BIOLOGY OF REPRODUCTION **65,** 1437–1443 (2001)

A Functional Composite Cis-Element for NFκB and RBPJκ in the Rat Pregnancy-Specific Glycoprotein Gene[1]

Chorng-Der Wang,[4] Geen-Dong Chang,[4] Yung-Kang Lee,[3] and Hungwen Chen[2,3,4]

Institute of Biological Chemistry,[3] Academia Sinica, Nankang, Taipei 115, Taiwan
Graduate Institute of Biochemical Sciences,[4] National Taiwan University, Taipei 106, Taiwan

ABSTRACT

The rat pregnancy-specific glycoprotein gene *rnCGM3* is primarily expressed in the placenta. Previously, three DNase I footprinting sites (FPI, FPII, and FPIII) were identified in the *rnCGM3* promoter region, a yeast one-hybrid screen was performed to identify the nuclear factors binding to the FPIII (5′-GCCTGGGAAAAAACTC-3′) element, and RBPJκ, a downstream effector of the Notch signaling pathway, was identified as one of the FPIII-binding factors. In the present study, the NFκB member p65 was identified as another FPIII-binding factor. Electrophoretic mobility shift assays showed that NFκB members, including p50 and p65, bound to the FPIII site. The core binding sequence in the FPIII element for p50 and p65 is GGGAAA, which overlaps with that for RBPJκ. Competition exists between p50 and RBPJκ for binding to the FPIII element. Transient expression analyses revealed that p65 significantly stimulated the expression of a reporter gene directed by the NFκB core sequence in the FPIII element. However, RBPJκ could block this stimulation. These results suggest that the regulation of *rnCGM3* expression involves both NFκB and RBPJκ, and they are mutually exclusive in the FPIII element.

gene regulation, placenta, pregnancy, syncytiotrophoblast, trophoblast

INTRODUCTION

Pregnancy-specific glycoproteins (PSGs) are selectively expressed in the placenta [1]. In situ hybridization has further revealed that PSG is synthesized by the syncytiotrophoblast and spongiotrophoblast in the human and rat placenta, respectively [2, 3]. The concentration of human PSGs in maternal circulation reaches as high as 200–400 μg/ml at term [4]. Based on the sequence identity at nucleotide and amino acid levels, PSG family members and the carcinoembryonic antigen (CEA) family members were categorized into one subfamily within the immunoglobulin (Ig) superfamily [1, 5]. The protein structure of PSG/CEA family members consists of a leader peptide, an Ig variable (V)-like N-terminal domain, various numbers of Ig constant (C)-like domains, and a carboxyl domain of varying length [1, 5]. The carboxyl domain determines whether the PSG/CEA family members are cell-surface bound or secreted molecules. The CEA subfamily members are cell-surface bound molecules, whereas the PSG subfamily members are secreted molecules [1, 5]. Multiple functions have been attributed to the CEA subfamily members, including cell adhesion, receptors for bacteria and mouse hepatitis virus, and inhibition of tumor growth [6]. Although the physiologic roles of PSGs are currently unknown, significant physiologic functions of PSGs are suggested based on the fact that low concentrations of PSGs are associated with poor pregnancy outcomes [7–10].

Because the expression levels of *PSG* are specifically high in placenta, additional transcription factors might be involved in the upregulation of PSG expression. Two rodent *PSG* genes, *rnCGM6* and *rnCGM3*, have been characterized, and both are primarily expressed in rat placenta [11, 12]. The protein structures of rnCGM6 and rnCGM3 contain three IgV-like domains, each preceded by a leader peptide, and an IgC-like domain [11, 12]. The 5′ flanking region of *rnCGM3* has also been sequenced, revealing a TATA box, CATAAA, located at nucleotides −226 to −221 upstream from the translation start site [11]. Moreover, DNase I-footprinting analyses three nuclear protein-binding sites at nucleotides −311 to −290 (FPI), −257 to −239 (FPII), and −108 to −93 (FPIII) [11]. The factor binding to the FPII site had been identified as C/EBPβ by expression cloning [13].

To further understand the molecular mechanism of *rnCGM3* expression, we recently performed a yeast one-hybrid screen to isolate the nuclear factors binding to the FPIII site. Because the expression patterns of the human and rat PSG genes are very similar, a commercial human placental cDNA library was used in this screen. One of the factors was identified as RBPJκ, the downstream effector of the Notch signaling pathway [14]. In the present study, we identified another FPIII-binding factor as the NFκB member p65. We also demonstrated that the other NFκB member, p50, could bind to the FPIII site and that p65 transactivated FPIII-driven promoter constructs. In addition, RBPJκ competed with p65 and p50 for binding to FPIII. Consequently, transactivation of the FPIII-driven promoter by p65 was suppressed by RBPJκ. Our data suggest that the FPIII site is a composite cis-element for RBPJκ and NFκB in the promoter region of *rnCGM3*.

MATERIALS AND METHODS

Library Screening and Characterization of cDNA Clones

For the yeast one-hybrid screen, six tandem copies of the FPIII element (5′-GCCTGGGAAAAAACTC-3′) were placed upstream of the minimal promoter of the yeast *HIS3* gene in vector pHISi-1 (Clontech, Palo Alto, CA) to generate the target reporter plasmid, $p(FPIII)_6HIS3$. The YM4271 yeast cells (*MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-903,* and *tyr1-501*) were transformed with 2 μg of linearized $p(FPIII)_6HIS3$ to select for positive reporter strains in a synthetic medium without histidine (SD/-His). One of the strains, YM4271FPIII, has a low level of HIS3 activity and did not replicate on SD/-His plates supplemented with 30 mM 3-aminotriazole (3-AT), a competitive inhibitor of the yeast HIS3 protein. For screening of FPIII-binding factors, the YM4271FPIII cells were transformed with 20 μg of a pACT2 human placental cDNA library (Clontech), in which the cDNAs are fused to the

[1]This work was supported by grants (to H.C.) from the National Science Council (89-2311-B-001-229) and Academia Sinica of Taiwan.
[2]Correspondence. FAX: 011 886 2 27889759;
e-mail: hwchen@gate.sinica.edu.tw

Received: 8 December 2000.
First decision: 26 January 2001.
Accepted: 18 June 2001.
© 2001 by the Society for the Study of Reproduction, Inc.
ISSN: 0006-3363. http://www.biolreprod.org

