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An Intestine-Specific Homeobox Gene Regulates Proliferation and Differentiation

EUNRAN SUH AND PETER G. TRABER*

Division of Gastroenterology, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6144

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Precise regulation of cellular proliferation, differentiation, and senescence results in the continuous renewal of the intestinal epithelium with maintenance of a highly ordered tissue architecture. Here we show that an intestine-specific homeobox gene, *Cdx2*, is a transcription factor that regulates both proliferation and differentiation in intestinal epithelial cells. Conditional expression of *Cdx2* in IEC-6 cells, an undifferentiated intestinal cell line, led to arrest of proliferation for several days followed by a period of growth resulting in multicellular structures containing a well-formed columnar layer of cells. The columnar cells had multiple morphological characteristics of intestinal epithelial cells. Enterocyte-like cells were polarized with tight junctions, lateral membrane interdigitations, and well-organized microvilli with associated glycocalyx located at the apical pole. Remarkably, there were also cells with a goblet cell-like ultrastructure, suggesting that two of the four intestinal epithelial cell lineages may arise from IEC-6 cells. Molecular evidence for differentiation was shown by demonstrating that cells expressing high levels of *Cdx2* expressed sucrase-isomaltase, an enterocyte-specific gene which is a well-defined target for the Cdx2 protein. Taken together, our data suggest that *Cdx2* may play a role in directing early processes in intestinal cell morphogenesis and in the maintenance of the differentiated phenotype by supporting transcription of differentiated gene products. We propose that *Cdx2* is part of a regulatory network that orchestrates a developmental program of proliferation, morphogenesis, and gene expression in the intestinal epithelium.

The intestinal epithelium develops from visceral endoderm through a series of complex transitions that begin between embryonic days 14 and 15 in mice and culminate after weaning in postnatal week 4 (reviewed in references 11, 12, 14, and 15). The fully developed intestinal epithelium is continually renewed from stem cells, located in intestinal crypts, that give rise to enterocytes, a variety of types of enteroendocrine cells, mucus-secreting goblet cells, and Paneth cells (5, 11, 12, 15). Spatially defined patterns of proliferation, differentiation and gene expression, cell migration, and senescence are maintained during rapid renewal of the epithelium. Experiments with transgenic mice have provided evidence that there are complex programs for directing the patterns of gene transcription in epithelial cell lineages and along the crypt-villus and craniocaudal axes of the intestinal tract (2, 7, 11, 12, 15, 24, 25). Our current understanding of the regulation of intestinal genes suggests that there are multiple regulatory elements that act in a combinatorial manner to direct developmental patterns of gene transcription. However, the transcription factors that direct these developmental patterns of gene expression and ultimately the differentiation of the intestinal epithelium have not been elucidated.

Several lines of evidence suggest that homeobox genes related to the *Drosophila melanogaster* gene *caudal* (27–29), with the designated abbreviation *Cdx*, may be involved in the regulation of intestinal differentiation. In the developing mouse embryo, *Cdx1* (9, 26) and *Cdx2* (17) genes are expressed at the time when visceral endoderm transforms into a simple columnar epithelium with nascent villi, a critical transition during intestinal development. In the adult mouse, both genes continue to be expressed at high levels in the epithelium of the small intestine and colon with undetectable levels of mRNA in other tissues (18, 37). In addition to the developmental pattern of *Cdx* gene expression in mice, a role in intestinal differentiation is suggested by the fact that intestine-specific genes are targets for the Cdx protein. We recently showed that Cdx2 is important for transcriptional activation of the sucrase-isomaltase (SI) gene promoter, a gene expressed only in small intestinal enterocytes (17). Moreover, Cdx2 binds to promoter elements of other intestine-specific genes and can act on enhancer elements linked to heterologous promoters (21). These findings raise the possibility that the Cdx2 protein regulates transcription of multiple intestinal genes.

From these observations, we hypothesized that *Cdx2* participates in a regulatory network that ultimately determines the intestinal phenotype. To examine this possibility, we investigated the effect of *Cdx2* on IEC-6 cells, an epithelial cell line derived from neonatal rat ileum (33). IEC-6 cells have characteristics of crypt-type intestinal cells but do not exhibit differentiated morphology or gene expression (33). We further show in this study that IEC-6 cells do not express *Cdx2* or *Cdx1* mRNA. When we forced expression of *Cdx2* in stably transfected IEC-6 cells, there were remarkable changes in both proliferation and differentiation. Initially, there was arrest of proliferation for several days followed by a period of growth with establishment of a stable culture of cells at a higher density than that of cells not expressing *Cdx2*. Multicellular structures then developed in the cells expressing high levels of Cdx2 protein, which contained a columnar epithelium with a morphological appearance consistent with intestinal epithelial cells. Moreover, these cultures expressed SI mRNA, a molecular marker of differentiation. These findings suggest that *Cdx2* may play a fundamental role during intestinal development and differentiation.

