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Mitogen-Activated Protein Kinase Activates Human Placental Lactogen-B Enhancer by an NF-IL6-Dependent Pathway

Anoop K. Brar, Randall G. Richards, You-Hong Cheng, Brian Richardson, Yuki Kanda, and Stuart Handwerger

Department of Pediatrics, University of Cincinnati College of Medicine and Division of Endocrinology, Children's Hospital Research Foundation, Cincinnati, OH

Computer analysis of the human placental lactogen-B (hPL-B) enhancer reveals two putative binding sites for the transcription factor NF-IL6, but the role of NF-IL6 in the regulation of the enhancer is unknown. Using gel mobility shift and supershift assays, we demonstrated that NF-IL6 binds to both enhancer sites. Transient transfection studies indicated that the transcription factor NF-IL6 stimulates hPL-B enhancer activity by 4.4-fold in primary cultures of human trophoblast cells and by 32.0- and 8.4-fold in JAR and BeWo choriocarcinoma cells, respectively. Overexpression of MEK (mitogen-activated protein [MAP] kinase kinase), which is known to stimulate phosphorylation of NF-IL6, induced a 3.6-fold increase in hPL-B enhancer activity. The induction by MEK was completely inhibited by an expression plasmid for a dominant/negative mutant of NF-IL6 or by mutation of the NF-IL6 binding sites on the enhancer. PD98059, an inhibitor of MEK, inhibited hPL release from cultured trophoblast cells by about 50%. Taken together, these results indicate that MAP kinase stimulates the hPL-B enhancer by an NF-IL-6-dependent pathway.

Key Words: Human placental lactogen; mitogen-activated protein kinase; NF-IL6; placenta; gene regulation.

Introduction

The secreted form of human placental lactogen ([hPL]; human chorionic somatomammotropin) is the product of two genes (*hPL-A* and *hPL-B*) of the placental lactogen/growth hormone gene cluster on chromosome 17. The two genes are 95% homologous, and the transcribed mRNAs are 98% similar in nucleotide sequence, encoding identical mature proteins. The proximal promoters of these genes share approx 95% nucleotide sequence identity, and enhancer elements located approx 2.3 kb 3' of the genes share 94% sequence identity (for review of the hPL gene *see* ref. *1*). The hPL-B enhancer is significantly stronger than the hPL-A enhancer, and interacts with both hPL promoters *(2)*. Numerous studies indicate that the hPL-B enhancer mediates, at least in part, the tissue-specific expression of the *hPL-B* gene *(1)*.

Studies of the regulation of the hPL-B enhancer indicate that members of the TEF family of transcription factors are important for enhancer activity. Cooperative interaction of TEF-1 and a placental-specific factor, chorionic somatomammotropin enhancer factor 1, stimulate hPL enhancer activity *(3)*. During trophoblast differentiation, hPL gene expression is induced in part by upregulation of TEF-5 and downregulation of TEF-1 *(4,5)*. TEF-1 also represses hPL promoter activity by interaction with a TATA-binding protein (TBP) that results in the inability of TEF-1-TBP to bind to the TATA element *(6,7)*. At present, other transcription factors important in the regulation of the hPL-B enhancer are unknown.

A possible role for the transcription factor NF-IL6 in the regulation of the hPL-B enhancer is suggested by several observations. NF-IL6 is abundant in placental cells *(8)*; and computer analysis of the hPL-B gene reveals two putative NF-IL6 response elements on the hPL-B enhancer, one located at nucleotide (nt) +3922 to +3931 and the other at nt +4038 to +4047 (Fig. 1A). The latter putative NF-IL6 response element partially overlaps a region of the enhancer that is protected by DNase I footprinting *(9)*. Furthermore, interleukin-6 (IL-6) and IL-1 have been shown to stimulate hPL promoter activity by an NF-IL6-dependent pathway *(10,11)*.

