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## Human *MUC2* Mucin Gene Is Transcriptionally Regulated by Cdx Homeodomain Proteins in Gastrointestinal Carcinoma Cell Lines\*

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**In intestinal metaplasia and 30% of gastric carcinomas, *MUC2* intestinal mucin and the intestine-specific transcription factors Cdx-1 and Cdx-2 are aberrantly expressed. The involvement of Cdx-1 and Cdx-2 in the intestinal development and their role in transcription of several intestinal genes support the hypothesis that Cdx-1 and/or Cdx-2 play important roles in the aberrant intestinal differentiation program of intestinal metaplasia and gastric carcinoma. To clarify the mechanisms of transcriptional regulation of the *MUC2* mucin gene in gastric cells, pGL3 deletion constructs covering 2.6 kb of the human *MUC2* promoter were used in transient transfection assays, enabling us to identify a relevant region for *MUC2* transcription in all gastric cell lines. To evaluate the role of Cdx-1 and Cdx-2 in *MUC2* transcription we performed co-transfection experiments with expression vectors encoding Cdx-1 and Cdx-2. In two of the four gastric carcinoma cell lines and in all colon carcinoma cell lines we observed transactivation of the *MUC2* promoter by Cdx-2. Using gel shift assays we identified two Cdx-2 binding sites at -177/-171 and -191/-187. Only simultaneous mutation of the two sites resulted in inhibition of Cdx-2-mediated transactivation of *MUC2* promoter, implying that both Cdx-2 sites are active. Finally, stable expression of Cdx-2 in a gastric cell line initially not expressing Cdx-2, led to induction of *MUC2* expression. In conclusion, this work demonstrates that Cdx-2 activates the expression of *MUC2* mucin gene in gastric cells, inducing an intestinal transdifferentiation phenotype that parallels what is observed both in intestinal metaplasia and some gastric carcinomas.**

There is consistent data indicating that in human stomach as well as in other organs mucin genes are expressed in a

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regulated cell- and tissue-specific manner and that altered mucin gene expression occurs in cancer and precancerous lesions (1). In normal gastric mucosa most studies show little or no expression of the intestinal mucin *MUC2* (2–9). In intestinal metaplasia, a preneoplastic lesion of the stomach characterized by the transdifferentiation of the gastric mucosa to an intestinal phenotype, there are alterations in the mucin expression pattern including *de novo* expression of *MUC2*, mostly in goblet cells (10). Thirty percent of gastric carcinomas, including all carcinomas of the mucinous type, also aberrantly express *MUC2* intestinal mucin (11, 12). The molecular mechanisms responsible for the regulation of *MUC2* transcription and expression are beginning to be elucidated. The structure of *MUC2* promoter was characterized (13, 14) and *MUC2* expression was reported to be regulated by methylation of the promoter (15–17) and by the Sp1 family of transcription factors (13, 18, 19). It has also been described that *MUC2* is transcriptionally activated by p53 (20) and, in tracheobronchial epithelial cells, by lipopolysaccharide from *Pseudomonas aeruginosa* (21, 22) and epidermal growth factor (19). However, information on *MUC2* transcriptional regulation in gastric cells, in relation with the overexpression of *MUC2* in intestinal metaplasia and gastric carcinoma, is essentially unknown.

The intestine-specific homeobox genes *Cdx-1*<sup>1</sup> and *Cdx-2* were also recently shown to be aberrantly expressed in intestinal metaplasia and in a subset of gastric carcinomas. Both in intestinal metaplasia and in gastric carcinoma, expression of *Cdx-1* and *Cdx-2* is closely associated with the expression of mucin *MUC2* (23). Altogether, these observations suggest that *Cdx-1* and/or *Cdx-2* play important roles in the aberrant intestinal differentiation program of intestinal metaplasia and some gastric carcinomas, partly due to *MUC2* regulation at the transcriptional level (23). This hypothesis is further supported by the direct involvement of *Cdx-1* and *Cdx-2* in the differentiation of intestinal epithelial cells (24), namely in transgenic models (25) and by the evidence showing that they act as transcription factors for several intestinal genes such as sucrase-isomaltase (24, 26, 27), lactase-phlorizin hydrolase (27–30), intestine phospholipase A/lysophospholipase (31), claudin-2 (32), and more recently  $\beta$ -1,3-galactosyltransferase T5 (33).

Since the promoter of *MUC2* contains some Cdx putative binding sites, we hypothesized that *Cdx-1* or *Cdx-2* might func-

<sup>1</sup> The abbreviations used are: Cdx-1, caudal-related homeobox factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIF1, sucrase-isomaltase footprint 1; 5'-UTR, 5'-untranslated region; EMSA, electrophoretic mobility shift assay; RT, reverse transcriptase.

TABLE I  
Sequences of the oligonucleotides used for RT-PCR, site-directed mutagenesis, and EMSAs

Primer sets used in RT-PCR analysis of gastric carcinoma cells<sup>a</sup> and colon carcinoma cells<sup>b</sup> are indicated. Sense oligonucleotides used for site-directed mutagenesis and EMSAs are shown. Antisense oligonucleotides were also synthesized. For EMSAs, sense and antisense oligonucleotides were annealed to produce double-stranded DNA. Positions of the Cdx-2 binding sites relative to *MUC2* transcription start site are indicated (36). Mutated nucleotides are bold, italicized, and underlined.