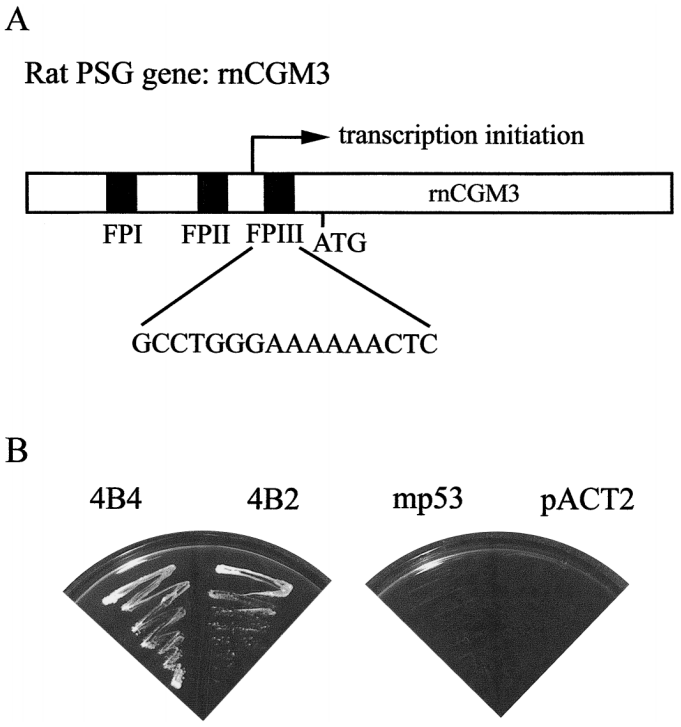

FIG. 1. Isolation of the FPIII-binding factor from yeast one-hybrid screen. **A**) Schematic representation of the promoter region of *rnCGM3*. The filled boxes denote the protected sites from the DNase I footprinting analysis. Sequence of the FPIII site is shown. **B**) In vivo interaction between FPIII and p65 clones (4B2 and 4B4) in yeast cells. Both 4B2 and 4B4 clones encode the NFκB member p65. The mouse p53 and the parental vector pACT2 are included as negative controls. The results shown are from one of three similar experiments.

GAL4 activation domain. Approximately 5×10^6 yeast colonies were screened in a synthetic medium minus histidine and leucine and containing 30 mM of 3-AT. Positive colonies were purified after two more rounds of streaking on selection plates. The pACT2 plasmids were rescued from the positive clones, retransformed into *Escherichia coli* strain HB101, and purified for sequencing.

Plasmid Constructs

The NFκB expression plasmids pCMVp65 and pCMVp50 were kindly provided by Dr. T.-H. Tan (Baylor College of Medicine, Houston, TX). The pCMVp5065 chimera construct was built by ligating the DNA-binding domain of p50 with the transactivation domain of p65 as previously described [15]. The coding region of *rRBPJκ-2N* was amplified by polymerase chain reaction (PCR) with the *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and cloned into pRcCMV to make the pCMVRBP plasmid. The plasmid $p(FPIII)_6$E1bCAT contains six FPIII sites fused to a minimal promoter element from the adenovirus *E1B* gene and the bacterial *CAT* (chloramphenicol acetyltransferase) coding sequence. Five copies of M34 (5′-GCTAGGGAAAAAACTC-3′) and four copies of M56 (5′-GCCTCCGAAAAAACTC-3′) oligonucleotides were inserted in front of the minimal promoter of *E1B* gene to generate the $p(FPIIIM34)_5$E1bCAT and $p(FPIIIM56)_4$E1bCAT reporter plasmids, respectively. All constructs were verified by DNA sequencing using the Sanger dideoxy chain-termination method [16].

Electrophoretic Mobility Shift Assay

To prepare NFκB-enriched nuclear extracts, cells were transfected with pCMVp65 or pCMVp50 or with both plasmids. Cells in a 100-mm culture plate were incubated with 10 ml of a calcium phosphate-DNA coprecipitate containing 25 μg of expression plasmid. Twenty plates of transfected cells were collected for nuclear extracts preparation 48 h after transfection. Nuclear extracts were prepared as described by Ohlsson and Edlund [17]. The p50 and p65 proteins in the nuclear extracts were detected by Western analysis with a p50-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a p65-specific antibody (Santa Cruz Biotechnology), respectively. In vitro transcription/translation of *p65* and *p50* cDNAs was performed using the TNT (transcription and translation)-coupled wheat germ extract system (Promega, Madison, WI). For the electrophoretic mobility shift assay (EMSA), end-labeled oligonucleotide FPIII probes (1 ng; $0.5–1 \times 10^5$ cpm) were incubated for 20 min at room temperature in binding reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% NP-40, 0.5 mM dithiothreitol, and 10% glycerol) containing 1 μg of poly(dI-dC) and 2 μg of nuclear extracts or 1 μl of in vitro-translated proteins. After incubation at room temperature for 20 min, the reaction mixtures were analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. For competition experiments, the unlabeled oligonucleotides were used at 50- to 200-fold molar excess and incubated with the cell extracts for 20 min on ice before adding radiolabeled probe. For comparison, the signals of binding complexes were quantitated by the image densimeter (Bio-Rad, Hercules, CA). Oligonucleotides used in EMSA were FPIII (5′-GCCTGGGAAAAAACTC), m8 (5′-GGGCACTGTGGGAACGGAAA), and κ light chain (5′-CAGAGGGGACTTTCCGAGAG).

Cell Culture, Transfection, and Reporter Gene Assays

The human embryonic kidney cell line 293 was obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in Hepes-buffered Dulbecco modified minimal essential medium supplemented with 4% fetal bovine serum, streptomycin (100 μg/ml), and penicillin (100 U/ml). The 293 cells in the 30-mm culture plate were incubated with 2 ml of a calcium phosphate-DNA coprecipitate containing 3 μg of reporter plasmid and various amounts of expression plasmid (see figure captions). The pRcCMV empty vector was included, if necessary, to maintain a constant amount of DNA in the transfection assay. Cells were harvested in the reporter lysis buffer (Promega) 48 h after transfection. CAT activity was assayed by incubating total cellular proteins in the reporter lysis buffer containing 4 mM acetyl coenzyme A and 0.1 μCi of [^{14}C]chloramphenicol. The assay was run for 1 h at 37°C with the amount of extracts required to convert 0.5–50% of the substrate to the acetylated forms. The acetylated compounds were separated from the chloramphenicol by thin-layer chromatography and quantitated by a bio-imaging analyzer (BAS-1500; Fujifilm, Minami-Ashigara-Shi, Kanagawa, Japan). Protein concentrations were measured using the BCA protein assay kit (Pierce, Rockford, IL). The Student *t*-test was used to determine statistical significance for differences between means. A P value of less than 0.05 was considered significant.

