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Overexpression of ZBP-89, a Zinc Finger DNA Binding Protein, in Gastric Cancer

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ZBP-89 is a Krüppel-type zinc finger protein that binds to the gastrin EGF response element (gERE). Sp1 binds to the same DNA element and transactivates gastrin promoter activity, whereas ZBP-89 competes for Sp1 binding and prevents EGF induction. Both transcription factors mediate growth factor signals originating from the EGF receptor and thus were studied in normal and neoplastic tissues or cell lines. When compared to normal tissue from the same patient, ZBP-89 protein expression was increased in neoplastic tissue from the stomach antrum and in malignant cell lines. RT-PCR analysis of ZBP-89 mRNA correlated with protein overexpression. Immunocytochemical studies confirmed that ZBP-89 expression is elevated in neoplastic tissue and chronic gastritis, whereas Sp1 expression was nearly unchanged. These results suggest that the transcription factor ZBP-89, like Sp1, may be a marker for neoplastic transformation in some gastric cancers.

Gastric cancer is the most common cancer in the world and is the leading cause of cancer deaths in developing countries (1). Nevertheless, morphologic classifications and molecular markers are not as precisely defined as for colon cancer. Gastric cancers are classified as well-differentiated adenocarcinoma or poorly-differentiated adenocarcinoma and represent the accumulation of multiple genetic alterations (2). These alterations vary depending upon the histologic subtype, indicating that these subtypes may evolve along different genetic pathways. Overexpression of the transcription factor Sp1 occurs in some gastric cancers, appears to correlate with EGF receptor overexpression and may be a marker for transformation (3). Sp1 is also expressed in the developing mouse stomach (4) and binds to GC-rich elements present in the promoters of several growth-related genes including TGFα, the EGF receptor, TGFβ and *c-erbB2* (5-9). Thus Sp1-mediated gene expression is potentially one mechanism by which cancers regulate the overexpression of growth factor receptors and ligands.

Recently, we have found that Sp1 and a second zinc finger protein called ZBP-89 bind to a GC-rich EGF response element in the human gastrin promoter (10). Gastrin is a hormone expressed in the stomach antrum that stimulates gastric acid secretion and epithelial cell growth (11). Changes in gastric pH, mucosal inflammation and developmental cues stimulate and repress gastrin gene expression (12,13). Gastrin is expressed by some pancreatic and colon cancers presumably as an autocrine growth factor for these malignancies (14-16). Consistent with the positive and negative expression of this hormone, Sp1 and ZBP-89 respectively stimulate and inhibit gastrin gene expression (10,17). Since ZBP-89 is a novel DNA binding protein that modulates growth factor signals, we studied the expression of this factor in cell lines and gastric malignancies using biochemical and immunocytochemical methods.

MATERIALS AND METHODS

Cell culture and tissue procurement. Normal human epidermal melanocytes (NHEM), normal human bronchial epithelial cells (NHBM) and normal human mammary epithelial cells (NMEC) were purchased from Clonetics (San Diego, CA) and cultured according to the suppliers instructions. All cell lines were purchased from American Tissue Culture Center except the MKN-45 cell line which was a kind gift from Dr. E. Tahara (18) and breast cancer cell lines Hs578t and SKBR3 which were gifts from Dr. Eric Fearon (University of Michigan Cancer Center). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) containing 8% horse

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Abbreviations used: RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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serum, 6% newborn calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2-95% air. Approval was obtained from the University of Michigan Institutional Review Board for Human Subjects prior to obtaining surgical specimens from the Tissue Procurement Core. Two stomach tumors were obtained at surgery along with normal tissue from two patients.

Western blot analysis. Nuclear protein was extracted from primary cell lines (NHEM, NHBM, and NHEC) and tumor cell lines (G361, A549, Hs578t, SKBR3, MKN-45, AGS, Kato III, HCT116, CoLoDM, and CaCo2) by detergent lysis (19). Whole cell lysates were extracted from surgical specimens using TRI Reagent (GibcoBRL). Fifty micrograms of nuclear extract or whole cell protein lysate was solubilized in Laemmli sample buffer at 95°C then applied to a 7.5% SDS-polyacrylamide gel, blotted to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), and probed with polyclonal rabbit antisera raised against the human form of ZBP-89 called htβ. This antisera was designated anti-htβ. The htβ antisera was raised in rabbits against a histidine-tagged fusion protein corresponding to the first 180 amino acids of htβ which is 96% identical in this region to ZBP-89 (10,20). A blot of whole cell extract from gastric tissue was resolved in parallel and probed with rabbit anti-Sp1 antibody (Santa Cruz Biotechnology). Protein gels resolved in parallel were also stained for protein in 0.01% Coomassie blue and destained in 5% methanol/5% acetic acid.