Primers used for RT-PCR	Sequence (5'→3')
<i>MUC2</i> <sup>a</sup>	S: CGA AAC CAC GGC CAC AAC GT AS: GAC CAC GGC CCC GTT AAG CA
<i>Cdx-1</i> <sup>a</sup>	S: GGA CCA AGG ACA AGT ACC GC AS: GGT GTT GCT GGG ACA CAG G
<i>Cdx-2</i> <sup>a</sup>	S: CCA GGA CGA AAG ACA AAT ATC GA AS: CTC TGG GAC ACT TCT CAG AGG
<i>GAPDH</i> <sup>a</sup>	S: ACC ATC TTC CAG GAG CGA G AS: GGA TGA CCT TGC CCA CAG
<i>MUC2</i> <sup>b</sup>	S: CTG CAC CAA GAC CGT CCT CAT G AS: GCA AGG ACT GAA CAA AGA CTC AGA C
<i>Cdx-1</i> <sup>b</sup>	S: CGG GCA CAC CGT CCT CGC CCG AS: CAT TGG AGA GGA GGT GGC CAG G
<i>Cdx-2</i> <sup>b</sup>	S: CGG CTG GAG CTG GAG AAG G AS: TCA GCC TGG AAT TGC TCT GC
28S <sup>b</sup>	S: GCA GGG CGA AGC AGA AGG AAA CT AS: TGA GAT CGT TTC GGC CCC AA
Primers used for site-directed mutagenesis	Sequence (5'→3')
Cdx-2 site -191/-187	AAG AAG GCT GCG <b><i>CGC TGC</i></b> CAG GGA GCC ATA AAG AGA TGA CCT CCG CGG AGG TCA TCT CTT TAT GGC TCC CTG <b><i>GCA CGC</i></b> CGC AGC CTT CTT
-177/-171	AAG AAG GCT GCG TTT ACC CAG GGA GCC <b><i>CGC TGG</i></b> AGA TGA CCT CCG CGG AGG TCA TCT <b><i>CCA CGC</i></b> GGC TCC CTG GGT AAA CGC AGC CTT CTT
Double mutation	CTG AAG AAG GCT GCG <b><i>CGC TGC</i></b> TCC CAG GGA GCC <b><i>CGC TAG</i></b> AGA TGA CCT CCG CGG AGG TCA TCT <b><i>CTA CGC</i></b> GGC TCC CTG <b><i>GGA CGC</i></b> CGC AGC CTT CTT CAG
Oligonucleotides used for EMSAs	Sequence (5'→3')
Probe	
SIF1	GGG TGC AAT AAA ACT TTA TGA GTA
wt -201/-161	GAA GGC TGC GTT TAC CCA GGG AGC CAT AAA GAG ATG ACC T
dm -201/-161	GAA GGC TGC <b><i>GGC GTG</i></b> CCA GGG AGC <b><i>CGC GTG</i></b> GAG ATG ACC T

tion as transcriptional regulators of *MUC2*. In this study we show that Cdx-2 is a regulator of *MUC2* expression both in gastric and intestinal cancer cells whereas Cdx-1 only transactivates *MUC2* in intestinal cells. The implications in intestinal metaplasia and gastrointestinal cell differentiation are discussed.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—Human gastric carcinoma cell lines were cultured at 37 °C in a humidified 5% CO<sub>2</sub> incubator. GP202, GP220, and MKN45 cells were maintained in RPMI 1640 medium (with Glutamax and 25 mM Hepes) supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). KATOIII cell line was maintained in RPMI 1640 medium supplemented with 20% fetal bovine serum and gentamicin (50 µg/ml). AGS cell line was cultured in Nutrient Mixture Ham's F12 (with L-Glutamine) supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). Human colon carcinoma cell lines HT-29 STD, Caco-2 and LS174T were cultured as described in Van Seuning *et al.* (34). Cell lines GP202 and GP220 were established at IPATIMUP (35).

**Reverse Transcriptase-PCR Analysis**—Total RNA from gastric carcinoma cell lines was isolated using Purescript RNA isolation kit (Gentra systems, Minneapolis) and treated with DNase. 5 µg of RNA were primed with random hexamers and reverse transcribed using Superscript III (Invitrogen) in a final volume of 20 µl. Four microliters of this mixture was PCR-amplified in a 25-µl reaction using AmpliTaq DNA polymerase (Applied Biosystems). The sequences of the primers used to amplify *MUC2*, *Cdx-1* and *Cdx-2* are indicated in Table I. *GAPDH* was used as an internal standard. The PCR reaction mixture was denatured at 94 °C for 2 min followed by 35 cycles at 94 °C for 45 s, 55 °C for 15 s, and 72 °C for 45 s for *MUC2*, *Cdx-1* and *Cdx-2* (25 cycles for *GAPDH*).

Total RNAs from colon cancer cells were prepared using the RNeasy mini-kit from Qiagen. Cells were harvested at 100% of confluence and 1.5 µg of total RNA was used to prepare cDNA (Advantage™ RT-for-PCR kit, Clontech) as described before (34). PCR was performed on 2 µl of cDNA using specific pairs of primers (MWG-Biotech, Germany) for *MUC2*, *Cdx-1*, *Cdx-2*, and 28 S rRNA as described in Table I. PCR

reactions were carried out in 50-µl final solutions (5 µl of 10× PCR buffer containing MgCl<sub>2</sub>, 4 µl 2.5 mM dNTPs, 10 pmol of each primer, 1 unit of Taq polymerase (Roche Applied Science)). Cycling conditions were as follows: 1) denaturation: 94 °C, 2 min for one cycle; 2) denaturation: 94 °C, 45 s; annealing: 60 °C, 1 min; and extension: 72 °C, 1 min for 30 cycles; and 3) final extension: 72 °C, 10 min. PCR products were analyzed on 2% ethidium bromide-agarose gels run in 1× Tris borate-EDTA buffer.

**MUC2/Luciferase Plasmid Construction and Transient Transfection Assays**—pGL3-*MUC2* promoter constructs covering the -947/-1, -2096/+27, and -2627/-1 regions of *MUC2* promoter were previously described and used to study *MUC2* regulation in the mucocellular NCI-H292 lung cancer cell line (19). -371/+27 and -947/+27 deletion mutants used in this study were prepared as described in Perrais *et al.* (19). For the transient transfection assays, gastric carcinoma cell lines were seeded at 2.5 × 10<sup>5</sup>/well in 24-well plates. Transfections and co-transfections were performed the next day by mixing 0.8 µg of the pGL3 construct of interest, and 0.4 µg of expression vector in the co-transfections, with tfx-50 reagent (Promega) (tfx-50:DNA ratio of 4:1) in 200 µl of serum-free and antibiotic-free medium. Cells were incubated with the transfection mixture for 1 h at 37 °C followed by the addition of 1 ml of complete medium. Total cell extracts were prepared after a 48 h incubation at 37 °C using 1× reporter lysis buffer (Promega), as described in the manufacturer's instruction manual. 20 µl of cell extract were mixed with 100 µl of luciferase assay reagent (Promega) to determine luciferase activity in a 1450 Microbeta luminescence counter (Wallac). The β-galactosidase activity was measured using 50 µl of cell extract. The luciferase activity of test plasmids is expressed as fold of induction of the test plasmid activity compared with that of the corresponding empty vector (pGL3 basic, Promega), after correction for transfection efficiency as measured by the β-galactosidase activity. Each plasmid was assayed in triplicate in two separate experiments. Transfection of colon carcinoma cell lines was performed as described in Perrais *et al.* (19).