* Corresponding author. Mailing address: Clinical Research Building, Ste. 600, 415 Curie Blvd., Philadelphia, PA 19104-6144. Phone: (215) 898-0154. Fax: (215) 573-2024. Electronic mail address: traberp@mail.med.upenn.edu.

MATERIALS AND METHODS

Construction of *Cdx2* conditional expression vectors. Two expression vectors, the LacSwitch System (Stratagene, La Jolla, Calif.) and a vector utilizing the sheep metallothionein promoter, were chosen for use in methods for directing the conditional expression of *Cdx2* in stably transfected cell lines. The LacSwitch system requires cotransfection of two plasmids, pOPRSVI, which contains a Rous sarcoma virus long terminal repeat promoter for directing expression of cloned genes and expresses the neomycin resistance gene, and p3′SS, which expresses the *lacI* gene and a hygromycin resistance gene. Expression of the *lacI* gene maintains low-level expression of genes cloned in pOPRSVI because of *lac* repressor-binding sequences located in pOPRSVI. When isopropyl-β-D-thiogalactopyranoside (IPTG) is added to the culture media, repression mediated by *lacI* is removed and the gene of interest is expressed. The complete coding sequence of the mouse *Cdx2* cDNA (37), including a Kozak consensus sequence, start codon, and termination codon, was synthesized by PCR and subcloned into the *Not*I site in pOPRSVI to yield the *Cdx2* expression construct pOPRSVCdx2. The nucleotide sequence of the expression plasmid was confirmed. The other inducible expression vector used was pMT-CB6$^+$, which contains the promoter of the sheep metallothionein I gene (32). This vector was a gift of Frank Rauscher, Philadelphia, Pa., and was constructed by inserting the promoter from pMT010/A$^+$ (6) into pCB6$^+$ (31). The coding sequence of *Cdx2* was amplified with oligonucleotides that incorporate *Hin*dIII restriction sites and was inserted into the *Hin*dIII site of pMT-CB6+ and sequenced, yielding pMT-Cdx2.

Cell culture and establishment of stable cell lines. IEC-6 cells were obtained from the American Type Culture Collection, Rockville, Md., and maintained under an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 0.1 U of insulin per ml. The cells were subcultured every 5 days. IEC-6 cells were transfected with pOPRSVCdx2 and p3′SS or with pMT-Cdx2, by electroporation at 250 V and 960 μF with a gene pulser (Bio-Rad, Hercules, Calif.). Clones resistant to selection medium containing 0.6 mg of G418 per ml and 0.3 mg of hygromycin B per ml for LacSwitch plasmids or 0.6 mg of G418 alone per ml for pMT-Cdx2 were isolated and screened for *Cdx2* expression by Northern (RNA) blot, RNase protection assays, and electrophoretic mobility shift assay (EMSA). For experiments with the LacSwitch cell lines, the cells were plated at a density of 5×10^3 cells per cm^2 and 24 h later the medium was replaced with Dulbecco's modified Eagle's medium containing 5% fetal calf serum with or without 4 mM IPTG. When cell lines transfected with pMT-Cdx2 were used, the cells were cultured in the presence of 50 μM $ZnSO_4$ to induce expression of *Cdx2* (1). The passage number of the cells after isolation of the stable lines was recorded.

Assessment of cellular proliferation and DNA synthesis. To assess the change in cell number after various times of cell culture, cells were plated in 12-well tissue culture plates at 5×10^3 cells per well. At 24 h after plating, medium containing either 4 mM IPTG or 50 μM $ZnSO_4$ was added, and the cells were collected by trypsin treatment at various intervals and counted with a hemacytometer. To assess DNA synthesis, cells were plated at 2×10^3 cells per well in 96-well tissue culture plates. After 24 h, the cells were serum starved for 24 h and then placed in serum-containing medium with or without the inducing agent. At various intervals, 1 μCi of [*methyl*-^{3}H]thymidine (25 Ci/mmol) was added to each well and the mixture was incubated for 1 h at 37°C. The cells were then lifted off the plate with trypsin, and cellular DNA was collected on glass filters (2.4-cm GF/C glass microfilters; Whatman, Maidstone, Kent, England) with a vacuum filtration cell harvester. Incorporation of tritium was measured with a scintillation counter.

Electron microscopy. For electron-microscopic analysis, cells were grown on tissue culture dishes for 17 days postconfluency (27 days), washed with phosphate-buffered saline, fixed at room temperature in 2.5% glutaraldehyde–3.2% paraformaldehyde buffered with 0.1 M sodium cacodylate (pH 7.4), postfixed in 2% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon. Ultrathin sections were examined in an electron microscope.