RT-PCR analysis. Total RNA was prepared from cell lines and fresh surgical specimens using TRI reagent (Molecular Research Center, Cincinnati, OH). RT-PCR was performed using the Super Script Preamplification System for First Strand cDNA Synthesis (GibcoBRL). First strand cDNA was synthesized from 8 μg of total RNA then 4 μl of first strand cDNA was simultaneously amplified using forward and reverse primers of ZBP-89 to produce an amplicon of 762 bp and commercial GAPDH primers to produce an amplicon of 452 bp (Clontech). The PCR product was verified by subcloning the amplicon into a TA cloning vector (InVitrogen) for sequencing. The ZBP-89 forward primer (1957-1985nt) was 5′-AAAGTGGAG-ATTAAGAGCAATCATGACA-3′ and the reverse primer (2722-2695nt) was 5′-CTCTATTATCATTTACATTCACCAAGGG-3′. PCR reactions were carried out in a Thermolyne cycler for 30 cycles after denaturing for 1 min at 94°C, annealing for 2 min at 55°C, and extending for 2 min at 72°C. RT-PCR products were resolved on a 2% agarose gel and quantified on a Fluoroimager (Molecular Dynamics).

Immunocytochemistry. Three micron sections were prepared from paraffin-embedded tissue fixed in 4% paraformaldehyde/PBS or 10% formalin. After sectioning, the specimens were deparaffinized in xylene and rehydrated through an alcohol series. Hematoxylin and eosin (H&E) stained specimens were used to confirm tissue morphology. Endogenous peroxidase activity was blocked by treating the sections with 0.3% hydrogen peroxide in 100% methanol. Nonspecific antigen binding was prevented by incubating the specimens for 1 h in blocking solution containing 5% normal goat serum, 1% BSA, 0.1% Triton X-100 and 0.1% Tween 20. A 1:100 dilution in blocking solution of IgG-fractionated ZBP-89 was incubated with the sections overnight. This antibody was raised against amino acids 1-521 of rat ZBP-89, is 88% identical to htβ and recognizes the same 89 kDa protein on immunoblots. This antisera was designated anti-ZBP-89. The primary antibodies were detected using biotinylated anti-rabbit antibody (1:200 dilution) and the Vectastain ABC Elite kit to detect antigen-antibody complexes using diaminobenzidine substrate. Negative controls for each section did not contain primary antibody and

