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ZBP-99 Defines a Conserved Family of Transcription Factors and Regulates Ornithine Decarboxylase Gene Expression

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Among transcription factors that regulate ornithine decarboxylase (ODC) gene expression are those that interact with GC-rich promoters, including Sp1 and ZBP-89. Sp1 functions as a transactivator and ZBP-89 as a transrepressor of both the ODC and gastrin promoters. This study reports the cloning and characterization of a second member of the ZBP family that also binds GC boxes. ZBP-99 contains four Krüppel-type zinc fingers that collectively share 91% amino acid sequence similarity and 79% sequence identity with those found in ZBP-89. In addition, there are highly conserved amino acid sequences in the carboxy-terminal segments of the two genes. In spite of their structural similarities, the two proteins are encoded at distinct loci, ZBP-89 on chromosome 3q21 and ZBP-99 on 1q32.1. The predicted open reading frame of ZBP-99 cDNA encodes a 99-kDa protein. Electrophoretic mobility shift assays showed that ZBP-99 protein specifically binds to the GC-rich promoter elements of gastrin and ODC genes. Northern blot analysis showed that a major ZBP-99 transcript of 5.6 kb is expressed ubiquitously at low levels, with elevated expression levels in placenta and in adult kidney, liver, and lymphocytes. Cotransfection of AGS gastric adenocarcinoma and HT-29 colon adenocarcinoma cells with a ZBP-99 expression construct and with an ODC reporter construct show that ZBP-99 repressed basal expression in the two cell lines by 80 and 60%, respectively. Collectively, the data suggest that ZBP-99 binds GC-rich promoters and may complement the activities mediated by ZBP-89.

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Gene Database: ZBP-99 cDNA is GenBank Accession Number AF125158.

Abbreviations used: EMSA, electrophoretic mobility shift assay; FISH, fluorescence *in situ* hybridization analysis; ODC, ornithine decarboxylase; ZBP, zinc finger binding protein.

Key Words: **ZBP; gene family; cellular proliferation; ODC; zinc finger.**

ZBP-89 is the prototype of a novel class of transcription factors, phylogenetically conserved in mammals, that contain a characteristic array of four Krüppel type zinc fingers (1). ZBP-89 represses the expression of several genes, e.g., gastrin (1) and ODC (2), and concomitantly exerts a negative effect on cell proliferation rates in GH_4 cells (3). Recently, evidence that ZBP-89 may also act as a transcriptional activator has emerged (4–6). Altered expression of ZBP-89 in malignant cells (7) suggests that its regulatory effects on cell proliferation have biological significance. This was recently confirmed by the observation that ZBP-89 up-regulates the expression of stromelysin (matrix metalloproteinase-3), a factor whose activity is associated with tumor metastasis (5).

The regulatory effects of ZBP-89 are mediated, at least in part, by its binding to GC-rich promoter elements whose sequence also encompasses binding sites for other transcription factors, e.g., the Sp1 transcription factor family (1, 2). Another GC-rich binding protein, ZBP-99, is described here. Its structural and functional homology to ZBP-89 indicates that it is the second member of the ZBP gene family. Like ZBP-89, we hypothesize that ZBP-99 may play important roles in regulating cell proliferation, differentiation, and oncogenesis.

MATERIALS AND METHODS

cDNA library screening and clone characterization. A yeast one-hybrid screen was used to isolate ZBP-99 cDNAs from a Jurkat cell line library as previously described (2). Additional clones were isolated from two Stratagene (La Jolla, CA) cDNA libraries. The human fetal brain library (catalogue number 937227) contained oligo(dT)-primed, unidirectionally cloned, sequences, and the Jurkat T-cell

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leukemia library (catalogue number 936219) contained both oligo(dT)- and random-primed clones. Both libraries were constructed in lambda ZAP II, and *in vivo* excision of pBluescript phagemids from positive lambda clones was performed by the manufacturer's protocol. Each library had an average insert size of 1 kb and contained about 2×10^6 primary clones. To avoid cross-hybridization with conserved sequences, we amplified a unique segment of ZBP-99 cDNA. The 380-bp fragment encompasses a segment encoding amino acids 98-223, upstream of the zinc finger region. Analysis of human genomic southern blots showed that the probe detected only single copy sequences. The fragment was labeled using standard random priming conditions to a specific activity of about 10^9 cpm/μg. For screening, about 10^6 pfu were plated and duplicate Hybond N+ (Amersham; Piscataway, NJ) filter replicates were prepared. The filters were hybridized in 0.5 M sodium phosphate buffer, pH 7.5, 7% SDS, and 5 mM EDTA at 68°C for 18–36 h.

