Key Role of the *Cdx2* Homeobox Gene in Extracellular Matrix–mediated Intestinal Cell Differentiation

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Abstract. To explore the role of homeobox genes in the intestine, the human colon adenocarcinoma cell line Caco2-TC7 has been stably transfected with plasmids synthesizing *Cdx1* and *Cdx2* sense and antisense RNAs. *Cdx1* overexpression or inhibition by antisense RNA does not markedly modify the cell differentiation markers analyzed in this study. In contrast, *Cdx2* overexpression stimulates two typical markers of enterocytic differentiation: sucrase-isomaltase and lactase. Cells in which the endogenous expression of *Cdx2* is reduced by antisense RNA attach poorly to the substratum. Conversely, *Cdx2* overexpression modifies the expression of molecules involved in cell–cell and cell–substratum interactions and in transduction process: indeed, E-cadherin, integrin-β4 subunit, laminin-γ2 chain, hemidesmosomal protein, APC, and α-actinin are upregulated. Interestingly, most of these molecules are preferentially expressed in vivo in the differentiated villi enterocytes rather than in crypt cells. *Cdx2* overexpression also results in the stimulation of *HoxA-9* mRNA expression, an homeobox gene selectively expressed in the colon. In contrast, Cdx2-overexpressing cells display a decline of *Cdx1* mRNA, which is mostly found in vivo in crypt cells. When implanted in *nude* mice, *Cdx2*-overexpressing cells produce larger tumors than control cells, and form glandular and villus-like structures.

Laminin-1 is known to stimulate intestinal cell differentiation in vitro. In the present study, we demonstrate that the differentiating effect of laminin-1 coatings on Caco2-TC7 cells is accompanied by an upregulation of *Cdx2.* To further document this observation, we analyzed a series of Caco2 clones in which the production of laminin-α1 chain is differentially inhibited by antisense RNA. We found a positive correlation between the level of *Cdx2* expression, that of endogenous laminin-α1 chain mRNA and that of sucrase-isomaltase expression in these cell lines.

Taken together, these results suggest (*a*) that *Cdx1* and *Cdx2* homeobox genes play distinct roles in the intestinal epithelium, (*b*) that *Cdx2* provokes pleiotropic effects triggering cells towards the phenotype of differentiated villus enterocytes, and (*c*) that *Cdx2* expression is modulated by basement membrane components. Hence, we conclude that *Cdx2* plays a key role in the extracellular matrix–mediated intestinal cell differentiation.

Homeobox genes encode nuclear transcription factors involved in patterning and cell differentiation during development of metazoans (McGinnis and Krumlauf, 1992). They have also been identified as a new class of protooncogenes (Maulbecker and Gruss, 1993), and substantial evidences indicate that homeobox gene alterations participate in tumor genesis (see Cillo, 1994). Considerable progress in understanding the function of homeobox genes arose from the finding that they regulate molecules involved in cellular interactions such as cell adhesion molecules and extracellular matrix components (Edelman and Jones, 1993). Homeobox genes themselves are regulated by other homeobox genes (Hayashi and Scott, 1990), retinoids (Mavilio, 1993; Gudas, 1994), and/or growth factors (Ruiz i Altaba and Melton, 1989; Pavlova et al., 1994).

Homeobox genes of the *caudal* family, *Cdx1* and *Cdx2*, are expressed in the intestinal epithelium (James and Kazenwadel, 1991). In *Drosophila*, *caudal* is involved in anterio-posterior patterning (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). In mammals, *Cdx1* was the first homeobox gene discovered in endodermal tissues (Duprey et al., 1988; Hu et al., 1993); it exhibits an increasing gradient expression along the longitudinal axis of gut (James and Kazenwadel, 1991; Freund et al., 1992), and the

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protein is predominantly expressed in undifferentiated crypt cells (Silberg et al., 1997). *Cdx2*, another *caudal*-related gene present in the intestine, encodes a nuclear transcriptional factor that is expressed in the differentiated enterocytes (James et al., 1994) and binds cis-elements present in the gene promoters of enterocytic markers such as sucrase-isomaltase (SI)[1], lactase-phlorizin hydrolase (LPH), apolipoprotein B, carbonic anhydrase 1, and calbindin D9K (Suh et al., 1994; Drummond et al., 1996; Lambert et al., 1996; Lee et al., 1996; Troelsen et al., 1997). An important role has been attributed to *Cdx2* in intestinal cell differentiation because this homeobox gene triggers SI expression and cell polarization in undifferentiated intestinal IEC cells in vitro (Suh et al., 1994; Suh and Traber, 1996). Furthermore, using xenograft models in which the cell fate of the fetal intestinal endoderm can be modulated by its association with mesenchymal cells of different origins (Duluc et al., 1994; Fritsch et al., 1997), we have observed a correlation between the level of *Cdx2* (and *Cdx1*) expression, and the small-intestinal–like versus colonic-like morphogenesis and differentiation of the grafted epithelial cells (Duluc et al., 1997). These observations suggest that homeobox genes of the *caudal* family participate in intestinal differentiation and that their expression is dependent on epithelial/connective tissue interactions.

The involvement of epithelial–mesenchymal cell interactions in the control of cell differentiation during intestinal ontogeny and during the continuous cell renewal in the mature organ has been demonstrated (Simon-Assmann and Kedinger, 1993; Simon-Assmann et al., 1995). Evidence of the functional role played by reciprocal cellular interactions was provided by grafting experiments of various tissue recombinants (Haffen et al., 1989; Kedinger et al., 1990; Duluc et al., 1994). The basement membrane at the interface between epithelial and mesenchymal cells participates in these interactions (Simon-Assmann et al., 1995). Of particular interest is the finding that laminin-1, consisting of α1/β1/γ1 chains and synthesized by both epithelial and mesenchymal cells (Simo et al., 1991, 1992*a*), promotes intestinal cell differentiation in vitro (Hahn et al., 1990; Vachon and Beaulieu, 1995; Basson et al., 1996). In addition, cells grown in the presence of anti–laminin-1 antibodies or cells in which the endogenous production of laminin-1 is inhibited by antisense RNA, fail to complete their differentiation, as assessed by the absence of enterocytic markers such as LPH and SI (Simo et al., 1992*b*; De Arcangelis et al., 1996).

In an attempt to approach the role of *caudal*-type homeobox genes in the intestinal epithelial cell behavior and in the response to the basement membrane, we have analyzed (*a*) the phenotypic changes resulting from the modification in the level of *Cdx1* or *Cdx2* expression in a human colonic adenocarcinoma cell line (Caco2-TC7; Chantret et al., 1994), and (*b*) the level of *Cdx2* expression in Caco2 cells cultured on exogenous laminin substratum or producing laminin-α1 chain antisense RNA.

1. *Abbreviations used in this paper*: EHS, Engelbreth-Holms-Swarm; FAK, focal adhesion kinase; HD1, component of hemidesmosome; LPH, lactase-phlorizin hydrolase; RT-PCR, reverse transcriptase-polymerase chain reaction; SI, sucrase-isomaltase.

Materials and Methods

Cells and Injections in Nude Mice

The Caco2-derived cell clone TC7 (Chantret et al., 1994) was grown under 5% CO_2, 95% air atmosphere in DME (GIBCO BRL, Courbevoie, France) containing 20% fetal calf serum, 1% nonessential amino acids, 1% penicillin, 1% streptomycin, and 0.1% gentamicin. Cells seeded at low density (1.2×10^4 cells/cm^2) were passaged every 7 d. For some experiments, they were cultured on coatings of laminin-1 (10 μg/cm^2) extracted from Engelbreth-Holms-Swarm (EHS) tumors, as described by Simon-Assmann et al. (1994*a*). Caco2 clones transfected with the plasmid pCB6/AS-LN producing laminin-α1 chain antisense RNA (De Arcangelis et al., 1996) were grown in the presence of 1.2 or 0.6 μg/ml G418 (geneticin; GIBCO BRL). RNA extraction, protein preparation, and immunocytochemical experiments were performed on cells grown for 5 d.

For grafting experiments, 10^7 cells were injected subcutaneously to *nude* mice and the tumors were recovered 7 or 12 wk after injection.

Adhesion and Aggregation Tests

Adhesion and aggregation tests were performed with transfected TC7 cell clones. Adhesion tests were performed as described previously (Simon-Assmann et al., 1994*a*) on microtiter wells coated or not with 1 μg/well EHS-extracted laminin-1 followed by an incubation with 1% bovine serum albumine (Vth fraction; Sigma, St. Quentin Fallavier, France). In inhibition studies with antiintegrin antibodies (Table I), the cell suspension was mixed with antiintegrin-β1 (1/8,100 final dilution), antiintegrin-β4 (1/25 final dilution) or antiintegrin-α6 (1/8,100 final dilution) antibodies before plating on the coated wells (Orian-Rousseau, V., personal communication). Changes in cellularity after 1 and 2 h were measured by the binding of crystal violet dye at pH 5.5 to fixed cultures at an optical density of 595 nm.

