GATA-Binding Proteins Regulate the Human Gonadotropin α -Subunit Gene in the Placenta and Pituitary Gland

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The human glycoprotein hormone α -subunit gene is expressed in two quite dissimilar tissues, the placenta and anterior pituitary. Tissue-specific expression is determined by combinations of elements, some of which are common and others of which are specific to each tissue. In the placenta, a composite enhancer confers specific expression. It contains four protein-binding sites: two cyclic AMP (cAMP) response elements that bind CREB, a trophoblast-specific element that binds TSEB, and a sequence motif, AGATAA, that matches the consensus binding site for a family of transcription factors termed the GATA-binding proteins. In pituitary gonadotropes, the cAMP response elements remain important for expression, TSEB is absent, and elements further upstream participate in tissue-specific expression. Here we establish a regulatory role for the GATA element in both the placenta and pituitary by demonstrating that a mutation of this element decreases α -subunit gene expression 15-fold in JEG-3 human placental cells and 2.5-fold in α T3-1 mouse pituitary gonadotropes. In JEG-3 cells, human GATA-2 (hGATA-2) and hGATA-3 are highly expressed and both proteins bind to the α -subunit gene GATA element. In α T3-1 cells, the GATA motif is bound by mouse GATA-2 (mGATA-2) and an mGATA-4-related protein. Cotransfection of hGATA-2 or hGATA-3 into α T3-1 cells activates the α -subunit gene threefold. These studies establish a role for the GATA-binding proteins in placental and pituitary α -subunit gene expression, significantly expanding the known target genes of GATA-2, GATA-3, and perhaps GATA-4.

The profile of genes distinguishing an individual differentiated cell type is determined by specific transcriptional regulatory proteins expressed in highly restricted patterns (38, 43). First identified as controlling erythroid cell-specific transcription of the chicken β -globin gene cluster, members of the family of transcriptional regulators called the GATA-binding proteins (45) bind to a GATA core motif within the broader consensus sequence WGATAR (W indicates T or A, and R indicates G or A). This sequence is present in the regulatory regions of many genes, including most erythroid cell-specific genes (17). Members of the GATA-binding protein family contain two copies of a unique, highly conserved zinc finger domain of the form C-X-N-C-X₁₇-C-N-X-C (39), which is involved in DNA binding.

Presently, the family of vertebrate GATA-binding proteins is composed of four proteins designated GATA-1 (16, 59), GATA-2 (64), GATA-3 (64), and GATA-4 (3, 31), each of which has a distinct tissue distribution and developmental profile. GATA-1 is expressed only in cells of hematopoietic lineages, specifically erythroid cells, mast cells, and megakaryocytes, and it activates many, if not all, erythroid cell-specific genes (17, 40, 60). GATA-1 plays an obligate role in erythroid cell differentiation since embryonic stem cells harboring a mutant GATA-1 gene fail to contribute to erythroid lineage in chimeric mice (47). GATA-2 is expressed in many different tissues and cell types, including erythroid, fibroblast, and endothelial cells (14, 36, 64). This protein activates an endothelial cell-specific gene, the preproendothelin-1 gene (62). In chickens, GATA-3 expression is restricted to adult reticulocytes, T lymphocytes, the embryonic brain, and the adult kidney (64). Human GATA-3 regulates the T-cell receptor

genes in T lymphocytes and has been proposed to play a role in regulating the development of the T-cell lineage (23, 30, 34). Lastly, two distinct factors, both termed GATA-4 (one from mice and the other from *Xenopus laevis*), are expressed in a restricted set of tissues including the heart, intestinal epithelium, primitive endoderm, and gonads (3, 31).

Our initial interest in the GATA-binding family of proteins stemmed from studies directed at determining the mechanisms controlling tissue-specific expression of the glycoprotein hormone α -subunit gene in different species (54). The α -subunit gene of the glycoprotein hormones is a single-copy gene expressed in the pituitary of all mammals as a component of luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone (19, 21), but it is known to be expressed only in the placenta of primate and equine species (7, 20). Humans and horses express this gene in the placenta as a component of human chorionic gonadotropin and pregnant mare serum gonadotropin, respectively. Within the placenta, expression of the α -subunit gene is specifically directed to trophoblast cells, while in the pituitary, expression is specifically directed to gonadotropes and thyrotropes.

The molecular basis for placenta-specific expression of the human and equine α -subunit genes has been studied by transfection of human placental trophoblast cell lines that express human chorionic gonadotropin (10, 54). The human gene contains a composite enhancer, positioned within 180 bp of the mRNA start site, that confers placental specificity. At least two distinct *cis*-acting regulatory sequences compose the enhancer, a cyclic AMP (cAMP) response element (CRE) and a trophoblast-specific element (TSE; also called the upstream regulatory element [URE]) (11, 28, 29). Present as an exact 18-bp direct repeat (12, 53), the two copies of the CRE each bind the ubiquitous protein CREB (22, 24). The TSE is located 30 bp upstream of the CREs and binds TSEB, a protein specific to the placenta (11, 55). In contrast, the equine α -subunit gene contains neither the TSE nor the CRE (18, 54).

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Instead, placental specificity is controlled by two GATA elements that bind a factor we originally designated α -ACT (54). The upstream (AGATAA) and downstream (TGATAA) equine α -ACT binding sites display exact homology to the GATA consensus sequence, indicating that α -ACT may be a member of the GATA-binding family of proteins. The equine upstream GATA element is perfectly conserved in the human α -subunit gene and positioned within the placenta-specific enhancer between the TSE and CREs. Thus, in addition to controlling equine α -subunit gene expression, α -ACT is likely to regulate transcription of the human gene as well.

