Kruppel-like Factor 4 (KLF4) Represses Histidine Decarboxylase Gene Expression through an Upstream Sp1 Site and Downstream Gastrin Responsive Elements*

Received for publication, July 29, 2003, and in revised form, December 5, 2003 Published, JBC Papers in Press, December 10, 2003, DOI 10.1074/jbc.M308278200

Wandong Ai, Ying Liu, Michael Langlois, and Timothy C. Wang‡
From the Division of Gastroenterology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Histidine decarboxylase (HDC) is the enzyme that catalyzes the conversion of histidine to histamine, a bioamine that plays an important role in allergic responses, inflammation, neurotransmission, and gastric acid secretion. Previously, we demonstrated that gastrin activates HDC promoter activity in a gastric cancer (AGS-E) cell line through three overlapping downstream promoter elements. In the current study, we used the yeast one-hybrid strategy to identify nuclear factors that bind to these three elements. Among eight positives from the one-hybrid screen, we identified Kruppel-like factor 4 (KLF4) (previously known as gut-enriched Kruppel-like factor (GKLF)) as one factor that binds to the gastrin responsive elements in the HDC promoter. Electrophoretic mobility shift assays confirmed that KLF4 is able to bind all three gastrin responsive elements. In addition, transient cotransfection experiments showed that overexpression of KLF4 dose dependently and specifically inhibited HDC promoter activity. Regulation of HDC transcription by KLF4 was confirmed by changes in the endogenous HDC messenger RNA by KLF4 small interfering RNA and KLF4 overexpression. We further showed that KLF4 inhibits HDC promoter activity by competing with Sp1 at the upstream GC box and also independently by binding the three downstream gastrin responsive elements. Taken together, these results indicate that KLF4 can act to repress HDC gene expression by Sp1-dependent and -independent mechanisms.

Histamine is a bioamine that plays an important role in many physiological processes, including allergy, inflammation, neurotransmission, and gastric acid secretion (1–3). Histidine decarboxylase (HDC) is the single enzyme that converts histidine to histamine (4). HDC is expressed in many different cell types, including mast cells, skin cells, platelets, and basophils. However, in the adult mammals, HDC is highly expressed in enterochromaffin-like cells, where the HDC activity is tightly regulated by a gut peptide hormone, gastrin (5). HDC regulation occurs at both the transcriptional and post-translational levels, the latter by proteolytic processing through the ubiquitin-proteasome pathway (6, 7).

HDC promoter activity is up-regulated by several different stimuli, including gastrin (6), phorbol ester phorbol 12-myristate 13-acetate (8–11), oxidative stress (12), thrombopointin (13), and Helicobacter pylori infection (14, 15). Whereas not all of the cis-acting DNA elements or the transcriptional factors involved in regulation of HDC transcription have been identified, three GC-rich gastrin responsive elements located downstream of the transcription initiation site have been characterized in the human HDC promoter region (16, 17). Through the use of Southwestern blot and UV cross-linking, the sizes of the three gastrin responsive element-binding factors have previously been assessed at 53, 33, and 110 kDa of apparent molecular mass, respectively (16, 17). Recently, a neural peptide pituitary adenylate cyclase-activating polypeptide has been reported to regulate HDC promoter activity in PC12 cells, and the response element of pituitary adenylate cyclase-activating polypeptide has been further mapped to the –177 to –170 region of the HDC promoter. Moreover, by using different protein kinase inhibitors, it has been shown that the mechanism of pituitary adenylate cyclase-activating polypeptide regulation is distinct from gastrin regulation of the HDC promoter (18). Interestingly, the DNA methylation state of the HDC promoter region has been also shown to control HDC gene expression in both the human and mouse (19, 20). The HDC promoter region was demethylated in the HDC-expressing cell lines, such as the differentiated mast cell line MC9 and the erythroleukemia cell line DS19. In low HDC expression cell lines, such as P815, the HDC promoter was also demethylated after induction through incubation of P815 cells in the peritoneal cavity of BDF1 mice, and forced demethylation treatment induced high expression of HDC mRNA in P815 cells (19). Although mutation of the Sp1-binding GC box in the human HDC promoter appear to disrupt the promoter activity, Sp1 binding to this GC box element does not appear to be influenced by DNA methylation at this site (20).

