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The Human Mucin MUC4 Is Transcriptionally Regulated by Caudal-related Homeobox, Hepatocyte Nuclear Factors, Forkhead Box A, and GATA Endodermal Transcription Factors in Epithelial Cancer Cells*[S]

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Nicolas Jonckheere[‡1,2], Audrey Vincent[‡1,3], Michaël Perrais[‡], Marie-Paule Ducourouble[‡], Anita Korteland-van Male[§], Jean-Pierre Aubert[‡], Pascal Pigny[‡], Kermit L. Carraway[¶], Jean-Noël Freund[‖], Ingrid B. Renes[**], and Isabelle Van Seuningen[‡4]

*From the [‡]INSERM, U560, Place de Verdun, Lille, F-59045, France, the Department of Pediatrics, Divisions of [§]Gastroenterology and Nutrition and **Neonatology, Erasmus MC and Sophia Children's Hospital, Rotterdam, 3015GE The Netherlands, the [¶]Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Florida 33136, and the [‖]INSERM, U682, 3 avenue Molière, Strasbourg F-67200, France*

The human gene *MUC4* encodes a large transmembrane mucin that is developmentally regulated and expressed along the undifferentiated pseudostratified epithelium, as early as 6.5 weeks during fetal development. Immunohistochemical analysis of Muc4 expression in developing mouse lung and gastrointestinal tract showed a different spatio-temporal pattern of expression before and after cytodifferentiation. The molecular mechanisms governing *MUC4* expression during development are, however, unknown. Hepatocyte nuclear factors (HNF), forkhead box A (FOXA), GATA, and caudal-related homeobox transcription factors (TFs) are known to control cell differentiation of gut endoderm derived-tissues during embryonic development. They also control the expression of cell- and tissue-specific genes and may thus control *MUC4* expression. To test this hypothesis, we studied and deciphered the molecular mechanisms responsible for *MUC4* transcriptional regulation by these TFs. Experiments using small interfering RNA, cell co-transfection, and site-directed mutagenesis indicated that *MUC4* is regulated at the transcriptional level by CDX-1 and -2, HNF-1α and -1β, FOXA1/A2, HNF-4α and -4γ, and GATA-4, -5, and -6 factors in a cell-specific manner. Binding of TFs was assessed by chromatin immunoprecipitation, and gel-shift assays. Altogether, these results demonstrate that *MUC4* is a target gene of endodermal TFs and thus point out an important role for these TFs in regulating MUC4 expression during epithelial differentiation during development, cancer, and repair.

MUC4 is a large transmembrane mucin with a very long glycosylated extracellular domain, which is expressed by epithelial cells in normal respiratory, gastrointestinal and genital tracts (1). In epithelial cancers MUC4 mucin is often overexpressed (2, 3) with, ultimately, consequences for the biological properties of tumor cells. Alterations may involve tumor cell recognition by immune cells, cell-cell homotypic interactions, cell interaction with extracellular matrix, tumor cell proliferative and metastatic properties, and alteration of ErbB2 signaling (4, 5). Moreover, it was shown that the expression of MUC4 was developmentally regulated in the respiratory and gastrointestinal tracts and varied with the degree of cell and tissue differentiation (6–12). In our laboratory, *MUC4* mRNA was found expressed in human embryos as early as 6.5 weeks of gestation in the primitive gut prior to epithelial cytodifferentiation, the expression being intense and located all along the undifferentiated stratified epithelium (6, 7). However, the molecular mechanisms governing *MUC4* expression during embryonic and fetal development are unknown.

Recently, we characterized the promoter region of *MUC4*, which is composed of a proximal and a distal promoter (13) and identified binding sites for transcription factors (TFs)[5] of the hepatocyte nuclear factor (HNF), GATA, and caudal-related homeobox (CDX) families in the distal promoter (14). We also recently showed that *MUC4* endogenous expression is regulated by HNF-1α and HNF-4α TFs in esophageal cancer cells (15). Altogether, these findings suggest that *MUC4* expression may be regulated by TFs responsible for cell differentiation programs leading to the formation of organs derived from the primitive gut.

HNF, GATA, and CDX TFs regulate regional cell differentiation of the endoderm and its derivatives during embryonic development (16–18). Their participation in maintaining a functional epithelium is the result of a tight spatio-temporal

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[1] Both authors participated equally to this work.
[2] Recipient of a Centre Hospitalier Régional et Universitaire de Lille-Région Nord-Pas de Calais Ph.D. fellowship.
[3] Recipient of an INSERM-Région Nord-Pas de Calais Ph.D. fellowship.
[4] To whom correspondence should be addressed. Tel.: 33-320-29-88-67; Fax: 33-320-53-85-62; E-mail: isabelvs@lille.inserm.fr.

[5] The abbreviations used are: TF, transcription factor; HNF, hepatocyte nuclear factor; CDX, caudal-related homeobox; E, embryonic day; P, postnatal day; RT, reverse transcriptase; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

regulation of cell- or tissue-specific genes in the lung (19–21) and intestine (22–25). Among these genes, we recently identified the secretory mucin MUC2 as a target of CDX-1/CDX-2 (26) and GATA-4 (27).

The GATA family consists of six members, GATA-1 to -6, that bind to the 5′-(A/T)GATA(A/G)-3′ nucleotide motif via their zinc finger domains. They are grouped into two subfamilies based on structural features and expression patterns. GATA-1, -2, and -3 are involved in hematopoiesis and neurogenesis. GATA-4, -5, and -6 are found mainly in heart and endoderm-derived tissues, including liver, lung, pancreas, stomach, and intestine, and are involved in regulation of cardiogenesis and gut development (28).

Hepatocyte nuclear factors belong to a heterogeneous family of TFs involved in a wide variety of biological pathways. Although important in liver development and function, they are also involved in visceral endoderm differentiation and found in kidney, pancreas, stomach, small intestine, and colon (29). HNF-1α and -1β are homeodomain proteins that form homo- or heterodimers and bind the consensus sequence 5′-GTTAATGATTAAC-3′. HNF-3α and -3β belong to the forkhead/winged helix DNA binding domain family. They bind the consensus sequence 5′-GATTATTGACTT-3′ as monomers (30) and are expressed in embryonic endoderm and in the adult intestine (31). In this paper we will use the new nomenclature FOXA1 (HNF-3α) and FOXA2 (HNF-3β). HNF-4α and -4γ are members of the steroid hormone receptor superfamily. They are zinc finger TFs and bind the consensus sequence 5′-TGGACTTAG-3′. HNF-4 has been implicated in early endodermal development and differentiation of the liver, kidney, pancreas, stomach, and intestine (32).

Homeobox CDX-1 and -2 are intestine-specific genes that bind the AT-rich consensus sequence (T/C)ATAAA(T/G) either as homo- or heterodimers (33). *In vivo* and *in vitro* studies suggest that these TFs are important in intestinal development, intestinal cell proliferation and differentiation, and in the control of intestinal identity (33–35).

In this paper we report that Muc4 mucin is expressed before and after cytodifferentiation in the lung and gastrointestinal tract of the developing mouse and that the apical surface expression observed prior to cytodifferentiation in the gastrointestinal tract is also observed in mucus-secreting goblet cells after differentiation. Furthermore, we report, by deciphering the molecular mechanisms of transcriptional regulation, that *MUC4* is a target gene of CDX-1/-2, HNF-1/-4, FOXA1/A2, and GATA-4, -5, and -6 TFs.

EXPERIMENTAL PROCEDURES

Animals—Pregnant female Balb/c mice (Charles River, Maastricht, the Netherlands) were housed at constant temperature and humidity on a 12-h light-dark cycle. The mice had free access to a standard pelleted diet (Special Diets Services, Witham, Essex, England) and tap water. Pregnant females were sacrificed by cervical dislocation, embryos were isolated and the lungs and intestine were excised at embryonic days (E) 15.5, E17.5, E18.5, postnatal days (P) 1.5 and P14.5, and adults. Lungs and intestine were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline and prepared for light microscopy. All the experiments were performed with the approval of the Animal Studies Ethics Committee of the Erasmus Medical Center (Rotterdam, the Netherlands). Cdx-1$^{-/-}$ (36) and Cdx-2$^{+/-}$ (37) mice were housed under standard laboratory conditions, as recommended by the Ethics Committee of the University Louis Pasteur (Strasbourg, France).

Immunohistochemistry—Immunohistochemistry was carried out as in Ref. 38. Muc4 expression at different stages of mouse development and in Cdx-1$^{-/-}$ and Cdx-2$^{+/-}$ mice was carried out using rabbit polyclonal HA-1 antibody (1:2000 dilution) that recognizes a C-terminal peptidic region of the ASGP-1 (MUC4α) subunit (39). Negative controls included staining sections without primary HA-1 antibody or by incubating with rabbit preimmune serum (supplemental data Figs. S1 and S7).

Cell Culture—Human pancreatic CAPAN-1 and CAPAN-2, colonic HT-29 STD, HT-29 5F12, LS174T, and Caco-2, gastric KATO-III, and respiratory NCI-H292 cancer cell lines were cultured as previously described (13, 40–43). All cell lines were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and cultured at 37 °C in a 5 (CAPAN-1, CAPAN-2, KATO-III, NCI-H292) or 10% (HT-29 STD, HT-29 5F12, LS174T, Caco-2) CO_2-jacketed incubator.