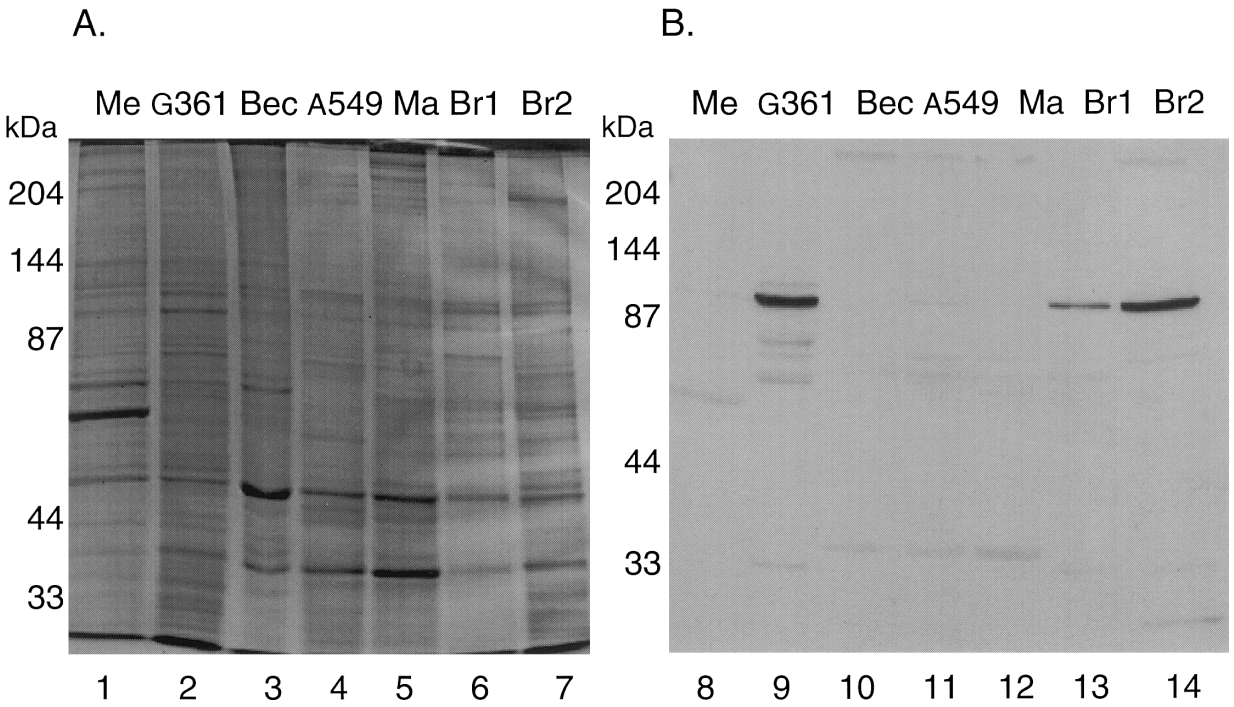

FIG. 1. ZBP-89 protein expression in melanoma, lung and mammary cell lines. (*A*) Coomassie blue protein stain of 20 μg protein resolved by SDS-PAGE. (*B*) Immunoblot analysis with anti-htβ antisera (see Methods). Fifty micrograms of nuclear extracts were resolved by SDS-PAGE and transferred to PVDF membrane. The predicted molecular size of ZBP-89 is 89 kDa. *Lanes 1* and *8,* Me-human melanocyte; *lanes 2* and *9,* Melanoma cancer cell line G361; *lanes 3* and *10,* Bec-human bronchial epithelial cell; *lane 4,* Lung cancer cell line A549; *lanes 5* and *12,* Ma-human mammary epithelial cell line; *lanes 6* and *13,* Br1-Breast cancer cell line Hs578t; *lanes 7* and *14,* Br2 Breast cancer cell line SKBR-3.

