BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 123–127 (1998)
ARTICLE NO. RC988585

Expression of Ets-1 Transcription Factor in Relation to Angiogenesis in the Healing Process of Gastric Ulcer

Masahiro Ito,* Toshiyuki Nakayama,* Shinji Naito,* Mutsumi Matsuu,* Kazuko Shichijo,* and Ichiro Sekine*,[1]

**Department of Molecular Pathology, Nagasaki University School of Medicine, Nagasaki 852-8523, Japan*

Received April 2, 1998

Ets-1 is a transcription factor known to control the expression of genes involved in extracellular matrix remodeling. The purpose of this study is to evaluate the expression of Ets-1 in the process of healing of ulceration in the rats. The time-dependent changes and distribution of Ets-1 in the margins of ulcer were examined. Ets-1 did not express in the normal gastric mucosa. In the marginal granulation tissue, fibroblasts and endothelial cells of capillaries were immunopositive for Ets-1. Ets-1 expression was significantly increased at the early phase, and returned to normal levels at the scarred phase. Serial sectioning revealed that fibroblasts and endothelial cells also expressed MMP-1. Protein levels and mRNA expression of Ets-1 were confirmed by Western blotting and RT-PCR. These findings suggest that Ets-1 plays an important role in angiogenesis in the early phase of ulcer healing.

Angiogenesis, the development of new blood vessels, is an important process in tissue development and wound healing. In ulcer healing angiogenesis is a key factor to determine the quality of healing. Ets-1 was originally characterized as the v-ets retroviral gene, 1 of the 2 oncogenes (v-myb and v-ets) in the avian leukemia retrovirus, E26 (1). The Ets family of genes encodes transcription factors for mesodermal cell development during the embryonal period (2, 3). Recently, it has been reported that Ets-1 appears to play an important role in angiogenesis, regulating the expression of proteases and the migration of endothelial cells (4). Ets-1 protein interacts with the urokinase-type plasminogen activator gene enhancer and with the promoters of metalloproteinases, the stromelysin-1 and collagenase-1 genes (5, 6). In the present study, we examined the expression of Ets-1 in the healing stage of gastric ulcer in relation to angiogenesis in the rat.

MATERIALS AND METHODS

Male Wistar rats were purchased from Charles River Japan (Atsugi, Japan) at aged 8 weeks of age for use in this study (n=42). The rats were housed in groups of 3 to 4 per cage in an air-conditioned room at 24°C (lights on from 7 AM to 7 PM), and allowed free access to food (laboratory chow F2, Japan CLEA, Tokyo) and tap water at the Laboratory Animal Center of Nagasaki University. Ulcers were induced in the fundus of the stomach by luminal application of 40% acetic acid (0.2 ml, 45 s) to an area clamped with a pair of forceps. Gastric specimens were obtained 0 (control), 1, 2, 3, 7, 14, 21, and 28 days after ulcer induction for histology, Western blot analysis, and RT-PCR to evaluate the expression of Ets-1 protein and ets-1 mRNA. In each experimental day four to six rats were used for analysis. For immunohistochemistry of Ets-1, MMP-1, CD34, and VEGF, gastric tissues were fixed with 4% paraformaldehyde solution. CD34 immunohistochemistry was performed to confirm neovasculization, because CD34 mRNA was strongly expressed by most of the vascular endothelial cells in developing organs (7).

Immunohistochemistry. Paraformaldehyde-fixed and paraffin-embedded tissues were cut into 4 μm sections, deparaffinized in xylene and rehydrated in phosphate-buffered saline. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then incubated overnight at 4°C with an optimal dilution (0.1 μg/ml) of a primary polyclonal antibody against Ets-1 (C-20, raised against the C-terminal domain of the Ets-1 protein; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The slides were sequentially incubated with an alkaline phosphatase-conjugated horse anti-rabbit immunoglobulin antibody, and the reaction products were resolved using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersberg, MD). Preabsorption of the primary antibody with excess recombinant Ets-1 peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight was used as a negative control. Adrenal gland tissue served as the internal positive control for Ets-1 immunostaining.