The ZBP-99 cDNA contig was complemented by electronic screening of expressed sequence tag (EST) databases. Unique sequences flanking the conserved zinc finger-encoding segment of ZBP-99 were used to query XREFdb (http://www.ncbi.nlm.nih.gov/XREFdb/; 8). EST clones 112831 (GenBank Accession Number AA297293) and 610235 (GenBank Accession Number AA176120) were identified with sequences upstream of the zinc finger domain; and clones 550274 (GenBank Accession Number AA085487) and 590764 (GenBank Accession Number AA158166) overlapped the downstream query sequence. The I.M.A.G.E. and T.I.G.R. consortia clones were obtained from Research Genetics (Huntsville, AL) and ATCC (Manassas, VA), respectively.

DNA sequencing and analysis. cDNA clones were sequenced using standard automated cycle sequencing with Applied Biosystems DNA Sequencers and dye-labeled terminators, according to the manufacturer's protocols. Sequences were aligned using MacVector version 6.5 (Oxford Molecular Group, Campbell, CA) and BLAST (9) analyses.

Somatic cell hybrid analysis. The National Institute of General Medical Sciences (NIGMS) human/rodent somatic cell hybrid mapping panel #2 (Coriell Institute, Camden, NJ) was analyzed by PCR analysis with primers based on human ZBP-99 cDNA sequence: 1034-FOR: 5′-GAGGACACATAGTGGAGAAAAGCC-3′ and 1345-REV: 5′-TCTACTGAGTAACTCT GCATGT-3′. A 312-bp amplimer resulted from both human and Chinese hamster control DNA templates. These could be discriminated by a hamster-specific Taq I restriction site that cleaved the amplimer into fragments of 209- and 103-bp. After analysis of the hybrid panel DNAs, the products were digested for 30 min with Taq I prior to analysis on 2.5% agarose gels. The amplification parameters were 94°C × 30 s, 60°C × 30 s, 72°C × 30 s for 35 cycles on a Perkin–Elmer 9600 thermal cycler.

Fluorescence in situ hybridization analysis (FISH). FISH was performed by Genome Systems, Inc. (St. Louis, MO) using standard procedures. Briefly, a bacteriophage P1 clone encompassing human ZBP-99 was labeled with digoxigenin-dUTP by nick translation, combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies and Texas Red avidin followed by counterstaining with DAPI. After initial localization to chromosome 1, a biotin-labeled probe specific for the heterochromatic region of chromosome 1 was co-hybridized with the labeled P1 clone to permit more precise localization of the ZBP-99 signal.

Northern blot analysis. RNA expression patterns were assayed using Clontech (Palo Alto, CA) poly (A)+ multiple tissue northerns, specifically the human 12-lane and human fetal II blots. The ZBP-99 specific PCR product used to screen the cDNA libraries (above) was also used for northern blot analysis. The random prime-labeled fragment (10^9 cpm/μg) was hybridized according to the manufacturer's suggestions. The blots were stripped and rehybridized with the β-actin fragment provided with the blots in order to normalize for RNA loading.

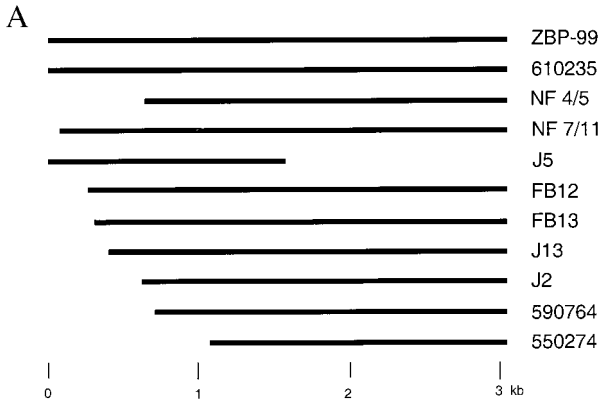

FIG. 1. Structural features of ZBP-99. (A) cDNA contig. DNA sequences from 10 independent clones were assembled into the ZBP-99 cDNA sequence. NF clones were isolated from a Jurkat library by a yeast one-hybrid screen. FB and J clones were isolated by hybridization from fetal brain and Jurkat libraries, respectively. The remaining clones were identified by electronic screening through the XREFdb. (B) cDNA sequence and predicted amino acid sequence. ZBP-99 cDNA (nucleotides 1-3029) and deduced amino acid (residues 1-895) sequences were derived by a minimum of 4 reads across the contig in panel A. ZBP-99 protein contains several structural domains, including glycine-rich (italics, underline), proline-rich (open rectangle), histidine repeat (bold), basic domains (dashed underline), and zinc finger region (shaded rectangle). (C) Comparison of conserved domains in ZBP-99 and ZBP-89. Comparison of human ZBP-89 (GenBank Accession No. AJ236885) and human ZBP-99 (GenBank Accession No. AF125158) amino acid sequences shows extensive homology within the zinc finger and carboxy-terminal regions. Sequence alignments were made using BLAST analysis.

