

Fetal Endoderm Primarily Holds the Temporal and Positional Information Required for Mammalian Intestinal Development

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Abstract. In rodents, the intestinal tract progressively acquires a functional regionalization during postnatal development. Using lactase-phlorizin hydrolase as a marker, we have analyzed in a xenograft model the ontogenic potencies of fetal rat intestinal segments taken prior to endoderm cytodifferentiation. Segments from the presumptive proximal jejunum and distal ileum grafted in nude mice developed correct spatial and temporal patterns of lactase protein and mRNA expression, which reproduced the normal pre- and post-weaning conditions. Segments from the fetal colon showed a faint lactase immunostaining 8–10 d after transplantation in chick embryos but not in mice; it is consistent with the transient expression of this enzyme in the colon of rat neonates.

Heterotopic cross-associations comprising endoderm and mesenchyme from the presumptive proximal jejunum and distal ileum developed as xenografts in nude mice, and they exhibited lactase mRNA and protein expression patterns that were typical of the origin of the endodermal moiety. Endoderm from the distal ileum also expressed a normal lactase pattern when it was associated to fetal skin fibroblasts, while the fibroblasts differentiated into muscle layers containing

α -smooth-muscle actin. Noteworthy, associations comprising colon endoderm and small intestinal mesenchyme showed a typical small intestinal morphology and expressed the digestive enzyme sucrase-isomaltase normally absent in the colon. However, in heterologous associations comprising lung or stomach endoderm and small intestinal mesenchyme, the epithelial compartment expressed markers in accordance to their tissue of origin but neither intestinal lactase nor sucrase-isomaltase. A thick intestinal muscle coat in which cells expressed α -smooth-muscle actin surrounded the grafts.

The results demonstrate that: (a) the temporal and positional information needed for intestinal ontogeny up to the post-weaning stage results from an intrinsic program that is fixed in mammalian fetuses prior to endoderm cytodifferentiation; (b) this temporal and positional information is primarily carried by the endodermal moiety which is also able to change the fate of heterologous mesodermal cells to form intestinal mesenchyme; and (c) the small intestinal mesenchyme in turn may deliver instructive information as shown in association with colonic endoderm; yet this effect is not obvious with nonintestinal endoderms.

DURING organogenesis, the alimentary tract develops as a closed tube comprising the pseudostratified endoderm surrounded by a coat of mesodermal cells. Concomitantly, the consecutive regions along the antero-posterior (A-P)¹ axis acquire positional information defining the presumptive esophagus, stomach, small intestine, and the colon; lateral buds form the lung, liver, pancreas, and the gallbladder. In the intestine, organogenesis is achieved with the progressive re-organization of the endoderm into a monolayered epithelium that lines basal crypts

in which cells proliferate, and villi where the cytodifferentiation occurs. From this stage onwards, the epithelium is continuously renewed from the crypt stem cells. This process includes the emergence of cell diversity within the epithelium (the appearance of the absorptive, enteroendocrine, goblet, and the Paneth cells), and the proper differentiation of these cell types based on the selective expression of their respective differentiation markers (Dauça et al., 1990; Kedinger, 1994). It has been demonstrated for the last years that cell differentiation of the intestinal epithelium is tightly dependent on the establishment of functional interactions with the adjacent mesenchyme (for reviews see Haffen et al., 1989; Simon-Assmann and Kedinger, 1993). For instance, the effect of glucocorticoids on the expression of digestive enzymes is thought to be mediated by the mesenchyme (Kedinger et al., 1989). Moreover, changing the secretory pattern of laminin by the mesenchyme in an in vitro cell co-

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1. *Abbreviations used in this paper:* A-P, antero-posterior; DI, distal ileum; LPH, lactase-phlorizin hydrolase; PC, proximal colon; PJ, proximal jejunum; SI, sucrase-isomaltase.

culture system caused respectively a precocious induction and an alteration in the expression of differentiation markers at the apical side of the epithelium (Simo et al., 1992).

Rodents represent an attractive model for studying intestinal ontogeny because their intestine is "immature" at birth, and is subsequently subjected to functional re-differentiation at weaning (Henning et al., 1987; Dauça et al., 1990). Indeed, in suckling animals the small intestine expresses throughout its length the enzymes required for milk digestion (i.e., Lactase-Phlorizin Hydrolase, LPH; EC 3.2.1.23-62), whereas those needed for the assimilation of the solid food of adults appear or rise at weaning (i.e., Sucrase-Isomaltase, SI, EC 3.2.1.10-48). Noteworthy, at this stage the functional regionalization downwards the A-P axis of the small intestine becomes obvious (Tsuboi and Castillo, 1989). The rat colon also shows re-differentiation during postnatal development. It shares structure and functions with the small intestine in neonates, whereas the mucosa flattens a couple of days after birth and stops expressing digestive enzymes such as LPH (Helander, 1973; Foltzer-Jourdainne et al., 1989; Freund et al., 1990).

It has been assumed from the expression pattern of several digestive enzyme activities in isografts of fetal intestinal implants, that the timing of intestinal development is primarily directed by an autonomous program (Ferguson et al., 1973; Kendall et al., 1979; Montgomery et al., 1981). Yet, modulation of this program may occur by extrinsic factors such as hormones and nutrients (Kendall et al., 1977; Kedingner et al., 1983; Yeh and Holt, 1986; Henning, 1987; Duluc et al., 1992). Using markers whose patterns are fixed transcriptionally at the onset of expression during endodermal cytodifferentiation in fetuses (Sweester et al., 1988; Cohn et al., 1992), Rubin et al. (1992) have shown that the functional differences acquired at birth along the A-P axis of the intestine also depend on this autonomous ontogenic program.

In the studies conducted up to now to address the autonomous timing of intestinal development, fetal explants were taken after the onset of endodermal cytodifferentiation, at days 17–20 of gestation (Ferguson et al., 1973; Kendall et al., 1977; Montgomery et al., 1981). In addition, these studies did not approach the establishment of the functional regionalization that progressively emerges during postnatal development, in particular at weaning. Furthermore, it has not been investigated in mammals whether the temporal and positional information needed for intestinal ontogeny is dictated by the endoderm and/or by the mesenchyme (Rubin et al., 1992; Gordon, 1993). Therefore, the aim of the present work was to analyze: (a) the autonomous developmental potencies of the small intestine and of the colon taken from 14-d-old rat fetuses, when the endoderm is still undifferentiated; (b) the ability of the fetal transplants to follow a correct spatial and temporal development corresponding to both pre- and post-weaning conditions; and (c) the respective involvement of the endoderm and of the mesenchyme in the determination of the temporal and positional information. Because intestinal explants rapidly degenerate *in vitro*, we have taken advantage of the model of xenograft of fetal intestinal segments in nude mice, which allows developmental analysis for up to 6–8-wk after implantation and may thus go beyond the normal weaning period (Winter et al., 1991). In addition, xenografts were combined with the model of tissular association of heterologous endoderm and mesoderm to define the

specific role of these embryonic anlagen in the determination of the temporal and positional information. This study was mainly addressed with respect to LPH because this enzyme is an interesting marker of the temporal and spatial development of the intestine, whose expression undergoes differential regulations at the transcriptional, posttranscriptional, and posttranslational levels (Duluc et al., 1993). In rats, the onset of expression of small intestinal LPH mRNA and enzyme occurs concomitantly with the cytodifferentiation of the fetal endoderm between days 17–20 of gestation (Rings et al., 1992). A maximum is reached during the perinatal period. Subsequently, the tenfold decline of enzyme activity occurring progressively in suckling animals and at weaning operates despite the maintenance of a high level of mRNA (Freund et al., 1989). In addition, the longitudinal distribution of this transcript is modified at weaning in that the LPH mRNA selectively disappears in the distal ileum but remains abundant in the jejunum and in the proximal ileum throughout adulthood (Freund et al., 1991a). In the colon, transient lactase gene and enzyme expression is temporarily restricted to the few days following birth, when the colonic mucosa exhibits villi structures (Foltzer-Jourdainne et al., 1989; Freund et al., 1990).