**Stable Transfections with Cdx-2**—The gastric carcinoma cell line, GP202, was transfected at confluence with 10 µg of the Cdx-2 expression vector (pRC/CMV-Cdx-2), or the empty vector pRC/CMV (mock

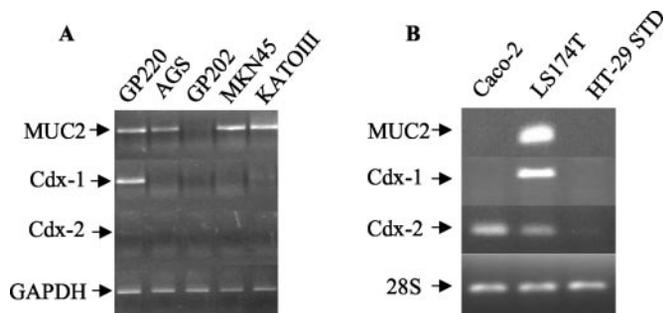


FIG. 1. Study of the expression pattern of the *MUC2*, *Cdx-1*, and *Cdx-2* mRNA in gastric and colon cancer cells. A, human gastric carcinoma cell lines (GP220, AGS, GP202, MKN45, and KATOIII) with GAPDH as an internal control. B, human colon carcinoma cell lines (Caco-2, LS174T, and HT-29 STD). 28 S was used as an internal control. PCR products were separated on a 2% agarose gel run in 1× Tris borate-EDTA buffer in the presence of ethidium bromide.

cells). Transfections were performed with tfx-50 reagent (Promega) (tfx-50:DNA ratio of 4:1) and the procedure was as previously described for transient transfections. After 48 h, transfected cells were selected with the antibiotic G418 (Sigma) at a final concentration of 0.5 mg/ml.

**Site-directed Mutagenesis**—QuickChange site-directed mutagenesis kit (Stratagene) was used to generate site-specific mutations in the two *Cdx-2* binding sites in the *MUC2* promoter constructs  $-371/+27$  and  $-947/+27$ . Single and double mutations were realized for each construct. Oligonucleotides containing the desired mutations were designed according to the manufacturer's instructions and their sequences are depicted in Table I.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extracts from GP220 cells were prepared as described by Van Seuning *et al.* (37) and kept at  $-80^{\circ}\text{C}$  until use. Protein content (2  $\mu\text{l}$  of cell extracts) was measured using the bicinchoninic acid method (Pierce), as described in the manufacturer's instruction manual. The sequences of the oligonucleotides used for EMSAs are indicated in Table I. They were synthesized by MWG-Biotech (Germany). SIF1 (sucrase-isomaltase footprint 1) probe, corresponding to an evolutionarily conserved *Cdx-2* cis-element of the sucrase isomaltase gene (26), was used as a positive control. Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and  $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ . Radiolabeled probes were purified by chromatography on a Bio-Gel P-6 column (Bio-Rad). Nuclear proteins (8  $\mu\text{g}$ ) were preincubated for 20 min on ice in 20  $\mu\text{l}$  of binding buffer with 1  $\mu\text{g}$  of poly(dI-dC) (Sigma) and 1  $\mu\text{g}$  of sonicated salmon sperm DNA. Radiolabeled DNA probe was added (60,000 cpm), and the reaction was left for another 20 min on ice. For competition experiments 50× excess of the cold probe was added 20 min before adding the radiolabeled probe. For supershift analyses, 1  $\mu\text{l}$  of the *Cdx-2* antibody (Biogenex) was added to the proteins and left for 30 min at room temperature before adding the radiolabeled probe. Reactions were stopped by adding 2  $\mu\text{l}$  of loading buffer. Samples were loaded onto a 4% non-denaturing polyacrylamide gel, and electrophoresis conditions were as described in Van Seuning *et al.* (34). Gels were vacuum-dried and autoradiographed for 48 h at  $-80^{\circ}\text{C}$ .

**Immunofluorescence**—GP220 and GP202 cells, *Cdx-2* transfected GP202 clones and GP202 mock cells were trypsinized, fixed in Preserv-Cyt solution (Cytoc) and layed on slides in a ThinPrep2000, according to the manufacturer. Cells were treated with neuraminidase, for 2 h at  $37^{\circ}\text{C}$ , and blocked with non immune serum diluted at 1:5 in a 10% bovine serum albumin solution for 20 min. Excess normal serum was removed and replaced with anti-MUC2 PMH1 antibody (10). Slides were incubated overnight at  $4^{\circ}\text{C}$ . After three washes with 1× phosphate-buffered saline, the slides were incubated at room temperature with fluorescein isothiocyanate-labeled secondary antibody (DAKO) diluted at 1:200 with a 5% bovine serum albumin solution, for 30 min in the dark, mounted in vectashield with DAPI (Vector, Burlingame, CA), and analyzed with a Leica DMIRE2 fluorescent microscope.

## RESULTS

**Expression of *MUC2*, *Cdx-1*, and *Cdx-2* in Human Gastric and Colon Carcinoma Cell Lines**—Expression of *MUC2*, *Cdx-1*, and *Cdx-2* was studied by RT-PCR. As shown in Fig. 1A, *MUC2* mRNA is expressed in all gastric carcinoma cell lines, except for GP202. *Cdx-1* is expressed in GP220 gastric carcinoma cell

