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ZBP-89, a Krüppel-Like Zinc Finger Protein, Inhibits Epidermal Growth Factor Induction of the Gastrin Promoter

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We have shown previously that a GC-rich element (GGGGCGGGGTGGGGGG) conferring epidermal growth factor (EGF) responsiveness to the human gastrin promoter binds Sp1 and additional undefined complexes. A rat GH_4 cell line expression library was screened by using a multimer of the gastrin EGF response element, and three overlapping cDNA clones were identified. The full-length rat cDNA encoded an 89-kDa zinc finger protein (ZBP-89) that was 89% identical to a 49-kDa human factor, htβ, that binds a GTGGG/CACCC element in T-cell receptor promoters. The conservation of amino acids between the zinc fingers indicates that ZBP-89 is a member of the C_2H_2 zinc finger family subclass typified by the *Drosophila* Krüppel protein. ZBP-89 is ubiquitously expressed in normal adult tissues. It binds specifically to the gastrin EGF response element and inhibits EGF induction of the gastrin promoter. Collectively, these results demonstrate that ZBP-89 functions as a repressor of basal and inducible expression of the gastrin gene.

The hormone gastrin stimulates both gastric acid secretion and gastric epithelial cell growth (36). Its expression and secretion in the gastric epithelium are tightly controlled by fluctuations in gastric pH. In addition, the tissue-specific expression of gastrin in the stomach antrum, duodenum, and fetal pancreas is developmentally regulated. Gastrin is expressed initially in the fetal pancreas, but after birth pancreatic expression is inhibited and expression in the stomach is activated (6). The fetal pattern of gastrin expression is recapitulated in islet cell tumors of the pancreas and duodenum, which express large quantities of gastrin as part of the multiple endocrine neoplasia syndrome (16). Thus, positive and negative regulatory mechanisms play important roles in the normal and pathologic expression of the gastrin gene.

Several DNA elements have been shown to regulate the positive and negative expression of the gastrin promoter. Studies of the human gastrin promoter in islet tumor cell lines identified a negative element (GasNE) at −100 to −93 that is also present in the beta interferon promoter (37). Flanking this element is an E-box motif that binds helix-loop-helix factors and functions as a positive element (35). In addition, we and others have identified a GC-rich element at −68 to −53, GGG GCGGGGTGGGGGG, that mediates both activation and repression of the promoter as well as inducible control by epidermal growth factor (EGF) and phorbol esters (8, 23). A minimum of three complexes including Sp1 bind to this element, designated the gastrin EGF response element (gERE) (24). Although Sp1 binds to the 5′ half-site of gERE, the other factors that recognize this element have not been characterized.

To identify other DNA-binding proteins capable of binding to gERE, an expression library was screened with a multimer of this DNA binding site. The three clones identified were closely related to a 49-kDa zinc finger protein called htβ (38). The htβ factor binds elements within two different T-cell receptor promoters and was isolated from a T-cell library by using a similar GC-rich element (GAAGTTGGGGGTGGTG). The alternative splice product reported here is an 89-kDa zinc finger-binding protein that is nearly twice the size predicted for htβ and is designated ZBP-89. Competition for binding of ZBP-89 by using a mutation of the gERE element demonstrated that ZBP-89 binds primarily to the 5′ half-site of gERE. In contrast to the modest T-cell receptor promoter activation observed with htβ, coexpression of ZBP-89 with a gastrin reporter construct specifically inhibited EGF induction of the gastrin promoter.

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MATERIALS AND METHODS

Expression cloning of ZBP-89. A λ Zap expression cDNA library was prepared with mRNA from a rat pituitary adenoma cell line (GH_4) (pBK phagemid vectors; Stratagene). The library represented cDNAs produced by both random and oligo(dT)-primed mRNA. The unamplified library was screened by the method of Singh et al. (32, 33) without guanidine HCl denaturation-renaturation. A multimerized gERE (GGGGCGGGGTGGGGGG) was labeled with [^{32}P]dCTP by using the Nick Translation System (Gibco-BRL). Three specific clones were identified after screening of 2.5×10^6 plaques plated at a density of 5×10^3 PFU per filter. The pBKCMV phagemid was excised from the λ Zap Express vector by helper phage coinfection (R408 strain). Single-stranded phagemids were rescued from *Escherichia coli* XLOR and subsequently transfected into *E. coli* DH5α to recover a double-stranded phagemid. Dideoxy sequencing of both the sense and antisense strands by using Sequenase (United States Biochemical Corporation) was carried out by creating specific oligonucleotides based on the new sequence.

Southern blot analysis. Genomic DNA isolated from GH_4 cells was digested with *Ava*I, *Bam*HI, *Sca*I, *Pst*I, *Eco*RI, *Hin*dIII, *Xba*I, and *Xho*I. Genomic DNA from pooled human leukocytes (Boehringer Mannheim) was digested with *Ava*I, *Bam*HI, *Sca*I, *Pst*I, *Eco*RI, and *Xba*I. Digests were resolved on a 0.6% agarose gel and transferred to a nylon membrane (Hybond-N$^+$; Amersham). Hybridization of blotted DNA to a radiolabeled 340-bp *Eco*RV-*Afl*II fragment of ZBP-89 (10^6 cpm/ml; Rediprime kit; Amersham) was carried out at 42°C overnight in hybridization solution consisting of 5× SSC (750 mM NaCl, 75 mM sodium citrate [pH 7.0]), 50 mM $NaPO_4$ (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 2.5× Denhardt's solution (0.05% bovine serum albumin, 0.05% Ficoll, 0.05% polyvinylpyrrolidone), 1 mM Na_2EDTA, and 200 mg of salmon sperm DNA per ml. The blot was washed three times in 0.1% SDS–0.2× SSC at 65°C.

Northern (RNA) blot analysis. mRNA was prepared by using the PolyATract system (Promega) after isolating total RNA from GH_4 cells by using TRIzol reagent (Gibco-BRL). Multiple tissue rat and mouse embryo Northern blots were purchased from Clontech. mRNA from GH_4 cells was resolved on a 1% agarose-morpholinepropanesulfonic acid-formaldehyde gel, transferred to a Hy-

bond-N^+ nylon membrane, and hybridized at 55°C overnight to the radiolabeled *Eco*RV-*Afl*II fragment in 0.1% SDS–50 mM $NaPO_4$ (pH 6.5)–5× SSC–1 mM Na_2EDTA–200 mg of salmon sperm DNA per ml–2.5× Denhardt's solution. Washes were performed at 55°C in 1% SDS–2× SSC. A riboprobe was prepared by using a truncated ZBP-89-pBKCMV expression vector as a template restricted with *Eco*RV and primed from the T7 promoter. A 2-kb human β-actin cDNA probe was labeled by random priming using the Rediprime kit. The multiple tissue blots containing 2 μg of mRNA per lane were first hybridized to the β-actin probe overnight at 55°C and then washed at 55°C in 1% SDS–2× SSC. After autoradiography, the blots were stripped and then reprobed overnight at 65°C with a riboprobe complementary to the coding sequence of ZBP-89 in 750 mM NaCl–50 mM NaH_2PO_4–5 mM Na_2EDTA (pH 7.4)–10× Denhardt's solution–100 mg of salmon sperm DNA per ml–2% SDS. Washes were performed at 65°C in 0.1% SDS–0.2× SSC.

Plasmid constructions. For expression in GH_4 cells, the pBKCMV expression vector containing the ZBP-89 cDNA was modified by restricting with *Nhe*I and *Afl*II to remove the *lacZ* promoter and 5′ untranslated regions (UTRs). The pBKCMV vector alone was modified by restricting with *Nhe*I and *Eco*RI to remove the *lacZ* promoter. For bacterial expression of the truncated ZBP-89 forms, 1.5- and 1.9-kb *Afl*II-*Eco*RI fragments were isolated from the unmodified pBKCMV expression vectors (designated B59 and B22) and subcloned into the *Sma*I and *Eco*RI sites of pGEX-3X (Pharmacia). The full-length ZBP-89 was isolated as a 3.5-kb *Afl*II-*Hin*dIII fragment from the unmodified pBKCMV vector and subcloned into the *Ecl* 136 and *Hin*dIII sites of pGEX-KG (14). All constructs were verified by restriction analysis and dideoxy sequencing. A gastrin reporter construct (240 GASLuc) was created by inserting 240 bp of 5′ flanking sequence including the human gastrin promoter and first exon upstream of the promoterless luciferase plasmid pGL2-B (Promega) as previously described (31).