RNA analysis. RNA was isolated from cell cultures with RNazol B as directed by the manufacturer (Biotech Laboratories, Inc., Houston, Tex.). RNase protection assays were performed with gel-purified probes as previously described (37). The riboprobe for *Cdx2* (220 bases) was prepared from pRc/CMV-Cdx2 linearized with *Sty*I (37). A riboprobe for SI was synthesized from pRSI-1, containing an 827-bp fragment of the partial rat SI cDNA, as previously described (38). The probe for *Cdx1* mRNA was generated from pRc/CMV-Cdx1, which was constructed from pEVRF1Cdx1 (26). The pEVRF1Cdx1 plasmid was digested with *Kpn*I, the ends were blunted, and then the *Cdx1* cDNA was excised with *Xba*I. The cDNA was cloned into the expression plasmid pRc/CMV (Invitrogen) which had been cut with *Hin*dIII, blunted, and then digested with *Xba*I. The resultant plasmid, pRc/CMV-Cdx1, was linearized with *Bgl*II to synthesize a riboprobe for RNase protection analysis.

EMSA. Nuclear proteins were isolated from cell cultures and EMSA was performed exactly as previously described (37).

RESULTS

Conditional expression of *Cdx2* in IEC-6 cell lines. To investigate the potential function of *Cdx2* in intestinal epithelial cells, the IEC-6 cell line was chosen for two important reasons. First, IEC-6 cells have certain characteristics of crypt-type intestinal cells but do not exhibit differentiated morphology or gene expression (33). Second, some investigators have found that these cells develop a few characteristics of differentiated enterocytes under certain culture conditions (3, 20). Thus, we reasoned that a specific molecular stimulus may be able to induce development of a differentiated phenotype in IEC-6 cells. After choosing IEC-6 cells as our model system, we used EMSA and the RNase protection assay to confirm that the line we obtained from the American Type Culture Collection did not express Cdx2 protein or mRNA (data not shown). Because another member of the *caudal*-related family *Cdx1* is also expressed specifically in the intestine, we used RNase protection analysis to show that IEC-6 cells do not express *Cdx1* mRNA (data not shown).

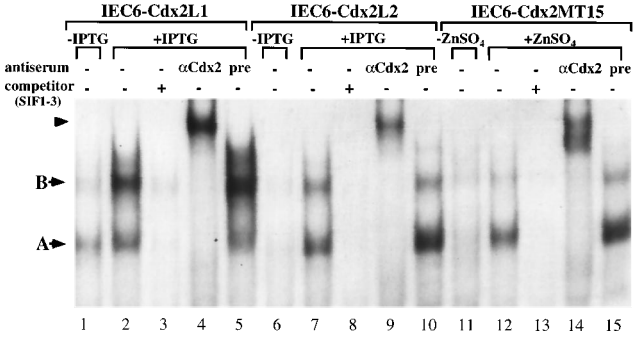

FIG. 1. Expression of Cdx2 protein in stably transfected IEC-6 cells. Nuclear proteins isolated from the indicated cell lines were analyzed by EMSA to assess for nuclear binding protein to the SIF1 DNA element of the SI promoter. The A and B DNA-protein complexes represent the interaction of one and two molecules of Cdx2 on the SIF1 DNA element, respectively. For competition experiments, a 100-fold excess of the same SIF1-3 double-stranded oligonucleotide was used. Anti-Cdx2 antibody (α-Cdx2) was used to perform supershift experiments demonstrating the presence of Cdx2 protein in the DNA-protein complexes. pre, preimmune rabbit serum. The arrowhead marks the position of the complex that supershifted with addition of anti-Cdx2 antibody.

Stable IEC-6 cell lines were isolated with both the LacSwitch System and the metallothionein promoter construct as described in Materials and Methods. Two cell lines expressing *Cdx2* were isolated after transfection with the LacSwitch vectors; we named these cell lines IEC6-Cdx2L1 and IEC6-Cdx2L2. In addition, four separate cell lines that had been transfected with only pOPRSV-Cdx2 and which constitutively expressed *Cdx2* were isolated. As negative controls for the LacSwitch vectors, three cell lines that had been transfected with pOPRSVI containing no insert were isolated. Of 14 cell lines that were isolated after transfection with pMT-Cdx2, 12 expressed Cdx2 protein and 2 did not. Nine negative control cell lines transfected with pMT-CB6$^+$ containing no insert were also isolated and analyzed.

