The Ets-binding site within the basal transcription element (BTE) of the rat prolactin (rPRL) promoter is critical for both basal and growth factor-regulated rPRL gene expression. Here we report the purification and identification of the factor that binds to the BTE. This factor was purified from GH3 pituitary nuclear extracts using ammonium sulfate fractionation, heparin-Sepharose and Mono Q chromatography, and BTE-affinity magnetic beads. We purified two proteins of 57 and 47 kDa and identified the 57-kDa protein by mass spectrometry as the Ets factor GABPα. Western blot analysis identified the 47-kDa protein as GABPβ1. Co-transfection of dominant-negative GABPβ1 blocks prolactin promoter basal activity by 85–88% in GH3 cells in the presence or absence of FGF-4. Additionally, expression of wild-type GABPα/β1 selectively activates a minimal BTE promoter 24–28-fold in GH3 cells, and this activation is dependent on the Ets-binding site. Finally, small interfering RNA depletion of GABP in GH3 cells results in the loss of prolactin protein. Thus, we have identified GABPα/GABPβ1 as a critical and functionally relevant Ets factor that regulates rPRL promoter activity via the BTE site.

The prolactin gene is selectively expressed in the lactotroph cells of the anterior pituitary gland. The proximal 425 bases of the rat prolactin (rPRL) promoter are sufficient to confer cell type-specific expression of the PRL gene (1). Within this region, DNase footprinting studies have identified three binding sites for the pituitary-specific POU homeodomain transcription factor Pit-1 (FPI, -III, and -IV) (2). Pit-1 has been shown to play a critical role in the cell type-specific expression of the PRL gene and the development of the lactotroph cell lineage. However, Pit-1 has also been shown to control the expression of the growth hormone (GH) and thyroid-stimulating hormone β genes in somatotroph and thyrotroph cells, respectively (3–5). Thus, other cis-elements and transcription factors must be involved in the cell type-specific expression of the PRL gene.

Further studies of the rPRL promoter have identified a region between −112 and −85 that is critical for the basal activity of the promoter (6–8). This basal transcription element (BTE) was subsequently shown to overlap with a cAMP-response element (9, 10); however, it was shown that the prototypical target CREB is unlikely to be involved at this site (8, 9). Similarly, studies (9) using the human PRL promoter have shown that the homologous A-site is critical for both basal and cAMP human PRL promoter activity, and that a ubiquitous 100-kDa protein distinct from CREB and Pit-1 binds to this site.

Recent studies have identified a binding site for the Ets family of transcription factors within the BTE. This Ets-binding site has been shown to be a critical cis-element for several growth factor signaling pathways, including fibroblast growth factors 2 and 4 (11), insulin (12, 13), insulin-like growth factor-1 (13), epidermal growth factor (14), and thyrotrpin-releasing hormone (15). By using electrophoretic mobility shift assays (EMSA), Stanley and co-workers (16) demonstrated that the EBS at −96 can bind to the Ets factors Elk-1, Sap-1, and GABPα/GABPβ1; this group has also shown that the CCAAT/enhancer-binding protein (C/EBPα) also binds to the BTE. However, the functional significance of any of these factors in pituitary cells remains unclear. We have characterized previously the Ets factors that bind to the BTE and have shown by EMSA and antibody supershift analysis that Ets-1 and GABPα/GABPβ1 can bind to the EBS of the BTE (17). In addition, UV cross-linking studies with GH3 nuclear extract and the BTE probe suggested that a protein of ~49–57 kDa binds to the BTE (17). However, transient transfection studies of a variety of Ets factors have failed to identify the functionally relevant protein. Thus, the exact identity of the protein that binds to the BTE and regulates rPRL gene expression remains unknown.

The Ets family of transcription factors is composed of more than 30 members that contain a highly conserved DNA binding domain (18). All members of the Ets family recognize and bind to a core 5′-GGA(A/T)-3′ DNA motif in a variety of promoters. Thus, the mechanism by which Ets factors achieve specific transcriptional responses is of great interest. Additional se-
sequences flanking the core are thought to contribute to binding specificity, in addition to the tissue-restricted expression of certain Ets factors (18). However, the primary mechanism for achieving specificity is thought to be through the interaction of Ets factors with other transcription factors bound to adjacent elements (19–21).

The rPRL promoter in GH3 pituitary cells represents an excellent model system to study the mechanisms by which Ets factors in combination with other classes of transcription factors at adjacent elements elicit specific transcriptional responses. Our laboratory has previously shown that Pit-1 selectively interacts with Ets1 at the rPRL promoter Ras-response element centered at −212, and this interaction may contribute to the cell type-specific expression of the PRL gene (22, 23). Thus, the identification and characterization of the Ets factor that binds to the BTE should further our understanding of the cell type-specific expression of the PRL gene.

In order to identify definitively the Ets factor that binds to the BTE of the rPRL promoter, a large scale purification scheme was undertaken. By using four purification steps, we have purified two proteins of 57 and 47 kDa from GH3 pituitary nuclear extracts. The 57-kDa protein was identified as the Ets factor GABPα by MALDI-TOF and MS/MS sequencing, and the 47-kDa protein was identified as GABPβ by Western blotting. By using transient transfection studies, we show that expression of dominant-negative GABPβ blocks basal rPRL promoter activity in the presence or absence of FGF-4. Co-transfection of wild-type GABPα/GABPβ activates the rPRL promoter in GH3 pituitary cells and selectively activates a minimal BTE promoter. Finally, siRNA against GABP results in the selective reduction of PRL protein levels. These studies show that GABPα/GABPβ is the critical nuclear factor that binds to the BTE and regulates basal rPRL promoter gene expression.

