Silencing of CDX2 Expression in Colon Cancer via a Dominant Repression Pathway*

CDX2 is a caudal-related homeobox transcription factor whose expression in the adult is normally restricted to intestinal epithelium. Mice heterozygous for germ line Cdx2 inactivation develop intestinal polyps, and the lesions lack Cdx2 expression. Prior studies indicate some human colon carcinomas also lack CDX2 expression. To address the role of CDX2 defects in colon cancer development, we analyzed CDX2 expression in 45 primary colorectal carcinomas. Four carcinomas lacked CDX2 expression, and three others showed aberrant cytoplasmic localization of CDX2, although no significant CDX2 gene defects were seen in the seven tumors. Marked reductions in CDX2 transcript and protein levels were seen in five of 13 colorectal cell lines, and nuclear run-off data indicated reduced transcription was a major factor in CDX2 silencing. Treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine and/or the histone deacetylase inhibitor trichostatin A did not restore CDX2 expression in CDX2-negative lines. However, consistent with a role for dominant repression mechanisms in CDX2 silencing, all somatic cell hybrids resulting from pairwise fusions between colon cancer lines with intact CDX2 expression and lines lacking Cdx2 had reduced Cdx2 transcripts and protein. A roughly 9.5-kb 5' -flanking region from the human CDX2 gene contained key cis elements for regulating transcription in colon cancer cells. Restoration of CDX2 expression suppressed proliferation and soft agar growth in the CDX2-negative HT-29 colon cancer cell line. Our findings suggest CDX2 inactivation in colon cancer results from defects in trans-acting pathways regulating CDX2 transcription, and CDX2 silencing contributes to the altered phenotype of some colorectal cancers.

Considerable progress has been made in defining some of the critical mutations and gene expression changes in colorectal cancer pathogenesis. Mutations in the adenomatous polyposis coli (APC), p53, and K-RAS genes appear to play prominent roles in the process, and defects in other oncogenes and tumor suppressor genes contribute in a more variable fashion to colorectal cancer development and progression (1). Besides the well established role of mutational mechanisms in tumor suppressor gene inactivation in cancer, a growing body of evidence indicates that epigenetic mechanisms play a prominent role in tumor suppressor gene inactivation in cancer in general and colorectal cancer specifically (2). These epigenetic mechanisms include hypermethylation at CpG dinucleotide sites in or nearby key cis-acting transcriptional regulatory elements as well as post-translational modifications (e.g. acetylation and methylation) of histones and perhaps other transcription factors and chromatin-associated proteins.

The discovery of specific germ line (constitutional) mutations that predispose to tumor development in man and/or the mouse offers the possibility of highlighting and clarifying genes and mechanisms involved in sporadic tumor development. Of some interest for the colorectal cancer field has been the observation that the majority of mice heterozygous for germ line inactivation of the Cdx2 gene (Cdx2+/−) develop from one to 10 polyps in their proximal colon and distal small intestine within the first 3 months of life (3, 4). Consistent with a possible tumor suppressor function for Cdx2, the epithelial cells in the polyps lose CDX2 protein expression. However, the mechanisms accounting for somatic inactivation of the remaining Cdx2 allele in the tumors arising in the Cdx2+/− mice remain obscure. Intriguingly the polyps in Cdx2+/− mice contain areas of keratinizing stratified squamous epithelium, similar to that seen in the forestomach and esophagus, as well as areas of epithelium resembling that seen in normal gastric mucosa (5). Based on these findings, it would appear the CDX2 protein functions as a key regulator of proliferation and differentiation in intestinal epithelial cells.

The CDX2 protein is a homeobox transcription factor, and it derives its name from the Drosophila homologous gene caudal (Cad). Cad plays an important role in segmentation and the formation of posterior structures in Drosophila such as the posterior midgut and hindgut (6, 7). A number of Cad-related genes have been identified in mammals, including at least two homologues in man termed CDX1 and CDX2. While CDX2 is rather broadly expressed in embryogenesis, in adult tissues of mouse and man, CDX2 expression appears to be essentially restricted to epithelial cells in the small intestine and colon (8–11). Like CDX2, CDX1 expression is restricted to the intestinal epithelium in the adult (12). Although there are similarities, the pattern of CDX1 expression in embryos differs from that of CDX2 (11), and Cdxi+/− mice have a phenotype distinct from that of Cdx2−/− mice (3, 13). Moreover, unlike Cdx2+/− mice, Cdxi+/− mice have not been reported to manifest a predisposition to intestinal tumors.