Aggregation tests were carried out according to the procedure described by Berndorff et al. (1994). Briefly, after dissociation, 3×10^6 cells were placed into 5 ml DME medium and agitated at 25°C; the number of single cells and small aggregates up to four cells were counted every 10 min. Aggregation was scored according to the decrease in the percent of isolated cells compared to those present in the suspension at the beginning of the experiment.

RNA Extraction, Northern Blots

RNA was extracted from cultured cells and tumors using Trizol Reagent (GIBCO BRL) according to the recommendations of the supplier. After cell homogenization, the mixture was centrifuged for 5 min at 12,000 *g* to discard genomic DNA contained in the pellet. RNA was treated at 37°C for 30 min with 0.25 U RNase-free DNase/μg RNA (GenHunter, Nashville, TN). RNA concentration was determined by optical densitometry at 260 nm.

Northern blot analysis was performed under standard condition, as previously described (Duluc et al., 1994), using ^{32}P-labeled probes (RediPrime kit; Amersham, Les Ulis, France).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Oligonucleotides were from Eurogentec (Seraing, Belgium). They derived from published genomic or cDNA sequences deposited in the GenBank/EMBL/DDBJ databank. Table II lists the oligonucleotides and the corresponding accession numbers in the databank.

Single-stranded cDNA was synthesized for one h at 42°C in 50 μl containing 6 μg RNA, 100 pmoles oligo-dT, 15 U AMV reverse transcriptase (Promega Corp., Madison, WI), 0.4 mM each dNTP, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 4 mM sodium pyrophosphate. To specifically amplify endogenous *Cdx1* mRNA in cells producing *Cdx1* antisense RNA, cDNA synthesis was primed with 100 pmoles of the Cdx1b primer. PCR reactions (100 μl) contained 2 μl of cDNA solution, 50 pmoles primers, 0.5 U Goldstar DNA polymerase (Eurogentec), 75 mM Tris-HCl, pH 9, 20 mM ammonium sulfate, 0.01% Tween 20, and 1.25 mM $MgCl_2$. Dynawax (Eurogentec) was used to separate the template cDNA and primers from the DNA polymerase during pipetting and before the first step of denaturation. PCR used a Thermojet apparatus (Eurogentec). Conditions were: 94°C, 30 s; 50°C, 45 s; 72°C, 45 s. For each pair of primers, reaction was performed for 16 to 38 cycles to overlap the range of cycles in which the amount of PCR product increased

Table I. Primary Antibodies

Antibodies	Specificity	Origin	Use
Mouse mAb N353	α-Actinin	Amersham (Amersham, England)	WB, IC
Mouse mAb 1	APC	Calbiochem (Meudon, France)	WB, IC
Rat mAb DECAM-1	E-Cadherin	Sigma-Aldrich Chimie	WB, IC
Mouse mAb 14	β-Catenin	Transduction Laboratories (Lexington, KY)	WB
Rabbit antiserum	CDX2	Dr. M. German (UCSF, San Francisco, CA)	WB
Mouse mAb 77	FAK	Transduction Laboratories	WB, IC
Mouse mAb HD1-121	HD1	Hieda et al., 1992	IC
Rat mAb GoH3	Integrin-α6	Immunotech (Marseilles, France)	IC, AT
Mouse mAb 4E9G8	Integrin-α6	Immunotech	WB
Mouse mAb K20	Integrin-β1	Immunotech	IC
Rabbit antiserum AB1937	Integrin-β1	Chemicon International	WB
Mouse mAb P4C10	Integrin-β1	GIBCO BRL	AT
Mouse mAb 3E1	Integrin-β4	GIBCO BRL	IC, AT
Rabbit antiserum 1024	Integrin-β4	INSERM U.381 (unpublished)	WB
Mouse mAb	Sucrase-isomaltase	Hauri et al., 1985	IC

Primary antibodies used for Western blots (*WB*), immunocytochemistry (*IC*), and adhesion tests (*AT*).

exponentially. As internal standard, RT-PCR was carried out with primers hybridizing to the mRNA encoding the 36B4 ribosomal protein. Control PCR was performed directly on RNA without the step of cDNA synthesis; no amplified DNA fragment was detected in this case. In addition, primers used to visualize *Cdx1*, *Cdx2*, *HoxC-8*, and LPH transcripts were chosen in different exons; no PCR product corresponding to the genomic fragments was obtained, demonstrating the absence of contaminating genomic DNA in the RNA samples.

PCR fragments were loaded on 3% agarose gels and analyzed using an Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). RT-PCR fragments were inserted in the pGEM-T plasmid (Promega Corp.) and sequenced using the T7 DNA polymerase sequencing kit (Pharmacia Diagnostics AB, Uppsala, Sweden) to confirm their identity.

Construction of Recombinant Expression Plasmids

General procedures to construct recombinant plasmids were as described by Sambrook et al. (1989). *Cdx1* and *Cdx2* cDNA fragments were inserted in the pCB6 expression vector (Brewer, 1994) that contains the cytomegalovirus promoter and the 3′-untranslated region and polyadenylation signal of the human growth hormone (a generous gift of Dr. G. Perrozi, INN, Rome, Italy).

The complete open reading frame of the murine *Cdx1* gene was reconstituted using pBH8 that contains a nearly full-length cDNA (Duprey et al., 1988), and pCDX1-34 that contains the 4-kb SmaI restriction fragment overlapping the 5′ region of the murine *Cdx1* gene (kindly provided by Dr. P. Duprey, Université Paris VII, Paris, France). The 1.1-kb EcoRI-BamHI fragment of pCDX1-34, comprising the *Cdx1* transcription start site and 0.3 kb of the transcription unit, was inserted in pUC19 (Pharmacia Diagnostics AB), and the resulting plasmid was cut with SphI to delete the upstream untranscribed segment. The NaeI-KpnI restriction fragment was then inserted between the corresponding sites of pBH8 to reconstitute the complete open reading frame of *Cdx1*, flanked at both extremities by HindIII sites. The resulting 1.6-kb HindIII fragment was inserted in both orientations in pCB6 to produce, respectively, the plasmids pCdx1-S and pCdx1-AS.

The murine *Cdx2* open reading frame was obtained by RT-PCR using two pairs of primers (Table II). The 5′ region was generated using primers Cdx2a and Cdx2c, and the 3′ region using Cdx2b and Cdx2d. RT-PCR conditions were as described above except that $MgCl_2$ concentration was 1.75 mM with primers Cdx2a/c, and 0.75 mM with Cdx2b/d. Both PCR fragments contained a unique AvrII restriction site corresponding to the single site included in the *Cdx2* open reading frame. This site was used to reconstitute an SphI-AvrII-SphI fragment overlapping the complete *Cdx2* open reading frame, and this fragment was inserted in the SphI site of pGEM (Promega Corp.). The 1-kb HindIII-BamHI fragment of the resulting plasmid was inserted between the HindIII-BamHI sites of pCB6, to give pCdx2-S in which the *Cdx2* cDNA was in sense orientation. To construct pCdx2-AS encoding *Cdx2* antisense RNA, the 0.52-kb AvrII-XbaI fragment of the 3′ cDNA region was inserted in the XbaI site of pCB6.

Cell Transfections

TC7 cells were transfected with the plasmids pCdx1-S, pCdx1-AS, pCdx2-S, and pCdx2-AS, or with the control pCB6 vector using DOTAP-Reagent (Boehringer Mannheim, Mannheim, Germany). Briefly, cells were seeded at 50% confluency in 10-cm^2 culture dishes. The next day, standard culture medium was replaced by 3-ml medium containing 3.5 μg of plasmid previously cut with the ScaI restriction enzyme, dissolved in 25 μl containing 20 mM Hepes, pH 7.3, and 150 mM NaCl, and incubated 15 min at room temperature with 15 μg DOTAP-Reagent. After 18 h at 37°C, selection was started by adding 1.1 mg/ml G418 to the culture medium. 20 d later, several clones were picked out by capillary duct aspiration and propagated individually. The remaining G418-resistant cell populations from each culture dish were pooled.