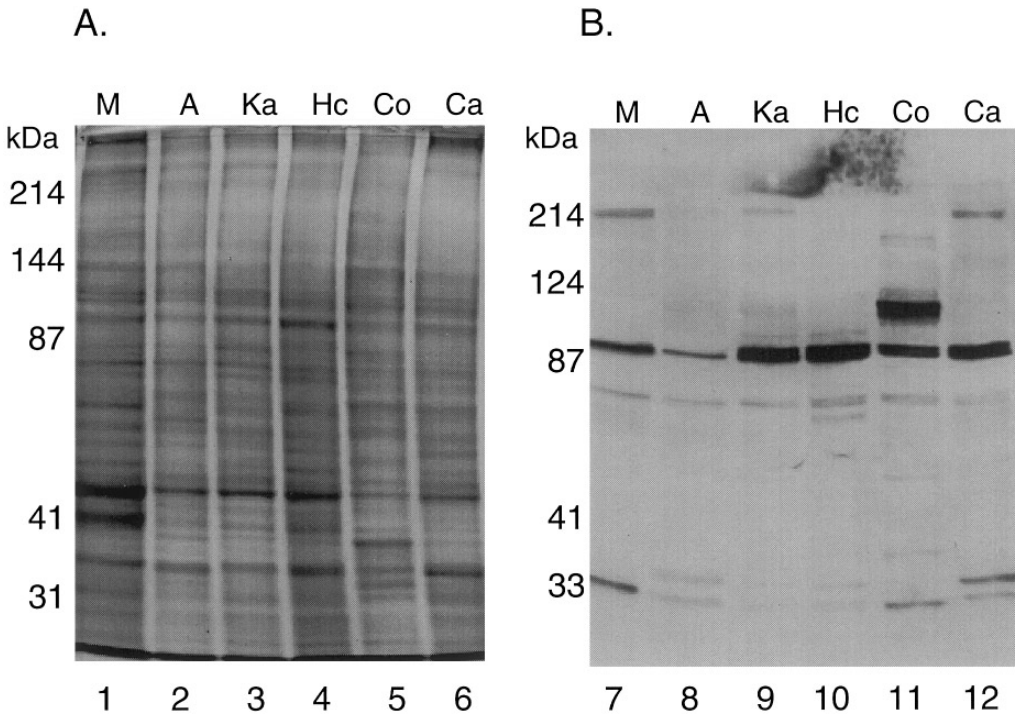

FIG. 2. ZBP-89 protein expression in gastrointestinal cell lines. (*A*) Coomassie blue protein stain of 20 μg of protein. (*B*) Immunoblot analysis with anit-htβ antisera (see Methods). Fifty micrograms of nuclear extracts were resolved by SDS-PAGE. *Lanes 1* and *7,* M-MKN-45 gastric adenocarcinoma cell line; *lanes 2* and *8,* A-AGS gastric adenocarcinoma cell line; *lanes 3* and *9,* Ka-KatoIII gastric adenocarcinoma cell line; *lanes 4* and *10,* Hc-Hct 116 colon adenocarcinoma cell line; *lanes 5* and *11,* Co-ColoDM colon adenocarcinoma cell line; *lanes 6* and *12,* Ca-CaCo2 colon adenocarcinoma.

were stained in parallel as described for the above sections. The tissue was counter-stained using Gill's formula #1 hematoxylin and eosin (Fisher) and photographed using a Zeiss Axiophot microscope.

RESULTS

ZBP-89 Protein Is Overexpressed in Cell Lines and Neoplastic Tissues

To determine whether there were differences in the expression of ZBP-89 in transformed cells, immunoblot analysis was performed on nuclear extracts isolated from primary human cell cultures and malignant human cell lines. Nuclear extracts were prepared from the cell lines of skin, lung and breast epithelial cell origin and resolved on an SDS polyacrylamide gel. Parallel gels were stained for protein to verify the quantity and integrity of the sample (Fig. 1A). The corresponding immunoblot containing the same amount of extract per lane was probed with anti-htβ antisera. The results shown in Fig. 1B demonstrate that the 89 kDa ZBP-89 protein was significantly elevated in three of the four malignant cell lines studied. The level of ZBP-89 protein expression in a human melanoma cell line (G361) and two human breast cancer cell lines (Hs578t, SKBR3) was elevated compared to the amount of ZBP-89 protein in the corresponding melanocyte and mammary primary cell cultures (Fig. 1B). In contrast, ZBP-89 was poorly expressed in primary epithelial and carcinoma cell lines from the lung (Fig. 1B). Since ZBP-89 binds to a GC-rich sequence in the gastrin promoter, we investigated whether gastrointestinal cell lines also overexpress this DNA binding protein. An immunoblot of nuclear extracts from three gastric (AGS, MKN-45 and Kato III) and three colon (Hct 116, CoLoDM, and CaCo2) cell lines revealed that these malignant cell lines also express significant amounts of ZBP-89 (Fig. 2). Collectively, these results suggested that ZBP-89 may be overexpressed in malignancies coincident with its ability to modulate growth factor signals.

To study the protein expression of ZBP-89 in normal and neoplastic gastrointestinal tissues freshly resected surgical specimens from patients with gastric cancer were retrieved by the University of Michigan Tissue Procurement Core and extracted for both protein and nucleic acids. Figure 3 shows the immunoblot of whole cell extracts prepared from the gastric specimens of two patients. A parallel gel with the same amount of protein was stained with Coomassie blue to verify the integrity of extracted proteins and that equivalent amounts of protein were loaded per lane (data not shown). The same amount of protein from the gastric tumors and adjacent normal tissue was blotted then