Loss of CDX1 and/or CDX2 gene and/or protein expression has been reported in a subset of primary colorectal cancers and cancer cell lines (10, 12, 14, 15). Our recent studies indicate CDX2 expression may be most commonly lost in poorly differ-
entiated colorectal carcinomas that show minimal gland-forming ability (16). In some prior studies, mutations in the CDX2 gene have not been found to account for loss of CDX2 expression (17–20), and the specific mechanisms underlying loss of CDX2 expression in colon carcinomas remain poorly understood. In an effort to obtain further insights into the means by which the CDX2 protein plays a role in colorectal cancer, we have pursued studies to identify mechanisms responsible for CDX2 gene inactivation in colon cancer. Here we report on evidence that loss of CDX2 expression in primary colon carcinomas and colorectal cancer-derived cell lines may result from defects in trans-acting pathways regulating CDX2 transcription. We also provide evidence of a significant role for CDX2 inactivation in the tumorigenic phenotype of some colorectal cancers.

EXPERIMENTAL PROCEDURES

Plasmids—To generate pBluescript II-KS (pBS)/CDX2 (2–164), a cDNA encoding CDX2 amino acids 2–164 with a BamHI site at the 5’-end and EcoRI site at the 3’-end was synthesized by PCR with forward primer 5’-GGGATCCGCTATCATCCTCCTCTGAACA-3’ and reverse primer 5’-TGAATTCTAGCTCGGCTGAGCCGCG-3’ using hexamer-primed cDNA from normal human colon tissue as template and then subcloned into pBS vector. A fragment of the human glycereraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was generated by PCR with forward primer 5’-AAAGCTTGAGAAGCGGACG-3’ and reverse primer 5’-TTCTCTACTGCTCTAGGGAT-3’ using hexamer-primed cDNA from Caco2 cells as template. The GAPDH PCR product was subcloned into pBS vector to generate pBS/GAPDH. A bacteriophage P1-derived artificial chromosomal (PACs) vector containing large human genomic DNA fragments (Genome System Inc., St. Louis, MO) was screened by PCR using primers derived from the CDX2 coding region (5’-AGAGCAAGAGAGGAAAATTCAAC-3’ [forward] and 5’-TTCTCTATGCTCGGCTGAGCCGCG-3’ [reverse]), and a PAC clone containing a DNA insert of about 120 kb harboring the full-length CDX2 coding region and 5’- and 3’-flanking regions was identified. A 9.4-kb fragment with XhoI sites containing sequence from the 5’-flanking region of CDX2 was cloned and inserted into the pGLO basic vector (pGLO) to generate pGLO/CDX2P9.5. The sequence of all inserts generated by PCR were verified by automated sequencing of the plasmid constructs.

Tumor Specimens and Immunohistochemistry—Forty-five formalin-fixed and paraffin-embedded primary colorectal carcinoma specimens were obtained and immunohistochemistry staining with an antibody against CDX2 essentially as described previously (16). Briefly, after formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated, antigen enhancement was performed by boiling slides in a 1.2% formaldehyde-agarose gels and transferred to Zeta-Probe GT membrane (Bio-Rad Laboratories, Inc., Hercules, CA) prior to hybridization. The dissected tissue.

Northern Blot Analysis—Total RNA was extracted from cells with Trizol (Invitrogen). For each sample, 10 μg of total RNA was separated on 1.2% formaldehyde-agarose gels and transferred to Zeta-Probe GT membrane (Bio-Rad) by capillary action. Transcript levels for CDX2 and GAPDH transcripts on Northern blots were generated by digesting pBS/CDX2 (2–164) with EcoRI and BamHI and pBS/GAPDH with EcoRI. The fragments were gel-purified and labeled with [α-32P]dCTP with the Rediprime II random priming labeling system (Amersham Biosciences). After prehybridization, membranes were hybridized in a Rapid-blot buffer (Amersham Biosciences) according to the manufacturer’s protocol. Signals were detected by exposure to BioMax-MS film (Eastman Kodak Co.) at ~80 °C with an intensifying screen.

Western Blot Analysis—Whole-cell extracts were prepared with RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM EDTA, 0.1% SDS, 1 mM PMSF, 1 mM sodium fluoride, 1 mM sodium vanadate) containing protease inhibitors (Complete Mini protease inhibitor mixture tablet, Roche Applied Science). Protein concentrations were determined by bichinonic acid protein assay kit (Pierce), and RIPA lysates containing 50 μg of protein were separated by electrophoresis in 8–10% SDS-polyacrylamide gels. After semi-dry transfer of the proteins to Immobilon-P membrane (Millipore, Bedford, MA), blots were incubated with Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 10% nonfat dry milk to block nonspecific antibody binding. Affinity-purified polyclonal rabbit antibody against CDX2 was used at 1:1,000 dilutions overnight with primary antibody at 4 °C. Affinity-purified polyclonal rabbit antibody to CDX2 was used at 1:100 dilution. After washing in phosphate-buffered saline, slides were incubated with biotinylated anti-rabbit IgG for 30 min (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). Antigen-antibody complexes were detected with the avidin-biotin peroxidase method using 3,3-diaminobenzidine as a chromogenic substrate (DAB substrate kit for peroxidase, Vector Laboratories, Inc.) as recommended by the manufacturer. Sections were lightly counterstained with hematoxylin and then evaluated by light microscopy. Genomic DNA from the neoplastic elements of the four carcinomas lacking CDX2 expression and the three with cytoplasmic CDX2 staining was obtained by microdissection of the elements and subsequent isolation of DNA as described previously (16). Briefly, five consecutive 5-μm formalin-fixed tissue sections were cut from each paraffin block, mounted on glass slides, and then weakly stained with hematoxylin. Neoplastic regions were carefully microdissected with 22-gauge needles under a microscope using adjacent hematoxylin- and eosin-stained sections as dissection guides, and genomic DNA was then extracted from the dissected tissue.

