Regulation of the Human Chorionic Gonadotropin α- and β-Subunit Promoters by AP-2*

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Production of the placental hormone, chorionic gonadotropin (CG), increases dramatically as cytotrophoblasts fuse to form syncytiotrophoblasts. The CG α- and β-promoters are both responsive to cAMP, although the kinetics of cAMP stimulation are different. In an effort to understand the mechanisms of coordinate induction of these genes, AP-2 binding sites were identified in the promoter regions of the α and βCG genes. AP-2 bound to the upstream regulatory element (−186 to −156 base pairs (bp)) in the α-promoter and to several different regions of the βCG promoter, including footprints 2 and 4B (FP2, −311 to −279 bp; FP4B, 221 to −200 bp). AP-2 antibodies induced supershifts of these complexes, confirming the identity of the protein-DNA complex. In JEG-3 cells, which contain abundant AP-2, mutations in these CGβ AP-2 sites reduced basal activity and decreased cAMP stimulation. In AP-2-deficient Hep-G2 cells, co-transfection of AP-2 stimulated expression of the CGβ promoter 10–20-fold, and the α-promoter was induced by 3–6-fold. Mutations that eliminate AP-2 binding to CGβ FP4B reduced AP-2 stimulation by more than 80%, whereas mutations in FP2 reduced AP-2 stimulation by less than 50%. Analyses of AP-2 mutants revealed a requirement for the DNA binding/dimerization domain and the amino-terminal proline-rich and acid-rich transactivation domains for stimulation of the CGβ promoter. Primary cultures of placent al cytotrophoblasts were differentiated into syncytiotrophoblasts in vitro to examine AP-2 expression by reverse transcriptase-polymerase chain reaction. AP-2 mRNA levels increased by day 2 and continued to rise in parallel with a marked increase in α and CGβ gene expression. We conclude that both the α and CGβ promoters contain binding sites for AP-2 and suggest that this transactivation factor provides a mechanism for coordinating the induction of these genes during placental cell differentiation.

Human chorionic gonadotropin (hCG) is a heterodimeric placental hormone encoded by separate α- and CGβ-subunit genes (1–3). It is a member of a family of hormones that are expressed in the pituitary (luteinizing hormone (LH), follicle-stimulating hormone, and thyroid-stimulating hormone) and the placenta (CG). The α-subunit is common to these hormones, and it is expressed in both pituitary and placenta. The CGβ gene is expressed almost exclusively in the placenta (3). The function of CG is to stimulate the corpus luteum in the ovary to produce progesterone during the early stages of pregnancy.

The dramatic exponential increase in CG expression in early pregnancy correlates with the formation of differentiated placental cells (4, 5). Trophoblast progenitor cells convert to proliferative cytotrophoblasts that invade the endometrium of the uterus. The cytotrophoblasts fuse to form nonmitotic syncytiotrophoblast cells. The production of CG is greatly enhanced upon the formation of syncytiotrophoblasts, which also produce a variety of other hormones including placental lactogen (5).

The cellular pathways that lead to activation of the α and CGβ genes have not been clearly established, although cAMP is able to induce expression of these genes in both placental cells (6) and choriocarcinoma cell lines (7, 8). Cyclic AMP-responsive DNA sequences have been characterized in the promoters of both genes (for review, see Ref. 9). In the α-gene, two identical repeats of a consensus CAMP response element (CRE) are located between −146 and −111 bp of the promoter (10–12). These CREs bind cAMP response element-binding protein (12–14) along with other members of the B-Zip family of transcription factors (15, 16). An adjacent element, termed the upstream response element (URE, −180 to −151), also contributes to basal expression and appears to contribute to placenta-specific expression of the α-promoter (12, 13, 17–20). The URE contains three overlapping protein binding sites referred to as the trophoblast-specific element (TSE, or URE2 (−187 to −159)) (12, 13, 18, 21), downstream domain (−172 to −151) (13, 19), and GATA (α-ACT, URE1) (−165 to −140) (22). Protein binding to the TSE and downstream domain are mutually exclusive (13, 19).