For immunohistochemistry of MMP-1, CD34 and VEGF, primary

[1] To whom correspondence should be addressed at Department of Molecular Pathology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Fax: +81-95-849-7108. E-mail: m-ito@net.nagasaki-u.ac.jp.

Abbreviations: VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; RT-PCR, reverse transcription polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; FGF, fibroblast growth factor; TGF, transforming growth factor; PDGF, platelet-derived growth factor; IL-1, interleukin 1; TNF, tumor necrosis factor.

0006-291X/98 $25.00

antibodies of MMP-1 (monoclonal, Fuji Chemical, Takaoka, Japan), CD34 (monoclonal, Novocastra, Newcastle, UK) and VEGF (polyclonal, Santa Cruz Biotechnology, Inc.) were purchased on a commercial basis. Secondary reagents were provided by the Vector Elite ABC kit, and diaminobenzidine with counterstain was used for color development. All cases were run with known positive controls for each antibody. For negative controls, the primary antibody was omitted for each run.

Western blot analysis of Ets-1 expression. Frozen gastric tissues crushed in liquid nitrogen were put in lysis buffer (1% sodium dodecyl sulfate [SDS], 1 mmol/L sodium vanadte, 10 mmol/L tris[hydroxymethyl] aminomethane, pH 7.4) repeatedly. Proteins obtained from lysates were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, MA) as recommended by the manufacturer. Detection of Ets-1 was performed with the polyclonal antibody (C-20, Santa Cruz Biotechnology, Inc.). Immunoreactive material was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

To evaluate specificity of the antibody for Ets-1 immunoprecipitation, preabsorption of the primary antibody with excess recombinant Ets-1 peptide (Santa Cruz Biotechnology, Inc.) was performed. Phorbol 12-myristate 13-acetate (PMA)-treated endothelial cells were used as a positive control (8). Endothelial cells were obtained from fresh bovine thoracic aorta and cultured in monolayers as described (9). PMA (100ng/ml) was treated for 2h, and then protein was extracted for Western blotting.

RT-PCR. Total RNA was prepared from gastric tissue using the acid guanidine phenol method. Total RNA (1 μg) was incubated at 37°C for 1 hour in 50 μl of reverse transcriptase buffer containing 20 units of RNAsin (Promega Corp., Madison, WI), 100 pmol of random hexamer primers (Boehringer Mannheim, Mannheim, Germany), and 400 units of Moloney murine leukemic virus reverse transcriptase (GIBCO/BRL). Reverse transcription was terminated by heating at 95°C for 10 minutes, and 20% of the resulting cDNA was removed for PCR. PCR samples were incubated with 50 pmol of each primer and 2.5 units of Taq DNA polymerase. The rat Ets-1 PCR primers were 5′-GGGTGACGACTTCTTGTTTG-3′ (sense) and 5′-GTTAATGGAGTCAACCCAGC-3′ (antisense). The human β-actin PCR primers were 5′-TCCTCCCTGGAGAAGACTA-3′ (sense) and 5′-AGTACTTGCGCTCAGGAGGA-3′ (antisense). The Ets-1 and β-actin primers are predicted to amplify 274 and 313 bp DNA fragments, respectively. Both primer pairs were chosen to span introns of their respective rat genes. Samples were subjected to 28 cycles of PCR amplification using a thermocycler. Each cycle included denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and primer extension at 72°C for 1.5 minutes. An aliquot of each amplification mixture was subjected to electrophoresis on a 2.0% agarose gel, and DNA was visualized by ethidium bromide staining.

RESULTS

Pathological Changes of Ulcer

One day after ulcer induction, gastric mucosa was necrotic macroscopically, and bleeding, edema, and inflammatory cell infiltration were observed in the submucosa microscopically. From the second day, fresh granulation was formed and new capillaries appeared in ulcer margins. On the 7th day, absorption of granulation and stromal fibrosis occurred and the regenerative epithelium extended from the margin. Mild inflammatory cell infiltration was observed. Between the 14th and 28th day (scar phase) the ulceration appeared to be healed macroscopically. The submucosal granulation was organized with fibrosis, and regenerative epithelium covered the area of ulceration completely.