RT-PCR—Total RNAs from cultured cells were prepared using the RNeasy mini-kit from Qiagen (Courtaboeuf, France). Total RNA (1.5 μg) was used to synthesize cDNA (Advantage™ RT-for-PCR kit, Clontech) as described (43). PCR was performed on 5 μl of cDNA using specific pairs of primers (MWG-Biotech, Ebersberg, Germany) for *MUC4* (44) and *28S* rRNA as the internal control. PCR were carried out in 50-μl final solutions (5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 4 μl of 2.5 mM dNTPs, 10 pmol of each primer, and 1 unit of *Taq* polymerase (Roche Diagnostics). 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 27 cycles; and 3) final extension: 72 °C, 10 min. PCR products were analyzed on 1.5% agarose gels containing ethidium bromide run in 1× Tris borate-EDTA buffer. PCR primers and annealing temperature to analyze TF expression are described in supplemental Table S1. A 100-bp DNA ladder was purchased from GE Healthcare.

Small Interfering RNA (siRNA) Assays—Cell seeding and cell transfection were performed as described before (15) with 100 nM human GATA-4, GATA-6, CDX-1, CDX-2, HNF-1α, HNF-1β, FOXA1, FOXA2, HNF-4α, or combinations of SMARTpool® siRNA, using 1 μl of DharmaFECT™ 1 transfection reagent (Dharmacon, Perbio Science, Brebières, France). Controls included mock-transfected cells, and cells transfected with siCONTROL™ GAPD siRNA or siCONTROL™ Non-Targeting siRNA Pool. Total RNA isolation and RT-PCR were as described above. siRNAs were assayed in triplicate in at least two separate experiments. *MUC4*/*glyceraldehyde-3-phosphate dehydrogenase* ratio was calculated by densitometric analysis using the GelAnalyst-GelSmart software (Clara Vision, Orsay, France).

pGL3-MUC4 Promoter Constructs and Site-directed Mutagenesis—The four pGL3-*MUC4* deletion mutants used in this study and that cover the distal promoter of *MUC4* were pre-

viously described (13). Plasmids used for transfection studies were prepared using the Endofree plasmid Maxi kit (Qiagen). QuikChange site-directed mutagenesis kit (Stratagene) was used to generate site-specific mutations. Oligonucleotides containing the desired mutations are shown in supplemental Table S2.

Transient Transfection Assays—Transfection experiments were performed using Effectene® reagent (Qiagen) as previously described (42). Luciferase activity was corrected for transfection efficiency by co-transfecting cells with pRL-TK vector (Promega). Total cell extracts were prepared after a 48-h incubation at 37 °C using 1× Passive Lysis Buffer (Promega). Co-transfection experiments were carried out in the presence of 1 μg of each pGL3-*MUC4* promoter fragment with either 0.5 μg of the expression vector encoding the TF of interest or 0.5 μg of empty control vectors as the reference. pCMV-FOXA1 and pCMV-FOXA2 were a kind gift of Dr R. Costa (University of Illinois, Chicago, IL). pCB6-HNF-1α, pCB6-HNF-1β, pMT2-HNF-4α, and pMT2-GATA-4 were a kind gift of Dr. S. Cereghini (UMR7622 CNRS, Paris, France). pSG5-HNF-4γ, pSG5-GATA-5, and pSG5-GATA-6 were a kind gift of Dr. J. K. Divine (Washington University, St. Louis, MO). pCMV-Cdx-1 and pCMV-Cdx-2 were previously described (45). Relative luciferase activity was expressed as -fold activation in samples transfected with vector expressing the TF of interest compared with empty vector. Each construct or combination was assayed in triplicate in three separate experiments. To study the effects of the TFs on endogenous expression of *MUC4* mRNA, cells were transfected with 4 μg of the expression vector of interest or empty control as previously described (27). Three independent experiments were carried out. *MUC4*/*28S* ratio was calculated by densitometric analysis as above.

Electrophoretic Mobility Shift Assay (EMSA)—Putative binding sites were identified using MatInspector (www.genomatix.de) software (46). Oligonucleotides used for EMSA (supplemental Table S3) were synthesized by MWG-Biotech. Annealed oligonucleotides were radiolabeled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]dATP (GE Healthcare) and purified by chromatography on a Bio-Gel P-6 column (Bio-Rad). Nuclear extracts were prepared as described before (47), and quantified using the bicinchoninic acid method (Pierce). Nuclear protein incubation with radiolabeled probes and competitions with unlabeled probes were as described in Ref. 26. For supershift analyses, 2 μl of the antibody of interest (anti-GATA-4, anti-GATA-6, anti-HNF-1α, anti-HNF-1β, anti-FOXA1, anti-FOXA2, anti-HNF-4α, and anti-HNF-4γ, 10× solutions (Santa Cruz Laboratories) and anti-CDX-2 (Biogenex, Alphelys, Plaisir, France) were added to the proteins and left for 1 h at room temperature before adding the radiolabeled probe. Electrophoresis conditions and gel processing were as described (42).

Chromatin Immunoprecipitation (ChIP)—Cells (3×10^6) were treated with 1% (v/v) formaldehyde for 10 min at room temperature and cross-links were quenched with glycine at a final concentration of 0.125 M for 5 min. ChIP experiments were then carried out as previously described (15). All antibodies were from Santa Cruz except GATA-4, GATA-5, and GATA-6, which were from R&D Systems. Primer information is given in supplemental Table S4.

Statistics—All values in this article are mean ± S.D. When indicated, Student's *t* test was used for statistical evaluations; a $p < 0.05$ was considered statistically significant.

RESULTS

Expression of Mouse Muc4 during Gastrointestinal and Lung Development—Expression and localization of Muc4 protein was analyzed by immunohistochemistry in stomach, small intestine, colon, and lung (Fig. 1). In the hindstomach, a weak expression was seen at the apical surface of the undifferentiated epithelium at E15.5, in the differentiated monolayer of the glandular stomach at E17.5 and in P1.5 neonates. Expression was stronger at the adult stage. In the small intestine, Muc4 was also present at the apical surface of the pseudostratified epithelium at E15.5. On days E17.5 (not shown) and E18.5 when the pseudostratified epithelium has undergone transition to a differentiated monolayer, Muc4 was confined to goblet cells of the villi and intervillus regions. This pattern was maintained in neonate (P1.5) and adult mice. In the colon, Muc4 was first seen at the apical surface at day E17.5, when goblet cells were not yet present. At E18.5 Muc4 was detected both in brush borders of crypt and surface epithelial cells and in goblet cells. Remarkably, in P1.5 neonates and adults, Muc4 was confined to goblet cells and absent from brush borders. In adults, it was restricted to goblet cells of the upper half of the crypts. In lung, Muc4 was seen at the apical surface of the pseudostratified epithelium at E15.5, of the columnar epithelial cells at days E17.5 and E18.5 (not shown), and the bronchiolar epithelium in neonates (P1.5) and adults.

MUC4 Expression in Epithelial Cancer Cell Lines—Because MUC4 has a cell-specific pattern of expression in respiratory and gastrointestinal tracts, we studied its mRNA expression and promoter regulation in a panel of epithelial cell lines of different origins (respiratory, gastric, colonic, and pancreatic) and phenotypes (enterocyte, mucus-secreting, and undifferentiated colonic cancer cells). *MUC4* mRNA was strongly expressed in respiratory NCI-H292, pancreatic CAPAN-1 and CAPAN-2, and to a lower extent in gastric KATO-III and intestinal HT-29 5F12 cell lines (Fig. 2*A*). The basal level of the TFs in the different cell lines is shown in Fig. 2*B*.

Regulation of MUC4 mRNA Expression by Endodermal Transcription Factors—To investigate whether endodermal CDX-1 and -2, HNF-1 and -4, FOXA1/A2, and GATA-4, -5, and -6 TFs regulate *MUC4* endogenous expression, knockdown assays were carried out with specific siRNA in CAPAN-1, HT-29 5F12, and KATO-III cell lines that expressed the TFs of interest (supplemental data Fig. S2). The strongest inhibitions of *MUC4* mRNA expression were observed with FOXA2, GATA-6, CDX-2, and HNF-1α siRNAs (75–80% inhibition, Fig. 2*C*). siRNA for HNF-1β and FOXA1 led to a 60% decrease of *MUC4* expression, whereas CDX-1, HNF-4α, and GATA-4 siRNA effects were milder (20–30% inhibition). Regulation of endogenous *MUC4* expression by HNF-4γ and GATA-5 could not be studied by the siRNA approach because none of the cell lines studied expressed these TFs or expression was extremely low (HNF-4γ in KATO-III). Forced expression of HNF-4γ and