The cAMP-responsive region in the CGβ promoter encompasses several protein binding domains between −311 and −200 bp (23–25). Maximal expression in placental cell lines and stimulation by cAMP requires this entire region, suggesting that it functions as a composite regulatory element (23).

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Nuclear extracts footprint two major regions (−311 to −274; −250 to −200) within this domain (23, 24). Neither region binds transcription factor cAMP response element-binding protein (23), nor are they competed by the CRE derived from the α-promoter (23, 24), suggesting distinct pathways for cAMP control of the two genes. Another B-Zip protein, c-Jun, negatively regulates both promoters, and it binds to the c-CRE and to the CGβ gene promoter between −245 and −220 bp (16). However, based upon antibody-mediated supershift studies, c-Jun does not appear to represent the major protein that binds to this region of the CGβ gene (16). Distinct sequences within the cAMP-responsive region of the CGβ promoter may share common binding proteins, since they cross-compete for protein interactions (24). In addition, the TSE region from the α-promoter also competes for these proteins, suggesting that the same transcription factors might be involved in the coordinate regulation of the two promoters (24).

Transcription factor AP-2 has been shown to mediate cAMP responses in other genes including metallothionein IIA (26), acetyl-CoA carboxylase (27), insulin-like growth factor-binding protein-5 (28), and RII protein kinase β (29) among others (16, 30). AP-2 has also been implicated in developmentally regulated gene expression in a variety of cell types including NT-2 and P19 teratocarcinoma cells (31, 32), primary neural cells (33), keratinocytes (34–36), and adipocytes (27). AP-2 is a 52-kDa protein that binds to DNA as a homodimer (37) and can associate with c-Myc, inhibiting transactivation by c-Myc (38). Several AP-2 variants have been identified (37, 39), some of which act as inhibitors of AP-2 gene activation through an undetermined mechanism (37). In this report, we examine a potential role of AP-2 in the regulation of the α and CGβ genes. We find that AP-2 binds to regulatory elements in both promoters and regulates the expression of these genes. AP-2 is also induced during placental cell differentiation.

**MATERIALS AND METHODS**

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared by the Shapiro method (40) modified by the addition of protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml N-α-aminobenzamidine) to the final dialysis buffer. Nuclear extracts (5–10 μg) were added to a 20-μl reaction containing: 2 μl of 10× buffer (200 mM HEPES, pH 7.9, 400 mM KCl, 10 mM MgCl₂, and 1% Nonidet P-40), 500 ng of dl-dC, and 1 μg of AP-2 or Sp-1 antibodies as indicated. Reactions were preincubated on ice for 30 min before the addition of 50 fmol of radiolabeled probe with or without unlabeled competitor DNA. Reactions were incubated at room temperature for 30 min before electrophoresis (180 V, 3 h) through nondenaturing 5% polyacrylamide gels in 0.5 × TBE (45 mM Tris borate, 1 mM EDTA).

Oligonucleotides for electrophoretic mobility shift assays are listed in Table I. Hybridized oligonucleotides were labeled by Klenow end filling. Klenow reactions included 5 pmol of annealed oligonucleotides, 1 μl of 10× Klenow buffer (Promega, Madison, WI), 4 μl of 25 mM dioxynucleoside triphosphates (without dATP), 3 μl of 25 mM PPdATP (3000 μCi/ml, DuPont NEN), and 1 μl of Klenow fragment (Promega). Reactions were incubated at 37 °C for 30 min before the addition of 5 μl of 25 mM dNTPs and incubation for an additional 10 min. After stopping the reactions, the solution was passed through Centricep columns (Adelphia, NJ) to remove unincorporated nucleotides.