To examine whether NF-IL6 regulates hPL-B enhancer activity, we have performed gel shift assays to determine whether NF-IL6 binds to the hPL-B enhancer, and transient transfection experiments to determine whether NF-IL6 transactivates the hPL-B enhancer. Because NF-IL6 can be activated by a mitogen activated protein kinase (MAPK)-dependent pathway in many cell types, we have also investigated the possible role of the MAPK cascade in activation of the hPL-B enhancer.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Anoop K. Brar, Division of Endocrinology, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: anoop.brar@chmcc.org

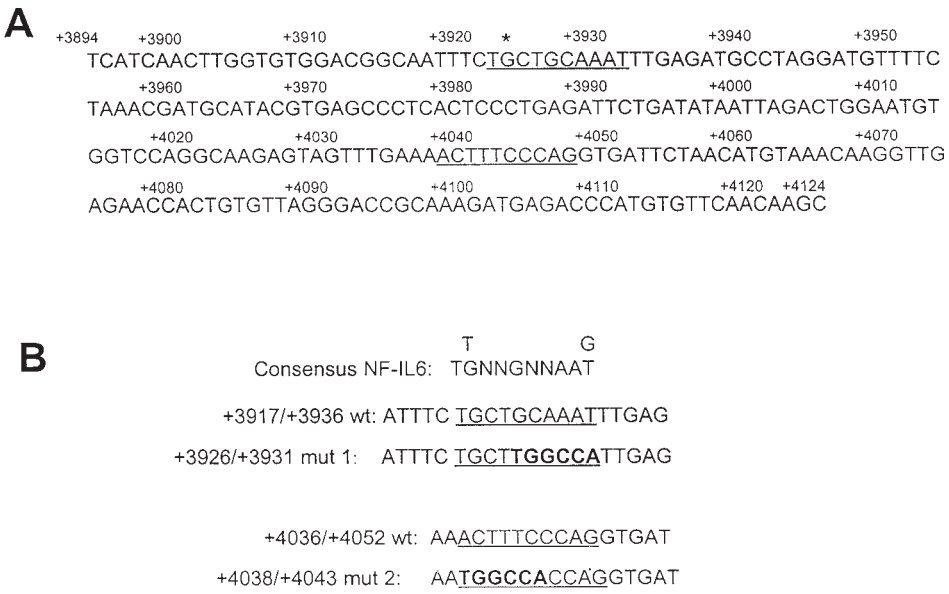

Fig. 1. (A) Locations of the putative NF-IL6 binding sites on the hPL-B enhancer. * indicates presence of a G instead of a C as reported by Jiang *(9)*. Nucleotide numbers are reported from the transcription start site of the hPL-B gene. The nucleotides comprising the NF-IL6 binding sites are underlined. **(B)** Sequences of the consensus NF-IL6 binding site and oligonucleotides encoding putative NF-IL6 consensus sites (underlined) in the hPLB enhancer. The wild-type (wt) oligonucleotides (+3917/+3936 and +4036/+4052) that were used in gel mobility shift assays are shown. Mutated bases in the NF-IL6 consensus site of the oligonucleotides (+3926/+3931 mut 1 and +4038/+4043 mut 2) used to generate mutant plasmids for transfection studies are set boldface.

Results

The locations of the two putative NF-IL6 response elements on the hPL-B enhancer are shown in Fig. 1A (underlined). One response element, identical in sequence to a consensus NF-IL6 response element, is located at nt +3922 to +3931; and the other, which differs in two nucleotides from a consensus response element, is located at nt +4038 to +4047 of the complementary DNA strand.

To determine whether oligonucleotides corresponding in sequence to the two putative NF-IL6 binding sites on the hPL-B enhancer are capable of binding to placental nuclear extracts, gel mobility shift assays were performed using nuclear extracts from normal trophoblast cells and labeled oligonucleotides corresponding in sequence to the two putative NF-IL6 binding sites (Fig. 1B; +3917/+3936 wt and +4036/+4052 wt). As shown in Fig. 2, each of the labeled oligonucleotides formed a single retarded band (lanes 1 and 4) that was markedly decreased in intensity when each reaction mixture was incubated in the presence of 50-fold molar excess unlabeled oligonucleotide (self-competitor, lanes 2 and 5). A single retarded band was also observed when the labeled NF-IL6 oligonucleotide was incubated with a nuclear extract of BeWo or JAR cells (data not shown). When the NF-IL6 oligonucleotides and the nuclear extract were incubated with a specific polyclonal antiserum to NF-IL6, the intensity of each retarded band was significantly reduced (lanes 3 and 6). In control experiments, incubation of the oligonucleotides and nuclear extract with normal nonimmune rabbit serum had no effect on the intensities of the retarded complexes (data not shown). Taken together, these studies indicate that NF-IL6 binds to both consensus sites on the hPL-B enhancer.