Expression constructs. For expression of ZBP-99 in mammalian cells, the cDNA insert of clone 610726 was excised using restriction sites in the multiple cloning region of pBS II KS (Stratagene, La Jolla, CA), and inserted into pCMV-SPORT1 (Life Technologies, Gaithersburg, MD). Specifically, a 3-kb, *Sma*I + *Xho*I fragment, encompassing 30 nt of 5′-UTR, 2685 nt of coding, and about 300 nt of 3′-UTR, was ligated into similarly digested and gel-purified pCMV-SPORT1. This placed the ZBP-99 gene downstream of the CMV promoter. DNA sequencing was used to confirm insert orientation and integrity.

Histidine-tagged fusion protein constructs were made to express ZBP-99 protein in bacteria. In this case, the cDNA insert of clone 610235 was excised with *Xho* + *Eco*RI and was ligated into *Xho*I + *Eco*RI digested pET28c(+) (Novagen, Madison, WI) to generate an amino-terminal 6XHis- and T7-tagged construct. A truncated form of ZBP-99, spanning amino acids 1 to 489, was inserted into pET28b(+) using PCR adapter primers. Primers Z99-AA1/*Sal*I/For: 5′-TCCGTCGACATGAAAATCGGCAGTGGGTTC-3′ and Z99-AA489/*Not*I/Rev: 5′-TGCGGCCGCTCATCTATTCTGGTAATGGTG-3′ were used under high fidelity amplification conditions to generate a 1.5-kb amplimer. The *Sal*I + *Not*I digested insert was ligated into pET28b(+) to generate an amino-terminal tagged construct. This form is truncated downstream of the zinc finger encoding domain and was used for EMSA assays since it was expressed and purified more efficiently than was a full length construct. Fusion protein was isolated from IPTG-induced *E. coli* strain BL21(DE3) (Novagen, Madison, WI) using the His-Bind enrichment system (Pierce Biochemical, Rockford, IL).