Control of cell-specific expression of the α -subunit gene in the pituitary differs from that in the placenta (5, 25). As in placental trophoblasts (JEG-3 cells), regulation of human α -subunit gene expression in α T3-1 pituitary gonadotrope cells involves the CREs (25, 51, 63); however, no TSE binding activity can be detected in gonadotrope or thyrotrope cell lines (25). In contrast, studies of pituitary cells implicate several regions upstream of the TSE (49, 63). The best-characterized element, called the gonadotrope-specific element (GSE), is located in the human gene from -223 to -197 and conserved in all mammalian α -subunit genes known thus far (25). The protein binding this element was found in α T3-1 cells (25) but not in thyrotrope cells, and the regions shown to confer thyrotrope-specific expression to the mouse α -subunit gene (44, 49) do not contain homology to the GSE. Recently, Barnhart and Mellon (4) demonstrated that the GSE-binding protein is the nuclear orphan receptor steroidogenic factor-1 (SF-1) (26; for review, see reference 46). Regions further upstream also play roles in pituitary gene expression. Schoderbek et al. (51) have shown that a mutation of the sequence from -337 to -330 of the mouse gene reduces expression in both α T3-1 and α TSH cells (1), but this region appears to have little effect in α T3-1 cells when it is deleted from the human gene (25) or in TtT-97 thyrotrope tumor cells when it is deleted from the mouse gene (44). Other regions of the mouse gene have also been implicated in activity in thyrotropes (-480 to -417, -254 to -177, and -177 to -120) (44) and gonadotropes (-445 to -438) (51).

A regulatory role for the human α -subunit gene GATA element was investigated by introducing point mutations into this element, as well as into the binding sites for CREB and TSEB, and analyzing the effect of each mutation on α -subunit gene expression by transfection into the placental cell line, JEG-3, and the pituitary gonadotrope cell line, α T3-1 (63). We found that a mutation of the α -subunit gene GATA element decreases transcriptional activity 15-fold in placental cells and 2.5-fold in pituitary gonadotrope cells. Thus, maximal activity of the placenta-specific enhancer requires not only a functional CRE and TSE but the GATA element as well. Furthermore, in JEG-3 cells, the α -subunit gene GATA element is bound by both human GATA-2 (hGATA-2) and hGATA-3, while in pituitary gonadotropes, it is bound by mouse GATA-2 (mGATA-2) and an mGATA-4-related protein, revealing that the GATA-binding family of transcription factors is involved in specific expression of the gonadotropin α -subunit gene in the placenta and pituitary.

MATERIALS AND METHODS

Plasmid constructions. The α -subunit gene enhancer mutants were constructed from the plasmid p α 224ACAT. This vector contains the α -subunit gene sequence from -224 to +60 cloned into the *Xba*I and *Xho*I sites of pTKCAT (54) so that the thymidine kinase promoter sequence is replaced with the α -subunit gene enhancer/promoter. p α 224ACAT contains

only one copy of the two 18-bp CRE repeats and was constructed by digesting p α 224CAT (11) with *Aat*II, which cuts at -136 and -118 in the human α -subunit gene to produce an 18-bp deletion. To introduce point mutations into the placenta-specific enhancer, a region spanning from -180 to -128 in the α -subunit gene (with one CRE removed), unique restriction enzyme sites flanking this domain were required. In p α 224ACAT, an *Xba*I site is present at -224. To create a unique cloning site downstream of -128, the base at position -102 was mutated from an A to a G to produce a *Kpn*I site in the α -subunit gene (from position -102 to -97: 5' GGATCC 3') overlapping a natural *Rsa*I site (GATC) at -101. Primers complementary to specific regions of the α -subunit gene were synthesized for use in PCR. The sense primer encoded the α gene sequence from -224 to -188 (with an *Xba*I linker directly 5' of -224). The antisense primer encoded the α sequence from -97 to -150, with the T to C substitution at -102 and the 18-bp deletion from -118 to -136 removing one CRE. Additionally, the 5' end of the antisense primer contained several GC residues so that the *Kpn*I site was not located at the very end of the PCR primer. Amplification of p α 224ACAT DNA with these primers produced a fragment that was subsequently digested with *Xba*I and *Rsa*I and then gel isolated. The PCR product was ligated to p α 224ACAT, which had been partially digested with *Rsa*I and completely digested with *Xba*I, to produce a cloning vector that had *Xba*I and *Rsa*I ends without the α -subunit gene sequence from -224 to -100. The product of this ligation, p α 224A(K)CAT, is a vector identical to p α 224ACAT except for the A to G substitution at -102 that generates the *Kpn*I site. Transfection of p α 224A(K)CAT and p α 224ACAT into JEG-3 cells revealed no difference between these two vectors for transcribing the chloramphenicol acetyltransferase (CAT) gene (data not shown). To produce α -subunit enhancer point mutations, four overlapping oligonucleotides that span the α -subunit gene from -224 to -97 were designed. Oligonucleotide 1 is identical to the sense primer used in the PCR described above. Oligonucleotide 2 encodes the antisense strand of the α -subunit gene from -196 to -155 and, therefore, contains the TSE. Oligonucleotide 3, which contains the GATA motif, encodes the sense strand of the α promoter from -165 to -140. Oligonucleotide 4 is identical to the antisense primer used in the PCR described above, and it contains the CRE. A second set of oligonucleotides, 2m, 3m, and 4m, are identical to oligonucleotides 2, 3, and 4 except that they contain the base substitutions in the TSE, GATA element, and CRE that are shown in Fig. 1A. Oligonucleotide 1 was annealed with different combinations of oligonucleotides 2 or 2m, 3 or 3m, and 4 or 4m to generate a partially double-stranded molecule reconstituting the α -subunit sequence from -224 to -97. 5' overhangs and gaps were filled in with T7 DNA polymerase (Sequenase), and nicks were sealed with T4 ligase. The resulting DNA fragments were digested with *Xba*I and *Kpn*I, isolated on an acrylamide gel, and ligated to p α 224A(K)CAT, which had been digested with *Xba*I and *Kpn*I. The products of these ligation reactions constituted the α -subunit gene enhancer mutants. Dideoxy DNA sequencing (48) confirmed the identity of each mutant.