To determine whether NF-IL6 transactivates the hPL enhancer, primary cultures of human trophoblast cells were cotransfected as described in Materials and Methods, with a plasmid containing the hPL-B enhancer coupled to a luciferase reporter gene (*phPLBE-Luc*) alone (control) or with an expression plasmid for NF-IL6 (pEF-NF-IL6) (Fig. 3). Another group of cells was cotransfected with *phPLBE-Luc* and the base vector pEF-BOS, without the NF-IL6 gene. Luciferase activity of the cells cotransfected with the NF-IL6 expression plasmid was 4.4-fold greater than that of cells cotransfected with the control ($p < 0.001$). A similar experiment in JAR and BeWo cells, which contain very little NF-IL6 mRNA *(11)*, showed an even more dramatic effect of overexpression of NF-IL6. Cotransfection of the NF-IL6 expression plasmid into JAR cells along with *phPLBE-Luc* resulted in a 34-fold increase in luciferase activity ($p < 0.0001$), and cotransfection into BeWo cells resulted in an 8.4-fold increase ($p < 0.01$) (data not shown).

Because NF-IL6 has been shown to be activated by a Ras-dependent MAPK cascade *(12)*, experiments were performed to determine whether the activation of the hPL-B enhancer by NF-IL6 is mediated via the MAPK cascade. In initial experiments examining the role of the MAPK cascade in hPL expression, primary cultures of trophoblast cells were cotransfected with *phPLBE-Luc* and a plasmid expressing MEK, a known activator of the MAP kinase

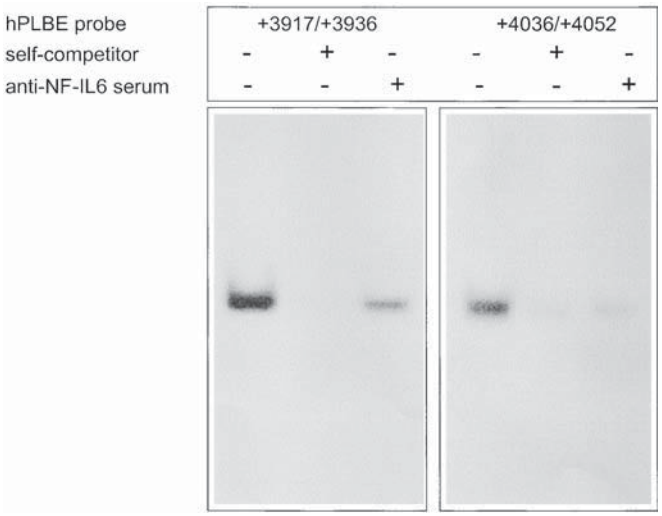

Fig. 2. Gel mobility shift assays of nuclear extracts from human trophoblast cells with radiolabeled oligonucleotides (+3917/+3936 and +4036/+4052) corresponding to NF-IL6 binding sites on the hPL-B enhancer. A single retarded band (lanes 1 and 4) formed with each of the oligonucleotides was markedly decreased in intensity when each reaction mixture was incubated with 50-fold molar excess unlabeled oligonucleotide (self-competitor, lanes 2 and 5). The intensity of each retarded band was also reduced in intensity on incubation of nuclear extracts with anti-NF-IL6 serum (1 μg of IgG), added subsequent to the addition of labeled oligonucleotide (lanes 3 and 6). Similar results were determined using nuclear extracts from JAR and BeWo cells.