B

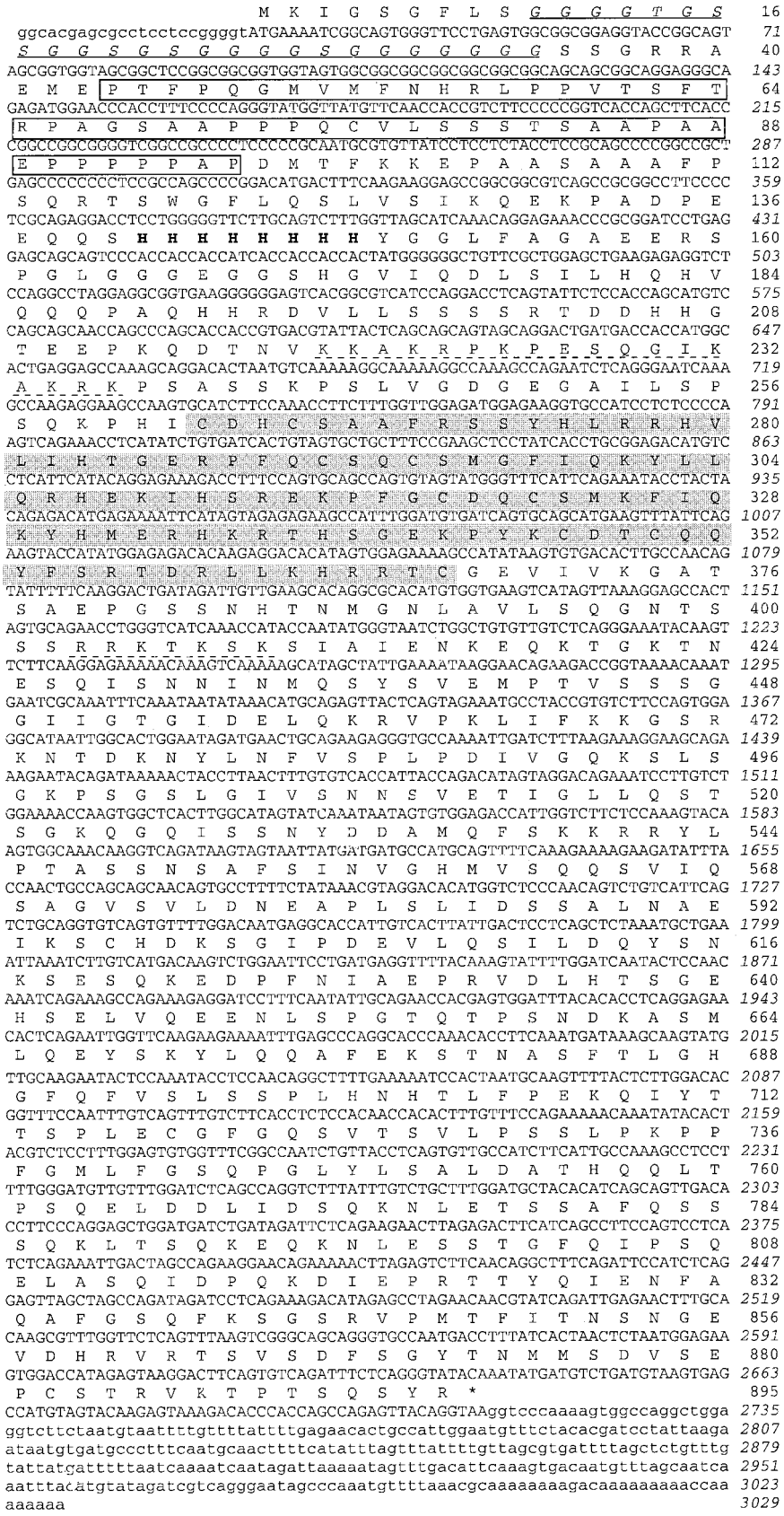

FIG. 1—*Continued*

C

ZINC FINGER REGION

79% identity, 91% similarity

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ZBP-99:  263 CDHCSAAFRSSYHLRRHVLIHTGERPFQCSQCSMGFIQKYLLQRHEKIHSREKPFGCDQC 322
             C+HC+AAFR++YHL+RHV IHTGE+PFQCSQC M FIQKYLLQRHEKIH+ EKPF CD+C
ZBP-89:  173 CEHCNAAFRTNYHLQRHVFIHTGEKPFQCSQCDMRFIQKYLLQRHEKIHTGEKPFRCDEC 232

ZBP-99:  323 SMKFIQKYHMERHKRTHSGEKPYKCDTCQQYFSRTDRLLKHRTCGE 369
              M+FIQKYHMERHKRTHSGEKPY+C+ C QYFSRTDR+LKH+R C E
ZBP-89:  233 GMRFIQKYHMERHKRTHSGEKPYQCEYCLQYFSRTDRVLKHKRMCHE 279
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CARBOXY-TERMINAL REGION

75% identity, 88% similarity

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ZBP-99:  528 SSNYDDAMQFSKKRRYLPTASSNS 551
             S+NYDDAMQF KK+RYL  AS+NS
ZBP-89:  461 STNYDDAMQFLKKKRYLQAASNNS 484
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49% identity, 68% similarity

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ZBP-99:  553 FSINVGHMVSQQSVIQSAGVSVLDNEAPLSLIDSSALNAEIKSCHD 598
             +++NVG + SQ SV Q+A  SV+D     S+++S ALN EIKS HD
ZBP-89:  487 YALNVGTIASQPSVTQAAVASVIDESTTASILESQALNVEIKSNHD 532

ZBP-99:  599 KSGIPDEVLQSILDQYSNKSESQKEDPFNIAEPRVDLHTSGEHSEL 644
             K+ IPDEVLQ++LD YS+K+  Q E  F++A+  V    S   SE+
ZBP-89:  533 KNVIPDEVLQTLLDHYSHKANGQHEISFSVADTEVTSSISINSSEV 578
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59% identity, 89% similarity

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ZBP-99:  654 TQTPSNDKASMLQEYSKYLQQAFEKST 680
             +Q  S+DKA+MLQEYSK+LQQA ++++
ZBP-89:  591 SQASSSDKANMLQEYSKFLQQALDRTS 617
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FIG. 1—*Continued*

Electrophoretic mobility shift assays. EMSA assays were performed as previously described (1; Merchant *et al.*). Briefly, double stranded oligonucleotide cassettes for the gastrin (gERE: =gatctGGGGCGGGGTGGGGGG) and ODC (ODC/−116 = gatctCCCCGCCCC TCCCCC) promoter elements were end-labeled with Klenow enzyme, and incubated with an enriched fraction of His-tagged ZBP-99 (1–489) fusion protein. Unlabeled gERE, ODC and Sp1 (gatctCCCGGCCGGGGCGGGG) were incubated at 200X molar probe excess in the competition experiments. The DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels.

Transient transfections. Human gastric adenocarcinoma cells (AGS) and colon adenocarcinoma cells (HT-29) were cultured in Dulbecco's Modified Essential Medium (DMEM) and McCoy's 5A media, respectively, as previously described (1). DNA was transiently transfected into sub-confluent monolayers using FuGENE 6 (BMB), according to the manufacturer's recommendations. Typically, 200 ng each of expression construct (pCMVSPORT-ZBP-99) and an ODC reporter containing 180 bp of human 5′-flanking sequence (p180ODCLuc; 3) were transfected per 35 mm well for 20 h. Basal expression was assessed in cells similarly transfected with the ODC reporter and empty pCMVSPORT vector. Extracts were prepared and assayed for luciferase expression as previously described (10).