For cotransfection experiments, hGATA-2 and hGATA-3 protein coding sequences were cloned into the cytomegalovirus (CMV) expression vectors pEV3S and pEVRF2 (41). A *Syl*I fragment from hGATA-2 cDNA (14), which retains the entire amino acid sequence of GATA-2 when the 5' overhangs are filled in with Klenow fragment except for the C-terminal glycine residue, was placed into pEV3S digested with *Kpn*I and treated with T4 DNA polymerase to remove the 3' overhang,

FIG. 1. Analysis of the transcriptional activity conferred by individual elements of the α -subunit gene enhancer in placental cells. (A) Point mutations introduced into the human α -subunit gene enhancer. The DNA sequence of the placenta-specific enhancer of the human α -subunit gene is presented with the CRE, TSE, and GATA core motifs shown in boldface. The 18-bp duplicated CRE directly downstream of -129 is not shown since it has been specifically removed from all reporter genes used for these studies. The single-base mutations introduced into the human α -subunit gene are shown above the DNA sequence and incorporate the equine and murine base substitutions within the core regulatory elements as well as several additional substitutions to further disrupt the DNA sequence. (B) DNase I protection analysis of protein binding to the CRE, TSE, and GATA sites within the α -subunit gene enhancer. ^{32} P-end-labeled α -subunit gene fragments containing the mutations described in panel A were incubated with 15 and 0 μ g of JEG-3 nuclear extract, partially digested with DNase I, and separated on a 6% sequencing gel. Point mutations within the CRE (C), TSE (T), and GATA element (G) for probe DNAs are indicated for each binding reaction. Protected regions are indicated by rectangular boxes. (C) Transfection of α -subunit genes with wild-type and mutant enhancers into JEG-3 cells. CAT expression plasmids were constructed containing the human α -subunit gene from -224 to +60 (with one of the two 18-bp CRE repeats deleted) with either wild-type or mutant CREs, TSEs, and GATA elements. The transcriptional activity of each mutated regulatory element along with different combinations of mutated elements was compared with the activity of the intact α -subunit enhancer by transfection into the placental trophoblast cell line JEG-3. The presence of a wild-type CRE, TSE, and GATA element in each transfected plasmid is indicated by a triangle, oval, and diamond, respectively. CAT activity values are corrected for levels of β -galactosidase activity from an internal control β -galactosidase expression vector and normalized to the activity of the wild-type α -subunit gene promoter. Values are the means of three independent transfection experiments, with error bars representing the standard errors of the means.

digested with *Bam*HI, and then digested with Klenow fragment to fill in the 5' overhang. The hGATA-3 protein coding sequence was produced by amplifying JEG-3 first-strand cDNA by using PCR with primers targeted to the N- and C-terminal ends of hGATA-3. Primers contained *Xba*I sites on their 5' ends to facilitate cloning into the *Xba*I site of pEVFR2 and incorporated single amino acid substitutions at the N-terminal (leucine substituted for methionine) and C-terminal (glycine replaced with valine) ends. The entire hGATA-3 fragment generated by PCR was sequenced to confirm that no

mutations were created by *Taq* DNA polymerase during amplification.

Cell culture, transfections, and CAT assays. JEG-3 cells were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, 5% equine serum, 4.5 mg of glucose per ml, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). α T3-1 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 4.5 mg of glucose per ml, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Transfections were performed with calcium phos-

phate precipitates (42) containing 10 μ g of plasmid DNA and 2.5 μ g of the internal control plasmid pUC-Lac (9), incubated for 5 h, and rinsed before the media were changed. Cells were harvested after 48 h. CAT assays were performed as previously described (52), and β -galactosidase assays were performed as described elsewhere (11). Units of CAT activity were normalized to the β -galactosidase activity of the internal control.

Oligonucleotides. For DNA binding studies, oligonucleotides were purified on acrylamide gels before use. Double-stranded DNA oligonucleotides corresponding to the following regions in the human α -subunit gene were used: TSE, from -180 to -157 bp, and GATA, from -162 to -142 bp. Both oligonucleotides contain 5' GATC 3' at their 5' ends.

Isolation and characterization of hGATA-3 cDNAs. Approximately 5 ng of JEG-3 poly(A)⁺ RNA and primers homologous to regions within the highly conserved zinc finger domain of the GATA-binding proteins were used in PCR to amplify putative GATA-binding factors in JEG-3 cells. The sense primer encodes the amino acids ECVNCGA, positioned within the amino-terminal zinc finger of hGATA-2 and hGATA-3, and it is a 20-mer with full degeneracy (256-fold) to represent all possible codon uses. The antisense primer encodes HN(V/I)NRPL, an amino acid sequence carboxy terminal to the second zinc finger domain in hGATA-2 and hGATA-3, and it is also a fully degenerate 20-mer (4,096-fold degeneracy). Following PCR, the expected 278-bp fragment was gel isolated and blunt-end cloned into the *EcoRV* site of Bluescript KS(+) (Stratagene). Dideoxy DNA sequencing (48) revealed that the PCR product is identical to hGATA-3. A λ -Zap (Stratagene) cDNA library prepared from JEG-3 poly(A)⁺ RNA was screened under high-stringency hybridization to the hGATA-3 zinc finger domain. Hybridizations were carried out for 24 h at 65°C in 1 M NaCl-50 mM Tris (pH 7.5)-1% sodium dodecyl sulfate (SDS)-1 $\times$ Denhardt's solution-100 μ g of denatured salmon sperm DNA per ml plus 10⁶ cpm of ³²P-labeled probe prepared by random hexanucleotide priming (specific activity, >10⁸ cpm/ μ g) per ml. Filters were washed twice with 1 $\times$ SSPE (0.18 M NaCl, 0.01 M NaH₂PO₄, 0.001 M EDTA)-0.1% SDS for 20 min at 65°C. Approximately 3.5 $\times$ 10⁵ PFU were screened, and eight positives were identified. Following restriction analysis and dideoxy DNA sequencing, only four unique clones remained, all encoding hGATA-3.