**Reporter Genes and Construction**—The α and CGβ luciferase constructs in the pA3LUC plasmid have been described previously (23, 25). Site-directed mutagenesis was performed using sequences that correspond to the mutant oligonucleotides used in gel mobility shift assays (Table I). Polymerase chain reactions were used to incorporate the mutations within the α and CGβ promoters (25). All site-directed mutations were sequenced to verify the mutation as well as the correct orientation and promoter sequence. Expression vectors containing wild type AP-2 and AP-2 mutants were driven by the Rous sarcoma virus promoter (41).

Transient Transfections—Transient transfections were performed using either the CaPO₄ (42) or lipid-mediated (43) methods. CaPO₄ reactions consisted of 4.5 μg of reporter plasmid, 250 μl of HEPES-buffered saline (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂PO₄, 1 mM dextrose, 21 mM HEPES, pH 7.05) and 10 μl of 2 mM CaCl₂. Expression vectors (300–600 ng) were used as indicated, and equal amounts of empty vector were added to keep the total amount of expression vector constant in different reactions. Lipid transfections were performed using l-α-phosphatidyethanolamine, dioleoyl (Sigma) and dimethyl-dioctadecyl-ammonium bromide (Sigma) lipids prepared by the ethanol injection method (44). Cells were harvested for luciferase assays 18–24 h after transfection (45).

**Western Blots**—Nuclear extracts are described above (40). Whole cell placental extracts were kindly provided by Dr. T. Woodruff (Northwestern University). Primary antibodies included 1:1000 AP-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 1:10,000 Sp-1 (Santa Cruz Biotechnology) and were added to nitrocellulose membranes for 60 min in solution containing albumin (5 mg/ml). After washing three times in 0.1% Tween phosphate-buffered saline, membranes were incubated with 1:10,000 secondary anti-rabbit antibody (Santa Cruz Biotechnology) in 3% milk phosphate-buffered saline. After washing, membranes were subjected to enzyme-linked chemiluminescence as described by the manufacturer (Amersham Corp.).

**RT-PCR Assays for mRNA Expression in Placental Cells**—Cytotrophoblast cells were isolated from human term placenta and cultured under conditions that allow fusion into syncytiotrophoblast cells (46). RNA was isolated from placental cells every 2 days over a 12-day period of culture (46). Total RNA (1 μg) was reverse transcribed (37 °C, 2 h) by the addition of 15 units of reverse transcriptase (Promega) in the presence of 10 pmol of random hexamer primers, 25 mM dioxynucleoside triphosphates (dNTPs) in 1 × MI buffer (67 mM Tris, pH 8.8, 6.7 mM MgCl₂, 16 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol) in a total volume of 20 μl. PCR reactions included specific primers for the α and CGβ genes, AP-2, and internal controls, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein 19 (RPL19). The final
PCR reaction (100 μl) included a 1-μl aliquot of the RT reaction product, 50 pmol of sense and antisense primers for AP-2, α, and CGβ, along with one of the controls, GAPDH or hRPL19, in 1 X MI buffer (25 μM dNTPs, 10 μl of Me₂SO, 0.5 μl of Taq DNA polymerase (Promega), and 0.1 μl of [32P]dATP (DuPont NEN). Cycle conditions were 96 °C for 30 s, 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. An aliquot (20 μl) of each reaction was subjected to polyacrylamide (6%) gel electrophoresis, and the specific products were quantitated using a Fujix 2000 phosphoimager (Fuji Medical Systems, Stamford, CT). Data were analyzed by one-way analysis of variance using Dunnett’s test.

Primers (Life Technologies, Inc.) were designed to span exon-intron boundaries to avoid amplification of genomic DNA. The primers include the following: α sense, 5’-CCAGAATGCACGCTACAG-3’, a antisense, 5’-CCGCCGTGTGGTTCTCCAG-3’, product 222 bp; hCGb sense, 5’-GTG-GAGAAGGAGGGCTGC-3’, hCGb antisense, 5’-GGCGGCAGAGTGCA-CATT-3’, product 232 bp; AP-2 sense, 5’-CTGCCAACGTTACCCTGC-3’, AP-2 antisense 5’-TAGTTCTGCAGGGCCGTG-3’, product 339 bp; GAPDH sense, 5’-GAGCCACATCGCTCAGAC-3’, GAPDH antisense, 5’-CTTCTCATGTTTCACACC-3’, product 430 bp.