Gastrin has also been shown to up-regulate gene expression through GC-rich DNA cis-elements in the promoter. For example, gastrin-induced activation of the chromogranin A promoter was mediated by a Sp1/Egr1 binding site (21). In addition, gastrin has been shown to up-regulate the vesicular monoamine transporter-2 promoter through a AP-2/Sp1 binding site (22). Three gastrin responsive elements in the HDC promoter are also GC-rich, and bind novel, yet undefined transcription factors. Whereas most of these gastrin responsive elements are GC-rich, they do not share significant homology with each other. In terms of transcription factors that bind to GC boxes, Sp1 was the first mammalian transcriptional factor to be cloned (23), and it binds to GC boxes (24), CACCC boxes (also

* The work was supported by an National Institutes of Health Grant RO1 DK-48077 (to T. C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Division of Gastroenterology, University of Massachusetts Medical School, Lakaze Research Bldg., Rm. 208, 364 Plantation St., Worcester, MA 01605. Tel.: 508-856-4778; Fax: 508-856-4770; E-mail: timothy.wang@umassmed.edu.
† The abbreviations used are: HDC, histidine decarboxylase; KLF, Kruppel-like factor; ODC, ornithine decarboxylase; 3-AT, 3-amino-triazole; EMSA, electrophoretic mobility shift assay; siRNA, small interfering RNA; GAS-RE, gastrin responsive elements; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
In addition, there is a requirement for KLF4 in p53-mediated activity (32) and up-regulation of several keratin genes (38–40) similar to those found in the Sp6 expression. Kruppel-like factors containing at least 20 identified members in mammals have the Sp1 binding GC box (25), as well as basal transcription elements.

KLF4, previously known as gut-enriched Kruppel-like factor 4 (KLF4), has been demonstrated by different laboratories that KLF4 both activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

In this study, we used yeast one-hybrid screening to identify KLF4 as one of the gastrin responsive element binding factors. In addition, we also demonstrated that overexpression of KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

KLF4 Represses the HDC Promoter

KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).

KLF4 Represses the HDC Promoter

KLF4 activates and represses gene transcription (32–36), and these activation and repression domains have also been mapped within KLF4 (30, 37). Analysis of KLF4 target genes reveals the function of KLF4 in inhibition of cell proliferation and promotion of terminal differentiation, which includes roles in down-regulation of ornithine decarboxylase (ODC) promoter activity (32) and up-regulation of several keratin genes (38–40). In addition, there is a requirement for KLF4 in p53-mediated G2/M cell cycle arrest upon DNA damage (41), and a requirement for KLF4 in the terminal differentiation of goblet cells in the colon (42). In addition, preliminary studies using a conditional gastric epithelium KLF4 knockout mouse model suggest that KLF4 is required for normal gastric epithelial proliferation and differentiation (43).
Fig. 1. Yeast one-hybrid screening identifies KLF4 as a gastrin responsive element-binding protein. A, gastrin responsive elements in the HDC promoter span from the transcriptional initiation site (+1) to the downstream 48th nucleotide (+48). Individual gastrin responsive elements and their locations are shown at the top. The specific mutations within each gastrin responsive element are shown at the bottom, which were used for further studies. B, probes used in yeast one-hybrid screening and positive confirmation. Two tandem copies of the full-length gastrin responsive element and three tandem copies of each individual were cloned into the reporter plasmid pHisI-1. C, confirmation of positives by yeast one-hybrid screening. Two plasmids from two positive clones (1 and 2) were transformed into GAS-RE (full), GAS-RE1, GAS-RE2, and GAS-RE3 reporter strains. The transformants from each transformation were patched onto His-Leu- dropout agar plates with or without 30 mM 3-AT, a competitive inhibitor of the yeast HIS3 protein (HIS3p) that is used to suppress the basal growth and for the positive selection. The plates were incubated at 30 °C for 2 to 3 days (30 mM 3-AT) or 4 days (0 mM 3-AT) before being photographed. Positive clone 2 showed the growth on 3-AT-containing His-Leu- dropout plates in single GAS-RE1, GAS-RE2, and GAS-RE3 reporter strains. Negative clone 1 did not grow on the same plate using the three single reporter strains, even though it grew the GAS-RE (full) reporter strain.

TG-3') and KLF4-3 (5'-CCCGGATTCCTTAAATGCGCTCTCAGTGTAG-3') for KLF4, KLF4-5ZF (5'-CCCGGATCCCTTGGATTACGC-GGGCTGC-3') and KLF4-3 for KLF4(ZF), KLF5-5 (5'-CCCGGATCCATGGCTACAGGTGCTAGC-3'), and KLF5-3 (5'-CCCGGATTCCTTGGATTACGC-GGGCTGC-3') for KLF5, KLF5-5ZF (5'-CCCGGATCCCTTGGATTACGC-GGGCTGC-3') and KLF5-3 for KLF5(ZF). The PCR production was digested with EcoRI and BamHI. The respective inserts were then cloned into pcDNA3.1/Myc-HisB (Invitrogen, catalogue number V800-20) precut with EcoRI and BamHI. The constructs were all confirmed by sequencing.

