Different Composite Regulatory Elements Direct Expression of the Human α Subunit Gene to Pituitary and Placenta∗

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To identify elements of the human α subunit gene necessary for cell-specific expression, we generated an array of block mutations spanning approximately 400 base pairs (bp) of promoter proximal region and examined them using transient transfection analysis in pituitary (αT3) and placental (BeWo) cell lines. Comparison of promoter activity in the two cell types revealed both common and unique elements required for transcription in pituitary and placenta. Two strong elements, the cyclic AMP response element (CRE) and the upstream regulatory element (URE), regulate expression of the α subunit gene in BeWo cells. In contrast, promoter activity in αT3 cells requires an array of weaker elements. These include the CREs, the URE, as well as two previously described elements, pituitary glycoprotein hormone basal element (PGBE) and gonadotrope-specific element (GSE), and two new elements we designated as the α basal elements 1 and 2 (αBE1 and αBE2). These new elements reside between −316 and −302 bp (αBE1) and −296 and −285 bp (αBE2) of the human α subunit promoter and bind distinct proteins designated αBP1 and αBP2, respectively. Southwestern blot analysis revealed that αBE1 specifically binds 54- and 56-kDa proteins. Additional studies disclosed several potential interactions between proteins that bind the CRE and proteins that occupy PGBE, αBE1, and αBE2, suggesting that gonadotrope-specific expression occurs through a unique composite regulatory element that includes components of the placenta-specific enhancer.

The glycoprotein hormones luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, and chorionic gonadotropin regulate a variety of key biological functions, including reproduction, pregnancy, and metabolism. These hormones belong to a family of heterodimeric proteins composed of a common α subunit and a specific β subunit (1). The α subunit is expressed in the pituitaries of all mammals as the product of a single-copy gene (2). In this tissue, expression occurs in two distinct cell types: gonadotropes, where it is a subunit of both follicle-stimulating hormone and luteinizing hormone, and thyrotropes, where it is a subunit of thyroid-stimulating hormone (3). In addition, the α subunit gene is expressed in the placenta of primates and horses (4, 5). Here, it is expressed in trophoblast cells as a subunit of chorionic gonadotropin in primates or as pregnant mare serum gonadotropin in horses. Because the human α subunit gene is expressed in both placenta and pituitary, examination of its promoter allows a comparative analysis of the mechanisms involved in directing expression of the same gene to two separate tissues. Such studies have been aided by the availability of both placenta and pituitary cell lines that express the α subunit gene.

Analysis of the human α subunit promoter in placental cell lines (BeWo or JEG3) has revealed the presence of a composite enhancer located within the first 180 bp of the promoter (6–13). This enhancer confers placenta-specific expression and consists of an upstream regulatory element (URE) and two juxtaposed (tandem) cyclic AMP response elements (CREs). Many earlier studies revealed a synergistic relationship between the URE and CREs in placenta, suggesting a functional interaction between the proteins that bind these elements (7, 9). Additional elements are also required for optimal expression in placenta cells and include the junctional response element (JRE) (14) and the CCAAT box (15).

The emerging model for gonadotrope-specific expression builds on the current model of placenta-specific expression. Although incomplete at this stage, transfection studies with mouse and human promoters containing deletion and clustered mutations have revealed the location of several elements important for expression in gonadotropes (16–18). These elements reside within the first 435 bp of the human promoter and 507 bp of the mouse promoter (17, 18).

In the human promoter, several elements have been identified as important for promoter activity in the pituitary gonadotrope cell line αT3. These include the gonadotrope-specific element (GSE) (18), the CREs, and the αACT element (17, 19). An additional element in the mouse α promoter, the pituitary glycoprotein hormone basal element (PGBE), has been identified as an important regulator of basal transcription in gonadotropes (17, 20). The significance of the PGBE in the human promoter is not known.