The stable cell lines derived from both inducible expression vectors that were used for detailed analysis expressed low constitutive levels of *Cdx2* mRNA (data not shown) and protein (Fig. 1). When induction medium was added to the cultures, there was a marked induction of *Cdx2* mRNA and protein, which persisted as long as the cells were cultured in induction medium (Fig. 1). High-level *Cdx2* expression was found up to 80 days of culture in the IEC6CdxL1 line, the last time point tested in our analysis (data not shown). Note that the A and B bands on EMSA represent monomer and dimer binding to the SIF1 element, respectively, since this SI promoter element contains two Cdx-binding sites (37). The DNA-protein com-

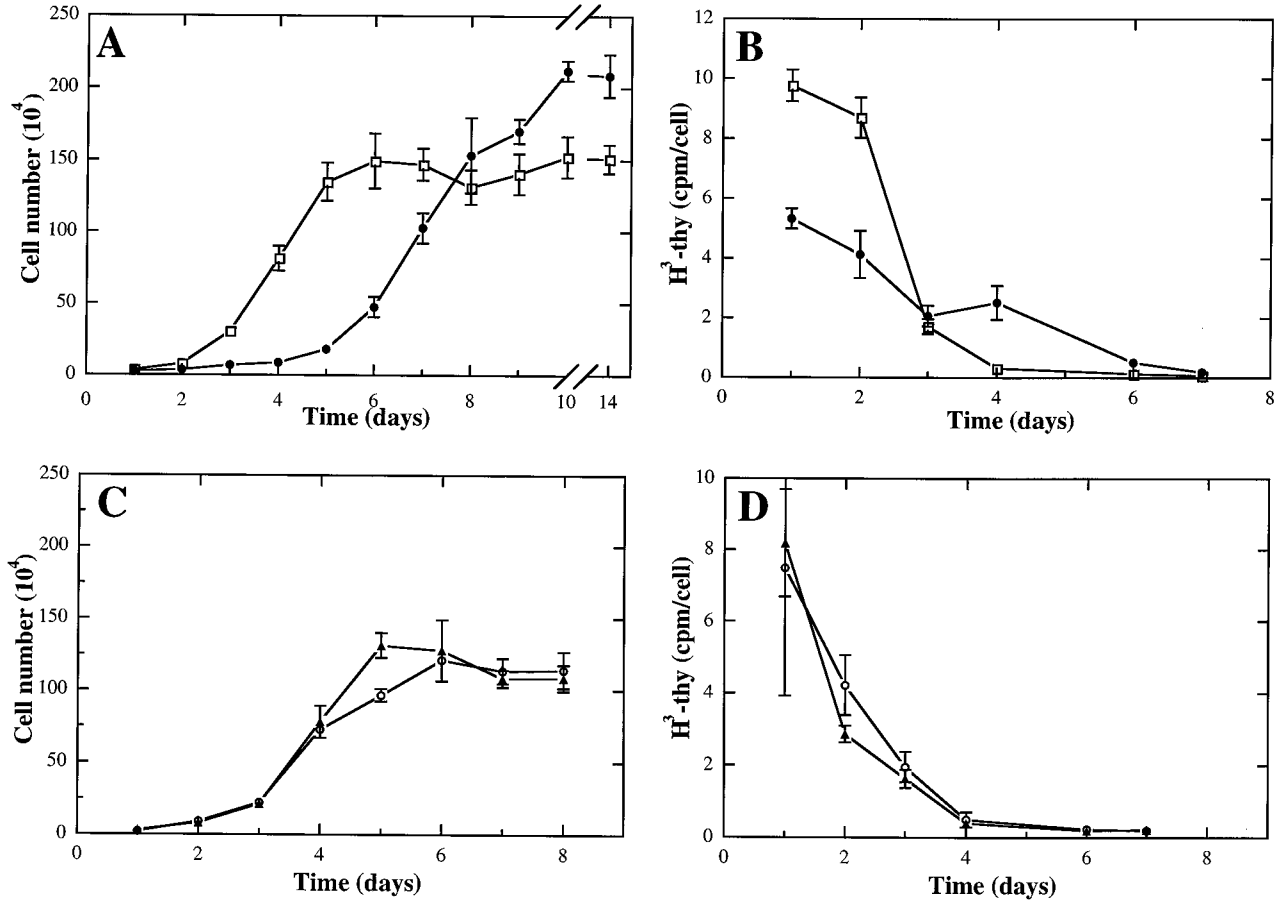

FIG. 2. Effect of Cdx2 expression on proliferation. (A and B) IEC6-Cdx2L1 cells. Symbols: ●, medium with IPTG; □, medium without IPTG. (C and D) IEC-6 cells. Symbols: ▲, medium with IPTG; ○, medium without IPTG. Cell number is expressed as mean ± standard deviation of three independent determinations. Radioactivity was normalized for cell number and expressed as mean ± standard deviation of three independent determinations. H^3-thy, [^{3}H]thymidine.

plexes that formed with nuclear extract from induced cell lines contained Cdx2 protein as shown by supershift analysis with specific anti-Cdx2 antibodies (Fig. 1). Thus, the stable IEC-6 cell lines express low constitutive levels of Cdx2 protein but have a marked increase in expression upon culture in induction medium. The cell lines derived from expression vectors without *Cdx2* cDNA inserts did not express Cdx2 protein (data not shown).