Binding Competition Assay for RBPJκ and p50

NH_2-terminally His-tagged rRBPJκ-2N (HisRBP) proteins were prepared using the baculovirus expression system. The insect cell line Sf9 (Gibco BRL, Grand Island, NY) was maintained in the serum-free medium, Sf-900II SFM medium (Gibco BRL) supplemented with 0.125 μg/ml of amphotericin B, 50 μg/ml of streptomycin, and 50 U/ml of penicillin, as suspension cultures at 28°C. The rRBPJκ-2N cDNA was subcloned into the transfer plasmid, pAcSGHisNT-B (Pharmingen, San Diego, CA). Recombinant baculoviruses were established using a commercial kit (Novagen, Madison, WI). Sf-9 cells were infected with the recombinant baculoviruses at a multiplicity of infection of 5 for 72 h. HisRBP proteins were purified using a cobalt affinity column (Clontech). The HisRBP proteins obtained were more than 95% pure and were used in this study. The recombinant p50 proteins were purchased from Promega.

A biotinylated oligonucleotide containing two copies of the FPIII sense-strand sequence was annealed with a complementary oligonucleotide. The annealed oligonucleotide, Biotin-$(FPIII)_2$, was attached to streptavidin-conjugated magnetic beads (Dynal Biotech, Oslo, Norway). For binding competition assay, the beads were incubated with 4 μg of HisRBP proteins and increasing amounts of p50 proteins in the binding solution (50 mM Hepes, pH 7.5, 10% glycerol, 0.05% NP-40, 50 mM NaCl, 5 mM EDTA, 10 mM $MgCl_2$) at 4°C overnight. After extensive washing with the binding solution, the reactions were analyzed by SDS-PAGE and Western assay with a monoclonal antibody specific to His tag (Clontech) and a polyclonal antibody specific to p50 (Santa Cruz Biotechnology).

RESULTS

Isolation and Characterization of the FPIII-Binding Factors

We performed a yeast one-hybrid screen of a human placental cDNA library to identify nuclear factors that bind to the FPIII site in the promoter region of the *rnCGM3* gene (Fig. 1A). Ten positive clones out of 5×10^6 yeast colonies screened were further characterized. Six of these