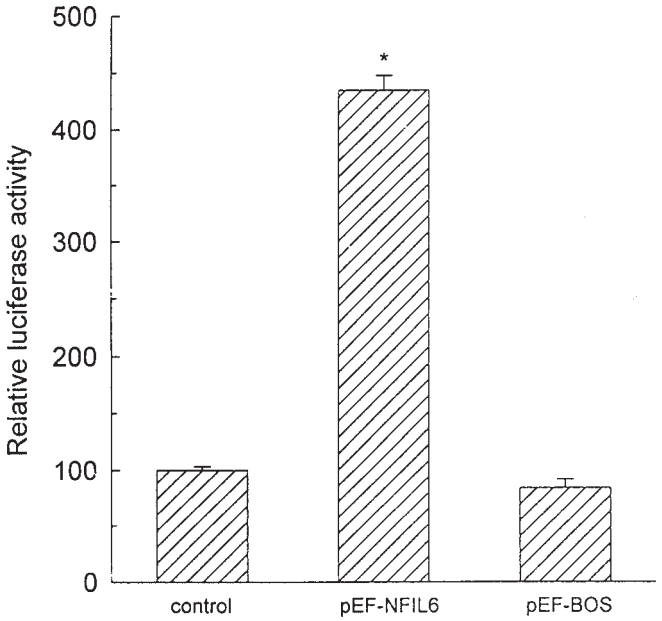

Fig. 3. Effect of overexpression of NF-IL6 on hPL-B enhancer activity. Primary cultures of human trophoblast cells were transiently transfected with *phPLBE-Luc* alone (control), *phPLBE-Luc* and pEF-NF-IL6 (an expression plasmid for NF-IL6), or *phPLBE-Luc* and pEF-BOS (the NF-IL6 carrier vector without the NF-IL6 gene). The results represent the mean ± SEM of triplicate wells. *p = <0.001 for *phPLBE-Luc* vs *phPLBE-Luc* + pEF-NF-IL6. Similar results were observed in two other experiments.

pathway. As shown in Fig. 4, overexpression of a constitutively active mutant of MEK (pMCL-R4F-MAPKK) in trophoblast cells cotransfected with *phPLBE-Luc* resulted in a 3.6-fold increase in luciferase activity ($p < 0.01$), whereas overexpression of a constitutively inactive mutant of MEK (pMCL-8e-MAPKK) had no significant effect. By contrast, cotransfection of the constitutively active mutant of MEK into BeWo or JAR cells with *phPLBE-Luc* resulted in no significant increase in luciferase activity (data not shown).

In a second set of experiments, primary cultures of human trophoblast cells were exposed for 4 d to the MAPK inhibitor PD98059 at concentrations of 1 and 10 μ*M* (Fig. 5). The cells exposed to 1 μ*M* PD98059 released 25.0% less hPL ($p < 0.05$) on d 4 of culture than control cells, and the cells exposed to 10 μ*M* PD98059 released 51.2% less hPL ($p < 0.001$).

To confirm that the activation of the hPL-B enhancer by the MAPK cascade is mediated, at least in part, by activation of NF-IL6, two sets of transient transfection studies were performed. The first set of experiments was designed to determine whether inhibition of NF-IL6 activity by overexpression of a dominant/negative mutant of NF-IL6 blocks the stimulation of hPL enhancer activity by MEK. The second set of experiments was designed to determine whether site-directed mutagenesis of the NF-IL6 response elements on the hPL enhancer prevents the stimulation of enhancer activity by MEK. As shown in Fig. 6, overexpression of a dominant/negative mutant of NF-IL6 (pEF-NFIL6 Δspl) completely blocked the effect of MEK (pMCL-R4F-MAPKK) on hPL-B enhancer activity. As anticipated, the dominant/negative mutant inhibited NF-IL6 (pEF-NFlL6)-mediated stimulation of hPL enhancer activity. Mutagenesis of either NF-IL6 binding site on the enhancer (mut1-*phPLBE-Luc* or mut2-*phPLBE-Luc*) completely prevented stimulation by MEK (pMCL-R4f-MAPKK) in trophoblast cells (Fig. 7).