CDX2 Mutation Analysis—The sequences of exon-intron boundaries of CDX2 were confirmed by sequencing of the CDX2-containing PAC. Eight pairs of primers were used to amplify CDX2 genomic DNA for the mutational analysis. Five pairs were used to amplify exon 1 sequences (1a–1e), one pair was used for exon 2, and two pairs were used for exon 3 (3a and 3b). The length of PCR products and the sequence of the primers used were as follows: exon 1a (200 bp, forward, 5’-CCCGGGACGACCTCCAG-3’, and reverse, 5’-CCTGTGTAACGCCGC- GTGATCC-3’), exon 1b (168 bp, forward, 5’-TGCCCAGCGAAGAATT- CCTGACG-3’, and reverse, 5’-GCCGCTAGCATCTGACTTCTC-3’), exon 2a (168 bp, forward, 5’-TCTGGCGCCAATGTGGCAG-3’, and reverse, 5’-GGGTTGTTGCGGATTGTA-3’), exon 1d (172 bp, forward, 5’-GGCCGGACCATGGTGCTAC-3’, and reverse, 5’-GGGAGGCACTGTCGCCC-3’), exon 1e (174 bp, forward, 5’-TGGCTGAAACCTGCTA- ACCCC-3’, and reverse, 5’-CTCTCCCAAGACCTCUCCTCAG-3’), and exon 2b (199 bp, forward, 5’-CCCCGAGCTGACAAGCTCTCCTC-3’, and reverse, 5’-AGTCGAGAGAAGAAGGAGTG-3’).

Cell Culture—All cell lines were obtained from the American Type Culture Collection (Manassas, VA). The exception of the following: HT-29/PGS-CDX2 and HT-29/PGS-neo cell lines, which were generated previously (21). All cell lines, except RKO, MCF-7, and BT-549, were propagated in Dulbecco’s minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum. RKO was grown in McCoy’s 5A medium, and MCF-7 was grown in minimum essential medium α (Invitrogen), both with 10% fetal bovine serum. BT-549 was grown in RPMI medium 1640 (Invitrogen) with 10% fetal bovine serum and 0.23 units/ml insulin from bovine pancreas (Sigma). HT-29/PGS-CDX2 and HT-29/PGS-neo were selected in medium containing 1 mg/ml and maintained in 400 μg/ml G418 (Invitrogen). Cell lines were maintained in appropriate medium and were treated with 1 or 2 μM 5-aza-2′-deoxycytidine (5-azaC) (Sigma) for 5 days to induce DNA demethylation before harvest. Cell lines were also treated with 0.5 μM trichostatin A (TSA) (Upstate Biotechnology, Lake Placid, NY) for 24 h to inhibit histone deacetylases. Wortmannin and insulin from porcine pancreas were purchased from Sigma. Cells were serum-starved for 16 h prior to insulin stimulation and/or wortmannin treatment. For continuous inhibition of phosphatidylinositol 3-kinase, medium from wortmannin-treated cells and control cells (Me2SO-treated) was replaced every 2–6 h because of the instability of wortmannin in aqueous medium (22).

The abbreviations used are: pBS, pBluescript II-KS; 5-azaC, 5-aza-2′-deoxycytidine; TSA, trichostatin A; GAPDH, glycereraldehyde-3-phosphate dehydrogenase; RIPA, radiomunoprecipitation assay; TES, 5-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PAC, P1-derived artificial chromosome; P13-K, phosphatidylinositol 3-kinase; LI, liver-intestine; CDX2P, CDX2 promoter.
secondary antibodies at 1:20,000 dilutions. Blots were subjected to enhanced chemiluminescence detection (Supersignal West Pico Chemiluminescent substrate, Pierce) and exposed to X-Omat film (Kodak).