Immunocytochemistry and Histology

Immunocytochemistry was performed on cells grown on coverslips for 5 d, or on 5 μm tumor cryosections. Cells were washed with PBS, fixed with 1% paraformaldehyde for 10 min, and permeabilized with 1% Triton X-100 for 10 min. To detect a component of hemidesmosome (HD1) protein, cells were fixed and permeabilized with methanol at −20°C for 5 min. Tumors were embedded in Tissue-Tek (Labonord, Villeneuve d'Asq, France) and frozen with isopentane cooled in liquid nitrogen. Cells and cryosections were incubated for 1 h with the primary antibodies (Table I) in PBS, and subsequently incubated with appropriate secondary antibodies conjugated with fluorescein isothiocyanate or Texas red (Pasteur Diagnostics, Paris, France; Interchim, Asnières, France; Amersham). Primary antibodies were omitted in controls. Cells or slides were mounted in glycerol/PBS/phenylenediamine, and observed with an Axiophot microscope (Zeiss, Oberkochen, Germany).

Morphological analysis was performed on tissues fixed in Bouin's solution, embedded in paraffin, and stained with hematoxylin-eosin or periodic acid-Schiff.

Protein Preparations, Western Blots

Cell membrane proteins were prepared as previously described (Simon-Assmann et al., 1994*a*) to analyze the membrane distribution of integrin-α6 and -β4 subunits, and according to Fabre and de Herreros (1993) to analyze E-cadherin. CDX2 protein was detected in nuclear extracts prepared as described by Bertrand et al. (1995) with slight modifications. Cells were harvested, resuspended in 10 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride, and homogenized with a tight-fitting Dounce homogenizer. Lysates were maintained 10 min on ice and centrifuged at 2,000 *g* for 10 min. The supernatant containing cytoplasmic and membrane proteins was immediately stored frozen at −70°C and further used to analyze the cellular level of APC, focal adhesion kinase (FAK), integrin-α6 subunit, integrin-β4 subunit, and α-actinin. The nuclear pellet was washed in 10 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.2 mM phenylmethylsul-

Table II. Synthetic Oligonucleotides

Gene	Name	Sequence	PCR product size	Accession number
			bp	
Cdx1	Cdx1a	gcgccaaggcggacgccctacgaat	252	M80463
	Cdx1b	tgtttactttgcgctccttggcccg		
	Cdx10	tcaccatgtacgtgggctatgtgct		
Cdx2	Cdx2a	ccaccatgtacgtgagctaccttct	557	U00454
	Cdx2c	tatttgtcttttgtcctggttttca		
	Cdx2b	cccagcggccagcggcgaaacctgt	549	
	Cdx2d	ttctcgcagcgtccatactcctcat		
HoxA-4	HoxA-4a	atgaagtggaagaaagaccacaaa	171	M74297
	HoxA-4b	tctagaagattatatggaggaggg		
HoxA-9	HoxA-9a	gacaagccccccatcgatcccaataa	237	X13536
	HoxA-9b	gttgattttcttcagtttcag		
HoxC-8	HoxC-8a	atgtttccatggatgagacccca	214	X07646
	HoxC-8b	ttgttctccttttttccacttcat		
HNF-1α	Hnf1αa	acagcgtcatcgagaccttcatctc	494	M57732
	Hnf1αb	atccagggcctctcagagagctcagca		
Laminin-α1	Ln3	aggactcggtcccaggacag	235	Haaparanta et al., 1991
	Ln4	gttgggctgaaagtccacaca		
Laminin-α3	Laminin α3a	tgtggggtgtctgaggaactt	646	X84013
	Laminin α3b	cttgcctgggtagagttagtg		
Laminin-β1	Laminin β1a	tgaagtggaacagctctccaagatg	412	M15525
	Laminin β1b	cttcttcagagcttcctttaccat		
Laminin-β3	Laminin β3a	gtgaagacagaggcagaggag	488	L25541
	Laminin β3b	actaaggcgggggatactgc		
Laminin-γ2	Laminin γ2a	acctccatccatccttccaac	435	S75987
	Laminin γ2b	gaagcaaggcagcaccatagt		
Integrin-α6	Integrin α6a	gactactgaatctgctaccaa	629	X53586
	Integrin α6b	ccacctcccaacacccttttt		
Integrin-β1	Integrin β1a	gtgaatgggaacaacgaggtc	756	X07979
	Integrin β1b	caggcaaatagcaaaataaga		
Integrin-β4	Integrin β4a	catcaccattgaatcccaggtgcac	449	X51841
	Integrin β4b	tggtgatgctgctgtactcgctttg		
FAK	FAKa	agcaaatgctgactgctgctcacgcc	211	L13616
	FAKb	tgtaagtgctggcgactgaggacac		
E-cadherin	E-cadherina	gaccaggactatgactacttgaaacg	408	Z13009
	E-cadherinb	atctgcaaggtgctgggtgaaccttt		
β-catenin	β-catenina	tgacagcaatcagctggcctggttt	370	X87838
	β-cateninb	caaagcaagcaaagtcagtaccatt		
Apc	Apca	attcagttctagcagctcaagcaaa	410	M74088
	Apcb	aagtatactatcaaatatggcttcc		
αActinin	αActinina	ttccaggccttcattgacttcatg	236	X15804
	αActininb	gtacagcgccgtggagaaggacat		
Sucrase	Sucrasea	ggtggtcacatcctaccatgtcaag	419	X63597
	Sucraseb	ccagttgatttctattggttcttct		
Lactase	Lactasea	cagaggagatttggtctgcattttgt	367	M61849
	Lactaseb	cttgcagtacttgtatgacagaaat		M61850
pCB6hgh	pCB6hgh	gcactggggaggggtcacagggta		A00501
36B4	PG193	atgtgaagtcactgtgccag	420	M17885
	PG194	gtgtaatccgtctccacaga		

fonyl fluoride, and resuspended in 20 mM Hepes/KOH, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride. After 30 min at 4°C under constant agitation, nuclear debris were centrifuged at 13,000 *g* for 5 min and the supernatant consisting in nuclear extract was frozen and stored at −70°C before analysis of CDX2 protein content.

Protein samples (50 μg) were incubated at 100°C for 5 min in Laemmli buffer containing 2% SDS and 100 mM DTT. For immunoblot of integrin-α6 and integrin-β1 subunits, DTT was omitted. Proteins were separated by SDS-PAGE and transferred overnight to nitrocellulose filters by electroblotting in 25 mM Tris-HCl, 192 mM glycine, pH 8.2, and 20% methanol. Filters were saturated for 1 h at 37°C in PBS containing 5% skimmed milk. For immunological detection, filters were incubated 4 h at room temperature with appropriate primary antibodies (Table I) in PBS containing 1% skimmed milk and 0.1% Tween 20, and subsequently with secondary anti–mouse, anti–rabbit, or anti–rat antibodies labeled by HRP (Amersham Buchler GmbH, Braunschweig, Germany; Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Detection by chemiluminescence was performed using Western blotting detection reagents (Amersham Buchler GmbH).

Measurements of Lactase and Sucrase Activities

Lactase (EC 3.2.1.23) and sucrase (EC 3.2.1.48) activities were determined in brush border proteins according to standard protocols (Simon et al., 1979). The results were expressed as specific activities (mU/mg protein).

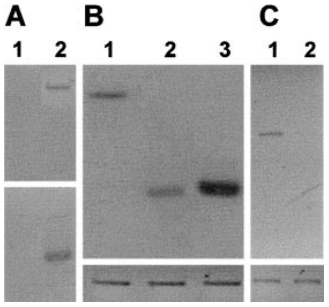

Figure 1. *Cdx1* and *Cdx2* expression in TC7 cells and in transfected cells cultured for 5 d. (*A*) Endogenous expression of *Cdx1* (*top*) and *Cdx2* (*bottom*) in TC7 cells. RT-PCR was performed on oligo-dT–primed cDNA for 24 cycles (lane *1*) or 32 cycles (lane *2*) with the oligonucleotide pairs Cdx1a/b and Cdx2b/c, respectively. (*B*) Transgene expression in transfected TC7 cells: expression of *Cdx1* sense RNA in pCdx1-S–transfected cells (lane *1*), of *Cdx1* antisense RNA in pCdx1-AS–transfected cells (lane *2*) and of *Cdx2* sense RNA in pCdx2-S–transfected cells (lane *3*); RT-PCR were made on oligo-dT–primed cDNA for 24 cycles using, respectively, the oligonucleotide pairs Cdx10/pCB6hgh, Cdx1b/pCB6hgh, and Cdx2b/pCB6hgh. The use of pCB6hgh as downstream primer allows to specifically detect the mRNA transcribed from the transfecting plasmid instead of the endogenous *Cdx1* or *Cdx2* mRNAs. (*C*) *Cdx1* expression in control pCB6-transfected cells (lane *1*) and in TC7 cells transfected with pCdx1-AS (lane *2*). RT-PCR was made for 32 cycles with the oligonucleotide pair Cdx1a/b on cDNA primed with Cdx1b. The use of Cdx1b as primer for cDNA synthesis allows the specific detection of endogenous *Cdx1* mRNA instead of the transcript synthesized from the recombinant plasmid pCdx1-AS. The lower panels in *B* and *C* show standard RT-PCR conducted for 22 cycles with primers designed to detect the 36B4 mRNA.