ZBP-99 protein expression. ZBP-99 protein expression was assayed by immunoblot and immunofluorescence assays. Polyclonal anti-ZBP-99 sera were raised in New Zealand white rabbits using a multiple antigenic synthetic peptide encompassing eight copies of S-S-R-T-D-D-H-H-G-T-E-E-P-K-Q-D-T-N-V-K-K-A-K-R-P-K-P-E, a unique segment of ZBP-99 protein predicted to possess high hydrophilicity and antigenicity levels (MacVector v. 6.5, Oxford Molecular Group, Campbell, CA). Immunoblot analyses were performed as previously described (1).

RESULTS

Isolation of cDNA encoding a zinc finger protein that regulates ODC expression. We previously used yeast one-hybrid selection to identify factors that regulate ODC expression (2). A Jurkat human leukemia cDNA library was screened with a GC-rich ODC promoter element (GC^N; 2) as the target binding sequence. Among several independent clones isolated were two related zinc finger transcription factors, ZBP-89, described previously (1, 2, 10) and ZBP-99, reported here. Partial ZBP-99 cDNA sequence initially was obtained from overlapping clones NF 4/5 and NF 7/11 (2). A more complete cDNA contig (Fig. 1A) was developed by additional hybridization screening of human fetal brain and Jurkat cDNA libraries, as well as electronic screening of EST databases using XREFdb (8). Three additional Jurkat clones (J2, J5, J13), two fetal brain clones (FB12, FB13) and four EST clones (IMAGE clones 610235–GenBank AA176120; 590764–GenBank AA158166; and 550274–GenBank AA085487; and TIGR clone 112831–GenBank AA297293) were incorporated

into the cDNA contig (Fig. 1A) and the assembled cDNA sequence is shown in Fig. 1B.

The 3-kb cDNA contains an open reading frame encoding a protein with a predicted size of about 99 kDa, including the conserved zinc finger domain. In addition to its ability to bind to the ODC target sequence in a yeast one-hybrid screen, ZBP-99 shares certain structural features with ZBP-89. Each protein contains four Krüppel zinc fingers (three C2H2-type and one C2HC-type) encompassing 105 amino acids, and sharing 91% amino acid sequence similarity and 71% identity (Fig. 1C). The zinc finger segments of the two proteins are flanked by short domains comprised primarily of basic amino acid residues (Fig. 1B, dotted underlines). The two ZBP family members also share homologous domains (between 68 and 89 percent amino acid sequence similarity) within their carboxy terminal regions (Fig. 1C). Unique to ZBP-99 are glycine-rich, proline-rich and histidine-repeat domains in its amino terminus (Fig. 1A). Similar homopolymeric segments have been seen in other transcription factors, including members of the Maf (11) and class III POU gene families (12). Like ZBP-89, the most upstream methionine codon lacks a consensus Kozak sequence and repeated efforts to isolate additional 5′-flanking sequences were unsuccessful, possibly due to the high GC nucleotide content. A poly (A) tail is seen at the 3′-end of the cDNA, but a consensus poly (A) signal was absent. Collectively, the cDNA and predicted amino acid sequence comparisons suggest that ZBP-99 is the second member of what appears to be a unique gene family. Therefore, we identified the genomic location of ZBP-99 to establish its relationship to the ZBP-89 locus.

Genomic localization. To determine the genomic localization of the gene encoding ZBP-99, we developed specific PCR primers that did not cross amplify with ZBP-89 or other sequences. We tested the unique PCR primers on human and hamster control DNAs from the Coriell Institute somatic cell hybrid mapping panel. Amplimers resulted from both templates suggesting that, like ZBP-89, ZBP-99 is phylogenetically conserved among mammals. A *Taq* I site in the hamster amplimer was used to distinguish it from the human product. Only the chromosome 1 hybrid contained a Taq I-resistant product, suggesting that ZBP-99 is on human chromosome 1 (data not shown). This localization was confirmed and refined by FISH analysis using a human BAC clone encompassing ZBP-99. The ZBP-99 probe hybridized at the distal region of the long arm of chromosome 1 in 68 of 80 metaphase cells. Two-color analysis, with a ZBP-99 and a chromosome 1 centromere-specific probe, placed the ZBP-99 locus at band 1q32.1, 60% of the distance to the telomere (data not shown). Since we previously showed that ZBP-89 is located at chromosome 3q21 (10), this result showed that the two family members are encoded at distinct loci. To begin comparison of ZBP-89 and ZBP-99 function, we next examined the ZBP-99 RNA expression pattern.