**EXPERIMENTAL PROCEDURES**

**EMSA**

The EMSA was performed essentially as described previously (17). Binding buffer contained 10 mM Hepes-KOH, pH 7.9, 50 mM KCl, 4% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, 200 μg/ml insulin, and 250 ng Poly(dI-dC) (Amersham Biosciences). Poly(dI-dC) was not included in gel shift reactions containing the DNA affinity-purified fractions. The sequence of the BTE probe used is (message strand only, linker sequences in lowercase): tgcgCTTAATGAGGGAATAGATAG, the putative Ets-binding site (GGAA) is in boldface, and the mutant BTE probe is identical except that the underlined sequences were changed to an XhoI site. Protein-DNA complexes were visualized by autoradiography, and gel shift activities were quantified using a PhosphorImager (Amersham Biosciences).

**Preparation of DNA Affinity Magnetic Beads**

Complementary oligonucleotides containing three repeats of the BTE sequence were annealed for the DNA-affinity purification step as described previously (17). The message strand was synthesized with a 5′-biotin containing a 6-carbon (6C) spacer, and the complementary DNA strand was identical, except it did not contain a biotin moiety (Invitrogen). The sequence of the 72-nucleotide BTE oligonucleotide used is (message strand only, Bst linker sequences in lowercase): 5′-Bio-6C-tgcgCTTAATGAGGGAATAGATAGtgcgCTTAATGAGGGAATAGATAGtgcgCTTAATGAGGGAATAGATAG-3′. Approximately 3 nmol of double-stranded (BTE), 5′-biotinylated oligonucleotide were coupled to 20 mg of M-280 streptavidin-coated magnetic beads (Dynal) according to the manufacturer’s instructions.

**Purification of the BTE Binding Factor (BTF)**

**Cell Culture and Preparation of Nuclear Extracts**—GH3 cells were grown in spinner culture at 37 °C in high glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% horse serum and 2.5% fetal bovine serum (Invitrogen) by the University of Colorado Health Sciences Center Tissue Culture Core Facility. Cells were grown to a density of 8–10 × 10^6 cells/ml and collected by centrifugation at 1500 × g. Nuclear extracts were prepared from a total of 40 liters of GH3 cells (24), with slight modifications, as described previously (17). 

**Ammonium Sulfate Fractionation**—BTF was purified from ~262 mg of GH3 nuclear extract derived from 40 liters of GH3 cells in four purification steps (Fig. 2). Ammonium sulfate powder (Fisher) was added to 50.5 ml of GH3NE on an ice bath at 4 °C to give a final saturation of 50% ammonium sulfate. Precipitated proteins were collected by centrifugation at 20,000 × g at 4 °C, and the pellet was resuspended in 20.5 ml of binding buffer (10 mM Hepes-KOH, pH 7.9, 7 mM KCl, 4% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Na3VO4, and 1× protease inhibitors) (Roche Applied Science). The 50% ammonium sulfate pellet and soluble fraction were dialyzed against binding buffer at 4 °C. The dialysate was cleared by centrifugation at 25,000 × g. Fractions were assayed for BTF binding activity by EMSA. Protein concentrations were determined using the Bio-Rad protein assay.

**Heparin-Sepharose Purification**—The 50% ammonium sulfate cut containing BTF activity was divided into five samples. Each sample was loaded onto a 5-ml Hi-Trap heparin-Sepharose column (Amersham Biosciences) and re-circulated at 4 °C for 20 min at 2 ml/min with a peristaltic pump. The flow-through was collected, and the column was washed with 10 ml of binding buffer at 1 ml/min. Bound proteins were eluted with a 50-ml 70–800 mM KCl linear gradient using the FPLC, with a flow rate of 1 ml/min, and 1-ml fractions were collected for each column. BTE binding activity eluted in two peaks, and the purification of peak 1 is the focus of this study because this peak contained unambiguous BTE binding activity. Peak 1 from five sequential heparin-Sepharose columns was pooled and dialyzed against binding buffer and then re-loaded onto the 5-ml heparin-Sepharose column in order to concentrate the sample. Heparin-bound proteins were step-eluted with
binding buffer containing 0.55 M KCl, and the concentrated peak fraction 1 was dialyzed against binding buffer.

Ion Exchange Chromatography—The peak heparin-Sepharose fraction 1 (5.1 mg) was loaded onto a 1-ml Mono Q column (HR 5/5, Amersham Biosciences) pre-equilibrated with binding buffer. The column was washed with 2 ml of binding buffer, and protein was eluted with 70–800 mM KCl linear gradient in 21 ml of binding buffer at 0.5 ml/min using an FPLC. Peak binding activity was present in fractions 38–41 (300 mM KCl). These fractions were pooled and dialyzed against 7 mM KCl binding buffer and clarified by centrifugation.

Magnetic DNA Affinity Purification—Twenty mg of magnetic beads coupled to 3 nmol of double-stranded BTE oligonucleotide was washed three times in binding buffer (10 mM Hepes-KOH, pH 7.9, 50 mM KCl, 4% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, and 200 µg/ml insulin, 1× protease inhibitors, Roche Applied Science). Approximately one-half (335 µg) of the peak Mono Q fraction (700 µg) was purified with the (BTE)3-coupled magnetic beads in three sequential purifications. For each purification, 20 mg of (BTE)3-coupled magnetic beads was incubated with 40, 70, or 225 µg of the peak Mono Q fraction for 30 min at room temperature and rotated every 3–5 min. The purification was done sequentially because the exact capacity of the (BTE)3-coupled magnetic beads was not known. The beads containing bound protein