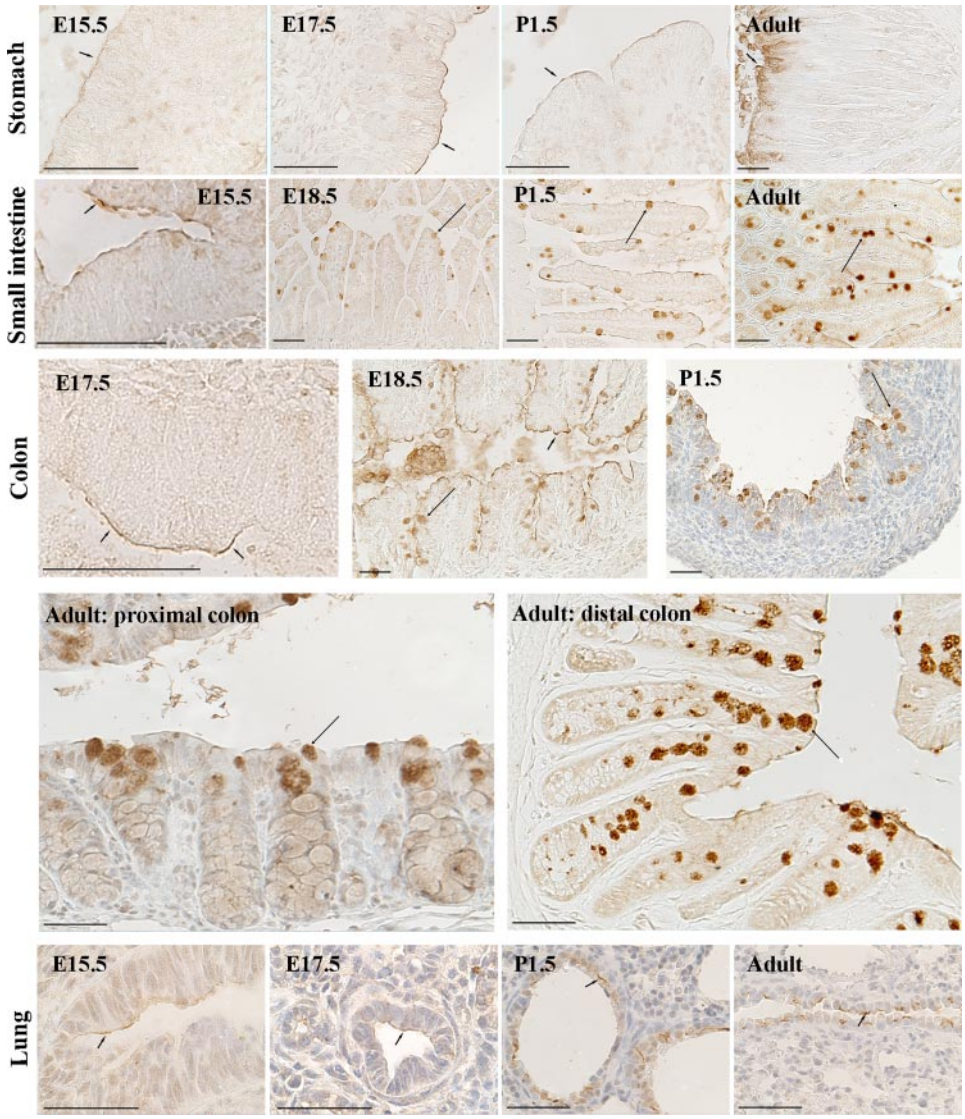

FIGURE 1. **Expression of Muc4 mucin in developing mouse stomach, intestine, and lung.** Immunohistochemical studies using the HA-1 antibody directed against MUC4 were performed on stomach, small intestine, colon, and lung on E15.5, E17.5, and E18.5, P1.5, P14.5, and in adult mice. *Arrowheads* indicate Muc4 staining at the apical surface of epithelial cells and *arrows* indicate Muc4 staining in the goblet cells. The *black bar* in each panel corresponds to 50 μm in length. Negative controls are shown in supplemental materials Fig. S1.

GATA-5 in NCI-H292 and CAPAN-2 *MUC4*-expressing cells led to a 5.5–7.5- and 1.5–2.0-fold increase of *MUC4* mRNA, respectively (Fig. 2*D*). Together, these results demonstrate that CDX, HNF, FOXA, and GATA TFs regulate *MUC4* mRNA expression in cancer cell lines of endodermal origin.

MUC4 gene expression is under the control of a proximal and a distal promoter (13). Analysis of the sequence showed a high density of putative binding sites for CDX, HNF, FOXA, and GATA TFs in the distal promoter (supplemental data Fig. S3). Three CDX binding sites (T90 and T60) contain the consensus sequence -ATAAAT- for CDX binding. The fourth site T164 is more degenerate. Among the HNF putative binding sites, T144 contains a consensus sequence for TF of the HNF-1 family, whereas T106 resembles a HNF-1 and FOXA binding site. T91 is more degenerate and resembles a FOXA binding site. The two GATA binding sites (T59 and T60) contain the consensus -GATA- binding sequence. To analyze the regulation of *MUC4* by these TFs at the promoter level, we studied their transactivating effects on four deletion constructs previously used in the laboratory (13, 15), which cover the distal promoter (supplemental data Fig. S3). Two of them (−2781/−2572 and −3135/−2572) contain the TATA box and the transcription initiation site and are transcriptionally active (13). The other two (−3135/−2837 and −3713/−3059) are more distal and do not contain canonical TATA box, but do contain AT-rich sequences. These two constructs do not have intrinsic transcriptional activity (13).

Regulation of MUC4 Distal Promoter by CDX-1 and CDX-2 Transcription Factors—As expected, activation of the *MUC4* promoter by intestine-specific CDX-1 (Fig. 3*A*) and CDX-2 (Fig. 3*B*) TFs was the strongest in intestinal cells. This activation occurred in a promoter region −3135/−2837 (*black bars*) that does not contain any consensus CDX binding site. Non-negligible activation of the promoter region containing either the T60 (*gray bar*, CDX-1/HT-29 STD) or the T90 (CDX-2/LS174T, *hatched bar*) CDX binding sites was also observed.

Regulation of MUC4 Distal Promoter by HNF-1α and -1β, FOXA1/A2, and HNF-4α and -4γ Transcription Factors—The strongest *MUC4* promoter activation by HNF-1α (Fig. 3*C*) and HNF-1β (Fig. 3*D*) was observed in HT-29 STD and KATO-III gastrointestinal cell lines in −3135/−2837 (T144 HNF binding site) and −3713/−3059 (T91 and T106 HNF binding sites) promoter regions. When HNF-4α and HNF-4γ were overexpressed, the effects were the most potent in the respiratory NCI-H292 cells (Fig. 3, *E* and *F*) in a region containing the T91 and T106 binding sites for HNF-4α (*hatched bar*) and a region devoid of HNF binding site (*white bar*) for HNF-4γ. Milder effects of HNF-4γ on different regions of the promoter were also observed in the other cell lines studied (supplemental data Fig. S4). Activation of *MUC4* promoter by FOXA1 and FOXA2 were the strongest in colonic cancer cell lines and implied different regions of the promoter for FOXA1 (Fig. 3*G*) and the −3135/−2837 region (T144 HNF binding site) for FOXA2 (Fig. 3*H*).

Regulation of MUC4 Distal Promoter by GATA-4, -5, and -6 Transcription Factors—Overexpression of GATA-4 led to the transactivation of the *MUC4* distal promoter in all the cell