Cell Culture and Transient Transfections—AGS-E cells and HEK293 cells were grown in complete medium (Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin) in a humidified atmosphere (5% CO2). AGS-E cells were generated by stable transfection of AGS cells (ATCC CRL 1739) with the CCKB receptor. Transient transfections were performed using Superfect® (Qiagen) according to the manufacturer’s protocol. AGS-E cells were seeded to ~60% confluence in 12-well plates. Each well was transfected with 0.005 g of total RNA was then used as a template to label the probes. [32P]CTP-labeled probes were incubated with 20 μg of total RNA for β-actin and 60 μg for HDC and then subjected to RNase digestion using a kit from Ambion (catalog numbers 1312 and 1415). Following electrophoresis on a 6% polyacrylamide denaturing gel with 8% urea, the gel was then dried and exposed to a Fuji phosphorimaging screen overnight, and the screen was scanned using a phosphor imager (Fuji FLA-5000). Three separate RNase protection assays were performed with similar results.

Luciferase Assays of HDC Reporter Constructs—After incubation with or without gastrin, cells were washed with PBS and frozen at −80 °C for at least 30 min to increase the efficiency of cell lysis. Cells were then transferred to room temperature and incubated with 250 μl of 1× passive lysis buffer (Promega) for 20 min with constant shaking. Fifty microliters of the cell lysate was then assayed in a Monolight luminometer, MonolightTM3010 (BD Pharmingen). Light units of each reporter were divided by the mean of the internal control Renilla luciferase to represent the relative promoter activity.

Western Blotting Analysis—KLF4-pcDNA3.1/Myc-HisB overexpression construct and the vector control were transfected into HEK293 cells similarly as described above. 48 h later after transfection, the cells were harvested and lysed with lysis buffer containing 10 mM Tris/HCl (pH 8.0), 300 mM NaCl, 1% Triton X-100, plus proteinase mixture (Roche Diagnostics, catalog number 10130500). The cells were sonicated briefly, and the supernatant was collected by centrifugation. About 100 μg of total protein was separated on 4–20% SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membrane. Western blotting analysis was then performed using anti-myc horse-radish peroxidase antibody (1:5000, Invitrogen, catalog number 46-7009) and anti-α-tubulin antibody (1:1000, Oncogene, catalog number CPE6-100UG) followed by a standard enhanced chemiluminescence (ECL) detection procedure.

RESULTS

Yeast One-hybrid Screening to Identify GAS-RE Binding Factors—To identify the nuclear factors that bind the gastrin responsive elements in the HDC promoter, a human lung cDNA library was screened using two tandem copies of full-length GAS-RE (full) as the bait that spans from +1 to +48 in the promoter (Fig. 1A). After two positive clones from the first round of screening were pulled out, they were further confirmed by using the individual gastrin responsive elements GAS-RE1, GAS-RE2, and GAS-RE3 reporter strains (respective reporter constructs are shown in Fig. 1B). As shown in Fig. 1C, only transfection of the plasmid extracted from clone 2 (but not from clone 1) into each reporter strain showed cell growth...
on the 30 mM 3-AT containing plate, whereas on plates lacking 3-AT, they showed similar growth, indicating that protein expressed from clone 2 can bind all three gastrin responsive elements (and clone 1 is not a true positive). After sequencing, the plasmid extracted from clone 2 was found to contain the in-frame 3′ sequence of the human KLF4 cDNA. From the yeast one-hybrid screening, in addition to KLF4 we found seven other plasmids (containing partial in-frame sequence of Baf60a, Mnf1, elf4B, elf6, PRED56, KLF2(KLF), and DC24) transactivated by reporter gene expression. We decided to concentrate our studies in this report on KLF4, because this zinc finger containing transcriptional factor is highly expressed in the gastrointestinal tract, and inhibits ODC promoter activity (32), an enzyme that is within the same functional group as HDC.

KLF4 Binds All Three GAS-REs by EMSAs—After we identified KLF4 as a potential factor binding to GAS-REs, we next confirmed this interaction through EMSAs. As shown in Fig. 2A, GAS-RE1 was radiolabeled and mixed with nuclear extracts from AGS-E cells, and the reaction was then loaded onto a 6% nondenatured polyacrylamide gel. One strong (C2), and two weak DNA-protein complexes (C1 and C3) were observed (Fig. 2A, lane 2). Two hundred-fold molar excess of different unlabeled oligos were used as competitors in EMSAs. As shown in Fig. 2A, unlabeled mutant GAS-RE1 competed with the major C2 complex to a lesser extent compared with wild type GAS-RE1 (lane 4 versus lane 3). The weak C1 and C3 complexes appeared to be nonspecific by these competition assays. In addition, unlabeled mutant bcn1 oligo competed with the C2 complex to a much lesser degree than the wild type bcn1 oligo (lane 6 versus lane 5), a DNA binding motif in the laminin B2 chain gene promoter (49) that binds strongly to KLF4 as previously reported (34). These data suggest that KLF4 is present in the C2 complex. The presence of KLF4 in this complex was confirmed by supershift with a KLF4 antibody (C4, in lane 8). This supershift was not seen when another antibody raised against a highly related zinc finger-containing transcriptional factor Sp1 was used (lane 7). Thus, from the EMSA data above, we have shown using both KLF4 binding oligo competition and anti-KLF4 antibody competition that KLF4 binds GAS-RE1.