### Table I

| Purification step       | Total protein | Total activity | Specific activity | Purification factor | Yield |
|-------------------------|---------------|---------------|------------------|---------------------|-------|
| GH3NE                   | 262           | 2.9 × 10^9    | 1.1 × 10^7       | 1                   | 100   |
| (NH₄)₂SO₄               | 160           | 3.1 × 10^9    | 1.9 × 10^7       | 1.7                 | 106   |
| Heparin-Sepharose (Pk 1)| 6             | 5.4 × 10^8    | 9.97 × 10^6      | 9.1                 | 19    |
| Mono Q                  | 0.7           | 1.2 × 10^6    | 1.7 × 10^4       | 15                  | 4     |
| DNA affinity            | 0.000561      | 1.5 × 10^6    | 2.8 × 10^11      | 25,000              | 5     |

* Protein concentrations were calculated by the Bio-Rad protein assay.
* Total activity was calculated by multiplying the PhosphorImager units present in 1 µl of the shifted extract by the total volume of the extract.
* Specific activity was calculated by dividing the PhosphorImager units of the shifted band by the amount of protein (µg) present in the gel shift reaction.
* Protein concentration was estimated from a silver-stained SDS-PAGE gel using bovine serum albumin as a standard.

**Fig. 3. Heparin-Sepharose purification of GH3NE.** A, elution profile of the heparin-Sepharose chromatography. The 50% ammonium sulfate fraction was purified by heparin-Sepharose, as described under “Experimental Procedures.” Proteins were eluted with a 50-ml linear gradient from 70 to 800 mM KCl, and 1-ml fractions were collected. Protein concentrations were determined by the Bradford protein assay and are shown by closed circles. Salt concentrations were determined by conductivity measurements using KCl standards, and the KCl gradient is depicted by closed squares. Specific activity units are shown by the open circles. B, gel shift assay of peak heparin-Sepharose fractions. One µl of input and 2 µl of each eluted fraction were incubated with the BTE probe to assay for BTF binding activity. Protein-DNA complexes were separated by nondenaturing gel electrophoresis. Peak binding activity was present in fractions 20–26 (∼300 mM KCl). Specific protein-DNA complexes are indicated by BTF, NS denotes nonspecific bands, and Free indicates free probe.
were washed three times, for 5 min each, with 500 μl of binding buffer containing 100 mM KCl. For the last two washes, 15 μg of poly(dI-dC) was added. Proteins were step-eluted using 100-μl aliquots of binding buffer containing 0.2, 0.3, or 1 mM KCl for 10 min each. The peak fractions (1 mM KCl) of the three sequential (BTE)α- affinity magnetic bead purifications (40-, 75-, and 225-μg inputs) were combined.

**Mass Spectrometric Analysis of Proteolytic Peptides**

For gel extraction and tryptic digestion, the peak BTE affinity-purified fractions were pooled and boiled in 2× SDS-sample buffer (60 mM Tris, pH 6.8, 2% SDS, and 10 mM dithiothreitol) and loaded onto a 1-mm thick, 1% SDS-10% polyacrylamide gel. The gel was stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma) in 25% isopropanol alcohol and 10% acetic acid for 2 h and destained with 10% acetic acid for 2 h. Protein bands and a control “blank” piece were excised from the gel, and in-gel trypsin digests were performed as described previously (25). Tryptic peptides were extracted from gel slices, concentrated, and desalted as described previously (25). For the LCQ analysis, the desalted extracts were completely dried in a vacuum centrifuge and resuspended in 5 μl of 0.1% formic acid.

**Mass Spectrometry Analysis**

MALDI-TOF mass spectrometry (Voyager-DE STR, PerkinElmer Life Sciences) in delayed extraction mode was used to identify the unknown proteins by peptide mass fingerprinting, as described previously (25). Briefly, one-tenth (0.5 μl) of each desalted extract was co-crystallized with matrix (α-cyano-4-hydroxy-trans-cinnamic acid, Hewlett-Packard) on a gold-coated sample plate, and the monoisotopic masses of des-Arg1-bradykinin (904.47 Da) and Glu(−)-fibropeptide B (1570.67 Da) were used to calibrate the instrument. The sum of 150 acquisitions for each sample was used to identify the peptide masses for each unknown protein. Proteins were identified using the Profound program (prowl.rockefeller.edu/cgi-bin/ProFound) searching the rodent, human, or eukaryotic NCBI nonredundant protein data bases.

Liquid chromatography electrospray ionization mass spectrometry with a quadrupole ion trap mass spectrometer (LCQ, Finnigan) coupled to a 1100 1100 high pressure liquid chromatography pump and stream splitter (Michrom BioResources) was used to obtain amino acid sequence, as described previously (25). Briefly, peptide fragmentation spectra were obtained for the 57-kDa protein as peptides eluted on a 100-μm (inner diameter) fused Silica C18 column (LC Packings) using an acetonitrile gradient at a flow rate of 300 nl/min. The amino acid sequences identified from the peptide fragmentation data were used to search the NCBI nonredundant protein database using the Sequest software (Finnigan) (26).

**Western Blot Analysis and UV Cross-linking Studies**

Western blot analysis of GH3NE (50 μg), the 50% ammonium sulfate cut (50 μg), the peak heparin-Sepharose (20 μg), Mono Q (8 μg), and pooled BTE-affinity fractions was performed as described previously (17). UV cross-linking studies of these same peak fractions were performed as described previously (17). The BTE probe used for UV cross-linking has been detailed previously (17). UV cross-linking studies of these same peak fractions were performed as described previously (17). The BTE probe used for UV cross-linking was detailed previously (17). Where indicated, competitor oligonucleotides were included at 400-fold molar excess.