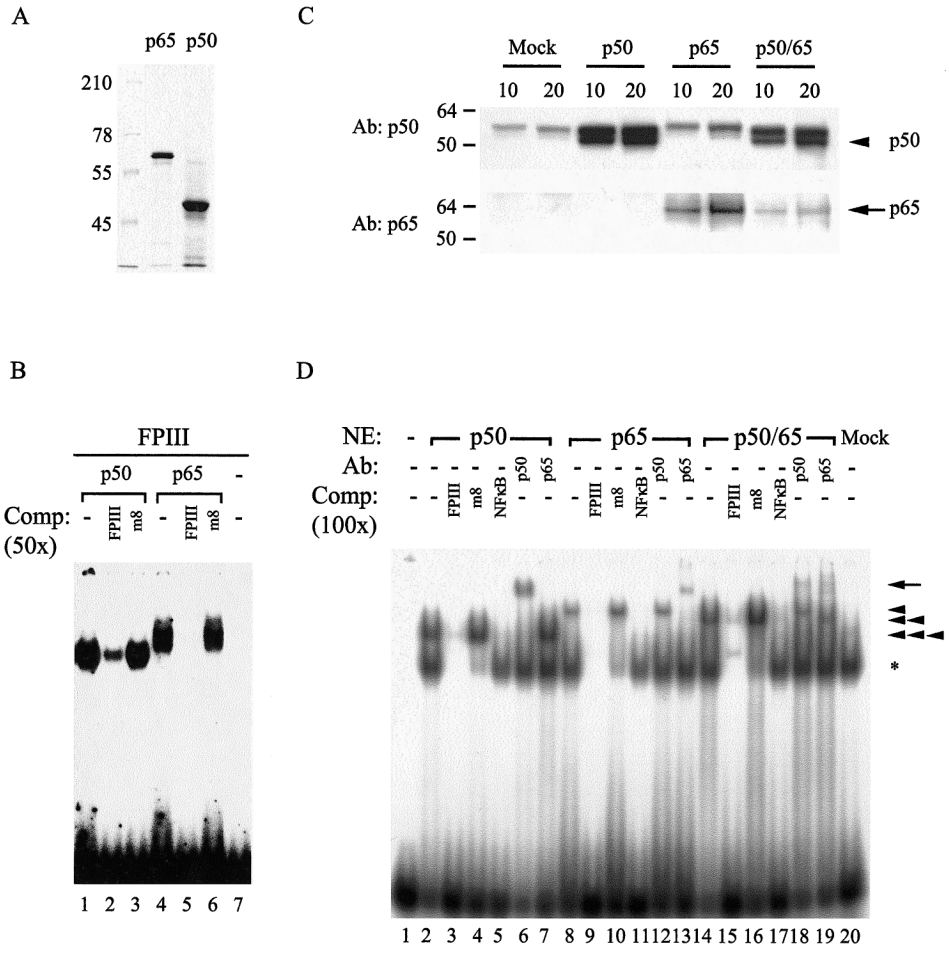

FIG. 2. Analysis of NFκB binding to the FPIII DNA element. **A**) In vitro translation of p65 and p50. Numbers on the left indicate the molecular mass (kDa) of protein markers. **B**) EMSA of in vitro-translated NFκB and radiolabeled FPIII probe. One microliter of in vitro-translated product was incubated with radiolabeled FPIII probe and analyzed. Lane 7 contains FPIII probe only. Comp, the presence or absence (−) of unlabeled FPIII or m8 oligonucleotide at 50-fold (50×) excess. **C**) Preparation of NFκB-enriched nuclear extracts. The human 293 cells were transfected with expression plasmids for p50 or p65 or both. Ten or 20 μg of nuclear extracts were analyzed by Western blots probed with antibodies specific to p50 or p65. The arrowhead and arrow indicate the p50 and p65 proteins, respectively. Mock indicates the nuclear extracts of 293 cells transfected with the parental expression plasmid. **D**) EMSA of NFκB-enriched nuclear extracts and the radiolabeled FPIII probe. Twelve micrograms of nuclear extracts were incubated with the radiolabeled FPIII probe in the absence or presence of various competitor fragments (Comp) or antibodies (Ab). The single arrowhead indicates the p65-FPIII complex (lanes 8, 10, and 12), the doubled arrowhead indicates the p50/65-FPIII complex (lanes 14 and 16), and the tripled arrowhead indicates the p50-FPIII complex (lanes 2, 4, and 7). The arrow indicates the supershifted NFκB-FPIII complex induced by antibodies (lanes 6, 13, 18, and 19). The asterisk indicates the complex formed between the constitutive nuclear factor RBPJκ and FPIII. The results shown are from one of three similar experiments.

clones encoded the NFκB family member p65. The other four clones encoded the RBPJκ, initiating at different nucleotides in the exon 2 region of the human *RBPJκ* gene [14]. The p65 clones are either the same clone or clones initiating at different nucleotides in the exon 1 region of the *p65* gene. Sequence analyses also indicated that these p65 clones encode N-terminally truncated polypeptides containing intact DNA-binding domains. To verify the specificity of the DNA-protein interaction in vivo, we reintroduced into the reporter yeast cells the two positive p65 clones (4B2 and 4B4). In addition, a plasmid containing the mouse *p53* gene, pGAD53m, and the parental vector pACT2 (containing the GAL4 activation domain) were also introduced into the yeast cells as negative controls. Colonies appeared only in the sectors of pACT2p65 (4B2 and 4B4) (Fig. 1B). Neither mouse p53 nor the GAL4 activation domain interacted with the FPIII element. These results demonstrate that p65 specifically binds to the FPIII element in vivo.

Analysis of NFκB Family Members Interacting with the FPIII Element

To investigate whether NFκB family members directly bind to the FPIII element, we performed an EMSA with a ^{32}P-labeled FPIII probe and in vitro-translated proteins of p65 and p50. The translated protein products all appeared to match the predicted molecular mass (Fig. 2A). Both p65 and p50 specifically bound to FPIII (Fig. 2B, lanes 1 and 4) because the unlabeled FPIII at a 50-fold excess could compete complex formation (Fig. 2B, lanes 2 and 5) but the unrelated oligonucleotide m8 could not (Fig. 2B, lanes 3 and 6). We also performed EMSA with rat placental nuclear extracts and the FPIII probe. However, the NFκB-FPIII complex was barely detected (data not shown). It is possible that the level of NFκB in the rat placenta extracts was too low for detection.

To prepare nuclear extracts enriched with NFκB, we transfected the human 293 cells with an expression plasmid encoding p65 or p50. The nuclear levels of exogenous p65 and p50 proteins were monitored by Western analysis. The p65 or p50 proteins were only detected in the nuclear extracts of transfected cells (Fig. 2C). EMSA was also performed using nuclear extracts prepared from the p65-, p50-, or p50/65-transfected cells and the ^{32}P-labeled FPIII probe. The p50/50 and p65/65 homodimers (lanes 2 and 8) and the p50/65 heterodimer (lane 14) specifically bound to FPIII (Fig. 2D). Both unlabeled FPIII and NFκB oligonucleotides block the corresponding complex (lanes 3, 9, and 15 for FPIII; lanes 5, 11, and 17 for NFκB). Moreover, antibodies against p50 or p65 supershifted the corresponding complex (Fig. 2D, lanes 6, 13, 18, and 19). In contrast, the NFκB-FPIII complex was not observed in the mock nuclear extracts (Fig. 2D, lane 20). A complex with a faster mobility was also observed in nuclear extracts of transfected and mock cells (Fig. 2D, asterisk), indicating the existence of a constitutive nuclear factor binding to FPIII. This constitutive nuclear factor is likely to be RBPJκ because an RBPJκ-binding oligonucleotide, m8 [18], blocked the formation of the

A

Wt	GCCTGGGAAAAAACTC
M34	GCtaGGGAAAAAACTC
M56	GCCTccGAAAAAACTC
M78	GCCTGGctAAAAACTC
M910	GCCTGGGAgcAAACTC
M1112	GCCTGGGAAAggACTC
M1314	GCCTGGGAAAAAtgTC

B

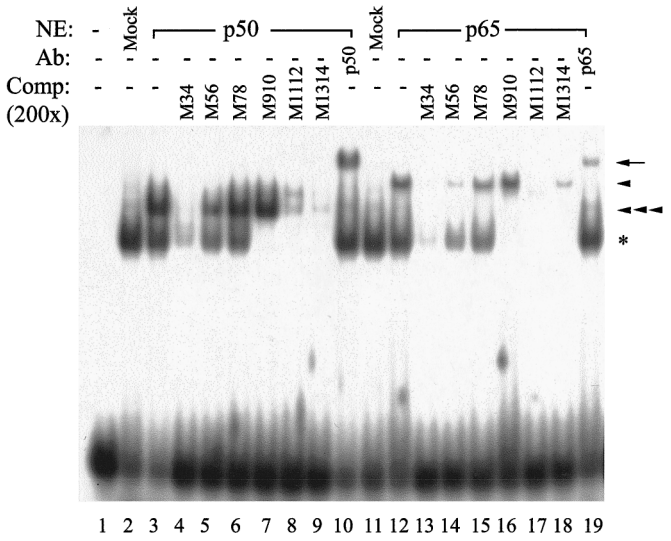

FIG. 3. Competition assay. **A**) Sequences for the mutant oligonucleotides. The mutated bases are written in lowercase letters. **B**) EMSA reactions were performed as described in Figure 2D, except that the concentrations of competitor fragments were 200-fold higher than those of the radiolabeled FPIII probe. The markers on the right are the same as described for Figure 2D. The results shown are from one of three similar experiments.

more quickly migrating complex but not the NFκB-FPIII complex (Fig. 2D, lanes 4, 10, and 16). The levels of NFκB-FPIII complex were increased in the presence of the m8 oligonucleotide. The signals for p50, p65, and p50/65 were increased by 5.6%, 36.6%, and 19.4%, respectively (Fig. 2B, compare lanes 2 and 4 for p50, lanes 8 and 10 for p65, and lanes 14 and 16 for p50/65). These data indicated that NFκB directly bound to FPIII and the constitutive factor, RBPJκ, competed with NFκB for FPIII.