Discussion

The results of this study indicate that the transcription factor NF-IL6 transactivates the hPL-B enhancer. Oligonucleotides with sequences corresponding to the two NF-IL6 binding motifs on the hPL-B enhancer bound to nuclear extracts of normal trophoblast, JAR, and BeWo cells; and the binding of the NF-IL6 oligonucleotide in each instance was inhibited by a specific NF-IL6 antiserum. Furthermore, overexpression of NF-IL6 in primary trophoblast, JAR, and BeWo cells resulted in a significant increase in hPL enhancer activity. The magnitude of the increase in enhancer activity in response to NF-IL6 overexpression was greater in the choriocarcinoma cells than in the primary trophoblast cells, probably because that choriocarcinoma cells express little or no NF-IL6 *(11)*.

Previous studies have indicated that NF-IL6 is activated by phosphorylation through multiple signal transduction

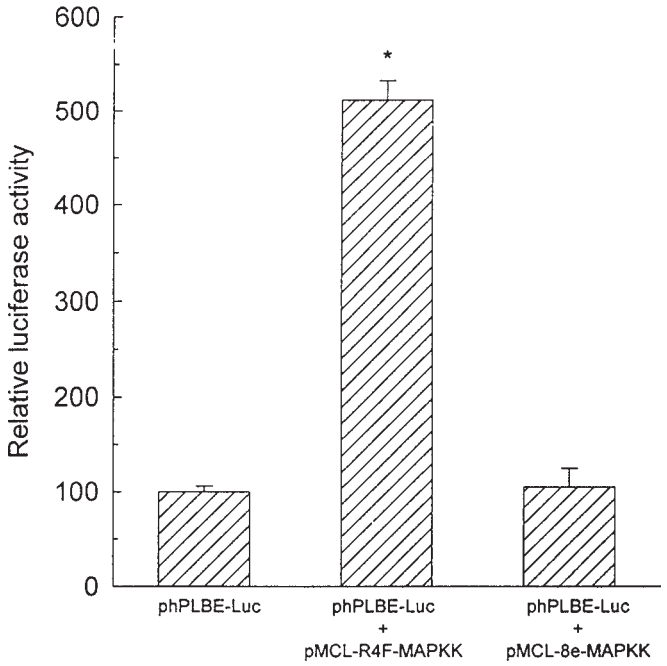

Fig. 4. Overexpression of MEK activates the hPL-B enhancer. Primary cultures of human trophoblast cells were cotransfected with *phPLBE-Luc* and either an expression plasmid for a constitutively active MEK (pMCL-R4F-MAPKK) or an expression plasmid for a catalytically inactive MEK (pMCL-8e-MAPKK). The results represent the mean ± SEM of triplicate wells. * p = <0.001 for *phPLBE-Luc* vs *phPLBE-Luc* + pMCL-8e-MAPKK. Similar results were observed in three other experiments.

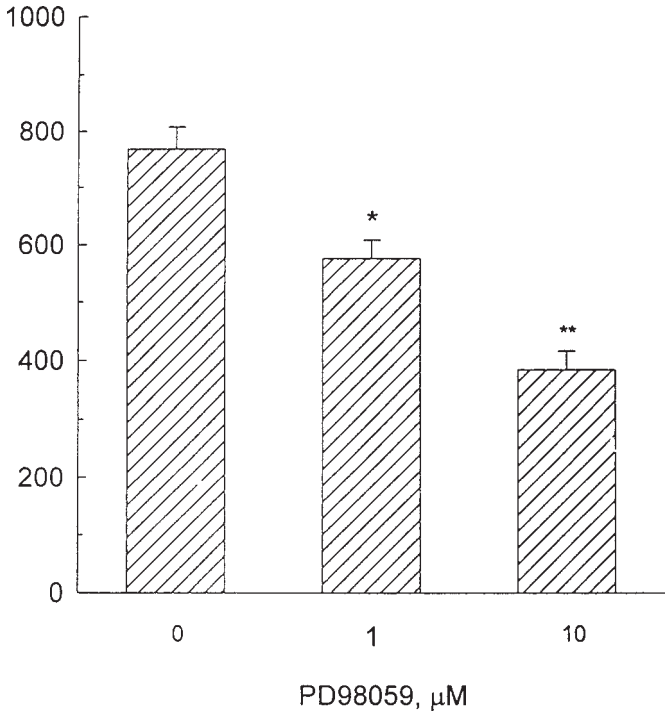

Fig. 5. The effect of PD98059 on hPL release. Primary cultures of trophoblast cells were incubated for 4 d in control medium or medium containing the MEK inhibitor PD98059 at concentrations of 0, 1, or 10 μM. The amount of hPL released during d 4 of culture was determined by radioimmunoassay. Each bar represents the mean of triplicate wells. The bars enclose 1 SEM. * p<0.05 and ** p<0.001 compared with untreated group.