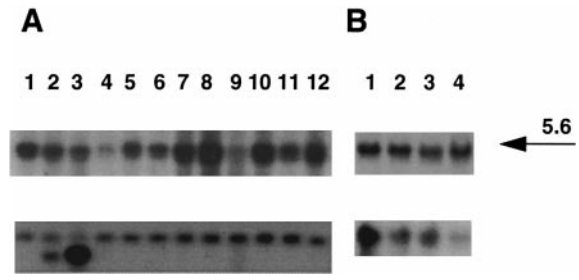

FIG. 2. Northern blot analysis of ZBP-99 mRNA expression patterns. Northern blots (Clontech) containing 2 μg of poly(A) RNA per lane were hybridized with a ZBP-99 probe (top panels), stripped, and re-hybridized with a β-actin probe (bottom panels; 2.0 and 1.8 kb transcripts). Panel A contains adult tissue RNA as follows: 1, brain; 2, heart; 3, skeletal muscle; 4, colon; 5, thymus; 6, spleen; 7, kidney; 8, liver; 9, small intestine; 10, placenta; 11, lung; 12, peripheral blood lymphocytes. Panel B contains fetal tissue RNA as follows: 1, brain; 2, lung; 3, liver; 4, kidney. The ZBP-99 transcript (arrow) is about 5.6 kb. The fetal Northern blot was exposed by overnight autoradiography, whereas the adult Northern blot required 2.5 days of autoradiography.

mRNA expression patterns. For Northern blot analysis, a ZBP-99-specific probe was developed from the region upstream of the highly conserved zinc finger domain. Southern blot analysis showed that this fragment detected single copy sequences, with no evidence of cross hybridization with ZBP-89 or other zinc finger genes (data not shown). The ZBP-99 probe was hybridized under high stringency conditions with multiple tissue northern blots. As shown in Fig. 2, ZBP-99 mRNA is ubiquitously expressed at low levels, with more abundant expression in placenta and adult kidney, liver and peripheral blood lymphocytes. The major transcript is about 5.6 kb suggesting that the cDNA sequence in Fig. 1 is missing about 2.5 kb of 5′ UTR. Additional expression data can be inferred from sequence homology with several ESTs (above), including 8-week embryo, HeLa cell, neuroepithelium and pancreas (8). This ubiquitous expression pattern was similar to that seen with 4.5 kb ZBP-89 transcript (1).

Protein expression. To further assess ZBP-99 structure and function, we examined its expression both *in vitro* and *in vivo.* We prepared a pET28(c)-ZBP-99 construct that encoded full length ZBP-99 with amino-terminal poly-histidine and T7 tags, such that the predicted fusion protein is about 102 kDa. After IPTG induction, bacterial protein extracts were subjected to nickel chelation chromatography to enrich for fusion protein. The source of *in vivo* expressed ZBP-99 was a nuclear protein extract of AGS cells. Immunoblot analysis of *in vitro* and *in vivo* expressed ZBP-99 is shown in Fig. 3. The eluate from nickel chelation chromatography (lane 1) is highly enriched for an ~100-kDa

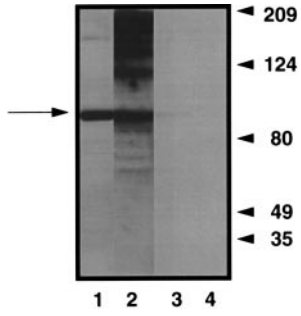

FIG. 3. Immunoblot detection of *in vitro* and *in vivo* expressed ZBP-99 protein. Rabbits were immunized with a multiple antigenic peptide encompassing amino acids 200-228 of ZBP-99 (MAP 200–228). An IgG-enriched fraction of the immune antiserum was diluted 200-fold prior to incubation with the electroblotted proteins. Secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG. Lanes 1 and 3 contain 1 μg of poly-histidine tagged bacterial fusion protein; lanes 2 and 4 contain 200 μg of AGS nuclear protein extract. Lanes 3 and 4 were incubated with immunodepleted ZBP-99 antiserum, i.e., after it was incubated with a 100-fold excess of MAP 200-228 and clarified by centrifugation with protein-A agarose. Small arrows denote molecular weight makers (kDa) and the large arrow indicates the major ~100 kDa ZBP-99 band.

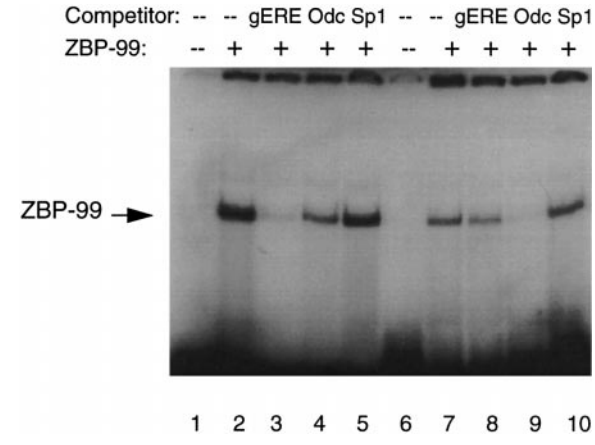

FIG. 4. ZBP-99 specifically binds to ODC and gastrin promoters. EMSA of ZBP-99 (1-489) binding to gERE (lanes 1–5) or the −116 to −110 ODC element (lanes 6–10). Lanes 1 and 6 contain extract from bacteria transformed with the empty pET vector. Lanes 2–5 and 7–10 contain extract from bacteria transformed with the ZBP-99 (1–489) expressed in the pET vector. The competitor nucleotides used (at 200×) include the gERE, ODC, and Sp1 elements. gERE = GGGGCGGGGTGGGGGG; ODC = CCCCGCCCCTCCCCC; and Sp1 = CCCGGCCGG GGCGGGG.

