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Changing intestinal connective tissue interactions alters homeobox gene expression in epithelial cells

Isabelle Duluc, Olivier Lorentz, Christine Fritsch, Cathy Leberquier, Michèle Kedinger and Jean-Noël Freund*

Institut National de la Santé et de la Recherche Médicale, Unité 381, Strasbourg, France

*Author for correspondence (e-mail: u.381@inserm.u-strasbg.fr)

SUMMARY

In segmented organs, homeobox genes are involved in axial patterning and cell identity. Much less is known about their role in non-segmented endoderm derivatives such as the digestive epithelium. Using a xenograft model of fetal intestinal anlagen implanted under the skin of *nude* mice, we have investigated whether the expression of five homeobox genes (*HoxA-4, HoxA-9, HoxC-8, Cdx-1* and *Cdx-2*) is modified when intestinal epithelium undergoes normal development or displays heterodifferentiation in association with heterotopic mesenchyme. In homotypic associations of fetal endoderm and mesenchyme that recapitulate normal development, the overall pattern of homeobox gene expression was maintained: *HoxA-9* and *HoxC-8* were the highest in the colon and ileum, respectively, and *HoxA-4* was expressed all along the intestine; *Cdx-1* and *Cdx-2* exhibited an increasing gradient of expression from small intestine to colon. Yet, grafting per se caused a faint upregulation of *HoxA-9* and *HoxC-8* in small intestinal regions in which these genes are not normally expressed, while the endoderm-mesenchyme dissociation-association step provoked a decay of *Cdx-1* in the colon. In heterotopic associations of colonic endoderm with small intestinal mesenchyme, the colonic epithelium exhibited heterodifferentiation to a small intestinal-like phenotype. In this case, we observed a decay of *HoxA-9* expression and an upregulation of *HoxC-8*. Additionally, heterodifferentiation of the colonic epithelium was accompanied by a downregulation of *Cdx-1* and *Cdx-2* to a level similar to that found in the normal small intestine. To demonstrate that mesenchyme-derived cells can influence *Cdx-1* and *Cdx-2* expression in the bowel epithelium, fetal jejunal endoderm was associated with intestinal fibroblastic cell lines that either support small intestinal-like or colonic-like morphogenesis. A lower expression of both homeobox genes was shown in grafts presenting the small intestinal phenotype than in those showing glandular colonic-like differentiation. Taken together, these results suggest that homeobox genes participate in the control of the positional information and/or cell differentiation in the intestinal epithelium. They also indicate that the level of *Cdx-1* and *Cdx-2* homeobox gene expression is influenced by epithelial-mesenchymal cell interactions in the intestinal mucosa.

Key words: Xenograft, Hox, Cdx, Endoderm, Mesenchyme, Differentiation

INTRODUCTION

The gastrointestinal tract arises from visceral endoderm and splanchnic mesenchyme, giving the digestive epithelium, lamina propria, and muscle cells. Reciprocal interactions between endoderm and mesenchyme derivatives are crucial for cell differentiation throughout development and at the adult stage (Yasugi, 1993; Kedinger et al., 1996). For instance, the mesenchyme dictates the morphology and cellular organization of the digestive mucosa, and epithelial cells instruct mesoderm-derived cells to develop typical intestinal mesenchyme and muscle structures (Kedinger et al., 1981, 1990). Up to weaning, a functional regionalization progressively emerges along the anterio-posterior (AP) axis of the gastrointestine, i.e. in the stomach, small intestine, and colon, while the digestive epithelium is continuously and actively renewed from stem cells (Duluc et al., 1993; Rubin et al., 1994; Crossman et al., 1994, reviewed by Gordon and Hermiston, 1994). Recent studies have emphasized that normal gastrointestinal development is recapitulated in a grafting model of fetal anlagen, suggesting that the developmental and positional information along the intestinal AP axis is fixed in the fetus, prior to endoderm cytodifferentiation (Rubin et al., 1992, 1994; Duluc et al., 1994).

Homeobox genes play a key role in the complex ontogenic processes that govern pattern formation and cell fate (Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992; Botas, 1993). For instance, morphogenetic transformations occur in homeobox gene 'knock out' mice or in animals showing ectopic homeobox gene expression (Wolgemuth et al., 1989; Joyner et al., 1991; Lufkin et al., 1991; Le Mouellic et al., 1992; Jegalian and De Robertis, 1992; Subramanian et al., 1995). The function of homeobox genes is well documented in segmented tissues, whereas much less is known about their role in organs derived from the non-segmented endoderm. In *Drosophila* and chicken, mesenchyme homeobox genes under the control of endodermic factors have been shown to con-

tribute to the positional information along the intestinal AP axis (Bienz, 1994; Yokouchi et al., 1995; Roberts et al., 1995). However, in the endoderm, the nature of positional signals remains elusive, but homeobox genes have been implicated because several of them are expressed in specific regions of the adult intestinal epithelium (James and Kazenwadel, 1991). For instance, *HoxA-9* (*Hox1.7*) mRNA is only detected in colonic epithelium, whereas *HoxC-8* (*Hox3.1*) transcripts are absent in the jejunum, present in ileum, and at a low level in the colon. Two *caudal*-related genes, *Cdx-1* (Duprey et al., 1988; Hu et al., 1993) and *Cdx-2* (James et al., 1994), exhibit an increasing gradient of expression along the intestinal AP axis (James and Kazenwadel, 1991; Freund et al., 1992; Hu et al., 1993; Meyer and Gruss, 1993; James et al., 1994).