RESULTS

AP-2 Binds to Regulatory DNA Elements in the α and CGβ Promoters—The α and CGβ regulatory elements that have been shown to bind proteins in JEG-3 nuclear extracts are depicted in Fig. 1A (12, 13, 23, 24). Previous studies have shown cross-competition by a subset of these elements, including the αURE/TSE and several of the CGβ footprinted elements, suggesting that these sequences may share common transcription factors (24, 47). The size of the URE/TSE binding protein (24) and the GC-rich nature of the DNA sequences shared by these elements raised the possibility that AP-2 might interact at these sites.

Electrophoretic mobility shift assays were performed using the following reagents defined in Fig. 1A, AP-2 or Sp-1 antibodies were added to extracts, as indicated at the top of the panel, for 60 min before the addition of radiolabeled DNA. The positions of antibody-induced supershifts of AP-2 and Sp-1 complexes are denoted by arrows. B, delineation of AP-2 and Sp-1 binding sites in CGβ FP2. Overlapping fragments spanning the FP2 sequence are depicted at the top of the panel. Added competitor DNA, sera, and antibodies are indicated at the top of the gel. As a control, the properties of Sp-1 binding to its consensus sequence are shown to the right of the gel. The positions of AP-2 and Sp-1 complexes are indicated by arrows. C, delineation of the AP-2 complex with FP2 by competition for Sp-1. Added competitor DNA, sera, and antibodies are indicated at the top of the gel. As a control, the properties of Sp-1 binding to its consensus sequence are shown to the right of the gel. The positions of AP-2 and Sp-1 complexes are indicated by arrows.

Fig. 3. Western blot analysis of AP-2 expression in placenta and various cell lines. Nuclear extract proteins (10 μg) for the indicated cell lines or whole cell extract proteins (10 μg) were subjected to 12% denaturing SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane that was sequentially probed with AP-2 (A) or Sp-1 antibodies (B). Molecular mass markers are shown at the left, and the location of the 52-kDa AP-2 and 110-kDa Sp-1 bands are shown by arrowheads.
JEG-3 nuclear extracts to assess whether a consensus AP-2 sequence competed for binding to the α and CGβ elements (Fig. 1B). Excess (100-fold) unlabeled AP-2 oligonucleotide inhibited binding to the αURE and CGβ FP4B. The lower part of the complex that binds to CGβ FP2 was also reduced by the AP-2 competitor. As a positive control, the AP-2 competitor was shown to compete well for protein binding to the homologous AP-2 sequence derived from the human metallothionein IIA (hMTIIA) promoter (−185 to −167) (26, 48). However, excess unlabeled AP-2 oligonucleotide did not compete with complexes that bind to the Sp-1 binding site or to CGβ FP4A, indicating that the competition is specific. These results suggest that AP-2 binds to sequences within both the α and CGβ promoters.

AP-2 antibody was used in supershift assays to confirm whether AP-2 was present in the protein complexes that bind to the α and the CGβ promoter elements (Fig. 2A). The AP-2 antibody supershifted the major complex binding to the αURE and to CGβ FP4B as well as proteins that bind to the control AP-2 element, hMTIIA. In contrast, the AP-2 antibody had no effect on protein binding to Sp1 element. The Sp1 antibody had no effect on protein binding to the putative AP-2 sites, whereas it caused a supershift of the Sp1 complex.