Effects of *Cdx2* expression on proliferation. *Cdx2* expression in the stable cell lines had a complex effect on proliferation. Following incubation of IEC6-Cdx2L1 cells with medium containing IPTG, there was inhibition of cell growth which persisted for nearly 3 days (Fig. 2A). The number of cells at the early time points (i.e., days 0 and 1) was similar for induced and uninduced cultures, indicating that this effect on cell number was not due to early death or loss of cells. Furthermore, measurement of incorporation of [^{3}H]thymidine into DNA indicated that there was also inhibition of DNA synthesis in the cultures induced to express Cdx2 protein (Fig. 2B). Several days after induction of Cdx2, cell growth resumed with the same log phase kinetics as in the uninduced cells, but the cellular density following confluence was greater for cells in which Cdx2 had been induced (Fig. 2A). The IEC6-Cdx2L2 and the IEC6-Cdx2MT15 cell lines showed similar cell growth kinetics, although the effects were less dramatic, with both showing a 50% reduction in cell number after 4 days in culture (data not shown). In contrast to the stable cell lines expressing Cdx2, incubation of the parental IEC-6 cells with medium containing IPTG had no effect on the rate of growth, incorporation of thymidine into DNA, and the final cell density at confluency (Fig. 2C and D).

Culture morphology of IEC-6 cell lines expressing Cdx2. The parental IEC-6 cells remained as a simple monolayer of polygonal cells for as long as 50 days in culture (Fig. 3A and B). In contrast, cell lines expressing Cdx2 developed striking morphological changes characterized by multicellular structures arranged in a lattice-like configuration. IEC6-Cdx2L1, IEC6-Cdx2L2, and IEC6-Cdx2MT15 cells that were induced to express high levels of Cdx2 developed a very dense network of multicellular structures (Fig. 3D, F, and H). In the IEC6-Cdx2L1 line, which expressed low constitutive levels of Cdx2, similar structures developed in the absence of induction media but their appearance was delayed and the density and complexity were lower (Fig. 3C). However, the uninduced IEC6-Cdx2MT15 cells did not develop any of the changes seen in the induced cultures (Fig. 3G). The cell culture morphology of the lines expressing Cdx2 was remarkably stable, with the same structure maintained for as long as 150 days in culture (IEC6-Cdx2L1 line; data not shown). Each of the four independent cell lines that constitutively expressed Cdx2 and each of the other 11 IEC6-Cdx2MT cell lines that expressed Cdx2 developed the same multicellular structures (data not shown). The control cell lines that were stably transfected with empty vector had the same morphology as did the parental IEC-6 cell line