To identify more specifically the nucleotides in the FPIII region important for NFκB binding, we performed competition experiments using p50- or p65-enriched nuclear extracts and a series of mutant FPIII oligonucleotides (Fig. 3A). Mutant oligonucleotides M78 and M910 could not compete efficiently with wild-type FPIII for p50 and p65 (Fig. 3B, lanes 6, 7, 15, and 16). The mutant oligonucleotide M56 could not compete efficiently with wild-type FPIII for p50 but competed partially with wild-type FPIII for p65 (Fig. 3B, lanes 5 and 14). This discrepancy was probably due to the fact that p50 prefers binding to the first half-site of the consensus sequence. These results suggest that a core sequence, GGGAAA, within the FPIII region is important for binding of p50 and p65. However, mutant oligonucleotides M910, M1112, and M1314 competed with wild-type FPIII for RBPJκ (Fig. 3B, lanes 7–9 and 16–18), suggesting that overlapping but distinct sequences define the binding sites for NFκB and RBPJκ.

Transactivation of the FPIII-Reporter Constructs by NFκB

To investigate whether binding of NFκB family members to their cognate sites in the FPIII region transactivates gene expression in vivo, transient expression experiments were performed. A reporter plasmid, $p(FPIII)_6$E1bCAT, was constructed consisting of six tandem copies of FPIII placed upstream of the minimal promoter of the adenoviral *E1B* gene. We examined the CAT activity after cotransfection of $p(FPIII)_6$E1bCAT with an expression plasmid containing either *p65* or *p50* into 293 cells. CAT expression directed by $p(FPIII)_6$E1bCAT was stimulated by p65 in a dose-dependent manner (Fig. 4A). Induction was 12.4- and 22.7-fold with 0.5 and 1 μg of pCMVp65 DNA over the empty vector, respectively. In contrast, no significant stimulation was observed with p50 (Fig. 4A). Because p65 and p50 can associate as a heterodimer, we also cotransfected pCMVp65 with increasing amounts of pCMVp50. Transactivation by p65 decreased with increasing amounts of p50 (data not shown). This phenomenon could be the result of transcriptionally inactive p50 dimers competing for binding with p65 dimers to limited FPIII sites on the CAT reporter plasmid. Therefore, the pCMVp5065 chimera construct, built by ligating the DNA-binding domain of p50 with the transactivation domain of p65, was tested. A dose-dependent transactivation by the p5065 chimera was observed (Fig. 4A). However, the p5065 chimera is less active than the p65 homodimer in transactivation (Fig. 4A).

To demonstrate that the transactivation of p65 depends on

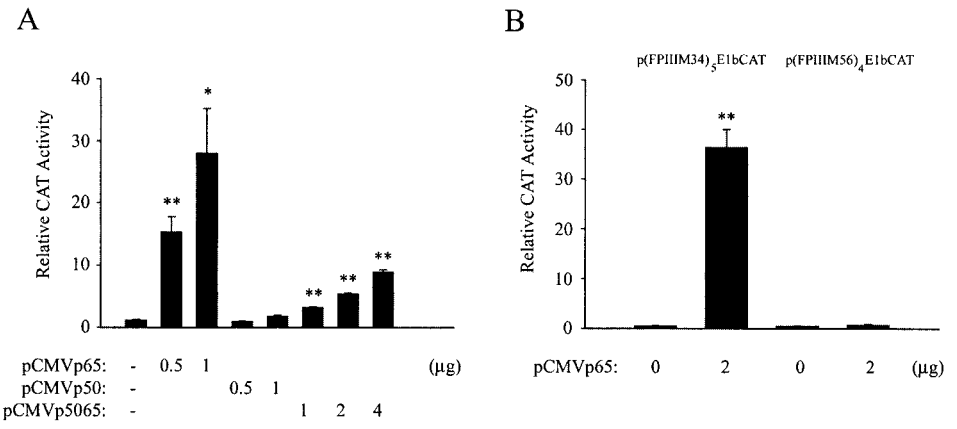

FIG. 4. Effect of NFκB on the FPIII-reporter construct. **A**) The human 293 cells were transfected with 3 μg of $p(FPIII)_6$E1bCAT reporter plasmid and the indicated amount (in μg) of another expression plasmid encoding p65 or p50 or the p5065 fusion protein. Values are mean and SEM obtained from three independent transfection experiments. **B**) Site-specific transactivation of FPIII-reporter constructs by p65. The human 293 cells were transfected with the mutant FPIII-reporter constructs $p(FPIIIM34)_5$E1bCAT and $p(FPIIIM56)_4$E1bCAT and 0.25 μg of pCMVp65. Values are mean and SEM obtained from three independent transfection experiments. Asterisks denote significant differences (*$P < 0.05$; **$P < 0.01$) between mock-transfected and expression plasmid-transfected groups.

the core sequence (GGGAAA) in the FPIII region, 293 cells were cotransfected with pCMVp65 and two mutant reporter constructs, $p(FPIIIM34)_5E1bCAT$ and $p(FPIIIM56)_4E1bCAT$. The construct $p(FPIIIM34)_5E1bCAT$ contains five copies of the M34 mutant oligonucleotide, and the construct $p(FPIIIM56)_4E1bCAT$ contains four copies of the M56 mutant oligonucleotide. The CAT activity from $p(FPIIIM34)_5E1bCAT$ induced by 1 μg of pCMVp65 was 65.7-fold greater than that of the control (Fig. 4B). No induction was detected with the $p(FPIIIM56)_4E1bCAT$ (Fig. 4B). These results suggest that p65 specifically transactivated reporter gene via the NFκ core sequence in the FPIII region.