Immunohistochemistry

Figure 1 shows a time-dependent changes of immunohistochemical Ets-1 staining in ulcerative lesions. Ets-1 did not express in the normal gastric mucosa. Ets-1 protein was detected in the nucleus of fibroblasts and in the nucleus of endothelial cells in the marginal granulation tissues. On close view, nuclei of endothelial cells in primitive ring-formed capillaries were immunopositive. Ets-1 expression significantly increased from days 1 to 7, and returned to control levels at the scarred phase. On the first day, gastric tissue was necrotic and Ets-1 expression was limited to a few cells in the submucosal layer. On the 3rd day, its expression was most enhanced in the fresh granulation. CD34 immunopositivity was observed in endothelial cells of capillaries in the fresh granulation from the 3rd to the 7th day after ulcer induction (Fig.2A), but not in mature blood vessels at the scarred phase. MMP-1 expression was present in endothelial cells and fibroblasts (Fig.2B), and its distribution and the time-course of immunopositive cells were similar to those of Ets-1. With serial sectioning, both MMP-1 and Ets-1 expression were detected in some of the identical cells. VEGF immunopositivity was observed in the scattered macrophages in the ulcer base from one day after ulcer induction (Fig.2C), and remained till 14 days after ulcer induction with gradual attenuation. A few endothelial cells and fibroblasts were also immunopositive for VEGF in the early phase. At the scarred phase VEGF immunopisitivity was limited to endothelial cells.

Western Blotting

The antibody recognized a single band (54 kd) in the positive control of PMA-treated endothelial cells. Preabsorption of the primary antibody with Ets-1 peptide was found to abolish immunoreactive products (Fig. 3A). Western blotting in the gastric tissues is shown in Fig.3B and this finding corresponded to the results of immunohistochemistry of Ets-1. In the normal gastric tissue, Ets-1 expression was not detected. On the 2nd day its expression increased significantly and reached a maximum on the 3rd day. The strong expression lasted till the 7th day. On the 14th day its expression again became weak.

RT-PCR

Weak expression of ets-1 mRNA was detected in the normal gastric tissue, whereas strong expression of ets-1 mRNA was observed in the ulcerative tissues, irrespective of time course (Fig. 4). β-actin mRNA, a control