Similar EMSA experiments using GAS-RE2 and GAS-RE3 as labeled probes (shown in Fig. 2, B and C) also showed that KLF4 is able to bind both GAS-RE2 and GAS-RE3. Incubation of labeled GAS-RE2 and GAS-RE3 with nuclear extracts resulted in two shifted bands (C1 and C2), but only one (C1) was specific and could be competed away by wild type but not mutant competitors. Incubation with the KLF4 antibody again resulted in a specific supershift (C3, Fig. 2, B and C) indicated the presence of KLF4.

KLF4 Dose Dependently and Specifically Inhibits the HDC Promoter Activity—Previously, it has been reported that KLF4 can act as a transcriptional activator and repressor in different systems (30–34). In addition, KLF4 is highly expressed in epithelia of the gastrointestinal tract. Therefore, because KLF4 binds to the HDC promoter as demonstrated by yeast one-hybrid screening and EMSAs, it was of interest to determine the effect of KLF4 binding on HDC gene regulation. To first test the transcriptional activity of KLF4 on the HDC promoter activity, the minimal 107-bp HDC promoter firefly luciferase gene reporter construct was generated. As indicated in Fig. 3A, the minimal HDC promoter spans from −59 to +48 relative to the transcriptional initiation site (shown by +1) and contains an upstream TATA-like box, an upstream GC box, and the three downstream gastrin responsive elements.

To test the effect of KLF4 on HDC promoter activity, transient cotransfection experiments with the minimal HDC re-

**Fig. 2.** KLF4 binds to all three gastrin responsive elements in the HDC promoter. A, EMSAs were carried out by mixing nuclear extracts from AGS-E cells with radiolabeled GAS-RE1 probe. Lane 1 shows the probe without nuclear extract, and lane 2 shows the result of the binding assay in the absence of competitors. Lanes 3–6 showed competition assays in the presence of a 200-fold molar excess of the indicated competitors (lane 3 for wild type GAS-RE1, lane 4 for mutant GAS-RE1, lane 5 for wild type bcn1 oligo, and lane 6 for mutant bcn1 oligo). Lanes 7 and 8 show the competition assay by 1.0 μl of Sp1 antibody (Santa Cruz: sc-59X) and 1.0 μl of KLF4 antiserum (Kindly provided by ChiChuan Tseng), respectively. C1, C2, and C3 indicate the DNA-protein complexes, and C4 indicates the supershift complex by KLF4 antibody. B, same as A except GAS-RE2 was used as the probe. C, same as A except that GAS-RE3 was used as the probe and C3 as the supershift complex.
porter plasmid and increasing amounts of KLF4 expression plasmid (pcDNA3-KLF4) were conducted. As shown in Fig. 3B, KLF4 dose-dependently inhibited HDC promoter activity, with ~60% inhibition seen with 1.0 μg of KLF4 plasmid. KLF4 function was further tested on the activity of the reporter construct containing the full human 1.8-kb HDC promoter (8) by cotransfection with the KLF4 expression plasmid or the vector control. Similar to the minimal HDC reporter (Fig. 3C), KLF4 also inhibited the promoter activity of the 1.8-kb HDC promoter, although the basal activity of the 1.8-kb promoter was much greater than the minimal promoter suggesting the presence of additional enhancer element(s) within the 1.8-kb fragment. Nevertheless, the -fold inhibition of KLF4 on both promoters was similar. Therefore, we used the minimal HDC promoter construct for further studies.