Competition of p50 and RBPJκ for FPIII Element

Previously, we identified RBPJκ as an FPIII-binding factor and demonstrated that the core sequence in FPIII for RBPJκ is CTGGGA [14], which overlaps with the core sequence (*GGGA*AA) for p50 and p65. In the present study, we tested whether NFκB and RBPJκ compete with each other for FPIII. Because p50 and p65 have similar binding characteristics and because pure p50 protein is commercially available, this factor was used in competition binding experiments. Four micrograms of HisRBP protein was incubated with a biotinylated FPIII fragment and increasing amounts of p50 protein. The HisRBP-FPIII complex was replaced by the p50-FPIII complex as the amount of p50 protein increased (Fig. 5). This result suggests that RBPJκ and p50 are mutually exclusive on the FPIII site.

Effect of RBPJκ on FPIII-Reporter Constructs

To determine whether RBPJκ competed with NFκB for binding to FPIII in vivo, we examined CAT expression of $p(FPIII)_6E1bCAT$ stimulated by p65 in the presence of rRBPJκ-2N. The CAT activity stimulated by p65 was suppressed when increasing amounts of rRBPJκ-2N were present (Fig. 6). These results indicate that the FPIII site in the *rnCGM3* promoter is a functional cis-composite element for NFκB and RBPJκ.

DISCUSSION

In this study, we identified NFκB as a potential FPIII-binding factor for *rnCGM3* expression. Several lines of evidence support this conclusion. First, the NFκB member p65 isolated from the yeast one-hybrid screen could interact with the FPIII site in vivo when retransformed into the yeast reporter strain. Second, both of the NFκB members p65 and p50 specifically bound to the FPIII fragment in vitro. Third, p65 could transactivate reporter-gene expression via the FPIII site. NFκB was originally described as the nuclear factor bound to an intronic enhancer element of the Ig κ light chain gene [19]. The mammalian NFκB is composed of a family of structurally related proteins, including c-Rel, Rel B, p65, p50/105, and p52/p100 [20]. NFκB exists as homo- or heterodimers that are sequestered in the cytosol with the inhibitory proteins IκBs through noncovalent interactions [20]. The latent NFκB can be released in the presence of extracellular stimulants, which leads to the degradation of IκBs [21]. Therefore, the sequestration of NFκB by IκB in the cytosol may be attributed to the low level of NFκB in the rat placental nuclear extracts used in this study.

We defined the core sequence for p65 and p50 as the GGGAAA motif within the FPIII site. The sequence of the FPIII site (GGGAAAAAAC) matches 7 of the 10 nucleotides in the consensus NFκB sequence, 5′-GGGPuNNPyPyCC-3′ [22]. Studies have shown that p50 prefers binding to the first half-site of the consensus sequence [23]. Although p50 binds to FPIII, it was not isolated in the yeast one-hybrid screen. There are several possible reasons for this finding: placenta may have a higher level of p65 than p50, the conditions in yeast nuclei may favor p65 binding, or the p65 activation domain, together with the GAL4 activation domain, may synergistically activate *HIS3* gene expression.

In our previous study, we identified RBPJκ, the downstream effector of the Notch signaling pathway, as a constitutive nuclear factor binding to the FPIII site in the *rnCGM3* promoter [14]. Notch functions as a transmem-

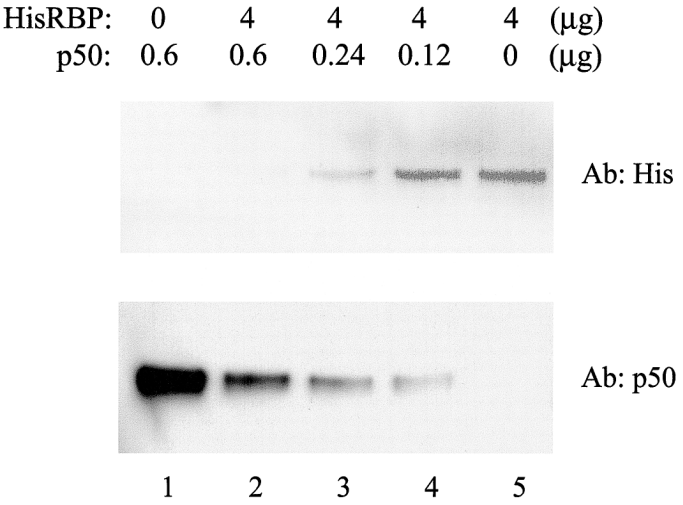

FIG. 5. Binding competition between p50 and RBPJκ. The biotinylated FPIII fragment, Biotin-$(FPIII)_2$, was incubated with 4 μg of recombinant HisRBP protein and the indicated amount of recombinant p50 protein. The Biotin-$(FPIII)_2$ fragment was purified by streptavidin-conjugated magnetic beads. The FPIII-binding proteins were detected by Western analysis with antibody (Ab) specific to the His tag or p50. The results shown are from one of three similar experiments.

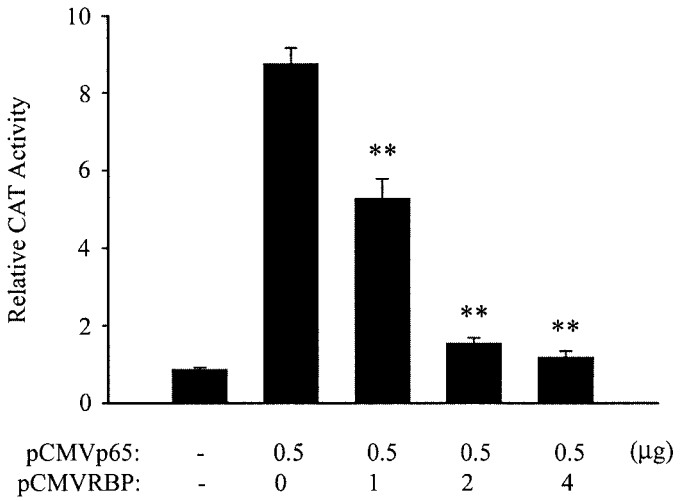

FIG. 6. Effect of RBPJκ on the transactivation of FPIII-reporter plasmid by p65. The human 293 cells were transfected with 3 μg of $p(FPIII)_6E1bCAT$ reporter plasmid and 0.5 μg of pCMVp65 and increasing amounts (in μg) of pCMVRBP. Values are mean and SEM obtained from three independent transfection experiments. Asterisks denote significant differences (**$P < 0.01$) between control (pCMVp65 alone) and pCMVRBP-cotransfected groups.