Although these studies add to our understanding of α subunit expression in gonadotropes, a more extensive analysis of the promoter is clearly needed to help identify the regions important for regulation of this gene. We therefore utilized an array of single and double block mutations spanning nucleotides −430 to −95 of the human α subunit promoter to further define the regulatory elements necessary for expression. These studies unveiled several potential interactions that collectively define a new composite regulatory element that controls pituitary-specific expression and established the presence of two new elements, αBE1 and αBE2, important for expression in gonadotropes.

The abbreviations used are: bp, base pair(s); PGBE, pituitary glycoprotein hormone basal element; GSE, gonadotrope-specific element; URE, upstream regulatory element; CRE, CAMP response element; JRE, junctional response element; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift analysis; TSE, trophoblast-specific element; αBE, α basal element.

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EXPERIMENTAL PROCEDURES

Reagents—Only the highest grade reagents were utilized. Reagents and vendors include the following: Tris-HCl, NaCl, MgCl2, glycerol, bisacrylamide, potassium acetate, sodium hydroxide, Triton X-100, and boric acid (Fisher); phenylmethylsulfonyl fluoride, EDTA, and Nonidet P 40 (Sigma); dithiothreitol, acrylamide, and SDS (Boehringer Mannheim); lipofectamine, HEPES, Dulbecco's modified Eagle's medium, horse serum, fetal bovine serum, penicillin/streptomycin, and Waymouth's media (Life Technologies, Inc.); nucleotides and poly(dI-dC) (Pharmacia Biotech Inc.); ethanol (Aaper, Shelbyville, KY); restriction enzymes and T4 polynucleotide kinase (Life Technologies, Inc. or Boehringer Mannheim); and radionucleotides were purchased from DuPont NEN.

Clone Lines—aT3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5% horse serum, penicillin, and streptomycin (Life Technologies, Inc.) (21). BeWo cells (ATCC) were grown in Waymouth's media supplemented with 15% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin.

DNA—All plasmid DNAs were prepared from overnight bacterial cultures using Qiagen DNA plasmid columns according to the supplier's protocol (Qiagen, Chatsworth, CA). Oligonucleotides were purchased from Midland Scientific (Midland, TX). Double-stranded oligonucleotides were generated by heating complementary oligonucleotides to 95°C in the presence of 10 mM Tris (8.0), 100 mM NaCl, and 1 mM EDTA and allowing them to cool slowly to room temperature. Radiolabeled oligonucleotides were generated using T4 polynucleotide kinase and T3–aP-ATP.

Clones—The human α subunit promoter was isolated from Hm(–1500/+45)CAT plasmid (12) by digestion with HindII and subcloned into the HindIII site of the luciferase reporter vector pGL2 Basic (Promega) to generate Hm(–1500)Luc. Hm(–845)Luc was generated by digesting Hm(–1500)Luc with BglII and religation. Hm(–485)Luc was generated by digestion of Hm(–1500)Luc with NsiI and Hoxl, blunting the DNA using a fill-in reaction with the Klenow fragment of DNA polymerase I, isolating DNA of the DNA fragment, and ligating of the DNA ends.

Generation of each of the mutant constructs was accomplished by bidirectional PCR using Deep Vent DNA polymerase (New England Biolabs, Beverly, MA). The reactions were performed using the buffer supplied by the manufacturer in the presence of 300 μM each dNTP, 100 pmol of each primer, 2 ng of Hm(–1500)CAT template, and 1.5 units of Deep Vent polymerase per 100-μl reaction. For each clone, two independent PCR reactions were performed: an upstream PCR reaction using a standard 5′-primer plus a 3′-mutant primer and a downstream PCR reaction using a 5′-mutant primer and a 3′-standard primer. The primer set for each mutant contained overlapping sequence within the mutation in which there was a common restriction enzyme site. The reaction mixtures were precipitated with ethanol, and the DNA was redissolved in the appropriate restriction enzyme buffer and digested with restriction enzymes compatible with both the Hm(–845)Luc cloning vector and the restriction enzyme common to both mutant primers. The products of the digestion were separated by electrophoresis on an 8–6% polyacrylamide gel in TBE and 5% glycerol. After staining, the bands of the appropriate size were excised and isolated. The new clones were generated by a triple ligation with each digested PCR product and a Hm(–845)Luc vector that was digested with either NsiI/SnaBl or SnaBl/PstI to generate Hm(–1500)Luc mutants, the upstream fragment, which extends from the BglII site at –845 to the HindIII site at approximately –1500, was subcloned into each mutant using the BglII site in the plasmid vector within the multiple cloning site. All clones were confirmed by sequencing using the Sequenase 2.0 sequencing kit from U. S. Biochemical Corp.