Materials and Methods

Animals

Wistar rat fetuses were delivered by Caesarean section at day 14 of gestation (the existence of a vaginal plug was designated as day 0). Swiss athymic nude mice (*nu/nu*) (Iffa Credo, France), adult Wistar rats from our own breed, and chick embryos were used as hosts for the grafts.

Tissular Association and Grafting Experiments

The intestine of 14-d-old rat fetuses comprising the presumptive small intestine and the colon was removed under the dissecting microscope. The small intestine was divided in four segments of identical length. The first and fourth segments designated respectively as the proximal jejunum (PJ) and distal ileum (DI), as well as the proximal half of the colon (PC) were grafted under the dorsal skin of nude mice. Alternatively, the fetal endoderm was separated from the mesenchyme by incubation in a 0.03% collagenase solution (Boehringer, Mannheim, Germany) for 1 h at 37°C followed by mechanical dissociation (Gumpel-Pinot et al., 1978). The purity of the dissociated endoderm and mesoderm has been checked morphologically. Endoderm and mesenchyme originating from distinct regions along the intestinal tract were re-associated as described by Kedingner et al. (1981). The resulting associations were allowed to assemble overnight on a gelified culture medium containing 2 g/l bactoagar (Difco, Detroit, MI) in MEM/Ham-F12 (GIBCO BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (GIBCO BRL) and 10% chick embryo extract (Wolff and Haffen, 1952). The chick embryo extract was prepared by mechanical homogenization of 9–10-d-old embryos and clarified by centrifugation. The tissular associations were then grafted under the skin of nude mice. For some experiments, the endoderm of the presumptive DI was associated to skin fibroblasts prepared from 19-d-old rat fetuses as described by Kedingner et al. (1990). The presumptive stomach and lung were also taken from rat fetuses at day 14, the endoderm was isolated as for the small intestine, and the fundic or lung endoderm was associated with intestinal mesenchyme and grafted under the skin of nude mice. Occasionally, grafting was performed into the coelomic cavity of 3-d-old chick embryos above the omphalo-mesenteric vessels or under the kidney capsule of adult rats. Unless otherwise stated, the grafts were recovered two and four weeks after transplantation for individual analysis.

mRNA Accumulation Analysis

Cytoplasmic RNA was extracted from the grafts (0.01 to 0.07 g of tissue) according to the method described by Auffray and Rougeon (1980). RNA was separated by electrophoresis on 1% agarose, 17% formaldehyde gels,

transferred to nitro-cellulose filters, and hybridized simultaneously to P³²-labeled cDNA probes for LPH (Duluc et al., 1991) and β -actin under standard conditions in 50% formamid, 5 \times SSC (1 \times SSC is 0.15 mM sodium chloride, 0.015 mM sodium citrate), 0.1% sodium dodecyl sulfate, 0.02% polyvinylpyrrolidone, 0.02% ficoll, and 10% dextran sulfate for 16 h at 42°C (Freund et al., 1990, 1991a). The filters were washed twice for 15 min at room temperature in 2 \times SSC, 0.1% sodium dodecyl sulfate and twice for 15 min in 0.2 \times SSC, 0.1% sodium dodecyl sulfate at 60°C. The radioactivity retained on the filters was revealed by autoradiography.

Immunohistochemical Detection of Differentiation Markers

Grafted specimens were embedded in Tissue-Tek II (Miles Inc., Elkhart, IN) and immediately frozen in liquid freon placed in a nitrogen bath. The sections (5 μ m) were cut with a Slee cryostat and transferred to glass slides coated with 1% gelatine and 2% paraformaldehyde. The presence of specific proteins was analyzed by indirect immunofluorescent staining. Cryosections were incubated 2 h at room temperature with mouse monoclonal antibodies against rat lactase (Quaroni and Isselbacher, 1985) and sucrase (Hauri et al., 1980) at a 1:75 dilution in PBS, or with mouse monoclonals against the amino terminal decapeptide of α -smooth-muscle-actin (Sigma Chemical Co., St Louis, MO; dilution 1:400). Other sections were incubated with a 1:200 dilution of rabbit polyclonal antiserum directed against rat pulmonary surfactant apoprotein A (Sakai et al., 1992) or with a 1:100 dilution of rabbit polyclonal antiserum raised against the murine gastric pS2 protein (a generous gift from Dr. M-C. Rio, INSERM U184, Strasbourg, France). The anti-pS2 antiserum enlightens the supranuclear cytoplasm of the gastric mouse and rat surface and glandular mucous cells, which is in accordance with the results obtained by in situ hybridization (Lefebvre et al., 1993). The slides were rinsed three times with PBS and then incubated for 2 h with sheep anti-mouse-immunoglobulin or goat anti-rabbit-immunoglobulin antibodies diluted to 1:200 and labeled with fluorescein isothiocyanate (Institut Pasteur, Paris, France; Nordic Laboratories, Turnhout, Belgium). Primary antibodies were omitted for the con-

trols. The slides were mounted in glycerol/PBS/phenylenediamine and observed with an AxioPhot Microscope (Zeiss).

Results

Autonomous Development of the Fetal Rat Small Intestine Grafted in Nude Mice

Small intestinal segments originating from the presumptive PJ and DI were dissected out from 14-d-old rat fetuses, implanted under the dorsal skin of nude mice and harvested for analyses 2 or 4 wk after transplantation. 20/21 jejunal and 20/21 ileal implants developed into rounded vascularized segments showing the expected intestinal morphology with a central lumen, well organized villi and crypts, and typical muscular layers. When possible, RNA analysis and immunohistochemistry were performed on the same specimen. 2 wk after implantation, the segments originating from the presumptive PJ and DI exhibited a high amount of LPH mRNA and intense immunofluorescent staining of the villi borders with the monoclonal anti-LPH antibodies; nascent crypts were devoid of labeling (Fig. 1). When the grafting period was prolonged for two additional weeks, samples deriving from the presumptive PJ still contained abundant LPH mRNA although the immunofluorescent staining of lactase protein was less intense. However, in the grafted specimens originating from the presumptive DI, the LPH mRNA and the enzyme were absent. The functional development of the epithelium in the transplants of the PJ and DI was assessed