Protein interactions with different domains within CGβ FP2 are illustrated in Fig. 2B. When the full-length FP2 fragment was used, multiple protein complexes were observed. The addition of either AP-2 or Sp1 antibody, but not preimmune AP-2 sera, appeared to shift part of the major protein complex, suggesting that both proteins might bind to FP2. The FP2 fragment was divided into overlapping segments FP2A, FP2B, and FP2C. AP-2 competitor DNA inhibited protein binding to the FP2A and FP2C fragments, and these complexes were also supershifted by AP-2 antibody. On the other hand, Sp1 competitor inhibited protein binding to FP2B, and Sp1 antibody supershifted this complex. Additional experiments confirmed that these interactions were specific, since the Sp1 antibody did not alter binding to FP2A and FP2C and the AP-2 antibody had no effect on the FP2B complex (data not shown). These experiments indicate that AP-2 binds to the 5’- and 3’-ends of the FP2, whereas Sp1 binds to the central region (−306 to −285 bp).

The interaction of AP-2 with the FP2 region was resolved further by performing combinations of oligonucleotide competitions and antibody supershift experiments (Fig. 2C). In the presence of Sp1 competitor oligonucleotide, several FP2 protein complexes were diminished, and the amount of the putative AP-2 complex was increased. The addition of AP-2 antibody in the presence of Sp1 competitor supershifted the residual protein complex. These findings support the idea that both Sp1 and AP-2 bind to FP2 and raise the possibility that these binding sites overlap partially.

**AP-2 Is Present in JEG-3 Nuclear Extracts and in Placenta**—Western blots were performed to determine whether AP-2 is present in cells that express the α and CGβ genes (Fig. 3). AP-2 antibody detected a 52-kDa protein in JEG-3 cells and extracts from whole placenta and trophoblasts. As controls, the same band was seen in HeLa cells but not in Hep-G2 cells, which
have been shown previously to be deficient in AP-2 (41). The same blot was reprobed with an Sp-1 antibody and verified that the nuclear extracts from each of the cell lines contained similar levels of Sp-1 protein (Fig. 3B). However, little Sp-1 was detected in the whole cell extracts from placenta or trophoblast cells (Sp-1 was seen in these extracts with longer exposure, data not shown). These results indicate that AP-2 is abundant in the placenta.

### Role of the AP-2 Binding Sites in the Function of the α and CGβ Promoters

Transient expression assays were performed in Hep-G2 cells to examine the effects of co-transfected AP-2 on the α and CGβ promoters (Fig. 4) (41). AP-2 stimulated 22-fold (Fig. 4A). Deletions to −1700 or −345 bp decreased stimulation to 10-fold, and subsequent deletions to −248 bp essentially eliminated AP-2 stimulation. The deletion between −345 and −248 bp includes FP2 but not

### Table I

Sequences of wild type and mutant oligonucleotides

| Oligonucleotide | Wild Type | Mutant 1 | Mutant 2 | Mutant 3 |
|----------------|-----------|----------|----------|----------|
| hCGb FP4A      | CATTTCCGGGAGGAGCACTCCGGGCAT | ATCCCTGGCTTGAAGGATGATG | ATCCCTGGCTTGAAGGATGATG | ATCCCTGGCTTGAAGGATGATG |
| hCGb FP4B      | AGCTTCGGGGGAGGAGCACTCCGGGCAGCT | TCGCCGCCGTCGGAGAACA | GTGCCGAGCACCTGCTGC | ACACACCTTCGCGGGCCCTATTT |
| hCGa 172ma     | ACACACCTTCGCGGGCCCTATTT | TCGCCGCCGTCGGAGAACA | GTGCCGAGCACCTGCTGC | ACACACCTTCGCGGGCCCTATTT |
| hCGb FP2       | AAAATAAGCCATTGTTGAAACAA | AAAATAAGCCATTGTTGAAACAA | AAAATAAGCCATTGTTGAAACAA | AAAATAAGCCATTGTTGAAACAA |

**Fig. 6.** Functional effects of mutations in AP-2 sites in the CGβ promoter studied in Hep-G2 cells. Hep-G2 cells were co-transfected with 4.5 μg of the indicated −345CGβ promoter mutants and 600 ng of an expression vector either with or without AP-2 cDNA sequences. A, the locations of different CGβ promoter mutations are depicted in FP2 and FP4B. B, CGβ promoter activity in the absence and presence of AP-2. Results represent mean ± S.E. of triplicate transfections. -Fold stimulation by AP-2 is shown above the bars.
FP4. An internal deletion of FP4 (345 d-bFP4) also eliminated AP-2 stimulation.