Double mutants were made by subcloning DNA fragments containing one mutation into a vector having the second mutation. The double mutant Hm(–1500/77/124) was made by subcloning the 600-bp SnaBl/BglII fragment of μ7 into the analogous site in Hm(–1500/μ14. Hm(–1500/77/124) contains the SnaBl-BglII fragment of μ7 cloned at the analogous position in Hm(–1500/μ14. Hm(–1500/77/124) contains the SnaBl-BglII fragment of μ7 cloned at the analogous position in Hm(–1500/μ14. Hm(–1500/77/124) contains the SnaBl-BglII fragment of μ7 cloned at the analogous position in Hm(–1500/μ14. Hm(–1500/77/124) contains the SnaBl-BglII fragment of μ7 cloned at the analogous position in Hm(–1500/μ14. The cloned was generated as described above. The mutated sequence within region 8 is altered slightly from the original μ8 mutation in that it has an 8-bp deletion. Removal of bases from region 8 is unlikely to alter the interpretation of the results, as the binding sites for the proteins in question are still fully disrupted, and there are no upstream elements that would be affected by the spacing change.

Transfection and Enzyme Analysis—aT3 cells were seeded onto 35-mm plates at a density of 170,000 cells per plate. The day after seeding, the cells were transfected using lipofectamine reagent according to the manufacturer's suggestions (Life Technologies, Inc.). Each construct (1.25 μg) was cotransfected with 0.42 μg of Rous sarcoma virus (RSV) long terminal repeat for transfection efficiency, in triplicate using 5 μl of lipofectamine. The cells were incubated with the lipofectamine/DNA solution overnight, which was then replaced with complete medium. Cells were harvested 48 h after removal of the DNA using 150 μl of lysis buffer (Promega). After removal from the plates, the lysate was centrifuged at 16,000 × g at 4°C, and the supernatant was assayed for both luciferase and β-galactosidase activities. BeWo cells were seeded onto 35-mm plates at a density of 85,000 cells per well. The day after plating, the cells were transfected and harvested as above, except 1 μl of lipofectamine was used for transfection.

Luciferase assays were performed using 10–20 μl of lysate and 100 μl of luciferase assay reagent (Promega). Emitted light was quantified as residual light units in a Berthold Lumat LB9501 luminometer (Berthold, Wildbad, Germany). The light units emitted by the human wild-type α subunit promoter were in the range of 1–3 × 106 residual light units. β-Galactosidase activity was quantified by luminometry as above using the Galacto-light assay system (Tropix, Bedford, MA). The light units emitted by β-galactosidase activity were in the range of 2–6 × 105 residual light units. The linear range of both assays was determined, and all data presented fell within those ranges. For each transfection, the luciferase/β-galactosidase activity of each construct was normalized to the luciferase/β-galactosidase activity of the wild-type promoter. The values were then averaged over a minimum of three independent experiments.

Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared as described elsewhere (22). Electrophoretic mobility shift analysis (EMSA) was performed as described previously with slight modification (23). 7 μg of αT3 nuclear extract was incubated with 25–50 fmol of radiolabeled double-stranded oligonucleotide in the presence of 10 mM MgCl2, 25 mM KCl, 0.5 mM ethidium bromide, 12.5 mM HEPES (7.9), 0.5% Triton X-100, 200 ng of Escherichia coli DNA, 200 ng of salmon sperm DNA, 10% glycerol, and 1 μg of poly(dI-dC):poly(dI-dC) in a 20-μl reaction volume. Reactions were incubated on ice 5 min prior to the addition of probe and then an additional 10 min after. Reactions were loaded on a 5% polyacrylamide gel in 0.25 × TBE and subjected to 240 volts for 3–4 h. The gels were dried and analyzed by autoradiography using Dupont's Reflections autoradiography film (DuPont NEN).