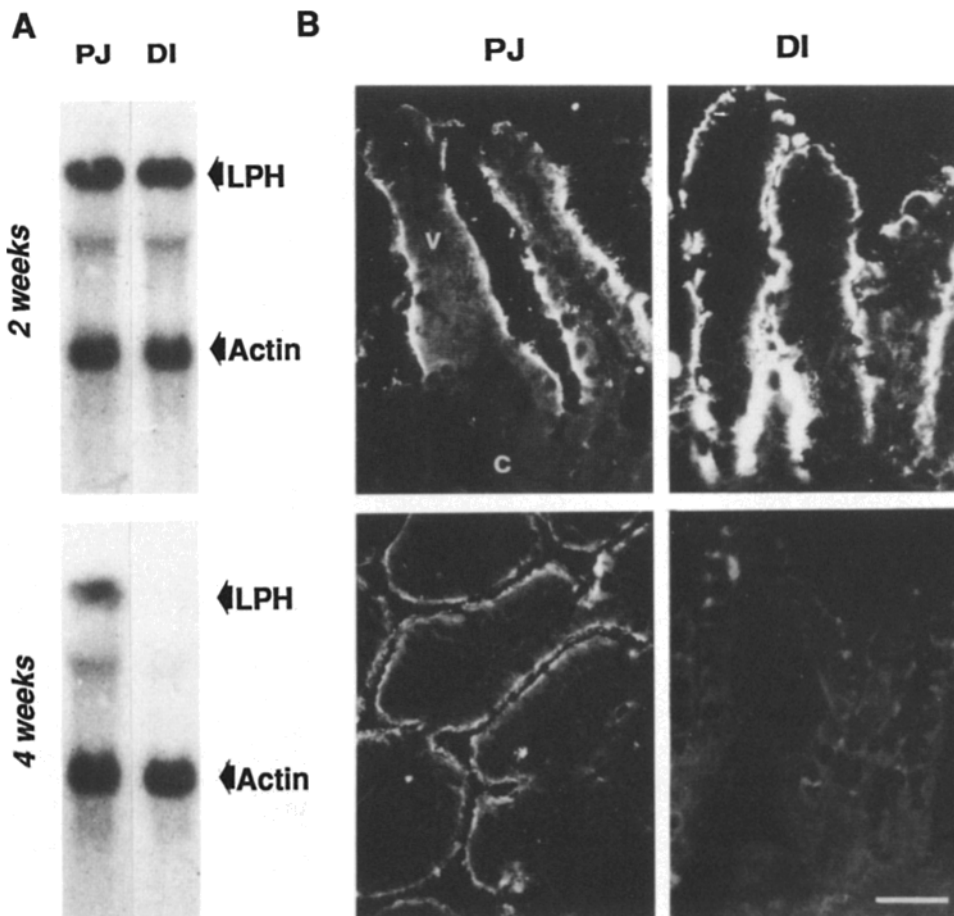


Figure 1. LPH mRNA and protein expression in xenografts of the presumptive PJ and the DI of fetal rats implanted in nude mice. LPH and β -actin mRNA accumulation (A), and immunofluorescent staining of the lactase protein (B) in intestinal segments originating from the presumptive PJ and DI of 14-d-old rat fetuses, transplanted for 2 wk (top) and 4 wk (bottom) under the skin of nude mice. 20 μ g of cytoplasmic RNA were loaded in each lane and hybridized simultaneously to the radiolabeled probes for LPH and β -actin. c, crypt; v, villus. Bar, 50 μ m.

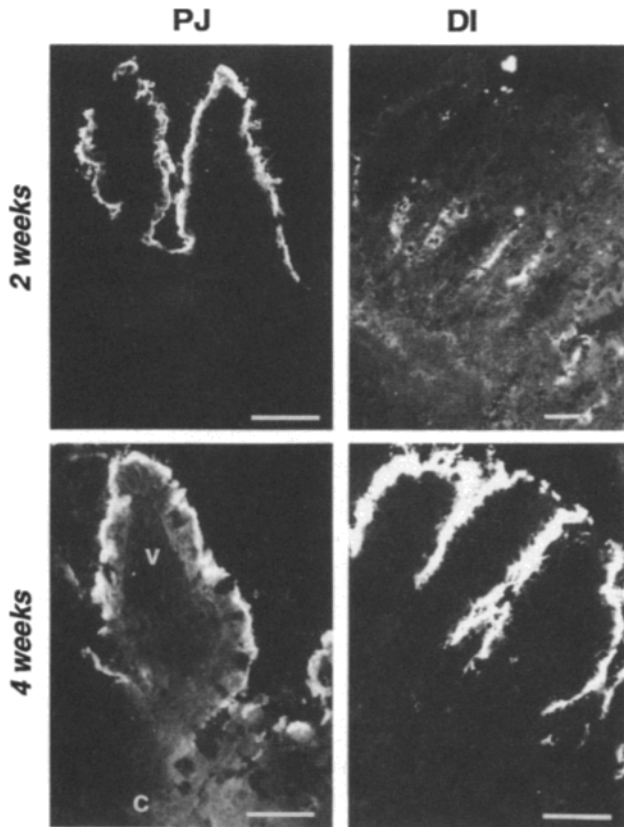


Figure 2. SI protein expression in xenografts of the presumptive PJ and DI of fetal rats implanted in nude mice. Immunofluorescent staining of the SI protein in intestinal segments originating from the presumptive PJ and DI of 14 day-rat fetuses, transplanted for 2 wk (top) and 4 wk (bottom) under the skin of nude mice. *c*, crypt; *v*, villus. Bars, 50 μ m.

using SI as marker because this protein is expressed over the whole length of the small intestine after weaning. In the grafting model in adult animals, precocious onset of SI expression has been reported (Ferguson et al., 1973), most likely as a consequence of the high level of endogenous glucocorticoids present in the hosts (Kendall et al., 1977; Keding et al., 1983; Yeh and Holt, 1986). As shown in Fig. 2, SI was already immunodetected in the 2-wk implants. Yet, at this stage all the enterocytes lining the villi were SI positive in the PJ, whereas SI-expressing enterocytes were confined to the villus base in the DI. This observation indicated that the onset of SI synthesis occurred according to an A-P progression. Indeed, in the DI implanted for 2 wk, unlike in the PJ, the resident SI nonexpressing enterocytes were not entirely replaced by the new population of cells synthesizing SI from the crypt mouth. 4 wk after transplantation, the whole surface of the villi was immunostained with anti-SI antibodies in the samples coming from the PJ and from the DI. As for LPH, the crypts were devoid of SI immunofluorescent labeling.

Altogether, these data provide evidence that small intestinal segments taken from rat fetuses prior to endodermal cytodifferentiation (and hence, prior to the onset of lactase expression) develop in a xenograft model and exhibit typical patterns of LPH mRNA and protein expression regarding to the position of the segments along the A-P axis of the small

intestine. Thus, the normal pre- and post-weaning conditions are reproduced despite the different hormonal status of the adult hosts compared with sucklings, and despite the absence of exogenous nutrients in the lumen of the transplants. In the glucocorticoid-rich environment of the adult host, the onset of expression of SI soon after transplantation mimics the precocious appearance of this enzyme, which occurs in suckling rats treated with glucocorticoids.

Autonomous Development of the Fetal Rat Colon Grafted in Nude Mice and in Chick Embryos

In the rat colon, a transient expression of lactase is obvious during the perinatal period when the mucosa exhibits a small intestinal-like structure. The question arose whether this transient phase results from an intrinsic developmental program or from a hormonal stimulation linked to birth. To try to answer this question, the presumptive PC was taken from 14-d-old rat fetuses and implanted for 6, 8, 10, and 12 d under the skin of nude mice. 17 of 23 of the implants developed. However none of them exhibited detectable levels of lactase. A faint and focal immunostaining with the anti-SI antibodies was observed at the apex of restricted groups of epithelial cells in two samples (not shown). When the grafting period was prolonged for 3 and 4 wk, LPH and SI could not be detected at the surface of the epithelium (see for instance Fig. 7 *d*).

In rats, the transient expression of lactase in the neonatal colon is inhibited by exogenous administration of hormones and growth factors (Foltzer-Jourdainne et al., 1989; Freund et al., 1990). To test whether the hormonal environment of the adult host may have prevented LPH expression in the implanted colon, the PC of fetal rats was grafted in the coelomic cavity of 3-d-old chick embryos. 11 out of 15 of the implants developed. No trace of lactase was found by immunohistochemistry for up to 8-d after implantation (Fig. 3 *a*). In three out of the four specimens grafted for 8 and 10 d, a faint and fuzzy LPH immunofluorescent staining was observed in the epithelium (Fig. 3 *b*). This signal was less polarized at the cellular apex of the grafts than in situ in the neonatal colon.