brane receptor, which can be activated by ligand stimulation [24]. The interaction between Notch and its cognate ligand results in proteolytic cleavage, with release and nuclear translocation of the intracellular domain of Notch protein (NotchIC) [25]. RBPJκ is identical to CBF1 and KBF2 and can associate with a variety of proteins, including the *Drosophila hairless* protein, the EBV nuclear antigen 2 (EBNA2), and NotchIC [26–29]. EBNA2 and NotchIC do not bind directly to DNA. However, the associated RBPJκ/EBNA2 and RBPJκ/NotchIC complexes can activate target genes through the RBPJκ site. Recently, potential RBPJκ binding sites have been identified within the NFκB sites in the promoter region of genes, including the mouse major histocompatibility complex, β_2-microglobulin, interleukin (IL) 6, NFκB2, and CYP2B1 [26, 30–34]. Moreover, Oswald et al. [33] demonstrated that RBPJκ binds to the NFκB binding sites in *Bcl-3, IκBα, IFNβ,* and *mRBPJκ.* Therefore, a subset of NFκB binding sites may also contain an overlapping RBPJκ site. In the present study, we collected evidence that the FPIII site of the *rnCGM3* promoter is also a composite element containing overlapping sequences for NFκB (GGGAAA) and RBPJκ (CTGGGA). In EMSA, when the endogenous RBPJκ proteins in the NFκB-enriched nuclear extracts were titrated out with an RBPJκ-specific oligonucleotide, formation of the NFκB-FPIII complex increased dramatically. In transient expressions, p65 activated CAT expression directed by $p(FPIII)_6$E1bCAT or $p(FPIIIM34)_5$E1bCAT by 22.7- or 65.7-fold over the control, respectively. This result suggests that p65 activates reporter gene expression via the NFκB core sequence in the FPIII element more efficiently when the RBPJκ core sequence is mutated. These results and those of the in vitro-binding competition assay using pure p50 and RBPJκ proteins (Fig. 5) indicate that NFκB and RBPJκ are mutually exclusive for binding to FPIII. We also observed a 1.5- to 2-fold stimulatory effect by p65 in JEG3 and BeWo cells on the reporter construct directed by a native *rnCGM3* promoter fragment (nucleotides −326 to −33 relative to the translation initiation site), which contains the FPIII element (data not shown). Thus, endogenous RBPJκ may obscure the positive effect of p65 using this native promoter construct.

The C/EBPβ transactivator is a positive regulator that binds to the FPII element of the *rnCGM3* promoter. Physical and functional interactions between NFκB and C/EBP families have been demonstrated in the control of cytokine gene expression such as that of IL-6 and IL-8 [35–38]. The regulatory region of IL-6 contains one NFκB and two C/EBPβ binding sites, and the two factors activate IL-6 synergistically [39]. The FPII and FPIII sites in the promoter region of *rnCGM3* contain recognition sites for C/EBPβ and NFκB, respectively, suggesting that the two factors play important roles in mediating *rnCGM3* expression. Both NFκB and C/EBPβ are responsible for regulating a variety of genes involved in inflammatory and immunologic responses, e.g., cytokines and acute phase protein genes. Although the physiologic functions of rnCGM3 are not known, *rnCGM3* may belong to this category of genes based on the mechanism of transcriptional regulations.

In our previous studies, we also demonstrated that RBPJκ represses reporter gene expression through the FPIII site and that NotchIC can counteract this repression [14]. Moreover, NotchIC enhances the transcriptional activation of C/EBPβ on *rnCGM3* [14]. The physiologic role of NFκB- and RBPJκ-mediated gene expression via the FPIII composite element in the *rnCGM3* promoter is not clear. Recently, a dynamic expression of *Notch* genes has been detected by in situ hybridization during trophoblast differentiation [40]. The Notch signaling pathway is involved in determination of the fate of many cell types, including the inhibition of myoblast differentiation, the maturation of T-cell development, and the singling out of neural precursor cells in *Drosophila* [41]. RBPJκ might repress *rnCGM3* expression until ligands for the Notch receptor induce the cleavage of the intracellular domain of the Notch receptor, which subsequently can associate with RBPJκ. The association of NotchIC and RBPJκ activates *rnCGM3* expression, which might be considered an outcome from trophoblast differentiation controlled by the Notch signaling pathway. As pregnancy proceeds, a variety of cytokines, such as IL-1, IL-2, IL-6, tumor necrosis factor α, transforming growth factor β, and interferon γ, are released from uterine epithelial cells, trophoblasts, and lymphocytes to modulate trophoblast proliferation and invasion and maternal immune responses. Therefore, these extracellular stimulants might activate the latent NFκB and consequently activate *rnCGM3* expression to maintain a high level of PSG proteins during pregnancy. The effects of C/EBPβ, NFκB, Notch, and RBPJκ on *rnCGM3* expression in trophoblasts at different gestation stages are currently under investigation.

ACKNOWLEDGMENT

We thank Dr. Hsou-min Li for critical reading of the manuscript.