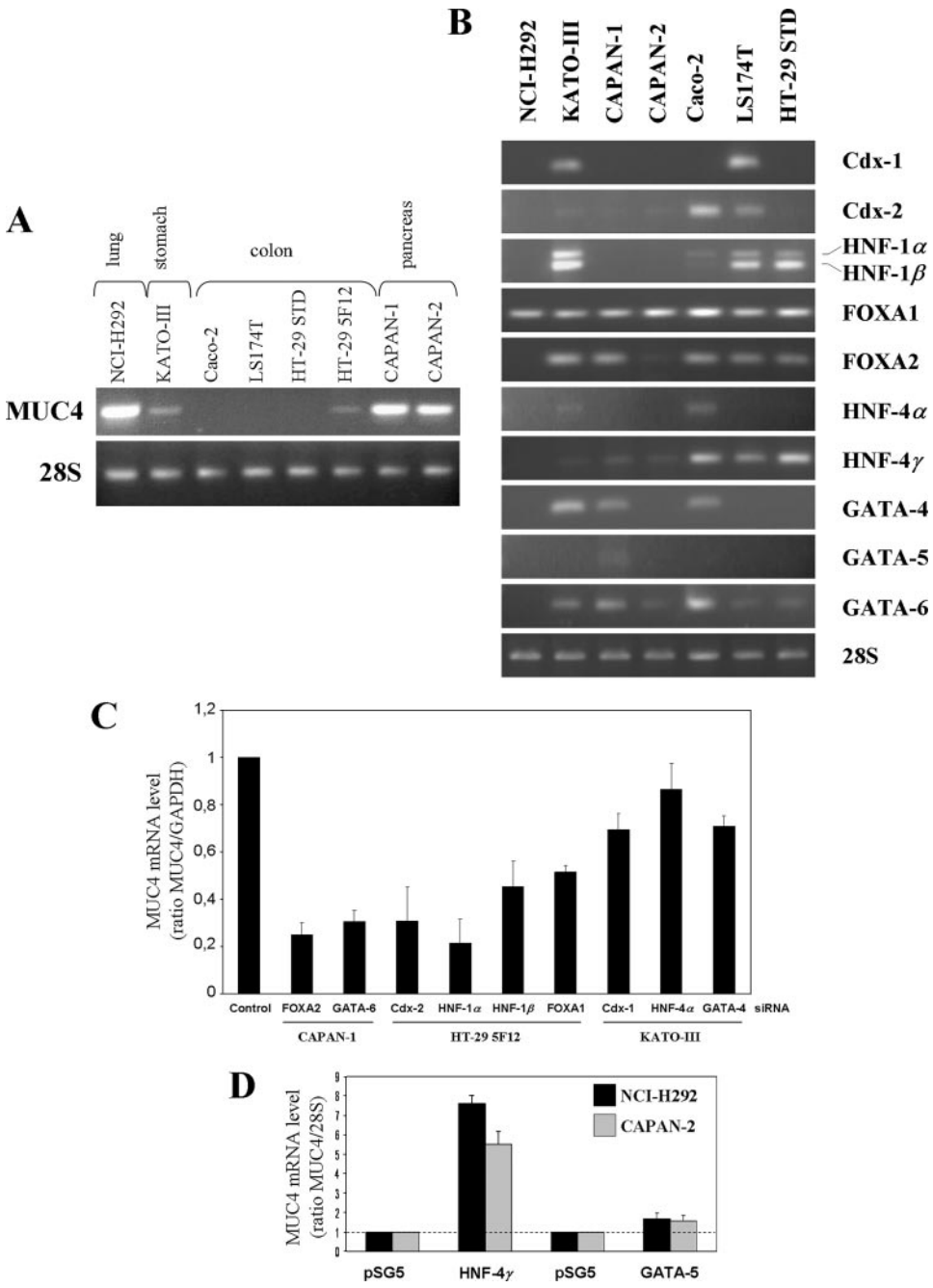

FIGURE 2. **Study of the expression of *MUC4* mRNA and its regulation by endodermal TFs in epithelial cancer cell lines.** *A*, expression of *MUC4* was studied by RT-PCR. *MUC4* (10 μl) and *28S* rRNA (2 μl) PCR products were analyzed on a 1.5% agarose gel. *B*, expression level of TFs in epithelial cancer cell lines by RT-PCR. 10 μl of PCR products were loaded and resolved on a 1.5% agarose gel. PCR primer information is given in supplemental Table S1. *C*, siRNA experiments targeting indicated TFs were carried out in cell lines expressing both *MUC4* and the TF of interest (FOXA2 and GATA-6 in CAPAN-1; CDX-2, HNF-1α, HNF-1β, and FOXA1 in HT-29 5F12; CDX-1, HNF-4α, and GATA-4 in KATO-III). *Error bars* represent the means of values obtained in triplicate in at least two separate experiments. Knockdown of TFs by their respective siRNA is shown in supplemental data Fig. S2. *D*, expression vector encoding HNF-4γ or GATA-5 was transfected in NCI-H292 (*black bars*) or CAPAN-2 (*gray bars*) cell lines. *MUC4* (10 μl) and *28S* (2 μl) PCR products were analyzed on a 1.5% agarose gel. The value obtained in the experiments performed with the control empty vector pSG5 was arbitrarily set to 1. *Error bars* represent the means of values obtained in three independent experiments. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

lines studied except for pancreatic CAPAN-2 (Fig. 3*I* and supplemental data Fig. S4). All the promoter fragments were stimulated by GATA-4 although there were cell-specific differences. For instance, the region −3135/−2572 (T60 GATA binding site) was strongly activated in pancreatic CAPAN-1 but not in LS174T (Fig. 3*I*). Transactivation of the *MUC4* promoter by GATA-5 (Fig. 3*J*) was the strongest in pancreatic CAPAN-2 and enterocyte Caco-2 but like GATA-4 activations could be seen in all the cell lines studied and implied different regions of the promoter (supplemental data Fig. S4). The same conclusions can be drawn for GATA-6 (supplemental data Fig. S4). Interestingly, in respiratory NCI-H292 cells a strong activation of the promoter region −2781/−2572 that does not contain any putative GATA binding site was observed (Fig. 3*K*).

Altogether the results obtained at the promoter level correlate well with those obtained at the mRNA level (see Fig. 2) and demonstrate that CDX, HNF, FOXA, and GATA TFs regulate *MUC4* transcription. To show whether they act directly on the *MUC4* promoter, we undertook to identify their binding sites by EMSA, show their *in vivo* binding to chromatin by ChIP, and confirm their functionality and role in regulating *MUC4* transcription by site-directed mutagenesis.

Identification and Functionality of the CDX cis-Elements Present in MUC4 Distal Promoter—The radiolabeled probes T60, T90 (two binding sites), and T164 are representative of the four CDX *cis*-elements located in the *MUC4* distal promoter (supplemental data Fig. S3). When incubated with nuclear extract from Caco-2 cells, which express CDX-2, the labeled probes T60 and T90 (Fig. 4*A*) produced retarded bands (*lanes 2* and *6*) that were competed away when preincubated with an excess of unlabeled probe (×50, *lanes 3* and *7*). Involvement of CDX-2 in the complex formation was then proved in supershift experiments carried out with an antibody specific for CDX-2 (*lanes 4* and *8*). Furthermore, *in vivo* binding of CDX-2 to the chromatin region encompassing T60 and T90 (Fig. 4*B*) was confirmed by ChIP. Unlike T60 and T90, no CDX-type binding was identified to the T164 probe (not shown).

Mutation of the T60 *cis*-element significantly abrogated (80% loss, $p < 0.05$) the transactivation of the *MUC4* promoter by both CDX-1 and CDX-2 in colonic HT-29 STD cells (Fig.

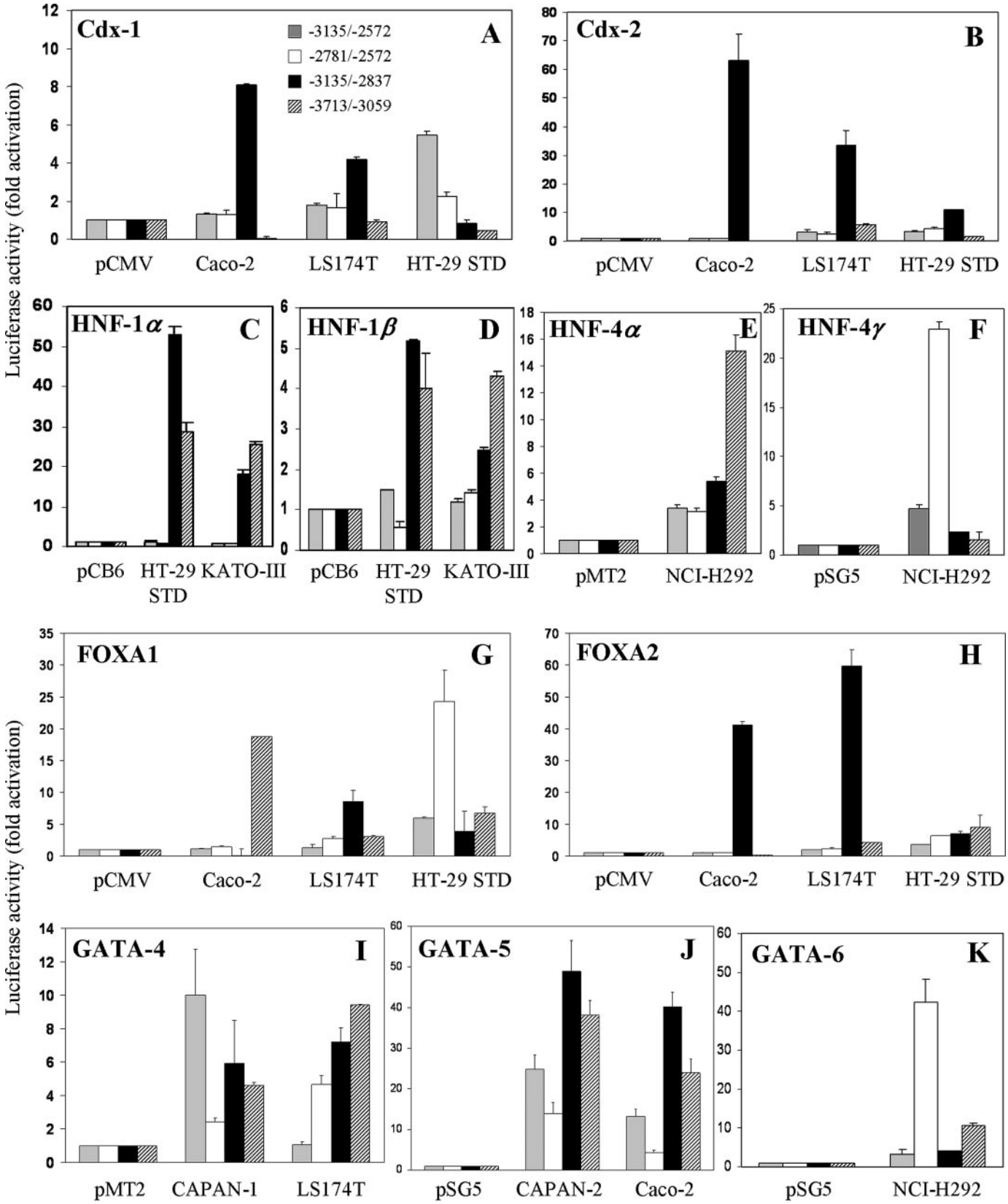

FIGURE 3. **Transcriptional regulation of *MUC4* distal promoter by endodermal transcription factors in epithelial cancer cell lines.** 1 μg of the pGL3-*MUC4* deletion mutants −3135/−2572 (*gray bars*), −2781/−2572 (*white bars*), −3135/−2837 (*black bars*), and −3713/−3059 (*hatched bars*) was transfected in the presence of 0.5 μg of the pCMV-Cdx-1 (*A*), pCMV-Cdx-2 (*B*), pCB6-HNF-1α (*C*), pCB6-HNF-1β (*D*), pMT2-HNF-4α (*E*), pSG5-HNF-4γ (*F*), pCMV-FOXA1 (*G*), pCMV-FOXA2 (*H*), pMT2-GATA-4 (*I*), pSG5-GATA-5 (*J*), or pSG5-GATA-6 (*K*) expression vectors. The luciferase activity obtained in co-transfections performed in the presence of 1 μg of pGL3-*MUC4* promoter construct and 0.5 μg of the control empty vector was arbitrarily set to 1. *Error bars* represent the means of values obtained in triplicate in three separate experiments.