Results

Transfection of TC7 Cells with Plasmids Expressing Cdx1 and Cdx2 Sense and Antisense RNAs

The human colon adenocarcinoma cell line TC7 corresponds to a spontaneously differentiating clone derived from the original Caco2 cell population (Chantret et al., 1994). 5 d after seeding, they expressed a low level of *Cdx1* and *Cdx2* mRNA: RT-PCR fragments were observed after 32 amplification cycles, but could not be detected by 24 cycles (Fig. 1 *A*).

TC7 cells were transfected with plasmids synthesizing *Cdx1* and *Cdx2* RNAs in sense (pCdx1-S and pCdx2-S) and antisense orientations (pCdx1-AS and pCdx2-AS). Controls were transfected with the pCB6 vector. G418-resistant cell populations were obtained with every recombinant plasmid except with pCdx2-AS. In this case, G418-resistant cells grew after transfection but were lost during the first subculture because they no longer spread onto the culture dish after trypsin treatment. Transgene expression in the pooled G418-resistant cell populations was analyzed by RT-PCR using appropriate murine-specific primers (Fig. 1 *B*): *Cdx1* sense and antisense RNAs, and *Cdx2* sense RNA were already detected at 24 cycles indicating a high level of transgene expression in transfected cells. The ability of *Cdx1* antisense RNA (produced in pCdx1-AS–transfected cells) to downregulate endogenous *Cdx1* mRNA expression was investigated by RT-PCR on cDNA primed with the Cdx1b primer. The amount of *Cdx1* mRNA was indeed lower in pCdx1-AS–transfected cells than in controls transfected with pCB6 (Fig. 1 *C*).

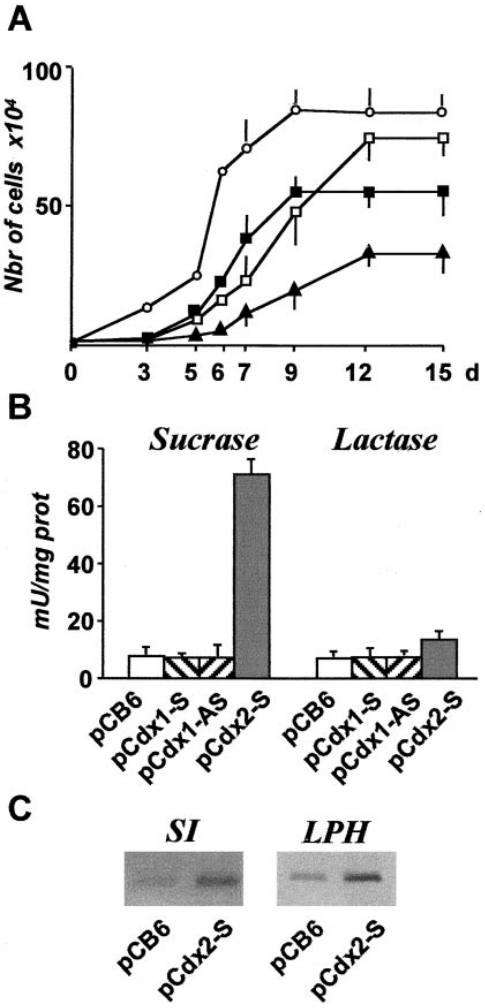

Figure 2. Growth and differentiation of the transfected cells. (*A*) Growth curves of TC7 cells transfected with the control plasmid pCB6 (○), with pCdx1-S (□), with pCdx1-AS (▲) and with pCdx2-S (■). Results are mean values of triplicate cultures; bars are standard deviations. (*B*) Sucrase and lactase specific activities in the brush border membranes of cells stably transfected with expression plasmids, and cultured for 10 d. Results are mean values of three experiments; bars are standard deviations. (*C*) RT-PCR analysis of sucrase-isomaltase (*SI*) and lactase-phlorizin hydrolase (*LPH*) mRNA expression in TC7 cells transfected with pCB6 or pCdx2-S. PCR amplification was carried out for SI (26 cycles) and LPH (30 cycles), respectively.

Growth and Differentiation of Cells Transfected with pCdx1-S, pCdx1-AS, and pCdx2-S

Growth and differentiation were analyzed in pooled cell populations transfected with pCdx1-S, pCdx1-AS, and pCdx2-S (Fig. 2). Compared to cells transfected with pCB6, onset of proliferation was delayed in the three cell populations, corroborating observations previously reported in *Cdx2*-overexpressing IEC cells (Suh and Traber, 1996). The cell number at the plateau was significantly lower in pCdx1-AS– and pCdx2-S–transfected populations than in controls, unlike pCdx1-S–transfected cells (Fig. 2 *A*). Sucrase and lactase activities, two markers of intestinal cell differentiation, were neither modified in pCdx1-S–transfected cells that overexpressed *Cdx1*, nor in pCdx1-

AS–transfected cells in which the endogenous level of *Cdx1* mRNA was reduced (Fig. 2 *B*). In contrast, overexpression of *Cdx2* in pCdx2-S–transfected cells caused a 9-fold and 1.7-fold increase of sucrase and lactase activities, respectively (Fig. 2 *B*). The corresponding mRNAs changed concomitantly (Fig. 2 *C*). This result is consistent with the fact that *Cdx2* encodes a transfactor that binds to the SI and LPH gene promoters (Suh et al., 1994; Troelsen et al., 1997).

During standard subcultures, we noted that pCdx2-S–transfected cells at confluency exhibited high resistance to detachment with trypsin. Indeed, a 20 min treatment with 0.01% trypsin at 37°C was required to dissociate efficiently pCdx2-S–transfected cells instead of 5 min for cells transfected with pCB6, pCdx1-S, or pCdx1-AS. This suggests that modifications of cell–substratum and/or cell–cell interactions occurred in *Cdx2*-overexpressing cells.

To obtain further insight into the phenotypic and molecular changes induced by *Cdx2* overexpression, cellular clones transfected with pCdx2-S were isolated by capillary duct aspiration and propagated individually. The selected C2S clone presented all the phenotypic changes described above as far as transgene expression, growth curve, resistance to trypsin, and brush border enzyme activities are concerned. In the following studies, the C2S clone has been compared to CB6, a clone transfected with the control plasmid pCB6. Western blotting performed using the polyclonal antiserum raised against the pancreatic CDX3 protein known to be identical with intestinal CDX2 (German et al., 1992; Laser et al., 1996) demonstrated a higher level of CDX2 in nuclear extracts of C2S cells compared to CB6 (Fig. 3 *A*). C2S cells also displayed a 2.2- and 18-fold higher lactase and sucrase activity than CB6 cells (data not shown).

Expression of Homeobox Genes in C2S Cells

In *Drosophila* (Dearolf et al., 1989) and more recently in mice (Subramanian et al., 1995), genes of the *caudal* family have been shown to control the expression of other homeobox genes. We have compared the expression of *HoxA-4*, *HoxA-9*, *HoxC-8*, and *Cdx1* in C2S and in control CB6 cells because these genes exhibit specific and distinct patterns of expression along the anterio-posterior axis of gut (James and Kazenwadel, 1991; Freund et al., 1992; Duluc et al., 1997). Fig. 3 *B* indicates that *HoxA-4* and *HoxC-8* expression was similar in both cell lines; in contrast, the level of *Cdx1* mRNA was markedly reduced in C2S cells whereas *HoxA-9* was upregulated in these cells compared to CB6. Similar variations were observed between the pooled populations of cells transfected with pCdx2-S and pCB6 (not shown). We have also analyzed HNF-1α mRNA expression because this nuclear factor, like CDX2, binds to the sucrase-isomaltase gene promoter (Wu et al., 1994). HNF-1α mRNA was present, though unchanged, in both cell types (Fig. 3 *B*).

