially one region between -1220 to - 1270 that contains three NF-IL6 binding sites in very close proximity. Such data prompted us to study this region as a possible site responsible for IL-6 mediated hPL gene expression.

MATERIALS AND METHODS

Cells

BeWo cells (American Tissue Culture Collection, Rockville, MD) were maintained in monolayer cultures in RPMI-1640 supplemented with 10% FCS. Before each experiment, the cells were trypsinized and replated at a density of 5.0×10^6 cells/well in 6 well plates in 2% FCS prior to transfection. The medium was changed at 24-48 h intervals. Control cells were exposed to medium containing 0.01% ethanol.

Plasmids

hPL genomic DNA fragments were kindly provided by Dr. John Parks (Emory University School of Medicine, Atlanta, Georgia). A 2.3 kb (-2300 to +2 bp) sequence of the hPL_3 (hCS-A) gene was isolated by cleaving the gene with Bam H1 and inserting the fragment into a chloramphenicol acetyltransferase (CAT) gene plasmid, pChlorAceE (US Biochemical, Cleveland, OH). The 2.3 kb Bam H1 fragment was ligated with Hind III linkers, digested with Hind III and ligated into the plasmid at a site 5' to the CAT reporter gene. A 1.1 kb (-1078 to +2 bp) sequence of the hPL_3 was isolated by cleaving the gene with Bam HI and Bgl II. A 0.5 kb (-496 to +2) sequence of the hPL_3 gene was isolated by cleaving the gene with Bam HI and Eco RI. The

orientation of each hPL insert was determined by restriction mapping. A fragment that contained three NF-IL6 responsive sites on the hPL promoter (-1078 [Bgl II] to -1241 [Nco I]) was ligated into a heterologous SV40 CAT vector. A β-galactosidase (β-Gal) expression vector under the control of the Rous sarcoma virus (RSV) promoter was used in the transfection studies as an internal control to determine the efficiency of transfection, and all results were normalized to amount of βGal expression.

Transfection and CAT Assay

BeWo cells were plated (0.5 x 10^6 cells /60 mm plate) 24 h before transfection. The medium was changed the following day, and the cells were subjected for 4 h to calcium phosphate coprecipitate (5 μg hPL construct, indicated amounts of CMV-NF-IL6, 2 μg RSV-βGal, 250 μl calcium phosphate and 250 μl hepes buffered saline). The cells were osmotically shocked with 20% glycerol in PBS for 2 min at room temperature and then washed with PBS. The cells were refed with complete medium. The medium was removed 48 h later, and 200 μl of 0.1 M Tris (pH 7.8) was added to the cells. The cells were then lysed by 3 cycles of repeated freeze-thawing. The cell debris was removed following a 10 min centrifugation in a microcentrifuge at 4° C. Aliquots of the supernatant were removed for protein determination (Bio-Rad Bradford assay) and β-Gal activity. The remainder of the supematants was heated to 65°C for 10 min to inactivate endogenous acetylases. CAT activity was measured by the liquid scintillation method (16), using 100 μg of cell lysate protein, 50 μl of 5 μM chloramphenicol (Sigma, St. Louis, MO) and 0.5 μCi of [^{3}H] acetyl coenzyme A (Amersham, UK). CAT activities were evaluated by calculating the slopes within a linear range of the response after 5 cycles of counting.

Gel Mobility Shift Assay

BeWo cells express very little or no endogenous NF-IL6 (our own observation). Therefore, BeWo cells were transfected with the CMV-NF-IL6 expression vector to overexpress NF-IL6. We were able to show by Northern analysis that CMV-NF-IL6 transfected cells expressed NF-IL6 mRNA compared to control cells transfected with vector minus insert for NF-IL6. 5μg of nuclear extracts (17) were incubated with 2 μg poly (I:C) for 20 minutes at room temperature in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 5 mM, $MgCl_2$ 1 mM dTT, 1 MM EDTA, and 15% glycerol. ^{32}P-labeled oligonucleotide corresponding to NF-IL6 (15) (5'AGCATTGCACAATCT-3' and its complement sequence) was then added (~30,000 cpm) and incubation was prolonged for 15 min (final volume, 20 ml). Where indicated, competing oligonucleotides were added along with the probe. The mixture was then directly loaded onto a 5% polyacrylamide gel in 0.5 x TBE (45 mM tris base, 45 mM boric acid, and 4 mM EDTA), prerun for 30 min at room temperature. The gels were then fixed, dried and exposed to x-ray film.

RESULTS

Effects of NF-IL6 on hPL promoter activity.