Homeobox gene function in segmented organs has been approached using experimental conditions in which morphogenesis is altered (Ispizua-Belmonte et al., 1991b; Morgan et al., 1992). In this study, we have investigated in xenograft models of fetal rat intestinal anlagen whether the expression of homeobox genes is modified when differentiation of the intestinal epithelium is redirected by heterotopic mesenchyme or fibroblastic cell lines. In the first model, fetal rat endoderm was dissociated from the mesenchyme, re-associated either with its own mesenchyme (i.e. homotopic associations) or with intestinal mesenchyme from another AP level (i.e. heterotopic associations), and subsequently grafted under the skin of *nude* mice. Homotopic grafts develop according to their original position along the bowel AP axis; however, in heterotopic grafts in which colonic endoderm is associated with small intestinal mesenchyme, the fate of the colonic epithelial cells is modified to form typical small intestinal-like structures of villi and crypts, with expression of sucrase-isomaltase (SI) and occasionally lactase-phlorizin hydrolase (LPH), two digestive enzymes normally restricted to the small intestine (Duluc et al., 1994). In the second model, fetal jejunal endoderm was associated with two different intestinal fibroblastic cell lines, one of which supports a glandular colonic-like development after grafting in the coelomic cavity of chicken embryos, whilst the other supports a typical small-intestinal-like phenotype assessed by morphology and expression of LPH (Fritsch et al., 1997).

We have analyzed the expression of five homeobox genes in the grafts: *HoxA-9*, *HoxC-8*, *Cdx-1* and *Cdx-2* were chosen according to their region-specific distribution along the adult intestine (James and Kazenwadel, 1991); *HoxA-4* was assayed because it is predominantly expressed in the fetal intestinal mesenchyme (Ispizua-Belmonte et al., 1991a), and causes megacolon when overexpressed (Wolgemuth et al., 1989).

MATERIALS AND METHODS

Animals and tissues

Wistar rats from our own breeding colony and Swiss athymic (*nu*/*nu*) mice from Iffa Credo (Les Oncins, France) were maintained under standard laboratory conditions. Intestines were recovered from 14-day or 20-day fetal rats delivered by Cesarean section, and from 5-day-old neonates or adult animals. The first and fourth small intestinal quarters, respectively, designated as the proximal jejunum (PJ) and distal ileum (DI), and the proximal half of colon (PC) were used for subsequent experiments.

Tissue dissociation, re-association and xenografts in *nude* mice

The method used to dissociate and re-associate rat fetal intestinal endoderm and mesenchyme has been developed in this laboratory and previously described in detail (Kedinger et al., 1981; Duluc et al., 1994). Briefly, PJ, DI or PC segments of 14-day rat fetuses were incubated for 1 hour at 37°C with 0.03% collagenase (Boehringer, Mannheim, Germany), and the endoderm was separated from the mesenchyme by mechanical dissociation. Homo- and heterotopic endoderm/mesenchyme re-associations were allowed to assemble overnight on gelified medium containing 2 g/l Bactoagar (Difco, Detroit, MI) in MEM/Ham F12 medium (Gibco BRL, Cergy-Pontoise, France), 10% fetal calf serum (Gibco) and 10% chick embryo extract. Intestinal associations were grafted under the skin of *nude* mice and recovered 5 weeks after implantation for individual analysis. In controls, intact intestinal segments were implanted in *nude* mice without dissociation, or tissue associations allowed to assemble in vitro were immediately analyzed without implantation in *nude* mice.

In 20 day fetuses, intestinal muscle layers were first recovered with forceps and the epithelium was separated from the lamina propria by incubation with 10 mM EDTA for 10 minutes. At later developmental stages (suckling, adult animals), the epithelium can no longer be separated cleanly from the mesenchyme.

Association of fetal endoderm and fibroblastic cell lines; xenografts in chicken embryos

The fibroblastic cell lines A1:F1 and F1:G9 have previously been isolated from the ileum of 5-day-old suckling rats and characterized for growth and differentiation properties (Fritsch et al., 1997).

Jejunal endodermal pieces prepared as described above from rat fetuses of 14 days were enveloped in confluent A1:F1 and F1:G9 fibroblast sheets, and cultured for 24 hours on gelified medium. The associations were then grafted in the coelomic cavity of chicken embryos (day 3 of development) and harvested 10 to 13 days later.