Expression of Molecules Involved in Cell–Cell and Cell–Substratum Interactions

Because several genes encoding cell adhesion molecules are the targets of homeoprotein regulation (Edelman and Jones, 1993), and because *Cdx2*-overexpression cells display a high resistance to trypsin treatment at confluency, we have compared the expression of a panel of molecules involved in cell–cell and cell–substratum interactions in C2S and CB6 cells. These molecules have been analyzed at the protein and mRNA levels by immunocytochemistry, Western blotting, and/or RT-PCR.

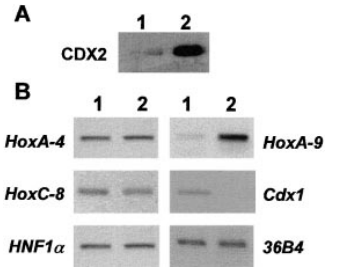

Figure 3. Homeobox gene expression. (*A*) CDX2 protein present in nuclear extracts of control CB6 (lane *1*) and in *Cdx2*-overexpressing C2S cells (lane *2*) grown for 5 d was revealed by SDS-PAGE and immunodetection with anti-cdx2/3 antibody. (*B*) *HoxA-4*, *HoxA-9*, *HoxC-8*, *Cdx1*, and *HNF-1α* mRNA expression in CB6 (lanes *1*) and C2S cells (lanes *2*). The mRNA levels were determined by RT-PCR. The number of PCR cycles was 32 for *HoxA-4*, 28 for *HoxA-9*, 30 for *HoxC-8*, 32 for *Cdx1*, and 26 for *HNF-1α*. Standard RT-PCR was performed for 22 cycles to detect the 36B4 mRNA.

Analysis of Proteins Belonging to the Cadherin–Catenin Complex in C2S Cells. We have analyzed two proteins of the cadherin–catenin complex, E-cadherin and β-catenin (Boller et al., 1985; Hermiston and Gordon, 1995), as well as APC, a microtubule-binding protein that also interacts with β-catenin and participates in the transduction processes (Hulsken et al., 1994; Vleminckx, et al., 1997).

Immunochemical labeling of E-cadherin was obvious at the periphery of both CB6 and C2S cells (Fig. 4, *A* and *B*). E-cadherin was slightly more expressed in *Cdx2*-overexpressing cells than in controls, as assessed by a modest increase (1.7-fold) in the intensity of the protein band detected by Western blot (Fig. 5), and corroborated at the mRNA level (Fig. 6). In contrast, β-catenin immunostaining and mRNA did not significantly change among the two cell lines (not shown). APC immunoreaction resulted in a punctated intracellular signal, that was increased in C2S compared to CB6 cells (Fig. 4, *C* and *D*); consistently, the APC protein band revealed on immunoblots (Fig. 5) and the corresponding RT-PCR signal (Fig. 6) were more intense in *Cdx2*-overexpressing cells than in control cells.

Expression of Laminin Constituent Chains and Integrin Subunits Varies in C2S Cells. We analyzed on one hand various constituent chains of laminin isoforms, α1, -α3, -β1, -β3, and -γ2, which are associated in laminin-1 (α1/β1/γ1) and laminin-5 (α3/β3/γ3), and on the other hand α6, β1 and β4 integrin subunits (for reviews see Mercurio, 1995 and Simon-Assmann et al., 1995).

As far as laminin chains are concerned, C2S cells displayed on one hand a downregulation of α1 chain mRNA and on the other hand an upregulation of γ2 chain mRNA compared to control cells (Fig. 6). In contrast, the mRNAs encoding the β1 and β3 laminin constituent chains were unchanged in both cell lines and that of the laminin α3 chain was undetectable (not shown).

Concerning integrin subunits, *Cdx2*-overexpressing cells displayed interesting changes in the α6, β1, and β4 mRNA content and/or in the amount or localization of the corre-

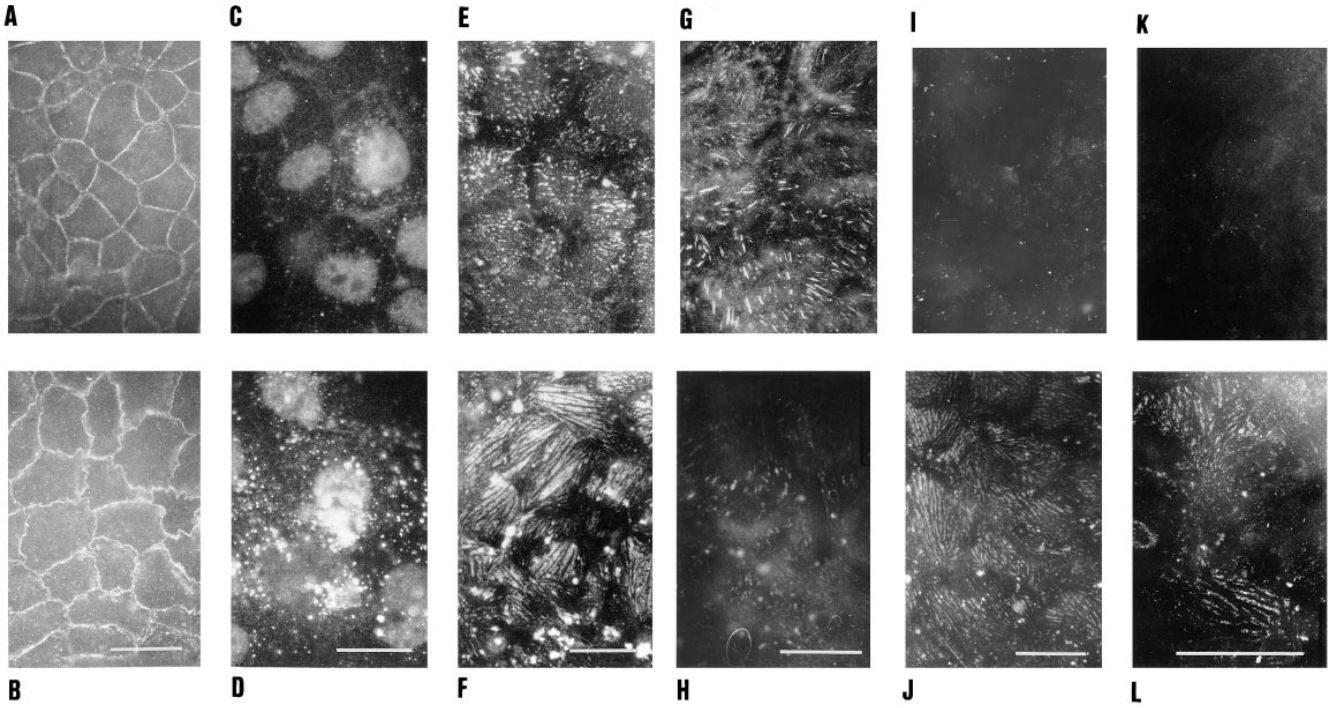

Figure 4. Immunodetection of E-cadherin (*A* and *B*), APC (*C* and *D*) integrin-α6 (*E* and *F*) integrin-β1 (*G* and *H*), integrin-β4 (*I* and *J*), and HD1 protein (*K* and *L*) in control CB6 (*A*, *C*, *E*, *G*, *I*, and *K*) and *Cdx2*-overexpressing C2S cells (*B*, *D*, *F*, *H*, *J*, and *L*) cultured for 5 d. Cells were fixed with 1% paraformaldehyde and permeabilized with 1% Triton X-100 (*A–J*), or fixed with cold methanol (*K* and *L*). Bars, 40 μm.

sponding proteins. Although the level of α6 mRNA (not shown) and the corresponding protein band revealed on immunoblots of whole cell extracts was similar in C2S and control cells, the intensity of the membrane-associated α6 protein band was significantly increased in C2S cells (Fig. 5). In parallel, immunostaining of the α6 integrin subunit on the ventral side of C2S cells was organized in parallel fibers and was more intense than that found in control CB6 cells, in which only short dots were visible (Fig. 4, compare *F* to *E*). These observations suggest that this integrin subunit was recruited in the membrane of C2S cells. An inverse observation was made for the integrin-β1 subunit since a rather weak labeling was detected at the base of *Cdx2*-overexpressing C2S cells, contrasting with the sharp basal staining of small fibers in control CB6 cells (Fig. 4, compare *H* to *G*). Consistently, the amount of integrin-β1 protein (Fig. 5) and mRNA (Fig. 6) was reduced in C2S cells compared to CB6; Northern blot quantification indicated a 2.5-fold decay of integrin-β1 mRNA in these cells (not shown). As far as the integrin-β4 subunit is concerned, it was hardly detected in control CB6 cells at both protein (Figs. 4 *I* and 5) and mRNA levels (Fig. 6), as previously reported for parental Caco2 cells (Basson et al., 1992; Fontao et al., 1997). However, immunostained patches were clearly visualized on the ventral side of C2S cells (Fig. 4 *J*), the integrin-β4 subunit protein band was clearcut on Western blots of C2S cell membrane extracts (Fig. 5), and the corresponding mRNA was detected in these cells (Fig. 6).