It has previously been established that KLF4 is a member of the Kruppel-like family and contains three zinc fingers at the carboxyl terminus. KLF5, also named as IKLF (intestinal Kruppel-like factor), is the Kruppel-like factor most closely related to KLF4. To test the specificity of the KLF4 inhibitory effects on the HDC promoter, both KLF5 and KLF4 expression plasmids were cotransfected with the minimal HDC promoter reporter construct. Whereas there was ~60% inhibition observed in conjunction with KLF4 expression, there was no significant change in promoter activity with KLF5 expression (Fig. 3D). In addition, there was no inhibitory effect observed when only the KLF4 zinc finger (amino acid residues from 388 to 470) or the KLF5 zinc finger (amino acid residues from 374 to 457) were used instead of full-length KLF4 or KLF5, consistent with published data showing that the inhibitory domain of KLF4 is outside of the zinc finger (30). These results are consistent with other reports indicating that KLF5 and KLF4 have different transcriptional functions (8), and further confirmed that the HDC promoter was inhibited specifically by KLF4. Finally, to demonstrate that transfection of the CMV-KLF4 construct resulted in increased production of the KLF4 protein, Western blotting analysis was performed. It confirmed high levels of KLF4 protein were expressed in the transfected cells (Fig. 3E).

Endogenous HDC Expression Was Attenuated by KLF4 Expression—To gain further insights into the regulation of the HDC promoter activity by KLF4, the endogenous HDC messenger RNA level was measured upon attenuating endogenous KLF4 expression. As shown in Fig. 4A, endogenous KLF4 messenger RNA was down-regulated by its specific siRNA, and was significantly increased by KLF4 overexpression as might be expected. Accordingly, the endogenous HDC mRNA level was up-regulated by KLF4 siRNA and down-regulated by KLF4 overexpression (Fig. 4A, bottom panel), consistent with our earlier transfection results with reporter gene constructs. Quantitative PCR was further performed, indicating that the 2-fold reduction of the endogenous KLF4 mRNA level by KLF4-specific siRNA corresponds with about a 3-fold increase of endogenous HDC mRNA level (Fig. 4B, left panel). In addition, overexpression of KLF4 results in over 1000-fold increase of KLF4 mRNA level (data not shown), corresponding with the ~3-fold decrease of endogenous HDC mRNA level. Moreover, inhibition of KLF4 by the gene-specific siRNA induced HDC...
mRNA production by using RNA protection assay (Fig. 4D, lane 6 versus lane 5), thus further strengthening the model of down-regulation of the HDC transcription by KLF4.

Sp1 Transactivates HDC Promoter through Upstream GC Box—It has been reported that one of the mechanisms by which KLF4 inhibits gene expression is through competition with other transcriptional activators, such as Sp1, which bind GC-rich sequences (32). In the minimal HDC promoter construct, there is a GC-rich region (GGGCGG) with high homology to the consensus Sp1 binding motif. Although Sp1 binding with this GC box was shown by supershift using Sp1 antibody in EMSAs, the effect of Sp1 in HDC promoter activity was not fully studied (20). To further test the binding of Sp1 with this element, EMSAs were performed using the HDC-Sp1 oligo (5′-AGGGGACCTTGAGGCGGAGCGTGAAGGGAGCTAAGGTCA-3′) as the probe. This EMSA probe contains the core GC box GGGCGG along with additional nucleotides at both 5′ and 3′ ends. In the absence of competitors, three DNA-protein complexes were observed (C1, C2, and C3 in Fig. 5A, lane 2). Competition studies suggested that these protein-DNA complexes appear to be specific. Unlabeled mutant HDC-Sp1 oligo (HDC-Sp1M, 5′-AGGGACTTGAGGGCGGAGCTTGAGCTTAAGGCTAAGGGAGCTAAGGTCA-3′) with minimal mutations in the core GC box barely competed with these complexes compared with wild type oligo (Fig. 5A, lane 4 versus lane 3). Importantly, the unlabeled mutant Sp1 consensus oligo competed with these complexes much less than the wild type Sp1 consensus oligo (Fig. 5A, lane 6 versus lane 5), suggesting that Sp1 or one of its family members such as Sp3 and Sp4 is present in these complexes. The presence of Sp1 in the C1 complex was further confirmed by disruption of this complex and a supershift C4 using the anti-Sp1 antibody (lane 7). As a negative control, anti-KLF4 antibody did not influence the composition of the DNA-protein complexes (lane 8). The disruption of C2 and C3 complexes and multiple supershifts (SS in lane 9) using anti-Sp3 antibody in the EMSA indicates that the Sp3 is in these two complexes. However, overexpression of the Sp3 construct did not influence HDC promoter activity (data not shown), suggesting that the differential effects of Sp1 and Sp3 exist even though they can bind similar DNA motifs, which is consistent with the previously reported data (50). Thus, these EMSA results show clearly the binding of Sp1 with the upstream GC box in the HDC promoter.