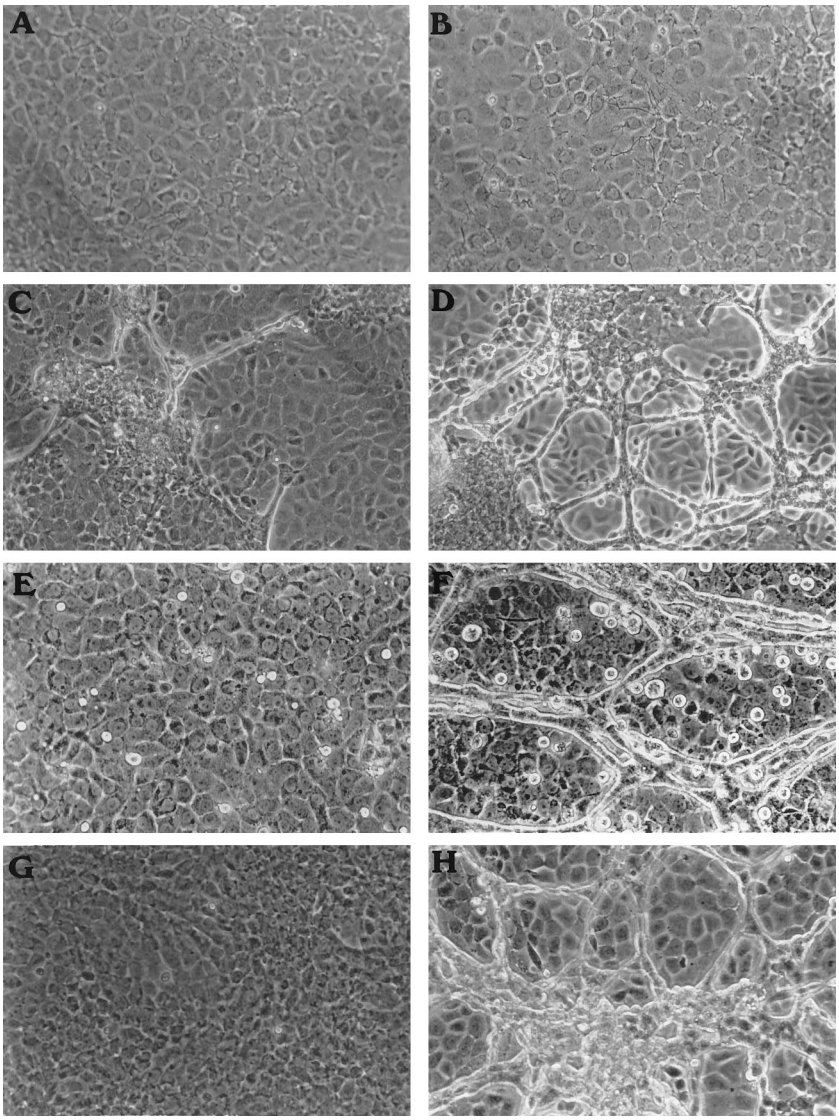

FIG. 3. Light morphology of cells grown in the presence or absence of IPTG for 27 days. (A) IEC-6 cells; (B) IEC-6 cultured in medium with IPTG; (C) IEC6-Cdx2L1; (D) IEC6-Cdx2L1 cultured in medium with IPTG; (E) IEC6-Cdx2L2; (F) IEC6-Cdx2L2 cultured in medium with IPTG; (G) IEC6-Cdx2MT15; (H) IEC6-Cdx2MT15 cultured in medium with 50 μM $ZnSO_4$. Magnification, ×200.

(data not shown). Thus, changes in culture morphology were consistently associated with Cdx2 expression.

Ultrastructural differentiation of IEC-6 cells expressing Cdx2. Electron-microscopic analysis of cell cultures revealed a marked degree of cellular differentiation in cells expressing high levels of Cdx2 protein. Parental IEC-6 cells showed a simple monolayer of flat epithelial cells with sparse microvilli and no evidence of cellular differentiation (Fig. 4A). Stable cell lines cultured in medium without IPTG, which expressed low levels of Cdx2, developed multicellular structures composed of layers of undifferentiated cells (Fig. 4B). In cultures induced to express high levels of Cdx2, the surface of the multicellular structures was covered with a columnar epithelium on a well-demarcated basal lamina that contained cells with characteristics of differentiated intestine (Fig. 4C to F). The predominant cell type in the columnar epithelium had a number of characteristics of intestinal enterocytes, including basally placed nuclei, tight junctions and desmosomes (Fig. 4D), well-developed microvilli with glycocalyx (Fig. 4D and F), and typical interdigitations of the lateral membranes (Fig. 4E). Remarkably, there were a few immature goblet-like cells with characteristic mucous granules (Fig. 4D), suggesting that at least two intestinal epithelial phenotypes may arise from the stable lines. Also, scattered through the epithelium were cells that appeared to be in different stages of degeneration.

An interesting characteristic of the undifferentiated cells located below the columnar epithelium was the production of exuberant extracellular matrix (Fig. 4G). In contrast, the parental IEC-6 cells produced only scant extracellular material (Fig. 4A). After culture for 150 days, electron microscopy revealed that the cells remained viable and the extracellular material had accumulated to even greater amounts (data not shown).

SI expression in IEC-6 cell lines. Cell cultures were examined for the expression of SI, a gene which is specific to the enterocyte lineage and is expressed only in the differentiated compartment of the intestinal crypt-villus axis (25, 38, 39). In addition to being a well-accepted marker for intestinal differ-