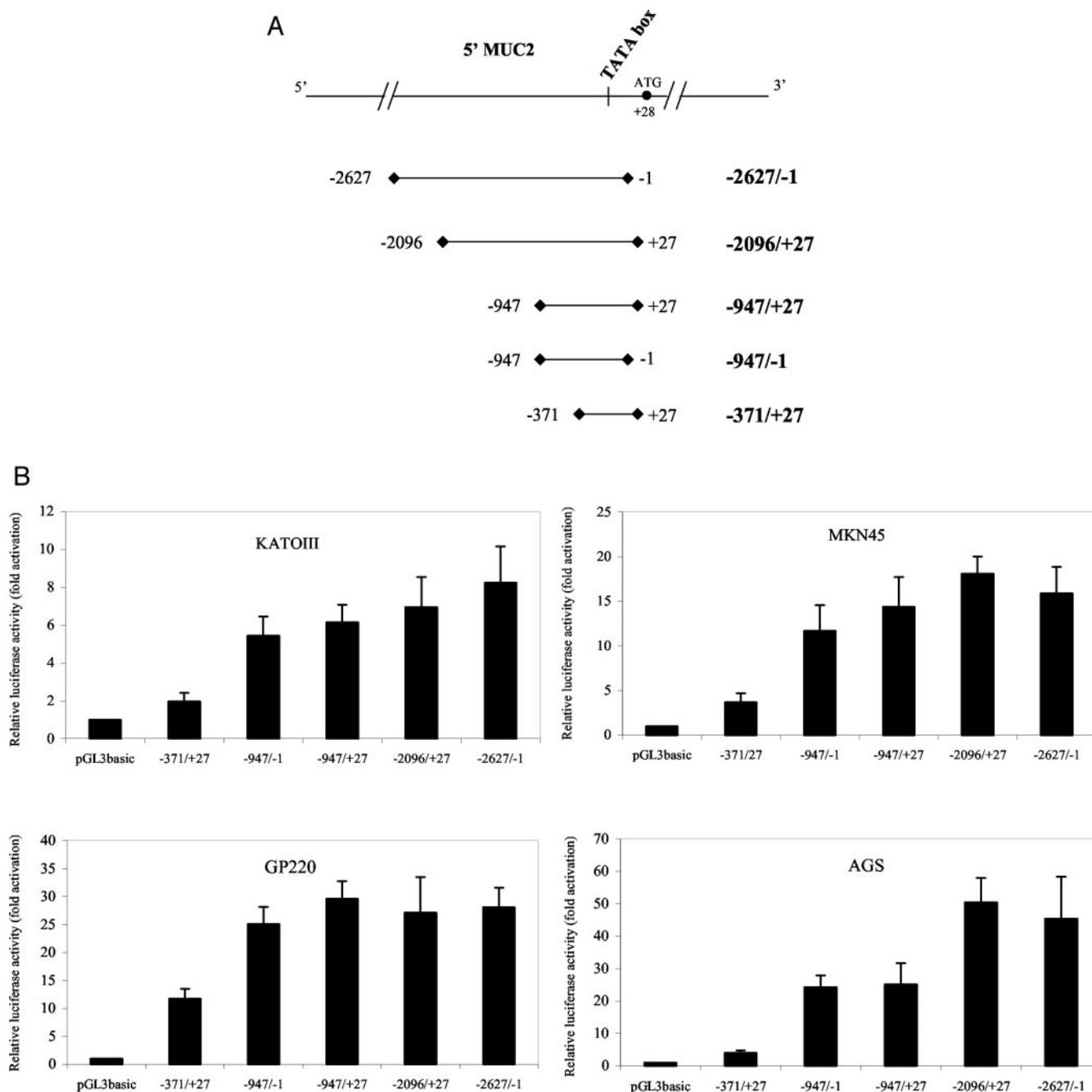
line. These cells also express low levels of *Cdx-2* (Fig. 1A). In colon carcinoma cell lines, *MUC2* is expressed in mucus-secreting LS174T cells and these cells also express *Cdx-1* and *Cdx-2*. Caco-2 enterocytes and HT-29 STD undifferentiated cells do not express *MUC2* or *Cdx-1*. *Cdx-2* is expressed in Caco-2 cells and to a lower extent in HT-29 STD cells (Fig. 1B).

**Characterization of the Promoter Activity of *MUC2* Gene in Gastric Cancer Cells**—A panel of deletion mutants covering 2.6 kb of the promoter of *MUC2* were constructed in promoterless pGL3 basic vector (Fig. 2A). They were used in transient transfection experiments in four gastric carcinoma cell lines (KATOIII, MKN45, GP220, and AGS) (Fig. 2B). Transient transfection of a pGL3 basic reporter construct containing nucleotides  $-371$  to  $+27$  of the *MUC2* gene resulted in low levels of luciferase activity. Addition of the next 576 nucleotides up to nucleotide  $-947$  ( $-947/+27$ ) led to a significant increase in promoter activity in all cell lines (about 4-fold activation on average) (Fig. 2B). Addition of the distal region up to nucleotide  $-2096$  ( $-2096/+27$ ) increased normalized luciferase activity by 2-fold in AGS cells and in MKN45 cells to a lower extent (1.2-fold). No further increase in activity was observed when the  $-2627/-1$  construct was used in either of the cell lines tested (Fig. 2B). The presence of the 5'-UTR in construct  $-947/+27$  did not modify the activity of the corresponding construct devoid of the 5'-UTR ( $-947/-1$ ). This suggests that the 5'-UTR does not play a major role in regulating *MUC2* gene in gastric cancer cells.

In conclusion, these results suggest that, in the four gastric cancer cell lines studied, essential positive regulatory elements for *MUC2* promoter activity are present within the  $-947/-372$  region. In AGS cells, a second distal active region, stretching over the  $-2096/-948$  nucleotides, contains enhancer elements.

***Cdx-1* and *Cdx-2* Transactivate the *MUC2* Promoter in a Cell-specific Manner**—The involvement of *Cdx-1* and *Cdx-2* in the intestinal development and differentiation and their role as transcription factors for several intestinal genes support the hypothesis that *Cdx-1* and/or *Cdx-2* could regulate *MUC2* transcription. Adding to this, four putative binding sites for *Cdx-1* and *Cdx-2* were identified in *MUC2* promoter at  $-177/-171$ ,  $-191/-187$ ,  $-1010/-1006$ , and  $-2614/-2610$  that all contain *Cdx* consensus sequence TTTAT/C (Fig. 3A). Co-transfection experiments were performed to study the biological effect of these transcription factors on *MUC2* transcriptional activity using pGL3-*MUC2* deletion constructs in the presence of expression vectors encoding *Cdx-1* or *Cdx-2*. The luciferase activity was compared with the one obtained in the co-transfection experiments carried out with the corresponding empty vector. *Cdx-1* did not have any significant effect on *MUC2* promoter activity in any of the gastric carcinoma cell lines, except for a 30% inhibition of the luciferase activity observed with the  $-371/+27$  fragment in AGS cells (Fig. 3B, black bar). In MKN45 (gray bars), co-transfection with *Cdx-2* induced luciferase activity of the four *MUC2* promoter constructs (4–7 fold induction) (Fig. 3B). In KATOIII cells (hatched bars), *Cdx-2* co-transfection resulted in a 2.5-fold transcriptional activation of the  $-947/+27$  construct. *Cdx-2* had no effect on the other two gastric carcinoma cell lines tested (GP220 and AGS), except for a 70% inhibitory effect with the  $-371/+27$  fragment in the AGS cell line (black bars).