**Plasmid Constructs and Transient Transfections**

The expression plasmids pSG5-Ets-1, pSG5-Ets-2, pTL2Eik-1, and pTL2Net, and the luciferase reporter constructs pA3–425rPRLluc and pA3–36rPRLluc have been described previously (23, 27). Other expression constructs used included the following: pCDNA3 ER (Frank Burton, University of Minnesota, Minneapolis, MN) (28); pCMXGABPα1 (Tom Brown, Pfizer) (29); pSG5ER81 (Ralf Janknecht, The Salk Institute, La Jolla, CA) (30); and MSVGBPβ1(D)550 (Laurent Schaeffer, Laboratory de Biologie Moleculaire et Cellulaire UMR, Lyon, France) (31). The minimal −36 rPRL promoters containing wild-type and mutant BTE inserts were constructed by inserting multiple copies of each double-stranded BTE oligonucleotide containing a SauI overhang into the SauI site of pA3–36rL, as described previously (23). The wild-type and mutant BTE oligonucleotides used were the same that used for EMSA as described above. The minimal promoters were sequenced by the University of Colorado Health Sciences Center DNA Sequencing Facility. Adherent GH3 or GH3T1 pituitary cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 12.5% horse serum and 2.5% fetal calf serum and maintained at 37 °C in 5% CO2. GH3 cells were transiently transfected by electroporation, harvested, and luciferase/β-galactosidase activities assayed as described previously (23). Cells were treated with or without 2 ng/ml FGF-4, 6 h prior to harvest as described previously (10).

**siRNA Transfections and Western Blot Analysis**

Double-stranded synthetic oligonucleotides directed against lamin A/C, GABPα, and GABPβ1 were prepared according to the manufacturer’s instructions (Dharmacon Research Inc.). The targeted sequences of GABPα and GABPβ1 were 5′-AACAGUGCCGAGACUGUAAU-3′ and 5′-AACAUUGGCAGCCUCUAAT-3′, respectively. The sequence of the lamin A/C oligonucleotide was as described previously (32). Adherent GH3 cells were seeded at 1 × 105 cells per 12-well plate. GH3 cells were transfected with 200 pmol of both GABPα and β1 oligonucleotides or 400 pmol of the lamin A/C oligonucleotide or no siRNA oligonucleotide using 10 μl of Effectene reagent (Qiagen). Cells were harvested in EB as described previously (10), and 75 μg of lysate was subjected to Western blot analysis as described above. Blots were probed with antibodies against α-tubulin (Oncogene), GABPα (provided by Dr. Tom Kristie, National Institutes of Health), or prolactin (provided by Dr. A. F. Parlow at the National Hormone and Pituitary Program). Protein levels were determined using an Alpha Imager.

**RESULTS**

**Purification of BTF**—The BTE is a key element of the proximal rPRL promoter that regulates both basal and growth factor-stimulated promoter activity (1, 6–10, 13–15, 33) (Fig. 1). We have characterized previously (17) a specific protein...
complex (complex A) present in GH3 nuclear extract that binds to the BTE of the rPRL promoter. This previous characterization suggested that an Ets factor is a component of complex A. However, the identity of the functionally relevant factor(s) that bind to the BTE remains unclear. In order to identify precisely the endogenous protein(s) that bind(s) to the BTE of the rPRL promoter, we set up a large scale protein purification scheme to purify BTF from GH3 pituitary cells. The four-step purification scheme used to purify BTF is shown in Fig. 2. Nuclear extracts were prepared from 40 liters of GH3 cells and purified by ammonium sulfate fractionation, heparin-Sepharose and Mono Q chromatography, and BTE-affinity magnetic beads. The presence of BTF binding activity in each fraction was monitored by EMSA using a radiolabeled BTE probe.

As an initial purification step, GH3 nuclear extract (GH3NE) was precipitated by a 50% saturated ammonium sulfate cut. BTF binding activity was enriched 1.75-fold in the 50% ammonium sulfate precipitate, and no detectable gel shift activity was present in the soluble fraction (Table I and data not shown). The 50% ammonium sulfate fraction was divided into five equal fractions, and each fraction was loaded sequentially onto a heparin-Sepharose column, and proteins were eluted using a 50-ml 70–800 mM linear KCl gradient. A representative protein elution profile and EMSA are shown in Fig. 3. Gel shift analysis of the heparin-Sepharose purified fractions shows that BTF binding activity is present in the input but not the flow-through fractions (Fig. 3B, lanes 1 and 2). BTF binding activity eluted from the heparin-Sepharose column at two different salt concentrations, the first peak of eluting in fractions 20–26 (peak 1, 300 mM KCl) (Fig. 3, A and B, lanes 6–12), and a second broader peak (peak 2) eluting at a higher salt in fractions 30–42 (peak 2, 500–600 KCl) (Fig. 3, A and B, lanes 17–29). Cumulative purification was 10-fold for peak 1 and 0.4-fold for peak 2 (Fig. 3A and Table I). Peaks 1 and 2 from the heparin-Sepharose column were pooled separately. Further purification of peak 2 did not yield specific BTF binding activity, and Western blot analysis showed that GABP was not present in peak 2 (data not shown). The purification of peak 1 is the focus of this study.

As a third purification step, peak 1 from the heparin-Sepharose column was pooled, dialyzed, and separated by Mono Q anion-exchange chromatography (Fig. 4). A linear gradient of
KCl (70–800 mM) was applied to the Mono Q column (Fig. 4A) and BTF binding activity eluted in fractions 38–41 (~400 mM KCl) (Fig. 4B, lanes 6–9). BTF binding activity was not detected in the flow-through or washes (data not shown). At this stage of the purification, a 19-fold increase in specific activity was obtained with a cumulative yield of 4% (Fig. 4A and Table I).