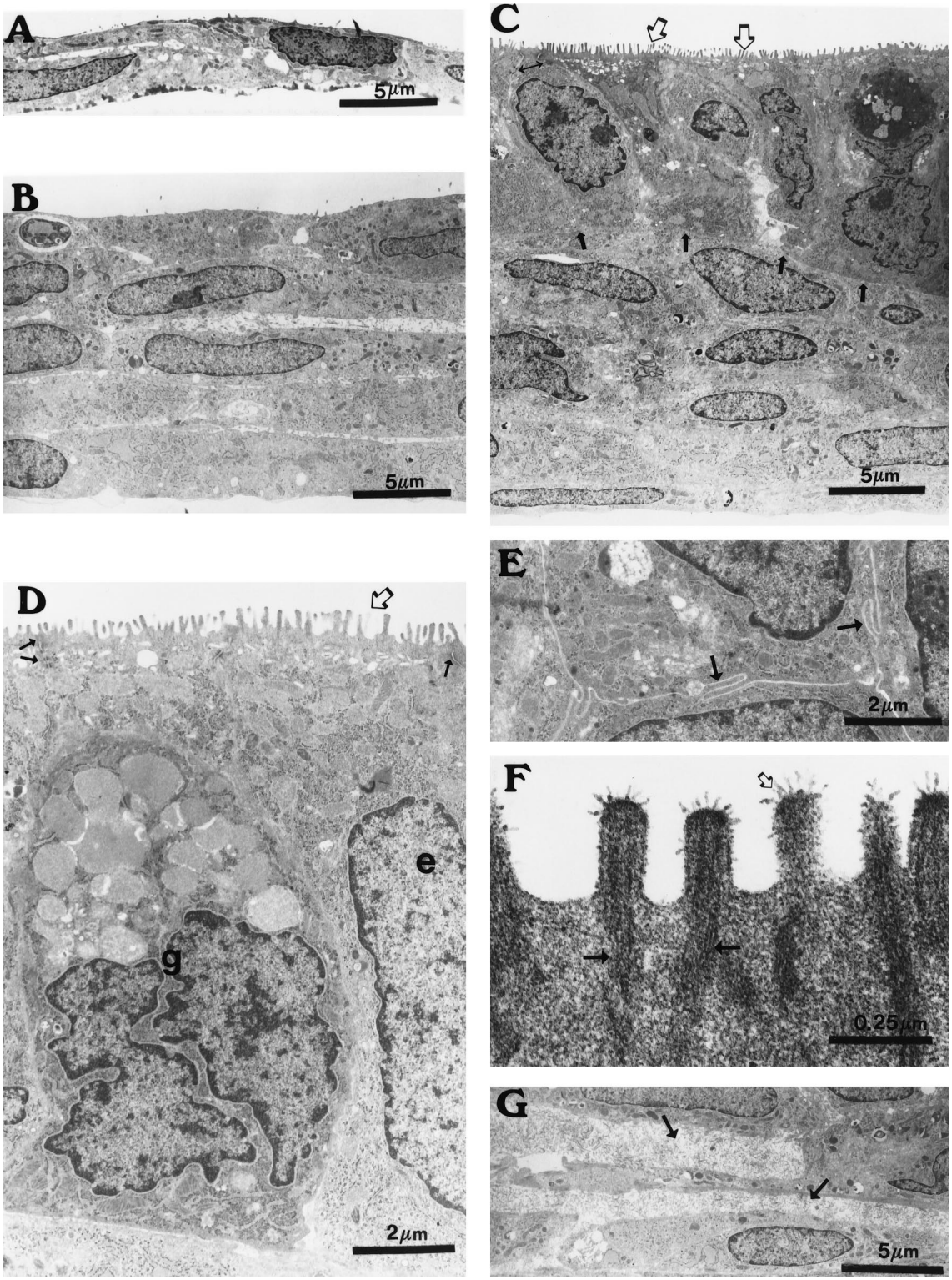

FIG. 4. Cdx2 induces ultrastructural differentiation in IEC-6 cells. Electron-microscopic analysis of cells harvested at a time point comparable to that shown in Fig. 3. (A) IEC-6 cultured in medium with IPTG; (B) IEC6-Cdx2L1 cells cultured in medium without IPTG; (C) IEC6-Cdx2L1 cells cultured in medium with IPTG (open arrows, microvilli; double-headed arrow, junctional complexes; solid arrows, basal lamina of columnar epithelium); (D) IEC6-Cdx2L1 cells cultured in medium with IPTG (g, goblet-like cell; e, enterocyte-like cell; open arrow, microvilli; small arrows, junctional complexes); (E) IEC6-Cdx2L1 cells cultured in medium with IPTG (arrows, interdigitations of lateral membranes of adjacent cells); (F) IEC6-Cdx2L1 cells cultured in medium with IPTG (open arrow, microvilli with glycocalyx; solid arrows, basal ends of the actin-containing microfilaments); (G) IEC6-Cdx2L1 cells cultured in medium with IPTG (arrows, extracellular matrix, including collagen fibrils).

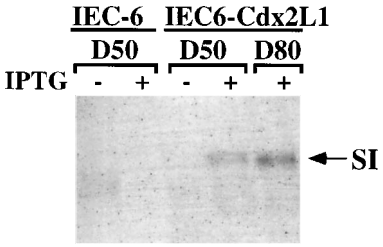

FIG. 5. Expression of tissue-specific genes. Total RNA from IEC6-Cdx2L1 was isolated at the indicated times. RNase protection assays for SI mRNA were performed as described in Materials and Methods.

entiation, the SI gene promoter contains a DNA regulatory element that specifically binds Cdx2 protein, which in turn activates transcription of the promoter (37). The parental IEC-6 cells did not express SI mRNA when cultured in the presence or absence of IPTG, even when the cells were maintained in culture for as long as 50 days (Fig. 5). IEC6-Cdx2L1 cells cultured in induction medium did not express SI mRNA out to 40 days in culture. However, when these cells were maintained in induction medium for 50 or 80 days, SI mRNA was expressed (Fig. 5). In contrast, the same cells cultured for 50 days in the absence of IPTG lacked SI mRNA (Fig. 5). Therefore, the expression of SI was a late event in the cell lines, occurring after evidence of morphological differentiation.