These results indicate that the fetal colon exhibits the transient postnatal small intestinal-like phase in the xenograft model in chick embryos, which suggests the autonomous nature of this process. Yet, the hormonal status of the host may interfere with this intrinsic program, as shown by the experiments performed in nude mice.

Prominent Role of the Endoderm in the Development of Small Intestinal Functions

Since the emergence of the regional specialization occurring in situ along the small intestine at weaning is reproduced in the xenograft model in nude mice, we have conducted experiments to investigate if the endoderm and/or the mesenchyme holds the positional information. For this purpose, intestinal segments from the presumptive PJ and DI of 14-d-old rat fetuses were dissected out, and the endoderm was dissociated from the mesenchyme. Jejunal endoderm was associated to ileal mesenchyme (ePJ/mDI) and ileal endoderm was associated to jejunal mesenchyme (eDI/mPJ). Both types of tissular association were grafted under the skin of nude mice. Absence of cross-contamination of epithelial

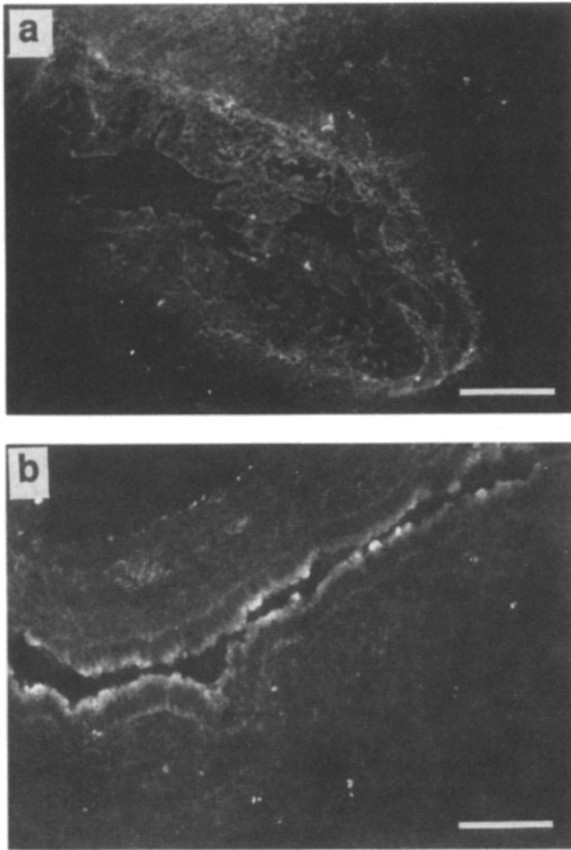


Figure 3. LPH protein expression in xenografts of the presumptive proximal colon (PC) of fetal rats implanted in chick embryos. Immunofluorescent detection of the LPH protein in intestinal segments originating from the presumptive PC of 14 day-rat fetuses implanted for 6 days (*a*) and 10 days (*b*) into the coelomic cavity of chick embryos. Bars correspond to 100 μm for (*a*) and 50 μm for (*b*).

cells in the mesodermal source has already been assessed (Kedinger et al., 1986). Histological examination further established the absence of mesodermal cellular cross-contamination in the dissociated endoderm (Fig. 4, *a* and *b*).

11 out of 18 ePJ/mDI implants and 16 out of 18 eDI/mPJ implants developed as rounded segments displaying a small intestinal structure with villi and crypts (Fig. 4 *c*). The tissular associations were analyzed for LPH expression 2 and 4 wk after implantation. As illustrated in Fig. 5, the LPH mRNA and the brush border membrane lactase were abundant in the ePJ/mDI and in the eDI/mPJ associations 2 wk after implantation. The situation was different 4 wk after implantation. Indeed, the transcript remained at a high level in the ePJ/mDI associations despite the decline of enzyme immunolabeling. However, in the eDI/mPJ associations grafted for 4 wk, the LPH mRNA and the protein could no longer be detected. As in the case of intact fetal intestinal segments, SI expression was obvious in both types of tissular association 2 and 4 wk after implantation (not illustrated). These results show that heterotopic associations between endodermal and mesenchymal anlagen originating from distinct regions along the fetal small intestine develop and express LPH mRNA and protein patterns that are typical of the regional origin of the endodermal moiety.

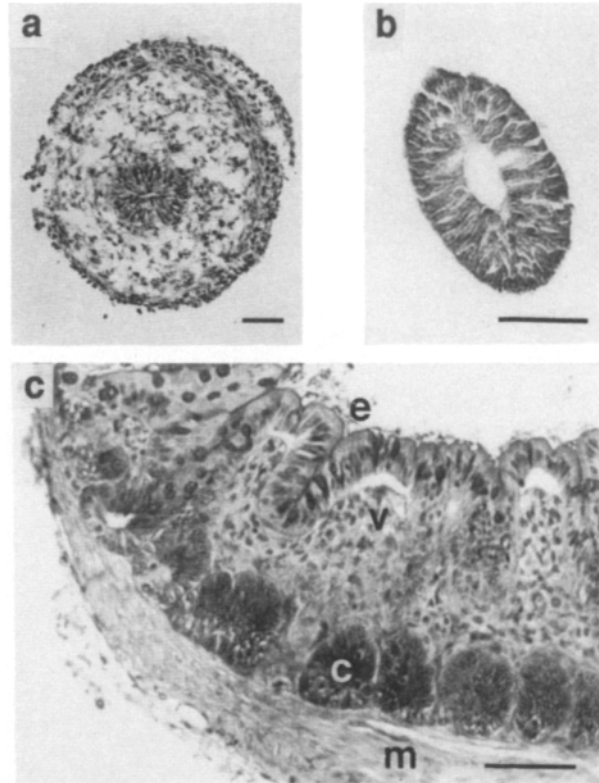


Figure 4. Morphological aspect of the PJ of 14-d-old rat fetuses (*a*), of the dissociated endoderm (*b*), and of ePJ/mDI associations developed for 2 wk in nude mice (*c*). PAS-Schiff staining has been performed on 5- μm sections. In *b*, note the absence of cellular cross-contamination of the endoderm by mesodermal cells. In *c*, the ePJ/mDI association developed typical small intestinal structures with villi (*v*) and crypts (*c*) lined by a single epithelium (*e*) composed of absorptive cells and goblet cells. A thick muscle coat (*m*) is surrounding the mucosa. Bar, 50 μm .