Expression of fusion proteins. ZBP-89, B59, and B22 were in vitro transcribed and translated from the pBKCMV vector as LacZ fusion proteins using the TNT Coupled Reticulocyte Lysate System (Promega). The expressed products were labeled with [^{35}S]methionine and primed from the T3 promoter. The labeled products were resolved on an SDS–7.5% polyacrylamide gel. All three forms of ZBP-89 were expressed as glutathione *S*-transferase (GST) fusion products from the pGEX expression vectors used to transform *E. coli* BL21(DE3) cells (Novagen). One liter of cells was grown overnight at 25°C in Luria broth containing 50 mg of ampicillin per liter. Three hundred milliliters of the overnight culture was used to inoculate 1 liter of fresh medium. After the culture reached an optical density of 0.4 at 600 nm (1 h at 25°C), the culture was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for an additional hour at 25°C. The cells were collected by centrifugation, resuspended in 5 ml of phosphate-buffered saline (PBS) containing 1% Triton X-100, 1 mM dithiothreitol, aprotinin (2 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM sodium metabisulfite, and then lysed at 4°C by sonication with two 15-s bursts at a setting of 30% output. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min at 4°C, and the fusion protein was removed from the supernatant by affinity chromatography. A 0.5-ml slurry of the glutathione-Sepharose 4B beads (Pharmacia) suspended in PBS was added to the supernatant and incubated with gentle rocking for 40 min. The beads were collected by centrifugation at 500 × *g* for 5 min and then washed three times by resuspension of the pellet in ice-cold PBS. The fusion protein was eluted by incubation of the beads for 10 min at 25°C in 0.5 ml of a solution containing 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. The elution step was repeated two more times, and the volumes were combined. The protein concentration was determined by the method of Bradford (5). The average yield per liter of IPTG-induced culture was 0.5 mg of affinity-purified protein. The fusion protein was stored at 4°C until use.

Generation of polyclonal antibody. Antibody to the first 184 amino acids of htβ was generated by immunizing rabbits with a histidine-tagged fusion protein. The cDNA encoding these 184 amino acids (550 bp) was generated by reverse transcription-PCR (RT-PCR). Total RNA was extracted from HeLa cells by using RNA STAT-60 (Tel-Test "B", Inc.), and 20 μg of RNA was reverse transcribed by using a mixture of 0.5 μg of oligo(dT) primer (Gibco-BRL), 0.5 μg of random primers (Gibco-BRL), and 200 U of SuperScript RNase H reverse transcriptase (Gibco-BRL). One-twentieth of the reverse transcription product was used for the PCR performed with primer 1 (5′ATGAACATTGACGACAAACTGGAAGG) and primer 2 (5′ATAGTTCGTTCTAAAGGCAGCATTGC) to generate the first 550 bp of htβ cDNA starting at the first ATG. The 550-bp PCR product was ligated to pCRII (Invitrogen) to create pCRII-htβ and sequenced by using Sequenase.

The htβ (550-bp) fragment was subcloned into pQE-30 (Qiagen) to generate a histidine-tagged protein containing the first 184 amino acids of htβ. Briefly, pCRII-htβ was digested with *Bam*HI and *Nsi*I, the DNA insert was gel isolated and ligated into pQE-30 digested with *Bam*HI and *Pst*I, and the recombinant plasmid was used to transform M15 cells. Transformants were grown to an optical density of 0.7 at 600 nm. IPTG was added to a final concentration of 2 mM, and the cells were allowed to grow for an additional 4 h. The cells were harvested, sonicated (30-s bursts followed by 1 min of cooling, repeated four times), and centrifuged at 10,000 × *g* for 20 min. The supernatant was incubated with 4 ml of Ni^{2+}-agarose for 1 h, and the resin was washed with 40 volumes of buffer A (50 mM phosphate [pH 8.0], 300 mM NaCl) followed by 40 volumes of buffer B (50 mM phosphate [pH 6.8], 300 mM NaCl, 10% glycerol). Histidine-tagged htβ was eluted from Ni^{2+}-agarose with 160 mM imidazole and dialyzed against PBS.

Purified histidine-tagged htβ was injected subcutaneously into New Zealand White rabbits with TiterMax (Vaxcel, Inc.) as an adjuvant. After 4 weeks, a blood sample was assayed for antibody production. The rabbits were given booster injections, and after another 2 weeks blood samples were withdrawn. The specificity of the antisera was examined by immunoblot analysis.

Immunoblot analysis. Nuclear protein was prepared from GH_4, ColoDM (a human colonic cell line), 293T (a transformed human embryonic kidney cell line), and HeLa cell lysates by detergent extraction (30). Heat-denatured nuclear protein (100 μg) was electrophoretically separated on an SDS–7.5% polyacrylamide gel and electroblotted to a nitrocellulose membrane. The membrane was blocked for 1 h in 100 mM Tris-Cl (pH 7.5)–0.9% NaCl–0.1% Tween 20 (TTBS) containing 5% nonfat dry milk and then probed for 1 h at 25°C with a 1:1,000 dilution of htβ immune serum in the blocking buffer with agitation. The membrane was rinsed in TTBS then probed for 1 h at 25°C with a 1:1,000 dilution of donkey anti-rabbit immunoglobulin G linked to horseradish peroxidase in TTBS. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL kit; Amersham).

EMSAs. Double-stranded oligonucleotide cassettes corresponding to gERE were end labeled with [^{32}P]dCTP by using Klenow enzyme as described previously (23). Radiolabeled probe (30,000 cpm/0.1 ng) was incubated at 25°C with bacterially expressed ZBP-89 or affinity-purified Sp1 (Promega) in a final volume of 20 μl containing electrophoretic mobility shift assay (EMSA) buffer (10 mM Tris-HCl [pH 7.9], 1 mM $ZnCl_2$, 100 mM KCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol). Unlabeled human metallothionein IIa Sp1 element (hMTIIa Sp1), wild-type gERE, or gERE mutants M5 (GGCGCGGG GTGGGGGG) and M6 (GGGGCGGGGCGGGGCG) were used as competitors at 15× the molar concentration of the probe. Sp1 antiserum (Santa Cruz Biotechnology), htβ antiserum, or both combined were incubated with GH_4 extracts 15 min before addition of the probe. DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel containing 45 mM Tris base, 45 mM boric acid, and 1 mM Na_2EDTA.

Cell culture and transfections. GH_4 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 8% horse serum, 6% newborn calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 and 95% air. The cells were cotransfected with 3 μg of the gastrin reporter plasmid or 1 μg of the serum response element core TK-Luc (40) (a kind gift from Jeffrey Pessin, University of Iowa) and 5 μg of modified pBKCMV expression plasmids, using the DEAE-dextran method as previously described (23). Ten nanomolar EGF was added 24 h after transfection. Cell lysates were assayed in triplicate for luciferase activity 3 h after EGF treatment and assayed for protein by the Bradford method (5). Luciferase specific activity represents light units normalized to cell protein mass. To confirm expression of the transfected ZBP-89 cDNAs, GH_4 cells were electroporated with 20 μg of the modified pBKCMV expression plasmids at settings of 960 μF and 0.25 kV, using a Bio-Rad electroporator. Pooled stable transformants were selected by using 400 μg of G418 (Gibco-BRL) per ml. Nuclear extracts were prepared as previously described for immunoblot analysis.

Nucleotide sequence accession number. The ZBP-89 cDNA sequence has been submitted to GenBank under accession number U30381.