To further test the effect of Sp1 binding with the GC box on the HDC promoter, an Sp1 expression plasmid was cotransfected with a HDC promoter reporter construct. With increasing amounts of the Sp1 expression plasmid, wild type HDC promoter activity was dose-dependently increased (Fig. 4B, black bars). However, in the presence of GC box mutations in the HDC promoter, previously shown to decrease Sp1 binding by EMSAs, basal HDC promoter activity as well as Sp1-induced HDC promoter activation was significantly reduced (Fig. 5B, white bars), indicating that Sp1 transactivates HDC promoter activity through binding with the upstream GC box. This up-regulation was further confirmed by the up-regulation of endogenous HDC mRNA after Sp1 overexpression using RNA protection assays (Fig. 4D, lane 4 versus lane 3).

KLF4 Competes with Sp1 in Regulation of HDC Promoter Activity—Functional studies showing an opposite effect on promoter activity by overexpression of KLF4 and Sp1 suggests a competition mechanism in regulation of the HDC promoter. To further explore this possibility, cotransfection of the Sp1 and KLF4 expression plasmids with the HDC reporter were performed. When the Sp1 plasmid was fixed to 1.0 μg, increasing amounts of KLF4 plasmid dose-dependently inhibited Sp1-induced HDC promoter activation (Fig. 6A). In contrast, in parallel cotransfection experiments where the exogenous amount of KLF4 expression plasmid was fixed, Sp1 was able to dose dependently reverse KLF4-mediated HDC promoter inhibition (Fig. 6B). These cotransfection experiments further strengthen the model of competition between Sp1 and KLF4 in transcriptional regulation.

GC Box in HDC Promoter Is Portable for KLF4 Inhibition—Because KLF4 competes with Sp1 in the regulation of HDC promoter activity, and Sp1 activates the promoter activity through the upstream GC box, it is suspected that this GC box also mediates KLF4 inhibition. If this model is correct, then this KLF4-mediated inhibitory function should be portable. To test this possibility, the GC box from the HDC promoter was cloned into an enhancerless promoter construct (pTATA-Luc+), and the effect of overexpression of the KLF4 construct was examined using transient cotransfection assays. As shown in Fig. 6C, the reporter activity as expected was significantly increased by the insertion of the GC box into the vector, because Sp1 (and possibly other members of Sp1 family) binds this DNA element resulting in transcriptional activation. This activation was largely abolished by the mutation in the core GC box, which further supports the activation effect of this GC box. More importantly, whereas there was minimal promoter inhibition when cotransfeting KLF4 with pTATA-Luc, there was 40% inhibition seen when cotransfeting the KLF4 with the GC box-containing promoter construct. Furthermore, mutations in the core GC box totally abrogated the KLF4-mediated inhibition (Fig. 6C). These data indicate that the GC box in the HDC promoter can also mediate KLF4-dependent inhibition, and that this inhibition is portable.

KLF4 Inhibits HDC Promoter Activity through Downstream Gastrin Responsive Elements—Because KLF4 binds all three downstream gastrin responsive elements by EMSAs, the relative importance of KLF4 binding to individual GAS-REs was then investigated. Mutant HDC reporter constructs with the upstream Sp1 binding site mutated (designated as Sp1M in Fig. 7) were first generated to avoid specific competition through this element. Mutations within each of three downstream gastrin responsive elements individually or in double or triple combinations were then introduced (designated as Sp1M-1M, Sp1M-2M, Sp1M-3M, Sp1M-12M, Sp1M-13M, Sp1M-23M, and Sp1M-123M in Fig. 7). Cotransfection experiments were then performed with these different mutant HDC reporters and KLF4 expression plasmid or the vector control. Among the different mutant HDC GAS-RE reporters, there was in general no significant change in the basal promoter activity when the vector control alone was cotransfected compared with the Sp1M reporter; the exception was the Sp1M-23M mutant reporter with mutations in both GAS-RE2 and GAS-RE3 along with the Sp1 mutation, which showed much higher basal activity. However, in the presence of background Sp1 binding site mutations (Sp1M), KLF4-dependent inhibition was partially relieved by GAS-RE mutations, single or in combination (Fig. 7). Interestingly, even though the KLF4 inhibition was partially relieved, the promoter activity was not fully recovered by these GAS-RE mutations. Nevertheless, the inhibition by KLF4 on the HDC promoter activity lacking an upstream Sp1 site indicates that KLF4 is able to inhibit HDC promoter activity in both Sp1-dependent and -independent manners.