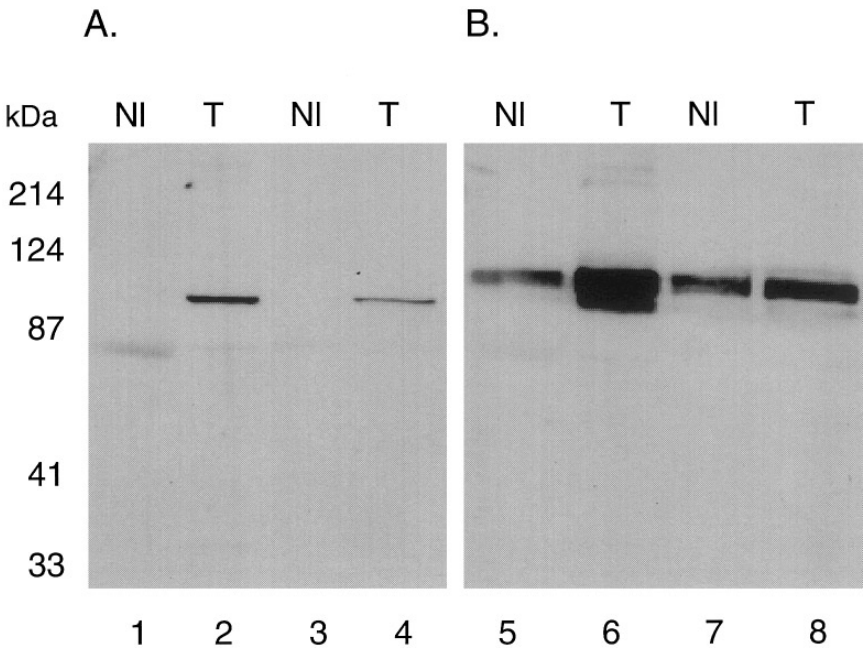

FIG. 3. ZBP-89 protein expression in gastric tissue. (*A*) Immunoblot analysis with anti-htβ antisera. (*B*) Immunoblot analysis with anti-Sp1 antibody. Fifty micrograms of whole cell extracts from normal and neoplastic tissue was obtained from patients with gastric cancer and resolved by SDS-PAGE on two parallel blots. One blot was probed with anti-htβ antisera; the parallel blot was probed with anti-Sp1 antibody. Nl, extracts from noncancerous tissue; T, extracts from gastric tumors. *Lanes 1* and *5* contain extracts from the noncancerous portion of the stomach from patient 1. The tumor from patient 1 was a well-differentiated adenocarcinoma and extracts from this tumore were resolved in *lanes 2* and *6*. *Lanes 3* and *7* contain extracts from the noncancerous gastric tissue of patient 2; *lanes 4* and *8* contain extracts from the malignant gastric stromal tumor derived from the same patient.

probed for ZBP-89 protein (Fig. 3A). The antibody recognized the expected 89 kDa protein in the sample derived from tumor; however, ZBP-89 protein was nearly undetectable in normal tissue. A parallel protein blot was prepared and probed with Sp1 antibody. The results demonstrated that Sp1 protein is also overexpressed in gastric tumors as described previously (3) (Fig. 3B). Collectively, these results demonstrated that ZBP-89 and Sp1 protein expression correlates with a transformed phenotype.

ZBP-89 mRNA Levels Are Elevated in Neoplastic Tissues and Cell Lines

To determine whether ZBP-89 overexpression in neoplastic cell lines and tumors was due to an increase in ZBP-89 gene expression, total RNA was subjected to RT-PCR. Primers specific for ZBP-89 were incubated together with GAPDH primers to control for the quality and quantity of total RNA used in the reaction. As predicted from the immunoblots, the cell lines derived from normal and neoplastic bronchial epithelium had nearly undetectable levels of ZBP-89 despite normal amplification of GAPDH mRNA (Fig. 4, lanes 2 and 3). ZBP-89 mRNA expression was detectable in normal mammary epithelial cell cultures and elevated in malignant breast cancer cell lines. The expression of ZBP-89 mRNA in the gastric and colonic cell lines was also elevated (Fig. 4, lanes 8-13). Total RNA extracted from gastric tissues was also subjected to RT-PCR and showed low but detectable levels of mRNA in normal gastric tissues (Fig. 5). However, there was an increase in ZBP-89 mRNA levels in the two gastric tumors (Fig. 5, lanes 3 and 5). These results correlated well with the protein expression of ZBP-89 in normal and malignant gastric tissue. Thus, the increase in ZBP-89 protein expression corresponded to an increase in ZBP-89 mRNA indicating that the changes were at the level of gene expression.