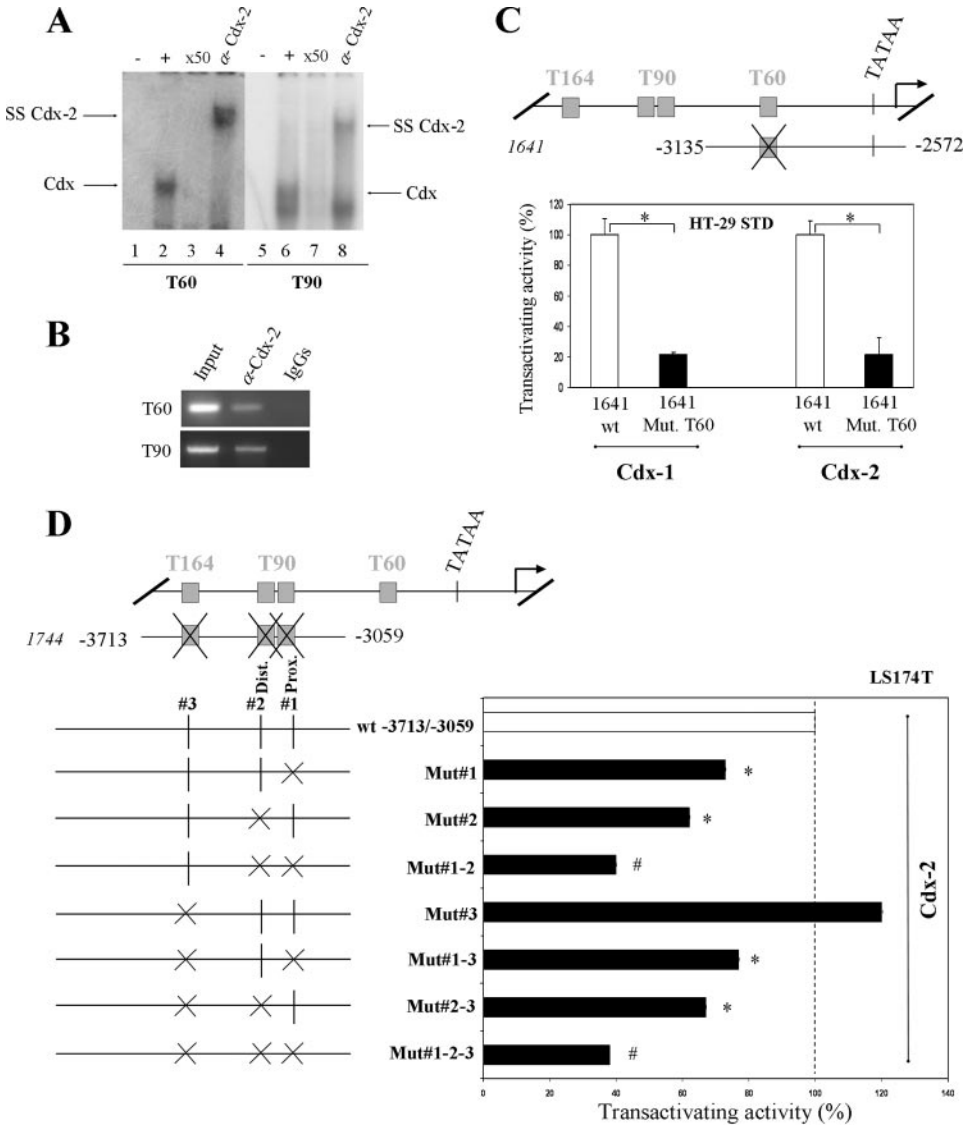

FIGURE 4. **Identification and functionality of the CDX *cis*-elements present in the *MUC4* distal promoter.** *A*, EMSA were performed with radiolabeled probes T60 and T90 incubated with 8 μg of Caco-2 nuclear extract (T60, *lane 2*; T90, *lane 6*). Cold competitions were performed with $\times$50 excess of unlabeled probes (*lanes 3* and *7*). Supershift experiment with 2 μl of anti-CDX-2 antibody (*lanes 4* and *8*). Probes alone (*lanes 1* and *5*). *B*, *in vivo* binding of CDX-2 to chromatin by ChIP. PCRs were carried out with specific pairs of primers covering the T60 and T90 binding sites, respectively (supplemental Table S4). PCR products (15 μl) were analyzed on 1.5% agarose gels. *IgGs*, negative control with rabbit IgGs. *C* and *D*, site-directed mutagenesis of CDX sites present in T60 (*C*), T90 (*D*), and T164 (*D*). Transient transfection experiments were performed in the presence of 1 μg of wild type or mutated forms of promoter constructs $-3135/-2572$ (*C*), $-3713/-3059$ (*D*), and 0.5 μg of pCMV-Cdx-1 or pCMV-Cdx-2 expression vectors. The transactivating activity obtained with the wild-type construct was arbitrarily set to 100%. *Error bars* represent the means of values obtained in triplicate in three separate experiments. *, $p < 0.05$; and #, $p < 0.01$.

4*C*). Single mutations in the T90 region (*mut#1* and *mut#2*) led to a significant ($p < 0.05$) 20–40% reduction of the transactivating effect of CDX-2 in LS174T cells and the double mutation (*mut#1–2*) led to 65% loss of transactivation, indicating an additive effect (Fig. 4*D*). Consistent with the absence of DNA binding, mutation of the T164 site (*mut#3*), alone or in combination with *mut#1* and/or *mut#2* did not alter *MUC4* promoter transactivation by CDX-2.

Identification and Functionality of the HNF cis-Elements Present in MUC4 Distal Promoter—The distal promoter of *MUC4* contains three putative HNF elements (supplemental data Fig. S3). T144 and T106 were recently identified as HNF-1α *cis*-elements (15). Here, we provide evidence by EMSA that T144 not only engages with HNF-1α (Fig. 5*A*, *lane 5*) but also with HNF-1β (*lane 6*) and that both TFs interact with the chromatin region encompassing T144 by ChIP (Fig. 5*B*). Incubation of the radiolabeled probe T91 with nuclear extract from gastric KATO-III cells, which express HNF TFs gave one retarded band (Fig. 5*C*, *lane 2*), which disappeared by competition with a $\times$50 excess of the corresponding unlabeled probe (*lane 3*). Use of the T91 radiolabeled probe in which the putative HNF binding site was mutated (Mut.T91) prevented DNA-protein complex formation (*lane 11*), confirming the specificity of the interaction. Inhibition of the shifted complex upon addition of anti-FOXA1 antibody in the reaction mixture confirmed the involvement of FOXA1 in the complex formation (*lane 6*). *In vivo* binding of FOXA1 to the chromatin region encompassing the T91 probe was then confirmed by ChIP (Fig. 5*D*).

Mutation of the T144 HNF-1 *cis*-element in the construct $-3135/-2837$ resulted in a 86 and 78% decrease of promoter transactivation by HNF-1α and HNF-1β, respectively (Fig. 5*E*). Mutation of that same *cis*-element did not alter the *MUC4* promoter transactivation by FOXA2 (supplemental data Fig. S5). The T144 *cis*-element appears thus essential in *MUC4* promoter regulation by HNF-1α and HNF-1β.

Mutation of the T106 HNF-1 element led to a 80% loss of transactivation by HNF-1α, HNF-1β, and FOXA2 but not FOXA1 (Fig. 5*E*). Mutation of the T91 FOXA element had a lower impact, alone (40–50% inhibition) or in combination with the T106 mutation (equivalent to T106 mutation), on the regulation by HNF-1α, HNF-1β, and FOXA2. On the other hand, the T91 mutation abrogated *MUC4* transactivation by FOXA1 and the double mutation T91/T106 was equivalent to the single T91 mutant. Altogether these results indicate that T106 participates in *MUC4* regulation by HNF-1α, HNF-1β, and FOXA2. T91 is more important in conveying *MUC4* activation by FOXA1. The T91 and T106 mutations partly affected promoter activation by HNF-4α or HNF-4γ (supplemental data Fig. S5). Absence of binding and