**Nuclear Run-off Assay**—Nuclei were obtained, and nuclear run-off assays were performed by a modification of procedures described previously (23). Briefly, 5 × 10⁶ cells were washed twice in ice-cold phosphate-buffered saline. Cells were then scraped and lysed in 1 ml of Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM CaCl₂, 2 mM MgCl₂, and 1% (v/v) Nonidet P-40) using a Dounce homogenizer (B pestle) until nuclei appeared free of membrane components by phase-contrast microscopy. Nuclei were harvested, resuspended in 200 μl of ice-cold glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, pH 8.0, and 40% (v/v) glycerol), and stored at −80 °C. Two hundred microliters of 2 °C reaction buffer with nucleotides (10 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.3 mM KCl, 5 mM dithiobiotiel, 1 mM ATP, 1 mM CTP, and 1 mM GTP) and 10 μl of [α-³²P]UTP (3000 Ci/mmol) (Amersham Biosciences) were added to 200 μl of nuclear suspension. The nuclear run-off transcription was allowed to proceed at 30 °C for 30 min with shaking. The reaction was then terminated by adding 0.6 ml of HSB buffer (10 mM Tris-HCl, pH 7.4, 0.5 mM NaCl, 50 mM MgCl₂, and 2 mM CaCl₂) containing 40 μg/ml RNase-free DNase I (Promega) and 10 μg of 0.2% SDS) containing 0.3 M NaCl. The ³²P-labeled RNA solution was precipitated by adding 1.5 ml of 1 M HEPES. Then RNA was precipitated by adding 0.6 ml of HSB buffer (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 50 mM MgCl₂, and 2 mM CaCl₂) containing 25 μg/ml RNase-free DNase I (Whortonning Biochemical Corp., Lakewood, NJ) at 30 °C for 5 min. Ten microliters of 20 mg/ml proteinase K and 200 μl of SDS/Tris buffer (0.5 mM Tris-HCl, pH 7.4, 125 mM EDTA, pH 8.0, and 5% SDS) were added and incubated at 42 °C for 30 min. After the mixture was extracted with phenol/ chloroform/isoamyl alcohol (25:24:1), 3 ml of 10% (v/v) trichloroacetic acid, 30 mM sodium pyrophosphate mixture. The filter was transferred to a siliconized glass scintillation vial and incubating with 1.5 ml of DNase I buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM CaCl₂) containing 25 μg/ml RNase-free DNase I at 37 °C for 30 min. The reaction was quenched by adding 45 μl of 0.5 M EDTA, pH 8.0 and 68 μl of 20% SDS, and RNA was eluted by heating at 65 °C for 10 min in 1 ml of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, and 1% (v/v) trichloroacetic acid, 50 mM sodium pyrophosphate mixture). The mixture was incubated further with 1.5 ml of elution buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8.0, and 1% SDS) at 65 °C for 10 min. The eluted RNA fractions were combined, and 4.5 μl of 20 mg/ml proteinase K was added followed by an incubation at 37 °C for 30 min. Following extraction of the mixture with phenol/chloroform/isoamyl alcohol (25:24:1), 0.75 ml of 1% NaCl was added, and the mixture was incubated for 10 min on ice. The reaction was terminated by addition of 1.5 ml of HEPES. Then RNA was precipitated by adding 0.53 ml of 3 M sodium acetate, pH 5.2 and 14.5 ml of 100% ethanol and incubating for 30 min on dry ice. Precipitated RNA was collected by centrifugation at 10,000 × g for 30 min and resuspended in 2 μl of 10 mM Tris-HCl, pH 7.5, 10 mM EDTA. Cells were lysed in 100 μg/ml RNase-free DNase I (Promega) and 0.2% SDS) containing 0.3 μl of NaCl. The ³²P-labeled RNA solution was hybridized to DNA immobilized on strips of nitrocellulose filters (BioRad). Five micrograms of linearized and denatured DNA plasmids such as pBS vector without insert as a negative control, pBS vector containing human GAPDH cDNA prepared in a 35-mm dish. Three different cell dilutions with the concentration of forward and reverse primers, a 200 μM concentration of each deoxyribonucleotide triphosphate, 1.5 μl of [α-³²P]dCTP (Amersham Biosciences), and 1 unit of Taq DNA polymerase (Invitrogen). DNA fragments for each polymorphic locus were amplified for 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min 30 s followed by a final extension for 10 min at 72 °C. PCR products were diluted 10-fold in denaturing buffer (95% formamide, 10 mM EDTA, pH 8.0, 0.05% xylene cyanol and 0.05% bromphenol blue) and denatured at 90 °C for 5 min. Two microliters of denatured sample were electrophoresed on 5% polyacrylamide gels containing 6 M urea, 30% formamide, and 1× Tris borate/EDTA electrophoresis buffer, and the polymorphic bands were visualized by autoradiography.