RNA extraction and Northern (RNA) analysis. Total RNA was prepared from tumor cell lines by the acid guanidinium thiocyanate-phenol-chloroform method (8). Poly(A)⁺ RNA was selected by oligo(dT)-cellulose (Stratagene Cloning Systems). RNAs were harvested approximately 48 h after the plating of cells. Northern blotting was performed as described by Maniatis et al. (37). Blots were hybridized with ³²P-labeled DNA fragments from hGATA-3, ~500 bp of sequence downstream of the *Pst*I site positioned in the 3' untranslated region; hGATA-2 (14), an ~800-bp *Eco*RI-*Sac*II fragment encoding the 5' end of the cDNA; mGATA-1 (59), an ~500-bp *Pvu*II-*Eco*RI fragment positioned C terminal to the Zn finger region; mGATA-2, ~200 bp of sequence C terminal of the *Bam*HI site positioned C terminal to the Zn finger domain; mGATA-3 (34), an ~800-bp *Eco*RI-*Pvu*II fragment encoding the 5' end of the cDNA; mGATA-4 (3); human *fos*; and human glyceraldehyde-3-phosphate dehydrogenase (15).

Nuclear extract preparation and DNase I protection analysis. Nuclear extracts were prepared according to the method of Dignam et al. (13), as described by Delegeane et al. (11), with the modification that ammonium sulfate precipitation was performed with 25 to 28% ammonium sulfate. DNA probes for the footprinting experiment were prepared from CAT expres-

sion plasmids used in the transient transfection experiments. End-labeled antisense fragments were generated by cutting with *Xba*I, filling in with the Klenow fragment of DNA polymerase I and [α -³²P]dATP (3,000 Ci/mmol), redigesting with *Xho*I, and lastly gel isolating the ~300-bp fragments. DNase I protection was performed with 5,000 to 10,000 cpm (i.e., ~1 to 2 fmol) of probe per binding reaction as previously described (54).

Gel retardation assays. The gel retardation assay was slightly modified from that of Sawadogo et al. (50). Binding reactions were performed in a 20- μ l mixture containing 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 5 mM spermidine, 1 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10% (vol/vol) glycerol, 0.25 μ g of bovine serum albumin per μ l, and 0.1 μ g of poly(dI-dC)·poly(dI-dC) per μ l. GATA DNA probe was prepared by end labeling the 25-bp double-stranded oligonucleotide encoding the α -subunit gene GATA site with T4 polynucleotide kinase and [γ -³²P]dATP (6,000 Ci/mmol). All binding reactions were carried out at room temperature, and each reaction mixture was preincubated 5 to 10 min before the addition of the probe (10,000 cpm, 1 to 2 fmol). To resolve complexes, the reaction mixtures were applied to 5% native acrylamide gels (30:1 ratio of acrylamide to bisacrylamide) in 0.25 $\times$ Tris-borate-EDTA-5% (vol/vol) glycerol.

RESULTS

The GATA motif present in the human α -subunit gene enhancer confers enhanced transcriptional activity in placental cells. To firmly establish a role for the GATA element in expression of the human α -subunit gene, single-base substitutions were introduced into the placenta-specific enhancer to specifically prevent the binding of CREB, TSEB, and α -ACT proteins. We designed the point mutations on the basis of the findings that none of these proteins bind to the analogous region of the murine α -subunit gene and that neither CREB nor TSEB binds to the equine gene (54) (Fig. 1A). Thus, comparison of the CRE, TSE, and GATA sequences in the human α -subunit gene to homologous regions in the murine and equine genes provides critical sequence information concerning the binding requirements for CREB, TSEB, and α -ACT. The murine and equine genes both have an identical, single-base substitution (i.e., a thymine substituted for a cytosine) in the fourth position of the core CRE sequence (TGATGTCA) that abolishes CREB binding. Similarly, the TSE core sequence, CCNNNGGG, determined by methylation interference studies with the human α -subunit TSE (27, 55), contains distinct and overlapping point mutations in the murine (TCTGAGGG) and equine (TCTAAGAG) genes that abolish TSEB binding. Lastly, the binding of α -ACT to the murine gene is prevented by a single-base substitution in the GATA analogous region of this gene (TGATAT) that disrupts the WGATAR consensus motif.

Though the binding sites are very closely spaced, all three proteins bind simultaneously to the α -subunit gene in vitro (54). To demonstrate that these mutations eliminate binding to individual elements without affecting binding of the adjacent proteins, we used α promoter DNA fragments containing these mutations as probes in DNase I protection studies with nuclear protein extract from JEG-3 cells, a human placental trophoblast cell line that produces both the α - and β -subunits of CG (35). Incubation of 15 μ g of extract with DNA probes containing individual mutations, followed by partial digestion with DNase I, revealed that each of the three mutations effectively eliminates binding (Fig. 1B). Furthermore, the removal of

individual factors from the DNA by mutation of each recognition site did not decrease or increase the intensity of the footprints produced by the remaining factors, indicating that these proteins can access their sites whether or not the neighboring sites are bound. Analysis of double mutations which contain only one functional binding site within the enhancer demonstrates that these three elements are bound independently by their respective proteins.

We assessed the effects of mutating the GATA element, TSE, and CRE on the expression of an α -subunit reporter gene (-224 to $+60$ linked to the bacterial CAT gene, containing a single CRE to facilitate mutagenesis) by transfection into JEG-3 cells (Fig. 1C). As expected, a mutation of either the CRE or TSE decreased α -subunit gene transcription, the magnitude of the reduction being approximately 12- and 2.5-fold, respectively. However, transcriptional activity decreased 15-fold because of the mutation in the GATA motif, demonstrating a strong contribution by this element to α -subunit gene expression. Additionally, the removal of more than one factor-binding site reduced expression even further. Transcriptional activity declined more than 20-fold compared with that of the intact α enhancer region for those reporter genes containing a single functional CRE, TSE, or GATA element. When all sites within the placenta-specific enhancer were destroyed, expression decreased approximately 90-fold, confirming the importance of the enhancer region in α -subunit gene activity. Furthermore, these data demonstrate that the three elements interact synergistically by activating α -subunit gene transcription to a level much greater than that predicted by adding the levels of expression produced by the individual regulatory elements.