To get further insight into the autonomous potential of the endoderm in determining the temporal and positional information, endoderm from the presumptive DI of 14-d-old rat fetuses was separated from the mesenchyme, associated to nonintestinal mesodermal cells, i.e., skin fibroblasts prepared from 19-d-old rat fetuses, and grafted under the skin of nude mice. Only 2 out of 11 of these associations developed with a very slow growing rate. Due to the difficulties encountered in these experiments to obtain a consistent development of the grafts under the skin of nude mice, associations comprising fetal skin fibroblasts and DI endoderm were implanted under the kidney capsule of isogenic rats. However the results were not markedly improved as only 3 implants out of 11 did develop under this condition. Because of the very small size of the 5 heterologous associations that developed under the skin of nude mice or under the kidney capsule of isogenic rats, the samples were exclusively analyzed by immunohistochemistry, 4–8 wk after implantation. They exhibited a monolayered epithelium with villi and crypt, while muscle cell layers expressing α -smooth-muscle actin had differentiated from the fibroblasts. SI expression was obvious in all cases (Fig. 6, *b* and *d*). However, in two samples, LPH-immunostaining appeared at the border of the villi (Fig. 6 *a*), whereas the staining was absent in the 3 other

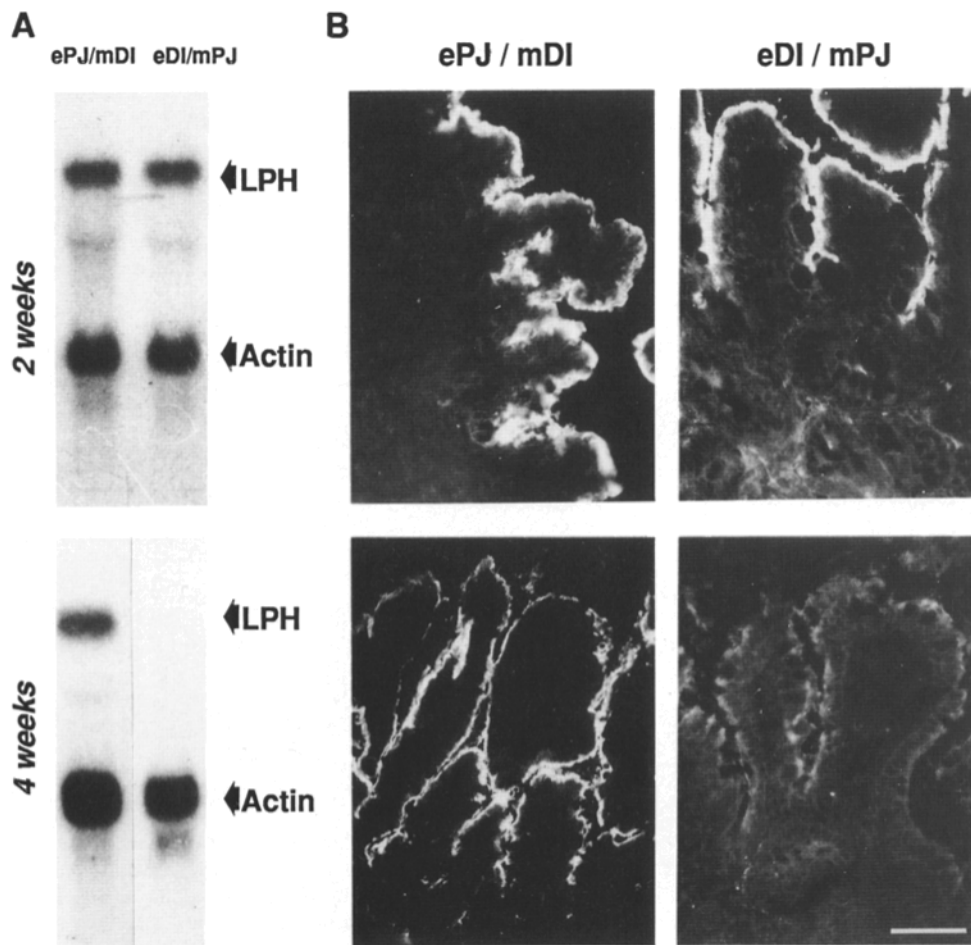


Figure 5. LPH mRNA and protein expression in epithelial-mesenchymal cross-associations of the presumptive PJ and DI of fetal rats implanted in nude mice. LPH and β -actin mRNA accumulation (**A**), and immunofluorescent staining of the lactase protein (**B**) in associations comprising the PJ endoderm and the DI mesenchyme (*ePJ/mDI*) or the DI endoderm and the PJ mesenchyme (*eDI/mPJ*) of 14-d-old rat fetuses. The associations were implanted for 2 wk (*top*) and 4 wk (*bottom*) under the skin of nude mice. 20 μ g of cytoplasmic RNA were loaded in each lane and hybridized simultaneously to the radiolabeled probes for LPH and β -actin. Bar, 50 μ m.

specimens (Fig. 6 *c*). Taking into account the very slow growing rate of the implants, the results concerning LPH in these heterologous DI endoderm/skin fibroblasts associations may correspond to those obtained with native fetal DI

grafted, respectively, for 2 and 4 wk under the skin of nude mice. Thus, the data suggest that the DI endoderm is able to express nearly normal expression patterns of LPH and SI when it lies in contact with heterologous nonintestinal

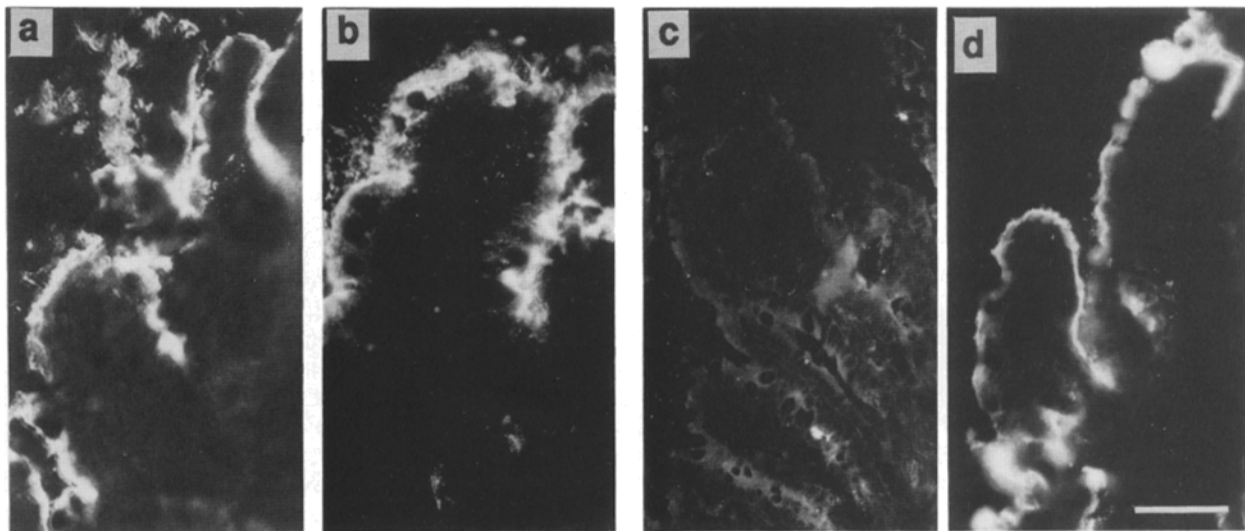


Figure 6. LPH and SI protein expression in heterologous associations of skin fibroblasts and DI endoderm of fetal rats implanted in nude mice. Immunofluorescent staining of lactase (*a* and *c*) and sucrase (*b* and *d*) proteins in two distinct specimens (respectively, *a* and *b*, and *c* and *d*) of heterologous associations comprising skin fibroblasts and DI endoderm of 14-d-old rat fetuses grafted for 6–8 wk in nude mice. Bar, 50 μ m.

mesenchyme. Yet optimal conditions for growth and development have not been found.

Heterodifferentiation of the Colonic Endoderm But Not of Nonintestinal Endoderms in Association with Small Intestinal Mesenchyme

We have demonstrated above that the endoderm of 14-d-old rat fetuses is the primary holder of the positional information along the small intestine. Yet, an inductive role of the mesenchyme may have been masked in these experiments, because of the prominent intrinsic potency of the endoderm moiety to follow its own program of development. Therefore, we have analyzed the fate of tissular associations comprising small intestinal mesenchyme and a heterologous endoderm either of intestinal origin, i.e. the colon, or of nonintestinal origin, i.e., the stomach and the lung.