Some Intracellular Proteins Are Increased in Cdx2-overexpressing Cells. The expression of intracellular molecules expected to mediate extracellular matrix signals was investigated: HD1, a component of hemidesmosomes that in-

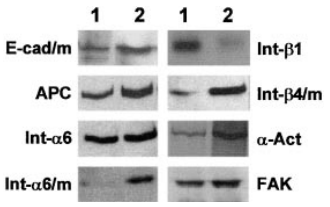

Figure 5. Protein expression in control CB6 (lanes *1*) and C2S cells (lanes *2*) cultured for 5 d and analyzed by immunoblotting experiments. 50 μg of proteins were loaded in each lane for electrophoresis on 7.5% polyacrylamide gels. α-Actinin (*α-Act*), APC, FAK, integrin-α6 (*Int-α6*), and integrin-β1 (*Int-β1*) were analyzed in whole cell extracts. E-cad/m, Int-α6/m, and Int-β4/m, respectively, designate E-cadherin, integrin-α6, and integrin-β4 present in cell membrane preparations.

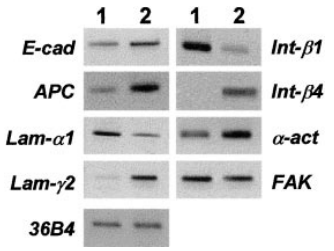

Figure 6. RT-PCR analysis of the mRNA expression of E-cadherin, APC, laminin-α1 chain, laminin-γ2 chain, integrin-β1 subunit, integrin-β4 subunit, α-actinin, and FAK in control CB6 (lanes *1*) and C2S cells (lanes *2*) cultured for 5 d. The number of RT-PCR cycles was 30 for E-cadherin, 26 for APC, 26 for α-actinin, 28 for laminin-α1, and -γ2, 26 for integrin-β1, 24 for integrin-β4, and 28 for FAK. Standard RT-PCR was made with 34B6 primers for 22 cycles.

teracts with α6β4 integrin and cytokeratins (Hieda et al., 1992; Sonnenberg et al., 1993; Fontao et al., 1997), the focal adhesion kinase pp125FAK (Hanks et al., 1992; Schaller et al., 1992), and α-actinin that connects β1-integrin to the actin network (Craig and Pardo, 1979).

We found a slight increase in the expression of α-actinin in C2S cells compared to control CB6 cells at the protein (Fig. 5) and mRNA (Fig. 6) levels. Immunostaining of HD1 protein was almost absent in control CB6 cells (Fig. 4 *K*), as previously shown in parental Caco2 cells (Fontao et al., 1997); however a clear staining was observed on the basal side of the C2S cells (Fig. 4 *L*). Finally, the tyrosine kinase associated with focal adhesions, pp125FAK, and the corresponding mRNA, were present without any obvious changes in C2S and CB6 cells (Figs. 5 and 6).

It is noteworthy that these variations were also recorded, but to a lower extent, in the pooled populations of pCdx2-S– and pCB6–transfected cells. Taken together, these results indicate that *Cdx2* overexpression provokes modifications in the level of expression or in the localization of molecules involved in cell–cell interactions, in cytoskeleton organization and transduction processes, and mostly in cell–substratum interactions.

Adhesion and Aggregation Properties of C2S Cells

The higher resistance to trypsin treatment displayed by *Cdx2*-overexpressing cells compared to control cells at confluency, and the modification of expression of molecules involved in cell–cell and cell–substratum interactions prompted us to investigate whether this behavior reflects modifications in the intrinsic adhesion and/or aggregation properties of the cells. For this purpose, C2S and CB6 cell adhesion to plastic or laminin-1 was compared after 1 and 2 h. Laminin-1 coatings increased the attachment of both cell types to the substratum (mean increase of 2.5-fold). However, C2S cells adhered two to three times less than control CB6 cells to plastic as well as to laminin. Adhesion inhibition studies, using antiintegrin antibodies, indicated that antiintegrin-β1 antibody caused an equal 85% inhibition of C2S and CB6 cell attachment to laminin-1. Antiintegrin-β4 and antiintegrin-α6 antibodies failed to inhibit cell attachment of both clones to laminin-1, as already observed in parental Caco2 cells (Orian-Rousseau, V., unpublished results). Aggregation tests indicated that 29% of the starting CB6 cell suspension formed aggregates containing more than four cells after 30 min under slow agitation, whereas the value increased to 49% for *Cdx2*-overexpressing C2S cells.

The higher capacity of C2S cells to form cell–cell contacts than the control cells could result from the increased expression of E-cadherin in these cells; it may also explain the higher resistance of C2S cells than CB6 cells to trypsin treatment at confluency. On the other hand, the lower capacity of C2S cells to adhere to the substratum could be linked to the decline of integrin-β1 expression, but seems to be independent on the rise of integrin-β4 expression, as confirmed by adhesion inhibition studies with specific antiintegrin antibodies.

Xenografts of C2S Cells

C2S cells were injected subcutaneously to *nude* mice and

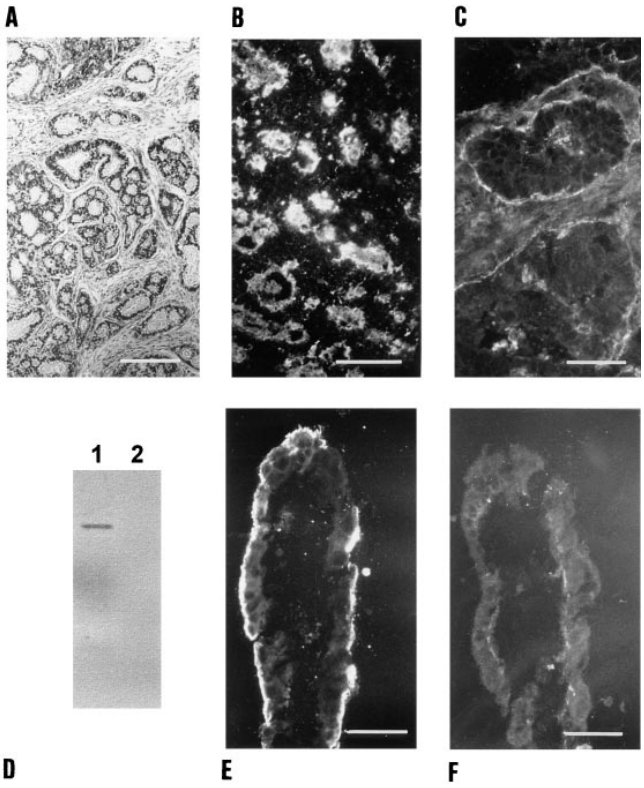

Figure 7. Injection of C2S cells to *nude* mice. Tumors grown from C2S cells (10^7) injected subcutaneously to *nude* mice were examined by standard histology (*A*). Cryosections were immunostained with antibodies against sucrase-isomaltase (*B*) and integrin-β4 chain (*C*). Transgene *Cdx2* mRNA expression was retained in seven week tumors, as shown by RT-PCR analysis using the primers Cdx2b/pCB6hgh (*D*); lane *1*: RT-PCR conducted with oligo-dT-primed cDNA, lane *2*: control PCR performed with RNA preparation instead of cDNA. Villus-like structure in a CS2 tumor grown 12 wk showing sucrase-isomaltase and integrin-β4 expression (*E* and *F*). Bars: (*A* and *B*) 200 μm; (*C*–F) 50 μm.

tumors were recovered after 7 and 12 wk. At 7 wk, expression of the *Cdx2* transgene under the control of the cytomegalovirus promoter was retained, as demonstrated by RT-PCR (Fig. 7 *D*). Tumors developed from the C2S cells were twice as large as those derived from control CB6 or parental TC7 cells. Despite the difference in size, both types of tumors displayed glandular structures with a single, polarized, epithelial layer lining a central lumen and delineated by stromal cells derived from the host (Fig. 7 *A*). Controls (not shown) and C2S tumors expressed SI on the apical side of the epithelial cells (Fig. 7 *B*), as well as integrin-β4 subunit at the interface with stromal cells (Fig. 7 *D*). 12 wk after implantation, C2S tumors displayed a fivefold greater weight than controls; they exhibited glands, sporadic villus-like structures (Fig. 7, *E* and *F*) and necrotic areas in the center of the tumors, unlike CB6 or TC7 tumors which were almost filled with stromal and granulation tissue. In the villus-like structures, SI (Fig. 7 *E*) and integrin-β4 subunit (Fig. 7 *F*) were detected, respectively, at the apical pole of the polarized epithelial cells and at the basal surface of the cells facing the stroma. The differences observed between C2S and CB6 tumors were also found

amongst the tumors developed from the pooled populations of transfected cells (not shown).