Western Blot Analysis—This analysis involved modification of a procedure described elsewhere (18). 10 μg of nuclear extract was rehydrated with 10 μl of 4× sodium dodecyl sulfate (SDS) loading buffer and 1 μl of 4× denaturing gel to a 10% resolving gel and transferred to nitrocellulose membrane (Schleicher and Schuell). Membrane-bound proteins were rehydrated in binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 0.1% Nonidet P-40) for 45 min at room temperature, and the filters were then blocked for 3 h at room temperature in binding buffer plus 5% nonfat dry milk. The membrane was then rinsed with binding buffer and hybridized overnight at 4°C in binding buffer containing 0.5% Carnation nonfat dry milk, 1 μg/ml salmon sperm DNA, and 1× 106 cpm/ml of probe. The membrane was then rinsed one time in binding buffer and washed for 30 min at room temperature in binding buffer, changing the buffer one time during the interval. The blot was analyzed by autoradiography as described above. Prestained molecular weight markers (Life Technologies, Inc.) were used to estimate the molecular weights of the proteins.

RESULTS

Unique Combinatorial Arrays of Regulatory Elements Are Important for Activity of the Human α Subunit Promoter in Placenta and Pituitary—To better define the regions of the α subunit promoter important for pituitary and placental expression, we have generated an extensive array of block mutations spanning nucleotides –430 to –95 of the human α promoter and placed each mutation within the context of the naturally occurring 1500-bp sequence located upstream of the transcrip-
Fig. 1. Sequence of the human α subunit gene promoter from −445 to −95. The sequence of the human α subunit promoter from bases −445 to −95 is given on the top line in upper case letters. The boxed regions indicate the numbers and positions of each mutation with the mutant nucleotides shown below the wild-type sequence in lower case letters. Also shown are the names of relevant response elements positioned next to the mutation number. These include PGBE (34), GSE (18), URE (7, 9, 10, 31, 35), CRE (6, 11, 12), and JRE (14).

Regulation of the α Subunit in Gonadotropes. Thus, while expression in the placenta appears to be controlled by two strong elements, expression in the pituitary is regulated by an array of weaker elements. Furthermore, these results show that distinct arrays of regulatory elements direct expression to placenta and pituitary.

A New Transcriptional Element Resides within Region 8 of the Human α Subunit Promoter—The scanning mutagenesis shown in Fig. 2B disclosed the presence of an undefined element(s) within the region disrupted by μ8 that is important for expression in αT3 cells. This element resides between bases −320 and −288 of the human promoter and is juxtaposed to the PGBE. To further define the site and to ensure that the diminished promoter activity is not the result of abrogating the binding of protein(s) to PGBE, several additional mutations were made (Fig. 3A). Transfection analysis indicated that three of the more restricted mutations within this region (μ8.1, μ8.2, and μ8.4) had considerable impact on promoter activity. A mutation that abuts μ8, μ9.1, also diminished promoter activity (Fig. 3B). These smaller mutations define a new region important for promoter activity that is distinct from the PGBE (20).

Sequence analysis of this region disclosed the presence of a potential Ets protein-binding site spanning positions −301 to −298. This family of transcription factors interacts specifically with sequences containing a common core tetranucleotide, GGAA, where both guanines are required for binding (24–29). A mutation that alters the essential guanines of this site (μ8.3) had little influence on promoter activity, suggesting that under basal conditions the transcription factor involved in activation of this promoter via this new element is not a member of the Ets family.

A Single Protein Complex Binds between Bases −316 and −302 of the α Subunit Promoter—EMSA using a radiolabeled probe spanning bases within region 8 revealed the presence of a single major shifted complex from αT3 nuclear extracts (Fig. 4A). Formation of this major protein complex, indicated on the gel as αBP1 (α binding protein 1), was inhibited by increasing concentrations of unlabeled homologous competitor DNA but not by unlabeled competitor DNAs containing the μ8 mutation sequence (Fig. 1) or a non-specific sequence. An oligonucleotide-containing sequence corresponding to the PGBE was also an ineffective competitor (Fig. 4B). Thus, αBP1 binds specifically to region 8 of the α subunit promoter and is distinct from proteins binding the PGBE.