Endoderm isolated from the presumptive PC of 14-d-old rat fetuses was associated either to PJ mesenchyme (ePC/mPJ) or to DI mesenchyme (ePC/mDI) and the resulting heterotopic associations were grafted under the skin of nude mice. They were analyzed by immunohistochemistry for the presence of LPH and SI proteins 6, 8, 10, 13, 15, 21, 30 and 60 days after implantation. 25 out of 30 of the ePC/mPJ associations and 23 out of 30 of the ePC/mDI associations developed. Noteworthy, they showed a typical small intestinal morphology with well differentiated villi and crypts, instead of the typical morphology of the adult colon characterized by the presence of a flat mucosa and deep glands. Up to 15 d after implantation, neither LPH nor SI proteins could be detected immunohistochemically in the monolayered epithelium (Fig. 7 *a*). However 5 out of 9 of the ePC/mPJ associations grafted for 21, 30, and 60 d exhibited SI positive cells at their apical side. A similar observation was obvious in 9 out of 10 ePC/mDI associations after the same periods of im-

plantation (Fig. 7 *b*). Despite the emergence of SI in the associations comprising colonic endoderm and small intestinal mesenchyme, LPH remained absent whatever the grafting period (Fig. 7 *c*). In control experiments, we found that intact fetal PC implanted for 3 and 4 wk under the skin of nude mice did not express SI, indicating that the appearance of this enzyme in colonic endoderm/small intestinal mesenchyme associations 3–4 wk after implantation did not result from the emergence of a small intestinal-like phenotype in the intact colon placed for long term in the xenograft condition (Fig. 7 *d*).

The presumptive lung and fundic endoderms taken from 14-d-old fetal rats were associated to the presumptive PJ mesenchyme and grafted under the skin of nude mice for 2 and 4 wk. The results obtained were identical whatever the grafting period. 9 out of the 10 associations comprising lung endoderm and PJ mesenchyme (eL/mPJ) showed a mixed structure which exhibited lung and intestine features. A monolayered epithelium lined protruding structures that resembled intestinal villi (Fig. 8, *a–c*). Yet, the epithelium abundantly expressed the pulmonary surfactant apoprotein A (Fig. 8 *a*) but neither LPH nor SI (Fig. 8 *b*). A thick muscle coat, like in the intact intestine, surrounded the grafts, and showed immunofluorescent staining with anti- α -smooth-muscle actin antibodies (Fig. 8 *c*). 10 out of the 12 associations comprising stomach endoderm and PJ mesenchyme (eS/mPJ) developed and exhibited a glandular structure characteristic of the gastric mucosa. The central lumen contained acidic secretions. The surface and glandular epithelium expressed the gastric pS2 protein but was devoid of LPH and SI, as shown by immunofluorescent staining with the respective antibodies (Fig. 8, *d* and *e*). A similar behavior was obtained when stomach endoderm was associated to mesenchyme originating from the presumptive DI (not shown).

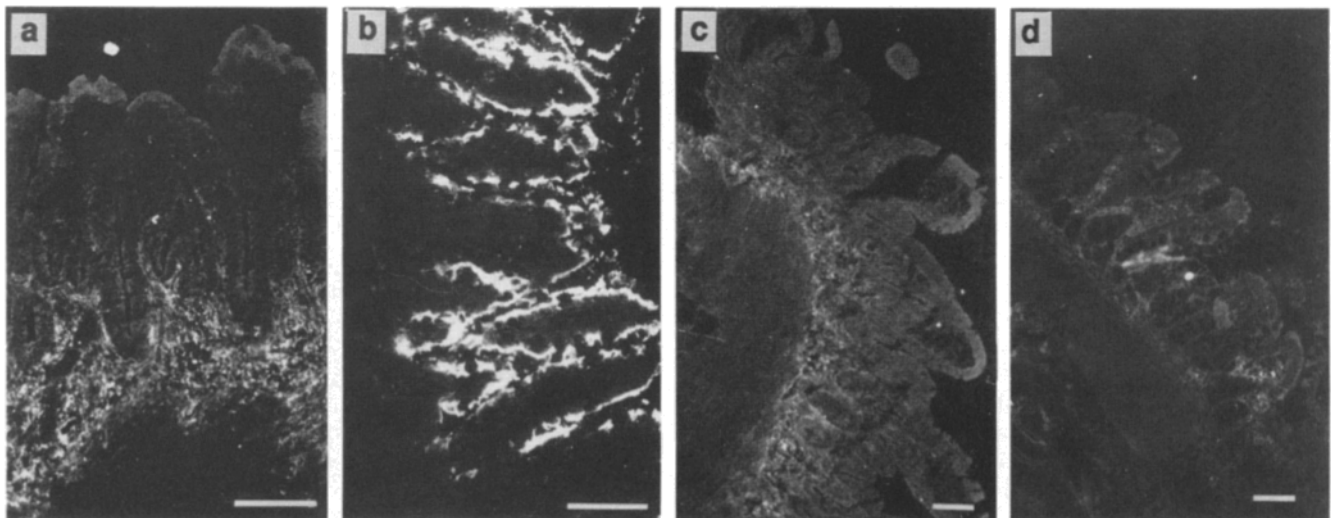


Figure 7. LPH and SI protein expression in heterologous associations of PC endoderm and DI mesenchyme of fetal rats implanted in nude mice. Immunofluorescent staining of sucrase (*a* and *b*) and lactase (*c*) proteins in heterologous associations comprising PC endoderm and DI mesenchyme of 14-d-old rat fetuses grafted for 2 wk (*a*) and 4 wk (*b* and *c*) in nude mice. The faint labeling found in *a* and *c* reveals a reaction of the lamina propria due to the presence of immunocompetent invading host cells with the second labeled antibody. *d* shows the absence of specific immunofluorescent staining of SI in intact colonic segments from fetal rats transplanted for 4 wk under the skin of nude mice. Bar, 100 μ m.