The GATA-3 and GATA-2 genes are expressed in JEG-3 cells. Because of the sequence homology between the GATA element in the α -subunit enhancer and the consensus GATA motif, we hypothesized that α -ACT might be a member of the GATA-binding family of proteins. Methylation interference data obtained for GATA-2 (62) revealed that this factor contacts the core GATA motif (AGATAA; base pairs contacted are underlined). Methylation interference analysis on the α -subunit enhancer GATA element with JEG-3 nuclear extract demonstrated that all the nucleotides which completely interfered with binding when methylated are located within the consensus GATA sequence in a pattern of protein-DNA contacts identical to that found for GATA-2 (data not shown), supporting the idea that α -ACT is a member of this family.

Since it was likely that α -ACT is a member of the GATA-binding protein family, we investigated whether this factor might be distinct from previously characterized GATA-binding proteins. Therefore, an approach directed at isolating α -ACT cDNA was taken. Degenerate oligonucleotides encoding regions of the highly conserved DNA-binding finger domain of the GATA-binding factors were designed and used for PCR (see Materials and Methods). Amplification of JEG-3 poly(A)⁺ RNA produced a DNA fragment encoding the GATA-binding protein finger domain. We subsequently isolated four overlapping cDNA clones from a JEG-3 cDNA library that specifically hybridize to the 278-bp PCR fragment. DNA sequence analysis demonstrated that all four cDNA clones were identical to hGATA-3.

GATA-3 expression patterns in chicken and murine tissues are similar in that the sites of most abundant expression are the embryonic brain and T lymphocytes (34, 64). Interestingly, mGATA-3 mRNA has also been detected in the murine placenta (34) and rat placenta (36a). Northern analysis of JEG-3 poly(A)⁺ RNA hybridized to the 3' untranslated sequence of hGATA-3 cDNA yielded an ~3-kb band that is

FIG. 2. Northern analysis of hGATA-2 and hGATA-3 expression in JEG-3 cells. To detect hGATA-2 mRNA, a Northern blot containing poly(A)⁺ RNA derived from JEG-3 (0.5 μ g), Jurkat (1.5 μ g), and HeLa (2 μ g) cells was hybridized to a ³²P-labeled cDNA probe encoding ~900 bp of hGATA-2 5' untranslated and amino-terminal coding sequence. To detect hGATA-3 mRNA, the same Northern blot was hybridized to a cDNA probe encoding ~500 bp of the 3' untranslated sequence of hGATA-3.

identical to the signal produced with RNA derived from Jurkat cells, a human T-cell line which expresses the gene for hGATA-3 but not for hGATA-2 (Fig. 2). Furthermore, no signal is detected with HeLa RNA, which contains mRNA for hGATA-2 but not for hGATA-3. Lee et al. (36) reported that hGATA-2 mRNA is present in JEG-3 cells, and to confirm this finding, we rehybridized the Northern blot from Fig. 2 (right panel) with hGATA-2 cDNA (14). JEG-3 poly(A)⁺ RNA produced a signal identical to that found with HeLa RNA, whereas no bands were detected with Jurkat RNA (Fig. 2, left panel). Thus, we conclude that JEG-3 cells express both the hGATA-3 and hGATA-2 genes.

GATA-3 and GATA-2 bind to the α -subunit gene GATA element in JEG-3 cells. Since hGATA-3 and hGATA-2 mRNAs are both present in JEG-3 cells, we investigated whether these two factors can bind to the α -subunit gene GATA site. Therefore, to further characterize α -ACT GATA-binding activity, we performed electrophoretic mobility shift analysis with JEG-3 crude nuclear extract and with the α -subunit GATA element as a probe. Gel shift analysis revealed that two protein-DNA complexes are produced with JEG-3 nuclear extract and the GATA DNA probe (Fig. 3A). In contrast, control lanes containing HeLa and Jurkat nuclear extracts showed only single gel shift complexes which represent binding by hGATA-2 and hGATA-3, respectively. Interestingly, the hGATA-2 and hGATA-3 gel shift complexes have slightly different relative gel mobilities. Furthermore, the two mobility shift complexes produced with JEG-3 protein extract migrate similarly to those yielded by HeLa and Jurkat extracts, suggesting that the JEG-3 complexes correspond to hGATA-2 and hGATA-3.

To further study this issue, we performed electrophoretic mobility shift assays with an antiserum raised against mGATA-2 (kindly provided by S. H. Orkin) that blocks mGATA-2 DNA binding and cross-reacts with hGATA-2 (Fig. 3B). The addition of mGATA-2 antiserum to a binding reaction mixture containing the α -subunit gene GATA probe and JEG-3 nuclear extract eliminated the upper protein-DNA complex yet had no effect on the lower band. No change in either gel shift complex is observed with preimmune serum. The control lanes revealed that the antiserum blocked binding by hGATA-2 in the HeLa extract but had no effect on hGATA-3 binding in the Jurkat extract. In both of the JEG-3

FIG. 3. Electrophoretic mobility shift analysis of the GATA-binding proteins in JEG-3 cells. (A) Two factors bind the α -subunit gene GATA site in JEG-3 cells. Binding assays were performed with a GATA oligonucleotide probe with nuclear extracts from JEG-3 (0.4 μ g), HeLa (1 μ g), and Jurkat (1 μ g) cells. As a specific competitor, unlabeled GATA oligonucleotide was added at an approximately 100-fold molar excess over the probe. (B) mGATA-2 antiserum identifies hGATA-2 as one of the two proteins binding to the α -subunit gene GATA element. Binding reactions were performed as described above except that, where indicated, 1 μ l of either mGATA-2 antiserum or preimmune serum was added to the reaction mixture. In order to completely resolve the protein-DNA complexes, gel electrophoresis was carried out for an extended period, which resulted in the free probe migrating out of the gel. The arrowhead indicates a presumed supershift complex.

and HeLa binding reactions in which the mGATA-2 antiserum was used, a weak band positioned high in the gel is present. This complex may represent a supershift consisting of the DNA probe, hGATA-2, and mGATA-2 antibody, suggesting that hGATA-2 DNA binding is not completely blocked by mGATA-2 antiserum. We conclude from these studies that the

upper gel shift complex yielded by the JEG-3 nuclear extract is produced by hGATA-2. Additionally, because the extension of time for this gel electrophoresis experiment allowed complete resolution of the protein-DNA complexes, it is clear that the lower JEG-3 mobility shift complex migrates identically to that produced by hGATA-3 in the Jurkat extract. Thus, given that hGATA-3 mRNA is present in JEG-3 cells, the lower complex very likely represents hGATA-3 binding to the probe.