Deletion constructs of the hPL promoter coupled to a CAT reporter gene were studied in transfection experiments for their responsiveness to NF-IL6. The hPL constructs (0.5/hPL-CAT, 1. 1/hPL-CAT and 2.3/hPL-CAT) were co-transfected into BeWo cells together with the expression vector for NF-IL6, and CAT activity was determined after 48 hours. As shown in Fig 1, progressive deletion of the 2.3 kb hPL promoter fragment resulted in a reduction in NF-IL6 stimulated CAT activity, suggesting that the DNA elements for NF-IL6 responsiveness are located

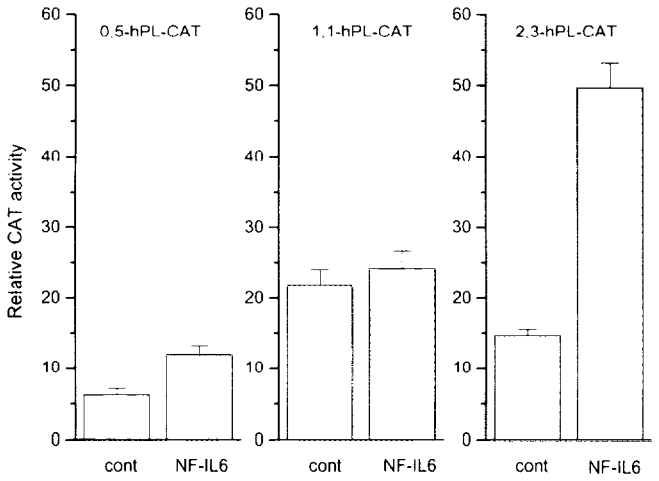

Fig. 1. NF-IL6 activates hPL promoter activity. BeWo cells were co-transfected with either a 0.5, 1.1 or 2.3 kb fragment of the hPL promoter coupled to a CAT reporter gene (hPL-CAT) and a NF-IL6 expression vector. CAT activity was normalized to β-Gal activity. Each bar represents the mean ± 1 SEM of triplicate cell cultures. Similar results were observed in another experiment.

in the -2.3 to -1.1 kb region of the hPL promoter. As shown in Fig 2, increasing amounts of NF-IL6 caused a dose-dependent stimulation of the 2.3/hPL-CAT construct.

Computer analysis of the hPL promoter indicates that the region between -1134 to -1097 bp contains 3 NF-IL6 responsive sites in close proximity (Fig 3). A fragment of the promoter containing this region was ligated into a heterologous SV40 CAT vector (NF-IL6/hPL-CAT) and a transfection experiments were performed to determine functional activity of the fragment. NF-IL6 stimulated CAT activity by approximately three fold (relative CAT activity for control, 9.3 ± 1.5; for NF-IL6 transfected cells, 27.7 ± 2.0). In contrast, no stimulatory effect was observed with the unligated CAT vector in response to NF-IL6, indicating that there are no NF-IL6 REs present in the vector.

Binding of NF-IL-6 to a region containing NF-IL6 responsive sites.

To determine whether NF-IL6 binds to the -1376/-1088 hPL fragment, mobility shift assays were performed using a labeled probe to this region. Preliminary studies indicated that BeWo cells express no or very little NF-IL6 mRNA, as assessed by Northern blot analysis (data not shown). Therefore, NF-IL6 was overexpressed in BeWo cells by transfection of the expression vector. Nuclear extracts were then prepared, and mobility shift assays were performed to detect

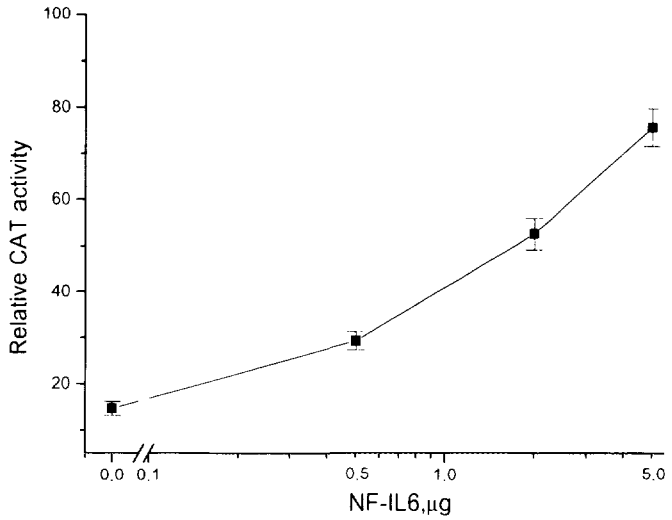

Fig. 2. Dose effects of NF-IL-6 on 2.3hPL-CAT transactivation. BeWo cells were co-transfected with the 2.3hPL-CAT construct together with the indicated amounts of NF-IL6. CAT activity was normalized to β-Gal activity. Each bar represents the mean ± 1 SEM of triplicate cell cultures. Similar results were observed in another experiment.

protein/DNA complex formation. As shown in Fig 4A, a retarded band was only present in nuclear extracts from cells overexpressing NF-IL6 and not from mocked transfected cells with a vector lacking the NF-IL6 insert. In addition, competition studies using unlabeled probe or a probe that