Reverse transcription and polymerase chain reaction (RT-PCR)

Tissue and grafts were frozen and stored at −70°C before RNA extraction. RNA was extracted with lithium chloride from intestinal segments of neonates and adult rats as previously described (Duluc et al., 1993). TRIzol™ solution (Gibco) was used to prepare RNA from fetal intestinal anlagen and from the grafts.

Oligonucleotides (Table 1) were from Eurogentec (Seraing, Belgium). Oligo-dT-primed cDNA synthesis was for 1 hour at 42°C in 25 μl of solution containing 4 μg RNA, 50 pmoles oligo-dT, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 4 mM Na pyrophosphate, 0.2 mM each dNTP and 15 units AMV reverse transcriptase (Promega, Charbonnières, France). PCR (100 μl) contained 1/10 of the cDNA mixture, 50 pmoles each primer, 75 mM Tris-HCl, pH 9, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1 mM $MgCl_2$, 0.2 mM each dNTP, and 0.5 unit of Goldstar DNA polymerase (Eurogentec). Conditions were: 94°C, 20 seconds; 50°C or 60°C, 30 seconds; 72°C, 30 seconds. DynaWax (Eurogentec) was used to separate cDNA and primers from the DNA polymerase during pipetting and before the first step of denaturation. PCR used a Thermojet apparatus (Eurogentec) for 24 to 38 cycles (see Table 1). RT-PCR fragments were inserted into the pGEM-T vector (Promega); nucleotide sequences were determined using the T7 Sequencing kit (Pharmacia, Orsay, France) to confirm the identity of the amplified cDNA fragments.

Control PCRs were carried out on RNA samples without cDNA synthesis by reverse transcriptase. The primers for *HoxC-8*, *Cdx-1* and β-actin were chosen in consecutive exons; no amplified fragment corresponding to genomic DNA was detected by RT-PCR.

Table 1. Synthetic oligonucleotides and experimental conditions used in this study

Gene	Name/sequence	Species	Reference	PCR linearity*	Cycling number	Hybridization temp. (°C)	Length (bp)
Cdx-1	CDX1a GTAAGACTCGGACCAAGGACAAGTA CDX1b AACTGTGTGGGAGGCATGGGCTGCG	Rat	Freund et al., 1992	32-38	34	50	250
Cdx-2	CDX2a CCCAGCGGCCAGCGGCGAAACCTGT CDX2b TATTTGTCTTTTGTCCTGGTTTTC	Mouse	James et al., 1994	28-34	30	50	100
HoxA-4	HOXA4a ATGAAGTGGAAGAAAGACCACAAA HOXA4b TATAGACGAGGGAATGGGTGTGAA	Rat	Gorski et al., 1994	32-38	34	50	250
HoxA-9	HOXA9a GACAAGCCCCCCATCGATCCCAATAA HOXA9b TTGTTGATTTTCTTCATTTTCAT	Guinea pig	Rubin and Nguyen-Huu, 1991	30-36	32	50	220
HoxC-8	HOXC8a ATGTTTCCATGGATGAGACCCCA HOXC8b TTGTTCTCCTTTTTCCACTTCAT	Mouse	Le Mouellic et al., 1992	30-36	32	50	230
β-Actin	ACTa ATATCGCTGCGCTCGTCGTCGACAA ACTb AACACAGCCTGGATGGCTACGTACAT	Rat	Nudell et al., 1983	24-30	26	60	406
LPH	LPHa TGCCCCTGGCCAGGGAAGATGA LPHb ATCAATGAATCGAACATAGT	Rat	Duluc et al., 1991	24-30	26	50	350
SI	SIa TGGGATGATGGCCAATCTAAAGAT SIb ATAGGGATTGTGTTCTGGAGGAAA	Rat	Broyart et al., 1990	24-30	26	60	450

*Number of cycles corresponding to a linear increase of the amount of PCR product.

Semi-quantitative analysis of mRNAs by RT-PCR

The number of amplification cycles resulting in a linear increase of the amount of PCR product was established for each pair of primers (Table 1). In these conditions, 1 μl of PCR mixture was dotted on nitrocellulose filters (Schleicher and Schuell, Dassel, Germany), incubated for 4 hours at 42°C in 10% formamide, 5× SSC (1× SSC is 150 mM NaCl, 15 mM Na citrate), 0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.1% SDS, 100 μg/ml denatured calf thymus DNA, and then hybridized overnight with 1 pmole Dig-labeled oligonucleotide. Oligonucleotides (20 pmoles) were labeled with Dig-11-dUTP (Boehringer, Mannheim, Germany) at 37°C for 30 minutes using 20 units of Terminal-deoxynucleotidyl-transferase (Boehringer) in 250 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.5, 5 mM $CoCl_2$, 25 mg/ml bovine serum albumin. Filters were washed 2× 15 minutes in 1× SSC at 42°C, 15 minutes in 0.5× SSC at 42°C and 2 minutes in 0.5× SSC at 50°C. They were subsequently incubated at 42°C for 1 hour in blocking solution containing 0.3% Tween-20 and 5% skimmed milk in PBS (125 mM NaCl, 0.3 mM NaH_2PO_4, 7 mM Na_2PO4). Anti-Dig Fab labeled with peroxidase (Boehringer, dilution 1:1,000) was added to the blocking solution and the filters were incubated for 3 hours at room temperature. They were then rinsed 2× 15 minutes in blocking solution, 3× 15 minutes in PBS containing 0.3% Tween-20, incubated for 2 minutes with luminol (Amersham, Les Ulis, France), and exposed to Hyperfilm (Amersham). Results were quantified using an Imaging Densitometer (Bio-Rad), related to actin, and expressed as arbitrary units.