pathways, including cyclic adenosine monophosphate–, protein kinase C–, and MAPK–dependent pathways (for review *see* ref. *13*). The intensity of the retarded band in supershift analysis was reduced but not completely competed using antiserum to NF-IL6. This may reflect the presence of other non-NF-IL6 proteins, or indicate that the total amount of NF-IL6 present is not blocked by the NF-IL6 antiserum. In the present study, we demonstrated that the activation of NF-IL6 in trophoblast cells is owing, at least in part, to activation of a MAPK-dependent pathway. We first noted that PD98059, which inhibits MEK activity in many intact cells *(14)*, blocked the release of hPL from primary cultures of trophoblast cells in a dose-dependent manner. PD98059 has a high level of selectivity for MEK and does not block other protein serine/threonine or tyrosine kinases *(15)*. Although this experiment did not indicate the mechanism by which MAPK inhibits hPL expression, a subsequent series of transient transfections in primary trophoblast cell cultures demonstrated that MEK (MAP kinase kinase [MAPKK]), which is known to stimulate MAPK activity, significantly increased hPL-B enhancer activity. Furthermore, the action of MEK was blocked by a dominant/negative mutant of NF-IL6 or by mutation of the NF-IL6 binding sites on the enhancer. By contrast, overexpression of MEK in JAR and BeWo cells had no effect on hPL-B enhancer activity, a finding compatible with the earlier observation that these cells have little or no NF-IL6 *(11)*.

In earlier studies, we demonstrated that apolipoprotein A-1 (apo A-1), the major apoprotein constituent of HDL, activates the hPL promoter by a MAPK-dependent pathway *(16)* and that IL-6 and IL-1 activate the hPL promoter by an NF-IL6-dependent pathway *(10,11)*. Apo A-1 stimulated a rapid (5-min) dose-dependent increase in MAPK activity that preceded the increase in hPL expression, and an stimulation by apo A-1 was blocked by the MAPK inhibitor PD98059. NF-IL6 mimicked the effect of IL-6 and IL-1, and deletion of the NF-IL6 binding sites on the promoter abolished the stimulation of the promoter by NF-IL6. Since stimulation of the hPL-B enhancer by MAPK-is mediated in part by NF-IL6, the results of the present study indicate that the activation of hPL gene expression by apo A-1, IL-6, and IL-1 may also be owing in part to activation of the hPL-B enhancer.

hPL gene expression is induced during the differentiation of cytotrophoblast cells to syncytiotrophoblast cells *(17,18)*. Since the MAPK signaling pathway *(19)* and NF-IL6 are implicated in the regulation of cellular differentiation *(20,21)*, it is possible that activation of MAPK and NF-IL6 may also be involved in the induction of trophoblast differentiation.