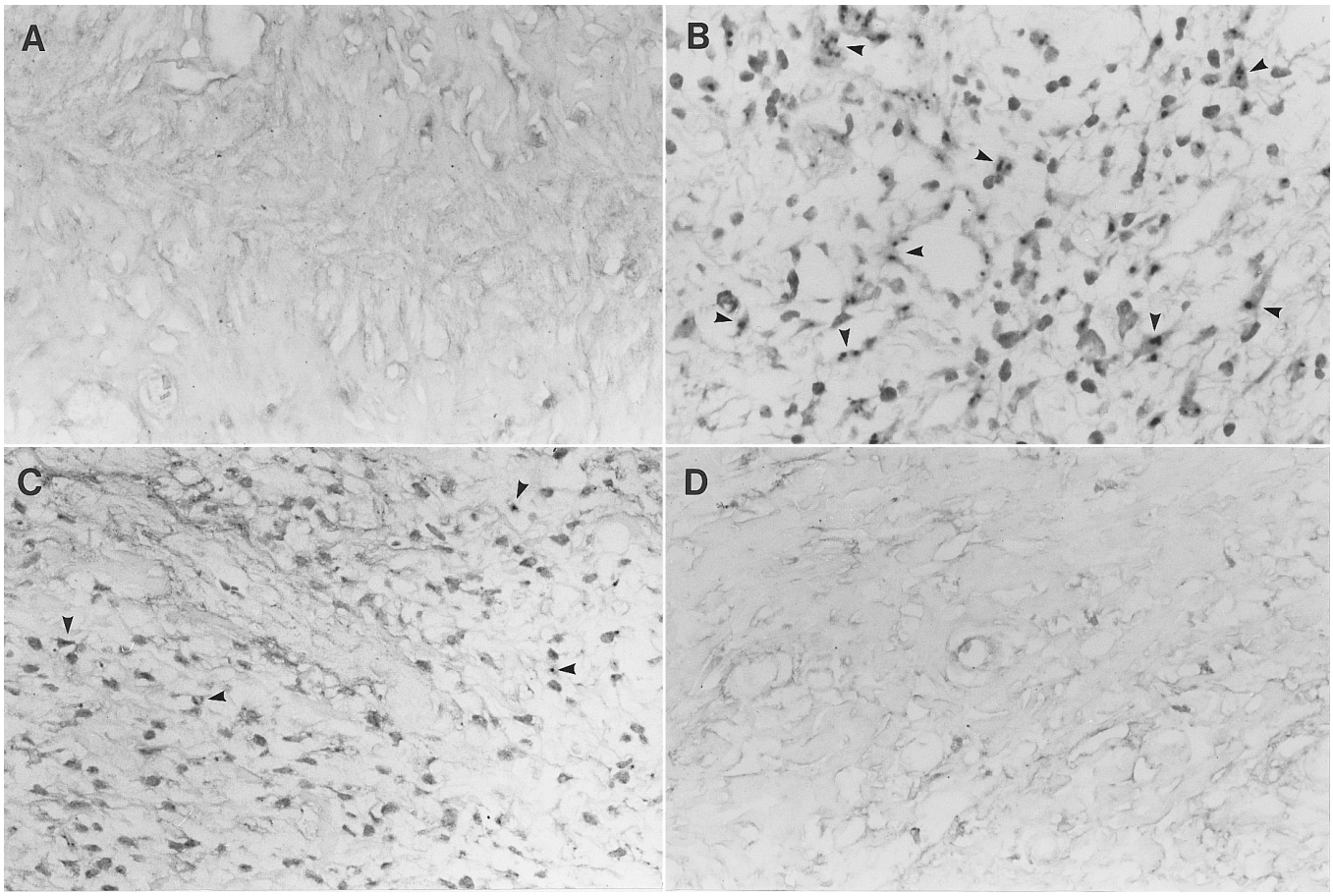

FIG. 1. Time-course of Ets-1 expression in the normal and ulcerative lesions by immunohistochemistry. Ets-1 did not express in the submucosa of normal gastric mucosa (A). Ets-1 expression significantly increased from days 1 to 7 (B, C), and returned to control levels at the scarred phase (D). On the 3rd day its expression was most enhanced in fresh granulation (B). Intranuclear expression was detected in endothelial cells and fibroblasts in the fresh granulation tissue (arrow heads). Some endothelial cells were positive in the newly formed capillaries. On the 7th day its expression was decreased (arrow heads) (C). After 28th day its expression was barely detected in the organizing granulation (D). (Original magnification ×200)

to demonstrate the equivalent amounts of tissue RNA, was used for cDNA synthesis and was detected in all the samples.

DISCUSSION

Angiogenesis, the sprouting of new blood vessels from existing vessels, occurs in many physiological and pathological processes, including embryonic development, wound healing, and tumor growth (10, 11). Ets-1 takes part in regulating angiogenesis by controlling the gene transcription of matrix metalloproteinase, whose activity is necessary for the migration of endothelial cells from pre-existing capillaries (4). In vitro experiments suggest that Ets-1 may activate the transcription of genes encoding collagenase 1, stromelysine 1 and urokinase plasminogen activator, proteases involved in extracellular matrix degradation. Although Ets-1 has been well studied in angiogenesis, its role in gastric ulcer healing is not known. The present study is the first demonstration of the expression of Ets-1 by gastric cells, especially at ulcer margins. In addition, these results confirmed a previous study showing that Ets-1 was produced by endothelial cells and fibroblasts in wound healing (4).