RESULTS

Molecular cloning of a gERE-binding protein. An unamplified phage expression library was prepared from mRNA isolated from GH_4 cells and screened by using a multimer of gERE. Three clones were sequenced after screening 2.5×10^6 phage plaques. Figure 1 shows the sequence of the full-length rat clone encoding ZBP-89 and two other clones containing C-terminal truncations. A poly(A) tail was not identified. The two additional clones were identical to ZBP-89 except for the sequentially shorter C-terminal domains and the absence of in-frame stop codons. Thus, the truncated forms of ZBP-89 probably originated from mRNA that was random primed at different positions along the C terminus. The first 143 nucleotides within the 5′ UTR of ZBP-89 differed significantly from one of the truncated cDNA clones, suggesting that this cDNA insert may be the product of alternative splicing. The open reading frame of ZBP-89 was 2,382 nucleotides long, which predicted a molecular mass of 89 kDa.

All three clones were 89% identical to the cDNA of a human CACCC element-binding protein called htβ (38). The open reading frame of htβ consists of 1,362 nucleotides that end at six in-frame stop codons. Thus, the predicted molecular mass of htβ is 49 kDa, slightly more than half the size of ZBP-89.

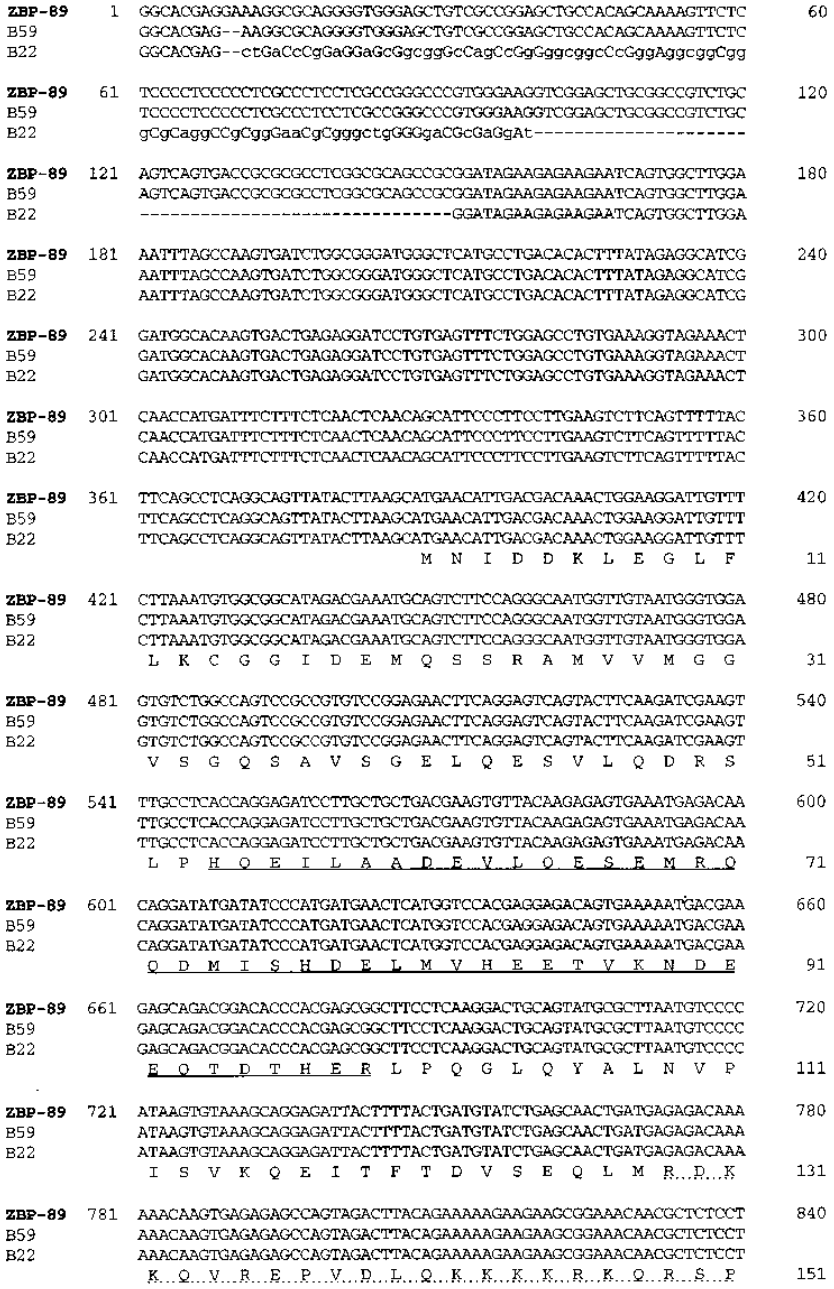

FIG. 1. Nucleotide sequences of ZBP-89, B59, and B22 and the corresponding amino acid sequences. The acidic (underline), two basic (dotted underline), and zinc finger (boldface) motifs are indicated. Differences in sequence between the B22 clone and the B59 and ZBP-89 clones are shown in lowercase. Absent nucleotides are indicated as dashes.

The discrepancy between the sizes of the proteins was due to two types of sequence variations (Fig. 2A). First, there were T-to-C transitions in several of the ZBP-89 codons that corresponded to stop codons in the human sequence. Second, two insertions at nucleotides 1647 and 1743 shifted the reading frame of ZBP-89. Thus, the terminal 35 amino acids within htβ differed significantly from ZBP-89 (Fig. 2B). The structural domains located within the first 330 amino acids consisted of an acidic, two basic, and four C_2H_2 Krüppel-type zinc finger motifs as described for htβ (Fig. 1 and 2C). Both of the basic domains contained nuclear localization signals at amino acids 141 to 148 and at 313 to 320 that are nearly identical to the nuclear localization signal (PKKKRKV) described for the simian virus 40 T antigen (10). Since the discrepancy between htβ and ZBP-89 was considerable, the sizes of ZBP-89 and the two cDNAs containing C-terminal truncations (B59 and B22) were confirmed by in vitro transcription-translation and in vivo after transfection into a transformed human embryonic kidney cell line (293T) (Fig. 3). All three clones migrated as doublets when translated in vitro, suggesting the presence of alternative translational start sites (Fig. 3A). The size of the major species detected by the htβ antibody was 89 kDa (Fig. 3B, lane 1). However, overexposure of the immunoblot revealed a doublet of ~50 to 60 kDa that correlated with the predicted size of htβ