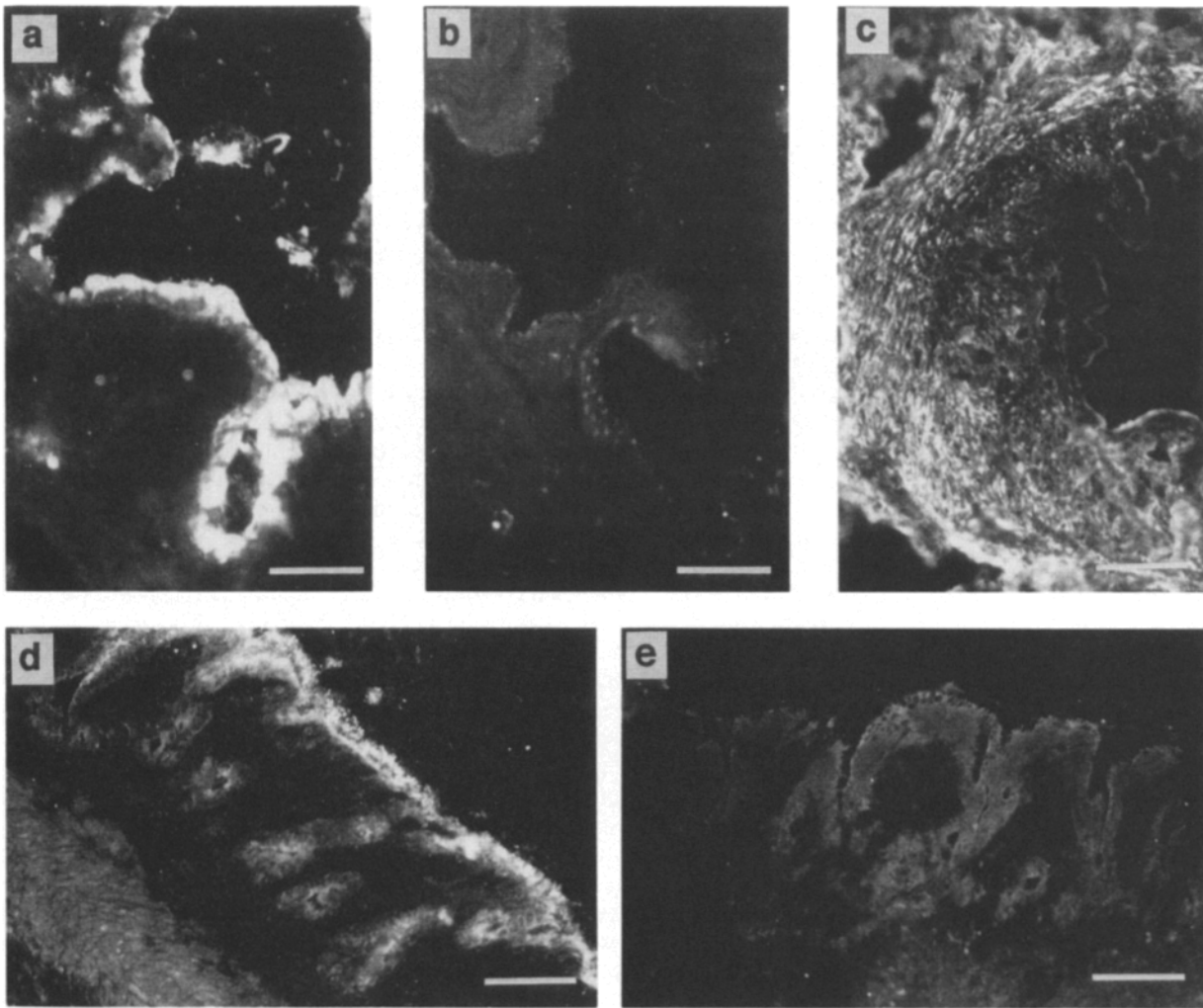


Figure 8. Expression of differentiation markers in heterologous associations of PJ mesenchyme and lung or stomach endoderm of fetal rats implanted in nude mice. Heterologous associations comprising lung endoderm and PJ mesenchyme (eL/mPJ; *a-c*) or fundic endoderm and PJ mesenchyme (eS/mPJ; *d* and *e*) were grafted for 4 wk in nude mice. Immunofluorescent staining was performed using primary antibodies against pulmonary surfactant apoprotein A (*a*), SI (*b* and *e*), α -smooth muscle actin (*c*) and pS2 (*d*). Bars: (*a*, *d*, and *e*) 50 μ m; and (*b* and *c*) 100 μ m.

Discussion

In rodents, the functional regionalization along the A-P axis of the intestinal tract essentially occurs at two distinct developmental stages: first, a proper identity progressively emerges in the small intestine and in the colon during endoderm cytodifferentiation around the perinatal period; second, the functional regionalization of the small intestine characteristic of the adult condition becomes obvious at weaning. Combining the use of two markers showing specific spatial and temporal patterns of expression, i.e., LPH and SI, and the techniques of xenografts, tissular dissociation, and heterologous association of fetal endodermal and mesodermal anlagen, the present study provides new data concerning the intrinsic process that governs the temporal development of the intestinal tract and the emergence of functional differences along its antero-posterior axis. (*a*) The onset of LPH expression during the perinatal period along the intestine, as well as the modifications of the enzyme and mRNA patterns occurring in neonates (in the colon) and up to weaning (along

the small intestine) are already determined in rat fetuses of 14 d. Hence, the spatial and temporal information needed for intestinal ontogeny up to the adult stage is fixed in the intestine prior to endoderm cytodifferentiation, at a stage where the intestinal stem cells cannot be identified as such. (*b*) In the presumptive small intestine, this spatial and temporal information is primarily held by the endoderm, which is additionally capable of inducing changes of the normal fate of heterologous mesodermal cells to form intestinal-specific mesenchymal-derived concentric layers. (*c*) The fetal colonic endoderm, but not the gastric or lung endoderms, shows heterodifferentiation toward a small intestinal pathway when it is associated with small intestinal mesenchyme.

While the onset of LPH expression in the small intestine of rat fetuses is most likely related to the activation of the gene transcription (Rings et al., 1992), the enzyme and mRNA patterns along the jejunioileum during postnatal development are mainly under posttranscriptional control (Duluc et al., 1993). The fact that fetal intestinal segments undergo correct development in the xenograft model sug-

gests that the ontogenetic autonomous program of the intestine includes regulatory paths acting at both transcriptional and posttranscriptional levels. Additionally, as these data show that the spatial and temporal pattern of LPH expression is primarily governed by the intrinsic program of development of the intestine, it implies that this pattern is essentially independent of the physiological status of the adult host. Hence, the regulatory effects exerted by thyroid hormones and by the dietary changes at weaning, respectively, on lactase enzyme activity and on the longitudinal distribution of the LPH mRNA (Paul and Flatz, 1983; Freund et al., 1991b; Duluc et al., 1992), should be considered as modulators of the intrinsic capacities of the intestinal cells. The situation is apparently different for SI, because this enzyme precociously appears when fetal small intestinal segments are transplanted in the glucocorticoid-rich environment of the adult host. Yet, glucocorticoids which participate *in vivo* to the maturation process occurring at weaning (Kedinger et al., 1983; Henning, 1987) may also be regarded as modulators of the intrinsic program of development, first because these hormones are not absolutely required for the emergence of SI at weaning (Martin and Henning, 1984), and second because the ability to respond to the glucocorticoid stimulation, *i.e.*, the presence of glucocorticoid receptors, is acquired by the intestine long before weaning, at the fetal stage (Kedinger et al., 1989).

Our study brings information on the transient phase of the neonatal colon ontogeny where its mucosa shows a small intestinal-like structure concomitant to a transient expression of LPH. The absence of lactase in colonic segments implanted in the hormone-rich environment of adult mice is consistent with the fact that LPH expression is abolished in the colon of rat neonates by the administration of factors such as thyroid hormones, glucocorticoids, or epidermal growth factor (Foltzer-Jourdainne et al., 1989; Freund et al., 1990). However, in an environment devoid of glucocorticoids and thyroid hormones, *i.e.*, the chick embryo, colon transplants are able to synthesize LPH. Thus, we conclude from the xenograft experiments in chick embryos that colonic development is governed by an autonomous ontogenetic program, and from the data obtained in nude mice that the transition from the small intestinal-like phenotype toward the typical colonic phenotype soon after birth is dependent in part on the hormonal environment. Lactase immunofluorescent staining in the transplanted colon is fuzzy and not strictly restricted to the apical membrane of the epithelial cells. This may correspond to an intracellular accumulation of the protein linked to a delayed processing of the lactase precursor. In rat neonates a delayed processing occurs in colonocytes compared with enterocytes (Büller et al., 1989; Freund et al., 1990).