For the final purification step, the peak Mono Q fractions (38–41) were pooled, dialyzed to 50 mM KCl, and approximately one-half of the pooled Mono Q fraction (340 μg) was incubated with magnetic beads coupled to three tandem copies of the BTE oligonucleotide. After incubation with the BTE-affinity magnetic beads, the flow-through was collected, and the beads were washed three times with buffer containing 100 mM KCl. To improve the efficiency of the purification, excess heparin-Sepharose and ammonium sulfate fraction and 2× SDS-PAGE sample buffer that only one strand of the BTE probe (Fig. 5A, lane 1 versus 2), and a small amount eluted in Wash 1 (Fig. 5A, lane 3). BTF binding activity was not present in the last two washes or the 0.2 M KCl fractions (Fig. 5A, lanes 4–7) but began to elute in the 0.3 M KCl fraction 1 and ended in the 1 M KCl fraction 1 (Fig. 5A, lanes 8–10). Approximately 25,000-fold purification was achieved by this step (Table I).

The purity of the BTE-affinity fractions was assessed by SDS-PAGE followed by silver staining. The silver stain of the BTE-affinity purified fractions shows that BTF binding activity was retained on the DNA affinity beads (Fig. 5A, lane 1 versus 2), and a small amount eluted in Wash 1 (Fig. 5A, lane 3). BTF binding activity was not present in the last two washes or the 0.2 M KCl fractions (Fig. 5A, lanes 4–7) but began to elute in the 0.3 M KCl fraction 1 and ended in the 1 M KCl fraction 1 (Fig. 5A, lanes 8–10). Approximately 25,000-fold purification was achieved by this step (Table I).

The purity of the BTE-affinity fractions was assessed by SDS-PAGE followed by silver staining. The silver stain of the BTE-affinity purified fractions shows that the input, flow-through, and washes contained a complex mixture of proteins (Fig. 5B, lanes 1–5). Analysis of the proteins present in the input versus the flow-through fractions revealed that two proteins of 47 and 57 kDa were retained on the BTE-affinity beads. The 47-kDa protein eluted between 0.2 M KCl and 1 M KCl fraction 1 (Fig. 5B, lanes 6–10). The 57-kDa protein eluted between 0.3 M KCl fraction 1 and 1 M KCl fraction 1 (Fig. 5B, lanes 8–10). The majority of gel shift activity was present in the 0.3 M KCl fraction 1 and 2 and the 1 M KCl fraction 1, where both the 47- and 57-kDa proteins were present (Fig. 5A). Binding activity was not detected in the 0.2 M KCl fractions where only the 47-kDa protein was present (Fig. 5, A and B). These data suggest that the 47-kDa protein does not bind to DNA, or that both proteins are required for efficient DNA binding.

UV Cross-linking of Purified Fractions—In order to determine which proteins binds to the BTE probe, UV cross-linking studies were done. An equal volume of each peak fraction was incubated with a BTE probe substituted with 5-bromo-2’-deoxyuridine and exposed to UV light, as described under “Experimental Procedures.” As observed previously (17), UV cross-linking of GH3NE with the BTE probe resulted in two different protein-DNA complexes: a distinct and specific protein-DNA complex that migrated with an apparent molecular mass of 64 kDa, and a more diffuse, nonspecific protein-DNA complex that migrated at ~50 kDa (Fig. 6, lane 10). Both protein complexes were present in the 50% ammonium sulfate fraction and the peak heparin-Sepharose and Mono Q fractions (Fig. 6, lanes 3–5). UV cross-linking of the BTE affinity-purified fraction shows that only the 64-kDa protein complex was present (Fig. 6, lane 6) and that this protein was specific to the BTE because addition of wild-type but not mutant BTE oligonucleotide competed for binding (Fig. 6, lanes 6–8). In estimating the molecular weight of the protein binding to the BTE probe, we assumed that during the denaturation of the protein-DNA complex in SDS sample buffer that only one strand of the BTE probe (~7 kDa) remained cross-linked to the protein. Therefore, we estimate the true molecular mass of the cross-linked protein to be ~57 kDa, which corresponds to the 57-kDa protein purified by DNA-affinity chromatography and visualized by silver staining (Fig. 5B, lanes 8–10). The co-purification of the 47- and 57-kDa proteins through this extensive purification scheme indicates that both proteins are likely to be involved in binding to the BTE, although UV cross-linking studies show that only the 57-kDa protein directly binds DNA.

Mass Spectrometry Identification of BTF—In order to identify the 47- and 57-kDa proteins that bind to the BTE, the peak fractions from the BTE-affinity purification were pooled and visualized by Coomassie staining. The 47- and 57-kDa proteins were excised from the gel and digested with trypsin in situ. The tryptic peptides were extracted from the gel slices and analyzed by mass spectrometry. Fig. 7A shows the MALDI-TOF spec-
The MALDI-TOF identification of GABP\(^{a}\)/H\(^{9251}\) was confirmed by MS/MS sequencing of the parent ion mass 1139.6 

The MALDI-TOF identification of GABP\(^{a}\)/H\(^{9251}\) was confirmed by MS/MS sequencing. As shown in Fig. 7B, one sequence tag (LNQPELVAQK), corresponding to the 1139.6 tryptic peptide identified by MALDI-TOF (Fig. 7A), matched the human and mouse GABP\(^{a}\) proteins in the nonredundant data base, confirming the identity of the 57-kDa protein as GABP\(^{a}\).