Immunocytochemical Localization of ZBP-89 and Sp1 in Gastric Carcinomas

Parraffin-embedded tissue was stained with anti-ZBP-89 or anti-Sp1 antibody. In normal gastric tissue from patient 1, ZBP-89 did not significantly stain the nuclei of surface mucous cells lining the gastric crypt (Fig. 6A). However, the nuclei of cells within the lamina propria were stained with anti-ZBP-89 antibody due to the presence of ZBP-89 protein in T lymphocytes (20). The cells within the lamina propria staining with anti-ZBP-89 antibody provided an internal positive control.

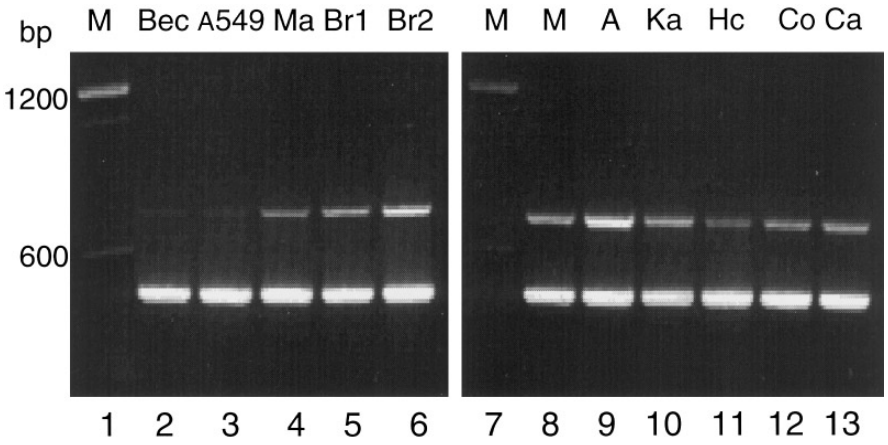

FIG. 4. RT-PCR analysis of ZBP-89 mRNA expression in cell lines. *Lanes 1-6* Lung and mammary cell lines; *lanes 7-13* gastrointestinal cell lines. Eight micrograms of total RNA was reverse transcribed. The cDNA was amplified simultaneously over 30 cycles for both ZBP-89 and GAPDH using specific primers. The expected ZBP-89 product is 762 bp and for 452 bp for GAPDH. *Lanes 1* and *7* contain 100 bp markers. *Lane 2,* Bec-bronchial epithelial cell; *lane 3,* Lung carcinoma cell line A549; *lane 4,* Ma-mammary epithelial cell line; *lane 5,* Br1-breast cancer cell line HS578t; *lane 6,* Br2-breast cancer cell line SKBR-3; *lane 8,* M-MKN-45 cell line; *lane 9,* A-AGS cell line; *lane 10,* Ka-KatoIII; *lane 11,* Hc-Hct 116; *lane 12,* Co-Colo DM; *lane 13,* Ca-CaCo2.

In addition, there was intense cytoplasmic staining of surface epithelial cells within areas of atrophic gastritis with intestinal metaplasia (Fig. 6A, inset). In the areas of gastric adenocarcinoma from the same patient, there was non-uniform immunostaining of the tissue with some nuclei staining for ZBP-89 protein and other nuclei without stain (Fig. 6B). Sp1 antibody stained the nuclei of cells in both the normal surface epithelium and lamina propria and most cell nuclei of the adenocarcinoma (Figs. 6C and D). There were no differences in the staining pattern for Sp1 in the areas of intestinal metaplasia and normal mucosa. The nuclei and cytoplasm of cells within the stromal tumor (patient 2) stained intensely for ZBP-89 protein (Fig. 6E). Similarly, Sp1 antibody immunostained the nuclei of tumor cells (Fig. 6F). Thus, the immunocytochemical staining pattern of ZBP-89 correlated well with the expression pattern observed on immunoblots and the RT-PCR analysis.