MMPs are believed to be active in connective tissue remodeling associated with various physiological processes and in pathological conditions, such as cancer and inflammation. The role of collagenase and gelatinases in the development and healing of acetic acid-induced gastric ulcer in rats was investigated (12, 13). Baragi et al. reported that the formation of acetic acid-induced ulcer is accompanied by an elevation of collagenase and gelatinase that gradually tends to return to control values during the healing phase (12). Maruyama et al. reported that interstitial collagenase showed the highest activity on the 3rd day and decreased on the 10th day in acetic acid-induced ulcers (13). These

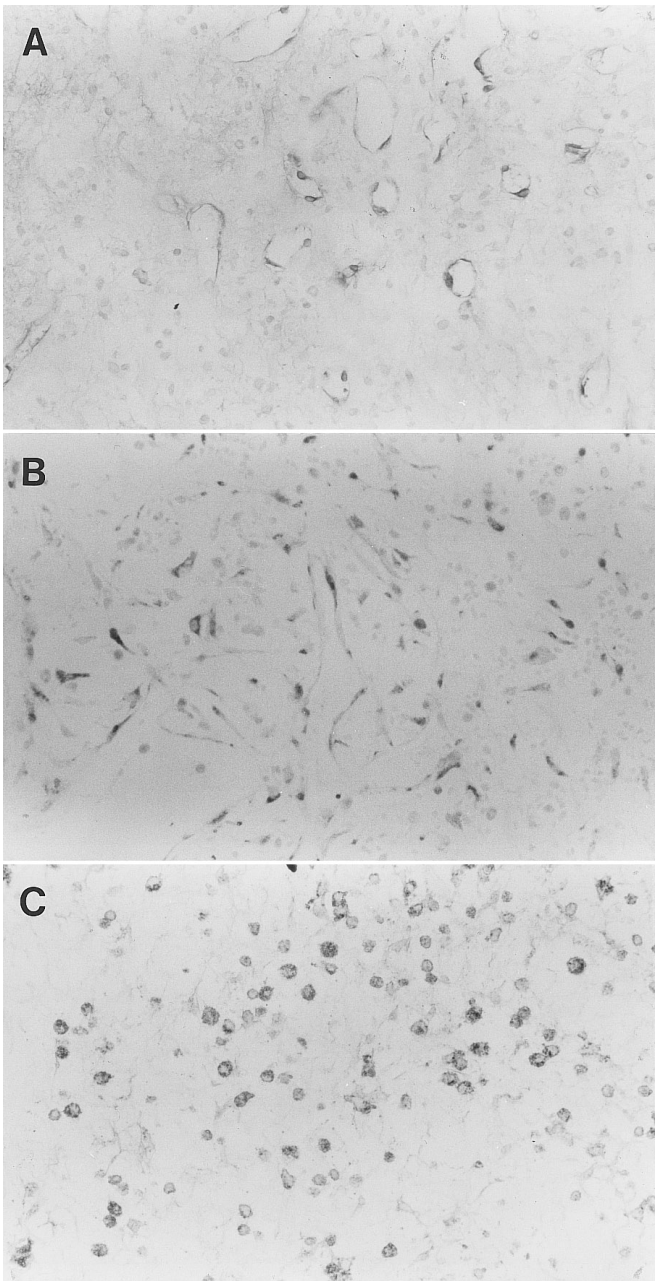

FIG. 2. Immunohistochemistry of CD34 in the 3rd day of ulceration (A). CD34 immunopositivity was observed in endothelial cells of capillaries in the fresh granulation. (Original magnification ×100) Immunohistochemistry of MMP-1 in the 3rd day of ulceration (B). MMP-1 immunopositivity was observed in endothelial cells and fibroblasts in the fresh granulation tissue of ulcer margin. (Original magnification ×100) Immunohistochemistry of VEGF in the first day of ulceration (C). VEGF immunopositivity was observed in the scattered macrophages in the ulcer base from one day after ulcer induction. (Original magnification ×100)

findings are similar to time-dependent changes of MMP-1 and Ets-1 expression in the healing process of acetic acid-induced ulcers in the present study, and support the hypothesis that Ets-1 stimulates MMPs

