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

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Patrícia Mesquita‡§, Nicolas Jonckheere††, Raquel Almeida‡, Marie-Paule Ducroux‡, Jacinta Serpa‡, Elisabeta Silva‡, Pascal Pigny‡, Filipe Santos Silva‡, Celso Reis‡, Debra Silberg‡**, Isabelle Van Steunenberg‡, and Leonor David‡‡‡

From the ‡Institute of Molecular Pathology and Immunology (IPATIMUP), University of Porto, Rua Dr. Roberto Frias s/n, 4200 Porto, Portugal, the ††Unité INSERM 560, Place de Verdun, 59045 Lille Cedex, France, the ‡‡Division of Gastroenterology, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the ‡‡‡Medical Faculty, University of Porto, 4200 Porto, Portugal

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 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 neoplastic 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 lipo-polysaccharide 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 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 phospholipaseA2lysophospholipase (31), claudin-2 (32), and more recently β-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-

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† To whom correspondence should be addressed: Institute of Molecular Pathology and Immunology (IPATIMUP), University of Porto, Rua Dr. Roberto Frias s/n, 4200 Porto, Portugal. Tel.: 351-22-5570753; Fax: 351-22-5570799; E-mail: pmesquita@ipatimup.pt.

Recipient of a CHRU de Lille-Region Nord-Pas de Calais Ph.D. fellowship.

‡ 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.
ion 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% CO₂ 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 t-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 Seuningen et al. (34). Cell lines GP202 and GP220 were established at IPATIMUP (35).

#### Reverse Transcriptase-PCR Analysis

Total RNA from gastric carcinoma cells was isolated using PureScript RNA isolation kit (Genta systems, Minneapolis) and treated with DNase. 5 μg of total 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 Cdx-2, 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 MgCl₂, 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 mucoepidermoid 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 × 10⁴/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 the corresponding empty vector (pGL3 basic, Promega), after transfection efficiency as measured by the pRL luciferase 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 replication vector containing the Neomycin resistance gene). Transfected cells were selected with 500 μg/ml of Geneticin (G418) for up to 14 days. Colonies were picked and expanded in culture. Clones harboring a single integrated copy of the expression vector were selected and used for further experiments. The effect of the Cdx-2 expression vector on the expression of the MUC2 gene was determined by RT-PCR analysis. It was shown that the expression of MUC2 is increased in the cells expressing Cdx-2 compared to the cells expressing the empty vector (pRC/CMV). This result indicates that Cdx-2 is a positive regulator of the MUC2 gene.

### Table I

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

<table>
<thead>
<tr>
<th>Primers used for RT-PCR</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC2</td>
<td>S: CGA AAC CAC GGC CAC A AC GT&lt;br&gt;AS: GAC CAC GGC CCC GTT AAG CA</td>
</tr>
<tr>
<td>Cdx-1</td>
<td>S: GGA CCA AGG ACA AGT ACC GC&lt;br&gt;AS: GGT GTT GCT GGG ACA CAG G</td>
</tr>
<tr>
<td>Cdx-2</td>
<td>S: CCA GGG CAA AAG ACA AAT ATC GA&lt;br&gt;AS: CTG TGG GAT CCT CTG AGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>S: ACC ATC TTC CAG CAG GGA&lt;br&gt;AS: GGA TGA CCT TGC CCA CAG</td>
</tr>
<tr>
<td>MUC2</td>
<td>S: CGG GCA AGC CTT CCT CCG&lt;br&gt;AS: CAT TGG AGA GGT GGT CCG G</td>
</tr>
<tr>
<td>Cdx-1</td>
<td>S: CGG CTT CAG CTT CCA CAG&lt;br&gt;AS: TCA GGC TGG AAA AGC AGC</td>
</tr>
<tr>
<td>Cdx-2</td>
<td>S: CGA GGG CCA AGC AGA AAA CT&lt;br&gt;AS: TGA GAT CTT TTC GGC CCC AA</td>
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</table>