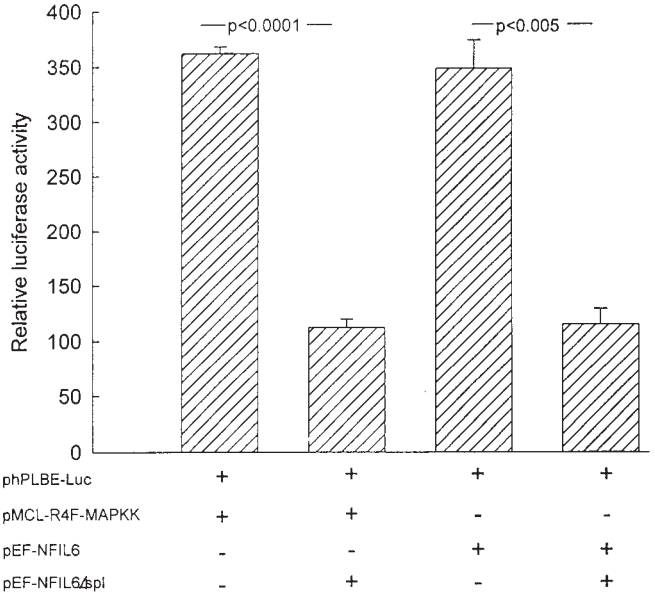

Fig. 6. Inhibition of MEK-activated hPL-B enhancer activity by a dominant/negative mutant of NF-IL6. Primary cultures of human trophoblast cells were transiently transfected with *phPLBE-Luc* and cotransfected with either an expression plasmid for constitutively active MEK alone (pMCL-R4F-MAPKK, bar 1) or in combination with an expression plasmid for a dominant/negative mutant of NF-IL6 (pEF-NFIL6Δspl, bar 2). In addition, some cells were transfected with *phPLBE-Luc* and cotransfected either with an expression plasmid for NF-IL6 alone (pEF-NFIL6, bar 3) or in combination with an expression plasmid for a dominant/negative mutant of NF-IL6 (pEF-NFIL6Δspl, bar 4). The results represent the mean ± SEM of triplicate wells. *p* values are shown over the bars. Similar results were observed in two other experiments.

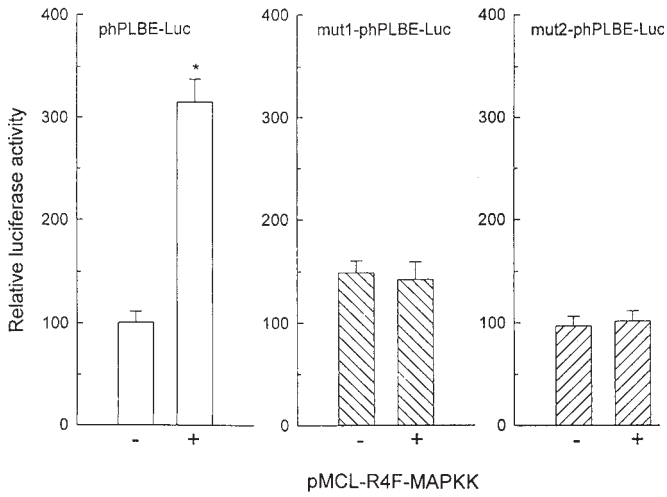

Fig. 7. Inhibition of MEK-activated hPL-B enhancer activity by mutagenesis of the NF-IL6 response elements on the enhancer. Six-well cultures of human trophoblast cells were transiently transfected with *phPLBE-Luc*, a mutant of hPLBE-Luc with a mutation of the NF-IL6 binding site at nt +3926 to +3931 (mut1-*phPLBE-Luc*), or a mutant of *phPLBE-Luc* with a mutation of the NF-IL6 binding site at nt +4038 to +4043 (mut2-*phPLBE-Luc*). Three of the culture wells in each set were cotransfected with empty plasmid lacking the MEK (–), and the other three wells were cotransfected with pMCL-R4F-MAPKK, an expression plasmid for a constitutively active mutant of MEK (+). The results represent the mean ± SEM of triplicate wells. *p = <0.0001 for *phPLBE-Luc* vs *phPLBE-Luc* + pMCL-R4F-MAPKK. Similar results were observed in two other experiments.

Materials and Methods

Plasmid Constructs and Expression Vectors

A 228-bp fragment (nt +3894 to +4124) of the hPL-B 3' flanking DNA, which maintains full enhancer activity in placental cells *(7)*, was obtained from Dr. John Parks (Department of Pediatrics, Emory University, Atlanta, GA). The hPL enhancer fragment (Fig. 1A) was ligated upstream of the luciferase reporter gene in the *Bam*Hl sites of pGL3-Basic (Promega, Madison, WI) to generate the plasmid (*phPLBE-Luc*). Although a minimal promoter was not cloned upstream of the reporter, the hPL enhancer significantly transactivated reporter activity. Expression vectors for a constitutively active mutant of MEK (MAPKK) (pMCL-R4F-MAPKK) and a catalytically inactive mutant of MEK (pMCL-8e-MAPKK) were obtained from Dr. Natalie G. Ahn (University of Colorado, Boulder) *(22)*. Expression plasmids for NF-IL6 (pEF-NFIL6) and a dominant–negative mutant of NF-IL6 (pEF-NF-IL6Δspl1) were obtained from Dr. Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan).