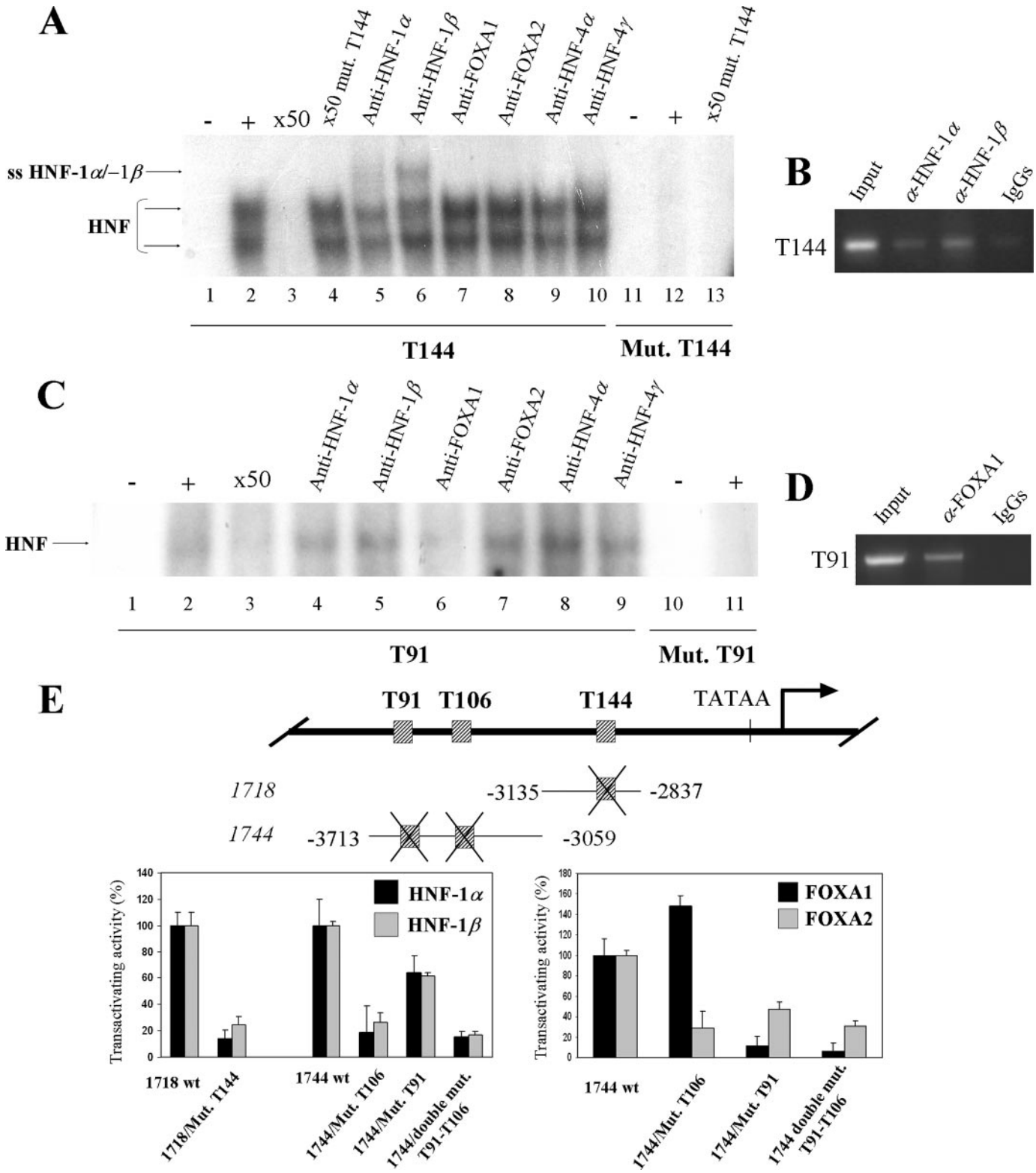

FIGURE 5. **Identification and functionality of the HNF *cis*-elements present in *MUC4* distal promoter.** EMSA were performed with radiolabeled T144 (*A*) and T91 (*C*) DNA probes and the corresponding mutated probes. The probes were incubated with 8 μg of KATO-III nuclear extract (T144, *lanes 2–10*; Mut.T144, *lanes 12* and *13*; T91, *lanes 2–9*; Mut.T91, *lane 11*). Cold competition was performed with $\times$50 excess unlabeled T144 or T91 (*lanes 3*). Supershift experiments were carried out by adding 2 μl of the antibodies as indicated (T144, *lanes 5–10*; T91, *lanes 4–9*). Radiolabeled mutated probes are Mut.T144, *lanes 11–13*; and Mut.T91, *lanes 10* and *11*. Probes alone are T144, *lane 1*; Mut.T144, *lane 11*; T91, *lane 1*; and Mut.T91, *lane 10*. *B* and *D*, *in vivo* binding of HNF-1α and HNF-1β (*B*) and FOXA1 (*D*) to chromatin by ChIP. PCRs were carried out with specific pairs of primers covering T144 (*B*) and T91 (*D*) binding sites, respectively (supplemental Table S4). PCR products (15 μl) were analyzed on 1.5% agarose gels. *IgGs*, negative control with rabbit IgGs. *E*, site-directed mutagenesis of the HNF sites present in T91, T106, and T144. Transient transfection experiments were performed in the presence of 1 μg of wild type or mutated forms of promoter constructs $-3135/-2837$ and $-3713/-3059$ and 0.5 μg of pCB6-HNF-1α, pCB6-HNF-1β, pCMV-FOXA1, or pCMV-FOXA2 expression vectors. The transactivating activity obtained with the wild type construct was arbitrarily set to 100%. *Error bars* represent the means of values obtained in triplicate in three separate experiments. All values (mutants *versus* wild type (*wt*) construct) were found significant with $p < 0.05$.

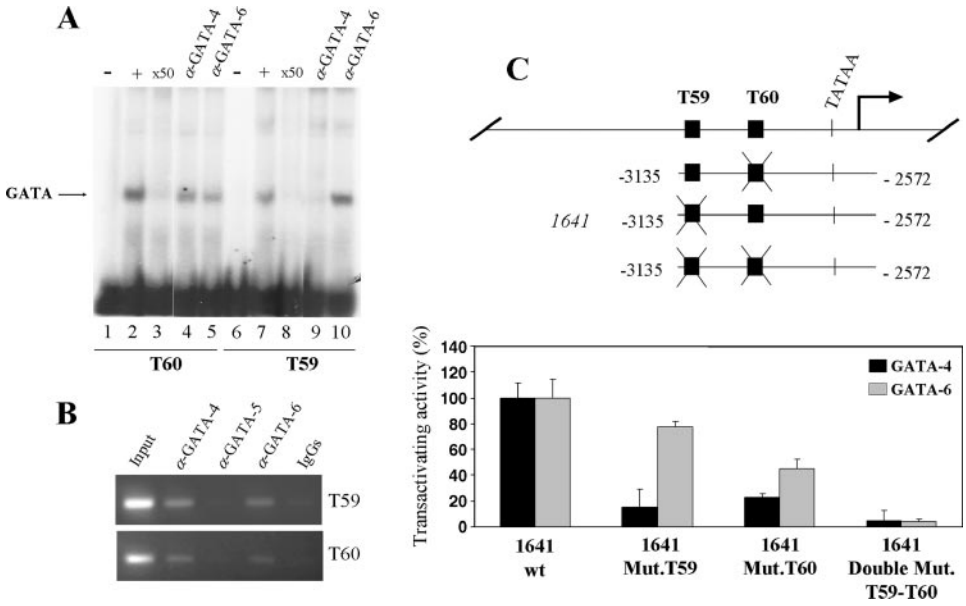

FIGURE 6. **Identification and functionality of the GATA *cis*-elements present in *MUC4* distal promoter.** *A*, EMSA were performed with T59 and T60 radiolabeled DNA probes. They were incubated with 8 μg of CAPAN-1 nuclear extract (T60, *lanes 2–5*; T60, *lanes 7–10*). Cold competitions were performed with ×50 excess of unlabeled T60 (*lane 3*) or T59 (*lane 8*) probes. Supershift experiments were carried out by adding 2 μl of the anti-GATA-4 (*lanes 4* and *9*) or anti-GATA-6 (*lanes 5* and *10*) antibodies, respectively. Probes alone are shown in *lanes 1* and *6*. *B*, *in vivo* binding of GATA-4, -5, and -6 to chromatin by ChIP. PCRs were carried out with specific pairs of primers covering T59 and T60 binding sites, respectively (supplemental Table S4). PCR products (15 μl) were analyzed on 1.5% agarose gels. *IgGs*, negative control with rabbit IgGs. *C*, site-directed mutagenesis of the GATA sites present in T59 and T60. Transient transfection experiments were performed in the presence of 1 μg of wild type (*wt*) or mutated promoter construct −3135/−2572 and 0.5 μg of pMT2-GATA-4 or pSG5-GATA-6 expression vectors. The transactivating activity obtained with the wild type construct was arbitrarily set to 100%. *Error bars* represent the means of values obtained in triplicate in three separate experiments. All values (mutants *versus* wild type construct) were found significant with $p < 0.05$.

partial loss of transactivation of the *MUC4* promoter by these two TFs indicate that their regulation mode is most likely indirect.

Identification and Functionality of the GATA cis-Elements Present in MUC4 Distal Promoter—Two putative GATA binding sites are present in the *MUC4* distal promoter (supplemental data Fig. S3). EMSA with T59 and T60 radiolabeled probes and nuclear extracts from CAPAN-1 cells (Fig. 6*A*), which express GATA TFs, revealed a retarded complex with both probes (*lanes 2* and *7*), that disappeared by competition with a ×50 excess of unlabeled probe (*lanes 3* and *8*). T59 binds GATA-4 because anti-GATA-4 antibody totally inhibited the shifted band (*lane 9*), whereas partial inhibition with antibodies suggested the binding of GATA-4 (*lane 4*) and GATA-6 (*lane 5*) to T60. *In vivo* binding of GATA-4 and GATA-6 to chromatin encompassing T59 and T60 binding sites was confirmed by ChIP (Fig. 6*B*).

Single mutations of T59 and T60, respectively, led to 87 and 80% decrease of GATA-4 transactivating effect in CAPAN-1 cells and the double mutation increased that loss up to 95% (Fig. 6*C*). Single mutations had more moderate effects on GATA-6 activity in KATO-III cells (20 and 60% loss for T59 and T60, respectively), whereas the double mutation led to a complete loss of transactivation by GATA-6. Together, these data demonstrate that GATA-4 and -6 regulate *MUC4* transcription by binding to two promoter elements.