Pituitary regulation of the human α -subunit gene is mediated in part by the GATA motif. In addition to placental trophoblasts, the human α -subunit gene is also expressed in the gonadotrope and thyrotrope cells of the pituitary. Analysis of the *cis*-acting sequences downstream of -224 in the human α -subunit gene has revealed that pituitary gonadotrope regulation involves the CRE and the GSE positioned at -223 to -197 (25). Interestingly, a truncation of the α gene sequence between the GSE and CREs, a region which contains the GATA motif, was shown to decrease reporter gene activity approximately twofold in α T3-1 cells (25). Therefore, to determine whether the GATA element confers transcriptional activity to the α -subunit gene in the pituitary, the α -subunit reporter genes described in Fig. 1 were transfected into α T3-1 cells, a murine pituitary gonadotrope cell line expressing the endogenous α -subunit gene (63). Specific mutation of the α -subunit gene GATA element decreased expression roughly 2.5-fold (Fig. 4), a finding consistent with the truncation analysis. Mutation of the CRE, a known regulator of the human α -subunit gene in the pituitary (25, 51), reduced activity approximately eightfold. When both the GATA element and CRE were mutated, reporter gene activity decreased approximately 18-fold, suggesting that these *cis*-acting sequences functionally interact and providing additional evidence that the GATA motif regulates transcription of the α -subunit gene in the pituitary.

Two GATA-binding proteins expressed in α T3-1 pituitary cells bind the α -subunit gene GATA element. To determine whether members of the GATA-binding family of transcription factors are expressed in α T3-1 cells, Northern analysis was performed on α T3-1 RNA with cDNAs of the murine GATA-binding proteins as hybridization probes. α T3-1 cells do not appear to contain mGATA-1 or mGATA-3, since mRNAs for these proteins were not detected in α T3-1 RNA (Fig. 5). However, the hybridization of α T3-1 RNA with mGATA-2 cDNA produced a signal identical to that yielded with RNA from murine erythroleukemia cells, a hematopoietic cell line expressing the GATA-2 gene (64). Furthermore, α T3-1 RNA hybridized to mGATA-4 cDNA produced an ~4-kb band that is slightly smaller than the signal yielded with RNA derived from mouse F9 cells treated with retinoic acid. Recent cloning of an mGATA-4 cDNA by Arceci et al. (3) revealed that this factor is present in murine F9 embryonal carcinoma cells and that retinoic acid treatment of F9 cells induces mGATA-4 mRNA levels. Analysis of mGATA-4 tissue distribution by these investigators did not reveal any alternatively spliced mGATA-4 mRNAs in different cell types; therefore, an explanation for the smaller mRNA detected by the mGATA-4 probe in α T3-1 cells is presently unknown. Thus, we conclude that α T3-1 cells express two GATA-binding proteins, mGATA-2 and an mGATA-4-related factor. The adult mouse pituitary also expresses mGATA-2, but the mGATA-4-related mRNA is not detectable (data not shown), indicating that it may be developmentally regulated since α T3-1 cells represent a developmental precursor to the adult pituitary gonadotrope (63).

To determine whether the GATA-binding proteins expressed in α T3-1 cells interact with the α -subunit gene GATA

FIG. 4. Analysis of the transcriptional activity mediated by the α -subunit gene GATA motif in pituitary cells. The α -subunit reporter genes described in Fig. 1 were transfected into the pituitary cell line α T3-1. The presence of a wild-type CRE, TSE, and GATA element in each transfected plasmid is indicated by a triangle, oval, and diamond, respectively. CAT activity values are corrected for levels of β -galactosidase activity from an internal control β -galactosidase expression vector and normalized to the activity of the wild-type α -subunit gene promoter. Values are the means of three independent transfection experiments, with errors bars representing the standard errors of the means.

element, electrophoretic mobility shift analysis was performed with α T3-1 nuclear extract and with the human α gene GATA element as a probe. Though our original analysis of the expression of the α -subunit gene in α T3-1 cells did not reveal a footprint over the GATA site (25), the more sensitive technique of gel shift analysis detected two specific protein-DNA complexes with α T3-1 nuclear extract and the GATA

probe (Fig. 6A). These bands are specific since unlabeled GATA oligonucleotide added to the binding reaction mixture completely eliminated both shifted bands, whereas a heterologous competitor had no effect. Incubation of nuclear extract from retinoic acid-induced F9 cells with the GATA DNA probe produced a major protein-DNA complex, which likely corresponds to mGATA-4 bound to the DNA probe (3), that migrated with the lower α T3-1 gel shift complex.

Because α T3-1 cells contain mGATA-2 mRNA, we investigated whether one of the two gel shift complexes generated with α T3-1 nuclear extract represents a complex of mGATA-2 and the GATA probe. Addition of the mGATA-2 antiserum described above (Fig. 3) to the electrophoretic mobility shift assays decreased the upper protein-DNA complex and produced a supershifted band positioned high in the gel (Fig. 6B). Preimmune serum had no effect on the upper gel shift complex, indicating that this band corresponds to mGATA-2 bound to the DNA probe. When added to the binding reaction mixture containing F9 nuclear extract, mGATA-2 antiserum decreased the minor gel shift band and produced a very weak supershifted complex, which is not surprising since mGATA-2 mRNA is present in these cells (Fig. 5). The properties of the presumptive mGATA-4-DNA complex from F9 nuclear extract were not affected by mGATA-2 antiserum, and this complex migrated identically to the lower gel shift complex from α T3-1 extract, providing additional evidence that a GATA-4-related factor is present in α T3-1 cells.