The same experiments performed in HT-29 STD, LS174T and Caco-2 colon carcinoma cell lines indicate that *Cdx-2* transactivates *MUC2* promoter in the three cell lines tested (Fig. 3C). In HT-29 STD (23-fold), LS174T (55-fold), and Caco-2 (15-fold), *Cdx-2* transactivation is much stronger with the smaller construct ( $-371/+27$ ) when compared with the longer construct ( $-2627/-1$ ) (Fig. 3C). One can note that the levels of

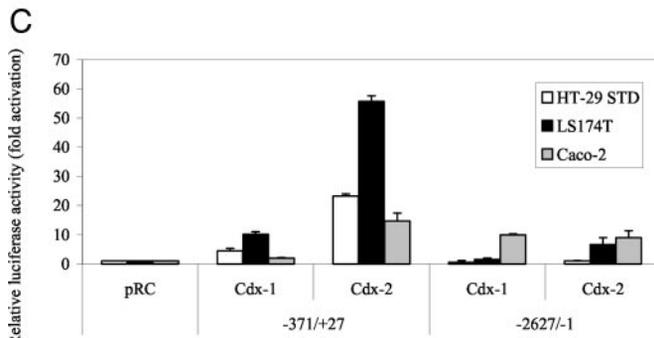
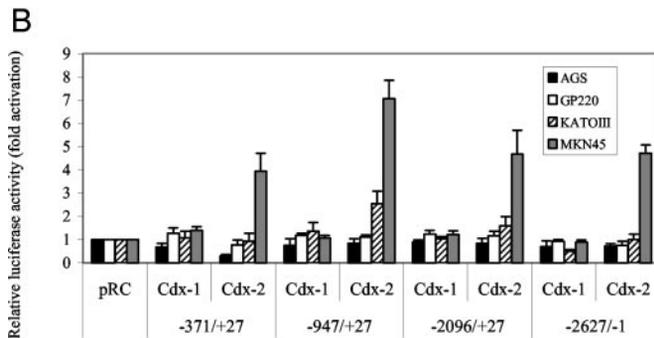
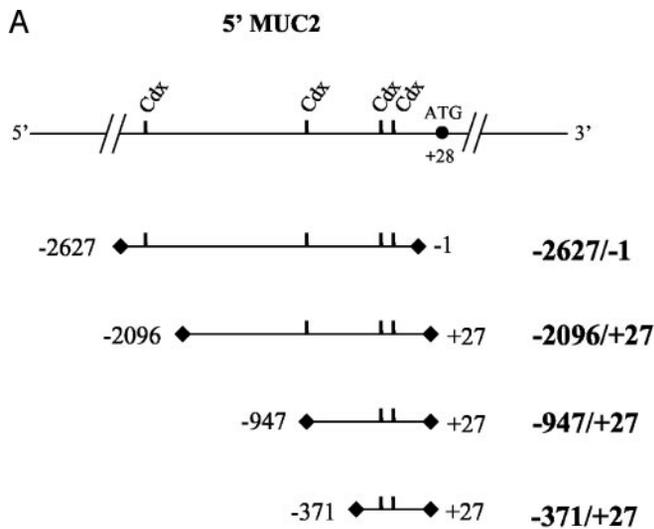


**FIG. 2. Analysis of the activity of the promoter of MUC2 gene by transient transfections.** A, schematic representation and localization of the different constructs covering 2627 nucleotides of the MUC2 promoter. B, transcriptional activity in gastric carcinoma cell lines (KATOIII, MKN45, GP220, and AGS). The values obtained in cells transfected with the empty vector were referred as to 1. The results are means  $\pm$  S.D. and represent two separate experiments in triplicate for each fragment.

activation vary greatly between the different cell lines indicating cell-specific activity of Cdx-2. Unlike gastric cells, we observed transactivation of MUC2 promoter by Cdx-1 in colon cancer cells. Transactivation was more efficient on the short construct  $-371/+27$  in HT-29 STD (4.5-fold) and LS174T (10-fold) cells whereas it was more active on the long construct  $-2627/-1$  in Caco-2 cells (10-fold). The transactivation is however much less important than with Cdx-2. From these studies it can be concluded that the MUC2 promoter is strongly transactivated by Cdx-2 in KATO-III and MKN45 gastric carcinoma cell lines as well as in all colon carcinoma cell lines. Cdx-1 appears more specific as it only transactivates MUC2 promoter in colon cancer cells.

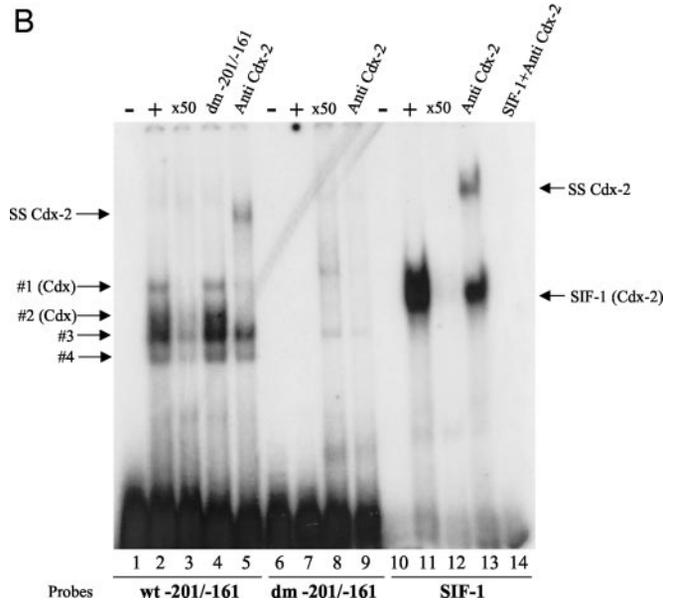
**Identification of Two Cdx-2 cis-Elements within the MUC2 Promoter**—In order to demonstrate that the Cdx-2 transcrip-

tion factor binds to the MUC2 promoter, EMSAs were performed in the presence of GP220 nuclear extracts and probe wild type  $-201/-161$ , that contains the two putative Cdx-2 binding sites at  $-177/-171$  and  $-191/-187$ , respectively (Fig. 4A). Incubation of probe wild type  $-201/-161$  with nuclear proteins led to the formation of four major shifted bands (Fig. 4B, lane 2, complexes 1–4). The specificity of these complexes was confirmed by the absence of retarded bands in the samples preincubated with 50 $\times$  excess of the cold probe (lane 3). On the contrary, cold competition with dm  $-201/-161$  probe, in which the two Cdx-2 binding sites were mutated, did not result in the inhibition of the four complexes (lane 4). Binding of Cdx-2 was confirmed by a total supershift of complexes 1 and 2 upon addition of anti-Cdx-2 antibody in the reaction mixture (lane 5). Involvement of the two Cdx-2 binding sites was confirmed by