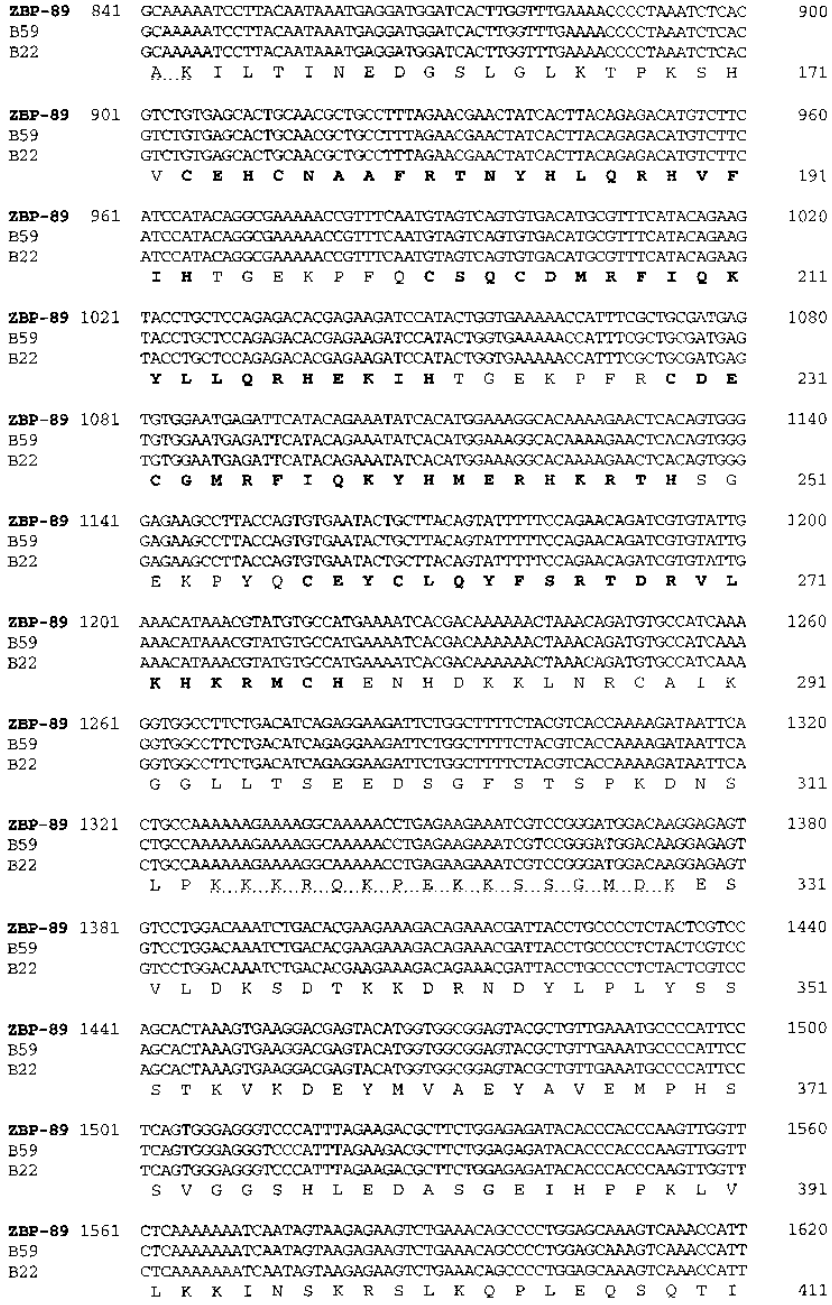

FIG. 1—*Continued.*

reported by Wang et al. (38). Transient transfection of each cDNA clone and detection of the expressed protein on immunoblots revealed the same pattern of expression predicted from in vitro translation (Fig. 3B). Thus, the molecular mass of ZBP-89 is about twice that of htβ.

To confirm the size of endogenous ZBP-89 in other cell lines, immunoblot analysis was performed on nuclear extracts from two additional human cell lines and GH_4 cells (Fig. 4). The results show that all three human cell lines express an 89-kDa protein. The predominant size of endogenous GH_4 ZBP-89 was ~120 kDa, and the protein comigrated with a similar species found in ColoDM cells. Therefore, the size of endogenous ZBP-89 was predominantly 89 kDa but varied as a function of cell type. The 120-kDa species may represent an alternative splice product, alternative start site, or posttranslational modification of ZBP-89.

Southern and Northern blot analyses. One major band and occasional minor bands were detected on Southern blots of both rat and human genomic DNA (data not shown). This result was consistent with the one major band detected on Southern blot analysis of human genomic DNA probed with htβ and suggests the presence of only one gene (38). Two transcripts corresponding to 4.1 and 7.4 kb were detected by Northern blot analysis of GH_4 mRNA (Fig. 5A). ZBP-89 mRNA was expressed by day 7 of mouse embryonic development and was ubiquitously expressed in adult tissues (Fig. 5B

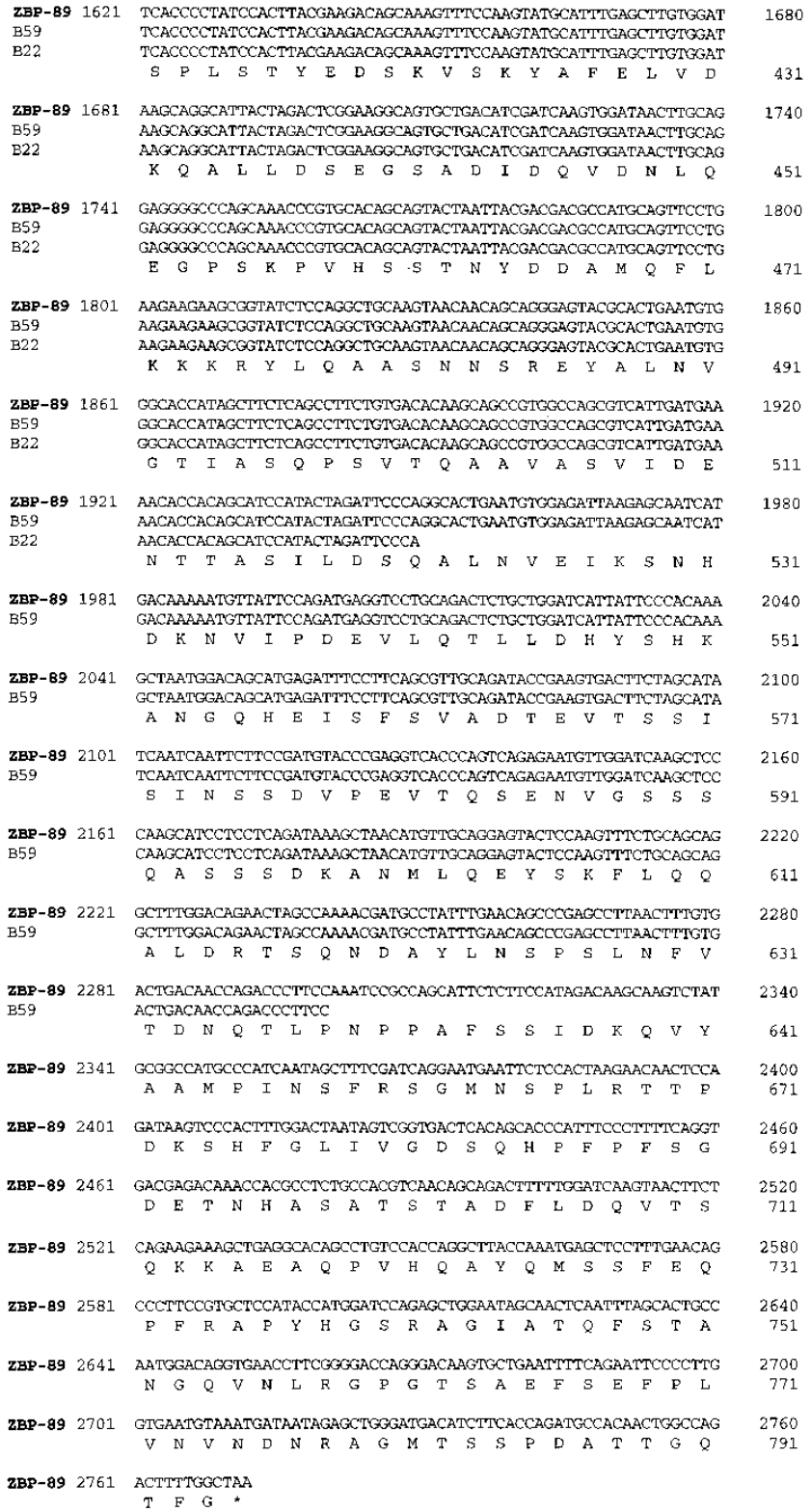

FIG. 1—*Continued.*

and C). However, several low-abundance transcripts smaller than 4.0 kb and larger than 7.5 kb were also detected upon overexposure. HeLa cells and three hematopoietic cell lines were used to study the expression of htβ and showed 4.2-, 7.6-, and 8.6-kb mRNA species of equivalent abundance (38).