RESULTS

Homeobox gene expression along the intestinal AP axis

The longitudinal distribution of LPH and SI mRNAs, two enterocytic differentiation markers (Fig. 1A), and of five homeobox mRNAs, *HoxA-4*, *HoxA-9*, *HoxC-8*, *Cdx-1* and *Cdx-2*, were determined by RT-PCR (Fig. 1B). In adult rats, LPH and SI mRNA were, respectively, detected in the proximal jejunum, and in the proximal jejunum and distal ileum, as previously reported (Freund et al., 1990; Duluc et al., 1993). Homeobox gene patterns were also fully consistent with those already reported using northern blots or RNase protection assays (James and Kazenwadel, 1991; Freund et al., 1992): *HoxA-9* was restricted to the colon, *HoxC-8* was predominantly expressed in ileum, and *HoxA-4* was present at a very low level all along the adult intestine; *Cdx-1* and *Cdx-2* mRNAs exhibited an increasing gradient of

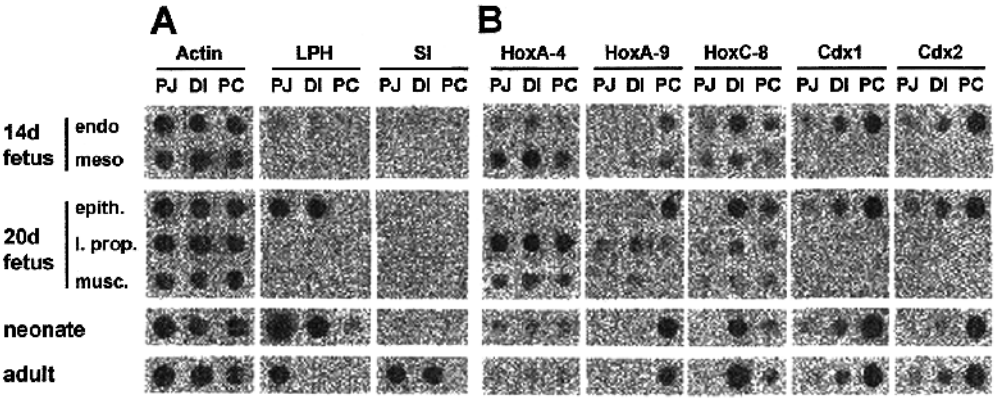

Fig. 1. Longitudinal expression of LPH and SI genes (A), and of the *HoxA-4*, *HoxA-9*, *HoxC-8*, *Cdx-1* and *Cdx-2* homeobox genes (B) in the proximal jejunum (PJ), distal ileum (DI) and proximal colon (PC) during development. β-actin was used as control. RT-PCR products were dotted on nitrocellulose, hybridized to specific Dig-labeled oligonucleotides, and revealed by chemiluminescent staining. The results correspond to a single series of samples representative of 3 or 4 experiments.

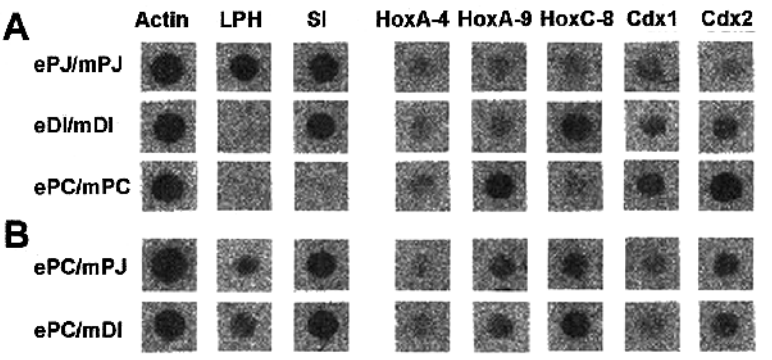

Fig. 2. Expression of β-actin, LPH, SI, *HoxA-4*, *HoxA-9*, *HoxC-8*, *Cdx-1* and *Cdx-2* genes in endoderm-mesenchyme associations grafted for 5 weeks to *nude* mice.
(A) Homotopic associations of jejunal (ePJ/mPJ), ileal (eDI/mDI) and colonic (ePC/mPC) anlagen;
(B) heterotopic associations comprising colonic endoderm and either jejunal (ePC/mPJ) or ileal (ePC/mDI) mesenchyme. Experimental details as in Fig. 1.

concentration along the intestinal AP axis. The data confirm the region specific distribution of homeobox mRNAs along the intestinal AP axis at the adult stage and validate the RT-PCR method used in this study.