**Reporter Gene Assays**—At 48 h prior to transfection, cells were seeded in 35-mm dishes. The transfections were performed with 4 μl of FuGENE 6 (Roche Applied Science)/μg of transfected DNA when the cells were at 50–80% confluency. To determine the transcriptional activity of the 5′-flanking region of the CDX2 gene, 0.5 μg of each CDX2 reporter gene construct and 0.5 μg of control plasmid pCH110 were used per 35-mm dish. At 40 h after transfection, the cells were collected and resuspended in reporter lysis buffer (Promega), and luciferase activity was measured with luciferase assay reagent (Promega) and a luminometer (model TD-20E, Turner Corp., Mountain View, CA). β-Galactosidase activities were determined by standard methods as a control for transfection efficiency.

**Cell Proliferation Analysis**—HT-29/PGS-CDX2 cells and HT-29/PGS neo cells were plated in 35-mm dishes at 2 × 10⁵ cells/dish. Two independent sets of experiments were carried out, each one performed with triplicate measurements of cell number at each of the respective days.

**Soft Agar Colony Formation Assay**—The ability of parental and transfected HT-29 cells to form macroscopically visible colonies [as determined essentially as described previously (25)]. Briefly, 1 ml 1× agarose (prepared in Dulbecco's minimal essential medium containing 20% fetal bovine serum and 0.6% Noble agar (Difco, Detroit, MI) was prepared in a 35-mm dish. Three different cell dilutions with the concentration of 1 ×, 3 ×, and 1 × 10⁵ cells/ml were prepared in Dulbecco's minimal essential medium containing 20% fetal bovine serum and 0.3% Noble agar, and 1 ml of each dilution was pipetted on the undersides of the layers in triplicate. After 8 weeks, colonies were fixed and stained with methylene blue.

**RESULTS**

**Lack of Evidence That CDX2 Expression Defects in Primary Colorectal Carcinomas Are Due to CDX2 Mutations**—To assess the frequency of CDX2 expression defects in primary colorectal cancers, we used immunohistochemistry to study CDX2 expression in 45 primary colorectal carcinomas. Studies of CDX2 expression in adjacent normal tissues from the carcinomas and in five sporadic colorectal adenomas were also undertaken. All normal colorectal tissues displayed strong nuclear staining for CDX2 only in epithelial cells without obvious evidence of a proximal to distal expression gradient in the colon and rectum or an expression gradient from crypt to surface epithelium (Fig. 1A). The adenomas studied also showed strong nuclear staining for CDX2 (Fig. 1B). While 38 of the 45 carcinomas showed strong nuclear staining in neoplastic elements, akin to that
The abnormalities in CDX2 expression were assessed by immunohistochemistry. While 38 of the 45 primary colorectal carcinomas showed strong nuclear staining in neoplastic elements, three tumors (nos. 1–3) had strong cytoplasmic staining for CDX2 in conjunction with nuclear staining, and four tumors (nos. 4–7) lacked any detectable CDX2 staining. To determine whether mutations in the CDX2 genes accounted for the abnormalities in CDX2 expression seen, genomic DNA from neoplastic cells of the seven tumors were obtained, and the CDX2 coding region and exon/intron boundaries were sequenced.

### Table I

| Tumor no. | CDX2 immunohistochemistry | Sequence alteration(s) |
|-----------|---------------------------|-----------------------|
| 1         | Strong cytoplasmic staining | Codon 34 CCC (Pro) to CTC (Leu) (heterozygous) |
| 2         | Strong cytoplasmic staining | Codon 27 GCG (Ala) to TCG (Thr) (homozygous) |
|           |                           | Codon 127 GCC (Ala) to GTC (Val) (homozygous) |
| 3         | Strong cytoplasmic staining | Codon 293 TCT (Ser) to CCT (Pro) (heterozygous) |
| 4         | Not detectable             | No alteration |
| 5         | Not detectable             | No alteration |
| 6         | Not detectable             | No alteration |
| 7         | Not detectable             | Codon 58 GGG (Ala) to GTG (Val) (heterozygous) |
|           |                           | Codon 135 GGG (Gly) to GAG (Glu) (heterozygous) |

**Fig. 1. Immunohistochemical staining of CDX2 in normal and tumor tissues from the colon.** Immunohistochemistry was carried out on formalin-fixed and embedded tissues using a polyclonal rabbit anti-CDX2 antibody. Representative immunohistochemistry results seen in normal colon mucosa and tubular adenoma are shown in A and B, respectively. A well-differentiated colon adenocarcinoma with strong cytosolic and nuclear staining for CDX2 is shown in C with the arrow indicating the region of the tumor enlarged in the inset. A poorly differentiated adenocarcinoma lacking CDX2 staining is shown in D with the arrow marking the region containing carcinoma cells. Normal colonic epithelial cells represent an internal control in the upper portion of D. Original magnifications, ×200 (A–D) (inset in C at ×400).