EMSA in the presence of competitor oligonucleotides that
contain mutations within the μ8 region was used to identify the bases important for binding of αBP1 (Fig. 5). Oligonucleotides μ2 through μ5, which contained mutations between bases -313 and -302, failed to compete the αBP1 signal, indicating that bases within this region are important for formation of the αBP1 complex. In contrast, oligonucleotides containing mutations downstream of base -302 (μ6 and μ7) effectively competed for αBP1 binding, while an oligonucleotide containing
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A Second Protein Complex Binds between Bases 285 and 312 of the Human α Subunit Promoter—As shown above, activity of the α subunit promoter was used in an electrophoretic mobility shift assay with αT3 nuclear extracts. Unlabeled competitors were added to the indicated assays and include homologous competitor (wt2) added in increments of 50×, 100×, and 200×, PGBE (200×), and nonspecific competitor (NS, 200×).

Mutations in Multiple Elements Suggests Transcription Factors Binding to Region 8 of the Human α Subunit Promoter. A, radiolabeled probe corresponding to bases –306 to –277 bp (wt2, 5′-TTATAGAAAGTGCAGCTTCAGAGAGTT-3′) of the human α subunit promoter was used in an electrophoretic mobility shift assay with αT3 nuclear extracts. Unlabeled competitors were added to the indicated assays and include homologous competitor (wt2) added in increments of 50×, 100×, and 200×, PGBE (200×), and nonspecific competitor (NS, 200×).

FIG. 4. Electrophoretic mobility shift analysis of proteins binding to region 8 of the human α subunit promoter. A, radiolabeled probe corresponding to bases within region 8 (wt, 5′-TGTCTCT-TGTATAGAAAGTGCAGCT-3′ of the human α subunit promoter was used in an electrophoretic mobility shift assay with αT3 nuclear extracts. Unlabeled competitors were added to the indicated assays and include wild type (wt), homologous competitor added in increments of 50×, 100×, 200×, 500×, 1000×, μ8 (oligonucleotide containing the sequence which to region 8 was mutated as shown in Fig. 1, 1000×), and nonspecific competitor (NS, 1000×). B, electrophoretic mobility shift assay using the radiolabeled wild-type oligonucleotide as probe and αT3 nuclear extracts. Unlabeled competitors were added to the indicated assays and include wild type (200×), PGBE (5′-ATATCAGGTACT-TAGCTAATTAAATGTCT-3′, 200×), and nonspecific competitor (NS, 200×).

FIG. 5. Electrophoretic mobility shift analysis of αBP1 using wild-type and mutant competitors. A, sequence of oligonucleotides used in electrophoretic mobility shift analysis. The box shows the wild-type promoter sequence from −319 to −277. The boxed area indicates the sequence of the oligonucleotide radiolabeled and used as a probe (wt). The base mutations are shown for each oligonucleotide. A dash indicates no change from wild type. B, a radiolabeled probe corresponding to bases −316 to −288 (wt) was used in an electrophoretic mobility shift assay with αT3 nuclear extracts. Competitors were added at a 100-fold molar excess. NS corresponds to an oligonucleotide having a nonspecific sequence.

Mutations upstream of −312, μ1, was capable of only partial competition. This indicates that the bases important for αBP1 binding lie between −316 and −302, defining the α basal element one (αBE1).

A Second Protein Complex Binds between Bases −296 and −285 of the α Subunit Promoter—As indicated above, αBP1 binding is not affected by mutations downstream of base −302. However, transfection analysis indicated that bases within this downstream region are important for promoter activity (μ8.4 and μ9.1, Fig. 3). This suggested that a second element resides within this more downstream region and binds a transcription factor distinct from αBP1. EMSA, using a radiolabeled oligonucleotide spanning through the 3′-portion of region 8 into region 9 (wt2), revealed the binding of a second protein complex, α binding protein 2 (αBP2, Fig. 6). Binding of αBP2 is competed by increasing concentrations of unlabeled wild-type competitor but not by either an oligonucleotide corresponding to the PGBE or a nonspecific oligonucleotide. Thus, αBP2 binds specifically to promoter sequences within this downstream oligonucleotide and does not correspond to proteins binding the PGBE.