Synergistic Activity of CDX-2, GATA-4, and HNF-1α TFs on MUC4 Promoter Activity—HNF-1α, GATA-4, and CDX-2 are known to cooperate synergistically to regulate intestine-specific gene expression. As *MUC4* is expressed in the intestine and regulated by these TFs (this report), we tested whether such a synergistic mechanism would exist for *MUC4*. Cell specificity was assessed by performing co-transfections in enterocytes (Caco-2), mucus-secreting (LS174T), and undifferentiated (HT-29 STD) colonic cancer cells on the construct −3135/−2837 (Fig. 7). A strong synergistic activation by HNF-1α and GATA-4 was observed in the three cell lines, whereas HNF-1α and CDX-2 specifically synergized in enterocyte Caco-2 cells. When combination of the three factors HNF-1α, GATA-4, and CDX-2 was tested, a significant synergistic effect was visualized in mucus-secreting LS174T cells ($p < 0.01$), whereas addition of CDX-2 to HNF-1α and GATA-4 had the opposite effect both in undifferentiated HT-29 STD and enterocyte Caco-2 cells.

DISCUSSION

Expression studies in human and rat have shown that MUC4 mRNA (6–11) and protein (12) is developmentally expressed, but the molecular mechanisms that are responsible for the specific pattern of expression of MUC4 have never been studied. Our studies in mouse (this report) indicate that the Muc4 mucin is expressed before and after cytodifferentiation in the lung and gastrointestinal tract. Clearly the spatio-temporal pattern of expression of Muc4 is organ- and cell-specific. These results are in agreement with previous data in human (12) and rat (48) and confirm the dual role of MUC4, especially in the intestine, as a membrane-associated protein before cytodifferentiation and both as a membrane-associated and secreted protein (localization in secretory granules of goblet cells) after cytodifferentiation. Altogether these studies indicate that MUC4 has a spatio-temporal pattern of expression both in developing human and rodents, which corrobates its important role in cytodifferentiation in both species.

In this paper we also demonstrate that the human mucin gene *MUC4* is regulated at the transcriptional level by transcription factors (HNF-1/-4, FOXA1/A2, GATA-4, -5, and -6, and CDX-1 and -2) involved in cell differentiation programs during embryonic development. HNF, GATA, and CDX TFs have a specific spatio-temporal pattern of expression during embryonic development and in adults and par-

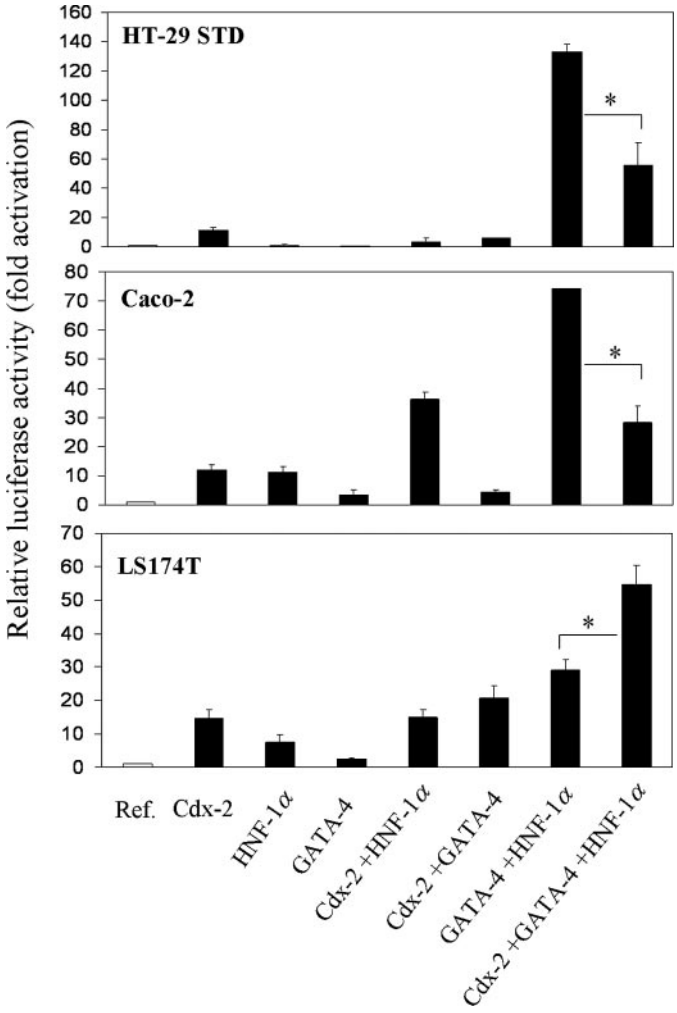

FIGURE 7. **Study of the synergistic effects between CDX-2, GATA-4, and HNF-1α on the transcriptional activity of *MUC4* distal promoter.** 1 μg of the pGL3-*MUC4* construct −3135/−2837 was transfected in the presence of combinations of 0.25 μg of pCMV-Cdx-2 and/or pCB6-HNF-1α and/or pMT2-GATA-4 as indicated on the figure (*black bars*). The transfections were carried out in colonic cell lines HT-29 STD, Caco-2 and LS174T. *Ref.* corresponds to the co-transfections performed with equimolar amounts of the corresponding empty vectors as controls; their values were arbitrarily set to 1 (*white bar*). *Error bars* represent the means of values obtained in triplicate in three separate experiments. *, $p < 0.05$.

ticipate in regional cell differentiation by regulating the expression of tissue-specific genes. It is known that they hierarchically regulate each other in a cell- and tissue-specific manner during development (49, 50) and that they act in various combinations to direct cell-specific transcription during cell differentiation. This is most likely why we did not always get complete inhibition of *MUC4* expression when we knocked down their expression one by one. We confirmed that hypothesis by using combinations of TFs in siRNA experiments that led to increased repression of *MUC4* (supplemental data Fig. S6). This is also in agreement with data obtained from targeted disruption of these TFs in mice that only resulted in moderate reduction of target gene expression (49).

CDX-1 and CDX-2, two intestine-specific TFs, preferentially transactivated the *MUC4* promoter in colonic cancer cell lines. CDX-2 mediates its effect via binding to two *cis*-elements (T60, T90) (Fig. 8) and reduction of Cdx-2 expression in the proximal colon of Cdx-$2^{+/-}$ mice led to a decrease of Muc4 mucin expression (supplemental data Fig. S7). These data indicate that CDX-2 plays a central role in regulating *MUC4* in the proximal colon. CDX-2 was very effective in activating *MUC4* expression in intestinal Caco-2 and LS174T cells, which are representative of two types of differentiated intestinal epithelial cells along the villi: the enterocytes and the goblet cells. Because CDX-2 is expressed in these two cell types (33, 35), we may hypothesize that *MUC4* as a target gene of CDX-2 plays a role in terminal differentiation of these two intestinal cell types. Our studies in human cancer cell lines suggested that CDX-1 activates *MUC4* expression. On the other hand, Cdx-1 loss of function in Cdx-$1^{-/-}$ mice leads to an increased expression of Muc4 toward the crypt bottom (supplemental data Fig. S7), which underlines the complexity and/or regional specificity of MUC4 regulatory mechanisms. Although the different results of CDX-1 and CDX-2 on MUC4 expression in cell transfection experiments and *in vivo* in mutant mice require further investigations, they illustrate the fact that both homeodomain TFs can have similar as well as distinct effects (45, 51, 52), the latter being related to their differential capability to interact with partners of the transcriptional machinery (53). Besides direct regulation via their binding to DNA *cis*-elements, CDX-1 and CDX-2 were found to strongly transactivate the promoter region −3135/−2837 that does not have apparent CDX binding sites. This could be explained either by a non-DNA binding mechanism or by indirect regulation via activation of another TF by CDX-2 as it has already been shown for another target gene of CDX-2 (54).

MUC4 transactivation by HNF-1α and HNF-1β is mediated by their direct binding to two HNF *cis*-elements (T106, T144) in the *MUC4* promoter (Fig. 8). In the small intestine, both TFs are highly expressed in the crypts (55), where MUC4 is also found, which suggests a role in the early steps of intestinal epithelial differentiation. We also showed that both TFs were able to regulate *MUC4* in all the cell lines tested regardless of their tissue origin, phenotype, or differentiation status suggesting that they may regulate *MUC4* in other organs in which they are expressed, such as stomach, colon, and pancreas (56). This is in agreement with recent data in which we showed that HNF-1α is a key regulator of MUC4 expression in esophageal cancer cells (15).