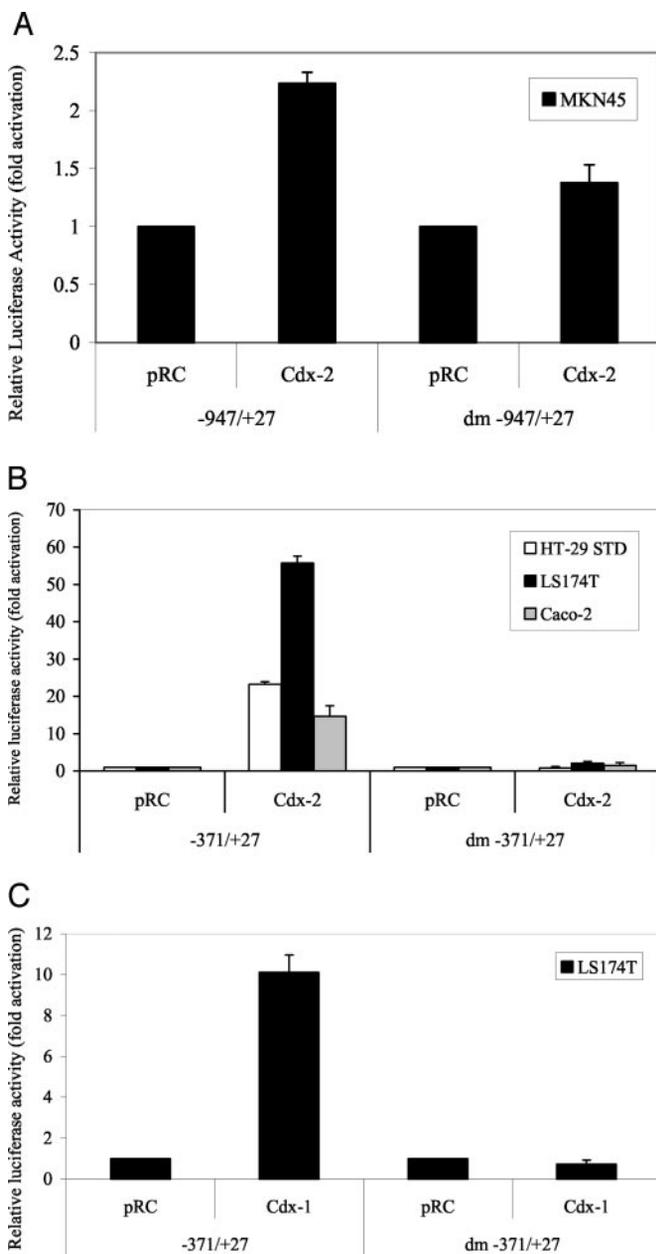
**FIG. 3. Regulation of the MUC2 promoter activity by Cdx-1 and Cdx-2 transcription factors.** A, schematic representation and localization of the different constructs used in co-transfection experiments with Cdx-1 and Cdx-2 expression vectors. Cdx putative binding sites are indicated. B, co-transfection experiments were performed in the presence of the MUC2 promoter constructs -371/+27, -947/+27, -2096/+27, and -2627/-1 in gastric carcinoma cell lines. C, co-transfection experiments were performed in the presence of the MUC2 promoter constructs -371/+27 and -2627/-1 in colon carcinoma cell lines. The values obtained in cells transfected with the empty vector were referred as to 1. The results are means  $\pm$  S.D. and represent two separate experiments in triplicate for each fragment.

the absence of shifted bands when nuclear proteins were incubated with radiolabeled dm -201/-161 probe (lane 7). SIF1 probe was used as a positive control to show Cdx-2 binding (lane 11) and supershift upon addition of anti-Cdx-2 antibody in the reaction mixture (lane 13). EMSA was also performed with the two distal putative Cdx binding sites located at -1010/-1006 and -2614/-2610 but no retarded band was visualized (not shown). Altogether these experiments show that Cdx-2 engages with two cognate cis-elements located at -177/-171 and -191/-187 within the promoter of MUC2.



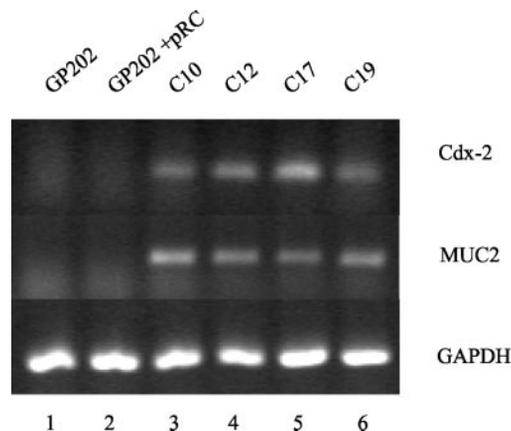
**FIG. 4. Identification of Cdx-2 cis-elements in the MUC2 promoter by EMSA.** A, the sequence covered by probe wt -201/-161 is boxed, and inside of it the two potential binding motifs for Cdx-2 are in bold and italicized. TATA box sequence is underlined. B, 8  $\mu$ g of nuclear extracts prepared from GP220 cells were incubated with the radiolabeled DNA probes as indicated. Lanes 1-5, wild type -201/-161, Cdx-2 binding sites at -177/-171, and -191/-187. Lanes 6-9, dm -201/-161, mutated Cdx-2 binding sites at -177/-171, and -191/-187. Lanes 10-14, SIF-1 (control), two Cdx-2 binding sites. Radiolabeled probe alone was loaded in the first lane of each series (lanes 1, 6, and 10). Supershift experiments were carried out by adding 1  $\mu$ l of antibody against Cdx-2 (lanes 5, 9, and 13). DNA-protein complexes observed with wild type -201/-161 (complexes 1-4), and with SIF-1 probes and supershifted complexes with anti-Cdx-2 antibody (SS Cdx-2) are indicated by arrows on each side of the autoradiogram.