GATA-2 and GATA-3 *trans* activate expression from a human α -subunit reporter gene. The hypothesis that GATA-binding proteins transcriptionally regulate the human α -subunit gene also involves determining a functional role for these proteins in α gene expression. To approach this issue, cDNA expression vectors containing the human CMV promoter directing expression of either hGATA-2 or hGATA-3 were cotransfected with reporter genes containing the human α -subunit gene enhancer/promoter with and without a functional GATA motif. Ideally, these studies would be performed with a cell type containing all of the proteins necessary for α -subunit gene expression except the GATA-binding factors, since reporter genes driven by the α -subunit gene enhancer/promoter exhibit very little transcriptional activity in heterologous cells.

FIG. 5. Northern analysis of the murine GATA-binding proteins expressed in α T3-1 cells. Total RNA derived from JEG-3 (2 μ g), α T3-1 (10 μ g), F9 (10 μ g) (treated for 96 h with 0.2 μ M retinoic acid), and murine erythroleukemia (MEL) (5 μ g) cells was hybridized to ^{32} P-labeled cDNA probes encoding mGATA-1, mGATA-2, mGATA-3, and mGATA-4. MEL RNA serves as a positive control for mGATA-1 and mGATA-2 mRNAs, while mGATA-4 mRNA is induced by retinoic acid in mouse F9 cells (3). In order to prevent cross-hybridization between the probe DNA and the highly conserved DNA-binding domains of the different GATA-binding proteins, all of the cDNA probes except for mGATA-4 did not contain the Zn finger domain.

FIG. 6. Electrophoretic mobility shift analysis of the GATA-binding proteins in α T3-1 cells. (A) Two factors bind to the α -subunit gene GATA element in α T3-1 cells. Binding assays were performed with a GATA oligonucleotide probe with nuclear extracts from α T3-1 cells (4 μ g) and F9 cells (4 μ g) treated with 0.2 μ M retinoic acid. As specific and heterologous competitors, unlabeled GATA and TSE oligonucleotides were added to the indicated binding reaction mixtures at an approximately 100-fold molar excess over the probe. (B) mGATA-2 antiserum identifies mGATA-2 as one of the two α T3-1 proteins binding to the α -subunit gene GATA element. Binding reactions were performed as described above except that, where indicated, 1 μ l of either mGATA-2 antiserum or preimmune serum was added to the reaction mixture. In order to completely resolve the protein-DNA complexes, gel electrophoresis was carried out for an extended period, which resulted in the free probe migrating out of the gel. The arrowhead indicates a presumed supershift complex.

Neither hGATA-2 nor hGATA-3 restored expression to the α -subunit reporter gene in HeLa and NIH 3T3 cells (data not shown), presumably because other important transcriptional regulators of the α -subunit gene, such as the placental protein TSEB and/or the pituitary factor GSEB, are not present in these cells. As for the cell types which normally express the α -subunit gene, overexpression of neither hGATA-2 nor hGATA-3 stimulated transcription from the α -subunit reporter gene in JEG-3 placental trophoblasts (data not shown); however, both GATA-binding proteins *trans* activated the α -subunit reporter gene in α T3-1 pituitary gonadotropes (Fig. 7). In α T3-1 cells, overexpression of hGATA-2 and hGATA-3 stimulated expression of the α -subunit reporter gene approximately five- and sixfold, respectively. However, the reporter gene carrying a mutant GATA motif exhibited twofold stimulation in response to the hGATA-2 and hGATA-3 expression vectors, an effect we believe to be nonspecific since these same expression vectors stimulated expression of the thymidine kinase promoter roughly twofold (data not shown). Therefore, overexpression of hGATA-2 and hGATA-3 specifically activated the α -subunit gene enhancer/promoter in *trans* approximately 2.5- to 3-fold. In support of these studies, an expression plasmid producing mGATA-3 (34) also stimulated activity from the α -subunit reporter gene in α T3-1 cells threefold (data not shown).

DISCUSSION

The family of GATA-binding proteins is composed of multiple members, each expressed in a distinct spatial and developmental pattern (45). Here we have established that this family of transcription factors is involved in placenta- and pituitary-specific gene expression. In the placental trophoblast cell line JEG-3, both hGATA-2 and hGATA-3 are expressed and bind to the human gonadotropin α -subunit gene GATA element and a mutation of this element decreases expression 15-fold. In α T3-1 pituitary gonadotrope cells, a mutation of the α -subunit gene GATA motif reduced transcriptional activity 2.5-fold and mGATA-2 and an mGATA-4-related protein were found to be expressed and to bind to this site. Furthermore, cotransfection of hGATA-2 or hGATA-3 into α T3-1 cells *trans* activated expression from an α -subunit reporter gene, demonstrating a functional role for members of the GATA-binding family of transcription factors in α -subunit gene regulation. It is not surprising that both hGATA-2 and hGATA-3 activated the α -subunit gene, since several groups have shown that these proteins interchangeably activate genes containing GATA motifs in cotransfections (14, 30, 34, 64). In contrast to the cotransfection studies with α T3-1 cells, cotransfection of hGATA-2 or hGATA-3 into JEG-3 cells did not activate the α -subunit gene. Thus, it is likely that in JEG-3 cells, hGATA-2 and/or hGATA-3 endogenous levels are not limiting for transcriptional activation, so increasing the level of GATA-binding protein produced no increase in expression. In support of this idea, Fig. 4 reveals that hGATA-2 and hGATA-3 mRNAs are present at much higher levels in JEG-3 cells than in the control cell lines HeLa and Jurkat. Additionally, 2 μ g of JEG-3 RNA hybridized to an mGATA-2 cDNA probe produced a significantly stronger signal than that yielded with 10 μ g of α T3-1 RNA (Fig. 5). The high levels of hGATA-2 and hGATA-3 mRNAs in JEG-3 cells also correlate with high protein levels since nuclear extracts derived from JEG-3 cells produced more abundant gel shift complexes than those from Jurkat, HeLa, and α T3-1 cells.