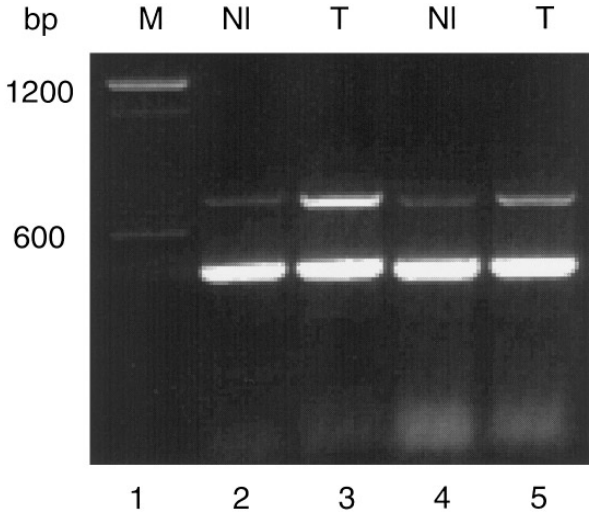

FIG. 5. RT-PCR analysis of ZBP-89 mRNA expression in gastric tissues. Total RNA was prepared as described in the Methods and amplified using both ZBP-89 and GAPDH primers for 30 cycles. *Lane 1* contains 100 bp DNA markers. *Lane 2,* cDNA amplified from the normal gastric tissue of patient 1; *lane 3,* cDNA amplified from the gastric adenocarcinoma of patient 1; *lane 4,* cDNA from the normal gastric tissue of patient 2; *lane 5,* cDNA amplified from the malignant gastric stromal tumor of patient 2.

DISCUSSION

ZBP-89 is a Krüppel-type zinc finger protein that competes with Sp1 for binding to GC-rich elements (10). The GC-rich element in the gastrin promoter that Sp1 binds also confers EGF induction suggesting that ZBP-89 may modulate growth factor signals. Both the protein and mRNA expression of ZBP-89 was elevated in malignant cells compared to primary cell cultures of the same tissue origin or normal tissue from the same patient. Although the primary cell cultures are capable of several cell divisions, these cells did not express significant amounts of ZBP-89 protein. Transformed cells expressed large amounts of the protein that also correlated with an increase in ZBP-89 gene expression. Chronic gastritis with intestinal metaplasia is a precursor to gastric carcinoma as illustrated by patient 1 (21) and is suggestive of a chronic infection with *Helicobacter pylori.*

Immunocytochemical staining revealed the presence of ZBP-89 protein in both the nucleus and cytoplasm. Cytoplasmic localization has been observed for other