In the fetus, the three *Hox* genes *HoxA-4*, *HoxA-9* and *HoxC-8* were expressed in the endoderm of 14-day fetuses and in epithelial cells of 20-day fetuses: the level of *HoxA-4* was low all along the AP axis; *HoxA-9* transcripts were not detected in the fetal small intestinal endoderm/epithelium, while present in the colon; *HoxC-8* mRNA was expressed at a relative high level in ileum and less in the colon. Notably, the longitudinal patterns observed in the fetus were superimposable on those obtained in adult animals, suggesting that epithelial cells are the primary source of *HoxA-9* and *HoxC-8* mRNAs in the adult intestine (James and Kazenwadel, 1991). *Hox* genes were also expressed in the mesenchyme and its derivatives: the lamina propria and muscle cell layers; they were more abundant in the lamina propria than in muscles, consistent with a primary role attributed to lamina propria cells in epithelial-mesenchymal interactions (Haffen et al., 1983; Simon-Assmann and Kedinger, 1993). In contrast to the *Hox* genes, *Cdx-1* and *Cdx-2* transcripts were restricted to fetal endoderm and epithelial cells, except for a very faint expression of *Cdx-2* in the colonic mesenchyme at the earliest fetal stage analyzed here (14 days). The latter observation could be related to the finding of Cdx-2 protein in the paraxial mesenchyme of 9.5-day fetal mice (Beck et al., 1995). The increasing gradient of expression of *Cdx-1* and *Cdx-2* along the intestinal AP axis of adult animals was already established in the fetus. As expected, LPH mRNA was restricted to the small intestinal epithelial cells, whereas SI mRNA was undetectable at the fetal stages.

Differentiation of the intestinal epithelium in homotopic and heterotopic grafts

Intestinal anlagen originating from the proximal jejunum (PJ), distal ileum (DI), and proximal colon (PC), were dissected out from 14-day fetuses to dissociate the endoderm from the mesenchyme. Homotopic re-associations comprising PJ, DI or PC endoderm and mesenchyme (respectively, designated ePJ/mPJ, eDI/mDI, and ePC/mPC), and heterotopic associations comprising PC endoderm and small intestinal mesenchyme (ePC/mPJ and ePC/mDI) were implanted for 5 weeks under the skin of *nude* mice. During this time, implants recapitulate intestinal development up to a stage that reproduces the typical adult differentiation. Morphological and immunohistological analyses of the xenografts have previously been reported (Duluc et al., 1994).

SI and LPH mRNA expression was examined by RT-PCR as marker of epithelial cell differentiation. Homotopic associations phenotypically developed according to their regional origin along the intestinal AP axis (Fig. 2A): indeed, both types of small intestinal grafts (ePJ/mPJ and eDI/mPI) exhibited SI mRNA, in contrast to colonic implants (ePC/mPC); LPH mRNA was present in jejunal (ePJ/mPJ), though absent in ileal associations (eDI/mDI), showing that the functional regionalization occurring normally at weaning along the jejuno-ileum was recapitulated under the grafting condition. In heterotopic associations of colonic endoderm and small intestinal mesenchyme (ePC/mPJ and ePC/mDI), heterodifferentiation of the colonic epithelium to a small intestinal-like phenotype was demonstrated by the presence of SI and LPH mRNAs (Fig. 2B), two transcripts selectively expressed in the small intestine of adult rodents.

Homeobox gene expression in homotopic and heterotopic intestinal grafts

The overall patterns of expression of *HoxA-9* and *HoxC-8* were retained in homotopic associations grafted for 5 weeks as compared to normal neonatal and adult tissues (Fig. 2A): *HoxC-8* mRNA was mainly detected in eDI/mDI, while *HoxA-9* displayed the highest level in colonic associations (ePC/mPC). However, both genes were also faintly transcribed in grafts corresponding to intestinal regions in which we normally failed to detect these mRNAs: a faint *HoxC-8* mRNA signal was present in ePJ/mPJ, and *HoxA-9* transcripts were found in both types of small intestinal samples (ePJ/mPJ and eDI/mDI). The typical anterio-posterior gradient of concentration of *Cdx-1* and *Cdx-2* was retained in ePJ/mPJ, eDI/mDI and ePC/mPC grafts. However, a slight decay of the amount of *Cdx-1* mRNA occurred in colonic associations (ePC/mPC), compared to normal colon (see also Fig. 4).

HoxA-9 and *HoxC-8* were expressed at an intermediary level in heterotopic associations in which colonic epithelium undergoes heterodifferentiation to small intestinal phenotype (Fig 2B), as compared to homotypic associations (Fig. 2A). This means that the expression of *HoxA-9*, normally restricted to the colon, was diminished in the colonic epithelium associated with small intestinal mesenchyme (ePC/mPJ and ePC/mDI vs ePC/mPC). However, this expression was still higher than that observed in the homotypic associations of small intestinal anlagen (ePC/mPJ and ePC/mDI vs ePJ/mPJ and eDI/mDI). *HoxC-8*, which is prominently expressed in ileal epithelium, was upregulated in grafts of colonic endoderm associated with ileal mesenchyme (ePC/mDI vs ePC/mPC).