Identification of a specific activity for the GATA sequence element in the α -subunit gene allows clarification of the

FIG. 7. *trans* activation of a human α -subunit reporter gene by hGATA-2 and hGATA-3. α -Subunit reporter genes (5 μ g; described in Fig. 1) were cotransfected into α T3-1 cells with 5 μ g of a CMV eucaryotic expression vector (control) or expression vectors containing either the hGATA-2 or hGATA-3 protein coding sequence under the control of the CMV promoter. CAT activity values are corrected for the levels of β -galactosidase activity from an internal control β -galactosidase expression vector. Values are the means of three independent transfection experiments, with error bars representing the standard errors of the means.

structure (and hence the nomenclature) of the placenta-specific enhancer region. The TSE was first defined as an enhancer element from -182 to -159 because of the borders of the DNase I footprint (11). A larger region from approximately -180 to -145 was termed the URE (28) because of its enhancer activity in JEG-3 cells. Jameson et al. (27) later partitioned the URE into two largely overlapping areas of activity called URE 1 (-172 to -147) and URE 2 (-177 to -156). They identified a binding activity (termed band 1) which bound URE 1 and was not inhibited by URE 2, placing it in the general region of the GATA element. However, this protein is not likely to be related to the GATA proteins that we have identified for three reasons. First, their migration patterns in gel shifts are dramatically different, with band 1 migrating near the free DNA probe, indicating a very small protein, and the GATA proteins migrating slowly, as is appropriate for larger proteins. Second, band 1 and what may be assumed to be TSEB (termed band 2) cannot bind simultaneously (27), while the GATA proteins and TSEB do bind simultaneously (54). Third, the methylation interference patterns for band 1 and the GATA proteins are quite different, with band 1 contacting nucleotides spread over the region from -181 to -142 . We have not detected band 1 in our experiments. Southwestern (DNA-protein) blotting with the entire URE region detected several proteins (58, 45, 42, and 41 kDa) which could not be detected with individual URE 1 or URE 2 probes (27). These sizes do not correlate with human GATA-2 (50 kDa), GATA-3 (48 kDa), or TSEB (56 kDa); however, size estimates on polyacrylamide gels and Southwestern blots are often not exact. Using 10-bp substitution mutations, Kennedy et al. (33) mutated the region from -170 to $+44$ and found that each of the seven mutations from -170 to -100 decreased expression approximately 10-fold. In this experiment, the TSE would not have been present because of the lack of the sequences upstream of -170 which are crucial for binding of TSEB (55) but the -160 to -150 mutation would have interrupted the GATA element. Here we have demonstrated that the placenta-specific enhancer is composed of the TSE, the GATA site, and two CREs. Within this, the URE can be thought to correlate with the combination of the TSE and GATA elements, while the clearest nomenclature is that referring to the individual elements.

Each of the *cis*-acting sequences contributes to the transcrip-

tional activity of the human α -subunit gene placenta-specific enhancer. The mutagenesis of this enhancer revealed that maximal expression in placental cells requires the GATA element, the CRE, and the TSE, which interact synergistically to activate transcription. In the native gene, the two CREs act synergistically in response to elevated levels of cAMP and bind protein cooperatively in vitro (2). Despite extensive investigation, we have not been able to show that proteins binding to the α -subunit TSE, GATA, and CRE bind cooperatively to naked DNA (1a). This may be because facilitated binding among these factors requires that DNA be packaged into nucleosomes since cooperative binding by GAL4 has been shown to be nucleosome dependent (58). Alternatively, the synergistic qualities of these elements may be conferred by protein-protein contacts that increase the transcriptional activation properties of these factors. DNA binding assays combined with in vitro transcription analysis with highly purified proteins and defined nucleosomal templates will provide further insight into the molecular mechanisms controlling expression of the human α -subunit gene.

Our studies demonstrate a new tissue-specific activity for hGATA-3. Although first shown to be present in adult reticulocytes, T lymphocytes, the embryonic brain, and the adult kidney (23, 30, 34, 64), we found hGATA-3 to be abundantly expressed in JEG-3 human placental cells. Furthermore, GATA-3 (and/or GATA-2) binding to the GATA element in the α -subunit gene is required for the full activity of the composite placenta-specific enhancer. GATA-3 may also regulate the placental lactogen gene in rat placental cells (36a) and has been found in mouse placental RNA (34), indicating a role for GATA-3 in activating multiple genes in placental trophoblast cells, analogous to its role in the hematopoietic cell lineages.

The presence in a single cell type of multiple members of a given transcription factor family with highly related DNA-binding specificities presents the problem of determining the mechanism of differential activation. The ability of proteins with the same DNA-binding specificity to direct distinct patterns of gene expression has been extensively studied in the case of mammalian POU homeodomain proteins Oct-1 and Oct-2. The ubiquitous Oct-1 and the related B cell protein Oct-2 differentially regulate gene expression through promoter-selective activation domains, which stimulate transcription

from specific promoters containing octamer motifs (57). For example, activation of the human U2 small nuclear RNA promoter is accomplished more effectively by Oct-1 than by Oct-2 (57), whereas Oct-2 activates the human β -globin promoter more effectively (32, 56). These different activation domain specificities are probably achieved by selective protein-protein interactions with different types of transcriptional initiation complexes. Similar issues arise for the multiple helix-loop-helix proteins expressed in the myogenic lineage (61). Multiple members of the GATA-binding family of transcription factors have been shown to be simultaneously expressed within the same erythroid progenitor cell (64) while regulating different processes (6). Here we have demonstrated that both GATA-2 and GATA-3 are present in placental trophoblasts, while both GATA-2 and a GATA-4-related factor are present in pituitary gonadotropes. In each case, both proteins can bind the α -subunit GATA element efficiently in vitro. Furthermore, both GATA-2 and GATA-3 proteins can activate the α -subunit gene in cotransfections in α T3-1 cells. It remains to be determined whether the GATA-binding transcription factors, which are highly homologous in their DNA-binding domains but very different in their overall primary structures, contain distinct promoter-selective activation domains that direct different programs of gene expression.

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