<table>
<thead>
<tr>
<th>Primers used for site-directed mutagenesis</th>
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<tr>
<td>Cdx-2 site</td>
<td>AAG AAG GCT CCG&lt;br&gt;GCG TGC&lt;br&gt;CAG GGA GCA CTA AAG AGA TGA CTT CCG&lt;br&gt;CGG AGG TCA TCT CTT TAT GGC TCC CTG GCA GGC CAC AGT CTG&lt;br&gt;AAG AAG GCT TGG TTT ACC CAG GGA GCC&lt;br&gt;GCG TGG&lt;br&gt;AGA TGA CTT CCG&lt;br&gt;CGG AGG TCA TCT CCA GGC GCC TCC GTG GAA GGA AGC AGC CTG&lt;br&gt;CTG AAG AAC GTG GCG GCG TTC CAG GAA GCC&lt;br&gt;GCG TAG&lt;br&gt;AGA TGA CTT CCG&lt;br&gt;CGG AGG TCA TCT CTA GGC GCC TCC CTG GAG ACC AGC CTT CAG</td>
</tr>
</tbody>
</table>

| Double mutation                          | AAG AAG GCT CCG<br>GCG TGC<br>CAG GGA GCA CTA AAG AGA TGA TTA TGA GTA<br>GA A GC TGG<br>TAC CCA GGG AGC CAT AAA GAG ATG ACC T<br>GA A GC TGC<br>GCC GTG<br>CCA GGC AGC<br>GCG GCC<br>GAG ATG ACC T |

<table>
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<tr>
<th>Oligonucleotides used for EMSAs</th>
<th>Sequence (5'→3')</th>
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<tbody>
<tr>
<td>Probe</td>
<td>GGG TGC AAT AAA ACT TTA TGA GTA&lt;br&gt;GGG GCC TCC GTG TAC CCA GGG AGC CAT AAA GAG ATG ACC T&lt;br&gt;GGA GCC TGC&lt;br&gt;GCC GTG&lt;br&gt;CCA GGC AGC&lt;br&gt;GCG GCC&lt;br&gt;GAG ATG ACC T</td>
</tr>
</tbody>
</table>

**REFERENCES**

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 and colon carcinoma cells 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 transcription start site are indicated (36). Mutated nucleotides are bold, italicized, and underlined.
Expression of MUC2, Cdx-1, and Cdx-2 in Human Gastric and Colon Carcinoma Cells—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 GP202 gastric carcinoma cells and Cdx-2 is expressed in all gastric cancer cells tested (Fig. 1A). 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 in the intestinal promoter constructs (4/−7) were identified in 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 TTATTC (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
activation vary greatly between the different cell lines indicating cell-specific activity of Cdx-2. Unlike gastric cells, we observed transactivation of \( \text{MUC2} \) promoter by Cdx-1 in colon cancer cells. Transactivation was more efficient on the short construct \(-2627/1 \) 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 \( \text{MUC2} \) 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 \( \text{MUC2} \) promoter in colon cancer cells.

**Identification of Two Cdx-2 cis-Elements within the MUC2 Promoter**—In order to demonstrate that the Cdx-2 transcription factor binds to the \( \text{MUC2} \) 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× 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
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 showed that Cdx-2 engages with two cognate cis-elements located at −177/−171 and −191/−187 within the promoter of MUC2.

**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 colon 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 cis-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/+27construct in LS174T (Fig. 5C), demonstrating that the Cdx-1 responsiveness of MUC2 promoter requires the same two Cdx binding sites.
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× Tris borate-EDTA buffer in the presence of ethidium bromide.

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 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 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 GP220 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 (dm −947/+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 to as 1. The results are means ± S.D. and represent two separate experiments in triplicate for each fragment.
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-κB, 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 sucrase-isomaltase (26), lactase-phlorizin hydro-lase (29), claudin-2 (32), and β-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

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 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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