seen in adjacent normal mucosa, three tumors had strong cytoplasmic staining for CDX2 in conjunction with nuclear staining (Fig. 1C), and four tumors lacked any detectable CDX2 staining (Fig. 1D). Consistent with our prior findings (16), all four tumors that had lost CDX2 expression were poorly differentiated lesions from the right side of the colon. All three tumors with cytoplasmic CDX2 immunoreactivity were well to moderately differentiated. Prior Western blot and immunohistochemical studies have indicated that the polyclonal antibodies against CDX2 have good specificity (16, 26), and independent studies have demonstrated that gastrointestinal cells with CDX2 immunoreactivity present only in the cytoplasm fail to express the CDX2-regulated gene liver-intestine (LI)-cadherin (21). As such, the cytoplasmic immunoreactivity for CDX2 may reflect a non-functional pool of CDX2 protein.

To determine whether mutations in the CDX2 gene accounted for the abnormalities in CDX2 expression seen, we obtained genomic DNA from neoplastic cells of the seven tumors and sequenced the CDX2 coding region and exon/intron boundaries. We found missense sequence alterations in four tumors (Table I). The missense substitutions found were not judged to be of obvious functional significance. In three of the four tumors, the substitutions affected only one of the two CDX2 alleles. Two of the four tumors with missense substitutions were found to have more than one missense substitution in the variant CDX2 allele, with two substitutions in one allele and three in another. Most sequence variants represented conservative substitutions (e.g. alanine to valine and arginine to lysine), and none of the missense substitutions were present in the DNA-binding (homebox) domain of the CDX2 protein. One of the seven tumors was heterozygous for a sequence change leading to a stop at codon 313. However, the significance of this sequence variation with respect to CDX2 expression and function was not obvious since only the 10 carboxyl-terminal amino acids of the protein would be predicted to be lost. In two of the seven tumors, no CDX2 sequence changes were found. Thus, our sequencing studies failed to offer conclusive evidence that somatic inactivating mutations in CDX2 play a role in the observed abnormalities in CDX2 expression and/or its subcellular localization.

**Decreased CDX2 Expression in Colorectal Cancer Cells Results from Transcriptional Defects**—To address potential mechanisms accounting for decreased CDX2 expression in colorectal cancers, we assessed levels of CDX2 transcripts and protein in colorectal cancer cell lines. Reduced or absent levels of CDX2 expression were seen in five of 13 cell lines (Fig. 2A), and reduced gene expression paralleled reduced protein expression. Sequence analysis of CDX2 in the LoVo, SW48, HCT116, HT29, WiDr, and SW480 cell lines was performed. Three of the cell lines (LoVo, SW48, and HCT116) displayed the microsatellite instability phenotype as a result of defects in mismatch repair function, and all three cell lines were homozygous for deletion of a G in a G7 sequence tract in the 3' region of the CDX2 coding region. The deletion is predicted to cause a frameshift leading to truncation of the last seven amino acids in the CDX2 protein product. However, two of the three cell lines with the presumptive frameshift mutation retained robust CDX2 expression (i.e. LoVo and SW48, see Fig. 2A). Prior sequence-based analysis of CDX2 in the RKO cell line indicates that it is heterozygous for a frameshift mutation that truncates the carboxyl-terminal 85 amino acids, and the mutant CDX2 protein has reduced functional activity compared with the wild type protein (27). All CDX2-expressing colorectal cancer cell lines showed predominant if not exclusive expression of CDX2 in the nucleus. To assess whether loss of CDX2 transcripts reflected reduced transcription or decreased transcript stability, we pur-
sued nuclear run-off assays. As shown in Fig. 2, B and C, while newly synthesized CDX2 transcripts were readily detected in the CDX2-expressing cell lines Caco2 and DLD-1, we failed to find robust CDX2 transcription in three colorectal cancer cell lines with reduced CDX2 transcript levels (i.e. HT-29, RKO, and WiDr) or the CDX2-negative breast cancer cell line MCF-7. These data indicate the reduced levels of CDX2 protein and transcripts in CDX2-negative colorectal cancer cells are due primarily to decreased rates of mRNA synthesis.