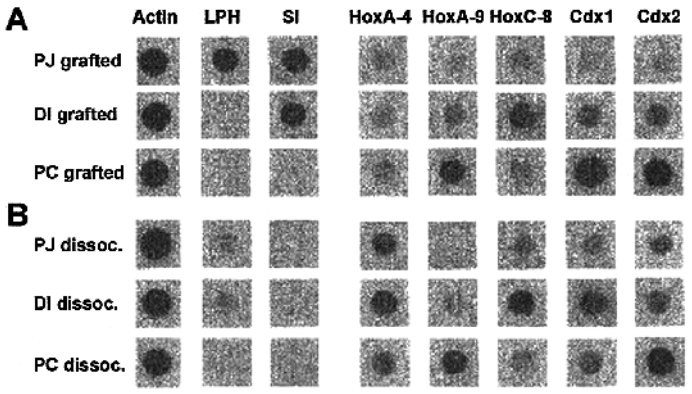

Fig. 3. Control grafts of undissociated tissue, and non-grafted homotypic associations. (A) mRNA expression of the β-actin, LPH, SI, *HoxA-4*, *HoxA-9*, *HoxC-8*, *Cdx-1* and *Cdx-2* in jejunal (PJ), ileal (DI) and colonic (PC) anlagen grafted for 5 weeks to nude mice without previous dissociation of the tissue. (B) mRNA expression in homotopic associations of fetal anlagen maintained in vitro for 16 hours on gelified medium. Experimental details as in Fig. 1.

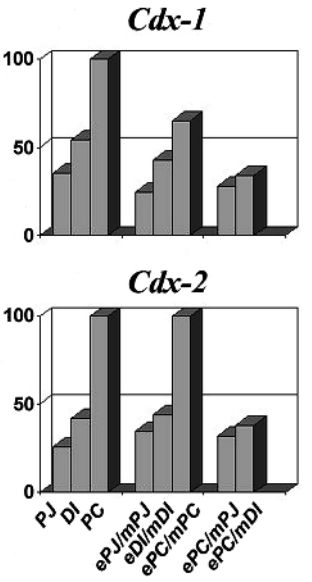

Fig. 4. Semi-quantitative analysis of *Cdx-1* (A) and *Cdx-2* (B) mRNA expression along the intestine of adult rats (PJ, DI and PC), in grafts of homotopic associations (ePJ/mPJ, eDI/mDI, ePC/mPC), and in heterotopic grafts comprising colonic endoderm and either jejunal or ileal mesenchyme (ePC/mPJ, ePC/mDI). β-actin was used as internal standard. Results are expressed as arbitrary units. 100 represents the value obtained for *Cdx-1* or *Cdx-2* mRNAs in the grafted proximal colon (PC).

Yet, *Hox C-8* mRNA was also detected in the associations of colon endoderm with jejunal mesenchyme, although this gene is only faintly detected in the homotypic jejunal associations (ePC/mPJ vs ePJ/mPJ). Finally, it should be emphasized that, compared to the control ePC/mPC grafts, the levels of *Cdx-1* and *Cdx-2* were diminished when colonic endoderm was induced to differentiate to a small intestinal-like phenotype in association with small intestinal mesenchyme (Fig. 2B). For *Cdx-1*, this decay was greater than that observed in homotypic colonic associations (Figs 2 and 4).

Influence of tissue re-association and grafting upon homeobox gene expression

We have shown that changes in the expression of homeobox genes occur in heterotopic associations of colon endoderm and small intestinal mesenchyme. Changes occur even with homotypic associations, in the small intestinal (*HoxA-9* and *HoxC-8*) and colonic anlagen (*Cdx-1*). Therefore, we have investigated the influence (i) of grafting conditions per se; and (ii) of the step of tissue dissociation-association, upon the expression of homeobox genes. For this purpose, RT-PCR were carried out on 5-week xenografts of intact fetal intestinal anlagen (non-dissociated PJ, DI and PC), and on endoderm-mesenchyme associations allowed to assemble in vitro on gelified medium, without implantation in *nude* mice. In both cases, SI and LPH expression was consistent with the normal patterns established, respectively, at the fetal and adult stages (compare Fig. 3 and Fig. 1A). LPH mRNA was faintly expressed in homotopic associations of small intestinal anlagen maintained 16 hours on gelified medium, though absent in colonic samples; SI mRNA was not detected at this stage. In 5-week grafts of intact intestinal segments, SI mRNA was present in both PJ and DI, whereas LPH mRNA was restricted to PJ; none of these transcripts appeared in PC.