Since it was possible that expression of the *Cdx2* gene or induction of morphological differentiation may have an effect on the expression of other members of the *caudal*-related gene family, we examined the expression of *Cdx1* mRNA by an RNase protection assay. *Cdx1* mRNA was not expressed in IEC6-Cdx2L1 cells after 6 and 40 days of culture in induction medium (data not shown).

DISCUSSION

The molecular mechanisms that orchestrate cellular transitions and changes in gene expression during intestinal epithelial differentiation and development are largely unknown. We found that expression of the *Cdx2* gene in undifferentiated intestinal cells resulted in pleomorphic effects on proliferation, morphogenesis, and gene expression. From these data, we propose that *Cdx2* is the first identified component of a regulatory network that coordinates the differentiation process in the intestine.

The arrest of proliferation of cells expressing Cdx2 protein is reminiscent of the effect of other transcription factors that induce differentiation, including myoD (13, 36), C/EBP (40), and Pit1/GHF1 (4). Interestingly, the proliferation arrest caused by induction of Cdx2 is released after several days, despite the continued expression of Cdx2, with resultant brisk growth to a high cell density. This finding suggests that the effect of Cdx2 expression in IEC-6 cells on proliferation is dependent on additional regulatory factors. Immediately after induction of Cdx2, there may be a direct or indirect effect on the cell cycle regulatory circuit. However, changes induced in the cells by a cascade of events initiated by Cdx2 expression may later block or modify the inhibitory effect of Cdx2 on proliferation. It is possible that modulation of the state of differentiation changes the cellular context such that the function of Cdx2 on proliferation is altered. This concept may be important for the in vivo function of the Cdx2 protein, since it has been shown that Cdx2 is expressed in both the proliferative and differentiated compartments of the mouse small intestinal epithelium (17). Additional investigation of this intriguing possibility will require probing the cell cycle with systems other than the conditional cell lines used in this study.

Following the growth phase and subsequent formation of a confluent layer of cells, the cell lines expressing Cdx2 developed multicellular structures containing differentiated epithelial cells. The differentiated cells appeared in the context of a complex culture architecture formed by the integration of undifferentiated cells underlying a differentiated columnar epithelium. In addition, a large amount of extracellular matrix was deposited between the undifferentiated cells and under the columnar epithelium. In this regard, it is interesting that culture of IEC-6 cells on a complex extracellular matrix (Matrigel) leads to the association of cells into multicellular bundles with some evidence of differentiation (3). The structural findings in the cell cultures suggest that there may be important cell-cell and cell-matrix interactions that are involved in the morphogenesis of IEC-6 cells expressing Cdx2. We speculate that proteins that are involved in these interactions may be regulatory targets for Cdx2. This would be consistent with evidence that homeobox genes modulate the transcription of morphoregulatory proteins such as cell adhesion molecules and growth factors (10). Moreover, extracellular matrix and mesenchymal cells are known to play an important interactive role in the differentiation of visceral endoderm (8, 23, 35).

The finding of SI mRNA in cell lines expressing high levels of Cdx2 provides molecular evidence that the morphologically differentiated cells express genes specific for the enterocytic phenotype. There may be a combination of mechanisms that lead to the expression of SI. The emergence of columnar enterocytes with microvilli suggests that there have been multiple changes in gene expression resulting in an altered molecular milieu within the cells. As a part of this differentiation process, multiple intestinal genes might be expected to be activated, including SI. On the other hand, the demonstrated function of Cdx2 on the SI promoter (37) suggests that Cdx2 plays a direct role in transcriptional regulation of the SI gene in differentiated cells. The late expression of SI mRNA in the cell cultures suggests that a later event is involved in facilitating the expression of the gene.

The complex effects of *Cdx2* gene expression on undifferentiated intestinal epithelial cells suggests that Cdx2 regulates the expression of genes early in the differentiation process as well as genes that are required for maintenance of the terminal differentiated state, such as SI. This combination of functions for homeobox genes is supported by evidence that *labial* directs cell specification of larval midgut cells in *D. melanogaster* (16) and that Pit1/GHF1 regulates development and cell-specific transcription in pituitary cells (41). In fact, Pit1/GHF1 may be the best example in mammals, since it has been shown to have effects on cellular proliferation (4), is responsible for directing developmental programs leading to the anterior pituitary cell phenotype (22, 34, 41), and directly activates transcription of cell-specific genes such as growth hormone and prolactin (30). As more examples of cell-specific transcription factors directing developmental processes are described (19), it may be that similar pleomorphic and developmental stage-specific functions will be a common feature. Taken together, our findings suggest that Cdx2 may be such a transcription factor for the intestine which plays a role in regulating the exquisite balance between proliferation and differentiation in the intestinal epithelium during development and morphogenesis.

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