KLF4 Inhibition and Gastrin Activation of HDC Promoter Are Separable Events—Previously, GAS-REs have been shown by our group to mediate gastrin-induced HDC promoter activation (8, 17). In this study, KLF4 was shown to bind to the GAS-REs and to inhibit HDC promoter activity. Thus, we investigated the relationship between gastrin activation and KLF4-mediated inhibition. Cotransfection experiments in
AGS-E cells were performed with the KLF4 expression plasmid and the different mutant HDC reporter constructs, containing mutations in the Sp1 binding GC box and in gastrin responsive elements (as described above). Cells were then treated with 10^{-7} M gastrin or left unchanged. Gastrin-mediated HDC promoter activation was reduced by mutation in GAS-REs (data not shown) as previously reported (17). Although KLF4-mediated inhibition was partially relieved by mutations in GAS-REs, all of the mutant HDC reporter constructs were responsive to gastrin treatment, with the Sp1M-23M construct showing the highest activation after gastrin treatment (Fig. 8). Taken together, these results suggest that gastrin-induced activation and KLF4-mediated inhibition of the HDC promoter activity are likely mediated by independent mechanisms.

**DISCUSSION**

In this study, we employed yeast one-hybrid screening to identify KLF4, a transcription factor highly expressed in the gastrointestinal tract with a molecular mass of 56 kDa, as one of the nuclear factors that binds to the GAS-RE domains in the HDC promoter. The molecular weight of KLF4 initially suggested to us that it was a good candidate for the GAS-RE1 binding protein (17), but further EMSA studies and co-transfection studies revealed that KLF4 in fact binds all three gastrin responsive elements. Cotransfection experiments showed that KLF4 dose dependently and specifically repressed HDC promoter activity and endogenous HDC mRNA expression was also regulated by KLF4. In vitro cotransfection studies and heterologous promoter studies supported a model of competition between Sp1 and KLF4 in regulation of HDC gene expression. Finally, mutations in the HDC promoter that abrogated KLF4-mediated inhibition were unable to block gastrin stimulation, suggesting that gastrin regulation and KLF4 inhibition on HDC promoter are likely distinct.

Our results in this study suggest that KLF4 inhibits HDC promoter activity by competition with Sp1 at the upstream GC box. This is consistent with previous reports showing that KLF4 represses cyclin D1 promoter (51), ODC promoter (32), and CYP1A1 promoter (52) through an Sp1-dependent mechanism. However, it has also been reported that under certain circumstances, KLF4 can activate gene transcription in combination with Sp1. For example, Sp1 and KLF4 have been reported to synergistically transactivate the rat laminin γ1 chain promoter (34) and the keratin 19 promoter (39). These differential transcriptional activities are consistent with the known function of KLF4 to inhibit proliferation by down-regulating genes that promote cell cycle progression and proliferation (such as cyclin D1 and ODC promoter), and to promote expression of differentiation genes (such as keratin 4 and keratin 19). A possible explanation for the opposite activity of KLF4 on different promoters may have to do with differences in primary structure of the promoters. It appears that in the repressed promoters, such as in cyclin D1, ODC, and CYP1A1, both Sp1 and KLF4 share one binding site, whereas in the activated promoters, there are separate Sp1 and KLF4 binding sites, such as in rat laminin γ1 and keratin 19 promoters. Presumably, KLF4 represses gene transcription by competing with Sp1...
within one KLF4/Sp1 binding site, whereas in the setting of separate binding sites, KLF4 is able to activate gene expression in synergy with Sp1. In the HDC proximal promoter region there is an Sp1 binding GC box, and there is no obvious separate KLF4 binding site in this region, which fit the one-site competition model as proposed above. However, this model cannot entirely account for the fact that KLF4 also binds to three downstream GAS-REs that likely leads to additional inhibition, although the binding sites are not all consensus CACCC motifs. Through competition with the Sp1 binding sites, KLF4 may inhibit gene transcription by displacing the recruitment of the Sp1 containing transcriptional activator complex to the proximal promoter site. In addition to direct competition with Sp1 for binding to the HDC promoter, KLF4 could mediate transcriptional repression through several additional mechanisms. First, physical interaction between Sp1 and KLF4 has already been shown by Zhang et al. (52). This interaction might disrupt the recruitment of the transcriptional coactivator complex, such as the cofactor (e.g. CRSP) required for Sp1-mediated gene transcriptional activation (53), resulting in transcriptional inhibition. Second, KLF4 might also interact directly with the coactivator complexes, leading to failure of the recruitment of the complexes to the Sp1 binding site or to the inhibition of the activity of the coactivator complexes. It should be noted that all of these possibilities are not mutually exclusive, and further experiments need to be done to address these possibilities.