EMSA in the presence of unlabeled competitor oligonucleotides containing mutations that span bases −306 to −285 further defined the region of αBP2 binding (Fig. 7). Mutations within μ3, which disrupted the binding of αBP1, had little or no effect on the binding of αBP2, as indicated by the ability of the μ3 oligonucleotide to compete effectively. However, mutations in μ7, which had no effect on the binding of αBP1, interfered with the ability of the αBP2 complex to bind DNA. Thus, the bases required for αBP2 binding are clearly distinct from those of αBP1. This conclusion is also supported by the observation that the wild-type oligonucleotide used as the probe to reveal αBP1 binding is not effective as a competitor for αBP2 binding, and conversely the oligonucleotide used to reveal αBP2 binding (wt2) does not compete for the binding of αBP1 (Fig. 5). Thus, a second element, distinct from αBE1, resides between bases −296 and −285 and defines the α basal element two, αBE2.

αBE1 Binds Both 54- and 56-kDa Proteins—Southwestern blot analysis using a radiolabeled probe corresponding to the wild-type oligonucleotide (see Fig. 5) unveiled the binding of two proteins having an approximate molecular mass of 54 and 56 kDa (Fig. 8). Both proteins bound specifically to this probe as demonstrated by the ability of unlabeled homologous competitor (wt), but not a competitor containing the mutation sequence through region 8 (μ8, Fig. 1), to diminish binding to the probe. Furthermore, oligonucleotides μ2 and μ3 did not compete for binding to these proteins, while μ7 did compete (see Fig. 5 for sequence of μ2, μ3, and μ7). This binding profile mimics that of the αBP1 complex observed by EMSA (Figs. 4 and 5), indicating that the 54- and 56-kDa proteins are components of the αBP1 complex.

Mutations in Multiple Elements Suggests Transcription Factors Binding the PGBE, the αBE, and the CRE Influence the Activities of Each Other—As shown above, activity of the α
subunit promoter in αT3 cells is not controlled by a single dominant element but by an array of weaker elements. This suggested that interactions between regulatory elements and their cognate transcription factors may play a key role in dictating cell-specific expression. To address this, a number of promoters with block mutations through two basal elements were evaluated for their effects on activity (Fig. 9).

Assessment of promoters with paired mutations in PGBE and GSE (μ7/μ11), αBE(1+2) and GSE (μ8/μ11), and GSE and CRE (μ11/μ14) indicated that mutations through the GSE had the same relative impact on promoter activity in the presence or absence of PGBE, αBE(1+2), or CRE. Thus, a mutation through the GSE decreased promoter activity by approximately 2.5-fold when mutated in the intact promoter or promoters with an additional mutation in PGBE, αBE(1+2), or CRE. In contrast, a paired mutation through PGBE/αBE (μ7/8) had no further impact on promoter activity than either of the individual mutations, suggesting that both sites must be present for either to be functional. Interestingly, paired mutations in PGBE/CRE (μ7/μ14) or αBE/CRE (μ8/μ14) resulted in a greater loss in promoter activity than the independent contributions of the individual elements. For example, in the intact promoter, a mutation through the CREs decreased promoter activity by approximately 6-fold, while in the absence of either PGBE or αBE(1+2), a second mutation through the CRE decreased promoter activity by an additional 50-fold. Moreover, in the intact promoter, a mutation through PGBE or αBE(1+2) decreased promoter activity by approximately 3-fold, while in the absence of the CRE an additional mutation in either of these elements decreased promoter activity by approximately 20-fold. Thus, mutations in multiple elements revealed that the transcription factors binding the PGBE, the αBE, and the CRE influence the transactivation abilities of each other, while the activity of the transcription factor binding the GSE does not appear to be influenced by the presence of the activators binding the other three elements.