ZBP-89 binds gERE. To determine the specificity of ZBP-89 binding to gERE, EMSAs with competition were performed with bacterially expressed ZBP-89 (Fig. 6). All three ZBP-89 fusion proteins bound gERE, and the binding was zinc dependent (Fig. 6A). It has previously been shown that the wild-type

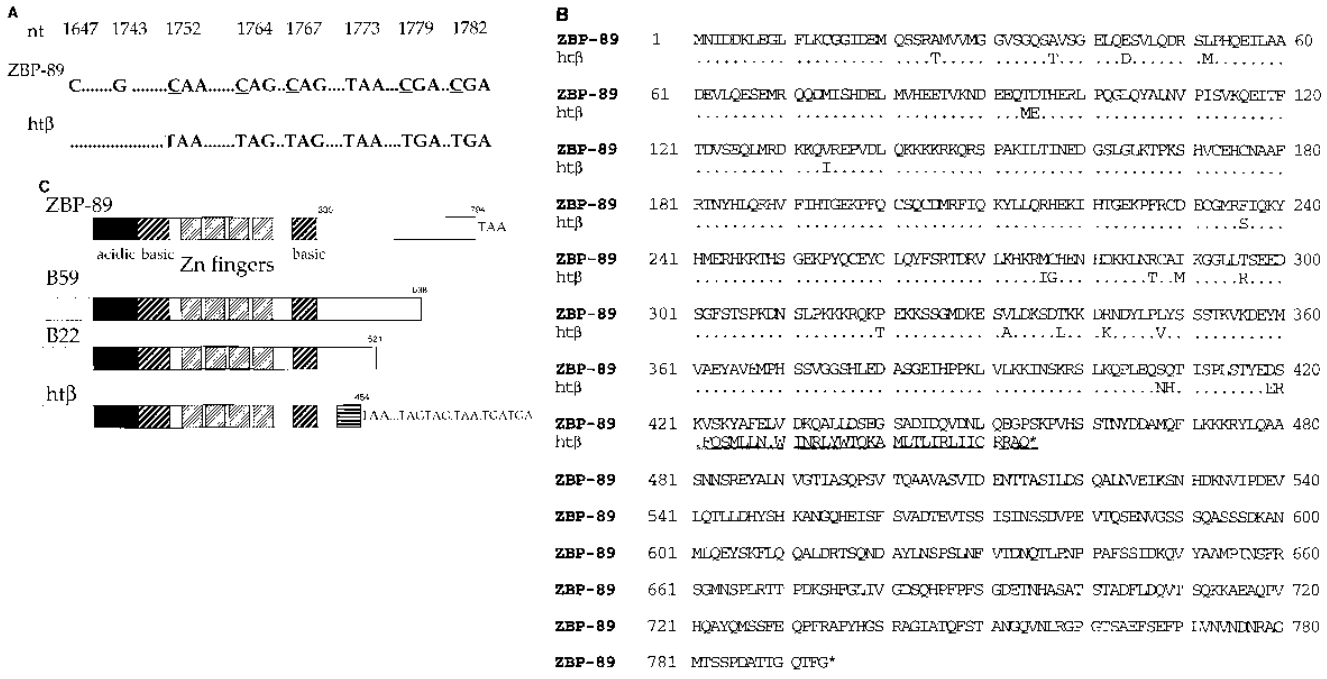

FIG. 2. (A) Comparison of C-terminal nucleotide differences between ZBP-89 and htβ. The T-to-C transversions in the rat sequence are underlined. Stippling represents breaks in the nucleotide sequence. (B) Comparison among the ZBP-89 and htβ amino acid sequences. Stippling indicates amino acid identity. The terminal 35 amino acids in htβ are underlined. *, location of stop codons. (C) Schematic representation (not to scale) of full-length ZBP-89, truncated forms, and htβ. The stop codons are shown. The acidic, basic, and zinc finger domains are labeled. The divergence in the amino acid sequences at the C terminus is indicated in htβ by horizontal lines.

gERE competes for complexes binding to both the 5′ and 3′ half-sites but that the hMTIIa Sp1 element does not compete for complexes occupying the 3′ half-site. The binding of GST–ZBP-89 fusion protein was competed for by gERE and M6 and slightly by the hMTIIa Sp1 element but less so by the M5 oligonucleotide containing 3′ base pair mutations. This result suggested that ZBP-89 preferentially bound to the 5′ half-site of gERE (Fig. 6B).

Competition for ZBP-89 by the 5′ half-site of gERE suggested that ZBP-89 might compete with Sp1 for binding to gERE. To test this hypothesis, Sp1 binding to gERE on EMSAs was competed for by increasing the concentration of ZBP-89 in the assay, and ZBP-89 binding was competed for by the addition of Sp1. The results show that both Sp1 and ZBP-89 competed for binding to gERE by the other protein; however, at least 50-fold more ZBP-89 was required to displace 50% of the Sp1 binding (Fig. 7).

Detection of endogenous ZBP-89 on EMSAs. To confirm the presence of endogenous ZBP-89 binding, htβ and Sp1 antisera were incubated with GH_4 nuclear extracts. Polyclonal htβ antiserum did not supershift the previously described gERE complexes, Sp1, or gastrin EGF response protein (gERP) 1 and gERP 2 on EMSAs (Fig. 8). However, it did specifically disrupt the binding of complexes 1, 2, and 3. The intensity of complex 1 decreased with htβ antiserum alone or with both antisera used together. This finding was consistent with the ability of the htβ antibody to disrupt rather than supershift cloned ZBP-89 (GST–ZBP-89). Furthermore, the result revealed that complex 1 is actually two different complexes, since only the upper portion of the complex was supershifted by Sp1 antibody, revealing another complex (1b) which decreased in intensity with the addition of htβ antiserum. Thus, Sp1 forms complex 1a and ZBP-89 forms complex 1b. Complexes 2 and 3 also contained ZBP-89 protein, since the binding activity of these complexes also decreased. Complexes 4 and 5 were not affected by either antiserum and have previously been designated gERP 1 and 2 (24).

ZBP-89 inhibits EGF induction of the gastrin gene. Since Sp1 has been shown to be important in conferring EGF induction, the EMSA results suggested that overexpression of ZBP-89 might regulate EGF induction of the gastrin promoter. To study the effect of ZBP-89 expression on the gastrin promoter in vivo, plasmids expressing ZBP-89 or the two trun-

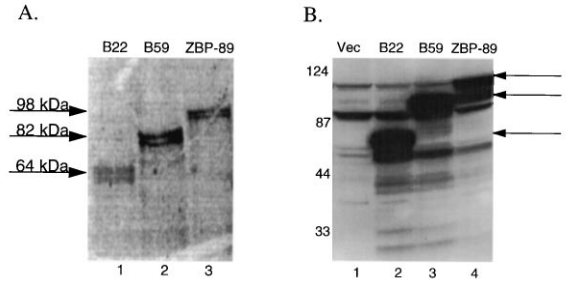

FIG. 3. (A) In vitro translation of ZBP-89–LacZ fusion proteins. In vitro transcription-translation of ZBP-89 cDNAs, including cDNAs for the truncated forms B59 and B22, was carried out as described in Materials and Methods. The [^{35}S]methionine-radiolabeled products were resolved on an SDS-polyacrylamide gel, dried, and then analyzed on a PhosphorImager (Molecular Dynamics). The molecular mass of each product is indicated. (B) Immunoblot of transfected ZBP-89 in mammalian cells. Each lane contains nuclear extract from 293T cells transiently transfected with 5 μg of expression vector by using $CaPO_4$ coprecipitation plus 100 μg of nuclear extracts from cells transfected with pBKCMV vector (lane 1), with the B22 cDNA (lane 2), with B59 (lane 3), or with ZBP-89 (lane 4). Nuclear extracts were resolved on an SDS–7.5% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and hybridized with a 1:1,000 dilution of htβ antiserum prior to chemiluminescence. Sizes are indicated in kilodaltons.