species, consistent with the predicted size of the fusion protein. The nuclear AGS extract (lane 2) contains multiple immunoreactive proteins, including one which co-migrates with the *in vitro* expressed protein. To better assess the specificity of the 100 kDa and larger proteins, we tested the extracts after depletion of the ZBP-99 antisera with an excess of purified ZBP-99 antigen, the MAP 200-208 peptide used to immunize the rabbits. Adding 100-fold antigen excess to IgG-enriched serum abolished its ability to detect the 100-kDa ZBP-99 protein in both bacterial (lane 3) and AGS (lane 4) extracts. In addition, immunodepletion removes reactivity with all the larger AGS proteins, suggesting that they are related to the 100-kDa species (lane 4). It is possible that this heterogeneity results from multiple transcriptional start sites in the ZBP-99 gene. Since efforts to amplify additional 5′ cDNA sequences have failed, we are investigating this possibility by analyzing genomic ZBP-99 sequence.

ZBP-99 binds ODC and gastrin promoter sequences. Since ZBP-99 was isolated by its ability to bind the GC-box of the ODC promoter, we confirmed the ability of the *in vitro* expressed ZBP-99 to bind ODC and gastrin promoter elements using electrophoretic mobility shift assays (EMSAs). As shown in Fig. 4, the zinc finger domain of ZBP-99 specifically binds both the gastrin (gERE; lanes 2–5) and ODC (lanes 7–10) promoter elements. Competition with unlabeled homologous oligonucleotides reduced the signal intensity for both probes (lanes 3 and 9). There is partial competition between the probes (lanes 4 and 8), and the Sp1 element (lanes 5 and 10) competes poorly for bound ZBP-99.

Regulatory effects of transiently expressed protein. Based on its structural similarity to ZBP-89 and its ability to bind the GC box in the ODC promoter, ZBP-99 may regulate ODC expression. ZBP-99 expressed from a CMV promoter was used to study the effect of ZBP-99 overexpression on ODC promoter activity in transiently transfected AGS and HT-29 cell lines. As shown in Fig. 5, co-expression of full-length ZBP-99 repressed basal ODC promoter activities in

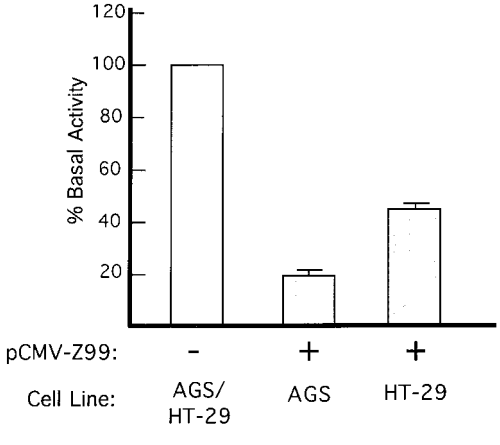

FIG. 5. ZBP-99 represses ODC expression in gastric and colon cells. AGS and HT-29 cells transiently cotransfected with the pCMVZBP-99 expression vector (+) and the pODC180Luc reporter construct. Basal expression levels (−) were assayed in cells cotransfected with empty pCMV and pODC180Luc. Relative light units were normalized for protein concentration and expressed as a percent of basal expression level. The mean ± SE for three independent experiments is shown.

both AGS and HT-29 cells by 80 and 60 percent respectively.

DISCUSSION

Overexpression of ODC is associated with increased cell proliferation and malignancy (13, 14). Accordingly, analysis of the regulation of ODC expression should yield insight into the processes that regulate both normal and neoplastic cell proliferation. Prior studies to identify transcription factors that bind to a GC-rich element in the ODC promoter revealed that the zinc finger protein, ZBP-89, represses the expression of ODC (2). ZBP-89 had also been shown to repress gastrin (1), a peptide hormone known to stimulate gastrointestinal cell growth. We describe here the cloning and characterization of ZBP-99, a second member of the ZBP gene family that also binds to GC-rich elements in the ODC and gastrin promoters. Moreover, both ZBP-89 and ZBP-99 similarly repress expression of their target genes.