REFERENCES

1. Chou JY, Plouzek CA. Pregnancy-specific β_1-glycoprotein. Semin Reprod Endocrinol 1992; 10:116–126.
2. Lei KJ, Sartwell AD, Pan CJ, Chou JY. Cloning and expression of genes encoding human pregnancy-specific glycoproteins. J Biol Chem 1992; 267:16371–16378.
3. Rebstock S, Lucas K, Thompson JA, Zimmermann W. cDNA and gene analyses imply a novel structure for a rat carcinoembryonic antigen-related protein. J Biol Chem 1990; 265:7872–7879.
4. Lin TM, Halbert SP, Spellacy WN. Measurement of pregnancy-associated plasma proteins during human gestation. J Clin Invest 1974; 54:576–582.
5. Hammarstrom S. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. Semin Cancer Biol 1999; 9:67–81.
6. Obrink B. CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. Curr Opin Cell Biol 1997; 9:616–626.
7. MacDonald DJ, Scott JM, Gemmell RS, Mack DS. A prospective study of three biochemical fetoplacental tests: serum human placental lactogen, pregnancy-specific beta 1-glycoprotein, and urinary estrogens, and their relationship to placental insufficiency. Am J Obstet Gynecol 1983; 147:430–436.
8. Mantzavinos T, Phocas I, Chrelias H, Sarandakou A, Zourlas PA. Serum levels of steroid and placental protein hormones in ectopic pregnancy. Eur J Obstet Gynecol Reprod Biol 1991; 39:117–122.
9. Masson GM, Anthony F, Wilson MS. Value of Schwangerschaftsprotein 1 (SP1) and pregnancy-associated plasma protein-A (PAPP-A) in the clinical management of threatened abortion. Br J Obstet Gynaecol 1983; 90:146–149.
10. Tamsen L, Johansson SG, Axelsson O. Pregnancy-specific β1-glycoprotein (SP1) in serum from women with pregnancies complicated by intrauterine growth retardation. J Perinat Med 1983; 11:19–25.
11. Chen H, Chen CL, Chou JY. Characterization of two promoters of a rat pregnancy-specific glycoprotein gene. Biochemistry 1994; 33: 9615–9626.
12. Chen H, Plouzek CA, Liu JL, Chen CL, Chou JY. Characterization of a major member of the rat pregnancy-specific glycoprotein family. DNA Cell Biol 1992; 11:139–148.
13. Chen H, Lin B, Chen CL, Johnson PF, Chou JY. Role of the transcription factor C/EBPβ in expression of a rat pregnancy-specific glycoprotein gene. DNA Cell Biol 1995; 14:681–688.
14. Chen H, Chong Y, Liu CL. Active intracellular domain of Notch enhances transcriptional activation of CCAAT/enhancer binding protein

β on a rat pregnancy-specific glycoprotein gene. Biochemistry 2000; 39:1675–1682.
15. Ruben SM, Narayanan R, Klement JF, Chen CH, Rosen CA. Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol Cell Biol 1992; 12:444–454.
16. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977; 74:5463–5467.
17. Ohlsson H, Edlund T. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell 1986; 45:35–44.
18. Tun T, Hamaguchi Y, Matsunami N, Furukawa T, Honjo T, Kawaichi M. Recognition sequence of a highly conserved DNA binding protein RBP-Jκ. Nucleic Acids Res 1994; 22:965–971.
19. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986; 46:705–716.
20. Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes Dev 1995; 9:2723–2735.
21. Beg AA, Finco TS, Nantermet PV, Baldwin AS. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol Cell Biol 1993; 13:3301–3310.
22. Kunsch C, Ruben SM, Rosen CA. Selection of optimal κB/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. Mol Cell Biol 1992; 12:4412–4421.
23. Urban MB, Schreck R, Baeuerle PA. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J 1991; 10:1817–1825.
24. Jarriault S, Le Bail O, Hirsinger E, Pourquie O, Logeat F, Strong CF, Brou C, Seidah NG, Israel A. Delta-1 activation of notch-1 signaling results in HES-1 transactivation. Mol Cell Biol 1998; 18:7423–7431.
25. Artavanis-Tsakonas S, Matsuno K, Fortini ME. Notch signaling. Science 1995; 268:225–232.
26. Brou C, Logeat F, Lecourtois M, Vandekerckhove J, Kourilsky P, Schweisguth F, Israel A. Inhibition of the DNA-binding activity of *Drosophila* suppressor of hairless and of its human homolog, KBF2/RBP-Jκ, by direct protein-protein interaction with *Drosophila* hairless. Genes Dev 1994; 8:2491–2503.
27. Dou S, Zeng X, Cortes P, Erdjument-Bromage H, Tempst P, Honjo T, Vales LD. The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. Mol Cell Biol 1994; 14:3310–3319.
28. Henkel T, Ling PD, Hayward SD, Peterson MG. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J_{κ}. Science 1994; 265:92–95.
29. Tamura K, Taniguchi Y, Minoguchi S, Sakai T, Tun T, Furukawa T, Honjo T. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-Jκ/Su(H). Curr Biol 1995; 5:1416–1423.
30. Israel A, Yano O, Logeat F, Kieran M, Kourilsky P. Two purified factors bind to the same sequence in the enhancer of mouse MHC class I genes: one of them is a positive regulator induced upon differentiation of teratocarcinoma cells. Nucleic Acids Res 1989; 17:5245–5257.
31. Kannabiran C, Zeng X, Vales LD. The mammalian transcriptional repressor RBP (CBF1) regulates interleukin-6 gene expression. Mol Cell Biol 1997; 17:1–9.
32. Lee SH, Wang X, DeJong J. Functional interactions between an atypical NF-κB site from the rat CYP2B1 promoter and the transcriptional repressor RBP-Jκ/CBF1. Nucleic Acids Res 2000; 28:2091–2098.
33. Oswald F, Liptay S, Adler G, Schmid RM. NF-κB2 is a putative target gene of activated Notch-1 via RBP-Jκ. Mol Cell Biol 1998; 18:2077–2088.
34. Plaisance S, Vanden Berghe W, Boone E, Fiers W, Haegeman G. Recombination signal sequence binding protein Jκ is constitutively bound to the NF-κB site of the interleukin-6 promoter and acts as a negative regulatory factor. Mol Cell Biol 1997; 17:3733–3743.
35. Kunsch C, Lang RK, Rosen CA, Shannon MF. Synergistic transcriptional activation of the IL-8 gene by NF-κB p65 (RelA) and NF-IL-6. J Immunol 1994; 153:153–164.
36. LeClair KP, Blanar MA, Sharp PA. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc Natl Acad Sci U S A 1992; 89:8145–8149.
37. Stein B, Baldwin AS Jr. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. Mol Cell Biol 1993; 13:7191–7198.
38. Stein B, Cogswell PC, Baldwin AS Jr. Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. Mol Cell Biol 1993; 13:3964–3974.
39. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S. Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci U S A 1993; 90:10193–10197.
40. Nakayama H, Liu Y, Stifani S, Cross JC. Developmental restriction of Mash-2 expression in trophoblast correlates with potential activation of the notch-2 pathway. Dev Genet 1997; 21:21–30.
41. Robey E. Notch in vertebrates. Curr Opin Genet Dev 1997; 7:551–557.