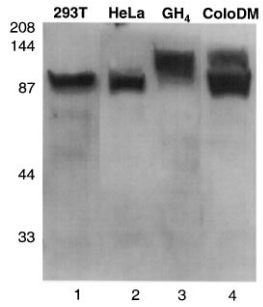

FIG. 4. Immunoblot of endogenous ZBP-89 expression in mammalian cells. Nuclear extracts from 293T, HeLa, GH_4, and ColoDM cells (lanes 1 to 4, respectively) were resolved on an SDS–4 to 15% polyacrylamide gradient gel and transferred to a polyvinylidene difluoride membrane. The blot was hybridized with a 1:1,000 dilution of htβ antiserum and detected by chemiluminescence.

cated forms (B59 and B22) from the cytomegalovirus promoter (pBKCMV) were cotransfected with the 240 GasLuc reporter constructs (Fig. 9). EGF stimulated gastrin promoter activity threefold in the presence of an empty expression vector. However, expression of full-length ZBP-89 depressed basal promoter activity and prevented significant induction by EGF, whereas expression of truncated ZBP-89 (B22) did not inhibit promoter induction. Although basal promoter activity decreased with cotransfection of B59, the promoter remained inducible by EGF. In contrast, coexpression of the ZBP-89 constructs had little effect on EGF induction of a heterologous promoter through the serum response element (Fig. 9). Thus, ZBP-89 abolished EGF induction of the gastrin promoter. To determine whether the ZBP-89 vectors were expressed in GH_4 cells, immunoblot analysis was performed on nuclear extracts from transfected cells. The results shown in Fig. 10 indicate that only the full-length ZBP-89 and B59 constructs were overexpressed. However, the B22 truncated form missing 273 C-terminal amino acids was poorly expressed in nuclear extracts. This result indicated that the absence of inhibition by the B22 construct correlated with poor nuclear expression.

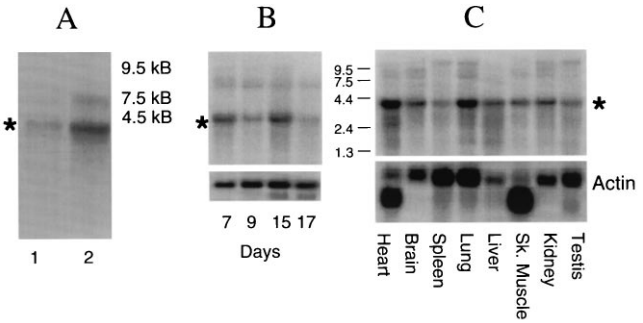

FIG. 5. Northern blot analysis. (A) Expression of ZBP-89 in GH_4 cells. Lanes 1 and 2, 6 and 12 μg, respectively, of mRNA from GH_4 cells. The Northern blot was hybridized to a radiolabeled ZBP-89 cDNA fragment. The 4.1-kb mRNA is indicated (*). (B) Expression of ZBP-89 in mouse whole embryos. The lanes contain 2 μg of mRNA isolated from 7-, 9-, 15-, and 17-day-old mouse embryos. The blot was hybridized to a β-actin cDNA probe at 55°C, stripped, and then hybridized at 65°C in 50% formamide to a riboprobe complementary to the B22 sequence. Sizes are indicated in kilobases. (C) The lanes contain 2 μg of mRNA isolated from rat heart, brain, spleen, lung, liver, skeletal (Sk.) muscle, kidney, and testis. The blot was probed with β-actin, stripped, and then reprobed with a riboprobe complementary to the B22 sequence.

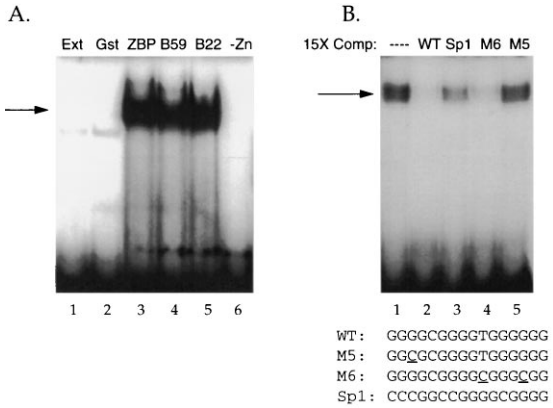

FIG. 6. ZBP-89 binding to gERE in EMSAs. EMSAs were performed as described in Materials and Methods, using a radiolabeled gERE probe at 30,000 cpm/0.1 ng and 0.5 μg of bacterially expressed protein. (A) Lane 1, bacterial extract alone; lane 2, GST vector alone; lane 3, GST–ZBP-89; lane 4, GST-B59; lane 5, GST-B22; lane 6, GST–ZBP-89 without 1 mM Zn^{2+}. (B) Competition for GST–ZBP-89 binding with no oligonucleotide competitor (lane 1) or 15× the molar competitor probe (Comp) concentration. Lane 2, wild-type gERE competitor (WT); lane 3, hMTIIa Sp1 competitor (Sp1); lane 4, gERE mutation 6 (M6); lane 5, gERE mutation 5 (M5). The arrow indicates the specific complexes formed by GST–ZBP-89 fusion protein and the gERE probe.

DISCUSSION

A GC-rich element that confers activation and repression on the gastrin promoter was used to screen an expression phage library. The three clones isolated were homologous to a human DNA-binding protein called htβ that binds to the consensus sequence GGGTGGGGG. The complementary strand is CCC CCACCC; therefore, htβ and presumably ZBP-89 are two of several CACCC element-binding proteins (38). Since the htβ-related factor ZBP-89 is not tissue specific and probably regulates a variety of genes through G-rich elements, we have named the factor ZBP-89, for 89-kDa zinc finger-binding protein, to emphasize structure rather than function. The results shown here indicate that ZBP-89 also preferentially recognizes the 5′ half-site of gERE and consequently is able to compete with Sp1 for binding to gERE. However, Sp1 has a greater affinity for the 5′ half-site of gERE, and the two proteins have similar relative migrations in EMSAs, which may explain why ZBP-89 is not readily detectable on gel shift assays. ZBP-89 depressed basal promoter activity and prevented the expected induction of the gastrin promoter by EGF. This result is in contrast to the modest effect of htβ on the T-cell receptor

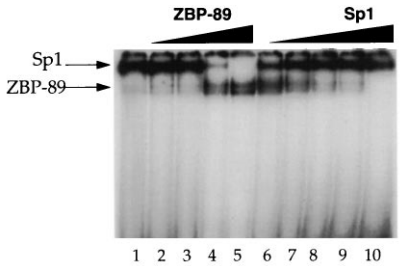

FIG. 7. Competition of Sp1 and ZBP-89 binding to gERE. The amount of affinity-purified Sp1 (lanes 1 to 5) or ZBP-89 (lanes 6 to 10) was held constant while various amounts of the other binding protein were added. Lanes 1 to 5 contain 5 ng of affinity-purified Sp1; 50, 100, 250, and 500 ng of ZBP-89 were added in lanes 2 to 5, respectively. Lanes 6 to 10 contain 100 ng of ZBP-89 to which 1, 5, 10, 25, and 50 ng, respectively, of Sp1 were added.

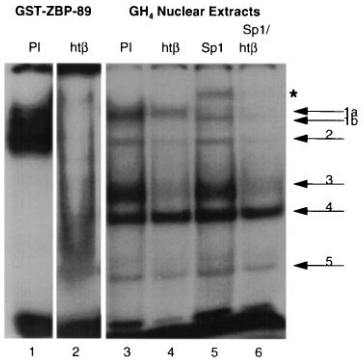

FIG. 8. Disruption of cloned and endogenous ZBP-89 binding by antisera. GST–ZBP-89 (25 ng) and GH_4 nuclear extracts (2 μg) were incubated for 15 min with 1 and 2 μl, respectively, of preimmune serum (PI; lanes 1 and 3) or 1 and 2 μl, respectively, of htβ immune serum (lanes 2 and 4). In addition, GH_4 nuclear extracts were incubated with 2 μl of Sp1 antibody (lane 5) or 1 μl of Sp1 and 1 μl of htβ antisera (lane 6). The extracts were then incubated with radiolabeled gERE probe and resolved on a 4% nondenaturing gel. The asterisk represents the Sp1 supershift. Complexes 1 through 5 are described in the text.

promoter. The discrepancy between the activities of the two factors may be related to the differences in the promoters and the cell lines used. Interestingly, the form of ZBP-89 missing the terminal 273 bp was poorly expressed in GH_4 cells. This truncated form contained putative nuclear localization signals but was missing a C-terminal serine-rich domain. Nevertheless, this result appears to be related to the cell type, since the same cDNA construct was well expressed in the 293T cell line.