FIG. 7. Electrophoretic mobility shift analysis of αBP2 using wild-type and mutant competitors. A, sequence of oligonucleotides used in electrophoretic mobility shift analysis. The top shows the wild-type promoter sequence between –306 to –277 and indicates the sequence of the oligonucleotide radiolabeled and used as a probe (wt2). The base mutations are shown for each oligonucleotide. A dash indicates no change from wild type. Note that oligonucleotide μ8 is not the same as the previous μ8 which has the entire sequence of the region 8 mutated. B, a radiolabeled probe corresponding to bases –306 to –277 (wt2) was used in an electrophoretic mobility shift assay with αT3 nuclear extracts. Competitors were added at a 100-fold molar excess. wt corresponds to an oligonucleotide with the sequence given in Fig. 5. NS corresponds to an oligonucleotide having a nonspecific sequence.

FIG. 8. Southwestern blot analysis of αBE1 binding proteins. 40 μg of αT3 nuclear extracts were resolved on an SDS-polyacrylamide gel with a 4% stacking gel and a 10% resolving gel and transferred to nitrocellulose membrane. Membrane-bound proteins were renatured, blocked, and probed with a radiolabeled probe corresponding to αBE1 (–316 to –288). Competitors, when included, were at a 200-fold molar excess to that of the probe. Unlabeled competitors include wild type (homologous), μ8 (corresponding mutation through region 8 as indicated in Fig. 1), and μ2, μ3, and μ7 (sequences of μ2, μ3, and μ7 are given in Fig. 5). The results shown from Southwestern blots in the presence of oligonucleotides μ2, μ3, and μ7 were from a different experiment than those done in the absence of competitor, wild-type competitor, or μ8. In this case, the control blot in the absence of added competitor and presence of wild-type competitor was nearly identical to the one shown here and therefore was not included. Migrations of molecular weight markers are indicated on the right.

A key objective of this study was to address how the α subunit gene achieves its highly restrictive pattern of expression. Our comparative analysis in BeWo and αT3 cells has revealed a number of differences as well as similarities between the regulatory codes that direct expression to placenta and pituitary. While placenta expression is predominantly controlled by two very strong elements, the URE and the CREs, pituitary expression is regulated by an array of weaker elements. This suggests that no single element is absolutely essential for α subunit gene expression in pituitary. Instead, mutational analysis of multiple elements indicated that there are a number of key interactions that are important for gonadotrope-specific expression. It is clear from our studies and others that the CREs of the human α subunit promoter play an important role in the expression of this gene in both placenta and pituitary (6, 17, 19, 30). In placental cells, the CREs bind homodimers of the ubiquitous protein CREB. We have preliminary data indicating that CREB binds the CREs in αT3 cells as well (data not shown). The CRE also plays a role in both placenta and pituitary expression of the human α subunit gene. In placenta, the CRE is actually a composite of several elements; the αACT, the trophoblast-specific element (TSE), and the URE1 (31). Here, the αACT element binds to hGATA-2 and hGATA-3 (19), while the TSE/URE1 forms an overlapping element that may bind two functionally interchangeable proteins, TSEB and UREB(31).

In αT3 cells, mutation of the URE had a considerably less dramatic effect on promoter activity than in BeWo cells, suggesting that it is less critical for pituitary expression than for placenta. Steger et al. (19) have shown that the activity associated with the URE in αT3 cells is due at least in part to the αACT element. This element both binds to and is activated by the transcription factor hGATA-2.

Gonadotrope-specific expression of the α subunit gene requires four additional elements that are not needed for expres-
The GSE was described previously for its role in regulating the human promoter and binds the orphan nuclear receptor steroidogenic factor-1 (SF-1) (18, 32, 33). The PGBE has only been described for its role in regulating activity of the mouse promoter, and one of the proteins that bind this element has been identified as the Lim-homeodomain protein, LH2 (17, 20, 34). Our studies show that PGBE is involved in the regulation of the human promoter as well. More importantly, we have shown through transfection analysis and binding studies the presence of two new elements, aBE1 and aBE2. These elements bind two different transcription factors, aBP1 and aBP2, respectively, both of which contribute to the function of the a subunit promoter. The binding of multiple transcription factors to this portion of the promoter is consistent with an earlier report indicating that these regions as well as PGBE are protected from digestion by DNase I (18).