**Western Blot Analysis of the Purified Fractions**—To confirm the MALDI-TOF and MS/MS identification of GABP\(^{a}\) and to determine whether the 47-kDa protein is GABP\(^{b}\), Western blots of the purified fractions with specific antibodies to GABP\(^{a}\) and \(\beta^{b}\) were performed. Western blot analysis with GABP\(^{a}\) antisera reveals that GABP\(^{a}\) is detected as a faint band (57 kDa) in GH3NE (50 \(\mu\)g), as observed previously (17). GABP\(^{a}\) is enriched in the 50\% ammonium sulfate fraction (50 \(\mu\)g) and further enriched in the peak fractions from the heparin-Sepharose (20 \(\mu\)g), Mono Q (8 \(\mu\)g), and BTE affinity (25 \(\mu\)l) purifications (Fig. 8). Similarly, Western blot analysis with specific antisera to GABP\(^{b}\) shows that expression of DNGABP\(^{b}\) was at least 10-fold lower than that of endogenous GABP\(^{b}\) (not shown). Results presented are a representative experiment from three transfections done in duplicate.

**Functional Analysis of GABP**—Previous studies (12, 17) have indicated that GABP\(^{a}\)/GABP\(^{b}\) binds to the EBS within the BTE; however, the functional role of GABP in growth...
factor- and basal-rPRL promoter activity at the BTE has been unclear and has been shown previously (12) to block insulin induction of the rPRL. The role of GABPα/GABPβ1 in basal and FGF-regulated rPRL promoter activity was tested by over-expression of a recently developed dominant-negative GABPβ1 (DN-GABPβ1) construct (31, 34). Deletion of the transactivation domain of GABPβ1 results in a dominant-negative effector of GABPα/GABPβ1-dependent responses. Specifically, GABPα, which lacks a transactivation domain, is still able to bind DNA and interact with DN-GABPβ1 but unable to activate transcription, because the transactivation domain of GABPβ1 is deleted (31, 34). Transfection of DN-GABPβ1 results in a highly specific and selective inhibitor of GABP-dependent responses because GABPα is the only known cofactor of GABPβ1 (35). Fig. 9 shows that co-transfection of DN-GABPβ1 blocks basal rPRL promoter activity by 85% and blocks rPRL promoter activity in the presence of FGF-4 by 88%. Of note, basal transcription of the ancestrally related and Pit-1-dependent rat growth hormone promoter is not affected by expression of DN-GABPβ1 (data not shown). Taken together, these data demonstrate that GABPα/β1 plays a key role in both basal and FGF-regulated rPRL promoter activity.

To determine the functional role of GABPα/GABPβ1 in the regulation of the rPRL promoter via the BTE, three or eight copies of the wild-type and three copies of the mutant BTE were cloned upstream of the unresponsive −36rPRL promoter and transfected into GHFT1 pituitary cells. As shown in Fig. 10, co-transfection of wild-type GABPα/GABPβ1 robustly activated both the (BTE)3 and (BTE)8 promoters 28- and 24-fold, respectively. Co-transfection of GABPα/GABPβ1 only modestly activated the (mutBTE)3 promoter and failed to activate the minimal −36rPRL promoter (Fig. 10). Thus, GABP activation of the BTE-containing promoters is dependent on exogenous GABP, the BTE, and an intact EBS within the BTE.

To test the specificity of Ets-factor activation of the BTE minimal promoter, other Ets factors that are expressed in pituitary cells were tested for their ability to activate the (BTE)3 minimal promoter in GH3 pituitary cells. As shown in Fig. 11A, the Ets factors Ets-1, Ets-2, ER81, Ehf, Elk-1, and Net failed to activate the (BTE)3 promoter, whereas co-transfection of GABPα/GABPβ1 activated the (BTE)3 promoter −6-fold. To ensure that the lack of Ets-factor activation of the (BTE)3 promoter was not due to lack of effector expression in GH3 cells, these same Ets constructs were transfected and tested for their ability to activate the proximal −425 rPRL promoter, which contains several consensus Ets-binding sites. Fig. 11B shows that Ets-1, Ets-2, GABP, and ER81 all activated the −425 rPRL promoter 1.5–4.5-fold, with the strongest activation mediated by GABP. However, as observed previously (23, 27), Net, Elk, and Ehf did not significantly activate the −425 rPRL promoter. Taken together, these data show that although several Ets factors, including GABP, Ets-1, Ets-2, and ER81, significantly activate the −425 rPRL promoter (Fig. 11), GABP is the only Ets factor capable of activating a minimal BTE promoter (Figs. 10 and 11). These data demonstrate that GABPα/GABPβ1 is the specific Ets factor that regulates basal rPRL promoter activity via the EBS within the BTE.

To establish further the role of GABP in the regulation of rPRL gene expression, small interfering RNA (siRNA) was used to knock down GABP protein levels, as described under “Experimental Procedures.” Because cycloheximide studies indicated that GABP protein turnover is prolonged (96 h, data not shown), extended siRNA incubations were necessary in order to reduce the expression of endogenous protein. Western blot analysis of GH3 cells transfected with GABP siRNA showed a significant reduction of GABPα protein levels by 30%.
at 168 h and by 50 and 60% at 216 and 240 h post-transfection, respectively (Fig. 12, A and C). To determine the effect of knocking down GABP on PRL expression, blots were probed with an antibody against PRL. Fig. 12, A and D, shows that PRL protein levels are reduced by 50, 70, and 80% at 168, 216, and 240 h post-transfection, respectively. GABPs or PRL protein levels were not affected by transfection of the control lamin A/C siRNA at any time point examined (Fig. 12, A and C). Because GABP also regulates the transcription of mitochondrial genes, the reduction of PRL expression by GABPs siRNA could be a nonspecific effect. To ensure the reduction in PRL expression by GABPs is specific, Western blots were probed with an antibody against α-tubulin. As shown in Fig. 12B, α-tubulin protein levels are unaffected by any siRNA condition tested, indicating that the reduction in PRL is specific and is not a result of depressed mitochondrial function. In conclusion, these data clearly show that knock down of GABPs results in a significant reduction of PRL protein levels, demonstrating a critical role for GABP in the regulation of PRL gene expression in GH3 pituitary cells.