Activation of *MUC4* by FOXA1 and FOXA2 was very strong in colonic cancer cell lines, suggesting intestinal specificity. Activation by FOXA1 was mediated by its direct binding to one HNF element (T91) in the *MUC4* promoter, whereas FOXA2 acts indirectly (Fig. 8). Indirect regulation by FOXA1 may also occur as we showed in HT-29 STD cells that it could induce strong activation of the −2781/−2572 promoter region, which does not contain any FOXA binding site. From that, it can be hypothesized that FOXA1 is able to recruit and/or activate a cell-specific TF that then interacts with the *MUC4* promoter. During embryonic development, FOXA TFs are expressed in the early steps of endoderm differentiation (30). In the adult intestine, FOXA1 is strongly expressed in intestinal crypts and decreases along the crypt-villus axis, whereas FOXA2 is found in the crypts (57) and in goblet

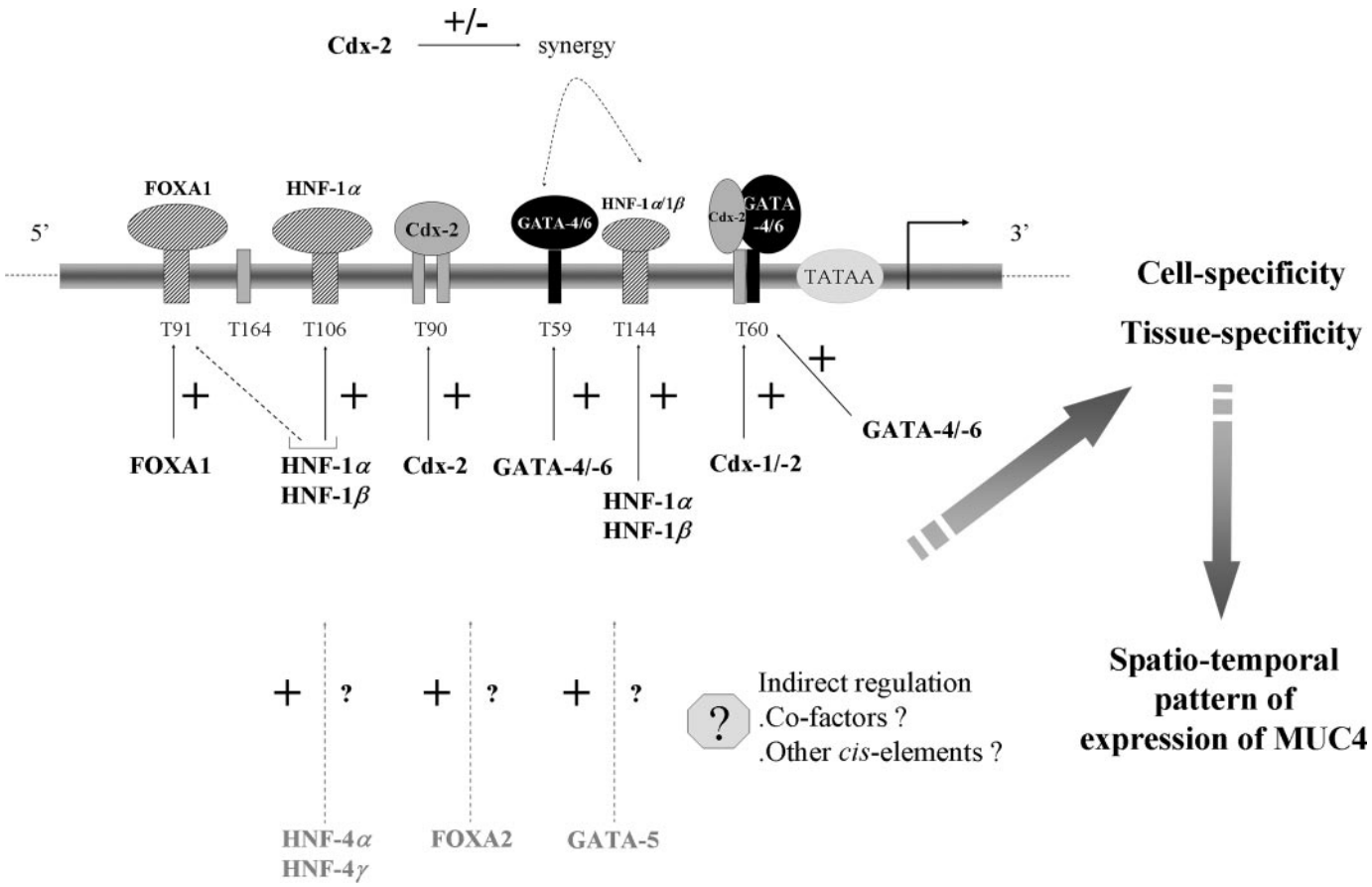

FIGURE 8. **Schematic representation of the regulation of *MUC4* distal promoter by HNF, GATA, and CDX transcription factors.** Transcription factors directly regulating *MUC4* transcription by engaging with their cognate *cis*-elements are indicated in *black* and those involved in indirect regulatory mechanisms (*dashed lines*) are in *gray*. Synergy between GATA-4 and HNF-1α was potentiated by CDX-2 in LS174T mucous secreting intestinal cells (+) but not in enterocyte-like Caco-2 or undifferentiated HT-29 STD cells (−). The different combinations of TFs engaged in *MUC4* regulation will lead to cell- and tissue-specific expression of MUC4 as well as be responsible for MUC4 spatio-temporal pattern of expression.

cells.[6] Thus, *MUC4* as a target gene of these TFs expressed both in the crypts and in the villi may play a role in both intestinal differentiation induction and terminal differentiation. In the lung, the same role could be played in differentiation of goblet cells, as FOXA2 was shown to be a major actor in lung epithelial cell differentiation and in regulating lung-specific genes (20, 21).

Zinc finger factors HNF-4α and -4γ showed strong transactivating activity in the respiratory cancer cell line and a non-negligible effect in pancreatic, gastric, and colonic cell lines. Our data suggest that their effects are indirect (transactivation of promoter regions devoid of HNF binding sites) and involve cooperation with co-factors (HNF-4α/HNF-1α and HNF-4α/GATA-6) (supplemental data Fig. S6). This is in agreement with previous results showing cooperation between these TFs to induce transcription of their target genes (58). In adults, HNF-4 is expressed in kidney, pancreas, stomach, and intestine (29). In the intestine, both TFs are expressed in the villi, whereas HNF-4α is also found in the crypts (49). They may thus participate to the complex regulatory system controlling *MUC4* expression along the crypt-villus axis.

GATA factors play essential roles in the development and function of endoderm-derived tissues (28). GATA-4 is required for the development of visceral endoderm and for proper differentiation of glandular gastric epithelial cells, whereas GATA-6 is essential for pulmonary development (59). In this paper we show that both TFs are important regulators of *MUC4* expression by directly mediating their effects via two *cis*-elements (T59, T60) (Fig. 8). Activation of a promoter region devoid of the GATA binding site by GATA-6 in NCI-H292 cells also suggests indirect regulation via the recruitment and/or activation of a cell-specific TF able to then activate the *MUC4* promoter. GATA-5 activation of *MUC4* transcription is indirect or requires other elements not present in the distal promoter as it did not bind to DNA. In the small intestine, GATA-4 expression increases along the crypt-villus axis, whereas GATA-6 expression is highly expressed in progenitor cells of the crypts (60), which suggests that the role of GATA-4 in intestinal differentiation is to activate terminal differentiation-specific genes, whereas GATA-6 participates in maintaining the pool of proliferating cells. GATA TFs are thus most likely important in deter-

[6] I. B. Renes and I. Van Seuningen, unpublished data.

mining the spatio-temporal pattern of expression of MUC4 during development and later on in adult respiratory and gastrointestinal tracts.

Until now, extensive work has been published regarding the regulation of enterocyte-specific markers, such as sucrase isomaltase, lactase, and fatty acid-binding protein by HNF, GATA, and CDX TFs (18, 22–25). In this work we showed that, like sucrase isomaltase, *MUC4* is synergistically activated by HNF-1α and GATA-4 in Caco-2 and HT-29 STD colonic cancer cells. The synergy is even stronger in the presence of CDX-2 in mucus-secreting LS174T cells. This could represent a combinatorial system able to drive MUC4 expression in the small intestine where the three TFs are co-expressed (61). This suggests that the MUC4 mucin can be added to the list of intestine-specific genes regulated by these TFs and as such plays a role in gastrointestinal differentiation.

Embryonic cells share common biological properties with tumor cells. HNF, GATA, and CDX TFs are known to participate in carcinogenesis, thus they may be responsible for some aberrant patterns of MUC4 expression in epithelial cancers. In general, MUC4 overexpression in epithelial cancers is associated with poor prognosis and tumor progression (2, 5, 62). Interestingly, among HNF, GATA, and CDX TFs, some have tumor suppressor functions (FOXA2 (63)), whereas others are thought to participate in tumor progression (GATA-6 (64, 65), HNF-1α (66), and FOXA1 (67)). Others like CDX-2 or GATA-4 have antagonist functions. CDX-2 is a tumor suppressor gene in colorectal cancer (68, 69), whereas its ectopic expression in intestinal metaplasia in the stomach and esophagus is considered as a preneoplastic condition (70). GATA-4 is overexpressed in pancreatic intraepithelial neoplasia (65) and down-regulated in gastric, colorectal, and lung tumors (64). Taken together, these data suggest that, depending on the function attributed to the TF in tumor progression and to the active oncogenic pathway(s) the TF is facing in a given cancer cell, activation of tumor-associated MUC4 mucin expression will have profound consequences on the tumor behavior. As we saw, multiple TFs may be able to activate *MUC4* in the same cell/organ, so the level of expression of these TFs will be an important factor in determining the level of MUC4 expression and consequently cancer cell fate toward either proliferation or differentiation.

In conclusion, we have shown in this report that MUC4 mucin has a spatio-temporal pattern of expression during development and is a target gene of CDX-1 and -2, HNF-1α and -1β, FOXA1/A2, HNF-4α and -4γ, and GATA-4, -5, and -6 transcription factors. The complexity of the mechanisms described in this report points out that *MUC4* is tightly regulated by these TFs and suggests that these TFs are responsible for the MUC4 spatio-temporal pattern of expression during embryonic development of gut endoderm-derived tissues. The regulatory mechanisms described in this paper will also help in a better understanding of *MUC4* regulation in epithelial cancers and epithelial repair during which cell differentiation is altered and recapitulates molecular mechanisms observed during development.

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