Southwestern blot analysis revealed that aBE1 binds two proteins of similar molecular weights, 54,000 and 56,000. The binding profile of these proteins mimics that of the aBP1 complex observed by EMSA (Figs. 5 and 8), indicating that the 54- and 56-kDa proteins are components of the aBP1 complex. The presence of two proteins by Southwestern blot analysis as opposed to the single aBP1 band observed by EMSA suggests that the 54- and 56-kDa proteins may comigrate as a single band on an EMSA gel or that aBP1 is a higher order complex consisting of both proteins. The relationship of the two proteins is not yet known, but it is possible that they are different forms of the same protein. Attempts to reveal aBP2 by Southwestern blot analysis were unsuccessful, suggesting that this protein may be a heterodimer incapable of binding aBE2 when resolved on a denaturing gel. It may also be that the conditions for Southwestern blot analysis are too harsh to reconstitute aBP2 binding activity.

Mutational analysis of multiple elements was used to identify potential interactions between regulatory elements and their cognate transcription factors that play a role in cell-specific expression. This analysis disclosed three types of interactions: independent, dependent, and compensatory (Fig. 9). The studies indicated that the GSE activates the promoter in a manner that is independent from PGBE, aBE1 (17), or CRE. That is, when a mutation is present in PGBE (µ7), aBE1 (17) (µ8), or the CREs (µ14), a second mutation in the GSE (µ11) has the same relative effect on promoter activity as it does when the rest of the promoter is intact. This implies that SF-1, the GSE binding protein, can activate transcription to the same degree in the presence or absence of the proteins binding the PGBE, the aBE1 (17), or the CREs.

This contrasts to what was observed when mutations were made in both PGBE (µ7) and aBE1 (17) (µ8). In this case, no further impact on promoter activity was observed with the double mutation (µ7/µ8) when compared to either mutation alone. This suggests that both sites must be present (and occupied) to get promoter activation, revealing a dependent or synergistic relationship between the proteins that bind these elements.

A third type of interaction was implicated by the results of the double mutations through the PGBE/CRE (µ7/µ14) and the aBE1/CRE (µ8/µ14). In the absence of either PGBE (µ7) or aBE1 (17) (µ8), a mutation in the CRE (µ14) had a much more dramatic effect on promoter activity than in the presence of these elements. The reverse is also true. That is, in the absence of the CRE, a mutation in either PGBE or aBE1 had a much more dramatic effect on promoter activity than in the presence of these elements.
of the CRE. This effect suggests that PGBE and \( \alpha \)BE become more important in the absence of a functional CRE and vice versa. It appears that the PGBE/\( \alpha \)BE-binding proteins can partially compensate for the loss of the CRE-binding proteins and that the CRE-binding protein(s) can partially compensate for the loss of the PGBE and \( \alpha \)BE-binding proteins. The fact that the binding of one protein can make up for the loss of the other suggests that the activation pathways of these proteins converge at some point to stimulate transcription and suggests the possibility that the proteins binding these elements share a common coactivator or member of the general transcription machinery.

A different way of interpreting these results is that proteins binding the CREs partially interfere with the ability of the proteins binding PGBE and PGBE (1+2) to activate transcription and vice versa. Thus, in the absence of one protein the other is a much stronger activator. Again, this suggests that proteins binding these elements interact with each other either directly or indirectly.

The double replacement mutagenesis strategy has disclosed several potential interactions between proteins that bind the CRE, PGBE, \( \alpha \)BE1, and \( \alpha \)BE2. This suggests that gonadotropespecific expression occurs through a unique composite element that, like the placenta-specific enhancer, includes the CRE. This provides incentive for future studies to identify the biochemical nature of these interactions.

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