Cdx2 Expression in TC7 Cells Grown In Vitro on Laminin Coatings

Extracellular matrix components of the basement membrane have been shown to potentiate intestinal cell differentiation (Simon-Assmann et al., 1995). Of particular interest, SI expression is increased in Caco2 cells grown in vitro on laminin coatings (Vachon and Beaulieu, 1995; Basson et al., 1996). *Cdx2* overexpression stimulates the expression of several differentiation markers, such as sucrase-isomaltase; thus, we analyzed whether the differentiating effect of laminin could correlate with an increase in *Cdx2* expression in Caco2 cells. For this purpose, TC7 cells were grown on standard culture dishes or on dishes previously coated with laminin-1 prepared from EHS tumors. RNA was extracted on day 5 of culture, and analyzed by RT-PCR for *Cdx2* expression; SI mRNA was used as a marker of cell differentiation. As shown in Fig. 8 *A*, laminin coatings promoted cell differentiation, assessed by the higher level of SI mRNA present in cells grown on laminin compared to plastic; noteworthily, the level of *Cdx2* mRNA also increased in cells cultured in presence of laminin-1. These results suggest that *Cdx2* expression is stimulated by laminin signaling.

Cdx2 Expression in Cellular Clones Deficient in Laminin-α1 Chain

To strengthen the above observations, we took advantage of Caco2 cell clones of the LAMα1-AS series established in our laboratory, in which endogenous expression of the laminin-α1 chain was inhibited by antisense RNA (De Arcangelis et al., 1996). Inhibition of laminin-α1 chain synthesis altered the processes of basement membrane assembly and cell differentiation, as shown by morphological and immunological observations.

We have analyzed the expression of *Cdx2* and SI as a function of the residual level of endogenous laminin-α1 mRNA present in three distinct clones of the LAMα1-AS series: LAMα1-AS12, LAMα1-AS20, and LAMα1-AS22, and in one control clone transfected with pCB6. RNA was extracted from cells grown for 5 d. Fig. 8 *B* shows that SI mRNA expression paralleled the amount of laminin-α1 mRNA present in the cells, confirming that inhibition of laminin-α1 chain by antisense RNA provoked a decay of SI gene expression. *Cdx2* expression also well correlated the amount of laminin-α1 mRNA: indeed, clones in which the endogenous production of laminin-α1 RNA was low, displayed a low amount of *Cdx2* mRNA, whereas this transcript was higher in clones synthesizing a higher amount of laminin-α1 RNA (Fig. 8 *B*). To further confirm these findings, an additional experiment was conducted on the LAMα1-AS12 clone, which shows a stringent phenotype of inhibition of laminin-α1 chain production (see De Arcangelis et al., 1996 and Fig. 8 *C*). This clone, originally grown in culture medium containing 1.2 mg/ml G418, was propagated for several passages in the presence of a low amount of G418 (0.6 mg/ml). After five passages, the endogenous level of laminin-α1 mRNA increased (Fig. 8 *C*), as already reported (De Arcangelis et al., 1996). Again, there was a parallel between the levels of laminin-α1 chain, SI, and *Cdx2* expression, because the upregulation of laminin-α1 mRNA was accompanied by an increase of the level of SI and *Cdx2* transcripts (Fig. 8 *C*).

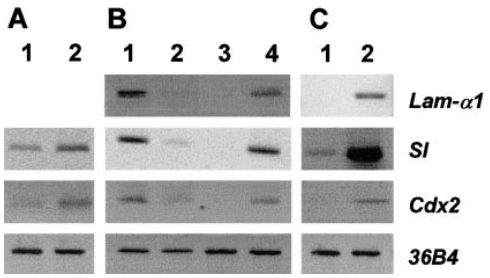

Figure 8. Correlation between laminin-α1 chain, *Cdx2* and SI expression. (*A*) TC7 cells grown on plastic dishes (lane *1*) or on laminin coatings (lane *2*). (*B*) Clones of the LAMα1-AS series (De Arcangelis et al., 1996): the clone pB6-18 was transfected with the control plasmid pCB6 (lane *1*); AS12 (lane *2*), AS20 (lane *3*), and AS22 (lane *4*) clones were transfected with the pCB6-AS LN plasmid. (*C*) The LAMα1-AS12 clone was grown in the presence of 1.2 mg/ml (lane *1*) or 0.6 mg/ml G418 (lane *2*). The number of PCR cycles was 30 for laminin-α1, 26 for SI, and 32 for *Cdx2.* Standard RT-PCR was carried out for 22 cycles with primers that detect the 36B4 mRNA.

Comparison of the Phenotypic Modifications Induced by Laminin-1 Coatings and Cdx2 Overexpression

To analyze whether the differentiation-promoting effect induced by laminin-1 can be linked to the phenotypic changes resulting from *Cdx2* overexpression, we analyzed the expression of molecules found to be affected by *Cdx2* overexpression, in TC7 cells grown on laminin-1 or on plastic, and in the LAMα1-AS12 clone cultured in medium containing a high or a low concentration of G418. This concerned three transcripts that were upregulated in *Cdx2*-overexpressing cells: the *HoxA-9*, APC, and integrin-β4 mRNAs; and two transcripts downregulated in these cells: the *Cdx1* and integrin-β1 mRNAs.

As shown in Fig. 9, TC7 cells grown on laminin coatings for 5 d displayed an upregulation of *HoxA-9*, APC, and integrin-β4 mRNA, and a downregulation of *Cdx1* and integrin-β1 transcripts, compared to cells cultured on plastic. Similar modifications in mRNA levels were observed in reverted LAMα1-AS12 cells producing endogenous laminin-α1 mRNA (grown in the presence of 0.6 mg/ml G418) compared to cells grown in the presence of 1.2 mg/ml G418. Immunocytochemical observations of integrin-α6 and -β4 subunits in TC7 cells grown on laminin confirm the stimulation of integrin-β4 expression, as highly positive patch-like structures appeared at the ventral side of the cells, and codistributed with α6 positive patches (not shown). In these cells, integrin-β1 subunits were hardly detected.

These data show that laminin-1 (of exogenous or endogenous origin) induces phenotypic changes similar to those observed when *Cdx2* homeobox gene is overexpressed, as far as the pattern of gene expression and the recruitment of integrin subunits on the cell base is concerned.

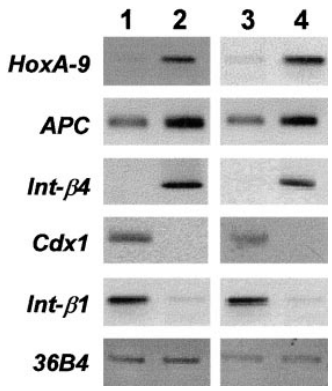

Figure 9. Comparison of phenotypic changes induced by laminin coatings and *Cdx2* overexpression. mRNA expression assessed by RT-PCR of *HoxA-9* (28 cycles), APC (26 cycles), integrin-β4 subunit (24 cycles), *Cdx1* (32 cycles), and integrin-β1 subunit (26 cycles) in TC7 cells grown on laminin coatings (lane *2*) versus plastic (lane *1*), and in the LAMα1-AS12 clone cultured in the presence of 1.2 mg/ml (lane *3*) or 0.6 mg/ml G418 (lane *4*). RT-PCR for 36B4 was carried out for 22 cycles.

Discussion

Two major conclusions arise from the present study. First, overexpression of a single homeobox gene, *Cdx2*, causes pleiotropic effects reproducing the properties of differentiated enterocytes lining the villi. These include a rise in differentiation markers such as sucrase-isomaltase and lactase-phlorizin hydrolase, as well as modifications in the expression pattern of molecules involved in cell–cell and mainly in cell–substratum interactions. Second, laminin-1, an extracellular matrix component of the basement membrane, is a potent regulator of the expression of *Cdx2.* These results suggest that the *Cdx2* homeobox gene plays a key role in the cascade of events involved in extracellular matrix–mediated intestinal cell differentiation.