AP-2 increased \(-846\) \(\alpha\)-promoter activity by 6.8-fold. This effect was reduced to 3–4-fold by deletion to \(-290\) or \(-180\) bp. Deletion to \(-156\) bp eliminates the \(\alpha\)URE and the AP-2 binding site. Deletion to \(-132\) bp eliminates one of the two CREs. These deletions both reduced basal expression and also reduced AP-2 stimulation to 1.3- and 2.5-fold, respectively. Deletion to \(-156\) bp eliminates the \(\alpha\)URE and the AP-2 binding site. Deletion to \(-132\) bp eliminates one of the two CREs. These deletions both reduced basal expression and also reduced AP-2 stimulation to 1.3- and 2.5-fold, respectively. A single point mutation (172M\(\alpha\)), shown previously to disrupt binding to the \(\alpha\)URE (19) (see below), decreased AP-2 stimulation further (0.9-fold).

Because deletion of CG\(\beta\) FP2 has been shown previously to eliminate cooperative interactions with more proximal sequences (23, 25), the roles of CG\(\beta\) FP2 and FP4B were examined further by creating point mutations within the individual domains. Electrophoretic mobility shift assays were used to determine the effects of the mutations on protein binding (Fig. 5A). Relative to the wild-type FP4B sequence, each of the CG\(\beta\) FP4B mutants (Table I) competed poorly for AP-2 binding. FP4B-m1 and FP4B-m3 showed little or no competition, whereas FP4B-m2 competed partially for AP-2 binding. The effects of mutations in CG\(\beta\)FP2 or the \(\alpha\)URE are shown in Fig. 5B. Consistent with the AP-2 supershift studies in Fig. 2B, fragments FP2A and FP2C, but not FP2B competed for AP-2 binding. The FP2-m1 mutation, which alters the binding site in fragment FP2A did not compete for AP-2 binding. The 172M in the \(\alpha\)URE eliminated AP-2 binding to this fragment of the \(\alpha\)-promoter (Fig. 5B).

JEG-3 cells have been used extensively for studies of \(\alpha\) and CG\(\beta\) gene expression (9). Because JEG-3 cells express abundant amounts of AP-2 (Fig. 3), they were used to assess the effects of the AP-2 mutations in the presence of the endogenous protein (Fig. 7). Each of the FP4 mutations greatly reduced basal activity, consistent with a role for endogenous AP-2 in the regulation of this site (Fig. 7A). The FP2 mutations caused an even greater decrease in basal activity, suggesting that the AP-2 and/or the Sp-1 sites in this region are also involved in basal expression.

AP-2 has been implicated in cAMP regulation of gene expression (26–28), and cAMP is known to induce the CG\(\beta\) gene. The effect of mutations of the AP-2 binding site on cAMP stimulation of CG\(\beta\) promoter activity were assayed after 18 h of treatment (Fig. 7B). The wild-type \(-345\) CG\(\beta\) promoter was induced 42-fold by cAMP (Fig. 7B). The AP-2 mutations in FP4 reduced cAMP stimulation to a variable extent (50–75% decrease). The FP2-m1 mutation, which eliminates AP-2 binding to the FP2A sequence, also decreased cAMP stimulation. In contrast, the FP2-m2 and FP2-m3 mutations, which disrupt Sp-1 binding, reduced basal activity but had little effect on cAMP stimula-
AP-2 Regulation of hCG

Chorionic gonadotropin gene expression in the placenta is a relatively recent evolutionary event, since its expression occurs almost exclusively in higher primates (9). In the case of the α-gene, modifications in the CRE sequence and in adjacent upstream regulatory elements appear to account for the ability of the α-gene to be expressed in the placenta as well as in the pituitary gland (17, 18). The CGβ genes appear to have duplicated and diverged from an ancestral LHβ gene (2). Although LHβ expression is restricted to the pituitary gland, the CGβ genes are expressed preferentially in the placenta, presumably reflecting the acquisition of new regulatory DNA sequences that direct placenta-specific expression (50).