**CDX2 Expression in Colon Cancer Cells Is Not Restored by Treatment with Inhibitors of DNA Methylation and/or Histone Deacetylation**—Hypermethylation of CpG dinucleotides in or nearby the regulatory regions of some tumor suppressor and candidate tumor suppressor genes has been implicated in loss of gene expression (2). In some cases, other mechanisms, such as chromatin condensation resulting from histone deacetylation, may cooperate with DNA methylation in silencing gene expression (28). To address the potential role of DNA methylation and histone deacetylation in silencing of CDX2 gene expression, we studied effects of the DNA methyltransferase inhibitor 5-azaC and the histone deacetylase inhibitor TSA on CDX2 expression in two colorectal cancer cell lines with very reduced or absent CDX2 protein expression. As shown in Fig. 3, CDX2 expression could not be induced in either cell line by treatment with 5-azaC alone or the combination of 5-azaC plus TSA. In contrast, in the RKO cell line, which lacks expression of the MLH1 mismatch repair protein as a result of epigenetic silencing, 5-azaC treatment alone or the combination of 5-azaC plus TSA readily restored MLH1 expression (Fig. 3A). Based on these results, we conclude that while promoter hypermethylation and histone deacetylation could perhaps play some role in the silencing of CDX2 expression, other mechanisms appear to play the crucial roles in initiating and/or maintaining CDX2 repression.

**A Dominant Repression Pathway Silencing CDX2 Expression in Colorectal Cancer**—We have previously had success in using somatic cell hybrid approaches to address potential mechanisms involved in transcriptional silencing of the E-cadherin tumor suppressor gene in breast cancer cells (24). To gain further insights into mechanisms underlying transcriptional repression of CDX2 in colon cancer cells, we analyzed somatic cell hybrids resulting from pairwise fusions between colon cancer cell lines with intact CDX2 transcription (CDX2+) and lines lacking CDX2 transcription (CDX2−). DLD-1 cells expressed CDX2 transcripts and protein (Fig. 2A), and a polyclonal G418-resistant population of DLD-1 cells was obtained by transfection of the cells with an expression vector containing a neomycin resistance gene. HT-29 and WiDr cells lacked detectable CDX2 expression (Fig. 2A). Each of these two CDX2− lines was transfected with a vector encoding resistance to the drug hygromycin in mammalian cells, and polyclonal hygromycin-resistant HT-29 and WiDr cell lines were subsequently derived. The G418-resistant DLD-1 line was fused to the hygromycin-resistant HT-29 line, and the G418-resistant DLD-1 line was also fused to the hygromycin-resistant WiDr line, yielding the “DLD-HT” and “DLD-Wi” hybrids, respectively. Hybrids were selected in G418 and hygromycin, and individual clones resistant to both drugs were isolated and expanded into stable lines. To confirm the hybrid lines retained genetic material from each parental line, PCR analysis with informative polymorphic microsatellite markers was carried out on genomic DNA from the parental and hybrid lines. The CDX2 gene has
been localized to chromosomal band 13q12.1–13q12.3 (29), and the nearby markers D13S1493 and D13S629 were used to study the parental and hybrid cell lines. All of the DLD-HT and DLD-Wi hybrid cell lines retained alleles from both CDX2 and CDX2– parents at both the D13S1493 and D13S629 loci (Fig. 4A and data not shown), analysis of the D13S629 alleles for DLD-HT hybrids and D13S1493 alleles for DLD-Wi hybrids). Western blot studies were carried out on lysates from parental and hybrid lines to evaluate CDX2 expression. None of the hybrid lines had detectable CDX2 protein expression, and Northern blot analysis demonstrated all hybrid lines had very reduced or absent CDX2 transcripts (Fig. 4B). The absence of strong CDX2 gene and protein expression in the DLD-HT and DLD-Wi hybrid lines indicates extinction of CDX2 expression is a dominant trait, consistent with the notion that a trans-acting repression pathway underlies silencing of CDX2 expression in CDX2– colon cancer cells.

Localization of cis-Acting Elements Regulating CDX2 Transcription—Because our somatic cell hybrid analyses suggested a trans-acting pathway played a primary role in loss of CDX2 transcription in colon cancer cells, we sought to define cis elements at the CDX2 locus that played a role in regulating transcription in CDX2+ versus CDX2– colon cancer cell lines. Based on the data base structure of the CDX2 gene and its presumptive transcription start site (29), we subcloned an ∼9.5-kb XhoI fragment from a CDX2-containing PAC clone into the reporter gene construct pGL3basic, generating the construct pGL3/CDX2P9.5 (Fig. 5A). The 9.5-kb CDX2 genomic DNA fragment contained sequences corresponding to −9207 to +287 with respect to the purported major transcription start site for CDX2. A second reporter gene construct, denoted pGL3/CDX2P0.4, containing only very minimal 5’-flanking sequences, and the transcription start site from the CDX2 gene was generated as a control. The pGL3/CDX2P0.4 construct shows only basal activity in the cell lines when compared with another control vector, pGL3 basic (data not shown). We then compared the transcriptional activity of the two CDX2 reporter constructs in 11 different cell lines. As shown in Fig. 5B, in transient reporter gene assays when normalized with the control pGL3/CDX2P0.4 construct for differences in transfection efficiency among the cell lines, the pGL3/CDX2P9.5 construct showed greater transcriptional activity in CDX2+ colorectal cancer cell lines than in CDX2– colorectal cancer lines or in the various cell lines derived from other non-colonic tissues. These data suggest elements in the 5’-flanking region of the CDX2 gene likely play a primary role in the response to positive (activating) factors regulating CDX2 gene expression in CDX2+ colorectal cancer cells as well as negative (repressive) factors that inhibit CDX2 gene expression in CDX2– colon cancer cells.