**Mutation of the Two Cdx-2 Binding Sites Abolishes Transactivation of MUC2 Promoter**—To examine the functional role of Cdx-2 in regulating MUC2 promoter activity, site-directed mutagenesis of the two Cdx-2 cis-elements located at -177/-171 and -191/-187 was performed. Mutations were introduced in promoter constructs -371/+27 and -947/+27. Co-transfection studies were performed in MKN45 cells in which MUC2 promoter (construct -947/+27) was efficiently transactivated by Cdx-2 (see Fig. 3B). Mutation of both sites resulted in the loss of the Cdx-2-mediated transactivation of the -947/+27 construct (MKN45 cells, Fig. 5A). The same result was observed in colonic cancer cells with -371/+27 construct (Fig. 5B). When mutation was performed on only one Cdx-2 binding site, no change in Cdx-2-mediated transactivation was observed (data not shown). Altogether, these results indicate that the two Cdx-2 sites are active and determinant to mediate transactivation of the MUC2 promoter by Cdx-2. Double mutation of these two Cdx binding sites also impairs Cdx-1 transactivation of -371/+27 construct in LS174T (Fig. 5C), demonstrating that the Cdx-1 responsiveness of MUC2 promoter requires the same two Cdx binding sites.



**FIG. 5. Effect of mutations of the two Cdx cis-elements on MUC2 promoter transactivation.** A, co-transfection experiments with Cdx-2 were performed in MKN45 gastric cells in the presence of  $-947/+27$  construct and the same construct mutated for Cdx binding elements located at  $-177/-171$  and  $-191/-187$  (dm  $-947/+27$ ) and in (B) colon carcinoma cell lines (HT-29 STD, LS174T, and Caco-2), in the presence of  $-371/+27$  construct and the same construct mutated for Cdx binding elements located at  $-177/-171$  and  $-191/-187$  (dm  $-371/+27$ ). C, co-transfection experiments with Cdx-1 were performed in LS174T colon carcinoma cell line in the presence of  $-371/+27$  construct and the same construct mutated for Cdx binding elements located at  $-177/-171$ , and  $-191/-187$  (dm  $-371/+27$ ). The values obtained in cells transfected with the empty vector were referred as to 1. The results are means  $\pm$  S.D. and represent two separate experiments in triplicate for each fragment.

**Stable Cdx-2 Gastric Carcinoma Cell Transfectants Overexpress MUC2**—Having shown *in vitro* activation of the MUC2 promoter by Cdx-2 *via* the binding on two cognate *cis*-elements, we then undertook to determine whether Cdx-2 may trigger MUC2 expression in gastric cells in which Cdx-2 expression was established. To this aim, we stably transfected GP202, a gastric carcinoma cell line that does not express MUC2 nor Cdx-2 (Fig. 6, lane 1), with either pRC/CMV-Cdx-2 expression vector (clones C10, C12, C17, and C19, lanes 3–6) or empty



**FIG. 6. Study of the expression of MUC2 in Cdx-2-stably transfected GP202 gastric carcinoma cells by RT-PCR.** Expression in wild-type cells (GP202, lane 1), in cells transfected with the empty vector (GP202+pRC, mock cells, lane 2) and in four Cdx-2 stable transfectants (clones C10, C12, C17, and C19, lanes 3–6). Cdx-2 expression was also analyzed in these cells by RT-PCR. GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in  $1\times$  Tris borate-EDTA buffer in the presence of ethidium bromide.

vector pRC/CMV (mock cells, lane 2). Expression at the mRNA level was studied by RT-PCR (Fig. 6). The results indicate that Cdx-2-transfected GP202 clones (lanes 3–6) express Cdx-2 and MUC2 mRNAs whereas mock cells do not (lane 2). MUC2 apomucin expression by Cdx-2 expressing GP202 transfectants was then confirmed by immunofluorescence studies (Fig. 7). As expected, MUC2 expression was found in MUC2-expressing GP202 cells (Fig. 7A) whereas no expression was seen in GP202 cells (Fig. 7B). On the contrary, MUC2 expression was detected in Cdx-2-expressing GP202 transfectants (Fig. 7C). In conclusion, these results demonstrate that stable expression of Cdx-2 in a gastric cell line, initially Cdx-2-negative, lead to MUC2 mRNA expression concomitant to MUC2 apomucin expression.

#### DISCUSSION

The molecular mechanisms responsible for the cell and tissue-specific expression of MUC2 are largely unknown. In the current study we demonstrate the direct involvement of the Cdx-2 homeodomain protein in the transcriptional regulation of the MUC2 gene in gastric and colon carcinoma cells. We have identified Cdx-2 as a major regulator of MUC2 expression and showed that Cdx-2 (i) binds to two cognate elements in the MUC2 promoter, (ii) regulates the activity of the promoter of MUC2 in a cell-specific manner, and (iii) is able to induce MUC2 expression when stably transfected in a gastric carcinoma cell line.

EMSA performed with nuclear extracts from a gastric carcinoma cell line that expresses Cdx-2 demonstrates the formation of two specific complexes with an oligonucleotide containing two adjacent Cdx-putative binding sites located at  $-177/-171$  and  $-191/-187$  upstream of the transcription initiation site of the MUC2 gene. The MUC2 promoter shares this feature (the presence of two closely located Cdx binding sites in the first 200 nucleotides of the promoter) with the promoter regions of other intestine-specific genes, such as sucrase-isomaltase (26), lactase-phlorizin hydrolase (29), claudin-2 (32), and  $\beta$ -1,3-galactosyltransferase T5 (33), that are transcriptionally regulated by the intestine-specific homeobox proteins Cdx-1 and Cdx-2. It was previously shown that Cdx-2 binds to the SIF1 element, a 22-base pair region that is completely conserved between the mouse and human genes, as either a monomer or a dimer, leading to the observation of two complexes with different molecular weights (26). We have shown that these two complexes are similar to the ones formed after interaction of

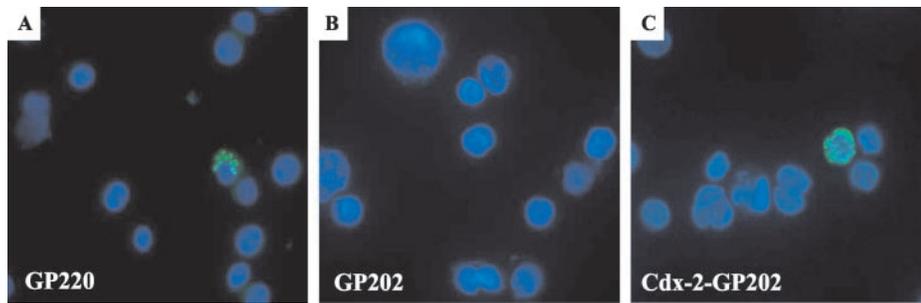


FIG. 7. Study of the expression of MUC2 apomucin in Cdx-2-stably transfected GP202 gastric carcinoma cells by immunofluorescence. DAPI staining for nuclear contrast (blue) and MUC2 staining (PMH1 monoclonal antibody, green) are shown. A, MUC2-expressing GP220 gastric carcinoma cells. B, MUC2-non expressing GP202 gastric cancer cells. C, Cdx-2 stable transfectant of GP202 gastric cancer cells (clone 12).