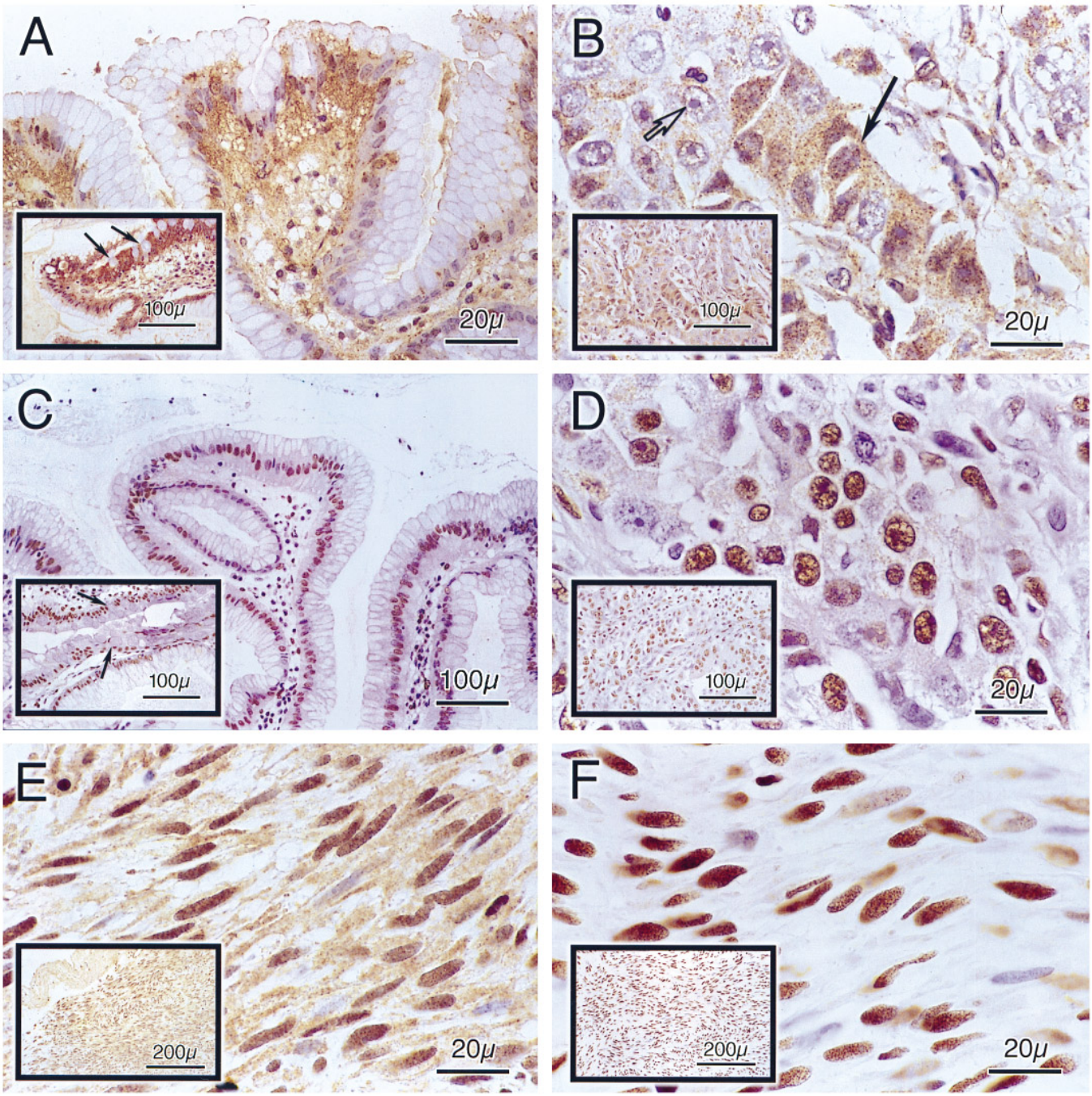

FIG. 6. Immunocytochemical staining pattern of ZBP-89 and Sp1 in noncancerous and cancerous gastric tissues. *A* and *C* noncancerous gastric tissue from patient 1. Arrows indicate areas of chronic atrophic gastritis with intestinal metaplasia. *B* and *D* represent the gastric adenocarcinoma resected from patient 1. *A* and *B* are stained with anti-ZBP-89 antibody. The open arrowhead in *B* indicates an unstained nucleus and the arrow indicates a stained nucleus. *C* and *D* are stained with anti-Sp1 antibody. *E* and *F* represent the malignant gastric stromal tumor resected from patient 2. *E* is stained with anti-ZBP-89 antibody and *F* is stained with anti-Sp1 antibody.

transcription factors, e.g., p53, cyclin D1, NFκB and the protooncogene *c-abl* (22-25). The role of cytoplasmic cyclin D1 is not known. However, the transcriptional activity of p53, NFκB and *c-abl* are all regulated by their subcellular location and nuclear translocation. Cytoplasmic pools of p53 translocate upon serum stimulation as a function of the cell cycle and mutant p53 accumulates in the nucleus of tumors (26). Cytoplasmic

pools of *c-abl* bind actin and are regulated by changes in the cytoskeleton (27). Moreover, changes in the cytoplasmic pools of *c-abl* also affect the activity of the nuclear pool. These examples may have relevance for ZBP-89 function in neoplastic cells since it has a negative effect on transcription from the gastrin promoter by competing with Sp1 (10).

Factors that correlate with malignant transformation may be under- or overexpressed. The most common mutation occurring in malignancies is an abnormal p53 transcription factor (28). Although p53 functions as a tumor suppressor gene to inhibit unregulated growth, mutated p53 genes produce a product that is readily detectable in malignant tissue (24). In contrast, other tumor suppressor genes do not express functional gene products when mutated, thus immunocytochemical staining for such tumor suppressor genes as WT1 and BRCA1 detect reduced or absent levels of these proteins (29,30). Activating mutations of oncoproteins are uniformly overexpressed in malignant tissues (31). At present, it is difficult to predict the significance of elevated ZBP-89 expression on cellular proliferation. Although the current antibodies detect primarily an 89 kDa factor, it is not know whether point mutations exist in the form expressed.

In summary, ZBP-89 and Sp1 are zinc finger DNA binding proteins that recognize GC-rich sequences. While Sp1 has been shown to be related to growth, the function of ZBP-89 has only recently been described. The results reported here suggest that ZBP-89, like Sp1, is significantly elevated in some gastric cancers and pre-malignant inflammatory states.

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