The DNA regulatory elements that control CGβ gene expression have been challenging to define. Mutational studies have suggested that several distinct elements may interact in an interdependent manner, a phenomenon that has made it difficult to clearly delineate discrete functional domains (23). Protein binding studies have therefore proven quite helpful for defining potential regulatory elements. DNase I footprinting analyses delineated several discrete binding sites, particularly between −311 and −200 bp (23, 24). More recently, it was found that several of these sites competed with one another, raising the possibility that a common protein was binding to multiple sites (24). Moreover, evidence that a key regulatory element (URE/TSE) for placental expression of the α-promoter also competed for binding to the CGβ elements suggested that this protein might be involved in the coordinate expression of the two genes (24). These findings have underlined the importance of identifying the factors that bind to the CGβ regulatory elements.

In this report, we provide several lines of evidence that a transcription factor that is immuno-reactive with AP-2 antibodies binds to the CGβ regulatory elements. Consensus AP-2 elements compete for binding to FP4B and for two of the complexes that bind to FP2. The identity of this protein as AP-2 is strengthened by the fact that an AP-2 antibody supershifts AP-2 of the complexes that bind to these sites. Similar data were found for the αURE site that competes for protein interactions with the CGβ elements, supporting the notion that this protein is shared in common by these sequences. The molecular mass of AP-2 is similar (52 kDa) to the size of the protein previously purified by affinity chromatography using the αURE sequence (24). Last, in AP-2-deficient Hep-G2 cells, co-expression of AP-2 stimulated expression of the α-promoter and, to a greater degree, the CGβ promoter. Taken together, these data suggest AP-2 may be a regulator of α and CGβ gene expression in the placenta.

AP-2 appears to interact with several CGβ elements, including FP2 and FP4B. Additional AP-2 sites were also identified several kilobase pairs upstream in the CGβ promoter. The proximal part of the CGβ promoter is very G-C-rich and has yet to be tested for AP-2 binding. FP2 is also bound by Sp-1, and it appears that AP-2 and Sp-1 may bind to partially overlapping elements, since competition for Sp-1 facilitated the binding of AP-2 (Fig. 2C). Previous studies revealed that c-Jun binds close to FP4A, which is adjacent to one of the AP-2 sites (FP4B) (25).

Fig. 9. Induction of AP-2 gene expression during trophoblast differentiation in vitro. RT-PCR was used to measure AP-2, α, and hCGβ mRNA levels during in vitro differentiation of primary cultures of placental trophoblasts. A, example of radiolabeled RT-PCR products for AP-2, α, and hCGβ during trophoblast differentiation. B, levels of specific mRNAs were corrected by comparison with an internal standard, GAPDH, as described under “Materials and Methods.” Results are from a representative PCR reaction and are expressed as percentage of maximal level. Maximal -fold inductions relative to day 0 were as follows: AP-2 (8-fold), α (16-fold), hCGβ (45-fold).

Domains of AP-2 Required for Induction of the CGβ Promoter—Several functional domains have been delineated in AP-2, including a DNA binding domain, a dimerization domain, and transactivation domains (41, 49). Transient co-transfection studies were conducted in Hep-G2 cells comparing wild type and mutant AP-2 expression vectors using the −345 hCGβ promoter as the reporter gene (Fig. 8). Deletion of the amino-terminal 50 amino acids of AP-2 (ΔN51) did not affect transactivation, but further deletion of the amino-terminal 165 amino acids (ΔN165) decreased CGβ promoter activation by 70%. The region of difference between the two deleted stretches includes the proline-rich and acidic activation domains. A further deletion that also removes the DNA binding domain (ΔN278) was inactive. Carboxyl-terminal deletion from 437 to 413 (ΔC413) had no effect, whereas deletion into the dimerization domain (ΔC390) completely eliminated AP-2 induction of the CGβ promoter. These results suggest that AP-2 induction of the CGβ promoter requires dimerization and DNA binding together with the transactivation domains, similar to studies performed with the hMTILIA AP-2 site (41).