NcoI
-1376 GCCATGGCCCAATCTTGGCTCACGGCAACCTC

-1344 TGCGTCCTGGGTTCAAGCGATTCTACCTCCTC

-1312 ACCCTCCGGAGTAACTGGGATTGCAGGCTTCT

-1280 GCCACCACACACAGCTACTTTTGTATTTTTAAG

-1248 AGATGGAGTTTTGCCATGTTGGCTAGTCTGGC

-1216 CTTGAACTCCTGACCTCAAGTGATCCACCCAC

-1184 CTCAGTCTTCCAAAGTGCTGGGATTACAGGCA

-1152 TGAGCCACCATGCCCGGCCTGGAGAAAGGACT
NF-IL6 NF-IL6
-1120 TTAAATGACGCAATGTGGGAAGAGCCAGGTTG
NF-IL6
-1088 TGGAGATCTG -1078
Bgl II

Fig. 3. A NcoI/Bgl II (-1376/-1078) hPL fragment showing the sequences of the three NF-IL6 sites.

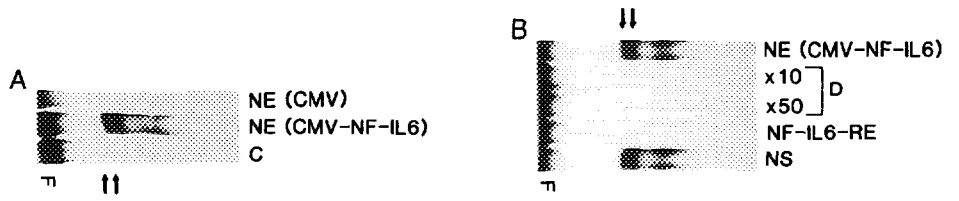

Fig. 4. (A) Mobility shift assays using the NcoI/Bgl II (-1376/-1078) hPL fragment as a probe and nuclear extract (NE) from BeWo cells that had been transfected to overexpress NF-IL6 or control plasmid minus insert (CMV). (B) Competition studies were performed by including unlabeled probe, a NF-IL6 RE and a non-specific (NS) probe. Fs designate the mobility of the free probe, and the arrows designate the mobility of the specific protein - DNA complexes.

has been previously shown to contain a NF-IL6- RE abolished the appearance of the retarded band (Fig. 4B). In contrast a non-specific probe of the same length was unable to abolish the band.

DISCUSSION

The present study demonstrates that the nuclear transcription factor NF-IL6 activates the hPL gene and that a region of the hPL promoter containing three NF-IL6 responsive sites (NF-IL6-hPL-CAT) confers NF-IL6 inducibility. The element binds NF-IL6 and is competed by both unlabeled NF-IL6-hPL fragment and by a natural NF-IL6-CRP fragment, indicating specificity of the NF-IL6-hPL element. These studies therefore indicate that IL6 mediated hPL gene expression is regulated, at least in part, via the type 1 IL-6 RE. However, the possibility that the type 2 IL-6 RE may also be activated by the presence of putative APRF binding sites on the hPL promoter can not be excluded.

Gene transcription is frequently regulated by a large number of nuclear factors that mediate their effects through regulatory response elements that respond to different signal transduction pathways. For example, two functionally distinct hormones, IL-6 and interferon-γ (IFN-γ), act through common DNA promoter elements that are responsive to transcription factors sharing the same binding sequence (18). Thus, IFN-γ has recently been reported to activate a novel IFN-stimulated gene factor 3 (ISGF3) p91-related transcription factor that is involved in the gp130-mediated signaling pathway (19). We suggest a similar situation occuring via the IL-6 and IL-1 transduction pathways for activating the hPL promoter (14) since the IL-1 inducible transcription factor NF-kB has recently been reported to bind to NF-IL6 DNA binding sequences and initiate transactivation (20). IL-1 may also be working via the MAPK cascade to phosphorylate NF-IL6,

which then binds to NF-IL6 REs. Further studies are necessary to elucidate whether the effects of IL-1 and IL-6 are utilizing a common or separate signaling pathways.

NF-kB, which was originally identified as a nuclear factor that binds to an enhancer element of the immunoglobulin gene (the kB motif), is thought to be involved in the expression of several viruses and cellular inducible genes encoding cytokines, immunoregulatory receptors and acute phase proteins. The major form of NF-kB is a heterodimer composed of 50 and 65 kDa units (p50 and p65). Because many immune response genes and the acute phase genes contain both the NF-IL6 and NF-kB sites, it is highly possible that cooperative interactions between NF-IL6 and NF-kB play an important role in the expression of these genes (15). Our data are in agreement with studies demonstrating dose-dependent effects of NF-IL6 on gene transactivation (21). In addition, NF-kB may also interact with NF-IL6 to ensure a synergistic activation of gene transcription (15, 21).

In summary, these studies strongly suggest that the effect of IL-6 on hPL gene expression is mediated, at least in part, by the binding of NF-IL6 to a region of the hPL promoter between -1134 and -1097 that contains three NF-IL6 responsive elements.

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