ZBP-89 clearly differs from the htβ factor described by Wang and coworkers (38) in terms of its size and function. The immunoblot demonstrated that ZBP-89 is the predominant form of this zinc finger protein that is expressed in human cells and presumably reflects complex transcriptional and posttranscriptional regulatory mechanisms such as alternative splicing.

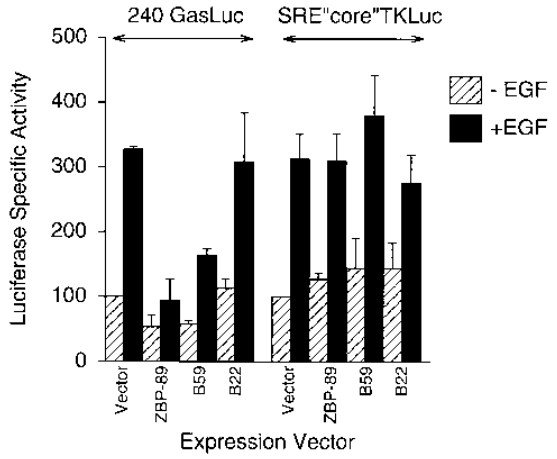

FIG. 9. EGF induction of gastrin promoter activity in the presence or absence of ZBP-89 expression vectors. The 240 GasLuc reporter construct containing the first 240 bp of the human gastrin promoter and the first exon were subcloned into a luciferase expression vector. pSRE-TK-Luc contains the core serum response element (SRE) from the *c-fos* promoter ligated upstream of the thymidine kinase promoter. The 240 GasLuc or pSRE core TK-Luc reporter constructs were cotransfected with 5 μg of pBKCMV alone or the ZBP-89 expression vectors. Twenty-four hours after transfection, the cells were stimulated with 10 nM EGF. Cell lysates were prepared 3 h after EGF treatment. The results are expressed as percentages of the luciferase specific activity (light units per milligram of protein) measured in the presence of the vector alone. The means of three experiments performed in triplicate ± standard errors are shown.

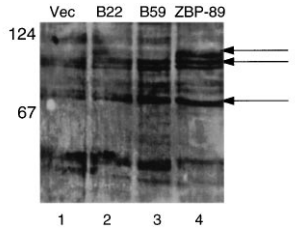

FIG. 10. Immunoblot of ZBP-89 expression in GH_4 cells. Twenty-microgram aliquots of ZBP-89 expression vectors were electroporated into GH_4 cells. Nuclear extracts were prepared from pooled stable transformants expressing the empty vector, B22, B59, or ZBP-89 and analyzed by immunoblot analysis. Two hundred micrograms of nuclear extract was denatured in Laemmli sample buffer and resolved on an SDS–7.5% polyacrylamide gel. Lane 1, extracts from cells transfected with the empty vector (Vec); lane 2, extracts from B22-transfected cells; lane 3, extracts from B59-transfected cells; lane 4, extracts from ZBP-89-transfected cells.

Other indicators of the existence of complex splicing mechanisms include the fact that the 5′ UTR of one of the clones has only 70% identity with ZBP-89 and htβ. Thus, the unique 5′ UTR may represent differential promoter usage or alternative splicing of a 5′ untranslated exon. Second, the locations of the stop codons identified in the htβ do not correspond to the location of the stop codon in ZBP-89. Both in vitro translation and in vivo immunodetection studies confirm that the size of ZBP-89 is 89 kDa. Thus, alternative splicing of a C-terminal exon would explain the difference in the predicted size of the htβ factor. Wang and coworkers concluded that the 7.6- and 8.6-kb mRNA species may be unspliced forms of the 4.2-kb mRNA. However, this statement does not adequately explain why the abundances of the three mRNA species were equivalent (38). Rather, the different mRNA species likely represent alternative splice variants. Alternative splicing of functional C-terminal domains is a common feature of some inducible transcription factors, e.g., NF-κB and STAT p91 (13, 29).

Mutational analysis of the EGF response element predicted that both half-sites are required to confer EGF induction (24). Both ZBP-89 and Sp1 were competed for by the same gERE mutation, suggesting that the two proteins may compete for binding to gERE. Since ZBP-89 does indeed compete with Sp1 for binding, this result is consistent with the ability of ZBP-89 to repress gastrin promoter induction. Furthermore, endogenous ZBP-89 binding to gERE comigrates with Sp1 on EMSAs, indicating the potential for in vivo competition between the two factors. The molecular mechanism of ZBP-89 inhibition is unknown but may represent competition with the transactivator Sp1 for binding to gERE, direct interaction with basal transcription factors through a specific domain, or a combination of both (20). The motifs of some *Drosophila* and mammalian transcriptional repressors include glutamine/proline-rich domains observed in the zinc finger proteins Krüppel and WT1 (the Wilms' tumor factor) and a polyalanine tract in the homeodomain factor even-skipped (4, 15, 22, 28). ZBP-89 does not contain polyglutamine, proline, or alanine tracts. Rather, putative functional domains within ZBP-89 are rich in glutamic acid (acidic domain), lysine (basic domains), and serine (C-terminal domain).

The serine-rich C-terminal domain of ZBP-89 is a 117-amino-acid region between amino acids 522 and 639. This serine-rich domain is not reminiscent of known amino acid motifs. However, 32 amino acids within this domain are 21% identical and 62% similar to an N-terminal domain within TAF110. Similarly, this domain is also 34% identical and 53% similar to a serine-rich motif within TFIIEβ. This observation may have

relevance since it has recently been shown that Krüppel regulates transcription through protein-protein interactions with TFIIB and TFIIEβ (27). In particular, a DNA-bound Krüppel dimer mediates transcriptional repression through its interaction with TFIIEβ.

The ZBP-89 factor is a member of the Krüppel zinc finger family. The highly conserved H-C links with the consensus motif TGEKPYX distinguish this group of transcription factors from other zinc finger proteins (7). Other members of this family include the Wilms' tumor gene product WT1, GLI, Sp1, Egr 1 and 2, EKLF, SRE-ZBP, and GATA-1, which bind the GC-rich sequences of growth-regulated genes (2, 9, 11, 17, 18, 21, 25, 34, 39). In specific instances, these factors have been shown to compete with Sp1 for binding to DNA (1, 19). Similarly, gERE consists of overlapping Sp1 and ZBP-89 binding sites. It is the overlapping sites, relative levels of Sp1 and ZBP-89 in the cell, and their relative affinities for DNA that likely determine the overall basal or inducible response of promoters that contain gERE. In addition to conferring inducible activation, G-rich elements are present in several tissue-specific promoters flanked by different transcription factor-binding motifs (3, 12, 26, 41). The flanking sequences within these motifs appear to modulate the function of this element, since these promoters are not known to be EGF responsive.

In summary, ZBP-89 is an alternative splice product of a CACCC-binding protein (htβ) that prevents the induction of gastrin by EGF. The mechanism of this repression may involve competition with the transactivator Sp1 or require novel transcriptionally active domains. ZBP-89 is ubiquitously expressed and likely regulates a variety of genes through G-rich elements. However, it remains to be determined how ZBP-89 exerts transcriptional repression or activation on other genes containing GGTGGG/CCCACC elements.