The increasing gradient of expression of *Cdx-1* and *Cdx-2* along the intestinal tract was recapitulated in intact PJ, DI and PC grafted 5 weeks (Fig. 3A). The result with *Cdx-1* contrasts with the diminished expression of this gene in homotypic PC associations (see also Fig. 4). *HoxA-9* and *HoxC-8* were preferentially expressed in PC and DI, respectively, consistent with the normal longitudinal pattern of these genes. However, a low amount of *HoxA-9* mRNA was also detected in small intestinal implants (PJ and DI), and *HoxC-8* was faintly expressed in PJ (Fig. 3A). This indicates that grafting per se slightly up-regulated *HoxA-9* and *HoxC-8* in small intestinal regions in which the expression of these genes was not normally detected.

In the samples which have been dissociated, re-associated but not grafted (Fig. 3B), the expression patterns of *Cdx-2*, *HoxA-4*, *HoxA-9* and *HoxC-8* were identical to those obtained in the normal intestine at the same stage (compare Figs 1B and 3B). However, *Cdx-1* expression was lower in the colonic samples consistent with the decay observed in colonic homotypic grafts (see also Fig. 2A). This indicates that the process of endoderm-mesenchyme dissociation and subsequent re-association causes a decline of *Cdx1* expression in the colon.

Effects of fibroblasts upon the expression of *Cdx-1* and *Cdx-2*

The previous results suggest that mesenchymal cells can alter the expression pattern of homeobox genes in the intestinal epithelium, in particular for *Cdx-1* and *Cdx-2*. To substantiate this hypothesis, we have used two cloned rat fibroblastic cell lines, A1:F1 and F1:G9, which support distinct patterns of morphogenesis and differentiation of bowel epithelium (Fritsch et al., 1997). When associated with fetal jejunal endoderm and grafted in the coelomic cavity of chicken embryos, the F1:G9 line supports typical small intestinal development of crypts and villi, whereas the A1:F1 line supports only glandular morphogenesis (Fig. 5A). RT-PCR using specific primers revealed a high level of LPH mRNA in grafts of endoderm with F1:G9 cells but only a weak expression in those with A1:F1 cells (Fig. 5B). This corroborates our previous immunocytochemical observations (Fritsch et al., 1997). The measurement of *Cdx-1* and *Cdx-2* mRNA by RT-PCR demonstrated that transcripts for both these genes were more abundant in grafts of the glandular phenotype (associated with A1:F1) than those developing the typical crypt-villous architecture (associated with F1:G9) (Fig.

5B). These results demonstrate that the levels of *Cdx-1* and *Cdx-2* homeobox gene expression correlate with the phenotype of the intestinal epithelium, and are dependent on inductive properties of the surrounding fibroblasts.

DISCUSSION

The restricted expression of *Hox* genes in the visceral mesoderm of *Drosophila* (Bienz, 1994, and references therein) and chicken (Yokouchi et al., 1995; Roberts et al., 1995) has suggested a role for these genes in intestinal morphogenesis. Yet, many of the functional regions along the intestinal AP axis do not correspond to clear anatomical boundaries (Murakami et al., 1994). The *HoxA-4*, *HoxC-8* and *HoxA-9* patterns reported here along the fetal rat intestinal mesenchyme are consistent with the *Hox* code established in mesoderm derivatives in the fetus (McGinnis and Krumlauf, 1992). To test the hypothesis that homeobox genes are also involved in patterning and cell fate of the intestinal endoderm/epithelium, as suggested by their specific longitudinal distribution, we have analyzed the expression of candidate homeobox genes in two xenograft models in which the epithelial cell fate can be manipulated by association with heterotopic mesenchyme or fibroblastic cell lines (Duluc et al., 1994; Fritsch et al., 1997).

The main result of this study concerned the expression of *caudal*-related *Cdx-1* and *Cdx-2* genes. Both transcripts were more abundant in the normal colon than the small intestine, and both were dowregulated to a level similar to that found in the small bowel when colonic epithelium heterodifferentiated to a small intestinal-like phenotype. In addition, both mRNAs were less abundant in grafts composed of fibroblasts that trigger small intestinal-like differentiation, than in grafts with fibroblasts supporting colonic-like glandular morphogenesis. However, some of the reduced expression of *Cdx-1* in the heterodifferentiated colonic epithelium resulted from the step of endoderm/mesenchyme dissociation. The correlation established between the cell fate of the intestinal epithelium and the level of *Cdx-1* and *Cdx-2* expression suggests an involvement of these homeobox genes in visceral endoderm patterning and/or cytodifferentiation. This is consistent with the role attributed to *Cdx-1* during early development of skeletal structures (Subramanian et al., 1995), and to *Cdx-2* in patterning extra-embryonic membranes (Beck et al., 1995). It could also be related to the finding that *Cdx-2* is downregulated in colon cancers cells which are often shifted toward a small intestinal-like phenotype (Zweibaum et al., 1984; Wiltz et al., 1991; Ee et al., 1995).