The NF-IL6 expression vectors were subcloned into pEF-BOS base vector. All recombinants were subjected to restriction digests and DNA sequencing for verification. Plasmids used for transfection were cloned in bacterial strain JM109 and isolated using a Plasmid Purification kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Sequence analysis of the hPL-B enhancer, performed twice, revealed a G at base +3823 instead of a C as reported previously *(23)*. The two putative NF-IL6 sites in the *phPLBE-Luc* plasmid were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutant oligonucleotides are shown in Fig. 1B (+3926/+3931 mut 1 and +4038/+4043 mut 2). DNA was isolated from each reaction, examined for the creation of an *Msc*I restriction site, and then sequenced to verify the mutations.

Gel Mobility Shift Assays

Nuclear extracts of primary cultures of human trophoblast cells and cell lines were prepared using a procedure essentially as described (*see* ref. *24*). Nuclear extract protein concentrations were determined by a Bradford assay (Sigma, St. Louis, MO) using bovine serum albumin as a standard. Gel shift assays were performed as previously described *(25)* using the oligonucleotides shown in Fig. 1B. A polyclonal antiserum to NF-IL6 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). In supershift experiments, the

antibody (1 μg of IgG) was incubated for 1 h at 4°C with nuclear extracts subsequent to addition of the radiolabeled probe.

Cell Culture

Third trimester placentas were obtained from women with normal pregnancies and deliveries. Cytotrophoblast cells were isolated by enzymatic disaggregation and cultured essentially as described previously *(26)* except that the cytotrophoblast cells were purified by negative CD-9 selection *(27)*. The protocol for obtaining placentas was approved by the Human Investigation Committees of the University of Cincinnati and the Children's Hospital Medical Center. JAR cells were grown in RPMI/10% fetal bovine serum (FBS), and BeWo human choriocarcinoma cells (ATCC CCL-98) were grown in Ham's F-12K supplemented with 15% FBS. In selected experiments, PD98059 (Calbiochem, San Diego, CA), which selectively blocks the activity of MEK *(15)*, was dissolved in dimethyl sulfoxide and then added to the incubation medium to obtain the desired final concentration. There was no effect of PD98059 on cell viability as determined by trypan blue exclusion. Conditioned medium was collected every 24 h and assayed for hPL by homologous radioimmunoassay as described previously *(28)*; the intra- and interassay variations were ±5% of the mean.

Transient Transfections

Transient transfection studies were performed using primary cultures of human trophoblast and JAR and BeWo choriocarcinoma cells. The transfection experiments using the choriocarcinoma cells were performed in triplicate by the calcium phosphate precipitation method as described previously *(24)*. The transient transfection studies of the trophoblast cells were performed in triplicate by the liposome method described by Golos et al. *(29)* beginning on d 2 of culture. Cells in each well were transfected for 4 h with 5 μg of *phPLBE-Luc* and either 0.5 μg of pRL-TK (Promega) for trophoblast cell experiments or 1.2 μg of pSV-β-galactosidase for JAR and BeWo choriocarcinoma cell experiments. pGL3B was used as filler DNA to ensure that the same amount of total DNA was transfected in each well. Primary trophoblast and choriocarcinoma cells were harvested 48 h and 24 h, respectively, after transfection. In the transfection studies with primary cells, the amount of luciferase activity by each test plasmid was normalized to the activity of pRL-TK using a dual luciferase reporter assay system (Promega). In the choriocarcinoma studies, luciferase activity was normalized to β-galactosidase activity using an enzyme assay system (Promega).

Statistics

Statistical differences between sample means were calculated by analysis of variance followed by planned orthogonal contrasts or by the Neuman-Keul's test, depending on the experimental design of each experiment.

Acknowledgments

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