DISCUSSION

This study demonstrates a key role for GABPs/β1 in the basal regulation of the rPRL promoter via the EBS in the BTE. Specifically, we used a biochemical approach to purify and identify the Ets factor that binds to the rPRL promoter BTE site, among the many Ets factors that are expressed in GH3 cells and have been shown to bind to the BTE. The data revealed that GABP is the selective and functionally relevant Ets member that binds to the BTE. GABP activation of the −36 rPRL promoter was dependent on the presence of transfected GABP, intact BTE control elements upstream of the minimal promoter, and an intact EBS within the BTE. Moreover, a dominant-negative GABPs/β1 construct almost completely blocked basal rPRL promoter activity but did not affect the basal activity of the evolutionarily related rGH promoter (Fig. 9 and data not shown). Furthermore, although several Ets factors activate the proximal −425 rPRL promoter in GH3 cells, GABPs/β1 was the only Ets member that significantly activated a minimal −36rPRL promoter containing multiple copies of the BTE. Finally, knock down of GABP using siRNA significantly and specifically reduced PRL protein levels.

The protein purification procedure used in the studies described here yielded an ∼25,000-fold purification with a cumulative yield of 5% (Table I), which was sufficient to identify the 57-kDa protein by MALDI-TOF and MS/MS sequencing as GABPs (Fig. 7). Of note, the sequence of rat GABPs has not yet been reported; however, the human and mouse isoforms are highly homologous. Insufficient purified material was obtained to identify the −47-kDa protein by MALDI-TOF or MS/MS sequencing, most likely due its wider range of elution (Fig. 5B). Nevertheless, we predicted the −47-kDa protein should be GABPs, because GABPβ is the only known cofactor of GABPs, and the predicted size of GABPs (48 kDa) is close to the calculated size of the −47-kDa purified protein (35, 36). The 47-kDa band was confirmed to be GABPβ by Western blot analysis (Fig. 8).

The identification of BTF as GABPs/GABPs/β1 is consistent

Western blots in A were stripped and re-probed for α-tubulin as described under "Experimental Procedures." C and D, graphical representation of siRNA knock down. The Western blots described in A and B were subjected to densitometry, and the GABPs and PRL levels were normalized to the α-tubulin levels. The percent of remaining GABPs and PRL was calculated based on the levels present in cells transfected with the lamin A/C oligonucleotide. Percentages reflect the average of three separate experiments, and error bars represent 1 S.D.
with the biochemical data presented in these and previous studies. GABPα/β is a ubiquitously expressed transcription factor (35, 36), and previous gel shift analysis with the BTE probe showed that BTE binding activity was present in both GH3 and HeLa nuclear extracts (17). These data indicated that BTF is not a pituitary-specific factor and suggested that BTF might be a ubiquitous protein (17). Additionally, previous antibody supershift studies showed that antisera to GABPα, -β1, and Ets-1 supershifted complexes containing BTF bound to the BTE probe (17). It is possible that the Ets-1 antibody is not as specific as previously thought and is able to recognize GABPα. Alternatively, small amounts of Ets-1 may bind to the BTE probe, but the level of binding could have been too weak to detect during the purification process. Finally, UV cross-linking studies with purified fractions show that only a protein of the predicted size of ~57 kDa specifically binds to the BTE probe (Fig. 6) (17), which is consistent with the ability of GABPα, but not GABPβ1, to bind DNA (37).

Although GABPα/β1 is ubiquitously expressed, we have shown that GABP is the Ets factor that binds to the BTE and contributes to the regulation of the tissue-specific expression of the rPRL gene. Indeed, GABPα/β1 has recently been shown to regulate the expression of a variety of tissue-specific genes. The mechanism of this tissue-specific regulation is unclear but is likely to involve co-regulation by other factors at adjacent DNA elements. For example, a functional interaction of GABP with C/EBPα is thought to contribute to the liver-specific expression of factor IX (38). Additionally, the association of GABP with c-Myb and C/EBPα is thought to regulate the expression of the neutrophil elastase gene in myeloid cells (39). With regard to the mechanism by which GABP regulates the pituitary-specific expression of the rPRL gene, it is possible that GABP interacts with other transcription factors that bind to adjacent and/or overlapping DNA elements. For example, GABP might interact with F2F, an FPII DNA binding activity that has yet to be identified (see Fig. 1) (7). Alternatively, GABP might interact with C/EBPα, which stimulates basal rPRL promoter activity and binds to DNA sequences overlapping the BTE (16).

The BTE site, however, not only contributes to basal rPRL promoter activity, it is also critical for a variety of hormonal and growth factor-regulated responses. The BTE contributes to the cAMP (1, 6, 8), FGF (10), insulin (11, 12), IGF-1 (13), epidermal growth factor (14), and thyroid-releasing hormone (15) responses. Although the role of the BTE site in mediating these responses is unquestionable, the identity of the precise transcription factors that transduce these signaling events directly through the BTE is less clear. The transcription factors that have been implicated in mediating the insulin response include GABP (11, 12) or Elk-1 in combination with C/EBPα (40). Here we present biochemical, transcriptional, and siRNA data that the BTE exhibits binding and functional selectivity for GABP. Taken together, these data suggest that the growth factor-dependent regulation of the rPRL promoter via the BTE is complex and may involve distinct Ets-factor binding to the BTE that is modulated by specific signaling pathways. Nevertheless, the studies presented here demonstrate a critical role for GABP in the basal regulation of the rPRL promoter.