Several structural and functional features of ZBP-99 establish it as a relative of ZBP-89, thereby defining the ZBP gene family. First, both proteins were isolated by their ability to bind to the GC box in the ODC promoter (2, this report). Comparison of their deduced amino acid sequences showed a striking degree of homology within the zinc finger domains (79% identity; 91% similarity), including the presence of three C2H2 fingers and a C2HC variant at zinc finger 4. Additional conservation was observed within similarly located basic amino acid domains flanking the zinc finger region and in the carboxy-terminal region that encompasses an activation domain in human (Law and Merchant, unpublished) and mouse (15) ZBP-89 homologues. The two family members are most divergent within their amino terminal regions, where ZBP-99 has unique glycine-, proline- and histidine-rich domains. ZBP-99 also contained a predicted tyrosine phosphorylation site between two basic domains downstream of the zinc finger region.

Interestingly, the antisense strand of ZBP-99 cDNA was highly homologous (90% sequence identity over 1606 nt) to the mouse interleukin 2 receptor mRNA (IL2R; GenBank Accession number M21977), suggesting that ZBP-99 and an IL2R-related gene might be overlapping and divergently transcribed, similar to the ORCTL2 and ORCTL2S genes (16). Regulated expression of the human IL2RA locus at chromosome 10p15–p14 is necessary to maintain regulated T cell proliferation (17), raising the possibility that ZBP-99 and an overlapping IL2R-related gene may coordinately regulate T cell proliferation. The precedent for ZBP gene involvement in T cell development was recently shown for ZBP-89, which activates the pre-T cell receptor alpha gene during T cell differentiation (4).

Initially, the striking homology within the zinc finger domains of ZBP-89 and ZBP-99 raised the possibility that the two variants could be encoded at the same locus (ZBP-89 maps to human chromosome 3q21). However, the subsequent identification of divergent sequences, and the localization of ZBP-99 to chromosome 1q32.1 suggest that the two genes arose from an ancestral gene by duplication and subsequent chromosomal translocation. Localization of ZBP-99 to chromosome 1q32.1 may reflect its biological significance in regulating genes involved in regulating lymphocyte proliferation (ZBP-99 was isolated from a Jurkat T-cell leukemia cDNA library), since this is a region frequently involved in chromosomal rearrangements in leukemia (18–20).

Additional ZBP-related loci have been identified in the mouse where the wild type homologue of ZBP-89, β-enolase repressor factor 1 (BERF-1), maps to chromosome 16 and a processed pseudogene variant, BERF-PS1, maps to chromosome 8 (21). We have shown that human ZBP-99 is expressed at both the mRNA and protein levels, thus excluding the possibility that it is merely a pseudogene derivative from ZBP-89.

Apart from the structural similarities, ZBP-99 shares several functional attributes with ZBP-89. The mRNAs of both family members are expressed ubiquitously at low levels, with modulated expression in a limited number of tissues and developmental stages. The major ZBP-99 transcript is 5.6 kb while the major ZBP-89 transcript is 4.5 kb. ZBP-99 mRNA is more abundantly expressed in a variety of fetal tissues, and ZBP-99 cDNA is present in an 8-week human whole embryo library (Law, D. and Merchant, unpublished). Moreover, the ZBP-99 transient transfection data show that ZBP family members isolated to date share the ability to repress the expression of genes that, in turn, stimulate cell proliferation. This suggests that one of the main biological functions of ZBP-99 (and ZBP-89) is to keep cellular proliferation rates in check. Collectively, these results suggest that ZBP-99 may regulate the expression of genes controlling both differentiation as well as cell proliferation.

The ability of ZBP-99 to bind to the GC-box of the ODC promoter and the loss of transrepression effects when this sequence is mutated, together, suggest that the regulatory effects of ZBP-99 are mediated, at least in part, by specific binding to GC-rich promoter elements in the ODC gene. Currently we are investigating whether all four zinc fingers are involved in DNA binding or whether one or more fingers may be involved in protein-protein interactions. Preliminary analyses of ZBP-89 (Law, D. and Merchant, unpublished) suggest that the latter may be the case.

Structural similarity to ZBP-89 also suggests that ZBP-99 may contain one or more transcriptional activation domains. While ZBP-89 frequently acts as a

transcriptional repressor (1, 2, 10), there are conditions when the protein acts in concert with other factors to activate gene expression (Law, D. and Merchant unpublished, Bai and Merchant, manuscript submitted). A similar effect has been noted in a heterologous reporter system for the mouse ZBP-89 homologue, BFCOL1 (15). Current studies in this lab, aimed at identifying protein-protein interactions, may help to elucidate the cellular signaling pathways that mediate ZBP-99 regulatory effects.

Lastly, several indirect observations, particularly the presence of faintly cross-hybridizing bands on ZBP northern and Southern blots and the detection of additional immunoreactive peptides on denaturing protein gels, suggest that there may be additional ZBP gene family members. Comparative studies will help to clarify the biologic functions of this unique zinc finger gene family.

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