Our study also demonstrated that KLF4 inhibits HDC promoter activity in an Sp1-independent manner through the downstream GAS-REs (Fig. 7), thus adding another layer of complexity to HDC gene regulation by KLF4. Although KLF4 could still inhibit promoter activity of HDC constructs lacking a functional upstream Sp1 binding site, in the absence of this Sp1 site the basal promoter activity was markedly reduced (data not shown). This suggests that inhibition through this
KLF4 Represses the HDC Promoter

Fig. 7. KLF4 inhibits HDC promoter activity through downstream gastrin responsive elements. Different minimal HDC reporter constructs with mutations in the Sp1 binding GC box and in each gastrin responsive element (see “Materials and Methods”) were cotransfected with vector (pcDNA3, empty bars) or KLF4 expression plasmid (pcDNA3-KLF4, black bars) into AGS-E cells. Statistical difference is indicated by a star (*) between the KLF4 overexpression plasmid and vector control using different mutant box. 1M/H11021p statistically significant (induced inhibition of the HDC promoter). Cells were starved overnight, then described in the legend to Fig. 7 were cotransfected with the KLF4 expression plasmid (pcDNA3-KLF4), Sp1M, 2M, and 3M indicate the mutations within GAS-RE 1, 2, and 3, respectively, and 12M, 13M, 23M, and 123M indicate the mutations in combination. The relative HDC promoter activity was calculated as described in the legend to Fig. 3B. Statistical difference is indicated by a star (*) between the KLF4 overexpression plasmid and vector control using different mutant HDC promoter reporters (p < 0.05). Partial relief of KLF4 induced inhibition of the HDC promoter activity by mutations in the GAS-REs in the presence of the Sp1 mutation in the promoter is also statistically significant (p < 0.05).

Fig. 8. KLF4 inhibition and gastrin activation of HDC promoter are separable events. Different HDC mutant reporters as described in the legend to Fig. 7 were cotransfected with the KLF4 expression plasmid (pcDNA3-KLF4). Cells were starved overnight, then treated with 10−7 M gastrin (black bars) or left unchanged (empty bars) for 24 h. The relative HDC promoter activity of each reporter was calculated as described in the legend to Fig. 3B. Statistical difference is indicated by a star (*) between gastrin treatment and control in the presence of pcDNA3-KLF4 using different mutant HDC promoter reporters (p < 0.05).

upstream Sp1 site likely plays a major role, whereas inhibition by KLF4 through the downstream GAS-REs may serve a more minor role. The portability of the GC box in the HDC promoter and the intact GC box-dependent inhibition by KLF4 (Fig. 6C) strongly suggests a general competition between Sp1 and KLF4 in regulation of gene expression. The GAS-REs mutations either individually or in combination only partially relieved KLF4 inhibition, suggesting that the functional mutations were not achieved, that KLF4 was binding to additional sites, or that an additional inhibitory mechanism was involved. The finding of KLF4 inhibition of transcription from a promoter without a cognate binding site (data not shown) would favor the last possibility.

In addition to the HDC promoters, gastrin also up-regulates several other promoters, such as chromogranin A (21), vesicular monoamine transporter subtype 2 (22), and heparin binding-epidermal growth factor (54). Importantly, it has been clearly shown that gastrin up-regulates the first two promoters through Sp1 binding sites, whereas in the case of heparin binding-epidermal growth factor promoter, Sp1 is not involved in gastrin regulation. In the human HDC promoter, the gastrin responsive elements are mapped downstream of the transcriptional initiation site (17), and the Sp1 binding GC box is located upstream. In the current study, we showed that even though the upstream Sp1 site is mutated, the HDC promoter is still up-regulated by gastrin treatment (Fig. 8). These data further suggest that even though gastrin up-regulates gene expression through GC-rich sequences, the mechanisms mostly likely are distinct. In addition, the response to gastrin stimulation remained intact when KLF4 binding sites were mutated (Fig. 8). The precise relationship between gastrin activation and KLF4 inhibition, which were mapped to the same region of the promoter, is currently unknown, but presumably involves different activation and repression complexes that share the same general sites but are not mutually exclusive. Further identification of nuclear factors/transcriptional factors that bind to three gastrin responsive elements in the HDC promoter and mediate gastrin responsiveness will help to delineate the mechanism.

Acknowledgments—We thank Dr. Chichuan Tseng for kindly providing pcDNA3-KLF4 plasmid, Sp1 plasmid, and anti-KLF4 antiserum. We also thank Dr. Raktima Raychowdhury for help with EMSAs and Dr. John V Fleming for insightful discussions.

REFERENCES
Kruppel-like Factor 4 (KLF4) Represses Histidine Decarboxylase Gene Expression through an Upstream Sp1 Site and Downstream Gastrin Responsive Elements
Wandong Ai, Ying Liu, Michael Langlois and Timothy C. Wang

doi: 10.1074/jbc.M308278200 originally published online December 10, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308278200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 30 of which can be accessed free at http://www.jbc.org/content/279/10/8684.full.html#ref-list-1