Cdx-2 with Cdx binding elements in the *MUC2* promoter, suggesting that complexes 1 and 2 seen in this report correspond to the binding of a dimer or a monomer, respectively.

In gastric cell lines, we observed cell-specific transactivation of the *MUC2* promoter by Cdx-2 in MKN45 and in KATOIII cells, whereas co-transfection with Cdx-2 induced luciferase activity in all colon carcinoma cell lines tested (HT-29 STD, LS174T, and Caco-2). Since two gastric carcinoma cell lines, GP220 and AGS, did not show transactivation of the *MUC2* promoter by Cdx-2 we hypothesized that Cdx-2 may require co-factors to be active, which are absent in these two cell lines and present in MKN45 and KATOIII gastric cell lines and in the three colon carcinoma cell lines. This suggests that cell-specific factors are involved in *MUC2* transcription, similar to what has been proposed for other target genes activated by Cdx-2 from enhancers in Caco-2 cells (38).

The interaction between zinc finger and homeodomain transcription factors has been reported in gene regulation as a way to synergistically induce transcription rate (39–42). The promoter of *MUC2* is known to be regulated by ubiquitous transcription factor Sp1 that belongs to the zinc-finger family (13, 18, 19) and the region surrounding the two Cdx-2 binding sites is GC-rich and binds Sp1 (1). We tried therefore to assess whether Sp1 could be one of the transcription factors cooperating with Cdx-2 to increase transcriptional activity of *MUC2*. To this aim, co-transfections in the presence of Cdx-2 and Sp1 expression vectors were performed in MKN45, KATOIII and colon carcinoma cells but synergistic activation of *MUC2* promoter was not observed (data not shown). Thus, consistent with the widespread expression of Sp1, this transcription factor does not seem to be responsible for the observed cell-specific Cdx-2-mediated regulation of *MUC2* promoter.

Other transcription factors of the zinc finger family such as GATA-4/-5/-6 have a cell-specific pattern of expression along the gastrointestinal tract and are important factors in the differentiation of gastrointestinal cells (43). Synergistic activity between GATA and Cdx factors has already been suggested for other intestine specific genes (30, 44) and Cdx-2 and GATA-4/-5 factors were recently suggested to be associated with gastric carcinogenesis (45). Future investigations will have to be performed to show whether GATA factors may be co-factors of Cdx-2 in *MUC2* regulation.

In MKN45 and KATOIII gastric carcinoma cell lines, we observed a higher transactivation effect of Cdx-2 on  $-947/+27$  construct. This construct contains the two Cdx-2 binding sites present in the smaller construct  $-371/+27$ , but includes an additional 576 nucleotides. Transfection experiments (see Fig. 2B) indicate that the  $-947/-372$  region is essential for transcriptional regulation of *MUC2* in KATOIII, MKN45, GP220, and AGS gastric carcinoma cell lines. Additional work will be needed to determine if other transcription factors with putative binding sites in the  $-947/+27$  region, such as NF- $\kappa$ B, are

participating into *MUC2* regulation in gastric cells, similarly to what has been shown in tracheobronchial epithelial cells (21, 22).

When we mutated either one of the two Cdx-2 sites in the  $-947/+27$  construct and co-transfected MKN45 cells with the Cdx-2 expression vector we did not observe any modification in the Cdx-2-mediated activation of *MUC2* (data not shown). Only mutation of both Cdx-2 sites decreased promoter activity to the level obtained with the empty vector. In colon carcinoma cell lines similar results were observed. Similar experiments performed with sucrose-isomaltase (26), lactase-phlorizin hydrolase (29), claudin-2 (32), and  $\beta$ -1,3-galactosyltransferase T5 (33) promoters have demonstrated that, although both Cdx-binding sites were required for full transcriptional activation of these genes, one of the sites was more active than the other. Our results imply that the two Cdx-2 sites are equally active to mediate the transactivation of the *MUC2* promoter.

From these results we conclude that *MUC2* is a Cdx-2 inducible gene in gastric cells, in agreement to the observed expression of *MUC2* in transgenic mice that ectopically express Cdx-2 in the stomach (25). This does not imply that the *MUC2* gene is only regulated by Cdx-2, as demonstrated by expression of *MUC2* in tissues and cells that do not express Cdx-2 (e.g. tracheobronchial epithelium) as well as also observed in gastric carcinoma cell lines.

Unlike Cdx-2, Cdx-1 does not have a significant effect on *MUC2* promoter activity in any of the gastric carcinoma cell lines studied. This is in contrast to the colon carcinoma cell lines (HT-29 STD, LS174T, and Caco-2) in which Cdx-1 activates *MUC2* promoter activity, *via* the same two Cdx-binding sites, although Cdx-2 remains a more potent activator. These observations suggest that Cdx-1 may cooperate with other factors to be transcriptionally active, and that these factors are present in colon carcinoma cell lines and absent in gastric carcinoma cells, as well as in other cells, including COS-7 cells (46). Our results on *MUC2* promoter in colonic cells are consistent with the previous observation that Cdx-2 is more efficient than Cdx-1 in the activation of the claudin-2 gene promoter in Caco-2 cells (32). However, *MUC2* regulation by Cdx-1 may also have important implications during intestinal development and cell differentiation since expression of Cdx-1 and *MUC2* were previously shown in intestinal stem cells of the crypts (47, 48). Consequently, *MUC2* activation by Cdx-1 in these cells may lead to their differentiation into goblet cells as the stem cells move upward toward the villi. In this work, *MUC2* clearly appears as a target gene of Cdx-1 in colonic cells. Therefore, this suggests a possible function for Cdx-1 in establishing goblet cell lineage during development. As the goblet cells migrate and differentiate, *MUC2* regulation may be then taken over by Cdx-2 that is more abundantly expressed in differentiated cells of the tip of the villi (49).

In summary, we have shown that *MUC2* is a target gene of

Cdx-2 both in gastric and colon cancer cells. This suggests that, in human gastric cells, Cdx-2 is capable of inducing MUC2 expression, a hallmark phenotypic change that occurs early in the transdifferentiation into intestinal metaplasia and gastric cancers showing intestinal differentiation.

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