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REFERENCES

1. Beech, C. A., and Gutierrez-Hartmann, A. (1989) Mol. Endocrinol. 3, 832–839
2. Gutierrez-Hartmann, A., Siddiqui, S., and Loukin, S. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5211–5215
3. Ryan, A., and Rosenfeld, M. (1997) Genes Dev. 11, 1207–1225
4. Theill, L., and Karram, M. (1995) Endocrinol. Rev. 16, 679–689
5. Treier, M., and Rosenfeld, M. (1996) Curr. Opin. Cell Biol. 8, 833–843
6. Iversen, R. A., Day, K. H., d’Emden, M., Day, R. N., and Maurer, R. A. (1990) Mol. Endocrinol. 4, 1564–1571
7. Jackson, S. M., Beech, C. A., Williamson, D. J., and Gutierrez-Hartmann, A. (1992) Mol. Cell. Biol. 12, 2708–2719
8. Liang, J., Kim, K. E., Schoderbeck, W. E., and Maurer, R. A. (1992) Mol. Endocrinol. 6, 885–892
9. Peers, B., Nalda, A. M., Monge, P., Vez, M. L., Belayew, A., and Martial, J. A. (1992) Eur. J. Biochem. 210, 55–58
10. Schweppe, R., Frazer-Abel, A., Gutierrez-Hartmann, A., and Bradford, A. (1997) J. Biol. Chem. 272, 30852–30859
11. Jacob, K., Ouyang, L., and Stanley, F. (1995) J. Biol. Chem. 270, 27773–27779
12. Ouyang, L., Jacob, K., and Stanley, F. (1996) J. Biol. Chem. 271, 10425–10428
13. Castillo, A., Tolon, R., and Aranda, A. (1998) Oncogene 16, 1881–1911
14. Jacob, K., Winingier, E., DeMimmi, K., and Stanley, F. (1999) Mol. Cell. Endocrinol. 152, 137–145
15. Wang, Y.-H., and Maurer, R. (1999) Mol. Endocrinol. 13, 1094–1104
16. Jacob, K., and Stanley, F. (1999) Endocrinology 140, 4542–4550
17. Schweppe, R., and Gutierrez-Hartmann, A. (2001) Nucleic Acids Res. 29, 1251–1260
18. Graves, B., and Petersen, J. (1998) Adv. Cancer Res. 75, 1–55
19. Wasylyk, B., and Norheim, A. (1987) in Transcription Factors in Eukaryotes (Papavassiliou, A. G., ed) pp. 251–284, Landes Bioscience, Austin, TX
20. Wasylyk, B., Hagman, J., and Gutierrez-Hartmann, A. (1998) Trends Biochem. Sci. 23, 213–216
21. Graves, B. (1998) Science 279, 1000–1002
22. Bradford, A., Wasylyk, C., Wasylyk, B., and Gutierrez-Hartmann, A. (1997) Mol. Cell. Biol. 17, 1065–1074
23. Bradford, A., Conrad, K., Tran, P., Ostrowski, M., and Gutierrez-Hartmann, A. (1996) J. Biol. Chem. 271, 24639–24648
24. Dignam, J., Lebovitz, R., and Roeder, R. (1983) Nucleic Acids Res. 11, 1475–1489
25. Lewis, T., Hunt, J., Aveline, L., Jonscher, K., Louie, D., Yeh, J., Resing, K., and Ahn, N. (2000) Mol. Cell. 6, 1343–1354
26. Eng, J., McCormack, A., and Yates, J., III (1994) J. Am. Soc. Mass Spectrum. 13, 976–989
27. Conrad, K. E., Oberwetter, J. M., Vallancourt, R., Johnson, G. L., and Gutierrez-Hartmann, A. (1994) Mol. Cell. Biol. 14, 1553–1565
28. Bochtler, M., Reinhaut, L., Sun, L.-Y., and Burret, F. (1998) Biochem. Biophys. Res. Commun. 246, 176–181
29. LaMarco, K., and McKnight, S. (1989) Genes Dev. 3, 1372–1383
30. Janke, N. (1996) Mol. Cell. Biol. 16, 1550–1556
31. Schaeffer, L., Ducert, N., Huchet-Deumas, M., and Changeux, J.-P. (1998) EMBO J. 17, 3078–3090
32. Elbashir, S., Harborth, J., Landeckel, W., Yalcin, A., Weber, K., and Tuschi, T. (2001) Nature 411, 494–498
33. Stanley, F. M. (1992) J. Biol. Chem. 267, 16719–16726
34. Bruguet, A., and Ruegg, M. (2000) J. Neurosci. 20, 5989–5996
35. Brown, T., and McKnight, S. (1992) Genes Dev. 6, 2502–2512
36. de la Brousse, F., Birkenmeier, E. T., King, D., Rowe, M., and McKnight, S. (1994) Genes Dev. 8, 1853–1865
37. Thompson, C., Brown, T., and McKnight, S. (1991) Science 253, 762–768
38. Boccia, L., Liliacer, D., Newcombe, K., and Mueller, C. (1996) Mol. Cell. Biol. 16, 1929–1935
39. Nishigrayou, I., Simkevich, C., Luo, M., Friedman, A., and Rosmarin, A. (1997) Blood 89, 4546–4554
40. Jacob, K., and Stanley, F. (2001) J. Biol. Chem. 276, 24931–24936
Purification and Mass Spectrometric Identification of GA-binding Protein (GABP) as the Functional Pituitary Ets Factor Binding to the Basal Transcription Element of the Prolactin Promoter

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