Lack of a Role for Phosphatidylinositol 3-Kinase Activity in Regulation of CDX2 Expression—In a recent study, it was shown that overexpression of the lipid phosphatase PTEN led to an increased level of CDX2 protein expression in a colon cancer cell line (30). Based on the known role of PTEN as a critical negative regulator of the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway (31), further studies were performed by the authors to address the role of PTEN and PI3-K in regulation of CDX2 with some evidence suggesting CDX2 transcription might be regulated in certain settings by PI3-K (PI3-K)/Akt signaling pathway (31), further studies were performed by the authors to address the role of PTEN and PI3-K in regulation of CDX2 with some evidence suggesting CDX2 transcription might be regulated in certain settings by PI3-K.
response to insulin treatment by Western blotting with the anti-phospho-Akt (Ser-473) antibody (Fig. 6A). As expected, wortmannin inhibited insulin-induced phosphorylation of Akt with potent inhibition of Akt phosphorylation at 100 nM and essentially complete inhibition at 500 nM. Arguing against a major role for activated PI3-K signaling in the repression of CDX2 expression in CDX2− colon cancer cells, wortmannin treatment failed to increase CDX2 expression in HT-29 cells (Fig. 6B). Thus, while overexpression of PTEN may have some effects on CDX2 expression in selected colon cancer cell lines, there is no definitive evidence that endogenous activation of PI3-K signaling is a general mechanism contributing to repression of CDX2 in colon cancer cells.

**Restoration of CDX2 Inhibits Proliferation and Tumorigenic Growth in Colon Cancer Cells**—To assess the biological consequences of CDX2 inactivation in colon cancer cells, we established a polyclonal HT-29 line with ectopic CDX2 expression (HT-29/PGS-CDX2) and polyclonal drug-resistant control line (HT-29/PGS-neo) (21). The HT-29/PGS-CDX2 cells had a more refractile appearance and a tendency to form multicellular aggregates (Fig. 7A). In addition, the in vitro proliferation of HT-29/PGS-CDX2 cells was reduced compared with the HT-29/PGS-neo cells, although both cell populations reached essentially the same saturation density (Fig. 7B). In contrast to HT-29 parental cells or control HT-29/PGS-neo cells, the HT-29/PGS-CDX2 cells failed to form colonies in soft agar (Fig. 7C). Altogether these results support the notion that CDX2 may be a potent suppressor of tumorigenic growth in some colon cancer cells, consistent with the fact that CDX2 expression may be selectively inactivated in a small but significant fraction of colon cancers.

**DISCUSSION**

In the studies presented here we have sought to address the mechanisms and significance of CDX2 inactivation in colorectal cancer. The intestine-specific homeobox transcription factors CDX1 and CDX2 are known to have critical functions in intestinal development, differentiation, and maintenance of the intestinal phenotype (11). Loss of CDX1 and CDX2 expression has been reported in some primary colorectal cancers and can-
uncovered via studies of Cdx2+/− mice, our studies demonstrated ectopic expression of Cdx2 in the Cdx2−/− HT-29 colon cancer cell line could not cause morphological changes but reduced proliferation in vitro and a failure to grow in an anchorage-independent fashion (i.e. inhibition of colony formation in soft agar). Our findings with HT-29 cells are essentially consistent with those of Mallo et al. (37) who studied the effects of Cdx2−29 or Cdx2 overexpression or tumorigenicity. At this time, the basis for the antiproliferative and tumor-inhibiting effects of Cdx2 in colorectal cancer cells remain unknown, although the effects are presumably mediated via downstream Cdx2-regulated target genes. To date, little is known about the identity of downstream genes regulated by Cdx2, although the LI-cadherin (cadherin 17) gene appears to be a direct target for regulation by Cdx2 (21). Consistent with the notion that Cdx2 has a critical function in regulating LI-cadherin in colon cancer cells, we found LI-cadherin expression was concordantly suppressed along with Cdx2 in the somatic hybrid cell lines studied here (data not show). Further studies to define specific signaling molecules and transcription factors responsible for Cdx2 repression as well as the gene expression changes resulting from loss of Cdx2 function will help to clarify the means by which Cdx2 inactivation contributes to colon cancer pathogenesis. The data should also inform understanding of the functions of Cdx2 in development and intestinal cell fate specification.

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