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REFERENCES

1. **Ackerman, S. L., A. G. Minden, G. T. Williams, C. Bobonis, and C.-Y. Yeung.** 1993. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc. Natl. Acad. Sci. USA **88:**7523–7527.
2. **Attar, R. M., and M. Z. Gilman.** 1992. Expression cloning of a novel zinc finger protein that binds to the c-*fos* serum response element. Mol. Cell. Biol. **12:**2432–2443.
3. **Bassel-Duby, R., M. D. Hernandez, M. A. Gonzalez, J. K. Krueger, and R. S. Williams.** 1992. A 40-kilodalton protein binds specifically to an upstream sequence element essential for muscle-specific transcription of the human myoglobin promoter. Mol. Cell. Biol. **12:**5024–5032.
4. **Biggin, M. D., and R. Tjian.** 1989. A purified Drosophila homeodomain protein represses transcription in vitro. Cell **58:**433–440.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brand, S. J., and P. J. Fuller.** 1988. Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. J. Biol. Chem. **263:**5341–5347.
7. **Bray, P., P. Lichter, H.-J. Thiesen, D. C. Ward, and I. B. Dawid.** 1991. Characterization and mapping of human genes encoding zinc finger proteins. Proc. Natl. Acad. Sci. USA **88:**9563–9567.
8. **Chung, D. C., S. J. Brand, and L. G. Tillotson.** 1995. Mutually exclusive interactions between factors binding to adjacent Sp1 and AT-rich elements regulate gastrin gene transcription in insulinoma cells. J. Biol. Chem. **270:** 8829–8836.
9. **Courey, A. J., and R. Tjian.** 1992. Mechanisms of transcriptional control as revealed by studies of human transcription factor Sp1, p. 743–789. *In* S. L. McKnight, and K. R. Yamamoto (ed.), Transcriptional regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10. **Dingwall, C., and R. A. Laskey.** 1991. Nuclear targeting sequences—a consensus? Trends Biochem. Sci. **16:**478–481.
11. **Drummond, I. A., S. L. Madden, P. Rohwer-Nutter, G. I. Bell, V. P. Sukhatme, and F. J. Rauscher III.** 1992. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. Science **257:** 674–678.
12. **Feo, S., V. Antona, G. Barbieri, R. Passantino, L. Calì, and A. Giallongo.** 1995. Transcription of the human β enolase gene (*ENO-3*) is regulated by an intronic muscle-specific enhancer that binds myocyte-specific enhancer factor 2 proteins and ubiquitous G-rich-box binding factors. Mol. Cell. Biol. **15:**5991–6002.
13. **Grumont, R. J., J. Fecondo, and S. Gerondakis.** 1994. Alternate RNA splicing of murine *nfkb1* generates a nuclear isoform of the p50 precursor NF-κB1 that can function as a transactivator of NF-κB-regulated transcription. Mol. Cell. Biol. **14:**8460–8470.
14. **Guan, K. L., and J. E. Dixon.** 1991. Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:**262–267.
15. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell **56:**573–583.
16. **Jensen, R. T., J. D. Gardner, J.-P. Raufman, S. J. Pandol, J. L. Doppman, and M. J. Collen.** 1983. Zollinger-Ellison syndrome: current concepts and management. Ann. Intern. Med. **98:**59–75.
17. **Kinzler, K. W., and B. Vogelstein.** 1990. The *GLI* gene encodes a nuclear protein which binds specific sequences in the human genome. Mol. Cell. Biol. **10:**634–642.
18. **Kitadai, Y., W. Yasui, H. Yokozaki, H. Kuniyasu, K. Haruma, G. Kajiyama, and E. Tahara.** 1992. The level of a transcription factor Sp1 is correlated with the expression of EGF receptor in human gastric carcinomas. Biochem. Biophys. Res. Commun. **189:**1342–1349.
19. **Lemaigre, F. P., D. A. Lafontaine, S. J. Courtois, S. M. Durviaux, and G. G. Rousseau.** 1990. Sp1 can displace GHF-1 from its distal binding site and stimulate transcription from the growth hormone gene promoter. Mol. Cell. Biol. **10:**1811–1814.
20. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59:**405–408.
21. **Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rauscher III.** 1991. Transcriptional repression mediated by the WT1 Wilms tumor gene product. Science **253:**1550–1553.
22. **Madden, S. L., and F. J. Rauscher III.** 1993. Positive and negative regulation of transcription and cell growth mediated by the EGR family of zinc-finger gene products. Ann. N. Y. Acad. Sci. **684:**75–84.
23. **Merchant, J. L., B. Demediuk, and S. J. Brand.** 1991. A GC-rich element confers epidermal growth factor responsiveness to transcription from the gastrin promoter. Mol. Cell. Biol. **11:**2686–2696.
24. **Merchant, J. L., A. Shiotani, E. Mortensen, D. Shumaker, and D. Abraczinskas.** 1995. EGF stimulation of the human gastrin promoter requires Sp1. J. Biol. Chem. **270:**6314–6319.
25. **Miller, I. J., and J. J. Bieker.** 1993. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the *Krüppel* family of nuclear proteins. Mol. Cell. Biol. **13:**2776–2786.
26. **Parmacek, M. S., H. S. Ip, F. Jung, T. Shen, J. F. Martin, A. J. Vora, E. N. Olson, and J. M. Leiden.** 1994. A novel myogenic regulatory circuit controls slow/cardiac troponin C gene transcription in skeletal muscle. Mol. Cell. Biol. **14:**1870–1885.
27. **Sauer, F., J. D. Fondell, Y. Ohkuma, R. G. Roeder, and H. Jackle.** 1995. Control of transcription by *Krüppel* through interactions with TFIIB and TFIIEβ. Nature (London) **375:**162–164.
28. **Sauer, F., and H. Jackle.** 1991. Concentration-dependent transcriptional activation or repression by Kruppel from a single binding site. Nature (London) **353:**563–566.
29. **Schindler, C., X. Y. Fu, T. Improta, R. Aebersold, and J. E. Darnell, Jr.** 1992. Proteins of transcription factor ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon α. Proc. Natl. Acad. Sci. USA **89:**7836–7839.
30. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17:**6420.
31. **Shiotani, A., and J. L. Merchant.** 1995. cAMP regulates gastrin gene expression. Am. J. Physiol. **269:**G458–G464.
32. **Singh, H., R. G. Clerc, and J. H. LeBowitz.** 1989. Molecular cloning of

sequence-specific DNA binding proteins using recognition site probes. BioTechniques **7:**252–261.
33. **Singh, H., J. H. LeBowitz, A. S. Baldwin, Jr., and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52:**415–423.
34. **Sukhatme, V. P., X. Cao, L. C. Chang, C.-H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. LeBeau, and E. D. Adamson.** 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53:**37–43.
35. **Tillotson, L. G., T. C. Wang, and S. J. Brand.** 1994. Activation of gastrin transcription in pancreatic insulinoma cells by a CACC promoter element and a 70-kDa sequence-specific DNA-binding protein. J. Biol. Chem. **269:** 2234–2240.
36. **Walsh, J. H.** 1994. Gastrointestinal hormones, p. 1–128. *In* L. R. Johnson, D. H. Alpers, J. Christensen, E. D. Jacobson, and J. H. Walsh (ed.), Physiology of the gastrointestinal tract. Raven Press, New York.
37. **Wang, T. C., and S. J. Brand.** 1990. Islet cell-specific regulatory domain in the gastrin promoter contains adjacent positive and negative DNA elements. J. Biol. Chem. **265:**8908–8914.
38. **Wang, Y., J. A. Kobori, and L. Hood.** 1993. The htβ gene encodes a novel CACCC box-binding protein that regulates T-cell receptor gene expression. Mol. Cell. Biol. **13:**5691–5701.
39. **Wu, Y., G. C. Fraizer, and G. F. Saunders.** 1995. GATA-1 transactivates the WT1 hematopoietic specific enhancer. J. Biol. Chem. **270:**5944–5949.
40. **Yamauchi, K., K. Holt, and J. E. Pessin.** 1993. Phosphatidylinositol 3-kinase functions upstream of Ras and Raf in mediating insulin stimulation of c-*fos* transcription. J. Biol. Chem. **268:**14597–14600.
41. **Yu, C.-Y., K. Motamed, J. Chen, A. D. Bailey, and C.-K. J. Shen.** 1991. The CACC box upstream of human embryonic epsilon globin gene binds Sp1 and is a functional promoter element in vitro and in vivo. J. Biol. Chem. **266:** 8907–8915.