AP-2 Gene Expression Increases during in Vitro Differentiation of Trophoblast Cells—Cytotrophoblast cells were isolated from human placenta and induced to undergo differentiation into syncytiotrophoblasts (46). RNA was extracted over the course of 12 days of differentiation, and the levels of AP-2 and of α and CGβ mRNA were analyzed by RT-PCR (Fig. 9). AP-2 mRNA levels increased 3-fold after 2 days of differentiation and continued to increase gradually during the 12-day period (8-fold increase). The α and CGβ mRNA levels also increased during the first 2 days and showed more marked stimulation between days 2 and 4 before reaching a plateau (maximal -fold increase for α was 16-fold and for CGβ was 45-fold). GAPDH and hRPL19 were used to normalize expression of the other mRNAs, and they did not change substantially during the differentiation process (data not shown). The finding that AP-2 mRNA levels increase in conjunction with the stimulation of the α and CGβ genes is consistent with a role for AP-2 in the regulation of these genes in the placenta.

DISCUSSION

The AP-2 mutant in the α-promoter did not affect cAMP stimulation (data not shown), consistent with the presence of other consensus CREs in this promoter (9).

AP-2 Regulation of hCG
Thus, Jun, AP-2, and Sp-1 have now been shown to interact with the CGβ promoter. Given evidence for combinatorial interactions among these sequences, an important question for future studies is to understand the mechanisms by which these regulatory elements interact. One possibility is that they may share transcriptional co-activators.

AP-2 has been suggested to mediate cAMP responsiveness in a variety of promoters (26–30). cAMP regulation of hCGβ was partially reduced by AP-2 mutants in FP4B (50–75% decrease). In FP2, the mutation that eliminates AP-2 binding (FP2-m1) decreased cAMP stimulation (65% decrease), whereas the mutations within the Sp-1 binding site (FP2-m2, FP2-m3) had less effect on cAMP stimulation. These findings support the idea that AP-2 plays a role in cAMP stimulation of the CGβ promoter. However, the fact that cAMP responsiveness is not eliminated by these mutations indicates that other sequences are probably involved in cAMP stimulation of the CGβ promoter (50).

AP-2 has been implicated in developmental regulation in several cell types, and it is intriguing to consider the possibility that AP-2 may participate in a developmental cascade during trophoblast differentiation. For example, the expression of keratin genes in the developing epidermis correlates with the presence of cells that express high levels of AP-2 (35). In NT-2 teratocarcinoma cells, retinoic acid induces AP-2 expression as these cells undergo differentiation (31). We found that AP-2 mRNA levels increased early in the process of trophoblast differentiation in vitro, and AP-2 protein levels are high in normal placenta. Because the CG genes are expressed very early during embryogenesis and implantation, it is of interest to determine whether AP-2 is already expressed at this time of development. Recently, the AP-2 gene was disrupted by tar-
teratocarcinoma cells, retinoic acid induces AP-2 expression as pregnancies proceeded to completion. It should be noted, potential effects on placental development were not evaluated, but one can speculate that there were no severe abnormalities as pregnancies proceeded to completion. It should be noted, however, that two additional AP-2-related genes (AP-2A, AP-2y) have recently been identified (37, 39), and it is possible that these genes may exert redundant functions in the placenta and other tissues. In addition, because the CGβ genes are not expressed in mice, it is not possible to evaluate potential effects of the null mutants on CGβ gene expression in the murine model.

In conclusion, we have shown that AP-2 expression increases as trophoblasts differentiate in an in vitro model. AP-2 appears to be a major regulator of the CGβ gene, and to a lesser degree, the α-gene. As such, it may represent one of several factors that coordinate the expression of these genes. Future studies will help to define other factors that function in conjunction with AP-2 to regulate the combinatorial elements in these genes.

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