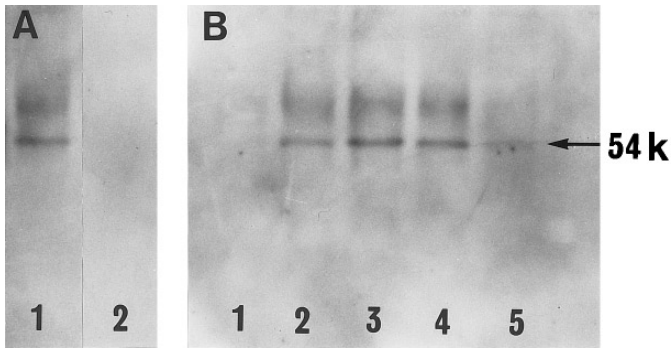

FIG. 3. Western blotting of Ets-1 protein in positive control (A) and ulcerative tissues (B). PMA-stimulated endothelial cells expressed Ets-1 protein with 54 kd (lane 1 in A). Preabsorption of the primary antibody with Ets-1 peptide was found to abolish immunoreactive product (lane 2 in A). In the normal gastric tissue, Ets-1 expression was not detected (lane 1 in B). On the 2nd day (lane 2 in B) its expression increased significantly and reached a maximum on the 3rd day (lane 3 in B). The strong expression lasted till the 7th day (lane 4 in B). On the 14th day its expression again became weak (lane 5 in B).

production to promote angiogenesis in the early phase of ulcer healing.

Many kinds of growth factors and cytokines are involved in ulcer healing, such as EGF, FGF, TGFα, TGFβ, VEGF, PDGF, and IL-1 (14-17). Four typical angiogenic growth factors, acidic FGF, basic FGF, VEGF, and EGF, induce the expression of Ets-1 mRNA in either human umbilical vein endothelial cells or human omental microvascular endothelial cells (4). EGF, bFGF, and PDGF are principally delayed ets-1 stimulators in human fibroblasts (18). VEGF is a multifunctional cytokine involved in angiogenesis, inflammation, and wound healing and plays a role in angiogenesis in the process of gastric ulcer healing (19). TNFα is known as an accelerative factor of mucosal injury by various kinds of ulcerogens (20). VEGF expression is enhanced

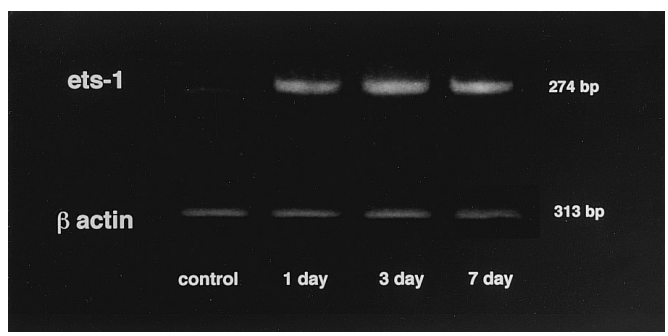

FIG. 4. RT-PCR analysis of ets-1 mRNA expression in normal and ulcerative gastric tissues using the specific primer pairs predicted to amplify fragment size on the right (ets-1: 274 bp, β-actin as internal control: 313 bp). Lane 1; normal gastric tissue. lane2-4; marginal tissue of gastric ulcer on the 1st (lane 2), 3rd (lane 3) and 7th day (lane 4) of ulceration.

by TNFα (19), and, interestingly, TNFα is one of the most potent c-ets-1 stimulators, inducing rapid and long-lasting increases of ets-1 mRNA and protein expression.(18). In the present study VEGF expression preceded Ets-1 expression in the early phase of ulcer healing. It is suggested that VEGF induces endothelial proliferation and consequently helps cell migration through activation of MMPs by Ets-1 up-regulation.