Although it is difficult to extrapolate results obtained from colonic adenocarcinoma cells to the normal intestinal epithelium, several features displayed by *Cdx2*-overexpressing Caco2 cells can be related to the properties of differentiated enterocytes. For instance, C2S cells show a significant rise of SI and LPH, two markers of the mature enterocytes. They also exhibit a stimulation of β4 integrin subunit, consistent with the fact that, in vivo, this basement membrane receptor chain exhibits an increasing gradient along the crypt–villus axis (Simon-Assmann et al., 1994*b*). APC, E-cadherin, and the laminin-γ2 chain, preferentially expressed by villus cells (Hermiston and Gordon, 1995; Nathke et al., 1996; Orian-Rousseau et al., 1996), are also upregulated in *Cdx2*-overexpressing cells. Of interest is the fact that *Cdx2* overexpression stimulates the synthesis and/or the recruitment at the cell base of α6 and β4 integrin subunits and of HD1. α6β4 integrin, HD1 together with laminin-5, which comprises the laminin γ2 chain, constitute the major components of type II hemidesmosomes concentrated at the base of villi enterocytes; studies in the future will investigate whether macromolecular hemidesmosomal structures, absent in parental Caco2 cells (Orian-Rousseau et al., 1996; Fontao et al., 1997), are actually assembled in *Cdx2*-overexpressing cells. The membrane recruitment of integrin-α6 subunits from an intracellular pool of molecules is consistent with observations made for other integrins during keratinocyte differentiation (Lenter and Vetsweber, 1994; Hotchin et al., 1995). Taken together, the present results indicate that *Cdx2* impels cells toward the phenotype of villi enterocytes, suggesting that this homeobox gene has a master function in the coordinate process leading to cell differentiation during the continuous renewal of the intestinal epithelium.

The pleitropic effects of *Cdx2* on cell differentiation contrast with the results obtained when *Cdx1* expression is modified in Caco2 cells. These differences in vitro may be related to the distinct patterns of expression of both genes in vivo, since CDX1 protein is restricted to undifferentiated crypt cells, whereas CDX2 is located in the nuclei of differentiating enterocytes (James et al., 1994; Subramanian et al., 1995). It is worth noting that the decrease of *Cdx1* expression by antisense RNA as well as the overexpression of *Cdx2* result in an inhibition of cell proliferation. This, together with the fact that the level of *Cdx1* mRNA is reduced in *Cdx2*-overexpressing cells, may suggest a negative regulatory effect of the CDX2 protein on *Cdx1* mRNA expression in enterocytes that pass the crypt–villus junction; positive and negative regulatory elements have been reported in the *Cdx1* gene promoter (Hu et al., 1993). A direct interaction between the CDX2 protein and promoter elements of enterocytic markers of differentiation has been demonstrated (Suh et al., 1994; Drummond et al., 1996; Lee et al., 1996; Lambert et al., 1996; Troelsen et al., 1997). However there is no information concerning the other genes studied here: E-cadherin, APC, α-actinin, the laminin-α1 and -γ2 chains, and the integrin-β1 and -β4 subunits. Recently, several genes belonging to *Hox* clusters have been proposed to be the targets of *Cdx1* during early development of skeletal structures (Subramanian et al., 1995), and we show herein that cells overexpressing *Cdx2* display an increase of *HoxA-9* mRNA level. It is therefore possible that the phenotype of *Cdx2*-transfected cells results from the direct interaction of CDX2 protein with the promoters of enterocytic differentiation markers, as well as from indirect effects mediated by secondary regulatory genes such as those belonging to the homeobox gene family.

The results obtained in this study are somewhat unexpected, as regards the possible link between *Cdx2* expression and colonic tumorigenesis. Indeed, on the one hand, transfected Caco2 cells producing *Cdx2* antisense RNA, as well as transfected intestinal IEC18 cells (personal data), attach poorly to the culture dish, suggesting that some alterations in cell–cell and/or cell–substratum interactions have occurred, like in many cancer cells. This is consistent with the propensity of *Cdx2*-overexpressing cells to form aggregates, while E-cadherin and APC, two tumor or invasion suppressor molecules, are upregulated (Frixen et al., 1991; Vleminckx et al., 1991; Nathke et al., 1996). We also found in these cells an important decrease in the level of *bcl-2* mRNA (our unpublished result), an anti–apoptotic protooncogene deregulated in many cancers (Reed, 1995). These data, together with the fact that there is a dramatic decay of *Cdx2* expression in human colonic tumors and in experimentally induced tumors in the rat (Ee et al., 1995; Mallo et al., 1997), suggest a tumor suppressor role of *Cdx2* in the gut. Confirmation of this has recently been provided by heterozygous *Cdx2* knock-out mice which develop multiple adenomatous polyps and metaplasia in the colon (Chawengsaksophak et al., 1997). However, on the other

hand, *Cdx2*-overexpressing cells injected in *nude* mice develop tumors larger than the control ones. Although no clear explanation can be put forward, this unexpected behavior may be related to the higher level of β4-integrin subunit and/or of laminin-γ2 chain in these cells. Indeed, both molecules are possibly linked to tumor formation or invasion (Pyke et al., 1994; Chao et al., 1996). Furthermore, the HT29 cell line expressing high levels of α6/β4 integrin and laminin-5 form significantly larger tumors in *nude* mice than the poorly expressing parental Caco2 cell line (Simon-Assmann et al., 1994; Orian-Rousseau, V., personal communication).

A major result of this work concerns the relationship between *Cdx2* expression and the presence of a basement membrane component: laminin-1. Various investigations have already been carried out, in particular in the liver and mammary gland, to explore the molecular basis of gene regulation by extracellular matrix components (Roskelley et al., 1995). They led to the identification of extracellular matrix–responsive elements in the gene promoters of specific cell differentiation markers (Schmidhauser et al., 1990, 1992; Juliano and Haskill, 1993). Using cell cultures on laminin-1 coatings and a collection of cell clones in which the endogenous production of laminin-α1 chain is inhibited by antisense RNA, we provide strong arguments in favor of a regulatory role played by extracellular matrix components, of endogenous or exogenous origin, on *Cdx2* expression. Since additionally, we observed modifications in the expression of some laminin chains and integrin subunits by *Cdx2* overexpression, we conclude that homeobox genes and molecules involved in cell–substratum interactions exert reciprocal controls during intestinal cell differentiation. The mechanism whereby laminin-1 regulates *Cdx2* in Caco2 cells remains unclear. In particular, we do not know whether *Cdx2* is directly controlled by transduction signals resulting from the binding of laminins to their integrin receptors, or whether this effect is indirectly exerted through a control of cell shape (Singhvi et al., 1994; Roskelly et al., 1995), or by promoting the binding of soluble factors to their membrane receptors (Streuli et al., 1995*b*; Miyamoto et al., 1996). The participation of integrins in mediating the effect of laminin-1 is suggested by the segregation of integrin subunits on the basal side of cells grown on laminin-1 coatings (our unpublished data). In parental Caco2 cells, the primary effect of laminin may involve integrins comprising the β1 chain, because integrin-β4 is virtually absent in these cells. Although the precise downstream signalling pathway is not known, it may require phosphorylation events including pp125FAK or ras-dependent protein kinases, and/or the activation of transcription factors such as STAT or NF-κB (Schlaepfer et al., 1994; Streuli et al., 1995*a*; Roskelley et al., 1995; Dehdar and Hannigan, 1996; Rosales and Juliano, 1996).

This study indicates that extracellular signals delivered by laminin-1 lead to a modification in the expression of the CDX2 homeoprotein, which in turn provokes changes (*a*) in the composition of the secreted basement membrane molecules (decay of laminin-α1 and increase of laminin-γ2 mRNAs) and (*b*) in the cellular attachment properties and transduction signals (modification of the repertoire of integrins: decay of integrin-β1 and stimulation of integrin-β4 subunits). Since several laminin chains and integrin subunits exhibit a specific distribution along the crypt–villus axis (Beaulieu, 1992; Simon-Assmann et al., 1994*b*; Perreault et al., 1995; Leivo et al., 1996), we speculate that, in addition to a possible intrinsic program of proliferation/differentiation held by the enterocytes themselves, a molecular code based on the spatial distribution of laminins and integrins may instruct cells to switch from proliferation to differentiation at the crypt–villus junction, during the continuous process of renewal of the intestinal epithelium. Although the complete cascade of events is far from being elucidated, the present results provide new insight to approach the molecular mechanisms of intestinal cell differentiation in relation with epithelial–mesenchymal cell interactions, extracellular matrix components and the control of homeobox gene expression. This study also opens the question whether the *Cdx2* decline observed in colon cancers results from regulatory changes associated with some alterations of extracellular matrix signaling, and/or with modifications of the transduction processes linked to oncogenic signaling.

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