Epithelial-mesenchymal interactions play a crucial role during intestinal organogenesis and in the mature organ for the continuous renewal of the epithelium from crypt stem cells (Haffen et al., 1989; Yasugi, 1993). We have combined the models of xenograft and of heterologous endodermal-mesenchymal association to investigate the respective role of these tissue anlagen in the determination of the temporal and positional information fixed by the ontogenetic program in the fetal small intestine. Heterotopic associations between endoderm and mesenchyme from the presumptive proximal jejunum and distal ileum develop and express lactase protein

and mRNA patterns according to the normal fate of the endodermal moiety. Similar results were obtained with heterotopic associations comprising small intestinal endoderm and colonic mesenchyme (unpublished results). The data reported here from associations of ileal endoderm and fetal skin fibroblasts further emphasize the self-differentiation potencies of the small intestinal endoderm, as proper differentiation occurs even in the presence of nonintestinal mesodermal derivatives. In addition, they show that the skin fibroblasts associated to an intestinal endoderm differentiate into typical intestinal mesenchyme expressing smooth muscle α -actin (Kedinger et al., 1990).

A possible inductive influence of the intestinal mesenchyme on the temporal and positional information held by the endoderm has been investigated using tissular associations comprising presumptive jejunal or ileal mesenchyme and endodermal segments originating from an anterior region (stomach, lung) or a posterior region (colon) to the small intestine. The results obtained with associations comprising stomach or lung endoderms indicate that the endodermal and the mesodermal moieties develop according to their own fate. Thus, the endodermal-deriving epithelial cells respectively express gastric pS2 (Lefèbvre et al., 1993) or pulmonary surfactant apoprotein A (Sakai et al., 1992) but neither intestinal LPH nor SI. In the associations with lung endoderm, morphogenesis, and cytodifferentiation are obviously dissociated since the overall morphology is of intestinal type whereas the cytodifferentiation of the epithelium is typical of the lung. Such a phenomenon has already been described in the gastrointestinal tract as well as in other systems (Yasugi et al., 1985). It is concluded from these results that the small intestinal mesenchyme taken from 14-d-old rat fetuses is not able to change the fate of the heterologous nonintestinal endoderm. The situation reported here in mammals differs from that described in birds as small intestinal mesenchyme of chick embryos is able to induce gizzard endoderm to switch its normal fate into an intestinal differentiation (Gumpel-Pinot et al., 1978; Haffen et al., 1982; Ishizuya-Oka and Mizuno, 1992). Two explanations can account for the discrepancies observed between mammals and birds. On one hand, the mammalian small intestinal mesenchyme at the stage preceding endodermal cytodifferentiation may not deliver any instructive information for the spatial and/or temporal development of the intestine. On the other hand, mammalian small intestinal mesenchyme, like in birds, may still exert an instructive influence, but the heterologous lung or stomach endoderms are insensitive to this effect. We believe the former hypothesis unlikely because the small intestinal mesenchyme of rat fetuses is able to exert an instructive inhibitory effect on the differentiation of chicken proventricular endoderm (Yasugi et al., 1989). The segregation of two endodermal cell lineages has been reported in chicken early during development in embryos having 15 to 20 somites: the one, at the level of the presumptive small intestine, invariably follows its own differentiation path irrespective to the mesenchyme to which it is associated; the other one, originating from the presumptive esophagus or stomach, is sensitive to an induction by small intestinal mesenchyme (Yasugi et al., 1992). In mammals, the segregation of endodermal cells early in development, and the consequence on their behavior, have not been investigated.

Unlike lung or stomach, fetal colon endoderm taken at day

14 of gestation exhibits phenotypic heterodifferentiation toward a small intestinal pattern when it is associated with small intestinal mesenchyme. The mucosa organizes into well differentiated villi and crypts, and the epithelium abundantly expresses SI. One explanation for this could be that the intrinsic potencies of the colonic endoderm lead to the development of a small intestinal phenotype, and that the colon mesenchyme exerts an instructive inhibitory effect on this endoderm leading the latter to express the typical colonic phenotype after the neonatal stage. Yet, we believe this unlikely because colonic mesenchyme is not able to change the fate of jejunal or ileal endoderms (unpublished results). Alternatively, the heterodifferentiation of the colon endoderm associated with small intestinal mesenchyme suggests that this mesenchyme is able to exert an instructive action on a heterotopic intestinal endoderm, i.e., the colon endoderm. Noteworthy, appearance of SI in these associations is delayed as compared to the expression of this enzyme in the small intestinal endoderm under the same grafting condition. This indicates that the inductive effect may result from a cascade of complex instructive actions and appropriate responses between the mesenchymal and endodermal moieties. SI expression in the colonic endoderm associated with small intestinal mesenchyme has been reported in some specimens grafted for 10 d in chick embryos and then incubated in vitro in the presence of dexamethasone (Foltzer-Jourdainne et al., 1989). These experiments suggest that one element of these interactions may involve glucocorticoids, possibly through the responsive small intestinal mesenchyme (Kedinger et al., 1989). Lastly, since the normal colonic mucosa transiently exhibits a small intestinal-like phenotype in rat neonates, the nature of the instructive action exerted by the small intestinal mesenchyme depends on the properties of the stem cells. Indeed, one can conceive two hypotheses: either the colon epithelium contains a single population of stem cells being responsible for the emergence of the small intestinal-like phenotype in newborns and later for the transition toward the typical colonic phenotype. In this case, the instructive action of the heterologous mesenchyme would consist of blocking the transition step. Alternatively, the colonic epithelium may be endowed with two sets of stem cells of small intestinal type and colonic type, respectively. In this case, the small intestinal mesenchyme would exert a dominant selection of the small intestinal type colonic stem cells. The data reported in this study may be extended to the human colon which also transiently exhibits a small intestinal-like phenotype in fetuses (Lacroix et al., 1984). Furthermore, small intestinal enzymes are expressed in colon cancers (Zweibaum et al., 1984). These findings emphasize the potential role of alterations of the epithelial-mesenchymal interactions in the emergence and development of human colon cancers.

In conclusion this study provides evidence that the temporal and positional information required to define the functional properties of each region of the intestinal tract throughout postnatal development is fixed in the presumptive intestine prior to endoderm cytodifferentiation. This intrinsic information is primarily carried by the endoderm which is also able to change the fate of heterologous mesodermal cells toward a typical intestinal mesenchyme. In turn, the small intestinal mesenchyme may exert an instructive effect as it causes heterodifferentiation of the colonic endoderm. This study does not identify the earliest stage at which

temporal and positional information is acquired by the endoderm. It also remains uncertain whether this early acquisition is intrinsic into the endoderm or mediated by interactions with the mesoderm. In chicken, initial information has been suggested to come from the endoderm, not from the mesoderm (Le Douarin and Bussonnet, 1966). Homeogenes expressed in a region-specific manner in the intestinal endoderm (Duprey et al., 1988; James and Kazenwadel, 1991; Freund et al., 1992) and mesoderm (Izpisua-Belmonte et al., 1991) are molecular candidates for the determination of the temporal and positional information, possibly via interactions with growth factors (Ruiz i Altaba and Melton, 1989). For instance, overexpression of *Hox-1.4* causes abnormal development of the colon (Wolgemuth, 1989). Adhesion molecules (Probstmeier et al., 1990), extracellular matrix proteins (Simon-Assmann and Kedinger, 1993) and oncoproteins (Nsi-Emvo et al., 1994) are also thought to participate in these events. Interestingly, it has been proposed in other organs than the intestine, that genes encoding adhesion molecules and extracellular matrix proteins may represent targets for the products of homeogenes and oncogenes (Jones et al., 1992; Kubota et al., 1992). In turn, epithelial-mesenchymal interactions can regulate homeogene expression (Takahashi et al., 1991). Thus, the experimental model used in the present study should help to get more insight into the molecular basis of the cellular interactions involved in morphogenesis, in the acquisition of positional information and finally in the functional development of the intestine.

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