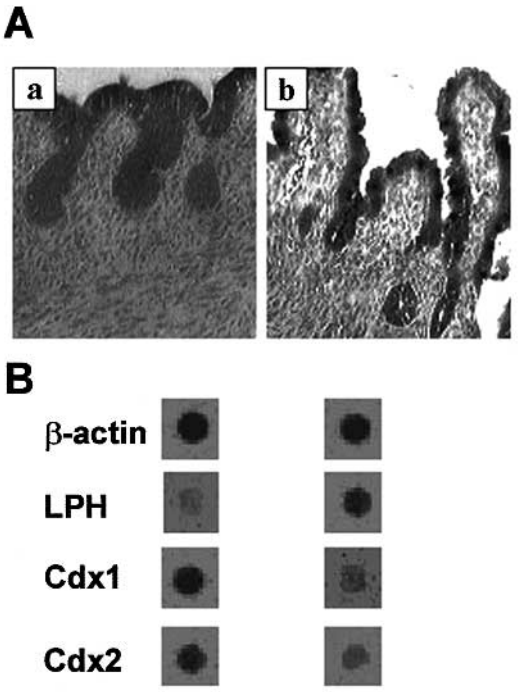

Fig. 5. Homeobox gene expression in jejunal endoderm associated with fibroblastic cell lines and grafted for 10 days in the chick coelomic cavity. (A) Morphological development of fetal jejunal endoderm associated to A1:F1 (a) or F1:G9 fibroblasts (b). Periodic acid-Shiff staining has been performed on 5 μm cryosections. ×20. (B) mRNA expression of β-actin, LPH, *Cdx1* and *Cdx2* genes in grafts comprising A1:F1 or F1:G9 fibroblasts. Experimental details as in Fig. 1.

It appears from this study that *Cdx-1* and *Cdx-2* levels in the intestinal epithelium are dependent on cellular interactions with underlying mesenchymal cells. Several molecules may be involved in epithelial-mesenchymal interactions in the gut: for instance growth factors of the TGFβ or Wnt families have been reported to be secreted by the visceral mesoderm (Capovilla et al., 1994; Graba et al., 1995). We have also shown in colon cancer cells in culture that *Cdx-1* and *Cdx-2* expression can be modulated by extracellular matrix components which are produced in a coordinate complementarity by both epithelial and mesenchymal cells (Kedinger et al., 1997; O. Lorentz and J.-N. Freund, unpublished data). Subsequent to the control exerted by the mesenchyme, the way by which *Cdx-1* and *Cdx-2* regulate intestinal cell fate is not clear: it is already established that the Cdx-2 protein acts directly as a transcription factor of typical enterocytic differentiation markers (Suh et al., 1994; Suh and Traber, 1996; Lambert et al., 1996; Lee et al., 1996; Drummond et al., 1996); alternatively *Cdx-1* and/or *Cdx-2* may also act indirectly through the control of other regulatory genes. For instance *Cdx-1* regulates *Hox* genes during axial skeleton development (Subramanian et al., 1995), and *Hox* gene expression has be shown to be altered by *Cdx2* overexpression in colon cancer cells in culture (Lorentz et al., 1996).

The results obtained with *Hox* genes are not as clear as those of the *caudal*-related genes. As one would expect, the colonic *HoxA-9* gene was downregulated when colon endoderm heterodifferentiated to a small intestinal-like phenotype; however this decrease did not reach the very low level observed in small intestinal grafts. As far as the *HoxC-8* gene is concerned, this was upregulated in heterotopic associations comprising colonic endoderm and ileal mesenchyme, consistent with a shift from colonic toward ileal differentiation. However, when associated with jejunal mesenchyme *HoxC-8* was also upregulated, inconsistent with the faint expression of these genes in homotypic associations of jejunal endoderm and mesoderm. *HoxC-8* knock out mice have not been reported to have an intestinal phenotype (Le Mouellic et al., 1992); nevertheless, changing the anterior boundary of expression of this gene results in stomach dismorphology, suggesting a role in gastrointestinal development (Pollock et al., 1992). Several hypotheses can account for the ambiguous results obtained with *Hox* genes.

First, it is most likely that additional genes to *HoxA-9* and *HoxC-8* are involved in the differentiation of gut epithelium (James and Kazenwadel, 1991), including for instance paralogue homeobox genes; functional interactions between paralogues have been reported in segmented organs (Condie and Capecchi, 1994). Second, translational regulation may occur, so that the amount of homeoprotein would not correlate with the amount of mRNA (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Third, *Hox* modifications may affect a limited population of epithelial cells (crypt or villus cells), sufficient to generate phenotypic changes but not resulting in clear differences in the overall level of gene expression in the whole sample.

In conclusion, we have established by changing epithelial-mesenchymal interactions a relationship between the cellular fate of the intestinal endoderm and the level of expression of two homeobox genes, *Cdx1* and *Cdx-2*. *HoxA-9* and *HoxC-8* expression were also modified upon colonic to small intestinal-like heterodifferentiation, though part of this effect was a consequence of the grafting procedure. Taken together, these results are the first indication that homeobox genes participate in the control of positional information and/or cell differentiation in the intestinal epithelium. They also show that expression of the *Cdx-1* and *Cdx-2* homeobox genes is influenced by epithelial-mesenchymal cell interactions.

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