In conclusion, we have shown the specific expression of Ets-1 at gastric ulcer margins and that Ets-1 expression by gastric fibroblasts and endothelial cells is up-regulated in the early phase of healing. These findings indicate the role of Ets-1 in gastric ulcer healing, which is through facilitating angiogenesis.

REFERENCES

1. Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983) *Nature* **306,** 395–397.
2. Kola, I., Brookes, S., Green, A. R., Grabber, R., Tymms, M., Papas, T., and Seth, A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7588–7592.
3. Vandenbunder, B., Pardanaud, L., Jaffredo, T., Mirabel, M. A., and Stehelin, D. (1989) *Development* **106,** 265–274.
4. Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. (1996) *J. Cell Physiol.* **169,** 522–531.
5. Vandenbunder, B., Wernert, N., Queva, C., Desbiens, X., and Stehelin, D. (1994) *Folia Biologica* **40,** 301–313.
6. Nerlov, C., De Cesare, D., Pergola, F., Caraccilo, A., Blasi, F., Johnsen, M., and Verde, P. (1992) *EMBO J.* **11,** 4573–4582.
7. Ito, A., Nomura, S., Hirota, S., Suda, J., Suda, T., and Kitamura, Y. (1995) *Lab. Invest.* **72,** 532–538.
8. Naito, S., Shimizu, S., Maeda, S., Wang, J., Paul, R., and Fagin, J. A. (1998) *Am. J. Physiol.* **274,** C472–480.
9. Shimizu, S., Nomoto, M., Yamamoto, T., and Momose, K. (1994) *Br. J. Pharmacol.* **113,** 564–568.
10. Millauer, B., Longhi, M. P., Plate, K. H., Shawver, L. K., Risau, W., Ullrich, A., and Strawn, L. M. (1996) *Cancer Res.* **56,** 1615–1620.
11. Nakayama, T., Ito, M., Ohtsuru, A., Naito, S., Nakashima, M., Fagin, J. A., Yamashita, S., and Sekine, I. (1996) *Am. J. Pathol.* **149,** 1931–1939.
12. Baragi, V. M., Qiu, L., Gunja-Smith, Z., Woessner, J. F., Jr., Lesch, C. A., and Guglietta, A. (1997) *Scand. J. Gastroenterol.* **32,** 419–426.
13. Maruyama, K., Okazaki, I., Arai, M., Kurose, I., Komatsu, H., Nakamura, M., and Tsuchiya, M. (1995) *J. Gastroenterol.* **30,** 301–309.
14. Abe, S., Sasano, H., Katoh, K., Ohara, S., Arikawa, T., Noguchi, T., Asaki, S., Yasui, W., Tahara, E., Nagura, H., and Toyota T. (1997) *Digest. Dis. Sci.* **42,** 1199–1209.
15. Cook, G. A., Yeomans, N. D., and Giraud, A. S. (1997) *Am. J. Physiol.* **272,** G1540–1549.
16. Watanabe, T., Arakawa, T., Fukuda, T., Higuchi, K., and Kobayashi, K. (1997) *Am. J. Pathol.* **150,** 971–979.
17. Ernst, H., Konturek, P., Hahn, E. G., Brzozowski, T., and Konturek, S. J. (1996) *Gut* **39,** 172–175.
18. Gilles, F., Raes, M. B., Stehelin, D., Vandenbunder, B., and Fafeur, V. (1996) *Exp. Cell. Res.* **222,** 370–378.
19. Takahashi, M., Kawabe, T., Ogura, K., Maeda, S., Mikami, Y., Kaneko, N., Terano, A., and Omata, M. (1997) *Biochem. Bioph. Res. Comm.* **234,** 493–498.
20. Ferraz, J. G., Tigley, A. W., Appleyard, C. B., and Wallace, J. L. (1997) *Am. J. Physiol.* **272,** G809–814.